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Estimation of Cell Concentration Using High-Frequency Ultrasonic Backscattering

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Abstract

Cell concentration is a crucial quantity for both clinical diagnostic examinations and cell culture studies. However, typical modalities for cell concentration measurements are either time-consuming or not cost-effective. In the present study, cell concentration is estimated using high-frequency ultrasonic backscattering. Validation tests indicate that the proposed method can differentiate red blood cells (RBCs) of various hematocrits. A 50-MHz ultrasound system with appropriate sensitivity is utilized to estimate cell concentrations from a small volume of RBCs suspended in saline, with hematocrits ranging from 1.66×10^{-4} to 10%, and fibroblasts, with concentrations ranging from 2×10^4 to 128×10^4 cells/mL. The backscatter strength and statistical distribution, characterized by the Nakagami parameter, are calculated from gated signals for quantitatively assessing the samples. Results show that the backscatter strength of RBCs linearly increases with increasing hematocrit level in the hematocrit range of 3 to 10%, which agrees well with results of previous studies. The backscatter strength of RBCS has an exponential relationship with the hematocrit level in the hematocrit range of 1.66×10^{-4} to 3%. The corresponding Nakagami parameter is sensitive to electronic noise as long as the signal-to-noise ratio decreasing follows with the decrease of RBC hematocrits at the concentration lower than 0.85%. The backscatter strength of fibroblasts exponentially increases with increasing fibroblasts concentration, which is consistent with results obtained from typical optical density measurements. A linear relationship, with correlation coefficient of 0.99, between the results of ultrasonic backscattering and those of the optical density measurements is established. High-frequency ultrasonic backscattering can be applied to sensitively estimate the concentrations of small volumes of cells.

Keywords: Cell concentration, High-frequency ultrasound, Ultrasonic backscattering, Nakagami parameter

1. Introduction

Cell cultures are typically used to investigate cellular properties, including cellular constituents, activities, and proliferation, for clinical applications or basic studies. Determining the number of cells provides a direct means of quickly assessing and characterizing many aspects of cellular properties. Thus, many techniques have been developed for cell counting, including hemocytometry, optical techniques, electronic cell counting, and flow cytometry [1-6].

A hemocytometer incorporated with a microscope is the most cost-effective and simplest device for cell counting. Although the hemocytometer employs a relatively easy protocol for cell counting, the procedure of subculture is tedious and is prone to measurement error while sampling the total number of cells during the dilution process and cell transfer [1,2]. The electronic counter is rapid and accurate but it is relatively expensive and requires a relatively large number of cell samples to achieve acceptable accuracy [5]. In addition to providing the functions of cell counting and sorting, flow cytometry can be utilized to detect antigens and molecular probes associated with cell activities. However, it is time-consuming and not cost-effective when a large number of cells are scanned [6]. Cellular viability can be determined by examining cells with colorimetric assays of tetrazolium salts such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT)

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[4]. However, the processes associated with these biochemical methods, such as staining and sonication, tend to cause cell death [3,4]. A cost-effective, flexible, rapid, and accurate technique for cell counting is thus desirable.

The propagation of ultrasound waves primarily depends on the elastic properties of the medium [7]. As an ultrasound wave propagates through a medium, part of the incident wave may be redirected into all directions by scatterers smaller than the acoustic wavelength. The strength of ultrasonic scattering is typically quantified as a scattering cross-section (σ_s). For weak scatterers, the corresponding ultrasonic scattering may be described by Rayleigh scattering theory [7,8], given as:

$$\sigma_s = \frac{k^4 a^6}{9}\left|\frac{\kappa_r - \kappa_o}{\kappa_o} + \frac{3\rho_r - 3\rho_o}{2\rho_r + \rho_o}\cos(\theta)\right|^2 \quad (1)$$

where θ denotes the angle between the transmitted and received ultrasound waves; k and a respectively represent the wavenumber and radius of a scatterer; κ and ρ are respectively the compressibility and density; and r and o correspond to the scatterers and the surrounding medium, respectively. According to Eq. (1), ultrasonic scattering is significantly affected by the ultrasound frequency and scatterer size. These properties enable ultrasonic backscattering to be able to differentiate hematocrits of red blood cells (RBCs) suspended in saline solution and other biological tissues [9-14]. Measurements of ultrasonic backscattering from RBC suspensions show that ultrasonic backscattering is a nonlinear function of hematocrit that is governed by the packing factor [9,10]. Ultrasonic backscattering of RBC suspensions tends to be proportional to the fourth power of hematocrit for hematocrit levels lower than 10%, as described in Eq. (1), for frequencies up to 90 MHz [15]. Extensive studies have been conducted on the effects of frequency, hematocrit concentration, size variation, and shear rate on the properties of ultrasonic backscattering from RBC suspensions and whole blood [16-21]. The hematocrit of RBCs in those studies [9,10,16,18-20] was measured using the centrifuge technique, whose accuracy is typically limited to 3%, making it unsuitable for measuring cells in low concentrations for cell culture studies. Measurements of ultrasonic backscattering from cells at low concentrations are difficult due to the acquired signals having very low signal-to-noise ratio (SNR). Ultrasonic backscattering and resolution increase with increasing ultrasonic frequency. Ultrasound with frequencies higher than 20 MHz thus provides better sensitivity for estimating low concentrations of a small volume of cells for cell culture studies.

Although high-frequency ultrasound provides better image resolution, the accompanying attenuation may greatly reduce the depth of wave penetration. A statistical analysis that considered the probability density function (PDF) of backscattered envelopes has been found to be less attenuation-dependent than other quantitative ultrasound parameters, such as ultrasound backscatter and velocity, and was thus used to correlate backscatter to the variation of blood coagulation [21]. The PDF of backscattered signals has been found to vary with the concentration and arrangement of scatterers in a resolution cell and that it may be categorized into pre-Rayleigh, Rayleigh, or post-Rayleigh distributions [22,23]. A general model, called the Nakagami distribution, was proposed to account for the statistics of ultrasonic backscattering and was found to have a low computation burden [22]. The Nakagami parameter, denoted as m, is a shape parameter that can characterize the PDF of backscattered envelopes (R). It is expressed as: [22]

$$m = \frac{[E(R^2)]^2}{E[R^2 - E(R^2)]^2} \quad (2)$$

where $E(\cdot)$ denotes the statistical mean of the backscattered envelope. The m parameter, with values less than 1, equal to 1, or larger than 1, corresponds to the statistics of pre-Rayleigh, Rayleigh, or post-Rayleigh distributions, respectively [22,23]. The m parameter has been validated to be capable of differentiating scatterer concentrations in studies that employed computer simulations, phantom experiments [24,25], and clinical assessments [26].

In the present study, a high-frequency ultrasound system was developed to measure ultrasonic backscattered signals from RBC suspensions and fibroblasts of various concentrations. A small volume of cells at low concentrations was estimated for cell culture studies. A series of experiments was performed on the RBC suspensions to verify the results with those of previous studies [9,10,18-20] and to test the signal conditions when measuring RBC suspensions with hematocrits of less than 3%. Cultured fibroblast models are frequently applied to investigate conditions associated with tissue repair and cellular phenomena. Ultrasonic backscattering and optical density measurements were thus obtained from fibroblasts of various concentrations. The results of ultrasonic backscattering and the statistical parameter are compared and discussed.

2. Materials and methods

2.1 Experimental arrangement

The developed high-frequency ultrasound system, shown in Fig. 1, mainly comprises a 50-MHz focused transducer (NIH Transducer Resource Center, USC, USA**)**, a pulser/receiver (Panametrics-NDT, 5900PR, Waltham, MA, USA), and an analog-to-digital converter card (Signatec, PDA-500, Corona, CA, USA). The aperture and -6 dB bandwidth of the high-frequency transducer are 2 mm and 35 MHz, respectively. The pulse echo response and characteristics of the employed transducer are shown in Fig. 2 and Table 1, respectively. All measurements were carried out in a distilled water tank with the temperature maintained at approximately 37 °C by a thermostat circulator.

2.2 Preparation of fibroblasts and porcine red blood cell suspensions

Human foreskin fibroblasts obtained from the cell line HS68 (Bioresource Collection and Research Center in Food Industry Research and Development Institute, Hsinchu, Taiwan) were prepared. The cells were cultured in a typical

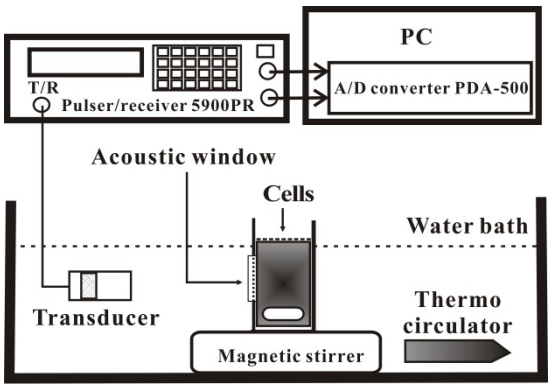


Figure 1. Block diagram of experimental arrangement.

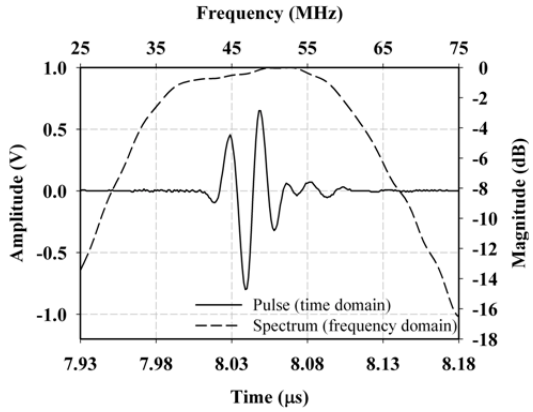
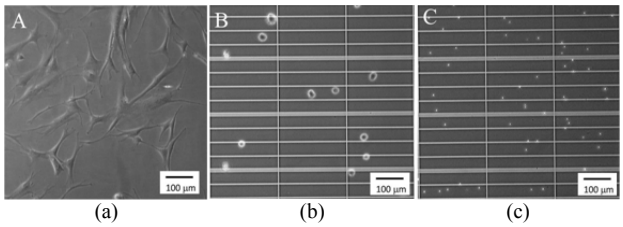


Figure 2. Pulse echo impulse response and spectrum of a 50-MHz transducer.

Table 1. Characteristics of the applied transducer.

Central frequency	48 MHz
-6 dB band width	35 MHz
f-number	1.5
Depth of focus	6 mm
Aperture size	4 mm

medium that consisted of Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel), 1% antibiotic/antimycotic solution (Gibco, Grand Island, New York, USA), 4 mM L-glutamine, and 1.5 g/L glucose. The temperature and CO_2 humidified atmosphere in the incubator were maintained at 37 °C and 5%, respectively. The medium of the cell culture was regularly replaced every three days. The seeded fibroblasts, with a size ranging from 20 to 30 μm, were obtained from the cell line between 13 and 15 passages. The cells were seeded in a Petri dish at an initial concentration of 1×10^4 cells/mL; the seeded cells were allowed to be grown to near 80% confluence. The suspended fibroblasts in the medium were prepared by a sequence of processes, including trypsinization and centrifugation. Concentrations ranging from 2×10^4 to 128×10^4 cells/mL were prepared for measurements. The concentration of fibroblasts was measured from cells stained with MTT. The assay was prepared by adding 0.2 mL of phosphate buffered saline (PBS) containing 0.05% of MTT, as described in a previous study [3]. The cells with MTT were kept in the incubator for 4 hours. The culture medium was then removed and subsequently replaced by 1 mL of dimethyl sulfoxide (DMSO) to solubilize the formazan. The optical density of each cell sample was measured at a 570-nm wavelength. Photographs of adherent and suspended fibroblasts are respectively shown in Figs. 3(a) and (b). In general, the shape and size of suspended fibroblasts were rounder and smaller than those of adherent cells.



(a) (b) (c)

Figure 3. Microscope photos of (a) adherent fibroblasts, (b) suspended fibroblasts, and (c) porcine RBC suspensions.

Fresh porcine blood was collected from a local slaughterhouse. A certain volume of acid citrate dextrose anticoagulant, with a concentration identical to those applied in previous studies [9,10,16,18-20], was added into the blood to prevent the collected blood from coagulating. The whole blood was centrifuged and washed twice in a buffer saline solution. RBC suspensions with hematocrits ranging from 1.66×10^{-4} to 10%, counted using a hemocytometer, were prepared by restituting the packed erythrocytes with a certain volume of saline solution. The MTT assay was not applied to measure the hematocrit level of RBC suspensions due to mammalian erythrocytes lacking a nucleus and organelles [27] to initiate the activity of mitochondria dehydrogenases for the colorimetry measurement [3]. Fibroblast concentrations of 2×10^4 to 128×10^4 cells/mL, which are equivalent to hematocrits of RBC suspensions ranging from 1.66×10^{-4} to 1.06×10^{-2}%, are frequently used for cell culture studies. A photograph of the suspended RBCs is shown in Fig. 3(c). The RBCs are much smaller than the suspended fibroblasts.

Samples of RBC suspensions or fibroblasts with a volume of 1.5 mL were prepared and stored in a rectangular plexiglass container equipped with an acoustic window. The suspended cells were stirred at 250 rpm using a magnetic stirrer to keep the cells homogeneously suspended in the container.

2.3 Data acquisition and analysis

For each measurement of RBC suspensions and fibroblasts, a total of 100 A-line ultrasonic radio-frequency (RF) backscattered signals, digitized at a 500-MHz sampling frequency and an 8-bit resolution, were acquired and stored for offline analyses. The backscatter strength, quantified by a root-mean-square (RMS) value, and the Nakagami statistical parameter (m) were calculated from gated signals from a region of interest (ROI) within the cell container. The RMS was calculated as:

$$RMS = \sqrt{\frac{1}{n}\sum_{i=1}^{n} x_i^2} \tag{3}$$

where n represents the number of A-lines and x_i is the signal corresponding to the i-th ultrasonic RF backscattered signal. The signal processing and parameter estimations were implemented using MATLAB software (MathWorks, Natick,

MA, USA). The data, expressed as the mean ± standard deviation (SD), represents the measurement of 5 samples of cells. Significant differences between the dietary groups were analyzed using one-way analysis of variance (ANOVA). A p value smaller than 0.05 was regarded to be significant. Data were statistically analyzed using SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

Measurements of RBC suspensions with various hematocrits were firstly performed to verify the ultrasonic backscattered signals and conditions associated with scatterers at low concentration. Figure 4 shows the RMS backscatter strength and m parameter of RBC suspensions as a function of hematocrit ranged from 1.66×10^{-4} to 10%. A hematocrit of RBC suspensions of between 1.66×10^{-4} and 1.06×10^{-2}% is equivalent to fibroblast concentrations of between 2×10^4 and 128×10^4 cells/mL, which are frequently used for cell culture studies [2]. The RMS backscatter strengths increased with increasing hematocrit. Results for RBCs with 3 to 10% hematocrits are consistent with those reported in previous studies [9,10,18]. However, the RMS values are difficult to be applied to differentiate RBC suspensions with hematocrits of 1.66×10^{-4} to 0.085% due to the inadequate SNR of the acquired signals.

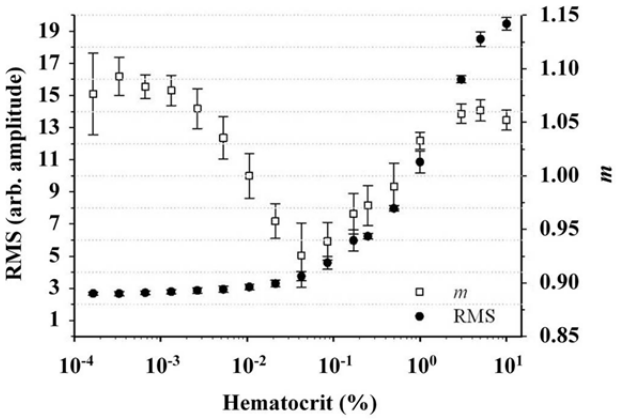


Figure 4. Ultrasonic backscattered signals (filled circles) and m parameter (empty squares) of RBC suspensions as functions of hematocrit concentration in the concentration range of 1.66 × 10-4 to 10%.

The m parameters associated with RBCs with hematocrits of between 1.66×10^{-4} and 2.66×10^{-3}% indicate that the acquired ultrasonic backscattered signals were dominated by noise, as shown in Fig. 4. As the SNR of RBCs at hematocrits of 2.66×10^{-3} to 0.85% increased, the associated m parameters decreased, indicating a variation of the PDF distribution with RBC concentration. A proportional relation between the m parameter and hematocrit exhibited in accordance with the SNR of ultrasonic backscattered signals were substantially improved while the corresponding hematocrits of red cells are higher than 0.85%. Despite sensitivity to noise, the m parameter only varied from 0.93 to 1 corresponding to the hematocrit of RBCs changed from 0.85 to 10%. This means that the PDF of ultrasonic signals backscattered from erythrocytes suspension tend to follow Rayleigh distribution. Previous studies demonstrated that the PDF of ultrasonic backscattered envelopes conforms to Rayleigh distribution when the number of scatterers in the resolution cell of the transducer is larger than 10 [28-30]. In this study, assuming that the resolution cell is a cylinder, the volume of the resolution cell is approximately 0.0017 mm^3. The volume of a porcine erythrocyte is approximately 68 μm^3 [11], and hence there should be approximately 200 erythrocytes randomly distributed in the resolution cell at a hematocrit of 0.85%. This indicates that the m parameter is difficult to apply for differentiating RBCs at such a low hematocrit.

Extensive measurements were conducted on fibroblasts with concentrations of between 2×10^4 and 128×10^4 cells/mL using both optical density and high-frequency ultrasound backscattering. The SNR of acquired signals from the suspended fibroblasts was satisfactory due to great increase in ultrasonic backscattered signal strength (suspended fibroblasts are about 20 times larger than RBCs in suspension), as shown in Fig. 4. Typical RF signals obtained from fibroblasts of various concentrations are shown in Fig. 5. The SNR of

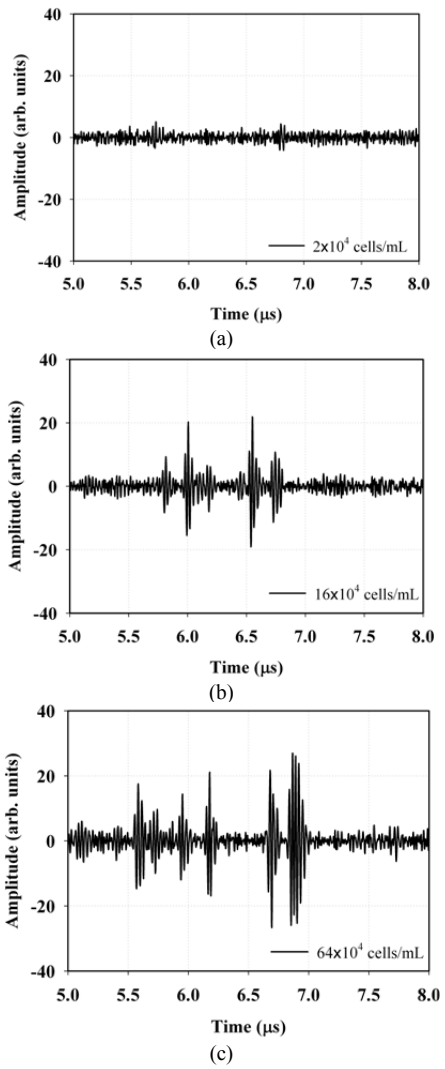


Figure 5. Ultrasonic backscattered signals of suspended fibroblasts with concentrations of (a) 2×10^4 cells/mL, (b) 16×10^4 cells/mL, and (c) 64×10^4 cells/mL.

fibroblasts was insufficient for the lowest concentration of 2×10^4 cells/mL. The SNR substantially increased with increasing fibroblast concentration. Figure 6 shows the RMS strength of fibroblasts as a function of concentration. The RMS strength increased corresponding to the increase of cell concentration from 2×10^4 to 128×10^4 cells/mL. The RMS strengths are significantly different ($p < 0.05$) between neighboring samples of fibroblasts for concentrations higher than 16×10^4 cells/mL.

Due to large difference in size, the backscattered signals of fibroblasts of a certain concentration are much larger than those of RBCs. For example, the RMS strength obtained from RBCs with concentration of 256×10^4 cells/mL is approximately equal to that obtained from fibroblasts with concentration of 64×10^4 cells/mL. The corresponding optical density results for fibroblasts as a function of cell concentration (in the range of 2×10^4 to 128×10^4 cells/mL) are shown in Fig. 7. The optical density range is from 0.13 to 2.66. The optical density results are also significantly different ($p < 0.01$) between neighboring samples of fibroblasts for concentrations higher than 8×10^4 cells/mL. There is a linear relation, with correlation coefficient of 0.99, between the results of Fig. 6 and Fig. 7, as shown in Fig. 8. Since the RMS values are affected by the SNR level of backscattered signals at low cell concentrations, the linear relation between the RMS strength and optical density does not pass through the origin. These results demonstrate that the measurement of ultrasonic backscattering with high- frequency ultrasound is very sensitive and as effective as optical density for quantifying cell concentrations.

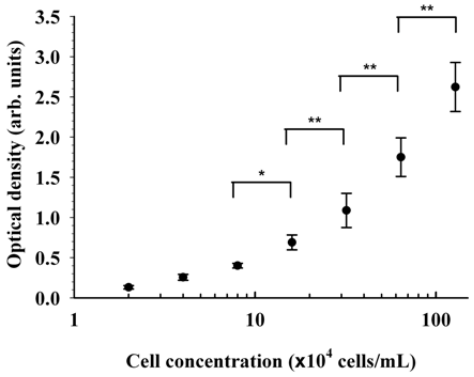


Figure 6. Ultrasonic backscattered signals of suspended fibroblasts as a function of cell concentration (*: $p < 0.05$, **: $p < 0.01$).

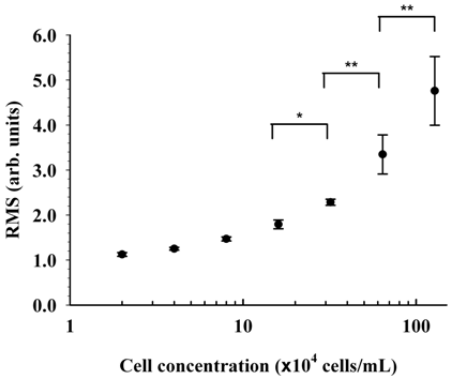


Figure 7. Optical density of suspended fibroblasts as a function of cell concentration (*: $p < 0.01$, **: $p < 0.01$).

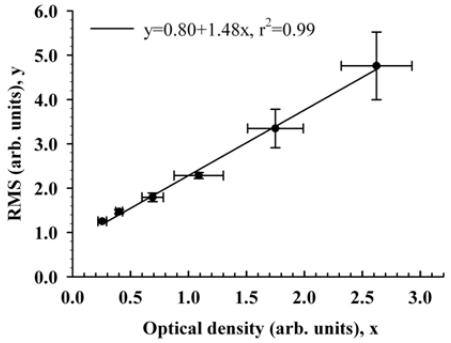


Figure 8. Empirically derived linear relationship between results obtained using ultrasonic backscattering and optical density.

Previous studies indicated that the ultrasonic backscattering of cells increases during the development of cell apoptosis, and that tends to be associated with changes of cell properties, such as nucleus size, density, and Poisson's ratio [31,32]. In the present study, the ultrasonic backscattering of RBC suspensions and fibroblasts increased with increasing concentration within the tested concentration range. As the concentration of RBC further increased, the tendency of corresponding ultrasonic backscattering were similar to those results associated with packing factor and attenuation in previous studies [9-10,18-19] The depth of high-frequency ultrasound penetration is limited by the accompanying attenuation and electronic noise. It is difficult to apply currently available techniques to measure cells of high concentrations due to backscattering signals needs to compensate attenuation. Moreover, the Nakagami parameter can properly differentiate RBC suspensions at hematocrits higher than 0.85% by characterizing the distribution of an ultrasonic backscattered envelope.

4. Conclusion

A 50-MHz ultrasound system was developed for measuring backscattered signals of RBC suspensions with hematocrits lower than 3%. The Nakagami statistical parameter was found to be sensitive to noise. Although the Nakagami parameter cannot easily be used for differentiating cells at concentrations used in a typical cell culture study, it can be used to comprehend the states of SNR of acquired signals. The backscatter strength of fibroblasts is much larger than that of suspended RBCs due to the former being larger. Measurements of backscatter strength and optical density were performed for fibroblasts as functions of cell concentration for concentrations of 2×10^4 to 128×10^4 cells/mL. A fairly linear relationship between the results acquired by these two methods was established. This study demonstrates that high-frequency ultrasonic backscattering is capable of sensitively detecting variations in cell concentration. To detect much lower concentrations of cells, the frequency of the employed ultrasound needs to be increased.

Acknowledgment

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