

Technical Notes

Analysis of Nonadherent Apoptotic Cells by a Quantum Dots Probe in a Microfluidic Device for Drug Screening

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This technical note describes a facile technique to screen some anticancer drugs and evaluate their effects on nonadhesive leukemic cells in an easily fabricated microfluidic device by utilizing the Annexin V conjugated quantum dots as apoptosis detection probes. The cell immobilizing structures and gradient-generating channels were integrated within the device which was fabricated in one-single step. The nonadhesive leukemic HL-60 cells can be felicitously immobilized and cultured on the dam structures at a proper lateral pressure. We then delivered Annexin V functionalized quantum dots which can readily bind to the outer membrane of apoptotic cells and distinguish the apoptosis from unaffected cells with single cell level resolution. The diffusion time of quantum dots reduced to 5 min before imaging. The capabilities of evaluating drug effect on HL-60 cell line have been shown in both population way and individual cell level. The technique presented herein can bridge the gap between the quantum dots based *in vitro* cell imaging and the analysis of individual apoptotic cell in a microfluidic system, allows an easy operating protocol to screen some clinically available anticancer drugs.

Apoptosis refers to a specific form of programmed or suicidal cell death, which takes place in tissues to guarantee the welfare of the whole organism through the elimination of unwanted cells.^{1,2} Dysfunction in apoptosis can lead to disease states including neurodegenerative diseases (e.g., Alzheimer, Parkinson), autoimmune diseases (e.g., lupus erythematosus, rheumatoid arthritis), and cancer.^{3–5} The techniques for detection apoptotic cell are therefore highly interesting, and much effort has been taken to measure apoptosis in a specific and a sensitive way. Rapid and sensitive detection of apoptotic cell is of considerable interest to

biological, pharmaceutical, and chemical researchers.^{6–8} Nowadays, a number of methods are available to determine programmed cell death.^{9–12} However, in most cases, expertise and sophisticated equipments were involved in these methods, which are very labor intensive and sample-consuming. Moreover, conventional techniques are not suitable for single cell level analysis or incapable of real-time monitoring for understanding the heterogeneity during apoptosis.

Microfluidic device have shown great potential toward realizing these goals in recent development.^{13–24} Albert van den Berg and co-workers successfully developed a silicon-glass microfluidic device which realized real time apoptotic dynamics measuring in a single cell level with the advantage of easily transporting cells in the channel and discriminating different dynamic stages during

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apoptosis.²⁵ Lin and co-workers have reported a cell-based high content screening method for multiparametric measurement of cellular responses using an integrated microfluidic device.²⁶ However, these techniques require relatively complex fabrication processes. Furthermore, the traditional fluorescent probes are prone to photobleaching and may hinder some further investigations when a long-term continuous fluorescence observation is needed. Moreover, none of these methods, compared with the method reported herein, have involved the nanotechnology such as quantum dots (Qdots) as apoptosis probes with intrinsic photostable fluorescence.^{27–31} Annexin V conjugated quantum dots have been successfully developed to recognize and image the apoptotic cells.^{32,33} It is also noticeable that Annexin V-Qdot bears a relatively large size (~20 nm), which may obstruct the real-time investigation for requiring extra time for diffusion.³²

Herein, we describes a method based on the Annexin V conjugated Qdots as the apoptosis detection probes to screen anticancer drugs in an easily fabricated microfluidic device. Our technique, as a newly developed microfluidic technique, can significantly reduce the diffusion time of Annexin V-Qdots for anticancer drug screening via assaying nonadhesive HL-60 cell apoptosis in a miniaturized scale. The technique presented herein can bridge the gap between the quantum dots based *in vitro* cell imaging and the analysis of the apoptotic cascade in the microfluidic system, which allows an easily operating way to screen some clinically available anticancer drugs. This method can be used in a number of biological systems, such as real-time monitoring apoptotic cascade and the evaluation of anticancer medication.

EXPERIMENTAL SECTION

Chip Fabrication. Fabrication of the master of the microfluidic device was accomplished by the established method.³⁴ Patterns used in these experiments were designed by Adobe Illustrator software and exported as AI files. After the file was processed by a high-resolution printer (2540 dpi), a film photomask was made. The film photomask was placed directly on the top of a printed circuit board (PCB, dimensions 10 cm × 15 cm × 0.16 cm, Kinsten glass-epoxy single sided PCB, Chiefskill, Shenzhen, China) and covered by a glass plate. After exposure under a UV lamp for 150 s, the PCB was placed in developing reagent (Chiefskill) for 5 min and the patterns were visible. The developed PCB was etched in etching reagent for 45 min, at room temperature without agitation. The PCB was thoroughly rinsed with tap water for 5 min after

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etching, and the remaining photoresist was washed away by acetone. The PCB master was polished by metal polishing reagent (Brasso, Reckitt & Colman, U.K.) to obtain a smooth surface. After the PCB master was thoroughly by DI water, we replica-molded polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) from the PCB master. The tips of two short silicone tubes were moistened by a little of the PDMS prepolymer mixture (curing agent/PDMS = 10:1) and mounted on the inlet positions of the PCB mold. After baked in the oven for 10 min (70 °C), a PDMS prepolymer mixture (curing agent/PDMS = 10:1) was poured on the mold. Holes at the outlets of the microfluidic device were cut through by a sharpened needle tip after curing for 2 h at 70 °C. These procedures can eliminate leakage of the unwanted PDMS prepolymer into the inlets of the chip before curing and make the connection with the syringe pump more convenient. The microchannels with the depth of 40 μm were used in these experiments. The whole device was formed by irreversibly bonding the PDMS sheet with an autoclaved plain glass slide (Changsha Shaoguang Chrome Blank Co., Ltd.) via oxygen plasma treatment by using a plasma cleaner (Harrick Scientific Corporation, Ossining, NY). Chips were coated with either fetal bovine serum (FBS) or a solution of 100 μg/mL human plasma fibronectin (Bender MedSystems GmbH, Vienna, Austria) for facilitating cell survival.³⁵

Cell Samples. Human promyelocytic leukemic HL-60 cells were obtained from Nanjing KeyGen Biotech Co., Ltd. HL-60 cells were cultured in phenol red-free RPMI-1640 medium (Gibco, Invitrogen Corporation CA) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. Supplements and antibiotics were purchased from Invitrogen. Cell cultures were maintained in a 5% CO₂ humidified atmosphere at 37 °C. Cell samples were centrifuged at 300g for 5 min, washed twice in phosphate buffered saline (PBS), and resuspended at a final cell density of 10⁷ cells/mL in PBS before the experiments.

Microchip Operation. The performance for generating concentration gradient of the microfluidic device was validated prior to the cell apoptosis experiment (see Supporting Information for more details).³⁶ During the experiment, the fluorescent intensity in cell chamber 1 shows no significant difference with background. This observation indicated that there was no FITC in chamber 1 that served as a negative control chamber in the experiment. Under the observation of microscopy, the suspended HL-60 cells were introduced into the microfluidic device from the cell/dye inlets and delivered to each culture chamber, respectively. Immobilization of suspension cells was achieved by the “sandbag”³⁷ microstructures as a cell trapping dam in the cell culture chambers. Because the sand-bag dam has a certain angle with the upstream gradient generator channel, the cells can be felicitously immobilized on the dam by proper lateral pressure (see Supporting Information for more details). Redundant cells which have not been trapped in the dam flowed away through the outlet of the channel. After cells were immobilized in the culture chambers, the syringe pump with two 1 mL syringes was

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used to provide flow to the two inlets.^{38,39} The cell culture medium with and without anticancer drugs were introduced into the microfluidic device at a flow rate of $0.1 \mu\text{L}/\text{min}$ which cannot disturb the cell function.^{40,41} Three representatives of clinically available antileukemic drugs, cycloheximide, etoposide, and camptothecin (Nanjing Sunshine Biotechnology Ltd., Nanjing, China), were selected as the model drugs to induce the apoptosis of HL-60 cells, respectively.

Annexin V-functionalized Qdots were prepared by the established method.³² Briefly, $2 \mu\text{L}$ of Qdots 605-streptavidin (Jiayuan Quantum Dots Corporation, Wuhan, China) were dispersed in $10 \mu\text{L}$ of PBS buffer; this solution was vortexed for 30 s and $10 \mu\text{L}$ of PBS buffer containing biotinylated Annexin V ($1 \mu\text{L}$) (Bender MedSystems GmbH, Vienna, Austria) was added. The resulting mixture was allowed to react for approximately 1 h at room temperature, and only the supernatant was added to a CaCl_2 -containing binding buffer. After introduction of the resulting Annexin V-Qdots solution into the cell culture chambers from the cell/dye inlet, the whole device was housed in the incubator for 5 min before observation. After examination, the Annexin V-Qdots label was detached by washing three times using the washing buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM MgCl₂), which differs from the binding buffer in that the calcium was replaced with magnesium. The device could then be reexamined to confirm that the Annexin V-Qdots was completely washed away. At a certain time point, the cells can be restained using the same method when a time-lapse analysis was taken.

Microscopy and Image. Excitations and filters were as follows: excitation filter 405 nm, LP 430 nm filter. When the device was mounted on the microscopy stage, the syringe pump was stopped. Phase-contrast and fluorescent images were acquired by a Nikon inverted microscopy (ECLIPSE TE-2000U, Nikon Corporation, Japan) equipped with a video camera (DS-U1, Nikon Corporation, Japan) and a self-made environment chamber that maintained the temperature at 37°C . After images were taken, the device was put back to the incubator and the syringe pump started again to continue the treatment with anticancer drugs. The collected data were analyzed using the Nikon imaging software NIS Elements (Nikon Corporation, Japan). Fluorescent intensity of individual cell was estimated by the program in the region of interest (ROI). For each image, the areas of each interested cell were manually defined three times over time, which gives an average intensity value \pm standard deviation.

RESULTS AND DISCUSSION

The design of our device and strategy is shown in Figure 1. An integrated poly(dimethylsiloxane) (PDMS) microfluidic device was used to generate the drug concentration gradients³⁸ and immobilize the nonadherent leukemic HL-60 cells. When the apoptosis was triggered by the anticancer drugs, the phosphatidylserine (PS) moieties flip out from the inside of the cell

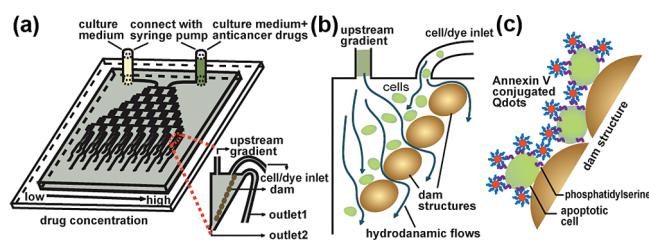


Figure 1. (a) Schematic drawing of microfluidic device with integrated gradient generator and cell trapping sand-bag dam structures, and the bottom sketch shows the geometry of the cell culture chambers. (b) A schematic drawing shows how the cells were hydrodynamically trapped on the sand-bag structure dam by a lateral pressure. (c) Schematic drawing depicts the detection of apoptotic cells immobilized on dam structures using the Annexin V conjugated quantum dots.

membrane.⁴² Annexin V functionalized Qdots were introduced from the cell/dye inlet and readily bound with the outer membrane of the apoptotic cells. This procedure can be used not only to analyze the cells treated with a different concentration of anticancer drug but also to distinguish the cells in the early apoptotic stage from nonapoptotic cells with single cell level resolution. The technique presented herein makes it possible for screening the anticancer drugs in a fully nanoprobe-assisted way.

To test the effect of anticancer drugs on HL-60 cells, at a certain time point, we manually counted the Qdots stained cells trapped in each of the culture chambers on the stage of inverted fluorescent microscopy. Only the first 180 cells were taken in each counting. Figure 2a–d illustrates a series of representative images obtained to characterize the proportional changes in the Annexin V-Qdots labeling of trapped cells over the eight culture chambers. It shows the proportion of Qdots-stained cells or the phosphatidylserine-positive cells stimulated by the anticancer drug gradient and was observed in a concentration-dependent manner. As shown in Figure 2e–g, at the concentration of $21.4 \mu\text{M}$ (24 h, 37°C), cycloheximide triggered the apoptosis in $27.0\% \pm 7.4\%$ in cells, while almost $78.0\% \pm 9.5\%$ cells presented apoptosis when the concentration (24 h, 37°C) increased to $50.0 \mu\text{M}$. Etoposide at 21.4 and $50.0 \mu\text{M}$ (12 h, 37°C) effectively induced apoptosis in $30.0\% \pm 11.0\%$ and $78.5\% \pm 6.3\%$, respectively. In addition, camptothecin at $2.57 \mu\text{M}$ (12 h, 37°C) effectively enhanced the apoptosis in $45.9\% \pm 4.8\%$ and when the concentration rose to $6.0 \mu\text{M}$ it caused almost $81.7\% \pm 7.3\%$ of cells. The above results reveal that compared with cycloheximide and etoposide, camptothecin shows a more potent effect on induction of apoptosis in the HL-60 cell line. However, the image taken from the anticancer drug nonaffected culture chamber 1 shows a slight fluorescence after 24 h cultured in the microfluidic device. We basically reckon this phenomenon was caused by unspecific binding of the Annexin V-Qdots on the cell membrane. Nonetheless, this could be alleviated in the future by using properly coated quantum dots which are not involved in the present method. Moreover, in comparison with the apoptosis detection in traditional cell culture plates or a Petri dish using Annexin V-Qdots, the incubation time dramatically reduced from 1 h to 5 min is presented herein.³² Therefore, our technique shows great capability of screening

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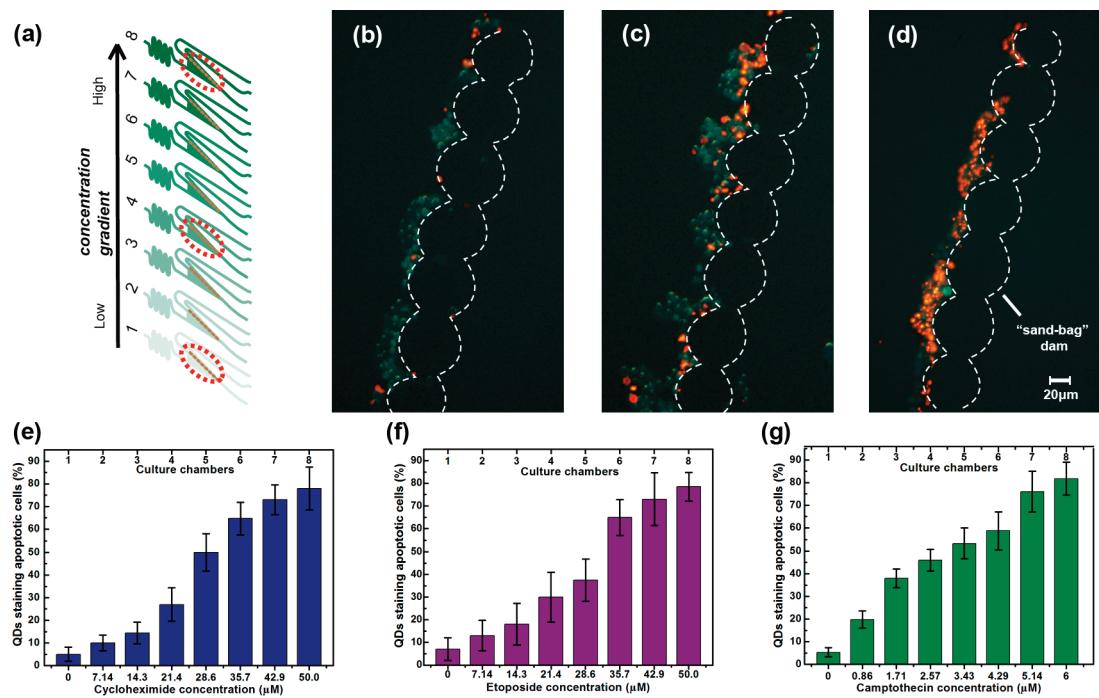


Figure 2. Analysis of cell apoptosis in the microfluidic device after anticancer drug treatment and Qdots staining. (a) A schematic drawing of the eight chambers in the microfluidic device. Each chamber contains a parallel sand-bag dam structure for trapping the nonadherent cells. The chambers 1, 4, and 8 where the images were illustrated in parts b, c, and d marked with a red dashed ellipse, respectively. (b–d) Composite images show immobilized HL-60 cells stained by Qdots, and cells with red fluorescence represent apoptosis. Images were taken from the cell culture chambers 1, 4, and 8 (24 h treated with CHX) corresponding to CHX concentrations of 0, 28.5, and 50.0 μ M, respectively. The trapping dam structure are marked as a white dashed curve. (e–g) Correlative analysis shows that the percentage of Qdots stained HL-60 cells was in dose-dependent fashion. Cells were incubated with the presence of cycloheximide, etoposide, and camptothecin gradients for 24, 12, and 12 h, respectively. Only the first 180 cells were taken into each counting, and the experiment was repeated four times, which gives an average percentage \pm standard deviation.

anticancer drugs on a nonadherent cell line by taking advantage of a significant reduction of the diffusion time in a microfluidic device.

It has been reported that the cell heterogeneity may be obscured in routine analysis by using a bulk measurement.⁴³ A study of an individual cell would reveal some important variations in the cell pathway dominated process, such as apoptosis. Since the cells have been immobilized on the dam, the cells could be regarded as semiaddressable and therefore the individual cell apoptosis could be analyzed at the given time points. If the phosphatidylserine (PS) moieties flip out from the inside of the cell membrane, Annexin V functionalized Qdots meet the PS and readily bind to the outer membrane of the cells, irradiating red fluorescence. It can be assumed that the fluorescence intensity could increase coincidentally with the number of binding Qdots, and this indicated the quantities of externalized PS which may facilitate the quantification of PS during cell apoptosis. To demonstrate this, we analyzed the fluorescence intensity of different cells trapped on the sand-bag dam at a given time. After treatment with the etoposide at a concentration of 50 μ M for 6 h, cells were imaged under the fluorescence microscope. Eight cells were picked, and the assay indicated that the intensity varies a lot from each of the picked cells. In Figure 3, cells numbered 2, 3, and 4 show comparatively high fluorescence intensity and may be deduced as the PS present adequately on the membrane of these three cells, which would be rapidly engulfed and degraded

by phagocytes *in vivo*.⁵ Cells numbered 1, 5, 6, 7, and 8 exhibit red fluorescence with less intensity than cells numbered 2, 3, 4 and they may be undergoing the early stage of apoptosis. Among these cells, number 2 shows the most brightness of fluorescence while the number 7 displays the weakest fluorescence. The fluorescence intensity varies a lot across these picked cells, which therefore vividly depict the single cell heterogeneity during the process of PS externalization when apoptosis was triggered. However, in some cases, it should also be noted that the cells inside the trap show more intense red fluorescence than the cells in the vicinity of the trap, due to the fact that the cells in contact with the dam structure suffered much shear stress and the clogging effect makes a topical increase of drug concentration. To alleviate this problem, we typically reduce the concentration of cell suspension before introduction into the device. At each introduction, the trapping of the cells was monitored under the microscope. Once the cells were trapped and sequentially formed a “mono-layer” on the dam, the introduction was instantly stopped by rapidly replacing the cell suspension solution with fresh PBS. This procedure cannot only feasibly trap cells on the sand-bag dam but also effectively eliminate the clogging problem during the cell culture process.

The current method is also available for analyzing the dynamic process of plasma membrane phosphatidylserine redistribution during apoptosis both in the population way and in the individual cell level by a time-lapse assay. We analyzed the time-lapse fluorescence images of cells trapped in the sand-bag dam structure

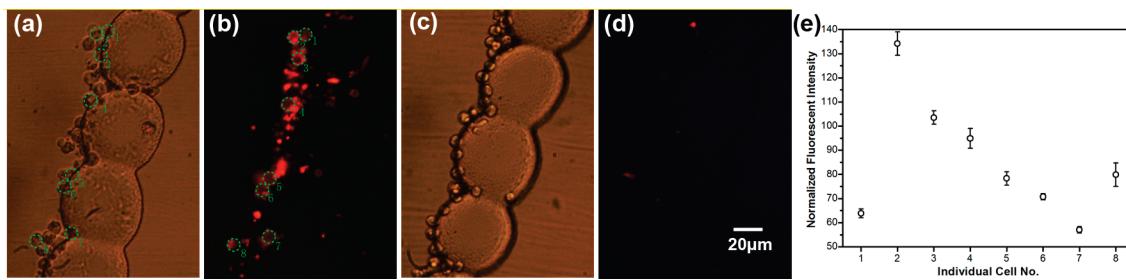


Figure 3. Cells that were undergoing apoptosis can be discriminated by Qdots-Annexin V. (a–d) Pairs of bright field images and fluorescent micrographs for cells trapped in chamber 8 (etoposide 50 μ M) and chamber 1 (no etoposide), respectively. The different cells were marked as green dashed circles. Parts a and b show that the trapped cells with a continuous presence of etoposide at 50 μ M for 6 h were stained by Qdots as apoptotic probes (show red fluorescence), and eight different cells were picked for fluorescent intensity measurement. Parts c and d illustrate the control group cells trapped in culture chamber 1 without etoposide treatment. (e) Fluorescence intensity analysis of eight picked cells. The ROI (region of interest) for each picked cell was manually defined three times in each image, which gives the average intensity value \pm standard deviation.

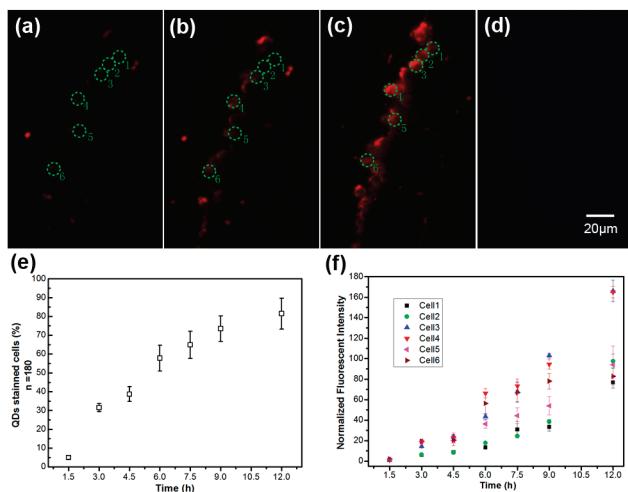


Figure 4. Time-lapse analyzes the Qdots-Annexin V stained cells at different time points treated with camptothecin at a concentration of 6 μ M. Six individual cells were picked to probe the apoptotic cascade in a single cell level. Fluorescent images a–c show the images of cells which have been previously trapped in the dam structures and were stained by Qdots at the time point of 3, 6, and 12 h (show red fluorescence), respectively. The different cells were marked as green dashed circles. (d) The image shows that cells trapped in chamber 1 as a control group without camptothecin treatment were Qdots negative at the time point of 12 h. (e) The Qdots stained cell percentages were increased in a time-dependent manner. The experiment was repeated five times, which gives an average stained population \pm standard deviation. (f) The fluorescence intensity of each individual cell was analyzed over time. The ROI (region of interest) was manually defined three times for each individual cell.

with the presence of camptothecin at a concentration of 6.0 μ M. As shown in Figure 4a–f, either the fluorescent intensity or the percentage of Qdots stained cells was increased in a time-dependent manner. After treatment with 6 μ M camptothecin for 3 h, 31.5% \pm 2.2% cells had red fluorescence, indicating the translocation of phosphatidylserine to the outside of the plasma membrane (Figure 4a,e). Except for the cells numbered 4, 5, and 6, other selected cells do not present red fluorescence, which suggests that those three cells were Annexin V negative. After the treatment with camptothecin for 6 h (Figure 4b,e), 57.8% \pm 6.8% cells went into apoptosis and the intensity of red fluorescence was apparently increased. A presentation of Qdots fluorescence took place in all the outer membrane of six selected cells. As

observed in the final step, at the time point of 12 h, there was a dramatic increase of both the percentage of population (81.5% \pm 8.2%) and fluorescent intensity (Figure 4c,e). In addition, as shown in Figure 4f, the fluorescent intensity of the six selected cells generally increased consistently with time, wherein the cells numbered 3 and 4 showed a most significant increase over time and thus indicated that these two cells represented a relatively more sensitive response to camptothecin. As for cells numbered 1 and 2, the intensity of which increased slowly during the time point 3–9 h and suggested that these two cells may lack the sensitivity to camptothecin when compared with numbers 3 and 4. Accordingly, cells numbered 5 and 6 represented a moderate susceptibility with camptothecin at the concentration of 6 μ M during a period of 12 h. Interestingly, the intensity profiles over 12 h of these randomly picked cells approximately show an exponential relationship, which may provide a hint that the PS translocation from the inner plasma membrane could fit exponentially during a long time investigation. Such a response distribution among the semiaddressed individual cells cannot be observed either with bulk analysis techniques or with traditional fluorescence dyes. Therefore, this kind of experiment can be used to screen the single tumor cell response to chemotherapeutic drugs in a long period of the apoptosis. During the process of apoptosis, the intracellular enzyme activities probably differ from each individual cell and may also be worthy of note.⁴⁴ However, in several cases, we find that some of the cells which previously have been trapped in the microdam were detached from the dam during the time-lapse experiment. The sand-bag structures serve as a physical barrier, confining cell movement in this case. An unwanted flow from the other side of the dams may cause the cells to depart from the place where they were previously immobilized. This raises an inconvenience in recognizing the cells formally taken into the time-lapse assay. Nevertheless, we are currently developing a more sophisticated device for cell trapping which may benefit the confinement of cells for real-time analysis. The device presented herein can still be reckoned as an alternative for cell immobilization in virtue of easy fabrication and a friendly operating way.

CONCLUSIONS

In this study, an integrated microfluidic device capable of screening anticancer drug has been presented for the first time with a strategy of analyzing apoptotic cells using biofunctionalized

Qdots. The incubation time for the Annexin V-Qdots sensing apoptotic cells has been significantly reduced because of the minimized reaction environment in the microfluidic device. Moreover, the method presented herein has exhibited the capability of evaluating the dose-dependent effect of different anticancer drugs on the cells while processing the ability to immobilize non-adhesive cells conveniently; it also allows monitoring the dynamic behavior of phosphatidylserine redistribution from the inner plasma membrane at the single cell level, which could be helpful in understanding the intracellular enzyme activities such as the aminophospholipid translocase which play an important role in apoptosis cascade.⁴⁴ We therefore believe that this method certainly potentiates the quantum dots utility in monitoring living cells for studying real-time biomedical problems such as anticancer pharmacological kinetics. In one word, this technique definitely paves a way for probing living cells with nanotechnology-based tools⁴⁵ in a microfluidic device and may find some potential in deeper cellular biological studies.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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