

# QUANTITATIVE ANALYSIS OF GENE EXPRESSION LEVEL OF INDIVIDUAL iPS CELLS BY USING ELECTROACTIVE MICROWELL ARRAY

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## ABSTRACT

We propose a method utilizing large number of arrayed microwells for quantitative measurement of GFP gene expression that is directly coupled with pluripotency marker-protein, Nanog, at the single-cell level among the cell population. To confine intracellular GFP, individual cells are trapped into microwells and lysed after closing the microwells by inducing electric potential to the electrodes at the bottom of each well. The microwell structure has a comparable size to the target cells making it possible to trap single cells and restrict the dilution of cell lysate. The individual intracellular GFP concentration is precisely quantified inside of the well-defined volume of microwells. Our method will be a useful tool for high-throughput and parallelized read-outs of gene expression level of individual cells in a large population of cells.

## KEYWORDS

Microwell array, Stem cell, Single cell analysis, Gene expression.

## INTRODUCTION

Gene expression levels are routinely measured on cellular ensembles with unavoidably averaging effects. However, the direct measurement at the single-cell level is required since cellular gene expression levels may fluctuate between individual cells. Especially, to understand heterogeneity on cellular differentiation of stem cells, gene expression level should be analysed at the single-cell level. Recently, a selection system for Nanog expression, which is associated with pluripotency of the stem cell, was developed for induced pluripotent stem cell (iPS) [1]. Undifferentiated Nanog iPS cells express GFP that is genetically encoded as a marker of Nanog expression. However, quantitative analysis of Nanog gene expression for individual cells has not been achieved due to the lack of experimental methodologies. Here, electroactive microchambers array [2] is proposed for the quantitative analysis of GFP gene expression of individual Nanog iPS cells to estimate Nanog gene expression level. To obtain confined intracellular materials of single cell, target cells are continuously introduced, and trapped into the microwell array by using DEP (Fig. 1). Microwells are closed by pressing a PDMS membrane for the isolation of individual cells. The trapped cells are lysed inside of the closed microwell with EP. Lysates of individual cells fill the closed microwells, allowing quantitative analysis of the signal from the intracellular materials. With the device, we have demonstrated quantification of GFP gene expression for individual iPS cells.

## EXPERIMENTAL

Our device consists of a microwell array surface and a patterned PDMS membrane, which are bonded to each other to form the microfluidic channel (Fig. 2a). First, we patterned interdigitated ITO electrodes on a glass substrate to attract cells by DEP and perform lysis by EP (Fig. 2b). A 500 nm thick layer of ITO was sputtered on a glass substrate for the electrodes. The shape of the electrodes were patterned using a positive-type photoresist (S-1813, Shipley far Ltd.), followed by etching of ITO. The microwell array was fabricated with a negative-type photoresist (SU-8 3010, MicroChem Co.) on the patterned ITO electrodes. In order to assemble the microwell array with the PDMS microfluidic channel, they were exposed to O<sub>2</sub> plasma to activate the surfaces using a Reactive Ion Etching machine (RIE-10NR, Samco Co.). Both were aligned and spontaneously bonded together. Cell line of iPS (MEF-Ng-20D-17) from Kyoto University, cultured on mouse embryo fibroblast (MEF) in plates coated with 0.1%

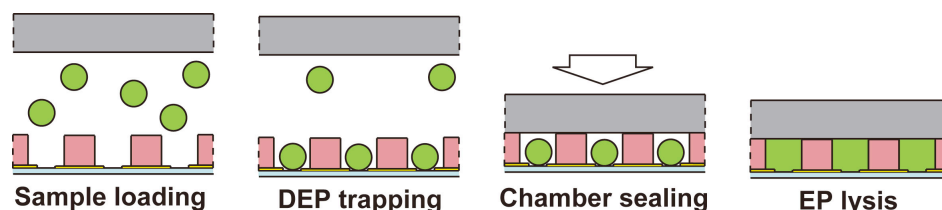
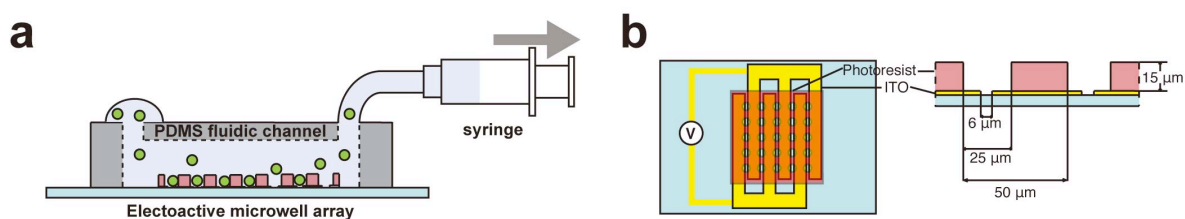


Figure 1: Experimental concept. Cells are introduced into the PDMS microfluidic channels and trapped into the microwell by DEP. Simply pressing a PDMS membrane closes microwells. Cells are lysed inside of the closed microwell by EP. Finally, fluorescence signal from the microchambers are acquired for the quantitative analysis.

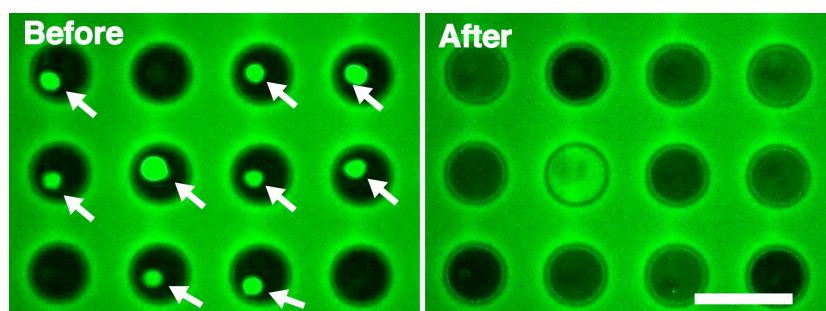


**Figure 2.** (a) Schematic illustration of the microfluidic device for trapping and lysing single cells. The device consists of a PDMS membrane attached onto a microwell array. Target cells or reagents for the biochemical reaction are introduced through an access port and delivered by the continuous flow induced by negative pressure. (b) Top and cross-sectional view of the microwell array part. The through-hole structure fabricated with the photoresist is aligned with the interdigitated ITO electrodes.

w/v gelatin (Sigma), was maintained in a complete medium of DMEM (Gibco) containing 15% Knockout Serum Replacement (KSR; Hyclone), 0.1mM 2-mercaptoethanol (Sigma), 1% MEM non-essential amino acids (Gibco), 100 U ml<sup>-1</sup> penicillin (Invitrogen), 100 U ml<sup>-1</sup> streptomycin (Invitrogen) and 1000 U ml<sup>-1</sup> recombinant human LIF (Wako, Japan), in a humidified incubator (37 °C, 5% CO<sub>2</sub>) with the medium changed every day. In order to induce cardiac differentiation, iPS cells were cultured in a medium without LIF containing 20% Fetal bovine serum (FBS, Invitrogen, Hyclone) with medium change every 2 days. Prior to DEP, the cells were dissociated into a single-cell suspension by TrypLE Select (Invitrogen) and the culture medium was changed with DEP buffer (10 mM HEPES, 0.1 mM CaCl<sub>2</sub>, 59 mM D-glucose and 236 mM sucrose; pH 7.35) to adjust the conductivity of the cell suspension medium (21.4 mS m<sup>-1</sup>) for pDEP. The DEP buffer contained final concentration of 1% (wt/vol) bovine serum albumin to block nonspecific cell adhesion.

## RESULTS AND DISCUSSION

We have demonstrated trapping and lysis of single iPS cells with electroactive microchamber array. The cell suspension of iPS was introduced into the device through an access port, and trapped into each microwell with DEP (Fig. 3). Since the cells were cultured to keep their pluripotency, the cells emit strong fluorescence signal of GFP. The inside of the closed microwell was filled with the fluorescence light after applying electric pulses due to the diffusion of the intracellular materials out of the cell. In order to induce cardiac differentiation, we added 20% fetal bovine serum (FBS) into the culture medium. After cultivation of iPS cells with FBS, we harvested the cells from the culture dish and measure GFP expression level with the electroactive microwell array device (Fig. 4). Measured GFP concentration is widely distributed from  $5 \times 10^5$  to  $3 \times 10^6$  molecules per cell for iPS cells (undifferentiated). Although they were cultured with the same condition in a culture dish, individual cells show large differences on a gene expression levels. The mean value of whole distribution is gradually decreased with the time. To evaluate our system, we directly compared the GFP expression level, obtained with the device, to the Nanog gene mRNA expression level, obtained with the conventional RT-PCR in bulk (Fig. 5). We note that mRNA levels and expression of GFP decrease with time in a similar manner.



**Figure 3.** Fluorescence image of closed microwell array before and after pulsing. (left) Single iPS cell array trapped into the microchambers before cell lysis. (right) Confined intracellular materials after lysis of trapped cells inside the closed microchamber. White arrows indicate trapped cells. Scale bar is 50 μm.

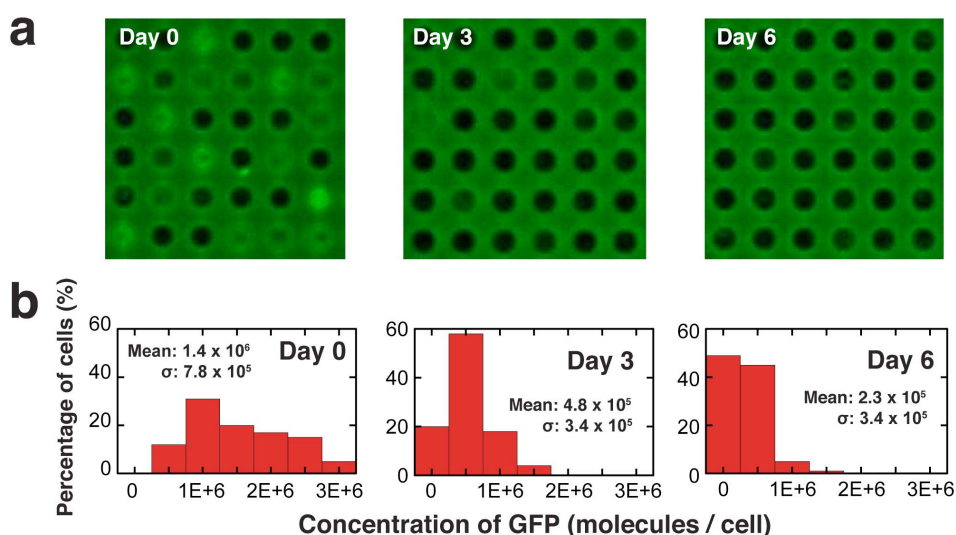


Figure 4. (a) Fluorescence images of tightly enclosed microchamber array containing intracellular materials of iPS cells. The cells were differentiated into the specific cell lines in a culture dishes before trapping. (b) Histograms of the distribution of intracellular GFP concentration of individual cells obtained by the quantification of fluorescence intensity of each chamber.

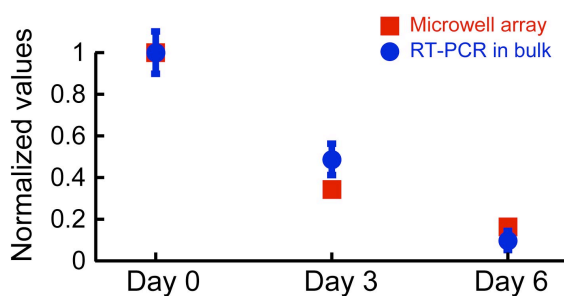


Figure 5. Comparison of GFP expression levels measured by microchamber array and mRNA levels for Nanog genes measured by quantitative RT-PCR in bulk. Both curves follow the same tendency showing a decrease with a good match.

## CONCLUSION

Our device allows quantitative analysis of gene express level at the single-cell level among a cell population. Such new concept of microwell array combined with highly sensitive analytical assays promises high-throughput and parallelized read-outs of intracellular analytes of individual cells in a large population of cells, while eliminating external manipulations.

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