

SOLID TUMOR SPHEROID FORMATION BY TEMPERATURE-CONTROLLED HIGH VOLTAGE ULTRASOUND IN A MULTI-WELL MICRODEVICE

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ABSTRACT

In the present work we demonstrate effective 3D growth of human hepatocellular carcinoma (HCC) HepG2 cell spheroids in parallel in a multi-well microdevice actuated with high voltage ultrasound in a temperature-controlled system. We compare the spheroid formation during continuous ultrasound exposure for one week where we formed spheroids in 59% of the wells, with the spheroid formation without ultrasound actuation, where we obtained 0% spheroids. Furthermore, we present an application of the tumor spheroids for investigating natural killer (NK) cells behavior against solid tumors.

KEYWORDS: Spheroid, Solid tumor, Ultrasound, Multi-well, Microchip, Natural killer cells

INTRODUCTION

In order to better understand the three-dimensional microenvironment of solid tumors *in vitro*, we need to mimic the 3D structure of the developing tumor. 2D cultures that are commonly used are inadequate to recreate this microenvironment and thus the outcomes of the experiments can be insufficient. Furthermore, solid tumor spheroids show increased drug resistance compared to 2D structures due to tight cell-cell contacts and interactions [1]. However, there are few reported methods for the controlled formation of many 3D spheroids in parallel [2].

With our multi-well microdevice we have previously shown that cell-cell contacts can be induced by ultrasonic standing waves in one hundred wells in parallel [3]. The method has also been used for synchronizing and real-time monitoring of interactions between natural killer (NK) cells and individual cancer cells [4]. In the present study, we use ultrasound in a novel temperature-controlled, microplate-based cell culture platform for highly parallel (up to one hundred) formation of solid tumor spheroids within 7 days. As a tumor cell model we use a HepG2 cell line that represents a human hepatocellular carcinoma (HCC). HCC is the most common malignancy of the liver and one of the most common and aggressive human cancers worldwide [5]. The future goal of the presented method is to use the solid tumor spheroids for optimizing chemo-therapy and immune-therapy protocols.

EXPERIMENTAL

The multi-well microdevice uses a ring-shaped ultrasonic transducer [6]. The device was integrated into a cell culture system (see Fig.1A), allowing control of the environmental conditions, while being operated by high voltage ultrasound (>100 V at 1.96 MHz) continuously for at least 7 days. The temperature in the multi-well microdevice (see Fig 1B, dashed circle) was regulated using an in-house built liquid heat exchanger capable of both cooling and heating (see Fig. 1B and C). Two different types of experiments were performed: with and without ultrasound actuation. The cells in both experiments were stained with the viability probe green calcein-AM and imaged using a confocal microscope. The resulting images were analyzed using MATLAB to calculate the tumor size (projected area) and fluorescence intensity of the calcein-AM (from the surface towards the center of the spheroid). Furthermore, the spheroids were subjected to natural killer (NK) cells stained with orange calcein-AM. The interactions between HCC spheroids and NK cells were imaged with high resolution confocal microscopy.

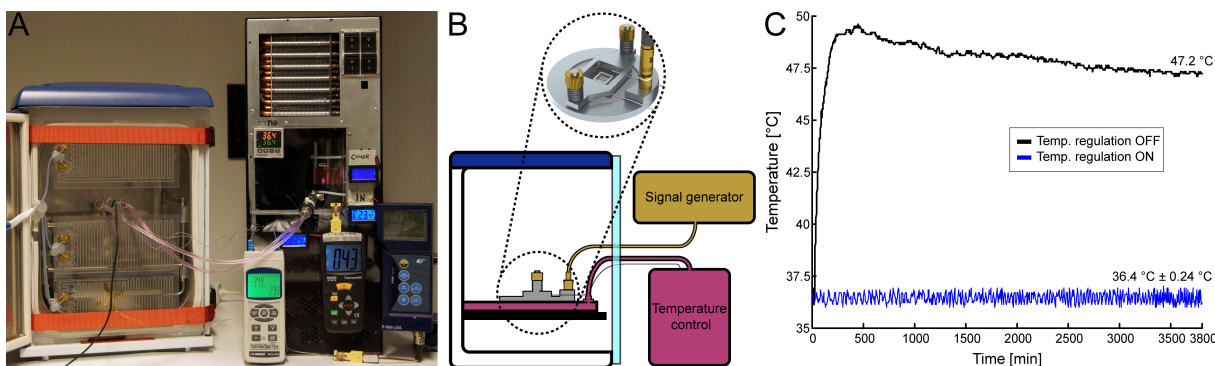


Figure 1: (A) The experimental setup with the cell incubator to the left and the temperature control system to the right. (B) The temperature of the multi-well microdevice (dashed circle) is regulated by controlling the temperature on the pink-colored plate inside the incubator using the temperature control system. Signal generator and RF amplifier shown in gold color. (C) The difference between temperature regulation ON and OFF with continuous ultrasound exposure. The regulation set-value was set to 36.4 °C. The temperature was measured on the multi-well chip.

RESULTS AND DISCUSSION

We show that 3D tumor HepG2 spheroids were successfully formed in the majority of the wells during continuous ultrasound exposure for one week (Fig. 2A). In contrast, the same cells were grown in monolayers (2D) in all 100 wells when the ultrasound was not applied (Fig. 2B). Fig. 2C shows the correlation between the penetration depths of the viability dye calcein-AM (Fig. 2A), and three different manually sorted cell aggregate categories: Spheroids (59%), inconclusive (26%), and not spheroids (14%). Here, the length of each curve (up to 120 μm) corresponds approx. to the radius of the spheroid. As seen in the diagram, when there were tight contacts formed between cells calcein could not penetrate far into the spheroid (red curves). This is clearly seen in Fig. 3A, where a high resolution confocal image of a spheroid slice reveals decaying fluorescence intensity over approx. three cell layers. Finally, we exemplify with 3D reconstructed confocal images, one application of the method where two natural killer (NK) cells interact with one spheroid (Fig. 3B).

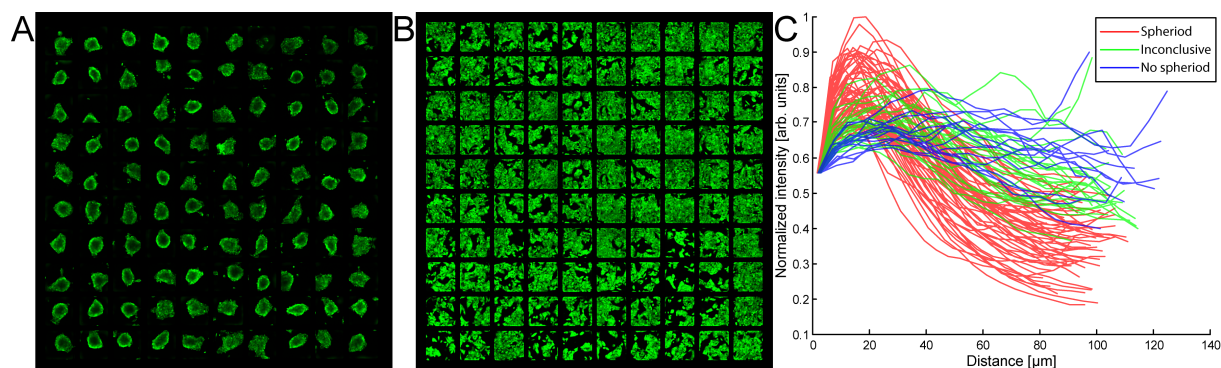


Figure 2: (A) Green calcein-AM stained HepG2 cell aggregates after 7 days of continuous ultrasound exposure (projected area of spheroids: $3 \cdot 10^4 \pm 5 \cdot 10^3 \mu\text{m}^2$). (B) Control experiment, 7 days culture without ultrasound, cells grow in monolayers. (C) Calcein-AM fluorescence intensity variations from the surface towards the center of each of the aggregates.

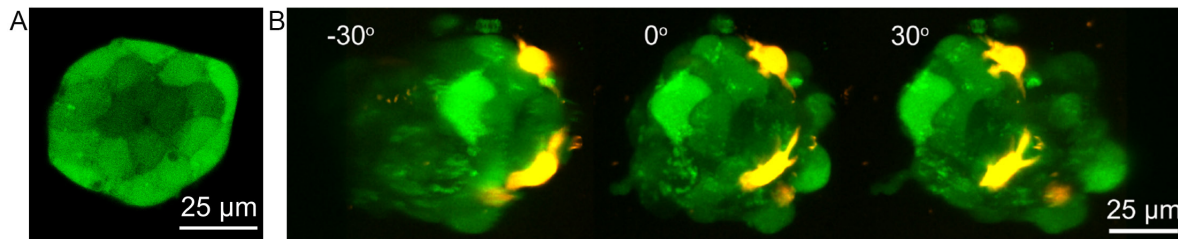


Figure 3: (A) 2D confocal image of a HepG2 spheroid. (B) 3D confocal image of natural killer cells (orange) interacting with a HepG2 spheroid (green).

CONCLUSION

As solid tumors grow in three-dimensions *in vivo*, formation of spheroids can be particularly important for cancer research. Multicellular tumor spheroids show increased resistance to drugs compared to monolayers due to tight cell-cell contacts and interactions. Thus, in future our device can be optimized for the controlled formation of large numbers of tumor spheroids for screening and characterization of anti-cancer drugs, but also for studying under what circumstances individual immune cells (such as NK cells) can penetrate and/or defeat solid tumors.

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