PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2018, 45(3): 363~374
www.pibb.ac.cn

Optimization of a Cell Electro-stretching Method and Analysis of The Effect of Doxorubicin on Stiffness of Leukemia NB4 Cells*

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Abstract Recent studies have suggested that the deformability of cells can be an effective biomarker to indicate the progression of diseases. Because of the obstacle of cellular heterogeneity, it was very difficult to make new cells stretch under the reported experimental conditions. In this paper, a cell electro-stretching method based on dielectrophoresis-coupled microfluidic chip was optimized and the effect of doxorubicin (DOX) on stiffness of leukemia NB4 cells was investigated using the established method. Firstly, positive dielectrophoresis (pDEP) method for cell capturing was optimized and validated, and the results demonstrated that it was easy to obtain pDEP effect of cells if the conductivity of cell suspension (σ_e) was so low as to make "cross-over" frequencies appear, meanwhile the frequency of the *E*-field *f* was adjusted in the range of the "cross-over" frequencies from Low-f_0 to High-f_0. Subsequently, three parameters affecting cell stretching were systemically discussed, and the results suggested that it was a prerequisite for cell electro-stretching that the conductivity of cell suspension (σ_e) was lower than that of cell cytoplasm (σ_c). In addition, increased voltage or decreased frequency of the E-field can also prompt cell stretching. Finally, the correlation between cell mechanical behavior and cell functional variation of NB4 cells was investigated *via* the optimized cell electro-stretching method, and the results showed that DOX treatment could make NB4 cells produce apoptosis and stiffen. Taken together, this study provides a novel cell electro-stretching method for the simple and efficient detection of cell deformability.

Key words electro-stretching, microfluidic, cell stiffness, positive dielectrophoresis, cross-over frequency
DOI: 10.16476/j.pibb.2016.0356

The biomechanical properties of a cell play an important role in many cell functions, such as cell differentiation, aging, apoptosis, metastatic potential of cancer cells and mechanotransduction [1–7]. Many evidences indicated that the occurrence and progression of diseases might be closely linked with the variation of cell mechanical properties [8–11]. For example, cell stiffness of metastatic cancer cells was more than 70% softer than that of the benign cells that line the body cavity [8]; the malignant cells (MCF-7) were indicated to be softer than benign cells (MCF-10A) [9]; the stiffness exhibited a decreasing trend when cells transformed from benign to malignant states[10–11]. To date, the change in cell stiffness has been utilized as a useful biomarker to identify the cells in different physiological or pathological stages[12].

Nowadays, there are a number of techniques that can be used to evaluate the biomechanical properties of a cell, which include micropipette aspiration, atomic force microscopy (AFM), magnetic tweezers, optical tweezers and DEP [13–17]. Both micropipette aspiration

* This work was supported by grants from National Science Fund for Distinguished Young Scholars of China (51425505), the Program of "One Hundred Talented People" of Shanxi Province, Outstanding Young Talents Support Plan of Shanxi Province and 131 Leading Talents of Shanxi Province.

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Received: November 15, 2016 Accepted: January 17, 2018

and AFM are contact-based approaches which could potentially cause damage to cells, and both magnetic tweezers and optical tweezers usually require complex, bulky and expensive equipment to conduct the experiments. Among these techniques, DEP is regarded as the most efficient way for non- contact cell manipulation and characterization [18–20]. The wide application of DEP method depends on the development of microfluidic technology to some extent, and the flatform of microfluidic with cell electro-manipulation functions is characterized by low cost and friendly manipulation.

In light of the advantages of DEP method, this technique has been increasingly adopted in recent years to provide a simple alternative approach for characterizing the mechanical properties of cells [18–20]. Although cell electro-stretching technology has been reported for many years, much work is still limited. It was found that different cells were stretched under different experimental conditions in different laboratories [18–22]. Most literatures only reported some fixed parameters and lacked flexibility in selecting the parameters. And they hardly provided the evidences of selecting the experimental parameters, nor did they offer more reference and guidance to new cell stretching to some degree. Along with cell heterogeneity, new cells stretching cannot be conducted under the reported experimental conditions at all and the broad application of cell electro-stretching technology was limited.

This paper presents an optimized cell electro-stretching method based on DEP-coupled microfluidic chip and discusses the stiffness variation of leukemia NB4 cells after drug doxorubicin treatment. A concept of the system of the microfluidic with cell electro-manipulation functions based on DEP, including cells, microelectrode and cell suspension, was raised for the first time. In cell electric experiments, NB4 cells were examined on a custom-designed microfluidic chip, which has a parallel stepped-shape microelectrode pair with different gaps. Cell electro-stretching consists of two main parts, immobilizing and stretching. In order to capture cells firmly, *via* the advantages of the designed microfluidic chip and the Jones' cell protoplast model theory [23], the optimized positive DEP (pDEP) method was obtained, applied and validated. In order to stretch cells effectively, the high efficiency of our microfluidic chip in cell stretching and three parameters affecting cell stretching were systemically discussed. Finally, through the optimized cell electro-stretching method, the correlation between cell mechanical behavior and cell functional variation of NB4 cells was investigated.

1 Materials and methods

1.1 Cell culture

Leukemia cell lines, NB4 cells, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Ordinarily, cells were saved and cultured in a humidified incubator with 5% CO_2 at 37℃. In order to execute cell stretching, cells were resuspended in an isotonic medium as which was used in the reported literature[24].

1.2 Dielectric model and electro-stretching principle of single cell in suspension

A cell in suspension may be seen as a basic electric model [23, 25] (Figure 1a). In the model, cell membrane is thought to be dielectric and there are innumerable disorganized ions with positive or negative charges inside and outside the cell. In the presence of nonuniform AC electric field, a cell's Maxwell-Wagner polarization effect occurs and the dipoles within the cell in suspension are generated[22–23] (Figure 1b). The induced dipoles, which arise from the free charges, can accumulate at the interface of a cell if its electrical properties differ from that of the medium. According to dielectric physics, at this time there is a classic Debye relaxation system in the microfluidic chip, and there is energy exchange between cells and the electric field during cell polarizing[25].

The well-known expression of the force, F, on a cell as an infinitesimal dipole in the electric field, is shown as the Expression (1)[23]:

$$F=\vec{P}\cdot\nabla\vec{E} \tag{1}$$

where P is the dipole moment, and ∇E is the gradient of electric field E. If the external electric field is uniform, no net force is exerted on a cell; if the external electric field is nonuniform, a cell subjects to the DEP force. So they are shown as the Expression (2)[23]:

$$\begin{cases}\nabla\vec{E}=0,\ F=0;\\ \nabla\vec{E}\neq 0,\ F_{\mathrm{DEP}}=2\pi r^{3}\varepsilon_{\mathrm{e}}\mathrm{Re}[f_{\mathrm{CM}}(\omega)]\nabla E_{\mathrm{rms}}^{2}\end{cases} \tag{2}$$

where r is the radius of a cell; ε_{e} is the permittivity of medium; E_{rms} is the root mean square value of the electric field; $f_{\mathrm{CM}}(\omega)$ is the Clausius-Mossotti (CM) factor; ω is the angular frequency of

electric field, and Re[$f_{CM}(\omega)$] is the real part of the CM factor. In addition, Re[$f_{CM}(\omega)$]>0 means that a cell can move towards the strong-E regions and show pDEP response, while Re [$f_{CM}(\omega)$]<0 means that a cell can move towards the weak-E regions and show nDEP(*i.e.*, negative DEP) response (see Figure 1c, d). Through pDEP effect, cells can be captured by the microelectrodes (Figure 1e).

Cell electro-stretching is characterized through the parameter of induced-dipole moment μ_c, and μ_c is shown as Expression (3)[21]:

$$\mu_c=4\pi\varepsilon_e r^3 \cdot E \cdot \frac{\sigma_c-\sigma_e}{\sigma_c+2\sigma_e} \quad (3)$$

where σ_e is the conductivity of cell suspension, and σ_c is the conductivity of cytoplasm. Therefore if a cell is subjected to an intermediate-frequency field in a weakly conductive medium (*i.e.*, $\sigma_e<\sigma_c$), the dipole moment μ_c is oriented in the direction of the field E. So the cell can be stretched along the field lines(Figure 1f).

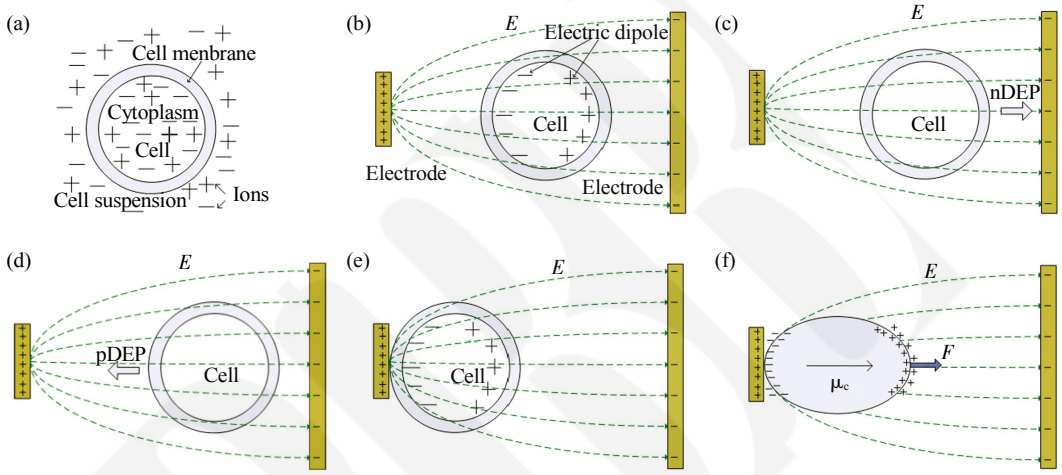

Fig. 1 Model and DEP effect of a cell in suspension

(a) A basic electric model. (b) A polarized cell. (c) nDEP effect of a cell. (d) pDEP effect of a cell. (e) A captured cell by the electrode. (f) The stretched cell.

2 Results

2.1 Optimization and validation of pDEP method for cell capturing

A microfluidic chip was designed as the tool to stretch cells in this work, as shown in the Figure 2a. Microfluidic chip is composed of microelectrodes and microchannel. The microelectrode (Figure 2b), which was made of indium tin oxide (ITO) glass slide, was designed as a parallel stepped-shape micro-electrode pair with five levels of gap-width of 20 μm, 30 μm, 40 μm, 50 μm and 60 μm, respectively. The microelectrode with different gaps aims to stretch many kinds of cells with different sizes on a chip and improve the universality of chip. Figure 2c shows the fabrication process of the microfluidic chip and details of chip fabrication can be referenced to our earlier work [26]. Electric field distribution in the microfluidic chip was simulated in Comsol Multiphysics 4.2 software. In this simulation, the relative permittivity of the medium was set to 80, and the conductivity 60 μs•cm^{-1}; an AC signal with amplitude of 5V_{pp} was applied to the microelectrodes. The simulation result of electric field distribution is shown in Figure 2d. Result shows that the strength of electric field is maximal in the edges of microelectrodes, and gradually weakens toward both sides. And the simulation result indicates that cells can be captured at the edges of electrodes(red in Figure 2d) when they subject to pDEP forces. So through the planar parallel microelectrodes, it is very suitable to intuitively observe DEP phenomena of cells.

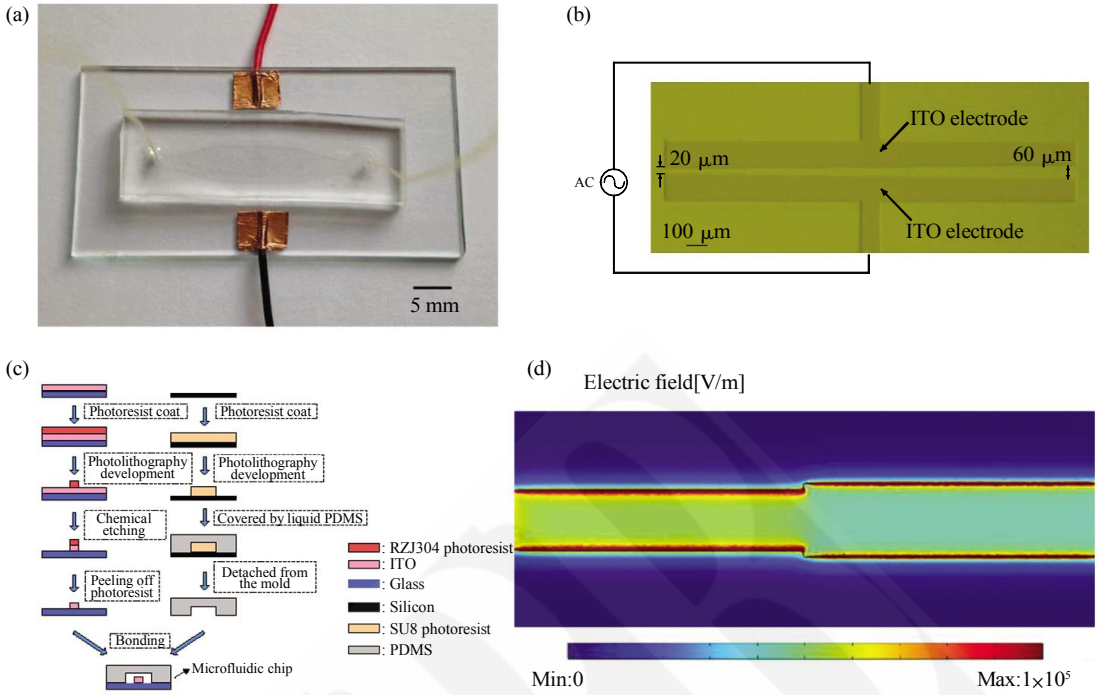

Fig. 2 A microfluidic chip for cell electro-stretching

(a) A microfluidic chip sample. (b) Image of microelectrodes under the microscope. (c) Fabrication process of the chip. (d) Simulation of the electric field distribution in the chip (red=max, blue=min).

An experimental flatform for observing cell electro-manipulation was firstly built, as shown in Figure 3a. Subsequently, we needed to test whether DEP technology could damage NB4 cells or not. DEP was used to capture cells and the viability of captured cells was tested by using SYTOX orange fluorescent dye. Under fluorescence microscope, dead cells emitted red fluorescence, while live cells did not, as shown in the top left and top right of Figure 3b, respectively. In the assay, cell suspension added with 0.5 μmol/L SYTOX orange fluorescent dye was injected into the microfluidic chip. NB4 cells were firstly captured at the edge of the electrodes by pDEP and photographed under the optical microscope, as shown in the below left of Figure 3b. Then through fluorescence microscope, captured NB4 cells were observed for 15 min and red fluorescence did not be found (see the below right of Figure 3b), indicating that DEP effect had not damaged NB4 cells. So the cell viability can be guaranteed in DEP experiments.

In order to observe DEP phenomena of NB4 cells, the designed microfluidic chip was prepared and the microelectrodes were supplied with a sinusoidal signal from a function generator, as shown in Figure 2a and b. The planar parallel microelectrodes are eminently suitable for intuitively observing DEP phenomena of cells. In the experiment, when the conductivity of cell suspension σ_e was 52.9 μs•cm^{-1}, the frequency of the *E*-field *f* was 1 MHz, and applied voltage *U* was $2V_{pp}$, NB4 cells were observed being captured at the edges of microelectrodes and pDEP phenomena appeared, as shown in Figure 3c and d. When the frequency was reduced to10 kHz, NB4 cells were observed detaching from the edges of microelectrodes and nDEP phenomena appeared, as shown in Figure 3e and f.

A concept of the system, including three key elements (i.e., cells, microelectrode and cell suspension), of the microfluidic with cell electro-manipulation functions based on DEP, was put forward for the first time. Only from the perspective of system to understand the microfluidic platform, cell electro-stretching method could be ascertained thoroughly. To investigate the basic working principle of this microfluidic system and obtain the pDEP

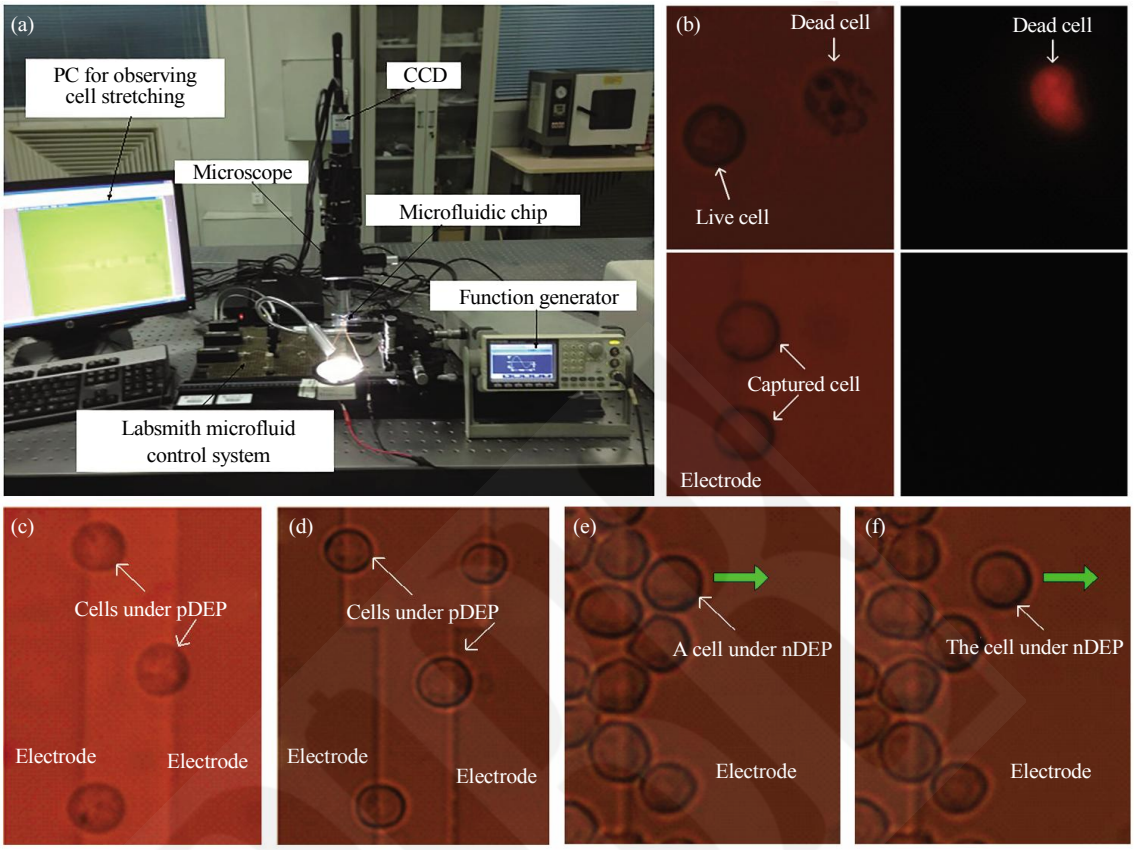

Fig. 3　Cell DEP experiments and the viability testing of NB4 cells

(a) An experimental platform for cell electro-manipulation. (b) The viability testing of NB4 cells; top left: live and dead cells under optical microscope; top right: the dead cell emitting red fluorescence under fluorescence microscope; below left: two cells captured by pDEP under optical microscope; below right: the captured cells not emitting red under fluorescence microscope. (c) and (d) pDEP phenomena of NB4 cells. (e) and (f) nDEP phenomena of NB4 cells. Green arrows represent the directions of cell movement.

experimental method of cells, DEP experiments were conducted and observed many times. It was found that the inherent relationship existed among the three key elements of cells，microelectrode and cell suspension. The following example will make it clear. As for as NB4 cells, the relationships between the real part of the polarization factor Re [K (f)] and frequency f at different conductivity of cell suspension σ_e (*e.g.*, *A*: σ_e=52.9 μs•cm^{-1}, *B*: σ_e=8500 μs•cm^{-1}) were found, as shown in Figure 4. Here, Re[K(f)] is also the real part of CM factor as the previously mentioned Re[$f_{CM}(\omega)$]; they are two functions with the same meaning but different variables(i.e., the former is f and the latter is ω). It is known that Re[K(f)] is bounded by Re[K(f)] ∈ [–0.5,1][19, 23], and pDEP response occurs if Re [K (f)] ∈ (0, 1), whereas nDEP response occurs if Re [K (f)] ∈ [–0.5, 0). In Figure 4, it was found that when σ_e was 52.9μs•cm^{-1} and f was in the range: 25 kHz<f<50 MHz, pDEP effect of NB4 cells occurred; whereas 10 Hz < f < 25 kHz, nDEP effect occurred; at this time the "cross-over" frequency f_0, which is defined by Re[K(f_0)]= 0, was about 25 kHz. When σ_e was 8 500 μs•cm^{-1}, only nDEP effect happened. In addition, we can hypothesize that there should be cell suspension with some conductivity value in the range from 52.9 μs•cm^{-1} to 8 500 μs•cm^{-1}, in which DEP effect of NB4 cells cannot appear and the cells are not affected by the electric field. These results are consistent with the reported results of MacQueen *et al*[27]. From Figure 4, in addition, it is found that, the higher conductivity of cell suspension σ_e is, the smaller real part of the polarization factor Re [K (f)] becomes. And a low conductivity of cell suspension corresponds to a "cross-over" frequency range of the E-field.

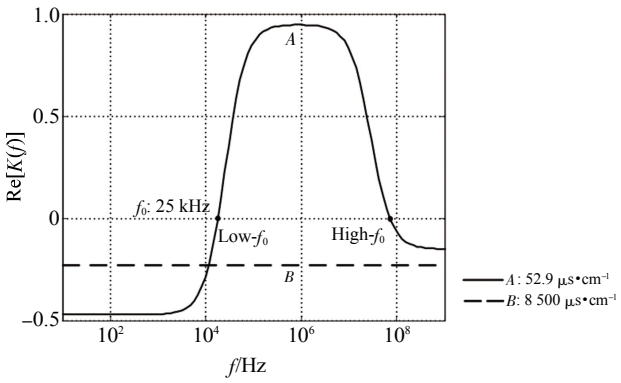

Fig. 4 Real part of the polarization factor, Re[K(f)], versus frequency, f, at different conductivity of cell suspension σ_e
(A: 52.9 μs•cm^{-1} and B: 8 500 μs•cm^{-1}), for NB4 cells.

During cell DEP experiments, a frequency sweep may be done to seek for the low or high "cross-over" frequency, *i.e.*, Low-f_0 or High-f_0 (Figure 4). When the frequency of the E-field is in the range from Low-f_0 to High-f_0, pDEP effect of cells can occur. As for NB4 cells, because the maximal frequency of our function generator (GWIUSTEK Taiwan AFG-3081) was limited to 50 MHz, only the low "cross-over" frequency appeared (e.g., 25 kHz). Sometimes, only the high "cross-over" frequency appeared (e.g., 13 MHz), when the DEP experiments of human esophageal squamous carcinoma KYSE150 were conducted. Therefore, to capture cells at the edge of the microelectrodes and make cells subject to pDEP forces, the conductivity of cell suspension σ_e should be adjusted to a lower level; meanwhile the frequency of the E-field should be selected in the intermediate-frequency range from Low-f_0 to High-f_0.

To validate the correctness of the optimized pDEP method and pDEP experimental parameters of cells, the Jones' cell protoplast model was discussed. NB4 cells were used as the sample cells. As previously mentioned, when the conductivity of cell suspension σ_e was 52.9 μs•cm^{-1}, the frequency (f) 1MHz and voltage (U) 2V_{pp}, NB4 cells were observed being captured at the edges of microelectrodes and pDEP effect occurred. Among the captured NB4 cells under the above experimental conditions, we took three cells as samples; *via* the ImageJ software, their radii were measured as: 6.77 μm, 7.08 μm and 7.37 μm, respectively. According to the Jones' cell protoplast model, the CM factor $f_{CM}(\omega)$, for living cells can be written as the Expression (4)[23, 28]:

$$f_{CM}(\omega)=\frac{\omega^2(\tau_e\tau_m-\tau_c\tau'_m)-1+j\omega(\tau'_m-\tau_e-\tau_m)}{2-\omega^2(\tau_c\tau'_m+2\tau_e\tau_m)+j\omega(\tau'_m+2\tau_e+\tau_m)} \quad (4)$$

An approximate expression for Re [$f_{CM}(\omega)$] of pDEP can be derived as the Expression (5)[23]:

$$\mathrm{Re}[f_{CM}(\omega)]_{pDEP}\approx\frac{\tau'_m-(\tau_e+\tau_m)}{\tau'_m+2(\tau_e+\tau_m)} \quad (5)$$

where $\tau_e=\varepsilon_e/\sigma_e$, $\tau_c=\varepsilon_c/\sigma_c$, $\tau_m=C_m r/\sigma_c$, $\tau'_m=C_m r/\sigma_e$; ε_e is the permittivity of cell suspension; ε_c is the permittivity of the cytoplasm; C_m is the effective capacitance of cell membrane, and r is the cell radius. Table 1 summarizes some experimental and classical protoplast model parameters of NB4 cells.

Table 1 Experimental and protoplast model parameters of NB4 cells (three cells were analyzed as samples)

Classic parameters[21, 25, 29]	Calculated parameters		
ε_0^*=8.854×10^{-12} F•m^{-1}	Cell *1*	Cell *2*	Cell *3*
$\varepsilon_c/\varepsilon_0$=78	r_1=6.77 μm	r_2=7.08 μm	r_3=7.37 μm
C_m=0.4 μF•cm^{-2}	τ_e=1.3×10^{-7}s	τ_e=1.3×10^{-7}s	τ_e=1.3×10^{-7}s
σ_c=0.01 s•cm^{-1}	τ_m=2.7×10^{-8}s	τ_m=2.8×10^{-8}s	τ_m=2.9×10^{-8}s
$\varepsilon_c/\varepsilon_0$=50	τ'_m=5.1×10^{-6}s	τ'_m=5.4×10^{-6}s	τ'_m=5.6×10^{-6}s
	τ_c=4.4×10^{-10}s	τ_c=4.4×10^{-10}s	τ_c=4.4×10^{-10}s

* ε_0 is the permittivity of a vacuum.

According to Table 1, Expression 4 and 5, the Re [$f_{CM}(\omega)$]$_{pDEP}$ of the three chosen NB4 cells were estimated. Through calculating, their average value was 0.916, which was in the range from 0 to 1. So pDEP effect of NB4 cells can occur under the above experimental conditions. *Via* the Jones' cell protoplast model, the experimental conditions for realizing pDEP effect of NB4 cells can be validated. So the findings

indicate that it is accessible to realize pDEP effect of cells rapidly and stably through our method. In a word, the first step of cell electro-stretching can be perfectly finished and cells can be immobilized well at the edge of the microelectrode instead of rotating and vibrating.

2.2 Investigation and optimization of experimental method for cell stretching

As we mentioned previously, a microfluidic chip with a parallel stepped-shape microelectrode pair with different gaps was designed in this work (Figure 2a). The parallel microelectrodes with different gap-widths are suitable for stretching cells with different sizes. Multiple cells can be captured and stretched simultaneously by our parallel microelectrodes (e.g., Figure 5), compared with traditional microelectrodes with tip-to-tip or tip-to-arc shape which only stretched a cell once[19–20]. And both left and right parts of parallel microelectrodes can be used to capture and stretch cells (e.g., Figure 5c and d). In addition, the length of the microelectrode section with 20 μm gap was designed to be 400 μm, and the distance between the centres of one cell and another adjacent cell along the electrode direction was estimated at about 24.7 μm; so about 16 cells can be stretched simultaneously in theory. Therefore, our designed microelectrodes are high efficient for cell electro-stretching.

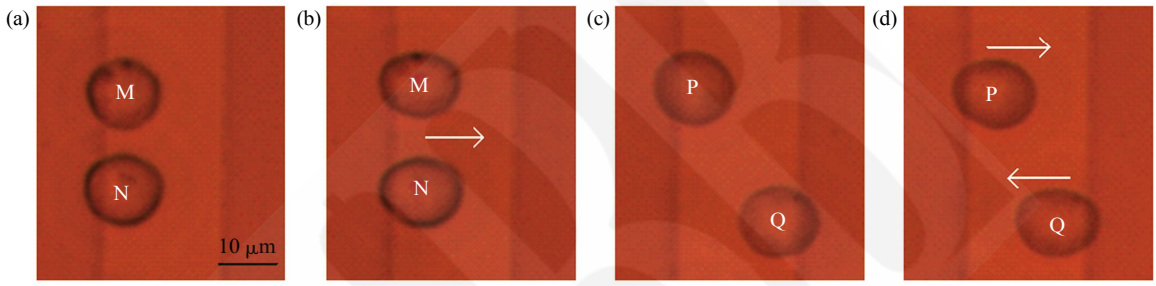

Fig. 5 Electro-stretching of NB4 cells based on the parallel microelectrodes

(a) Two cells named M and N were captured at the left edge of parallel microelectrodes. (b) They were stretched right simultaneously. (c) A cell named P was captured at the left edge of microelectrodes while another cell named Q was captured at the right edge. (d) The P and Q cell were stretched simultaneously in an opposite direction.

For effectively realizing cell electro-stretching and making preparations for a flexible choice of experimental parameters in the application, three important parameters such as conductivity of cell suspension, the applied voltage and frequency of the *E*-field, which could affect the deformation of cells, were roundly investigated in this paper.

Firstly, in order to investigate how the conductivity of cell suspension affects the deformation of cells, three groups of experiments about varied conductivity of cell suspension (i.e., *A*, *B* and *C* group) were arranged. *A* group: under the condition of $4V_{pp}$ and 1 MHz, 17 NB4 cells that suspended in cell suspension with the conductivity of 50 μs•cm^{-1} were stretched for 1 min; *B* group: under the condition of $4V_{pp}$ and 1 MHz, 19 NB4 cells that suspended in cell suspension with the conductivity of 52.9 μs•cm^{-1} were stretched for 3 min; *C* group: NB4 cells that suspended in cell suspension with the conductivity of 8 500 μs•cm^{-1} were tried to stretch. The stretching results of three groups of experiments were compared, as shown in Figure 6a. Here, the strain is defined as $(r-r_0)/r_0$, where r_0 denotes the radius of the initial cell shape, and $(r-r_0)$ denotes cell deformation. The result shows that cells are liable to deform when the conductivity value of cell suspension is relatively lower.

In favor of cell stretching, cell suspension is required to be not only low but also isotonic. Cells should be suspended in an isotonic solution (left of Figure 6b) before being stretched. In principle, any isotonic solution with low conductivity can be used to stretch cells. As for the made-up isotonic solution, the osmolality of solution needs to be measured by an osmometer, or other indispensable measures must be taken in the absence of osmometer. Here a simple method was provided to test the availability of the made-up solution. That is, firstly to measure the diameters of cells suspended in cell culture medium and obtain the average diameter of cells; then to measure cells suspended in the made-up solution and

average the diameters of cells. If the average sizes of cells in the above two kinds of solution are nearly equal, the made-up solution is available. Otherwise, to reduce the conductivity of the solution for cell stretching, deionized water was occasionally added into cell suspension. The consequence was that cell suspension became anisotonic; both cells and nuclei became bigger (right of Figure 6b); cells were damaged more or less. So at this time, the made-up solution was unavailable.

Secondly, in order to investigate how the applied voltage affects the deformation of cells, the strain of NB4 cells has been analyzed as a function of voltage which was stepwise increased (e.g., $3V_{pp}$, $4V_{pp}$, $5V_{pp}$ and $6V_{pp}$), followed by a stepwise unload phase, as shown in Figure 6c. The duration of each voltage step ($1V_{pp}$) during the loading or unloading was 3 min. Meanwhile, the frequency was chosen at 5 MHz. The result shows that the deformation of cells increases with applied voltage and vice versa, which is consistent with a previous study[30].

Thirdly, in order to investigate how the frequency of the *E*-field affects the deformation of cells, the corresponding experiment was executed. In the experiment, the applied voltages were increased in steps of $1V_{pp}$, e.g., $2V_{pp}$, $3V_{pp}$, $4V_{pp}$, $5V_{pp}$ and $6V_{pp}$. Every voltage kept unchanged for 3 min. Meanwhile, the frequency was set at 1 MHz or 5 MHz. The strains of NB4 cells at the different frequencies, e.g., 1 MHz and 5 MHz, are showed in Figure 6d. The results show that, at the same voltage the higher frequency is, the smaller deformation of cells is, and vice versa. And the analyzed tendency is consistent with a previous study[20]. In summary, the experimental results indicate that lower conductivity of cell suspension, increased applied voltage and decreased frequency of *E*-field can apparently promote cell deformation.

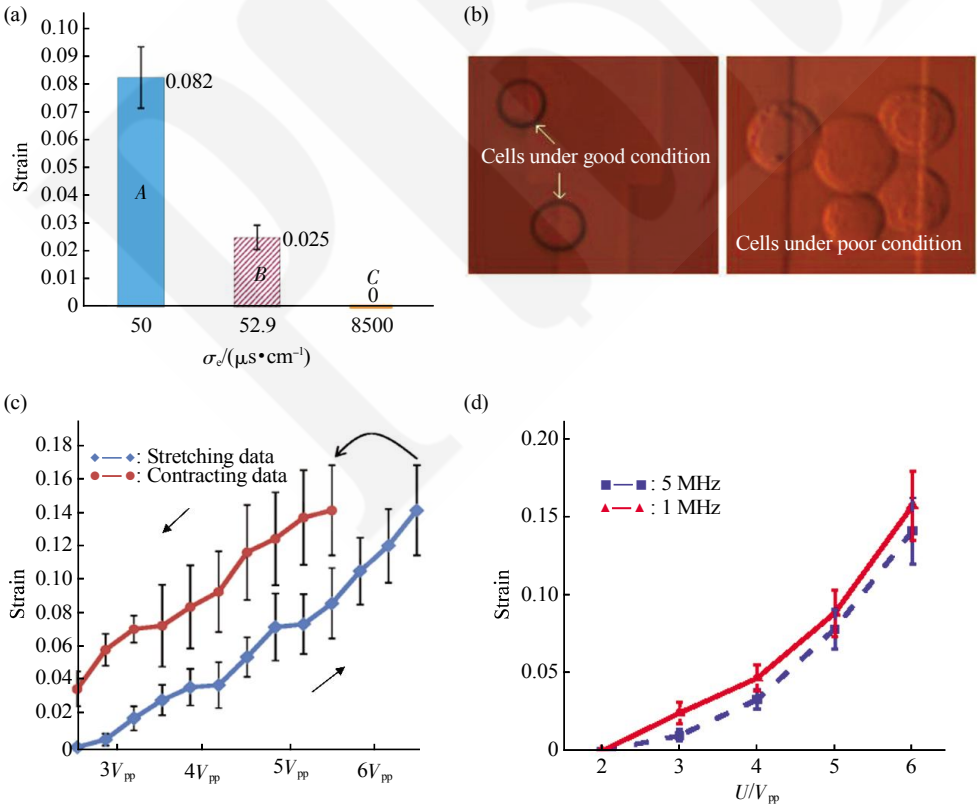

Fig. 6 Investigation of three parameters of affecting cell deformation and different cell morphologies

(a) Strain versus conductivity of cell suspension, σ_e (*A*: 50 μs•cm^{-1}; *B*: 52.9 μs•cm^{-1}; *C*: 8500 μs•cm^{-1}); (b) Left: NB4 cells suspending in isotonic solution; right: NB4 cells suspending in anisotonic solution; (c) Strain versus voltage, *U* (*U*: stepwise increase from $3V_{pp}$ – $6V_{pp}$ in steps of $1V_{pp}$ per 3 min, and followed stepwise decrease from $6V_{pp}$ – $3V_{pp}$); the one-way arrows indicate the step direction (n=15); (d) Strain as a function of voltage at two frequency, 1 MHz and 5 MHz; Sample size is twenty cells at 1 MHz or at 5 MHz, respectively. Strain values are mean±SE.

2.3 Application of the optimized cell electro-stretching method

Via the optimized cell electro-stretching method, the correlation between cell mechanical properties and cell functional variation was studied. Cellular stages of leukemia NB4 cells were altered by using drug doxorubicin(DOX), which was used to cure leukemia[31]. NB4-DOX cells derived from the NB4 cells which were treated by using 0.05 μmol/L DOX for 48 h. In order to analyze the stiffness difference between NB4 and NB4-DOX cells, we selected the same experimental conditions to conduct their stretching manipulations. Here we selected the cell stretching scheme which is characterized by variable voltage and constant frequency. The experimental parameters were selected based on the following considerations. Because the average diameter of NB4 cells was about 14 μm, only the microelectrode section with gap-width of 20 μm needed to be observed in the experiment. In this present study, the range of the "cross-over" frequencies was from 25 kHz to 50 MHz, so a frequency of 1.2 MHz may be selected for capturing and stretching of NB4 cells. According to the previous discussion, the applied voltage of $2V_{pp}$ can capture NB4 cells and $3V_{pp}$ or larger can make the cells deform. So $2V_{pp}$, $3V_{pp}$ or larger were selected to test NB4 cells. An isotonic medium with the low conductivity of 52.9 μs•cm^{-1} because of its little damage to cells, was selected to resuspend NB4 cells. And NB4 cells were observed to almost stop stretching after 3 min at a certain voltage.

In the experiment, timing started when the applied voltage was tuned to $2V_{pp}$. From 0 to 3 min, the applied voltage was set at $2V_{pp}$; from 3 min to 6 min, it was at $3V_{pp}$; from 6 min to 9 min, it was at $4V_{pp}$. After three independent tests of NB4 and NB4-DOX cells were conducted, respectively, the average strain of 33 NB4 cells and 30 NB4-DOX cells were analyzed and compared, as shown in Figure 7. It was found that at least applied voltage of $4V_{pp}$ can make NB4-DOX cells be stretched obviously, whereas $2V_{pp}$ or $3V_{pp}$ can hardly make them be stretched. By contrast, some NB4 cells started to be stretched at $2V_{pp}$ and most NB4 cells were obviously stretched at $3V_{pp}$ and $4V_{pp}$. The averaged strain of NB4 cells was 0.023 at 3 min after applied voltage was tuned to $4V_{pp}$, while that of NB4-DOX cells was 0.014.

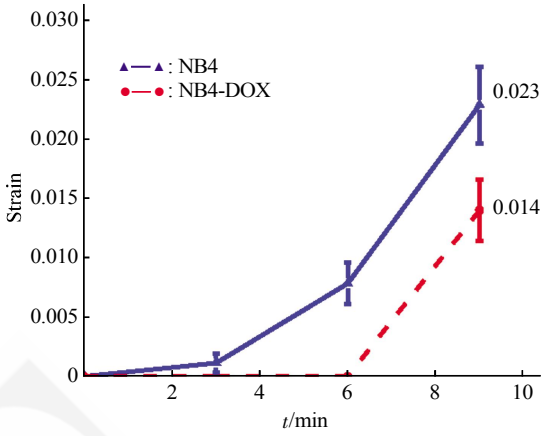

Fig. 7　Strain versus time, t (0<t<3 min: U=2V_{pp}; 3 min<t<6 min: U=3V_{pp}; 6 min<t<9 min: U=4V_{pp}), for NB4 and NB4-DOX cells, respectively

Values are mean±SE.

SAS 9.2 software was used to evaluate the statistical difference between the deformation behaviors of NB4 and NB4-DOX cells. Statistical significance was identified by two-sample independent *t*-test at 95% confidence level. For instance, Table 2 shows the running results of SAS about the differences between the deformation behaviors of NB4 and NB4-DOX cells at $3V_{pp}$ and $4V_{pp}$. From Table 2, when the applied voltage was $4V_{pp}$, *P*-value of *t*-test was 0.0379 which was smaller than 0.05, indicating that there was a significant difference between their deformations at $4V_{pp}$. For the same reason, there also was a significant difference between them at $3V_{pp}$. Therefore, the experimental results pointed out that the deformations of NB4 cells were significantly larger than those of the drug-treated group ($P < 0.05$), indicating that DOX treatment cause NB4 cells to stiffen significantly.

Table 2　SAS statistical results about t-test of the deformation difference between NB4 and NB4-DOX cells at 3V_{pp} and 4V_{pp}

Applied voltage	*P*-value of F-test	Equality of variances	*P*-value of *t*-test
$3V_{pp}$	<0.0001	unequal	<0.0001
$4V_{pp}$	0.1267	equal	0.0379

3 Discussion

Our optimized cell electro-stretching method aims to ensure high efficiency and reliability of cell electro-stretching. This paper emphasized the high efficiency of the improved microfluidic chip and the flexibility of selecting the experimental parameters. Surmounting the obstacle of cell heterogeneity, our method is a universal electro-stretching method for most cells in some sense.

Compared with the reported chip for cell electro-stretching [18-22], a microfluidic chip with a parallel stepped-shape microelectrode pair with different gaps was designed and improved. This paper discussed the outstanding advantages of our designed microelectrodes in cell electro-stretching. Firstly, *via* the parallel microelectrodes, DEP phenomena of cells can be observed easily; secondly, multiple cells can be captured and stretched simultaneously by the parallel microelectrodes; thirdly, the size difference between two adjacent gap-widths of our designed microelectrodes (i.e., 20 μm, 30 μm, 40 μm, 50 μm and 60 μm, respectively) is only 10 μm in turn, so the different gaps were sensitive to stretching of cells with different sizes.

A concept of the system of the microfluidic with cell electro-manipulation functions based on DEP, was presented for the first time. From the perspective of system to understand the microfluidic platform, besides ascertaining cell electro-stretching method thoroughly, we also expect, in near future, to realize the other cell electro-manipulations (e.g., cell sorting, cell fusion, cell rotation and so on) based on the complicated chip-structures. But the other literatures of cell electro-stretching have not concerned this respect[18-22].

This paper roundly revealed the experimental method of selecting pDEP parameters and its theoretical foundation. The optimized pDEP method pointed out that the experimental parameters for capturing cells were not unique, and the rationality of the selected pDEP experimental parameters can be validated through the Jones' cell protoplast model theory in this paper. However, other most literatures have hardly elucidated the flexibility and evidence of selecting the pDEP parameters[18-22]. The experimental results also indicated that the parameters of cell stretching was in an appropriate range, rather than a fixed value; while most literatures [18-19, 21-22] only reported some fixed parameters to stretch cells. As the prerequisites of cell deformation, the conductivity of cell suspension is required to be low, but the isotonicity of cell suspension is easily overlooked. Here this paper emphasized this respect.

Several research groups have revealed some biomarkers reflecting the chemotherapeutic effects of anticancer drug doxorubicin (DOX) on leukemia cells. For instance, He *et al.*[32] reported that high expression levels of Ikaros may be a potential biomarker to predict leukemia patients' chemotherapeutic effect and found that overexpression of Ikaros could enhance DOX-induced apoptosis of leukemia NB4 cells. Deng *et al.*[33] indicated that a biomarker, intrinsic fluorescence of DOX, was detected to obtain the information about DOX uptake and membrane surface P-glycoprotein expression in single leukemia K562 cells. Hu *et al.*[34] used cell viability as a biomarker to study the effects of doxorubicin with different concentrations on the acute leukemia (HL-60) cells. Here a distinctive biomarker, cell mechanical properties, was discussed in our study. From the perspective of cell mechanical properties, it was found and demonstrated that DOX can make leukemia NB4 cells produce apoptosis and stiffen. As mentioned before, through the optimized cell electro-stretching method, we ensured high efficiency and reliability of the tests of cell mechanical properties at the single cell level and compared the mechanical behaviors of NB4 cells before and after DOX treatment. And *via* the theory of statistical significance, drug-treated leukemia cells which are different significantly from the untreated leukemia cells in the stiffness can be easily obtained and studied. So it is expected to explore the low-dose and short-term chemotherapeutic regimens in the treatment of leukemia, which will make a significant sense to clinical reference in the future. Our method is worth spreading in clinical practice for drug discovery and screening quickly and inexpensively.

In summary, this paper demonstrated an optimized cell electro-stretching method and a correlation existed between cell mechanical properties and cell functional variation of leukemia NB4 cells, based on DEP-coupled microfluidic chip. A concept of the system of the microfluidic with cell electro-manipulation functions based on DEP, was raised for the first time. Results demonstrate that it is easy to capture cells at the microelectrodes, if the conductivity of cell suspension σ_e is so low as to make

“cross-over” frequencies appear; meanwhile the frequency of the *E*-field f is in the range of the “cross-over” frequencies from Low-f_0 to High-f_0. It is easy to stretch the captured cells, if the conductivity of cell suspension σ_e is lower than that of cell cytoplasm σ_c (i.e., $\sigma_e<\sigma_c$) and cell suspension is isotonic, and if the applied voltage is increased or the frequency is decreased.

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细胞电拉伸方法的优化和阿霉素对白血病 NB4 细胞的硬度影响分析 *

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摘要 研究表明，细胞的变形能力能够作为一个有效的生物标志，用来指示疾病的发展状况．由于细胞异质性的障碍，发现利用已报道的实验条件对新细胞进行电拉伸很困难．本论文中，一种基于介电泳 - 微流控的细胞电拉伸方法得到优化，并利用此优化方法进行了阿霉素对白血病 NB4 细胞硬度影响的调查．首先，用于细胞捕获的正向介电泳(pDEP)方法被优化和证实．结果指出，如果细胞缓冲液的电导率足够地低，低至细胞的截止频率能够出现，同时，电场的频率被调至高低截止频率范围之间时，细胞的 pDEP 效应容易实现．其次，系统地讨论了影响细胞变形量的三个参数．结果指出，细胞缓冲液的电导率低于细胞质的电导率是细胞电变形的先决条件，此外，增大电压或减小频率也能促使细胞变形．最后，借助优化的细胞电拉伸方法，调查了细胞机械特性和细胞功能变化之间的关系．结果表明，阿霉素使 NB4 细胞产生凋亡而变硬．总之，本文提出了一种新的细胞电拉伸方法，以便对细胞的变形能力进行简单有效的检测．

关键词 电拉伸，微流控，细胞硬度，正向介电泳，截止频率

学科分类号 TN405 **DOI**: 10.16476/j.pibb.2016.0356

* 国家杰出青年科学基金(51425505)，山西省百人计划，山西省青年拔尖人才和山西省 131 领军人才资助项目.

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收稿日期：2016-11-15，接受日期：2018-01-17