On-chip immunofluorescence analysis of single cervical cells using an electroactive microwell array with barrier for cervical screening

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ABSTRACT

Several specific tests for cervical screening have been developed recently, including p16/Ki67 dual immunostaining for diagnosing high-risk human papillomavirus positive squamous intraepithelial lesion in the cervix. However, manual screening of cells in an entire glass slide is currently a standard clinical procedure for quantification and interpretation of immunocytochemical features of the cells. Here, we developed a microfluidic device containing an electroactive microwell array with barriers (EMAB) for highly efficient single-cell trapping followed by on-chip immunofluorescence analysis with minimum loss of the sample. EMAB utilizes patterned electrodes at the bottom of cell-sized microwells to trap single cells using dielectrophoresis (DEP) and cell-holding structures behind the microwells to stabilize the position of trapped cells even without DEP. Using the device, we evaluated the performance of p16/Ki67 dual immunostaining of HeLa cells on the chip. The device shows 98% cell-trapping efficiency as well as 92% cell-holding efficiency against the fixed HeLa cells, and we successfully demonstrated high-efficiency on-chip immunofluorescence analysis with minimal loss of sample. p16/Ki67 dual immunostaining using EMAB may be useful for complementary tests for cervical screening in confirming the histopathological diagnosis.

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INTRODUCTION

It is well known that cancer has highly heterogeneous cell subpopulations.^{1,2} The origins of the subpopulations that contribute unequally to carcinogenesis remain a subject of controversy when considering multiple carcinogenesis in some types of cancers.³ The same is true in cervical carcinogenesis, and the persistent infection of high-risk human papillomavirus (HPV) in the cervix is clearly a pivotal promoter in causing cervical precancerous lesions and cancer. The molecular pathogenesis of cervical cancer has been well studied and was found to be extremely complicated and heterogeneous.^{4,5}

To date, cervical cancer screening has traditionally been based on cervical cytology. Given the etiological relationship between

HPV infection in the cervix and cervical carcinogenesis, HPV DNA testing has been currently applied into clinical practice, and it could predict the risk of cervical cancer by detecting the high-risk HPVs, such as types 16 and 18. However, HPV DNA testing itself provides more sensitivity but less specificity compared with cervical cytology, and hence, the number of high-risk HPV-positive women for unnecessary colposcopy-guided cervical biopsy is increased. Therefore, biomarkers for diagnosis and treatment need to be established for accurate detection and for the prevention of false positives.

p16 (also known as p16INK4a) is a cyclin-dependent kinase inhibitor that is overexpressed when the cell-cycle regulatory

retinoblastoma protein is inactivated by high-risk HPV E7 oncoproteins, and p16 expression is considered to be a surrogate marker during cervical carcinogenesis.⁶ On the other hand, Ki-67 is a marker of cell proliferation, which is a key feature of malignant progression and is highly correlated with the grade of dysplastic change in the epithelium.^{7,8} p16 and Ki-67 have long been used in histology to detect and confirm the presence of cervical precancerous lesions.^{9–11} The diagnostic accuracy of p16/Ki-67 dual staining of cytology samples has recently been tested,^{12–14} and the studies concluded that this method had similar sensitivity and significantly higher specificity when compared with HPV testing among women with normal and abnormal cytology.^{15,16}

Based on these results, CINTec PLUS p16/Ki-67 dual staining cytology (Roche, Tucson, USA) is currently available for testing of abnormal cervical cytology of women for further management and treatment. Fully automated p16/Ki-67 dual staining of cytology on the industry-leading VENTANA instruments (Roche) is also available and has proven to achieve diagnosis faster than manual staining methods. In addition, Wentzensen and von Knebel Doeberitz have observed good performance of p16/Ki-67 dual staining cytology for cytologists without any previous experience.¹⁷ However, large amounts of cells are aggregated when collected cells are transferred to the microscope slides for the conventional methods of liquid-based cytology, which hinders imaging and reliable analysis of individual cells. Fluorescence-activated cell sorting (FACS), a high-throughput and quantitative method for identification of individual cells, could be used for the suspended cells after immunostaining. However, sequential centrifugations and reagent changes, required for staining suspended cells, possibly cause a critical loss of the limited number of target cells, which is not well suited for widespread high-throughput analysis of suspended cells.

Microfluidic approaches have been employed for the efficient immunostaining by taking advantage of the high surface-to-volume ratio of microfluidic devices.^{18–21} However, previous microfluidic systems are mainly targeting analysis of tissue samples and cannot be used for assaying suspended cells without individual cell trapping. Recently, hydrodynamic single-cell trapping methods have been developed for arraying suspended single cells and employed for the observation of cellular responses.^{22–24} Highly improved single-cell trapping yield has been achieved by the optimization of flow profiling.^{25,26} However, a practical problem of physical structure-based trapping is that the cell bodies physically block the flow stream when the cells get trapped into the physical structures. Since the size of the microfluidic channel is comparable with that of cells, its blocking causes a drastic increase in the resistance. This would prevent an efficient exchange of various reagents required for the assay of trapped cells. We have developed a microfluidic device containing an electroactive microwell array (EMA) for trapping single cells using positive dielectrophoresis (PDEP), followed by the analysis of trapped single cells.^{27–29} Although we successfully demonstrated efficient single-cell trapping and analysis, the device could not be used for on-chip immunostaining requiring various reagents because the high conductivity of the reagents used for the assay was not compatible with PDEP. The trapped cells were flushed away during the sequential change of reagents.

In the present study, we present an advanced microfluidic device containing an electroactive microwell array with barriers

(EMAB) for highly efficient single-cell trapping, followed by on-chip immunofluorescence analysis with minimal loss of trapped cells. EMAB allows for highly efficient trapping of single cells into cell-sized microwells using PDEP, and the trapped cells stably maintain the position because of the cell-holding structure (barriers) behind the microwells even without DEP. We evaluated the performance of EMAB on single-cell trapping by trapping formalin-fixed HeLa cells (cervical cancer cell line) and on cell holding by monitoring the release of trapped cells after turning off DEP. The feasibility of the on-chip immunofluorescence analysis was demonstrated by carrying out p16/Ki-67 dual staining on the device.

MATERIALS AND METHODS

Device fabrication

EMAB was fabricated with the three-step fabrication processes of electrodes, microwells, and barriers. First, the interdigitated indium tin oxide (ITO) electrodes were fabricated on a glass substrate with conventional photolithography and wet etching processes. The shape of the interdigitated electrodes was patterned on an ITO coated glass substrate (GEOMATEC Co., Japan) using a positive-type photoresist (S1813; Shipley Far Ltd., USA). The ITO was wet-etched by dipping the photoresist patterned substrate into an ITO etchant (ITO-02; KANTO KAGAKU Co., Japan) for 5 min at 40 °C. Next, the remaining photoresist was removed by using acetone and isopropyl alcohol. The microwells for single-cell trapping were then fabricated with a photoresist on the patterned ITO electrodes. A negative-type photoresist (SU-8 3005, MicroChem Co., Newton, MA) was coated on the ITO substrate to a thickness of 4 μm and then soft-baked at 95 °C for 2 min. A chromium photomask that was patterned with the microwell array was aligned with the patterned ITO electrodes and exposed to ultraviolet light. The substrate was then post-expose-baked and was subsequently developed using an SU-8 developer (propylene glycol monomethylether acetate; GODO Co., Ltd., Japan) and rinsed with isopropyl alcohol (Wako Pure Chemical Industries, Ltd., Japan) following the manufacturer's instruction. Finally, the barriers for cell holding were fabricated on the microwell array. To fabricate barriers, the microwell substrate was coated with SU-8 3010 to a thickness of 12 μm. The substrate was then soft-baked and a chromium photomask patterned for the barrier in an array was aligned with the microwells in an array and exposed to ultraviolet light. The substrate was then post-expose-baked and subsequently developed and rinsed with isopropyl alcohol following the manufacturer's instruction. One microwell array contains 5000 microwells with barriers.

The polydimethylsiloxane (PDMS) microfluidic channel for cell delivery was fabricated through a standard replica-molding process. A mold master was fabricated with a negative-type photoresist (SU-8 3035, MicroChem Co., USA). SU-8 3035 was coated onto a silicon wafer to a thickness of 40 μm. The wafer was then soft-baked, and a chromium photomask patterned for the fluidic channel was aligned with the silicon wafer and exposed to ultraviolet light. The wafer was then post-exposure-baked, developed, and rinsed following the manufacturer's instruction. The mold master was exposed to CHF₃ plasma and coated with a fluorocarbon layer using a reactive-ion etching machine (RIE-10NR; Samco Co.,

Japan). A prepolymer of PDMS (Siloport 184; Dow Corning Toray, Co. Ltd.) was mixed with a curing reagent (at a 10:1 mass ratio) and poured into the mold master. The PDMS was cured at 75 °C for 2 h and then removed from the mold. After making holes on the PDMS to serve as access ports to the flow channels, the PDMS channel and the microwell substrate were exposed to O₂ plasma using a reactive-ion etching machine and bonded together. Before use, the device was washed with ethanol and water, and subsequently filled with a low conductivity buffer for DEP.

Cells and reagents

HeLa (CCL-2) uterine cervical cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium was DMEM (Gibco) supplemented with fetal bovine serum (10%, Gemini Bio-Products) and penicillin-streptomycin solution (1%, Sigma Chemical Co.). Cultured cells were trypsinized and the suspended cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries Ltd.) for 5 min at room temperature. Formalin-fixed HeLa cells were stocked in phosphate buffered saline (PBS) at 4 °C. Before using the cells, fixed cells were resuspended in a low conductivity buffer [1% nonionic surfactant, Pluronic F-68 (PF-68, Gibco), dissolved in MilliQ water] to induce PDEP.

Experimental setup

The microfluidic device was mounted on the x-y translational stage located on an inverted microscope (IX 71; Olympus, Japan). Cells in the device were monitored with a digital CCD camera (ORCA-R2; Hamamatsu Photonics, Japan) installed on the microscope. The electric potential for DEP trapping was applied to the ITO electrodes using a function generator (WF1948; NF Corp., Japan). The outlet of microfluidic device was connected to a gas-tight glass syringe (Hamilton Company, USA), and the syringe was operated with a precisely controlled syringe pump (MFS-SP1; Microfluidic System Works Inc., Japan).

Cell trapping

Fixed cells, applied at an access port of the device, were delivered into the microfluidic channel by withdrawing a gastight glass syringe using a syringe pump. The cells were trapped into the electroactive microwells by applying electric potentials to the ITO electrodes. The ratio of the trapped cells to the cells flowing over the microwells was calculated. After trapping, the DEP force was turned off to verify the effect of barriers on the cell holding without DEP, so that the trapped cells can be stably held on a microwell by the barrier. The number of remaining cells was determined after 1 min with various flow rates to calculate the cell-holding efficiency, a percentage ratio of the remaining cells to the trapped cells.

On-chip immunostaining

On-chip immunostaining of HeLa cells targeting Ki-67 and p16 proteins was carried out using a standard immunostaining protocol. The reagents for immunostaining were sequentially loaded onto the inlet of the microfluidic device after turning off DEP.

After trapping fixed cells, 0.05% tween 20 (Sigma) in PBS (PBST) was introduced into the device to wash out the remaining buffer. Trapped cells were permeabilized with 0.3% Triton X-100 in PBS for 5 min and blocked with 3% BSA in PBS for 60 min. They were stained with a cocktail of primary antibodies for Ki67 (1:500, ab15580) and p16 (1:500, ab16123) for 60 min. After washing with PBST for 20 min, secondary antibody for Ki67 (Alexa Fluor 488, ab150105), p16 (Alexa Fluor 594, ab150080), and DAPI (300 nM, Thermo Fisher Scientific) were sequentially introduced.

RESULTS AND DISCUSSION

Design and working principle

EMAB consists of a PDMS microfluidic channel for cell delivery and a microwell array substrate for cell trapping. The microwell array substrate contains a large number of cell-sized electroactive microwells (4 μm in thickness and 24 μm in diameter) for deterministic single-cell trapping and barriers (12 μm in thickness) behind the microwells for holding the cells [Fig. 1(a)]. Each microwell contains a pair of ITO electrodes at the bottom of the microwell array to induce DEP. The distance between the electrodes is 10 μm. The PDMS microfluidic channel (40 μm in height) allows us to introduce cell suspensions or reagents for efficient analysis [Fig. 1(c)].

To trap the cells, EMAB utilizes DEP, a phenomenon in which a force is exerted on a particle (cell) when it is immersed in a nonuniform electric field. The DEP force (F_{DEP}) can be approximated by

$$F_{DEP} = 2\pi\epsilon_e a^3 \text{Re}[K(2\pi f)] |\nabla|E|^2, \quad (1)$$

$$K(2\pi f) = \frac{\epsilon_{cell}^* - \epsilon_e^*}{\epsilon_{cell}^* + 2\epsilon_e^*}, \quad (2)$$

where $\epsilon^* = \epsilon + \frac{\sigma}{2\pi f}j$ is the complex permittivity, and ϵ_e , a , f , E , and σ are the permittivity of the medium, radius of cell, frequency, electric field strength, and conductivity, respectively. The subscripts *cell* and *e* represent the cell and the external medium. When the real part of the polarization factor, $\text{Re}[K(2\pi f)]$, is larger than 0, cells are attracted toward the strong electric field (PDEP). Conversely, when $\text{Re}[K(2\pi f)]$ is less than 0, cells are directed away from the strong electric field (negative DEP, NDEP). The real part of the polarization factor for the target cells can be controlled by adjusting the conductivity of the external medium and the frequency of the applied electric fields.

In the present configuration of the device, PDEP should be induced on the cells to attract them to the high electric field regions at the bottom of microwells. The fixed cells were used for the demonstration because the clinical samples are fixed immediately after collection for the preservation in a usual clinical setting. DEP responses on live or fixed cells with respect to the conductivity of the external medium and the frequency of the applied electric field were investigated by using the ITO electrode (Table 1). The live cells showed PDEP responses at a megahertz-order electric field with moderate conductivity of 280 and low conductivity of 33 and 1.7 mS/m. When the cells were fixed with 4%

FIG. 1. Design of the EMAB. (a) Cell trapping and holding using EMAB. Cells are trapped into the cell-sized microwells using PDEP. The barriers behind the microwells hold the position of the trapped cells even without DEP. (b) A photo of the fabricated device. Thin microwells were aligned with the interdigitated electrodes patterned on the glass substrates. The barriers were fabricated behind the microwells. Scale bars are 50 μm . (c) Immunostaining procedure in the device. Immunostaining was carried out by sequentially loading cell suspensions and required reagents into the microfluidic device.

paraformaldehyde, the fixed cells showed PDEP responses with low conductivity of 33 and 1.7 mS/m. However, the fixed cells showed nDEP when they were suspended in moderate (280 mS/m) and high conductivity buffers including PBST, which were required to stain the cells. This result indicates that DEP should be turned off during immunostaining because it cannot be used for cell trapping, since it would push the cells away from the microwells for the high conductivity buffer. To overcome this issue, barriers were fabricated behind the microwells to hold the trapped cells after turning off DEP.

Cell-trapping and holding efficiency of EMAB

The inherent efficiency of EMAB on cell trapping and holding was investigated by using fixed HeLa cells, a human cervical cancer cell line. The trapping and holding efficiency for microwells with/without barrier were investigated. Fixed HeLa cells stained with DAPI (around 600 cells) were introduced into the device, and the

cells were trapped into the microwell array by using PDEP. Without DEP, no cells were trapped into the microwells at the present flow rate of 2 $\mu\text{l}/\text{min}$. Cells were trapped in the microwell using a PDEP by applying a sinusoidal electric potential (peak-to-peak voltage, $V_{pp} = 5\text{ V}$ at 1 MHz) to the electrodes (Fig. 2). No significant damage to the fixed cells was observed with the present experimental condition. Microwells were gradually occupied by single cells (movie in the supplementary material). When a microwell was occupied with a cell, a second cell was prevented from entering. The cell-trapping capability of the device highly relies on the total number of microwells in the microfluidic device. To improve the cell-trapping capabilities of the device, the number of microwells should be increased.

To evaluate cell-trapping efficiency, the number of cells flowing over the microwell array (N_i) was determined for 3 min, and the number of trapped cells (N_t) was determined after 3 min. Cell-trapping efficiency was calculated from $N_t/N_i \times 100\%$. Figure 3 shows the cell-trapping efficiency as a function of the magnitude of

TABLE I. DEP responses of live or fixed HeLa cells suspended in a medium having various conductivities with respect to the frequency of applied electrical potential. The HeLa cells were fixed with 4% paraformaldehyde (N, negative DEP response; P, positive DEP response).

Medium	DEP responses of HeLa cells							
	10 kHz		100 kHz		1 MHz		10 MHz	
	Fixed	Live	Fixed	Live	Fixed	Live	Fixed	Live
PBST	N	N	N	N	N	N	N	N
Conductivity: 1.6 S/m								
10% PBS, 1% PF-68	N	N	N	N	N	P	N	P
Conductivity: 280 mS/m								
1% PBS, 1% PF-68	N	N	P	P	P	P	P	P
Conductivity: 33 mS/m								
MilliQ, 1% PF-68	P	P	P	P	P	P	P	P
Conductivity: 1.7 mS/m

FIG. 2. Time-lapse images of an EMAB during fixed HeLa cell trapping. Yellow arrows indicate cells trapped into the microwells using PDEP. When a cell occupied a cell-sized microwell, a second cell was prevented from entering the same microwell because of space restriction. Scale bars are 50 μm .

electrical potentials for EMA and EMAB. Cell-trapping efficiency was increased with the magnitude of electrical potentials. EMA shows slightly better trapping efficiency than EMAB at the lower electrical potential of 6 V. However, both EMA and EMAB show very good trapping efficiency at the higher electrical potential of 12 V, where trapping efficiencies of EMA and EMAB were $98 \pm 2\%$ and $98 \pm 1\%$, respectively.

The cell-holding abilities of EMA and EMAB were evaluated by turning off the DEP force after cell trapping. The cells were introduced into the device and trapped by using PDEP for 3 min, and the number of trapped cells (N_t) was counted. Next, DEP was turned off and the buffer was flowed with

various flow rates for 10 min, and the number of remaining cells in the microwell array (N_r) was counted. Cell-holding efficiency of the device was calculated from $N_r/N_t \times 100\%$. Figure 4 shows the cell-holding efficiency with respect to the various flow rates. The cell-holding efficiency was gradually decreased with the increase of the flow rate because the shear stress by the bulk flow in the microfluidic channel pushed the cells away from the microwells. The cell-holding efficiency of EMAB ($84\% \pm 9\%$) was much higher than that of EMA ($41\% \pm 13\%$) at the flow rate of $5 \mu\text{m}/\text{min}$. The results indicate that the barriers behind the microwells efficiently hold the position of the trapped cells even without the DEP.

FIG. 3. Cell trapping efficiency as a function of electrical potential used to induce DEP. Fixed HeLa cells were trapped into the microwells with or without barriers by applying various electrical potentials. The number of trapped cells was determined after cell trapping. The cell-trapping efficiency increased with electrical potential.

FIG. 4. Cell-holding efficiency as a function of flow rate. After trapping, the DEP force was turned off and the number of remaining cells was counted after 1 min. The holding efficiency significantly decreased with an increase in flow rate for the microwells without a barrier. On the other hand, EMAB showed a good holding efficiency because the barriers physically held the cells.

On-chip immunostaining using EMAB

The feasibility of EMAB on stable cell holding was demonstrated by carrying out on-chip immunostaining for fixed HeLa cells targeting p16 and Ki67 proteins. The p16 and Ki67 proteins have emerged as important biomarkers for the detection of high-risk HPV associated with dysplastic changes in the cervix. Fixed HeLa cells suspended in low-conductivity buffer were introduced into the microfluidic device and trapped into EMAB by inducing PDEP. Afterward, complex immunostaining procedures, including washing, permeabilization, blocking, and staining, were carried out by sequentially loading required reagents into the inlet of the device after turning off DEP. The reagents were delivered into the microfluidic channel at a flow rate of 2 μ l/min and sequentially reacted with the trapped cells. The trapped cells were successfully stained on the EMAB, and, especially, localization of Ki67 proteins in the nucleus was clearly detected in the HeLa cells (Fig. 5). To evaluate the cell staining efficiency, fixed HeLa cells stained with DAPI were trapped, and the number of trapped cells in the appropriate location in the microfluidic device was counted. After on-chip immunostaining, the number of remaining cells at the same location was counted. The EMAB showed very high on-chip staining efficiency of 99%, where 360 out of 362 cells were retained and successfully stained on the EMAB.

To demonstrate the feasibility of the present device for the different type of cancer cells, on-chip immunostaining for

SK-OV-3 cells (ovarian cancer cell line) was carried out, where SK-OV-3 cells lacked p16 expression.³⁰ The fixed SK-OV-3 cells were trapped into the EMAB by applying 9 V at 1 MHz to the electrodes. The EMAB shows good trapping efficiency of $97\% \pm 3\%$ for the SK-OV-3 cells. The trapped cells were stained by sequentially loading required reagents for washing, permeabilization, blocking, and staining for DNA, p16, and Ki67 proteins. The p16-negative SK-OV-3 cells showed positive signals for Ki67 but negative signals for p16 (Fig. S1 in the [supplementary material](#)). These results indicate that the fixed SK-OV-3 cells were properly stained in the device, and the present system could be used for the staining of suspended cells by just sequentially loading cell suspension and required reagents.

We successfully demonstrated on-chip immunostaining of suspended cells by using the EMAB microfluidic device. The present device eliminates laborious centrifugation and resuspension process, which shortens the whole processing time (4 h) compared to the conventional immunostaining process (5 h). Moreover, thanks to the high surface-to-volume ratio of the microfluidic device, a much smaller volume of reagents was required for the immunostaining. For instance, only 120 μ l of the primary antibody was required for the microfluidic device but 1 ml for conventional bulk immunostaining. Finally, no additional process was required to spread the cells on the microscope slide, which would cause the loss of target cells and aggregation of processed cells. These unique features of the EMAB would be beneficial for the immunostaining of suspended cells, which would facilitate reliable analysis of suspended clinical samples for the liquid-based cytology.

CONCLUSION

In this report, we have presented a novel microfluidic device that utilizes EMAB allowing highly efficient single-cell trapping and stable holding of trapped cells without attraction forces. On-chip immunofluorescence analysis using the EMAB was successfully demonstrated by sequentially loading target cells and required reagents for staining with minimal loss of sample. To that end, a microfluidic device has been efficiently utilized to develop the present system, as it allows for precise control of reagent application, facile imaging, and the ability to manipulate single cells with well-defined geometries. Future studies to improve the device and clinical trials to investigate the usefulness of the device are warranted.

SUPPLEMENTARY MATERIAL

See the [supplementary material](#) for the movie of cell trapping and the result of on-chip immunostaining of SK-OV-3 cells.

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FIG. 5. Single-cell immunostaining assay of HeLa cells. After trapping, the DEP force was turned off and required reagents for the immunostaining procedure (permeabilization, blocking, and labeling) were introduced sequentially. Only single cells were trapped into EMAB and target proteins including Ki67 and P16 were successfully stained on the microfluidic device. The Ki67 proteins, a nuclear protein expressed in proliferating mammalian cells, were localized in the nucleus. Meanwhile, the P16 proteins were expressed in both the nucleus and the cytoplasm. The merged image clearly shows that the positions of Ki67 proteins overlap with that of DNA (blue) indicating that the Ki67 proteins (red) are localized in the nucleus, but the stained area of P16 proteins (green) is larger than that of Ki67 proteins indicating that P16 proteins are expressed in both nucleus and cytoplasm. Scale bar is 100 μ m.

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