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Towards nonspecific detection of malignant cervical cells with fluorescent silica beads**

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Abstract

To date, the methods of detection of cancer cells have been mostly based on traditional techniques used in biology, such as visual identification of malignant changes, cell growth analysis, specific ligand-receptor labeling, or genetic tests. Despite being well developed, these methods are either insufficiently accurate or require a lengthy complicated analysis. A search for alternative methods for the detection of cancer cells may be a fruitful approach. Here we proposed a novel method for detection of cancer cells in vitro, which is based on non-specific adhesion of silica beads to cells. First, we use atomic force microscopy (AFM) to study adhesion of single silica beads to malignant and normal cells cultured from human cervix. We found that adhesion depends on the time of contact, and can be statistically different for malignant and normal cells. Using these data, we develop an optical method utilizing fluorescent silica beads. The method is based on the detection of difference in the number of adherent particles. The method has been tested using primary cells cultured from cervical tissues of three healthy individuals and three patients with cervical cancer. The method shows sufficiently high sensitivity for cancer to make it interesting to perform further statistical tests.

Keywords

cell-silica interaction; cervical cancer cells; atomic force microscopy; non-specific tagging

1. Introduction

The challenges for scientists working in the area of biomedical technology are often multidisciplinary in nature. Recent advances in this field are a result of inter-disciplinary research involving physics, chemistry, molecular biology, engineering and medicine. [1] Research efforts over the years have resulted in the development of DNA chips, [2,3] miniaturized biosensors [4–6] and bioMEMS. [7,8] These smart microsystems have found applications in gene expression profiling, drug delivery and clinical diagnostics. In

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particular, the development of highly sensitive probes for detection of cancer has attracted considerable attention in biology and medical research fields.

Cervical cancer is the second leading cancer in women worldwide and infection with high risk human papillomavirus (HPV) is the most significant risk factor in its etiology. [9] HPV causes a common sexually transmitted infection among both women and men. The objective of screening for cervical cancer is to prevent persistent HPV infection and death by detecting and treating high-grade squamous intraepithelial lesions, which are precursor lesions for invasive cancer. A simple and effective screening method is of prime and utmost importance especially in many developing countries where cervical cancer rates are particularly high. In the United States, an estimated 12,900 cases of cervical cancer and 4,400 deaths occur annually.

The Papanicolaou (Pap) smear test has proven to one of the most successful methods of cancer detection over the years [10]. Although the Pap test is the most widely used cancer screening method in the world and its impact in the incidence of cervical cancer is well known from a historical perspective, recent reports suggest that the sensitivity of Pap smear is 50–60%, with the relative proportion of sampling to screening errors being 2:1. [11] The tests may be further complicated by high unsatisfactory rates, preparation artifacts and unnecessary cost interventions. Each year in the United States alone approximately 3.5 million Pap smears are classified equivocal, out of which 75% of women do not have cancer. The economic constraints in developing countries have prompted alternative methods of screening cancer including visual inspection after application of 3–5% of acetic acid [12] and Lugol's iodine. [13] The major disadvantage of these tests is low specificity. Given the considerable variation in the way these tests are applied and interpreted in different settings, there is no standard universally accepted definition of the test results. It remains to be seen if the specificity can be improved by further developments in test definitions and training strategies.

Demands for the development of a universal testing and screening method have generated a high scientific and industrial interest in the past. Several methods such as receptor molecules that undergo color changes, [14] and magnetic [15] or fluorescent tags [16,17] have been developed and reported. Among the techniques mentioned, detection using fluorescence is an important non-isotopic method. Fluorescent labels have played an important role in the field of biomedicine, such as the detection of materials inside or outside of the cell, [18] hybridization and sequencing of nucleic acids, [19] and clinical diagnosis at the early stage. [20] These conventional fluorescent techniques have the following limitations: reduction in the emission intensity due to photobleaching, toxicity of some of the fluorescent dyes, and finally, detection sensitivity is low because only a few fluorophores can be coupled to one biomolecule in conventional fluorescent label methods.

In the present study, a non-conventional fluorescent silica probe is reported for detection of cervical cancer. The method is comparatively non toxic due to the encapsulation of the fluorescent dye within the silica particles, and it does not use any modification of the silica surface for tagging. The reported method has a relatively high specificity; it is simple, comparatively inexpensive, and straightforward in interpretation.

The rationale of the method described in this paper comes from two recent papers in which the cell surface was studied by means of atomic force microscopy (AFM). [21,22] It was found [22] that when an AFM probe, a several micron silica particle started deforming the cell it interacted first with a “brushy” layer on the cell surface, which is comprised of microvilli, microridges, and various polysaccharides of the glycocalyx. It was shown that these surface brush layers are significantly different for malignant and normal cells [21]. This

makes it plausible to expect differences in adhesive properties of these two types of cells. Such a difference, however, was not observed in the measurements reported in our earlier report [21]. The reason for that is discussed in this work.

In the present work we demonstrate that the adhesion depends on the duration of the contact between the silica particles and cell surface. As a result, the adhesion can still be statistically different for cancer and normal cells. Due to the difference in adhesion, a different number of silica particles adhere to malignant versus normal cells. Ultrabright fluorescent silica beads [23,24] are used to make the detection of silica particles easy. Instead of counting the number of adherent particles, one can measure the total amount of fluorescent light coming from a particular area. The measurement of fluorescent light is straightforward because the particles emit strong fluorescence signal. It does not require a high sensitivity spectrometer typically used for measuring fluorescence. A fairly simple instrumental setup described here is capable of measuring such fluorescence.

2. Results and Discussion

2.1. The measurement of adhesion: silica particle-cell interaction

The adhesion forces between silica probes and cell surfaces were found by processing the retraction force curves collected with AFM. Contrary to our expectations, the adhesion extracted from the data collected in [21] does not show a statistically significant difference in the adhesion to cancerous versus normal cells, Figure 1. The average adhesion for all normal cells is 0.39 ± 0.20 nN and 0.54 ± 0.16 nN for cancerous cell. Presumably the adhesion phenomenon is more complicated.

Here we hypothesized that the adhesion can depend on the time of contact between the silica probe and cell surface. Figure 2 shows a representative behaviour of the retraction force curves collected with AFM. The AFM probe, a spherical silica particle deforms the cell, stays at that point for a defined period of time, and then is pulled back. The contact times were 0 (no waiting in the point of contact), 1, 5, 10, 30 and 60 seconds. The averaged adhesion values (and one standard deviation) are shown in Figure 3. One can see from Figure 3 that the adhesion is greater for the cancer cells for short contacts. However, with the increase of contact time, adhesion increases considerably for the normal cells while increasing only moderately for cancer cells. It should be noted that the adhesion also depends on the force that the AFM probe exerts on cell. The forces used to obtain data in Figures 2 and 3 were about four times lower than used to obtain Figure 1. (This was done to avoid possible disturbance of the cells exposed to a long action of the silica probe.) Thus, the lack of a statistically significant difference in the adhesion shown in Figure 1 is presumably related to a “bad” choice of the time of contact and the load force used in ²¹.

Can the observed time-dependent behaviour be explained by the brush parameters found in [21]? The difference in the surface “brushy” layer between cancerous and normal cervical cells are the protrusions and corrugations of the cellular membrane like microvilli, microreages, and cilia [21]. The silica surface is rich in hydroxyl groups which interact with polysaccharides of the glycocalyx layer which is coating the membrane. Moreover, silica interacts with the lipids of the plasma membrane. Thus, the areas of contact between the cellular membrane and a silica particle are different for cancerous and normal cells. This situation is schematically presented in Figure 4 (the different brush structure is taken from [21]). The silica-cell surface interaction has the following components: hydrogen (attraction), van der Waals (attraction), steric (repulsion), and weak electrostatic (can be both attractive and repulsive) interactions with silanol groups of the hydrated silica surface of the probe. For example, proteoglycans of the glycocalyx seem to be only weakly negatively charged; [21] a strong competitive charge could prevent molecules from sticking

to the negatively charged silica. The time dependence of adhesion could be explained by “sea grass effect”. Because of thermal motion, the longer brush can envelope the silica surface, diffusing over the surface to find the sticky points (active head groups or charge concentrations) more effectively with time than the shorter brush. As a result, the area of contact between silica and cell, and consequently, the adhesion increases with time. In the beginning, the silica sphere penetrates more easily through a relatively rare long cancerous brush, while the brush of normal cells, being almost two times denser, keeps the silica ball repelled for some time (The repulsion by the brush of normal cells is ~ 3 times stronger due to the steric repulsion, eq.(1) in ref. [21]). This situation is shown in Figure 4a. After several seconds, the silica AFM probe presumably becomes enveloped by the long brush of normal cells, but it can still be repelled by the fairly dense and short second (inner) brush of cancer cells, Figure 4b. As a result, the adhesion to normal cells increases dramatically as compared to the adhesion to cancer cells.

2.2. Can the difference in the adhesion be used to detect cancer cells?

To answer the question of this subsection, a simple experiment was performed. Silica particles were dispersed over the normal and malignant cells. The particles were allowed to precipitate on cells in a cell culture dish for a definite period of time. After that the particles were removed by a controlled washing of the cells with HBSS buffer (using an orbital shaker (for more detail see the Experimental section, in which a similar procedure using fluorescent particles is described)). The force acting on cells from the silica particles during precipitation was definitely much smaller (~ 0.8 pN for of gravity) than the one used in the AFM measurements described previously (~ 3 nN). Keeping in mind the “sea grass” mechanism, a longer time of contact was used to compensate for the smaller force. It was found that about 120 sec waiting time before removing the particles was an optimum to see lower adhesion to normal versus cancer cells. Figure 5 shows an example of single cells either surrounded by silica particles (small round circles) in the case of normal cells (Figure 5a) and covered by the silica particles adhered for the case of cancer cells (Figure 5b). When waiting longer, the particles develop so strong adhesion that it was too hard to remove by a controllable washing. Therefore, in this work we will consider only the times/conditions when the adhesion is stronger with malignant cells.

It should be stressed that because the mechanism of detection is based on nonspecific adhesion, one cannot expect to see particles adherent to one type of cell but not to the other. Moreover, a small number of particles could even adhere to the culture dish (if it exposed to the particles), which is heavily covered with proteins adsorbed from the growth medium. Thus, one can expect any differences to be based on detection of a statistically different number of adherent particles.

While a clear difference is seen in Figure 5, to see if one can distinguish cancer and normal cells, one needs to be able to make a quantitative conclusion. It is not that easy to do with optically neutral silica particles. Furthermore, we need to perform a statistical analysis for a large number of the cells as well as for the cells taken from different individuals. It is not impractical to do manually. One would need to create software capable of distinguishing between cells and silica particles to calculate the amount of particles adherent per unit area of the cell surface. While in principle, this could be done, an easier approach is to use fluorescent silica particles. This is described in the next sections.

2.3 Spectrofluorometric and Image analysis of malignant and normal cervix cells

Using the ultra bright fluorescent silica particles described in the Experimental section, we can now develop a simple method to distinguish between cancer and normal cells *in-vitro*. Being silica from outside, these particles interact with the cells as we described in the

previous section. Therefore, it is plausible to expect that the fluorescent silica particles can also be used to distinguish between cancer and normal cells.

When using fluorescent particles, instead of counting the number of particles, one can measure the amount of fluorescent light coming from a particular area. The measurement of fluorescent light is rather straightforward because of the strong fluorescence signal from each particle. It does not require a highly sensitive spectrometer (due you mean spectrophotometer for visible light?) typically used for measuring fluorescence. A fairly simple instrumental setup capable of measuring such fluorescence is described here.

The confluent cells exposed to the silica particles were first imaged both with optical microscopy and fluorescent microscopy. Reflection optical images of cancer cells (CXT2, left) and normal cells (HCX28, right) exposed to silica probes are shown in Figure 6. One can see a fairly clear difference. To quantify this difference, we measured the fluorescent spectra as described previously. To make these spectra independent of a specific spectrometer, we made absolute measurements by calibrating the spectrometer in the energy units. The results are shown in Figure 7. Different spectra are shown for different areas on the cell culture dishes.

To get the total energy per unit area, we integrated the area under each spectral curve. This was done for the experiment described above as well as for cells taken from other individuals. Figure 8 shows the resulting statistics for cells taken from 3 healthy individuals and 3 cancer patients. Both average and one standard deviation are shown in the graph. The statistics were taken based on the analysis of three cell culture dishes per individual, 10–15 measured areas per each dish. Each measured area was approximately ~3 mm in diameter, which includes several thousand cells. One can see that on an average the emissions from the cancer cells are about 2 times stronger than that from normal cells (Figure 7). Despite the fact that the difference is obvious, the overlap between HCX30 and CTX2 cases is not negligible to speak about truly unambiguous detection of cancer.

To test the method against any artefacts related to a possible alteration of cells when they are close to confluent density, we tested rather low density cell populations. Combined transmission/fluorescent optical images are shown in Figure 9 for the case of a representative malignant (CXT2) and normal (HCX28) cells. To quantify the results of Figure 9, we cannot use just the fluorescence because there are different amounts of cells in each single measurement. Therefore, we did geometrical calculations of the areas occupied by the cells versus the areas occupied by the particles adherent to cells. The ratios of the area of the adherent particles to the cell area are shown in Figure 10. ANOVA tests showed a statistical difference at the $P < 0.05$ level.

3. Conclusions

A new method for detection of cancer cells is proposed. The method is based on the measurement of adhesion of silica beads to malignant versus normal cells cultured from human cervix. The difference was first studied with a help of atomic force microscopy (AFM). Then the method was expanded to use fluorescence silica particles, and the corresponding difference in the number of adherent particles was detected by a simple fluorometry method. The difference in the adhesion is non-specific, and is due to the alteration of the surface of the cells, which was previously found by means of atomic force microscopy and confocal scanning laser microscopy.

This method has been tested using cells taken from three healthy individuals and three patients with cervical cancer. The method shows reproducible statistical differences between

cancer and normal cells. Nevertheless, more statistical data are needed to determine the actual efficiency of the method, and to speak about unambiguous detection of cancer.

4. Experimental Section

Cell Culture

Primary cultures of human cervical epithelial cells were prepared by a two-stage enzymatic digestion of cervical tissue as described [25] and cells were maintained in keratinocyte serum free medium (Invitrogen, Carlsbad, CA). Cervical cancer cells were isolated from primary cervical carcinomas as described². All human tissue was obtained from the Cooperative Human Tissue Network. Informed consent was obtained from patients according to their published guidelines (<http://chtn.nci.nih.gov/phspolicies.html>). Normal cervical cells were used at passages 2 to 4 when they were actively growing and carcinoma cell lines were used at passages 30 to 40. This allows us to avoid possible confusion between cancer and normal cells. All cells were plated in 60 mm tissue culture dishes and dishes were used for experiments when cells were 80 to 100% confluent. Epithelial cells adhered tightly to the bottom of the tissue culture dishes.

Atomic force microscopy

A Nanoscope Dimension 3100 (Digital Instruments/Veeco, Inc., Santa Barbara, CA) atomic force microscope (AFM) was used in the present study. A standard cantilever holder cell for operation in liquids was employed. To measure the cell adhesion, the force-volume mode of operation was utilized. The AFM software version used was version 5.12, release 4. All scanning and measurements related to rigidity were performed on viable cells maintained in Hank's balanced salt solution (HBSS) within 2–3 hours after removal of growth medium.

To avoid the problem of nonlinearity and the creep error of the AFM scanner, a special NPoint *close-loop scanner* ($200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m} \times 30\text{ }\mu\text{m}$, XYZ) was used in this study. Close loop is paramount because it provides the linearity required for quantitative description of the force curves with such extended scan range. A relatively large vertical Z-range was particularly important because the cell height was $\sim 10\text{--}15\text{ }\mu\text{m}$. Adding the ramp size needed to collect the force curves (at approximately a half of the maximum range), one can easily get a number required for Z-scan range (ramp size) close to the full extent of the scanner.

AFM probe preparations

A V-shaped standard narrow $200\text{ }\mu\text{m}$ AFM cantilever (Digital Instruments/Veeco, Santa Barbara, CA) was used throughout the study. A $5\text{ }\mu\text{m}$ diameter silica ball was glued to the cantilever by epoxy resin using the AFM built-in micromanipulator. [22,26,27] Attachment on the silica ball was controlled by optical microscopy. Such a modified probe was an essential part in this study for the following reasons:

1. this silica probe mimics the individual fluorescent silica particle used for the cell labeling;
2. the probe has a considerably larger area of contact with the cell compared to a typical commercial probe (apex radius of curvature $\sim 5\text{--}50\text{ nm}$) commonly used for such measurements. The larger area of probe-cell contact results in averaging local variation in rigidity compared to that measured with the regular sharp probe. This leads to a lesser variation of the collected data. Consequently, one needs a smaller number of measurements to gather necessary statistics. This is important because of the limited time (2–3 hours) for measurements when the cells were alive in the HBSS solution;

3. excessively sharp probes may give smaller sensitivity than needed to detect surface molecular brushes, formation of the cell surface, see below;
4. using the 5 μm probes, we caused less potential disturbance of the cell during the scanning [27];
5. using a micron size silica ball as the AFM probe, we presumably do not overstress the cell surface (staying in the linear stress-strain regime) as it can be in the case of the sharp AFM tip, [28] and consequently, we may use the classical elastic models to describe deformations of the cell body. After each series of measurements, the probe was changed. In some cases, it was possible to clean it in acid/base solutions. The radius of the probe and its cleanliness were tested by scanning the reversed grid (TGT1, NT-NGT, Russia). The cantilever spring constant was measured using the built-in option of the Nanoscope software (resonance method) before gluing the probe. In each experiment, the AFM cantilever sensitivity was calibrated against a rigid substrate, a small piece of silicon wafer immersed in the HBSS solution in the Petri dish. Performing these sensitivity calibration tests, we are also able to check the AFM probe against any possible contamination. For example, if the silica ball was covered with a noticeable organic contaminant, it would be immediately seen on the calibration force curves as a large specific adhesion.

Adhesion measurements with AFM

Force volume mode of AFM operation was used. It allows automatically recording a large numbers of force curves simultaneously with topography. Force curves include information on both approaching and retracting force curves. If the AFM probe sticks to the cell, it deflects down to the cell when retracting. The adhesion force is found as the maximum (negative) deflection of the cantilever during the retraction multiplied by the cantilever spring constant.

The time-dependent adhesion forces between the silica sphere attached to the AFM cantilever and cell surfaces were measured in HBSS solution on viable cells. Using a built-in AFM software option, we kept the AFM probe in contact with the cell surface for a predefined time, from zero to 60 seconds. After that the probe was retracted and the force of adhesion was recorded.

Fluorescent silica beads

Recently a new one step self-assembly of nanoporous silica particles with encapsulated organic dyes has been developed, [23,24,29] in which the dyes are physically entrapped inside silica matrix. These particles have silica matrix, in which there are hexagonally packed cylindrical channels. Comparing fluorescence of these particles with the brightest micron-size particles assembled from aqueous compatible quantum dots [30] encapsulated in polymeric particles of similar size ($\sim 1.2 \mu\text{m}$) reported recently, [6] one can find that the particles (scaled to the same size) are about 170–260 times brighter. This makes the particles the brightest tags presently available.

The synthesis of this particles is described in the corresponding references. Briefly, it is a fairly straightforward one step synthesis. Tetraethylorthosilicate (TEOS, 99.99+ %, Aldrich), cetyltrimethylammonium chloride (CTACl, 25 wt.-% aqueous solution, Pflatz & Bauer), formamide (99 %, Aldrich) and hydrochloric acid HCl (37.6 wt. % aqueous solution, SafeCote), Rhodamine 6G (R6G) perchlorate dye (Sigma-Aldridge, Inc.) were used. All chemicals were used as received. The surfactant, acid, dye, formamide, and distilled water (Corning, AG-1b, $1\text{M}\Omega\text{-cm}$) were stirred in a polypropylene bottle at room temperature for 2 hours, after which TEOS was added and the solution stirred for ca. 5 min.

The solution was then kept under quiescent conditions for 3 days. The molar ratio of H₂O:HCl:formamide:CTACl:R6G:TEOS was 100:7.8:9.5:0.11:6×10⁻⁴:0.01:0.13. The materials so formed were washed by centrifugation to avoid damaging of the surface, washed with copious amounts of water, and mixed with HBSS solution for the further use on the cell culture.

Detection of Affinity of Fluorescent Silica Particles to Cells

After carefully washing the particles to remove any traces of the synthesizing chemistry, the particles were added to HBSS buffer solution to form a colloidal dispersion (~20 g/litre). The cells in the 60 mm culture dishes were washed twice with HBSS solution for 2 min each. The cells were then exposed to 1 ml of the colloidal dispersion for 2 min. During this 2 minute period, the dishes were subjected to initial 30 seconds of shaking on a Boekel Scientific Ocelot 260300F at minimum speed, to ensure uniform distribution of the colloidal solution and to minimize particle agglomeration. The cells were then washed with HBSS solution two times thoroughly using the same Boekel shaker, to remove excess particles that had not adhered to the cells. The culture dishes were then dried in air at ambient room temperature.

It is important to note about the density of cells in the Petri dish. A control experiment of the affinity was carried out for a light density population of cells. That was done only to verify that a heavy density population (up to confluent density) of cells behave similar to the light density population. The majority measurements were done on cells that just reached confluency in the cell culture dish bottom. This allows us to exclude the possible affinity of the particles to the Petri dish bottom as well as dealing with different total areas of coverage of cells in different dishes. In such a case, the processing of the results becomes fairly simple. The amount of adherent fluorescent particles is linearly proportional to the fluorescent signal.

Spectrofluorometric measurements of the particles attached to cells and imaging of cells

An efficient assembly was setup to record optical images of cells and record emission intensity of the fluorescent particles (Figure 11). An Olympus BH2-UMA microscope was connected to a JVC TK-1280U colour video camera. The images of cells were captured using FlashBus MV version 3.91 software. To record the fluorescent emission, an optic fibre (UVIR1000) with a C type adapter was used to connect the microscope to an Ocean Optics Inc, USB 2000 spectrometer. A Cyonics air cooled argon ion laser was used as the light source. It should be noted that the use of laser light here is not necessary but helpful. An advantage in using laser is a fairly strong fluorescence signal, and consequently, very fast measurements can be done. A 500nm notch filter (Omega Optical) was utilized to filter the laser light. The spectrographs were recorded using OOIBase32 software. To average the emission over the culture dish area, the focus knob was rotated three full turns in counter clockwise direction (increase in the distance between the objective and sample). This procedure was maintained the same for spectrofluorometric measurements of all samples.

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Figure 1.
Histograms of adhesion of silica particles to the cells averaged per each cell.

Figure 2.

A representative behavior of the retraction force curves. The AFM silica probe stays in contact with the cells for different periods of time (from zero to 60 seconds) before retracting. The curves are artificially shifted vertically for better visualisation.

Figure 3. Time-dependent adhesion between a silica particle and cell surface. The AFM silica particle, probe stays in contact with the cells for a definite period of time (from zero to 60 seconds) before retracting.

Figure 4.

A schematic of interaction between a silica particle and brushing cell surface. The areas of contact between the cellular membrane and a silica particle are different for cancerous and normal cells. (the different brush structures are drawn according to the results obtained infrom [21]). (a) The initial contact of a silica AFM probe and cell surface. (b) After a period of contact, the silica AFM probe becomes enveloped by the long brush of normal cells, but it can still be repelled by the fairly dense and short second (inner) brush of cancer cells.

Figure 5.

Optical phase images of single normal (a, c) and cancer (b, d) cervical cells exposed to 5 μm silica spheres (small round circles). Some particles could still adhere to the Petri dish. This however can be excluded either by software processing (excluding non-cellular areas from consideration) or using confluent cell coverage.

Figure 6.
Reflection optical images of CXT2 (left) and HCX28 (right) exposed to silica probes.

Figure 7.
Absolute flux of fluorescence for (a) CXT2 and (b) HCX28 cells exposed to silica probes.

Figure 8. Statistics of the total energy of fluorescent signal per unit area for cells taken from 3 healthy individuals and 3 cancer patients. Both average and one standard deviation are shown in the graph.

Figure 9.

Combined transmission/fluorescent optical images of (a) CXT2 and (b) HCX28 exposed to silica probes. Inverted contrast is shown. Dark spots indicate the bright fluorescent silica beads, whereas the cells are seen as grey spots.

Figure 10. Distribution of ratio of the total particle area to the area of the cells ($A_{\text{PARTICLE}}/A_{\text{CELL}}$). (a) cancer (CXT2) and (b) normal (HCX28).

Figure 11.
A schematic diagram of the optical measurement system.