# Quantification of biochemical changes in three-dimensional cultured cancer spheroids by high-frequency backscatter and envelope analysis

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## 1. Introduction

Engineered three-dimensional (3D) tissue culture platforms (spheroids) are useful tools for duplicating biological phenomena, replacing two-dimensional cell culture-based lab experiments. However, to date, no readily available applications achieve 3D label-free observations inside the spheroid in daily biological lab practices (i.e. easily accessible imaging approaches are in high demand). Ultrasound (US) could be a suitable alternative because of its low-cost, label-free, and non-invasive imaging capability. In addition, quantitative ultrasound (QUS) is a matured technique that provides the non-invasive and label-free internal evaluation of the tissues.

This study shows the applicability of QUS in evaluating the internal biochemical changes induced by chemical or biological nature. Specifically, this paper focuses on two different categories of QUS methods: envelope-statistic based approach and backscatter spectral quantification. Employing established methods for validation, fluorescent images with immunostaining from the spheroid's center were also captured to monitor biochemical alterations.

## 2. Materials and Methods

### 2.1 Spheroid preparation

Green florescent protein-labeled human breast adenocarcinoma (MDA-MB-231) cells were used for spheroid formation. Spheroids were prepared according to the established fast-fabrication protocol[1)]. Briefly, a cell-suspended collagen solution was dispensed onto a superhydrophobic multiwell plate and transferred to a 48-well plate at a certain incubation period. Primary spheroids were approximately 2 mm in diameter. Cultured spheroids were divided into three groups based on the different cultured mediums: cultured in the conventional cell culture medium (control), cultured in the medium with blebbistatin diluted with dimethyl sulfoxide (DMSO) to inhibit actomyosin contractility, and cultured in the medium with the DMSO (n = 3 for each day and each medium)[2)]. The spheroids were incubated from day 0, the day they were made, until day 7 regardless of their treatment. Independent of the ultrasound-measured spheroids, spheroids for immunohistochemical observations were also created. Cell nuclei, F-actin and myosin light chains were stained on cryosections for fluorescence imaging.

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### 2.2 Data acquisition

On each day of culture, the three-dimensional radio-frequency (RF) signals from three groups were acquired. A custom ultrasound laboratory scanning system with a 20-MHz single element transducer was used for this study. The scanning apparatus featured a single-element, spherically focused, ultrasound transducer (PT20-6-12.7, Toray Engineering, Japan) with a 6.0-mm focal length and 12.7-mm aperture. The transducer had a center frequency of 20.0 MHz and a -6 dB bandwidth that extended from 4.1 MHz to 31.0 MHz. The theoretically predicted axial and lateral resolutions of the imaging system were 82.5 and 38.97 μm, respectively. The 6-dB depth of field was measured to be 1.60 mm extending from 11.58 to 13.18 mm. The transducer was excited by a pulser/receiver unit (5073PR, Olympus) and RF echo signals were digitized using a 10-bit at a sampling frequency of 208 MHz.

Each spheroid was placed in a water tank filled with Dulbecco's Modifies Eagle Medium (DMEM). In the water tank, the spheroid was placed on the polydimethylsiloxane (PDMS) substrate as an acoustic absorber. Scan vectors were uniformly spaced by 48 μm in X and Y directions across the entire scan volume to acquire complete full-volume 3D data from each spheroid. During the experiments, the focus of the transducer was positioned on the medium/spheroid interface. All measurements were conducted at room temperature.

### 2.3 QUS analysis

Two different approaches were used to characterize and quantify the internal changes of spheroids[3)]. For an envelope-statistic based analysis, Nakagami shape parameter (μ) was estimated using Nakagami distribution. The effective scatterer diameter (ESD) and acoustic concentration (EAC) were computed with a spherical Gaussian scattering

model as backscatter spectral quantification. Prior to the QUS parameter computation, the spheroid was semi-automatically segmented in three dimensions to crop out the PDMS substrate and medium region.

## 3. Results and discussion

First, the morphological and immunohistochemical characteristics were compared with the fluorescence microscope images and corresponding ultrasound B-mode images (Fig.1). Spheroid in the control group contracted as the culture day elapsed while the contractility was relatively small in the blebbistatin group. Hypoechoic areas due to the cell necrosis were also confirmed at the center of the image at day 7 control group. The QUS estimates (Fig.2a: ESD, 2b: EAC, 2c: Nakagami μ) depict the culture day-dependent changes in QUS parameters. Specifically, the spheroids cultured with blebbistatin showed different trends in ESD and EAC. On the contrary, Nakagami μ did not show the obvious changes among groups. Among the three groups, lower EAC in the blebbistatin group indicate the acoustic impedance contrast differed from the remaining groups. Consistent Nakagami μ indicate the number density of scatterers per resolution cell did not show significant change. This was specifically confirmed after day 3 when the spheroids generally began their contraction.

This is likely due to changes in the interference state of sound waves caused by variations in cell density and contractility within the spheroid. The consistent Nakagami μ suggest that necrosis does not occur, maintaining cellular activity while cell contractility changes.

In conclusion, these results suggest that the QUS can be a powerful tool to characterize internal biochemical changes through physical phenomena.

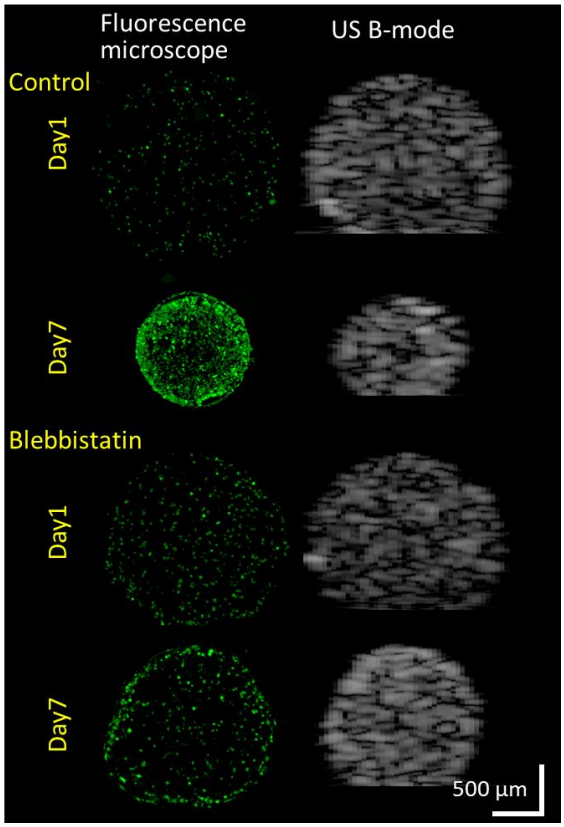


Fig.1 Ultrasound B-mode images and GFP fluorescent image of the represent control group (top) and myosin inhibition with blebbistatin group (bottom) on culture days 1 and 7.

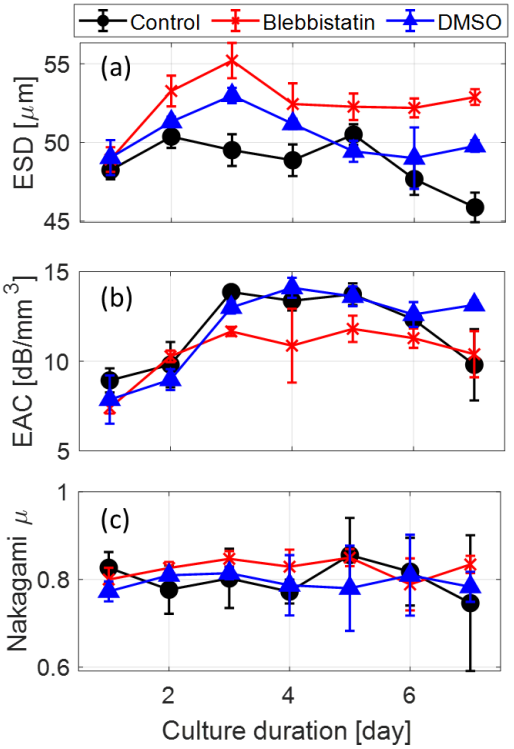


Fig. 2 Changes in the ESD (a), EAC (b) and Nakagami μ (c) averaged across entire spheroid (n=3).


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