Study of dynamic process of acetic acid induced-whitening in epithelial tissues at cellular level

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Abstract: Acetic acid, inducing transient whitening (acetowhitening) when applied to epithelial tissues, is a commonly used contrast agent for detecting early cervical cancer. The goals of this research are to investigate the temporal characteristics of acetowhitening process in cervical epithelial tissue at cellular level and develop a clear understanding of the diagnostic information carried in the acetowhitening signal. A system measuring time-resolved reflectance was built to study the rising and decay processes of acetowhitening signal from the monolayered cell cultures of normal and cancerous cervical squamous cells. It is found that the dynamic processes of acetowhitening in normal and cancerous cells are significantly different. The results of this study provide insight valuable to further understand the acetowhitening process in epithelial cells and to encourage the development of an objective procedure to detect the early cervical cancers based on quantitative monitoring of the dynamic process of acetowhitening.

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OCIS codes: (290.1350) Backscattering; (120.5820) Scattering measurements; (160.4760) Optical properties; (170.4580) Optical diagnostics for medicine

References and Links

1. T. C. Wright, R. J. Kurman, and A. Ferenczy, "Cervical Intraepithelial Neoplasia," in *Blaustein A. Pathology of the Female Genital Tract* (Springer-Verlag, New York, 1994).
2. U.S. Preventive Services Task Force, *Guide to clinical preventive services: report of the U.S. Preventive Services Task Force* (Williams & Wilkins, 1996).
3. M. C. Anderson, J. A. Jordan, A. R. Morse, and F. Sharp, *A Text and Atlas of Integrated Colposcopy: for Colposcopists, Histopathologists and Cytologists* (Chapman & Hall Medical, 1992).
4. M. F. Mitchell, "The Accuracy of Colposcopy," Clinical Consultations in Obstetrics and Gynecology **6**, 70-73, (1994).
5. M. Ronne, "Chromosome preparation and high resolution banding techniques. A review," J. Dairy Sci. **72**, 1363-1377, (1989).
6. S. Yamada, D. Wirtz, and P. A. Coulombe, "Pairwise Assembly Determines the Intrinsic Potential for Self-Organization and Mechanical Properties of Keratin Filaments," Mol. Biol. Cell **13**, 382-391, (2002).
7. R. Lambert, J. F. Rey, R. Sankaranarayanan, "Magnification and Chromoscopy with the Acetic Acid Test," Endoscopy **35**, 437-445, (2003).
8. C. Smithpeter, A. Dunn, R. Drezek, T. Collier, and R. Richards-Kortum, "Near Real Time Confocal Microscopy of Cultured Amelanotic Cells: Sources of Signal, Contrast Agents, and Limits of Contrast," J. Biomed. Opt. **3**, 429–436 (1998).
9. B. W. Pogue, H. B. Kaufman, A. Zelenchuk, W. Harper, G. C. Burke, E. E. Burke, D. M. Harper, "Analysis of Acetic Acid-Induced Whitening of High-Grade Squamous Intraepithelial Lesions," J. Biomed. Opt. **6**, 397-403, (2001).
10. C. Balas, "A Novel Optical Imaging Method for the Early Detection, Quantitative Grading, and Mapping of Cancerous and Precancerous Lesions of Cervix," IEEE Trans. Biomed. Eng. 48, 96-104, (2001).
11. R. S. Cotran, V. Kumar, and T. Collins, *Robbins Pathologic Basis of Disease* (W. B. Saunders, 1999).
12. M. A. Stanley, "Culture of Human Cervical Epithelial Cells", in *Culture of Epithelial Cells*, R. I. Freshney and M. G. Freshney, eds. (Wiley-Liss, 2002).

1. Introduction

Cervical cancer is the second most common cancer in women [1]. Nearly 500,000 new cases of cervical cancer are diagnosed each year worldwide. If the cervical cancer is detected early, the morbidity and mortality associated with the disease can be significantly reduced. The screening tests for early detection of the neoplastic growth at cervix include Pap smear and colposcopy [2]. After an abnormal Pap smear, the colposcopy is applied. An important diagnostic tool in colposcopy is the acetic acid technique that involves applying 3-5% acetic acid to the cervix to induce the acetowhitening effect and create the contrast between precancerous lesions and normal tissues. In general, abnormal areas appear white, while normal areas do not change in appearance. The acetowhitening effect is temporary and reversible [3].

The detection of early lesion through colposcopy is based on a few subjective criteria such as the rapidity of the acetowhitening processes and the length of its duration, the degree of acetowhiteness when the change of color reaches maximum, and the sharpness of the demarcation line between the precancerous lesions and normal tissues. In expert hands, colposcopic diagnosis is reported to have a high sensitivity (80-90%), at the expense of a significantly low specificity (50-60%) when differentiating abnormal tissues from normal tissues [4]. The diagnostic accuracy is highly dependent on physician's experiences and varies from individual-to-individual physician because colposcopy is a subjective procedure. Therefore, an objective approach based on quantitative measurement of the acetowhitening process is desirable to improve the performance of colposcopy in detecting early cervical lesions.

Although colposcopy has become a routine diagnostic procedure since it was first introduced by Hinselmann in 1925, the mechanism of acetowhitening is still not fully understood. The hypotheses of physical origin of the acetowhitening effect include coagulation of the nucleoprotein and polymerization of cytokeratins by adding acetic acid solution [3, 5-7]. These alterations increase refractive index mismatching between the nucleus and cytoplasm and the fluctuations in the refractive index in the nuclei and cytoplasm. Therefore, more light are scattered backwards. In a study of the acetic acid induced effects in the cell suspensions, the confocal and phase-contrast images were taken before and after the application of acetic acid. A dramatic increase of signal in the nuclei and the intracellular contents was found after applying acetic acid [8]. The results provide evidence supporting the hypotheses.

Recently, quantitative monitoring of the acetowhitening process in cervical tissue was investigated by two groups [9, 10]. It was found that the intensity of backscattered light from the normal cervical tissue was relatively stable before and after the application of acetic acid solution. On the contrary, the backscattered light from high-grade early lesions (CIN II/III) increased quickly after applying acetic acid solution and then decreased gradually over a few minutes. These studies indicated that quantitative measurement of acetowhitening process could make the colposcopy procedure objective and potentially improve its performance.

In this work, we build a time-resolved reflectance measurement system to study the temporal characteristics of acetowhitening effect at cellular level. The dynamic processes of acetowhitening in normal and cancerous cervical squamous cells are investigated quantitatively. Our goals are to develop further understanding of acetowhitening phenomena and provide an insight of the dynamic process of acetowhitening for tissue diagnosis.

2. Materials and methods

2.1 Cell sample preparation

Squamous cell carcinoma, which develops from the squamous cells in epithelium of the ectocervix, compromises about 75%-90% of all cervical cancers [11]. The second most common cervical cancer is adenocarcinoma, which presumably arises in the endocervical gland. Ten percent to 25% of cervical carcinomas constitute adenocarcinomas, adenosquamous carcinomas, undifferentiated carcinomas, or other rare histologic types [11].

Thus, our study was focused on the dynamic processes of acetowhitening in normal and cancerous cervical squamous cells because squamous cell carcinoma is the most common cervical cancer.

To monitor the temporal change of acetowhitening signal under a certain condition, the position of cells must be fixed over the time period of measurement and the concentration of acetic acid must be well controlled. Therefore, the monolayered cells cultured on standard tissue culture dishes, instead of cell suspensions, were used as samples in the experiments. Cancer cell samples were cultured from the commercially available human cervical squamous cancer cell line, and normal cell samples are made with the primary cell culture from the normal human ectocervical tissue specimen.

Human cervical squamous carcinoma cell line (SiHa) was obtained from American Type Cell Collection (ATCC, VA). The SiHa cells were first cultured to confluent and then subcultured for later use. All subsequent passages were performed at a split ratio of 1:4. Experiments were conducted when the cells reached confluent in a 35mm tissue culture dish.

Primary normal human ectocervical keratinocytes, the major cell components of a stratified squamous epithelium [12], were derived from the normal ectocervical tissue specimen obtained from the hysterectomy surgery. The standard trypsinization procedures were used to isolate the keratinocytes. Briefly, a fresh ectocervical specimen was transferred into a 25.0 caseinolytic units per ml solution of dispase (GIBCO) and incubated over 18 hours at 2 to 8°C. After incubation in dispase, the epithelial layer was separated from the specimen and placed into a solution of 0.05% Trypsin-0.53mM EDTA (GIBCO). It was then incubated at 37°C for approximately 15 minutes with pipette up-and-down for every 2-3 minutes to aid in cell dissociation. Following incubation, trypsin activity was stopped by adding 10mL Soybean Trypsin Inhibitor (GIBCO) solution. The cells were washed and seeded at cell density of $1-3 \times 10^4/cm^2$ in a T75 flask with 15ml culture medium. A monolayered normal cell culture was used in the experiment when the keratinocytes reached confluent in a tissue culture dish of 35 mm diameter. This work has been approved by the Ethical Committee of Prince of Wales Hospital, the Chinese University of Hong Kong. Consent letters were obtained from all the patients enrolled in this study.

2.2 Experiments

The schematic of the system to record the time-resolved reflectance from a cell culture sample is shown in Fig. 1. The sample was placed at the sample port of an integrating sphere of 6 inches in diameter (IS-060, Labsphere, Inc.). The illumination sources were three lasers with wavelengths at 457nm, 532nm, and 670nm, respectively. They produced a RGB illumination on the sample. An optic fiber system was used to conduct the lasers to the integrating sphere and generate a collimated illumination of 6 mm in diameter on the sample. The powers of the three lasers on the sample were precisely controlled at 20 mW. The total reflectance signals from the sample were measured using a color detector (RGB 3 channels, MCS3AT, Laser Components). The signals were pre-processed with an amplifier and a lowpass filter, and digitized with a computer controlled data acquisition system. The responses of RGB channels were calibrated with a power meter (Dual-Channel Power Meter, 2832-C, Newport Corp.).

In the study of the dynamic process of acetowhitening effect at a certain acetic acid concentration of interest, a pair of cell cultures growing under the identical conditions was used for time-resolved measurement of total reflectance and standard phase-contrast microscopy, respectively. To measure the acetowhitening signals from a sample in rising course, the culture medium was completely removed and the culture medium with acetic acid was quickly added into the sample. The total reflectance signals were recorded over the period of 5 minutes immediately after adding the medium with acetic acid. The medium with acetic acid was removed completely after recording the acetowhitening signal in rising course and the fresh medium was added into the sample for the measurement of acetowhitening signals in decay course. The acetowhitening signal was calculated by subtracting the recorded time-resolved reflectance in rising and decay courses from the baseline, the reflectance signal from the sample before applying medium with acetic acid. To obtain additional information on the

dynamic process of acetowhitening, the phase-contrast images were taken from the second sample. The procedure to change the medium for taking phase-contrast images in rising and decay courses was the same as that in the measurement of acetowhitening signals in rising and decay courses. It should be noted that the culture medium was used in this experiment instead of PBS solution because the culture medium could prevent the detachment of the cells from culture dish after adding acetic acid.
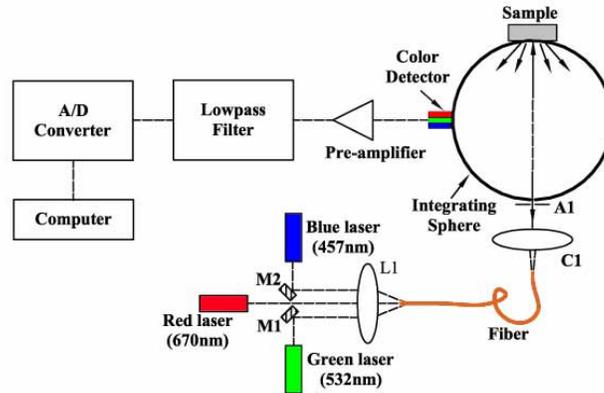

Fig. 1. Schematic of time-resolved reflectance measurement system. L1: Focus Lens (f = 35mm); C1: Fiber Optic Collimator; A1: Aperture Stop; M1, M2: Mirrors.

3. Results and discussions

Typical acetowhitening signals and phase-contrast images recorded in rising and decay courses are shown in Fig. 2. The movies in the figure display the results with applying the medium with 0.3% and 1.2% acetic acid, respectively. The acetowhitening signals shown in the figure were recorded in red channel. As can be seen, there is a sharp increase of acetowhitening signal in rising course, the first 300 seconds after applying the medium with acetic acid. A decrease of acetowhitening signal is observed in the decay course, the time period after removing the medium with acetic acid and adding fresh culture medium. The rising rate of the acetowhitening signal is proportional to the acetic acid concentration. On the contrary, the decay rate decreases with the increasing of acetic acid level.

The phase-contrast images in the movies were taken under the same condition as the measurements of acetowhitening signals. A phase-contrast image provides the variation of optical pathlength across the imaged object. Therefore, the intensity changes of phase-contrast signals in rising and decay course represent the change of refractive index induced by acetic acid. The movies of phase-contrast imaging in Fig. 2 show clearly that the major change of refractive index in rising and decay courses always occurs within the nuclei and small changes can be observed in the cytoplasm. This is consistent with the results obtained from the measurements in cell suspensions [8] and supporting the theory that the nuclear proteins are altered.

The results displayed in Fig. 2(a) show that the acetowhitening signal induced by the 0.3% acetic acid could return back to the baseline, the reflectance signal level before the cells were exposed to acetic acid. The phase-contrast images in the decay course also confirm that the original form of cells could be recovered after the medium with acetic acid were replaced with the fresh medium. However, it was found that the acetowhitening signal could not return back to the baseline after applying the medium with acetic acid level higher than 1.2%. The phase-contrast images also show that the original form of the cells could not be recovered after the cells were exposed to the medium with high acetic acid level. The results in Fig. 2(b) indicate that the acetowhitening effect is no longer reversible when cells are exposed to acetic acid of high concentration.
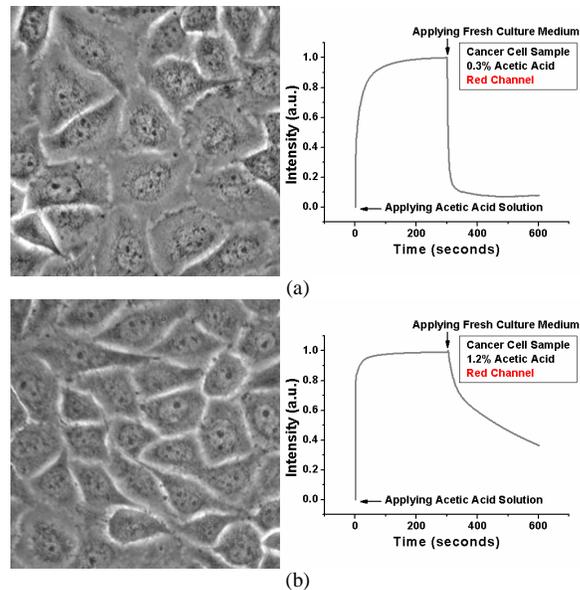
Fig. 2. Acetowhitening signals (right) and phase-contrast images (left) recorded from cancer cell samples in rising and decay courses. (a) (1.228MB) Concentration of acetic acid in medium: 0.3%; (b) (962KB) Concentration of acetic acid in medium: 1.2%.

Typical reflectance signals recorded from RGB channels are shown in Fig. 3(a) to demonstrate the wavelength-dependence of acetowhitening signal. The signals were recorded from a cancer cell sample with adding the medium of 0.6% acetic acid. As can be seen, though RGB signals follow the similar courses after the application and clearance of the medium with acetic acid, the intensity of acetowhitening signal is dependent on the read-out wavelength. In the measurements on all the cancer and normal samples with adding the acetic acid of concentration up to 5%, the recommended concentration in colposcopy examination, the magnitude of blue channel signal was always found to be the highest and the red channel signal was always the lowest.

The comparisons of RGB acetowhitening signals recorded in rising and decay courses are displayed in Fig. 3(b)(c). The signal recorded in red channel was used as a reference. The signals of green and blue channels versus the signal of red channel shown in the figure demonstrate excellent linear relationship among the RGB signals in both rising and decay courses. In addition, the linear relationship between RGB acetowhitening signals were generally found in both cancer and normal cell samples after applying the medium with 0.2% to 5% acetic acid. This shows that the monitoring of dynamic processes of acetowhitening effect is not sensitive to the read-out wavelength. Therefore, following investigation on the dynamic processes of acetowhitening is based on the analyses of signals measured from red channel.

In the study of the dependence of acetowhitening effect on the acetic acid concentration, the acetowhitening signals were measured from the cancer and normal cell samples with applying the medium of acetic acid concentration in the range from 0.1% to 5%. The movie in Fig. 4(a) displays the acetowhitening signal in rising and decay courses as a function of acetic acid concentration. The results show that the acetowhitening effect and its dynamic process are strongly dependent on acetic acid concentration.

The magnitude of acetowhitening signal as a function of concentration of acetic acid is shown in Fig. 4(b). When acetic acid concentration is lower than 0.2%, the peak magnitude of the acetowhitening signal is small and almost negligible. The signal increases tremendously with a little increase of acetic acid concentration to 0.25%, suggesting a threshold effect in the acetic acid induced cellular changes. An important finding is that the magnitude of

acetowhitening signal reaches to maximum when the acetic acid concentration is about 0.6%, a factor of 5~10 lower than that commonly used in clinical practice. The magnitude of acetowhitening signal starts to decrease gradually with further increasing of acetic acid concentration.
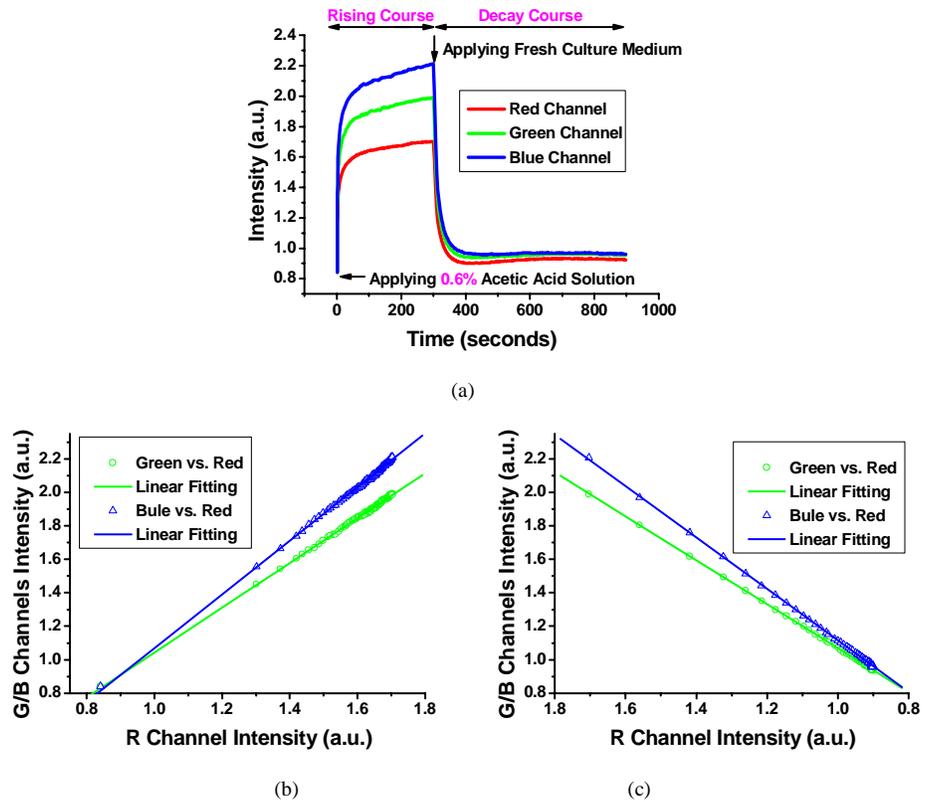

(a)

(b)   (c)

Fig. 3. Wavelength-dependence of acetowhitening signal. (a) RGB reflectance signals recorded from a cancer cell sample with the application of 0.6% acetic acid solution; (b) Comparison of RGB signals in the rising course; (c) Comparison of signals in the decay course.

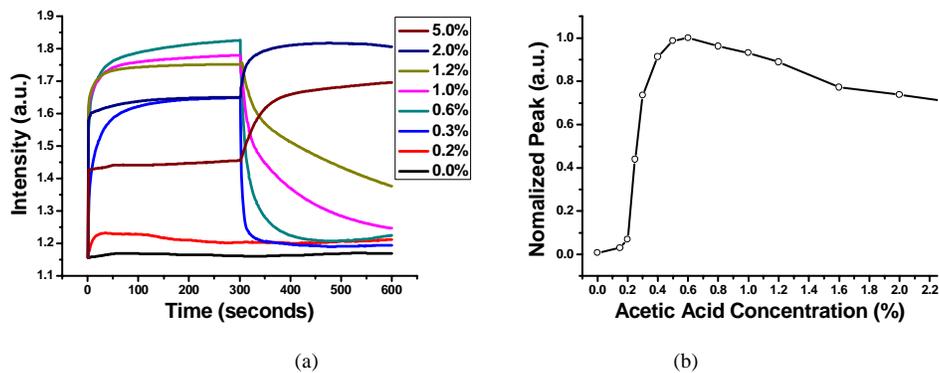(a)   (b)

Fig. 4. Acetowhitening signals of cancer cell samples at different acetic acid concentrations. (a) (203KB) Movie of the acetowhitening signals as a function of acetic acid concentration; (b) The peak value of acetowhitening signals as a function of acetic acid concentration.

It was found that the decay of acetowhitening signal with applying the medium of high acetic acid concentration (>2%) presented different course from that with applying the medium of low acetic acid concentration (<1.2%). As shown in Fig. 4(a), instead of monotonically decaying the signal recorded at high acetic acid level increases in the beginning of decay course and then decreases slowly. The increase of the signal may be caused by the dilution of acetic acid in cells in the beginning of decay course because the maximal magnitude of the signal appears at 0.6% acetic acid concentration. Moreover, it was found that the acetowhitening signal could not completely decay back to the baseline when the acetic acid concentration was at 1.2%, showing that the acetic acid induced cellular changes were no longer reversible and a damage of cell became permanent at high acetic acid level.
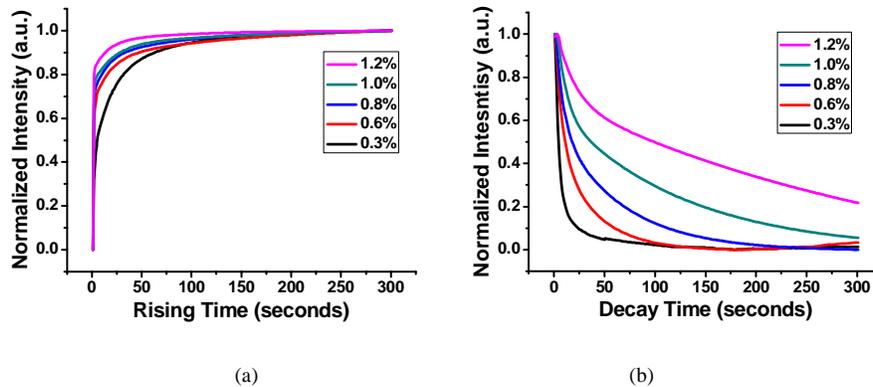

(a)   (b)

Fig. 5. Comparison of the acetowhitening signals of cancer cell samples when applying the medium with different acetic acid concentrations. (a) Normalized signals in the rising courses; (b) Normalized signals in the decay courses.

The normalized acetowhitening signals that present the rising and decay rates of the signal as a function of acetic acid concentration are displayed in Fig. 5. The signals recorded from the measurements with applying the medium of acetic acid concentration over 1.2% are not analyzed because the acetowhitening is no longer a reversible process. In the rising course, the signal increases rapidly after adding the acetic acid solution. The difference in rising rate becomes small when the acetic acid concentration is over 0.6%. On the contrary, the difference in decay rate between the signals recorded at different acetic acid levels is significant. The decay courses with the acetic acid concentration in the range from 0.3% to 1.2% can be clearly differentiated. The acetowhitening signal at low acetic acid level decays much more quickly than that at higher acetic acid level.

The dynamic process of acetowhitening effect in normal cells shows the characteristics similar to those in cancer cells. The representative results are displayed in Fig. 6. The acetowhitening signals measured from the normal cell samples follow the rising and decay courses similar to those measured from cancer cell samples. The threshold effect was observed at the acetic acid concentration 0.2%, the same as cancer cells. The maximal acetowhitening signal appeared at 0.5% acetic acid concentration. The decay course became no longer monotonic when concentration of the acetic acid is over 1% as shown in Fig. 6(a). The signal can not completely decay back to baseline when acetic acid concentration is over 1.6%, indicating that the threshold of permanent damage induced by acetic acid in normal cell is higher than that in cancer cell.

In principle, the differences in rising and decay courses of the acetowhitening signals can be used as quantitative indicators to differentiate cancer cells from normal cells. However, the rising course is generally very rapid and difficult to measure accurately. In this study, we focus on analyses of decay courses of acetowhitening signals recorded from normal and cancer samples in the range of acetic acid concentration 0.2% - 1%, where the acetowhitening process is completely reversible in both normal and cancer cells.

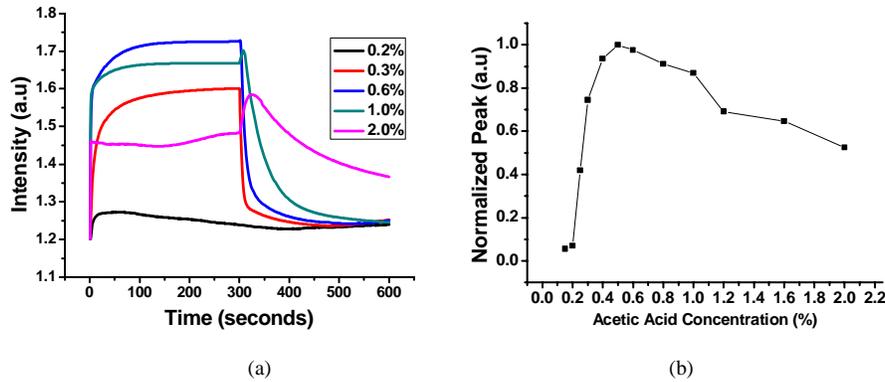(a)   (b)

Fig. 6. Acetowhitening signals of normal cell samples at different acetic acid concentrations. (a) The acetowhitening signals as a function of acetic acid concentration; (b) The peak value of acetowhitening signals as a function of acetic acid concentration.

A single exponential model was used to fit the acetowhitening signals in decay courses. The model consists of an exponential term to quantify the decay process of acetowhitening effect and a constant term to present the baseline. The decay time constants calculated from measured acetowhitening signals are displayed in Table 1. Each mean time constant and standard deviation were obtained from the measurements of three samples. As can be seen, the decay time constants measured from cancer cells are not significantly different from normal cells when the acetic acid concentration is lower than 0.4%. However, the decay time constant of normal cells becomes significantly shorter than that of cancer cells at the acetic acid level over 0.6%, indicating the existence of optimal working window for colposcopy procedure to maximize the contrast between normal and cancer cells without causing cell damage.

Table 1. The decay time constants for normal and cancer cell samples calculated from a single exponential model.

Acetic acid Concentration	Time Constant (sec.)	
	Normal Cell	Cancer Cell
0.3%	4.78±1.12	5.07±0.91
0.4%	4.90±1.95	6.54±0.13
0.6%	6.09±0.95	17.09±2.16
0.8%	25.46±3.32	47.49±9.50
1.0%	41.19±0.02	94.51±5.09

It has been noted that the accuracy in fitting the decay of acetowhitening signal with single exponential model is not satisfactory, especially the experimental data measured from the samples with applying acetic acid of concentration higher than 0.6%. The coefficient of determination, r^2, for the fitting with single exponential model was in the range from 0.96 to 0.98. To identify the source of error and improve the fitting quality, a dual-exponential model was used to fit the decay of acetowhitening signal. The model consists of three terms to present fast decay process, slow decay process and baseline, respectively. The representative fitting results are displayed in Fig. 7 for a comparison of single exponential and dual-exponential models in the analyses of decay process of acetowhitening effect. In general, it was found that the dual-exponential model appears to fit the experimental data measured from both normal and cancer cell samples very accurately. The r^2-value of the fittings was over 0.996. This demonstrates that the decay processes of acetowhitening effect in normal and cancer cells may be determined by two different mechanisms.
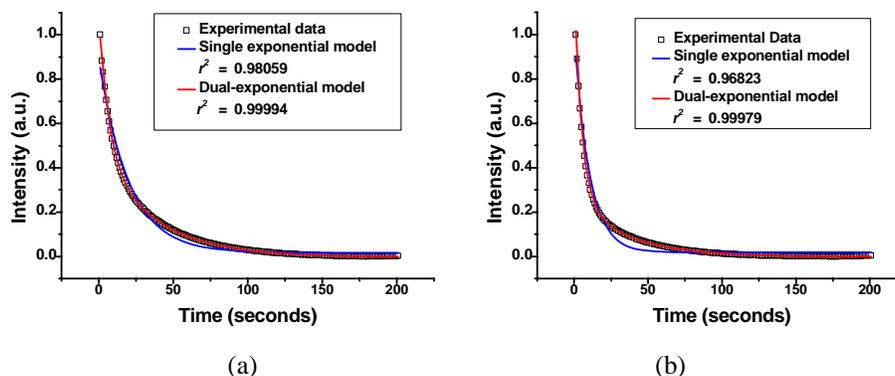
Fig. 7 Comparison of single and dual-exponential models in fitting the decay of acetowhitening signals. (a) Cancer cell sample, 0.6% acetic acid; (b) Normal cell sample, 0.6% acetic acid.

Table 2. Analyses of decay process of acetowhitening effect in normal and cancer cells using dual-exponential model.

Acetic acid Concentration	Normal Cell			Cancer Cell		
	τ_1 (sec.)	τ_2 (sec.)	a_1/a_2	τ_1 (sec.)	τ_2 (sec.)	a_1/a_2
0.3%	2.44 ±0.80	22.88 ±3.78	5.56 ±2.04	3.39 ±0.49	18.46 ±5.23	4.95 ±1.44
0.4%	3.61 ±0.33	30.80 ±2.18	5.31 ±1.58	5.21 ±1.52	22.24 ±3.93	4.39 ±1.16
0.6%	6.89 ±0.56	51.92 ±6.13	5.14 ±0.27	6.35 ±0.94	28.77 ±8.74	1.08 ±0.22
0.8%	16.01 ±1.99	88.03 ±4.52	4.48 ±0.26	7.50 ±0.90	66.76 ±2.20	0.83 ±0.16
1.0%	34.69 ±2.39	152.32 ±5.99	5.87 ±0.65	10.55 ±1.47	126.97 ±3.82	0.66 ±0.07

The calculated time constants and the relative contributions of two exponential terms to the decay signal are listed in Table 2. τ_1 and τ_2 are the time constants describing fast and slow decay processes, respectively. a_1 and a_2 are the amplitudes of two exponential terms, respectively. The a_1/a_2–value provides the quantitative description of the relative weight of fast and slow decay processes. It was found that time constants of fast and slow decay processes increase with increasing of acetic acid concentration in normal and cancer cells. The decay process of acetowhitening effect in normal cells is always dominated by the fast decay term because the a_1/a_2–values remain high (4.5 ~ 5.9) in the range of acetic acid concentration 0.3% - 1%. The decay process of acetowhitening effect in cancer cells is dominated by the fast decay term at low acetic acid concentration. However, the weights of fast and slow decay processes reach to even when acetic acid concentration is 0.6%. With further increasing of acetic acid level, the slow decay process becomes dominant. Overall, there is not significant difference in decay time constants and a_1/a_2–value between normal and cancer cells at low acetic acid level. However, the difference in a_1/a_2–value between normal and cancer cells becomes significant when acetic acid concentration is over 0.6%, which suggests that a_1/a_2–value could be an effective indicator for the differentiation of the normal cells from cancer cells.

The possible physical origins of acetowhitening effect are the coagulation of nucleoprotein and polymerization of cytokeratins in cytoplasm caused by the increase of H^+ concentration in cell [7]. To investigate the dependence of acetowhitening effect on H^+ concentration, the pH-values of all the acetic acid medium solutions used in the experiments

were measured with a standard pH-meter (Φ340 pH / Temp Meter, Beckman Coulter, Inc.). The measured pH-value and corresponding H^+ concentration as a function of acetic acid concentration in the medium are shown in Fig. 8. A nonlinear relationship between H^+ concentration and acetic acid concentration was found in the acetic acid concentration lower than 0.2%. It may be due to the buffer capacity of culture medium that neutralizes the H^+. However, the H^+ concentration starts to increase linearly with further increase of acetic acid concentration. The results indicate that the observed threshold-like effect of acetic acid induced changes in cell might be caused by the buffer capacity of culture medium.
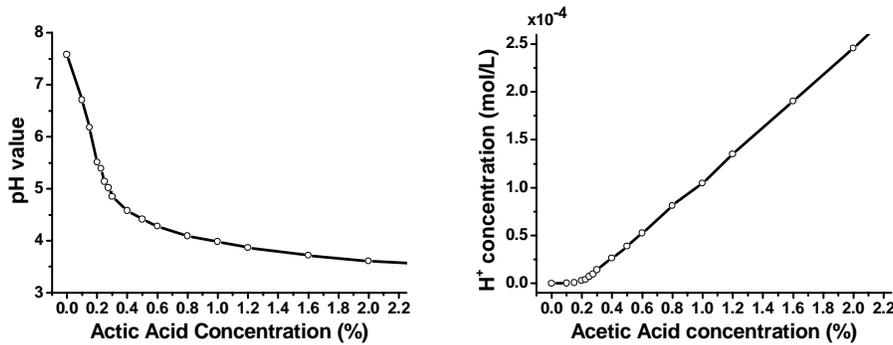

Fig. 8. (a) pH value and (b) H^+ concentration (mol/L) of the acetic acid solution as a function of the concentration of acetic acid at room temperature 25°C.

The acetowhitening effect has been observed in normal and cancer cell cultures, which is consistent with the results obtained in the cell suspensions [8]. However, several key observations in this study do not agree with what is observed in the measurements on tissue *in vivo*. Clinically, the colposcopically normal cervical tissue generally does not change its appearance though the abnormal cervical tissue appears white after applying 3~5% acetic acid, the concentration a few times higher than the possible cell damage threshold (~1%). It is well known that low concentration acetic acid does not work as well as higher concentration acetic acid *in vivo*. The acetic acid induced whitening would be very little at 1%, and this is likely due to the fact that *in vivo* there is a penetration problem, which also dilutes the acetic acid concentration to much lower levels when the acetic acid diffuses down to several cell layers into the epithelium. In addition, the rising and decay processes of acetowhitening effect observed from abnormal cervical tissue are significantly slower than cancer cell samples measured in this work [9, 10]. These may be caused by the difference in the application and clearance of acetic acid between cell culture and tissue. The processes of application and clearance of acetic acid in cell cultures are almost a step-function of time, while the delivery and clearance of acetic acid in tissue are determined by the diffusion of acetic acid in tissue, which is generally slower. The diffusion is the major factor to slow down the rising and decay processes of acetowhitening in tissue. It remains unclear whether the differences in diffusion and penetration of acetic acid between the normal and abnormal cervical epithelial tissues contribute to the contrast for the diagnosis of acetowhitening technique.

4. Conclusions

We investigated the dynamic process of acetowhitening effect in normal and cancerous cervical squamous epithelial cells. The measured acetowhitening signal, corresponding to the changes in cells induced by acetic acid, is strongly dependent on the applied acetic acid concentration. The acetowhitening signals of normal and cancer cells increase rapidly with increasing of acetic acid concentration and reach to the peak when the concentration is at 0.6% for cancer cells and 0.5% for normal cells, respectively, a factor of 5~10 lower than the

acetic acid concentration in the solution used in standard colposcopy procedure. The signal intensity starts to decrease gradually with further increasing of acetic acid. An important finding shows that the acetowhitening signal can not decay back to the baseline when acetic acid level is higher than 1.2% for cancer cells and 1.6% for normal cells, respectively, showing the possible cell damage because the acetic acid induced cellular changes are no longer reversible. The threshold of cell damage by acetic acid is a factor of 2~4 lower than the acetic acid concentration in the solution applied to cervix during the clinical examination. The results provide the evidence that the exact concentration of acetic acid in colposcopically examined tissue should be much lower than that in the acetic acid solution applied to the tissue surface. The acetic acid may be significantly diluted when it penetrates into the tissue.

The study of the wavelength-dependence of the acetowhitening signal shows that the signal intensity is inversely proportional to the read-out wavelength, while its temporal characteristic in rising and decay courses is not sensitive to the wavelength. This means that the quantitative monitoring of the dynamic process of acetowhitening effect can be based on the measurement at single wavelength. The quantitative analyses of the dynamics of acetowhitening effect show that the differences in decay course between the normal and cancer cells are significant. The differences can be used as quantitative indicators to differentiate the normal cells from cancer cells. Moreover, it was found that the decay of acetowhitening signal could be precisely described with dual-exponential function. This demonstrates that the dynamics of acetowhitening process in decay course may be determined by two mechanisms. Finally, the acetowhitening signals were observed in both normal and cancer cell samples, which is not consistent with the clinical observation of colposcopy. It may be due to the difference in the diffusion of acetic acid between normal and precancerous epithelial tissues. In addition, the ectocervical keratinocytes, used as the normal cells in this study, were extracted from the lower layer of normal squamous epithelium. Though they are the major cell components of a stratified squamous epithelium, their responses to acetic acid may not be exactly the same as the normal epithelial cells in the upper layers of normal squamous epithelium.

In future work, a light scattering spectroscopy method will be used to study the changes of scatterer size and refractive index distribution in cells in the rising and decay courses. The diffusion of acetic acid in epithelial tissue will be investigated. These studies will provide more detailed information and understanding on the acetowhitening processes in epithelial tissues.

Acknowledgments