Full-field optical coherence microscopy for identifying live cancer cells by quantitative measurement of refractive index distribution

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Abstract: The feasibility of identifying cancer cells by measuring the refractive index (RI) distribution across a single live cell with ultrahigh resolution full-field optical coherence microscopy (FF-OCM) is presented. The FF-OCM is utilized to quantify integral RI distributions of unmodified cells without any cell treatments and used as a biophysical indicator for diagnosing cell malignancy. Firstly, the physical thickness distribution of the cell adherent to a culture dish is measured by taking a series of 0.6 µm resolved en-face tomograms. Subsequently, from the en-face image of the bottom surface of the cell or the top surface of the dish, the phase gain image of the cell is extracted. Then, from these two measurements the axially averaged RI map of the cell is extracted. The implemented FF-OCM system had a 0.8 µm axial resolution and the phase measurement sensitivity of the system was around 124 mrad. With the system, RI maps of several living cell lines of normal and cancer cells were constructed and quantitatively analyzed. The experiments showed that cancer cells had higher RI than normal ones. This approach using the FF-OCM has significant potential for cancer diagnosis and dynamic cell analysis as in situ label-free biophysical assay.

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1. Introduction
For the last few decades, cancer identification using a live single cell has been a subject of considerable interest in cancer diagnosis. Because knowledge of the nature of viable cells provides valuable insight into the biomechanics of sub-cellular structures, many scientific efforts have been made to reveal the biophysical properties of cancer cells for monitoring onset and prognosis of human cancer [1–7]. Recently, refractive index (RI) of a live cell has gained much attention as an attractive indicator of cell abnormality. The cell RI attributable to optical interaction of light field with cellular organelles is informative for quantifying chemical composition within cellular structures, so that the potential application has been considered in cell physiology and pathology. In cancer biology, it is well known that the RIs
of cancer cells are relatively higher than normal cells [8–10], which is believed to be a good criterion for quantitative diagnosis of cell malignancy.

There have been a number of optical approaches to measure the RI of an individual living cell including the classical manner based on solution matching [11], micro-chip refractometry based on Fabry-Perot resonant cavity [12,13], and refractometer with microfluidic devices [14]. These optical assays provided typically an effective RI, the RI value averaged throughout the whole cell body, but did not give the local RI or RI distribution. Combination of phase microscopy with confocal microscopy has allowed one to obtain the RI distribution of a viable cell [15,16]. One was used to get the physical thickness of the cell and the other was utilized to get the phase shift induced by the cell. However, the use of two independent systems inevitably brought many mismatching problems in specimen location and image magnification.

In this paper, we firstly demonstrate the feasibility study of cancer cell identification using cell RI distribution. By using full-field optical coherence microscopy (FF-OCM) as extension of optical coherence tomography (OCT) [17], the RI distribution of a live single cell could be quantitatively measured. With a series of FF-OCM en-face images of a cell, we could get not only the physical thickness but also the optical thickness of the cell at a time, which allowed getting the RI map of the cell without suffering from the pixel and magnification mismatch problems of the earlier schemes. We show that the proposed FF-OCM enables quantitative assessment of the malignancy of living cells.

2. Material and Methods

2.1 Descriptions of FF OCM system

The schematic of the FF-OCM for cell RI imaging is shown in Fig. 1, similar to previous arrangements [18–20]. Low-coherent light emitted from a fiber-bundle incorporated with a 100 W halogen illuminator is directed to the interferometer through a beam shaper. The beam is split by a non-polarized beam splitter (BS) and passes through the reference arm and the sample arm of the system and then is tightly focused by a pair of identical water-immersion microscope objectives (UMPLFL 100XW, 1.0 NA, Olympus, MO). The beams back-scattered and -reflected at both arms are projected onto a two-dimensional image sensor such as a charge-coupled device (CCD). In the sample arm, cells cultivated on a flat dish filled with a culture medium are placed on a high precision moving stage. The reference mirror (RM) is sinusoidally oscillated with a piezo-electric transducer (PAZ020, Thorlabs, PZT) to get temporally phase-shifted interference signals. The time-varying interference signal is integrated by a CCD camera (CCD1020, 12 bit, 20 fps, Vosskühler) over a quarter of the PZT modulation period. The en-face image at a particular depth along the sample is extracted with the sequentially acquired four CCD images [18,20]. The 220 nm ultra-broadband spectrum of a halogen lamp gave a 0.8 µm axial resolution in water as the inset of Fig. 1 shows, and the details could be found in our previous works [21,22].

2.2 Principle and Procedures of RI measurement

The RI measurement of a transparent specimen can be made by measuring both the physical thickness and the optical thickness of the specimen in general. Assuming that an object is placed on, or actually adherent to, a plate having a flat surface, then the light field reflected from the bottom of the object or from the top surface of the plate has phase gain due to the RI of the object. Of course, the beam reflected from the top surface of the object has only the height information of the object. Therefore, the physical thickness profile of the object is simply obtained from the stack of FF-OCM en-face images [21] when the object is adherent to a flat surface. In experiment, a series of en-face images of the cell were taken with steps of 0.6 µm in z-axis using a high-precision linear stage.

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Fig. 1. Schematic of the FF-OCM system, BS: beam splitter; MO: microscope objective; NDF: neutral density filter; WG: window glass; AM: angled mirror; PZT: piezo-electric transducer; RM: reference mirror; L: lens; CCD: charge-coupled device.

When a cellular specimen is adherent to a dish filled with a culture medium, at a particular transverse position \((x, y)\), the phase gain difference between the beams reflected from the dish surface through the specimen and the culture medium only is expressed as

\[
\Delta \phi(x, y) = 2 \frac{\Delta \phi}{\lambda_0} (R_{\text{cell}}(x, y) - R_{\text{medium}}(x, y)) t(x, y),
\]

where, \(\lambda_0\) is the center wavelength in free space; \(R_{\text{cell}}\) and \(R_{\text{medium}}\) are the RIs of the cell and the surrounding medium, respectively; \(t(x, y)\) is the physical thickness of the cell at a position \((x, y)\) across the cell. The first 2 in equation is the round trip factor. In the FF-OCM imaging, when the \textit{en-face} image of the sample bottom, thus, the top surface of the dish, is taken, the obtained image is usually down-curved due to the RI difference between the sample and the medium [22]. Since the physical thickness of an adherent cell is tens of microns, when the RI difference is on the order of \(10^{-2}\), the optical thickness difference is merely on the order of a hundred nanometers. Thus, the single \textit{en-face} image taken at the top surface of the dish gives the RI distribution across the whole area of the cell. We note that the coherence gating length of the implemented system was 0.8 \(\mu\)m as shown with the inset of Fig. 1. Further, the RI distribution of the cell is then obtained from Eq. (1) with the measured phase gain difference and the physical thickness as,

\[
R_{\text{cell}}(x, y) = \frac{\lambda_0}{4\pi \Delta \phi(x, y)} + R_{\text{medium}}(x, y).
\]
Of course, the RI of the culture medium is measured before the cell measurement.

2.3 Measurement of phase sensitivity

The phase detection performance of the implemented system was estimated by measuring the thickness, thus the phase gain $\Delta \phi$, of a known sample. The height variation of the sample should be fine enough so that the whole structure could be captured with a single en-face image of the system. A USAF 1951 resolution target (NT58-198, positive, Edmund) was used because the metal bars were finely patterned on a flat surface with thickness of tens of nanometers. The target was imaged in distilled water, and only one en-face image was taken at the surface of the target plate. Figure 2(a) shows the extracted phase map of the target (group 6). From the phase map, the surface profile of the target was obtained and plotted in Fig. 2(b). The center wavelength of the light source was 650 nm, and 1.333 was used as the RI of the distilled water. A line image of the surface profile was taken along the dotted red line of Fig. 2(b) and compared with the one taken with an atomic force microscope (AFM, XE-200, 0.05 nm depth resolution, PSIA) as shown in Fig. 2(c). The mean value of the height was 84.45 nm for the FF-OCM, which was well matched with the one of 84.07 nm obtained with the AFM. And the root-mean-square (RMS) deviations were 1.54 nm for the FF-OCM and 1.13 nm for the AFM, respectively.

Further, to check the measurement stability, a series of measurements were made at a particular point of the target but at different times. Figure 2(d) shows the time-elapsed phase fluctuation (closed circles); it has 124 mrad RMS deviation, which corresponds to 4.8 nm thickness fluctuation (open circles) in water. Considering the fine flatness of the chrome printing of the resolution target and the high resolution of the AFM, we can say that the thickness measurement variation across a single en-face image of FF-OCM is fine enough for cell measurements. For getting a series of en-face images, however, due to the system and environment instability, we should think of appreciable phase fluctuation among images. For the cell RI measurement, considering a cell thickness of 10 µm (when we can assume a fixed thickness), the 124 mrad phase fluctuation induces $\delta n \approx 10^{-3}$ RI fluctuation. It is small enough because the RI differences between main cellular organelles are typically on the order of 0.01 [11,23,24]. However, for the cell measurement, we should think of the accuracy in the cell thickness measurements, which includes the accuracies of the depth scanning and the positioning of the cell bottom plane. A brief error analysis is given with the actual data obtained from a living cell at section 4.
2.4 Cell preparations

For cell culture, we took normal and cancer cells from the same origin of cell lines to exclude any RI variation influenced by different cell types. In this study, two groups of cell lines were used.

2.4.1 RK3E and RK3E-ras

The rat kidney epithelial cell line RK3E was obtained from American Type Culture Collection (CRL-1895) and a k-ras-transformed RK3E cell line (RK3E-ras) [25] was kindly provided by Dr. Eric Fearon (University of Michigan Medical School, USA). The cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 10 %, 5 % Fetal Bovine Serum (FBS) and 100 U/mL penicillin streptomycin (Gibco) at 37 °C with 5 % CO₂. The cells (2 × 10^4/ml) were seeded on a 60-mm plastic culture dish. A period of 48 h was allowed for adherence of cells onto the glass substrate before measurements.

2.4.2 Immortalized Normal Oral Keratinocyte (INOK) and YD-10B

Human immortalized normal oral keratinocyte (INOK) cells were kind gifts from Dr. E. C. Kim (Wonkwang University, Iksan, Korea) and were maintained in KGM medium (Invitrogen). Cells were cultured at 37 °C in a humidified atmosphere with 5 % CO₂ [26]. YD-10B cells, human oral squamous cell carcinoma (OSCC), were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were incubated in the RPMI medium containing 10 % FBS and 100 U/mL penicillin streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in atmosphere of 5 % CO₂ [27].
3. Experimental results

3.1 RI imaging of living single cells

A series of en-face images of the cell adherent to a plastic culture dish filled with a culture medium were consecutively captured with 0.6 µm depth steps. Each image was obtained by averaging 10 raw images, which took 2 seconds. For the cell viability and keeping the native state, the total measurement time of a single cell was kept less than 1 minute. The RI of the culture medium was measured to be ~ 1.336 with a conventional Abbe refractometer (DR-M4, ATAGO). Figure 3(a) shows the FF-OCM image of a single RK3E cell, taken at 2.4 µm below the apex of the cell. The image is in a logarithmic scale and coded with 4096 gray levels, which clearly reveals the cell surface (S) and the oval-shaped nucleus (N). From a stack of FF-OCM tomograms the height map of the cell was obtained as Fig. 3(b), and from the single en-face image taken at the surface of the dish the phase map of the cell was obtained as Fig. 3(c). From them the RI map of the cell was extracted as Fig. 3(d). The three maps are presented with pseudo color coding in a linear scale. Note that some parts of the cytoplasm (n = 1.350 ~ 1.405) had larger RI than the cytoplasm (n = 1.350 ~ 1.365). This is coincident to the claims recently reported [24,28]. In the figure, the RI values in most parts of the cell were less than 1.36 except some local regions. The mean RI was calculated to be 1.353 ± 0.008, which was commonly found in most normal cells [15,29,30].

The same measurements were repeated with a single RK3E-ras cancer cell. Figure 4(a) shows the en-face image taken at 6 µm below the apex of the cell. Many of small particles with high refractive indices were observed in the cytoplasm, which might be cytoplasmic components such as lipid droplets, lysosomes or circular organelles. From the measured physical thickness map (Fig. 4(b)) and the phase map (Fig. 4(c)), the RI map was extracted as Fig. 4(d). Large portions of the cell had RI values over 1.36 and the local parts with high RI of 1.417 were well matched with the locations of small particles in Fig. 4(a). The RI mean was 1.371 ± 0.014, much higher than the normal one of Fig. 3(d) and similar to those of other cancer cell studies [14,16,23].

In Eq. (2), the RI is calculated by dividing the phase map with the thickness map. Therefore, at the edge of the cell, and out of the cell boundary also, a small error can cause an extreme RI. To eliminate this singularity problem and to reduce the marginal background noise, the phase maps were truncated in Figs. 3 and 4.
Fig. 3. (a) En-face (XY) FF-OCM image (in log scale), (b) contour image of cell height profile, (c) phase image, and (d) quantitative RI map of a RK3E cell. In (a), white scale bar is 10 µm; S, cell surface; N, nucleus.

Fig. 4. (a) En-face (XY) FF-OCM image (in log scale), (b) contour image of cell height profile, (c) phase image, and (d) quantitative RI map of a RK3E-ras cell. White scale bar in (a) is 10 µm.
3.2 Quantitative comparison between normal and cancer cells

The RI distribution of a cell under imaging was analyzed pixel by pixel of the CCD. Only the CCD pixels giving RI values larger than the medium RI (1.336) were counted, however. The histograms of the RIs measured with the RK3E and the RK3E-ras cells are shown with Figs. 5(a) and 5(b), respectively. Interestingly, in the case of the RK3E-ras cell, the portion of pixels with RIs more than 1.36 was 77.8 %, whereas the RK3E case showed only 17.3 %. The RI increment of the cancer cell roughly corresponds to conventional assertion that the rapid cell division and proliferation activity of cancer cells lead to high concentration of protein molecules within cellular organelles and result in the increase of cell RI [16,31].

Fig. 5. Histograms of the RIs measured by every pixels of the CCD for (a) RK3E and (b) RK3E-ras cells.

For checking the reliability of the cell RI measurements, independent measurements for five different single cells for each cell line group have been performed. Table 1 shows that there exists clear difference between the RIs of the normal (RK3E, INOK) and the cancer cells (RK3E-ras, YD-10B). Especially, considering only the portions over 1.36 RI, more remarkable difference is quantitatively observed.

Table 1. List of RIs measured with normal and cancer cells for two cell line groups. For each cell type, five different single cells were measured independently.

| Cell line | Refractive index (RI) (mean ± standard deviation) | Portion of pixels (≥ 1.36 RI) (%) |
|-----------|-----------------------------------------------|----------------------------------|
| RK3E      |                                               |                                  |
| Cell 1    | 1.347 ± 0.006                                 | 5.1                             |
| Cell 2    | 1.348 ± 0.005                                 | 4.2                             |
| Cell 3    | 1.351 ± 0.008                                 | 9.5                             |
| Cell 4    | 1.358 ± 0.011                                 | 39.5                            |
| Cell 5    | 1.353 ± 0.008                                 | 17.3                            |
| RK3E-ras  |                                               |                                  |
| Cell 1    | 1.373 ± 0.016                                 | 80.1                            |
| Cell 2    | 1.366 ± 0.011                                 | 72.2                            |
| Cell 3    | 1.372 ± 0.018                                 | 70.2                            |
| Cell 4    | 1.378 ± 0.014                                 | 85.8                            |
| Cell 5    | 1.371 ± 0.014                                 | 77.8                            |
| INOK      |                                               |                                  |
| Cell 1    | 1.343 ± 0.007                                 | 0.7                             |
| Cell 2    | 1.348 ± 0.005                                 | 0.1                             |
| Cell 3    | 1.345 ± 0.006                                 | 0.1                             |
| Cell 4    | 1.351 ± 0.008                                 | 0.1                             |
| Cell 5    | 1.344 ± 0.009                                 | 9.9                             |
| YD-10B    |                                               |                                  |
| Cell 1    | 1.369 ± 0.010                                 | 75.2                            |
| Cell 2    | 1.371 ± 0.011                                 | 83.0                            |
| Cell 3    | 1.378 ± 0.010                                 | 95.8                            |
| Cell 4    | 1.377 ± 0.013                                 | 86.3                            |
| Cell 5    | 1.372 ± 0.011                                 | 90.6                            |
4. RI measurement error consideration

For measuring the cell thickness distribution, a series of FF-OCM images were taken with axial steps of 0.6 µm and using a 100 nm high-precision linear stage. The FF-OCM system itself, as the inset of Fig. 1 was shown, had a 0.8 µm axial resolution in water. Because of such limitations, the cell thickness measurement had a limited accuracy. Further, as was discussed with the experiment of Fig. 2, due to the system and environment instability, the phase error in successive measurements was 124 mrad.

For the cell height measurement, we need to locate the bottom and the top surfaces of the cell. For the bottom surface location, we locate the top surface of the culture dish, on which the cell is adherent. In general, if we could have a narrow enough scanning step, we could locate the center of the point spread function (PSF) of the system with the accuracy much finer than the width of the PSF. Even though the width of the system PSF was 0.8 µm and the scanning step was 0.6 µm, we could locate the center of the PSF with the accuracy of 1/5 of the scanning step, which gives 0.12 µm positioning error. The same thing happens with the top surface of the cell, each tomogram gives the heights in a ring shape. The data between the tomograms were obtained by interpolating and surface profile smoothing. Considering the positioning accuracy of the linear stage, 0.1 µm, therefore, we can say that the total accuracy of the thickness measurement was about 0.2 µm.

From Eq. (1), in general, we have the error in the relative RI, \( \delta RI = \delta (RI_{cell} - RI_{medium}) \), with the errors in the thickness measurement \( \delta t \) and the phase measurement \( \delta \phi \) as

\[
\frac{\delta RI}{RI} = \frac{\delta t}{t} + \frac{\delta \phi}{\Delta \phi}.
\]

For the RK3E-ras cell of Fig. 4, at the middle of the cell where the thickness was high, we have measured the mean thickness of \( t = 8.18 \mu m \) and the mean phase \( \Delta \phi = 6.28 \) radian. And the mean RI of the cell was 1.375. Considering the RI of the culture medium, 1.336, the mean RI difference becomes \( \Delta RI = 0.039 \). Substituting these values into Eq. (3), we have the accuracy in getting the RI difference as \( \delta RI = 1.72 \times 10^{-3} \). Of course if there is an error in the culture medium RI, the absolute cell RI is affected; however, the relative RI distribution is not affected. Further, Eq. (3) says that the accuracy becomes poor at the edge of the cell, where the thickness is small.

The perturbation in the phase-shifting piezo/reference mirror causes another error. Even though we used a high precision closed-loop feedback-controlled PZT (PAZ020, 5 nm resolution, Thorlabs), we think that some of the time-elapsed phase measurements error was caused by this. In general, the error in the phase of the reference mirror of a FF-OCM system makes it difficult to extract the envelope function from interference fringes [21,22]. By using a reference laser interferometer, of course, we can reduce the phase measurement error dramatically [32].

5. Discussion and Conclusions

The identification of live cancer cells by using refractive index (RI) contrast imaging has been demonstrated with ultrahigh resolution full-field optical coherence microscopy (FF-OCM). The proposed scheme gives the axially averaged RI map of a viable cell by measuring the physical thickness and the round trip phase gain simultaneously. This FF-OCM based methodology enables to locally monitor the physiological reaction of regional cellular components and get high through-put cytofractometry. The quantitative RI measurements have been achieved for adherent normal and cancer cells. The experiment proved that cancer cells were associated with higher percentage of large RI distribution. As a result, we can say that the RI assessment of a cell might be a key indicator to efficiently discriminate the cell malignancy. Despite somewhat limited analysis for two cell line groups, we showed good reproducibility of the measurements and confirmed the validation of the proposed scheme. We can also conclude that the RI measurement scheme can be extended to detection of
precancerous and preinvasive cancer changes as well. We expect that its potential applications will provide quantitative and objective assessments of cancer cells in cell physiology and pathology, and further improve the efficiency of biomedical diagnosis in cancer biology. However, to comment on the detail cause of the RI change especially in terms of the cell components, we need to improve the accuracy of the system, which is under preparation.

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