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Citation: *AIP Advances* **4**, 047120 (2014); doi: 10.1063/1.4871758

View online: <http://dx.doi.org/10.1063/1.4871758>

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Development of a thermal sensor to probe cell viability and concentration in cell suspensions

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(Received 16 January 2014; accepted 7 April 2014; published online 15 April 2014)

This paper presents a novel biothermal sensor to probe cell viability and concentration of a cell suspension. The sensing technique exploits the thermophysical properties of the suspension, so no labeling of suspended cells is required. When the sensor is periodically heated, the amplitude and phase of the thermal signal are dependent on the thermal properties of the cell suspension, particularly the thermal conductivity k . We measured k of HeLa, hepatocyte, and NIH-3T3 J2 cell suspensions with various concentrations and viabilities. The results demonstrate that the k of a cell suspension has a strong correlation with its concentration and viability. Accordingly, k can be employed as an index of cell concentration and viability. Furthermore, without data processing to obtain k , the electric signal that reflects the thermal response of the sensor can be used as a tool to probe viability of a cell suspension in real time. The proposed thermal sensing technique offers label-free, non-invasive, long-term, and real-time means to probe the viability and concentration of cells in a suspension. © 2014 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License. [<http://dx.doi.org/10.1063/1.4871758>]

I. INTRODUCTION

In cell culture study and cell response analysis, the importance of monitoring and controlling cell quality is indisputable.¹ Cell concentration and cell viability are two parameters that determine the quality of cell cultures, and are required to achieve reproducible cell seeding, and to define the appropriate times to control environmental conditions and to harvest cells. Several techniques can be used to measure cell viability and concentration.²⁻⁷ To probe cell viability, conventional optical methods use colorimetric dyes which stain live or dead cells specifically. For example, trypan blue (C34H28N6O14S4) is a diazo dye that selectively passes through the plasma membranes of dead cells, so dead cells appear blue under an optical microscope.⁸ Dye-based methods have the drawback that the dye may influence cellular activity, so the sample cannot be further tested or used. These methods also have other disadvantages including photobleaching, low signal-to-noise ratio, and complex procedures.⁹ Therefore, label-free, real-time, long-term and non-invasive cell viability probing techniques are desirable. Recently, an alternating current (AC) impedance measurement technique has emerged as an alternative to optical monitoring.⁹⁻¹² The technique is real-time, long-term and non-invasive when using AC of high frequency and low amplitude. However, the method can only assess the viability of cells attached to the sensor surface. In this paper, we demonstrate a novel biophysical technique applicable to cells suspended in buffer solutions. Analysis of cell

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suspensions is often required, and may be more convenient than cellular-level analysis in some cell studies as well as in many clinical applications. The technique based on the thermophysical properties of the cell suspension enables label-free and non-invasive measurement of concentration and viability of suspended cells.

Because the composition of the cell suspension changes due to cell metabolic processes, mass exchanges between the cells and the extracellular fluid, or both,¹³ the thermal property of the cell suspension should change according to the concentration of cells in it. Furthermore, the thermal conductivity k of a single cell changes (generally increases) when the cell dies.¹⁴ Similarly, death of suspended cells in a solution would change the chemical composition of the buffer (base fluid). When cells die, they lose their ability to maintain homeostasis, so water and extracellular ions flow into the cell and cause it and its organelles to swell and rupture, releasing cytoplasmic contents into the extracellular fluid.¹⁵ We suggested that this process alters the thermal network of cell suspensions. We exploit this process to measure cell concentration and viability by using the three-omega (3ω) method¹⁶⁻²¹ to measure the thermal conductivity k of cell suspensions. The method is a well-known thermal analysis technique that is suitable to measure k of liquids that are electrically-conducting, or small-volume, or both.²¹⁻²⁴ k of a cell suspension has a strong correlation with the cell viability and concentration, meaning that the 3ω thermal sensor can be used to measure cell viability and concentration. Furthermore, we also propose a real-time probing technique that can measure cell viability and concentration in real time by directly monitoring the voltage signal induced by the thermal response of the heater. At a single modulation frequency, the k of the cell suspension affects the sensor's amplitude and phase, and these changes can be correlated with cell viability and concentration by pre-calibrating the sensor. Accordingly, the cell viability and concentration can be monitored in real time from the voltage signal.

II. MATERIALS AND METHODS

A. Measurement principle

We used the 3ω scheme to measure k of cell suspensions with various concentrations and viabilities. The microfabricated sensing device is composed of a line heater/sensor on a glass substrate. When AC power of frequency ω is applied to the line heater, it generates heat at frequency 2ω . The heat induces oscillation of temperature and resistance at 2ω . The resistance multiplied by the current results in a voltage oscillation with frequency 3ω . The average temperature of the sensor can be expressed analytically and used to determine k of the liquid sample (k_l) in which the sensor is immersed.¹⁶ If $qb \ll 1$, where $1/q = \sqrt{k/2\omega C}$ is the thermal penetration depth (m), C is volumetric heat capacity of the sample ($\text{J} \cdot \text{m}^{-3} \cdot \text{K}^{-1}$), and b is half of the heater width (m), then k_l ($\text{W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$) of a liquid sample can be determined from the slope of the thermal-response curve in a plot of the real (in-phase) part of temperature T_{real} vs. $\ln(\omega)$:

$$k_s + k_l = -\frac{P}{2l\pi} \frac{d \ln \omega}{dT_{real}}, \quad (1)$$

where k_s is the thermal conductivity of the sensor substrate, P is the heating power (W) and l (m) is the length of the heater.²¹

To assess the status of a cell suspension in real time, we can continuously monitor the voltage signal of the thermal sensor at a single frequency. By pre-calibrating the sensor, the signal can be correlated with k of the liquid sample, i.e., with cell viability, concentration, or both. Either the amplitude or the phase of the voltage signal can be used in this real-time measurement technique; in the present work, the amplitude of the output signal was used. The modulation frequency is chosen to optimize its sensitivity and temporal resolution in the real-time sensing.

B. Sample preparation

The cultures tested contained HeLa cells, rat hepatocytes or NIH-3T3 J2 fibroblasts. The HeLa cells were obtained from American Type Culture Collection (ATCC). Hepatocytes were isolated from

adult female Sprague-Dawley rats (Central Lab. Animal Inc., Korea) weighting 140–160 g, using a modified two-step collagenase perfusion procedure.^{25,26} NIH-3T3 J2 fibroblasts were obtained from ATCC. HeLa cells were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (Gibco) and $100 \mu\text{g} \cdot \text{ml}^{-1}$ penicillin-streptomycin (Gibco). Culture medium was replaced twice per week and cells were passaged in 1:10 dilutions at least once per week. Cells were cultured at 37°C in a 95% air / 5% CO_2 atmosphere. Hepatocytes were suspended in hepatocyte culture medium that consisted of DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), $0.1 \text{ mg} \cdot \text{ml}^{-1}$ glucagon (Sigma), $7.5 \text{ mg} \cdot \text{ml}^{-1}$ hydrocortisone (Sigma), $10 \text{ mg} \cdot \text{ml}^{-1}$ insulin (Sigma) and $200 \mu\text{g} \cdot \text{ml}^{-1}$ penicillin-streptomycin (Gibco) and stored at 4°C . Dead hepatocytes were obtained by incubating isolated hepatocytes with added 10-mM acetaminophen for 24 h at 37°C in 90% air / 10% CO_2 atmosphere. This acetaminophen-induced hepatocyte death can be considered as apoptosis in a short term.^{27,28} NIH-3T3 J2 fibroblasts were cultured in DMEM (Gibco), supplemented with 10% bovine calf serum (Gibco) and $100 \mu\text{g} \cdot \text{ml}^{-1}$ penicillin-streptomycin (Gibco). Culture medium was replaced twice per week and cells were passaged at dilutions of 1:10 at least once per week. Cells were cultured at 37°C in a 95% air / 5% CO_2 atmosphere. Dead NIH-3T3 J2 cells were prepared by necrosis of the cells incubated in a suspension at 4°C for 24 h.

In all cultures, cell viability was checked using the trypan blue exclusion test. Cells and trypan blue were mixed in a 1:1 (v/v) ratio, and the mixture was loaded into a hemocytometer. Using an optical microscope, unstained and stained cells were counted as viable and nonviable, respectively. These results were used to provide known values against which the biothermal measurements could be compared.

C. Experimental setup

A thin-film Au/Ti strip heater/sensor was fabricated on a Borofloat glass substrate (Schott 33) using the lift-off process. A SiN dielectric layer of 200-nm thickness was deposited using plasma-enhanced chemical vapor deposition to prevent electrical leakage. The temperature of samples for each measurement was set at 4°C . In the measurement system (Fig. 1), an electrical current with a frequency ω from an internal lock-in amplifier (Stanford Research Systems, model SR810) was supplied to an Au strip. The ω component of the signal was eliminated by adjusting the gain of a differential amplifier (Analog Devices, model AD620) with an input impedance $10 \text{ G}\Omega$, leaving only the 3ω signal that bears the thermal information. The 3ω signal was measured using the lock-in amplifier. The amplitude and phase of the temperature signal were then determined from the signal. The measurement was repeated at modulation frequencies from 0.7 to 1000 Hz. Measurements were repeated ten times for each cell suspension. The accuracy of the measuring technique was tested by measuring the thermal conductivity of deionized water, ethylene glycol, and methanol. The deviations from reference values²⁹ were all $< \pm 1.5\%$. Because the density of cell was higher than that of buffer, a magnetic stirrer was used while k was measured to prevent sedimentation of cells. The 3ω signal is unaffected by the flow because the thermal wave penetration is confined to the vicinity of the surface of the sensor where the flow effect is negligible.³⁰

III. RESULTS AND DISCUSSION

The k of HeLa cell suspension was as a function of concentration (Fig. 2). At a relatively low concentration of $1.4 \times 10^7 \text{ cell} \cdot \text{ml}^{-1}$, k was close to that of the buffer ($0.563 \text{ W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$). As the cell concentration increased, k decreased almost linearly to $0.5 \text{ W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$ at $8.0 \times 10^8 \text{ cell} \cdot \text{ml}^{-1}$. The maximum standard deviation (sd) was 2.82%, and the average sd was 1.35%. The dashed line in Fig. 2 shows a simple linear regression fit. The change in k of the suspension cannot be explained by a simple mixing rule: because k of an individual cell is similar to that of the buffer solution, increase or decrease in cell population cannot affect the effective k significantly. Although cells in a suspension are not active, i.e., without activation of migration in a suspension, they exchange molecules and ions with the extracellular fluid and may excrete metabolic byproducts. Consequently, we suppose that the k variation is largely due to compositional changes that lead to an increased fraction of low- k substances in the solution.

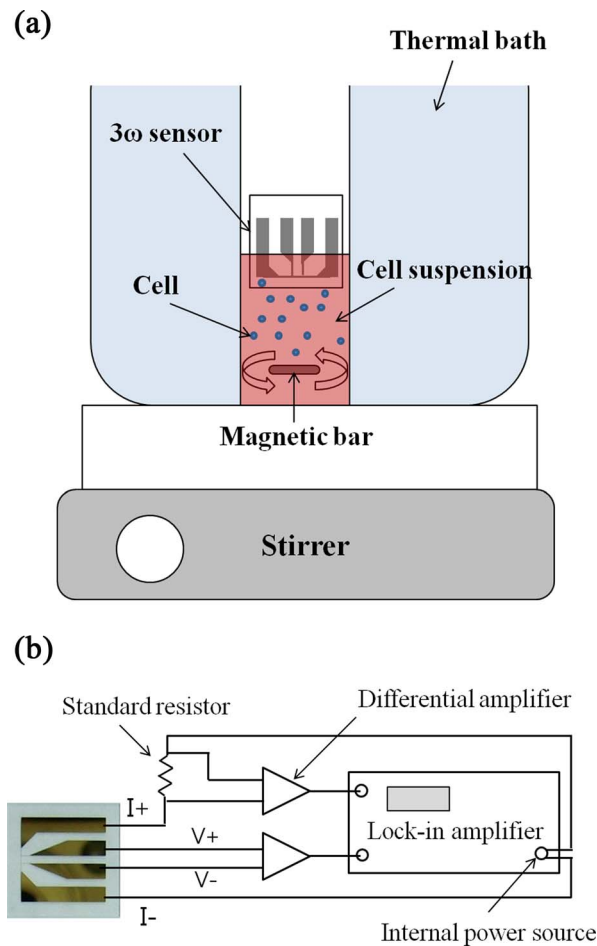


FIG. 1. (a) Schematic diagram and (b) electrical circuit of measurement setup.

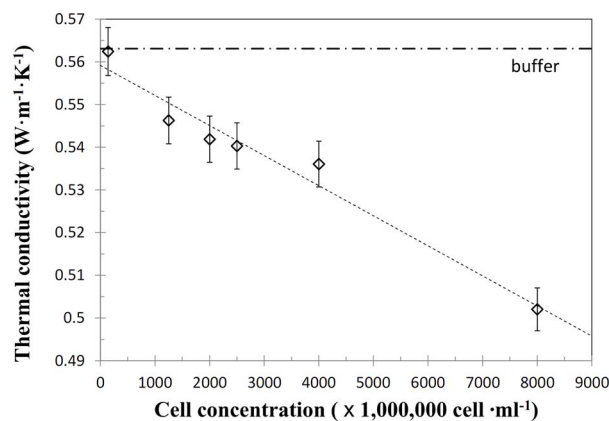


FIG. 2. Thermal conductivity of HeLa cell suspension with various cell concentrations.

The death a cell arrests its homeostatic abilities, so water and extracellular ions flow into it in response to concentration gradients, causing it to swell and ultimately rupture, thereby its cytoplasm contents into the extracellular fluid. This release should change the composition of the fluid and thus the effective k of the suspension. To test this hypothesis, we prepared hepatocyte cell suspensions that had various viabilities. The trypan blue exclusion test was performed to check the viability of these

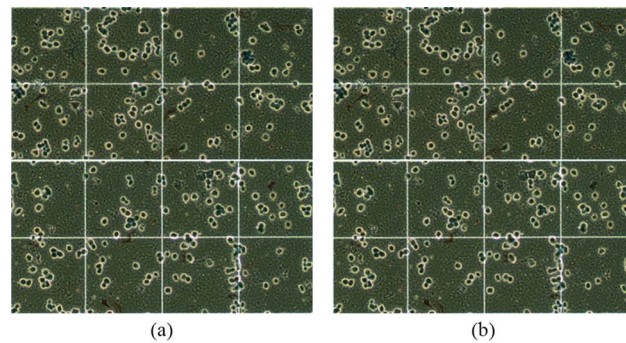


FIG. 3. Photographs of (a) live and (b) dead hepatocyte cells labeled using the trypan blue staining method.

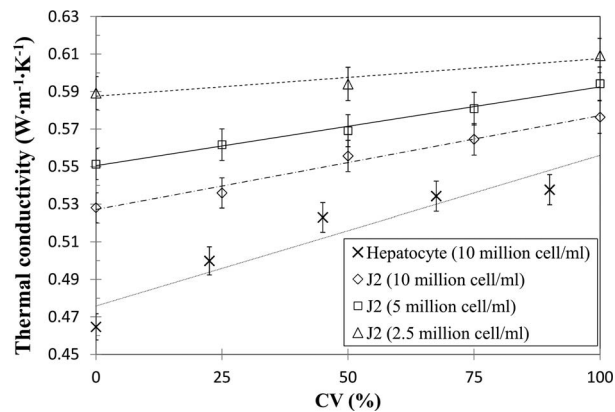


FIG. 4. Cell viability effect on k of hepatocyte and NIH-3T3 J2 cell suspensions.

samples (Fig. 3). The viabilities of live and dead cell suspensions were ~ 90 and $\sim 0\%$ respectively. To obtain cell suspensions of various viabilities, these suspensions were mixed at volume ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. Therefore the viabilities of each mixture were 90, 67.5, 45, 22.5, and 0% respectively. The k of a cell suspension with 90% viability was $0.537 \text{ W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$. As viability decreased, k of the suspension decreased, and when the viability was 0%, k was $0.465 \text{ W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$ (Fig. 4). The maximum sd was 3.03% and the average sd was 1.57%. This result demonstrates that increase in the population of dead cells induces a decrease in k of the suspension.

To verify that this phenomenon occurs generally, we also measured k of NIH-3T3 J2 fibroblast suspensions with various viabilities. We also observed whether the treatment process for preparing dead cells affected the results. The viabilities of live and dead cell suspensions were about 100 and 0%, respectively. These suspensions were again mixed at volume ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. As cell viability or concentration of NIH-3T3 J2 cell suspension decreased, k decreased (Fig. 4). The maximum sd was 2.97% and the average sd was 1.59%. Although the type of cell death was necrosis, unlike hepatocytes in which it was apoptosis, the change in k was similar to that of the hepatocyte suspension. This means that the decrease in k was mainly caused by morphological changes of the cell during death and that it occurred after both apoptosis and necrosis. Therefore, we suggest that the k value can be interpreted as a general index of cell viability because k is a physical property that is not affected by environmental conditions except temperature. The slope of the relation between k and viability decreased as cell concentration decreased, so this trend places limits on the sensitivity of the measurement. Measurement accuracy limits use of this probing technique to cell suspensions with concentrations $> 2.5 \times 10^7 \text{ cell} \cdot \text{ml}^{-1}$.

The result is noteworthy because the k of an individual cell increases when it dies, (Fig. 5),¹⁵ whereas k of the cell suspension decreased; this means that the decrease in k of the cell suspension was caused by a mechanism other than the change in k of individual cells contained in the suspension.

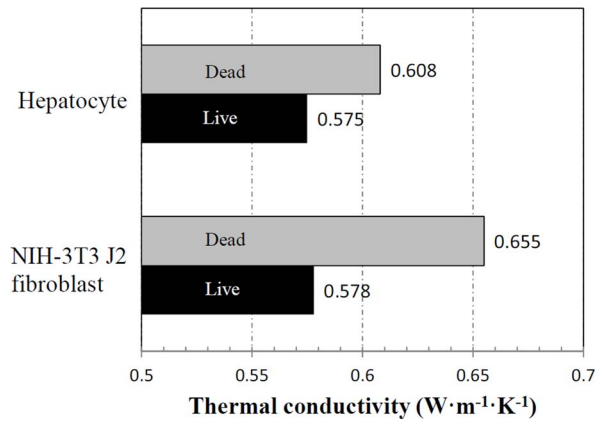


FIG. 5. Thermal conductivities of single live and dead cell (hepatocyte and NIH-3T3 J2 fibroblast).

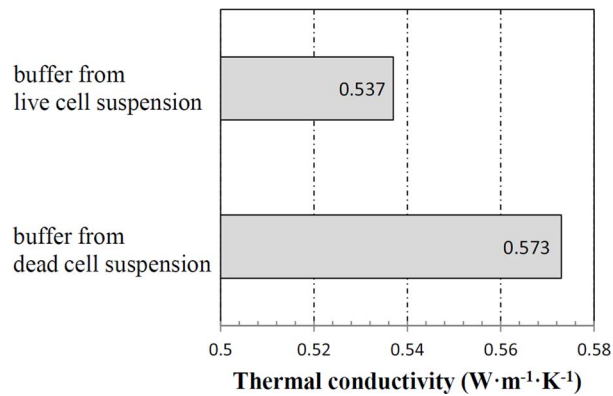


FIG. 6. Thermal conductivities of pure buffers after eliminating live cells and dead cells.

To clarify the physical mechanism responsible for reduction of k of the cell suspension by cell death, the k values of the buffer solutions were measured after eliminating both the live and dead hepatocyte cells from the sample solution with 1.0×10^7 cell ml^{-1} . A centrifuge (600 rpm, 5 min) was used to separate the cells from the solution and the measurements were conducted at a room temperature of 20°C . The k values from the buffer solutions with live and dead cells were 0.573 and $0.537 \text{ W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$, respectively (Fig. 6). These results are similar to the k values of 100 and 0% CV measured at 4°C . This supports our hypothesis that if cells are dead, water and extracellular ions flow into them, causing the cells to rupture and release their cytoplasm contents into the extracellular fluid. Although the k of cells is altered when they die, their volume fraction is too small to affect the thermal property of the suspension: when the mean volume of a cell is 10 μl , the volume fraction of the cells is only 1% at a concentration of 1.0×10^7 cell ml^{-1} . Consequently, the decreased k in dead cell suspensions is probably mainly due to the reduced k of the buffer, not to the k of the cells in it.

The probing technique described above has the drawback that the viability and concentration cannot be assessed in real time because the k measurement takes several minutes, e.g., $7 \sim 8$ min in this work, to obtain thermal responses at different frequencies. To overcome this problem, we propose an alternative method that uses the thermal response at a single frequency instead of scanning signals over a range of frequencies. In the single-frequency scheme, the raw voltage signal is directly correlated with cell viability and concentration. The voltage signal determined by the thermal properties of the suspension is mainly affected by k of the medium. As k of the suspension decreases, heat dissipation from the sensor lessens, and the temperature and voltage of the sensor rise accordingly. In this manner, we can measure the viability and concentration in real time. To demonstrate this real-time sensing method, voltage signals were measured for each viability

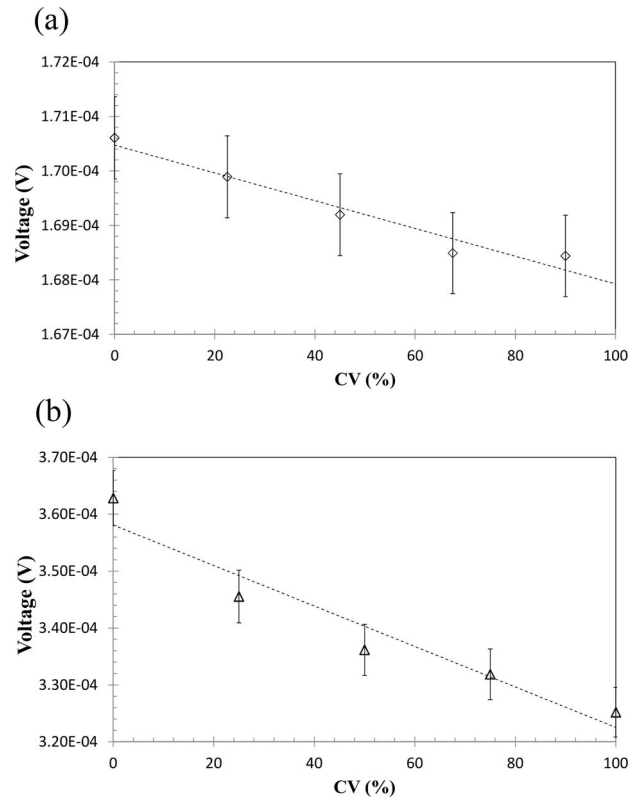


FIG. 7. Real-time voltage signal of (a) hepatocyte and (b) NIH-3T3 J2 cell suspension at 12 Hz with various cell viabilities.

sample at a single frequency (12 Hz). The AC sensing technique is much more effective than DC heating to detect the change of environment.³¹ As frequency decreases, the amplitude of the signal increases; therefore the signal-to-noise ratio increases, but the time required to acquire the voltage signal increases as frequency decreases. We selected 12 Hz as an optimum frequency to give a response that is accurate and sufficiently fast to allow continuous real-time monitoring of viability. The amplitude of voltage signal increased as cell viability decreased (Fig. 7). This means that the temperature of sensor increases because k , which measures the ability of cells to transport passively-applied thermal energy, decreased as cells died. This technique is less accurate than the absolute thermal-conductivity measurement but can monitor cell viability in real time without needing any data analysis to calculate k .

IV. CONCLUSION

We employed the 3ω thermal-analysis scheme to measure the change in effective k of cell suspensions while varying the cell concentration and viability. The results demonstrated that k of cell suspension has a strong correlation with cell concentration and cell viability. We thus suggest that measurement of thermophysical properties can serve as an effective tool to assess the viability of suspended cells. Regardless of the cell death process (apoptosis or necrosis), death of cells resulted in significant reduction of thermal conductivity of the suspension. This quantitative technique based on k change is non-destructive and label-free but required several minutes to analyze a sample. Therefore, we also proposed another single-frequency sensing method that can be used for real-time monitoring of cell viability and concentration. The real-time technique directly correlates the voltage response of the thermal sensor with cell viability and concentration. Because the biothermal sensor developed in this work can easily miniaturize be integrated in lab-on-a-chip devices, it can be used in various micro-Total Analysis Systems as well as in cell studies.

ACKNOWLEDGMENTS

This work was supported by the NRF Basic Research Programs (No. NRF-2010-0017848, No. 2011-0028845).

- ¹ K. B. Konstantinov, *Biotechnol. Bioeng.* **52**, 271–289 (1996).
- ² R. Dittmar, E. Potier, M. Van Zandvoort, and K. Ito, *Tissue Eng.: Part C* **18**(3) 198–204 (2012).
- ³ C. L. Harding, D. R. Lloyd, C. M. McFarlane, and M. Al-Rubeai, *Biotechnol. Prog.* **16**, 800–802 (2000).
- ⁴ S. Ingebrandt, G. Wrobel, S. Eick, S. Schäfer, and A. Offenhäusser, A., “TRANSDUCERS and EUROSENSORS '07 - 4th International Conference on Solid-State Sensors,” *Actuators and Microsystems* **4300252**, 803–806 (2000).
- ⁵ K. Konstantinov, S. Chuppa, E. Sajan, Y. Tsai, S. Yoon, and F. Golini, *Trends Biotechnol.* **12**(8), 324–333 (1994).
- ⁶ M. C. O'Brien and W. E. Bolton, *Cytometry* **19**, 243–255 (1995).
- ⁷ E. M. Strohm, G. J. Czarnota, and M. C. Kolios, *IEEE. T. Ultrason. Ferr.* **57**(10), 2293–2304 (2010).
- ⁸ W. Strober, *Curr. Protoc. Immunol. Appendix* (John Wiley & Sonx, Inc., 2001).
- ⁹ C. D. James, N. Reuel, E. S. Lee, R. V. Davalos, S. S. Mani, A. Carroll-Portillo, R. Rebeil, A. Martino, and C. A. Applett, *Biosens. Bioelectron.* **23**, 845–851 (2008).
- ¹⁰ J. P. Carvell and J. E. Dowd, *Cytotechnology* **50**, 35–48 (2006).
- ¹¹ O. I. Guliy, V. D. Bunin, D. O'Neil, D., Ivnitski, and O. V. Ignatov, *Biosens. Bioelectron.* **23**, 583–587 (2007).
- ¹² M. Thakur, K. Mergel, A. Weng, S. Frech, R. Gilabert-Oriol, D. Bachran, M. F. Melzig, and H. Fuchs, *Biosens. Bioelectron.*, **35**, 503–506 (2012).
- ¹³ A. Krogh, A.-L. Lindberg, and B. Schmidt-Nielsen, *Acta Physiol. Scand.* **7**, 221–237 (1944).
- ¹⁴ B. K. Park, N. Yi, J. Park, and D. Kim, *Appl. Phys. Lett.* **102**, 203702 (2013).
- ¹⁵ U. Ziegler and P. Groscurth, *Physiology* **19**, 124–128 (2004).
- ¹⁶ D. G. Cahill, *Rev. Sci. Instrum.* **61**, 802–808 (1990).
- ¹⁷ S. M. Lee and S. I. Kwun, *Rev. Sci. Instrum.* **65**, 966–970 (1994).
- ¹⁸ S. M. Lee and D. G. Cahill, *J. Appl. Phys.* **81**(6), 2590–2595 (1997).
- ¹⁹ I. K. Moon, Y. H. Jeong, and S. I. Kwun, *Rev. Sci. Instrum.* **67**(1), 29–35 (1996).
- ²⁰ F. Chen, J. Shulman, Y. Xue, C. W. Chu, and G. S. Nolas, *Rev. Sci. Instrum.* **75**, 4578–4584 (2004).
- ²¹ S. R. Choi, J. Kim, and D. Kim, *Rev. Sci. Instrum.* **78**, 084902 (2007).
- ²² S. R. Choi and D. Kim, *Rev. Sci. Instrum.* **79**, 064901 (2008).
- ²³ B. K. Park, J. Park, and D. Kim, *Rev. Sci. Instrum.* **81**, 066104 (2010).
- ²⁴ B. K. Park, N. Yi, J. Park, and D. Kim, *Rev. Sci. Instrum.* **83**, 106102 (2012).
- ²⁵ P. O. Seglen, *Methods Cell Biol.* **13**, 29–83 (1976).
- ²⁶ J. C. Dunn, M. L. Yarmush, H. G. Koebe, and R. G. Tompkins, *FASEB J.* **3**, 174–177 (1989).
- ²⁷ R. H. Pierce, C. C. Franklin, J. S. Campbell, R. P. Tonge, W. Chen, N. Fausto, S. D. Nelson, and S. A. Bruschi, *Biochem. Pharmacol.* **64**, 413–424 (2002).
- ²⁸ L. P. James, P. R. Mayeux, and J. A. Hinson, *Drug Metab. Dispos.* **31**(12), 1499–1506 (2003).
- ²⁹ D. R. Lide, *CRC Handbook of Chemistry and Physics*, 81st ed. (CRC, New York, 2000).
- ³⁰ J. Hong, Y. S. Chang, and D. Kim, *Meas. Sci. Technol.* **21**(10), 105408 (2010).
- ³¹ S. R. Choi, J. Hong, and D. Kim, *Sens. Actuators A* **150**, 40–45 (2009).