Cytometric Analysis of Upregulated Functional Gene Expression in Primary Cells by On-Chip Staining

Application Note

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Introduction

Tumor cell lines or immortalized cells have been a standard tool for the elucidation of biochemical pathways as well as for drug testing. However, indeterminate changes in the phenotype may occur during immortalization of a cell [1]. Work with primary cells is inherently limited by source availability and life span in culture. Flow cytometry offers extensive analytical opportunities but generally requires high cell numbers for an experiment. Here, we describe on-chip antibody staining assays with the Agilent 2100 bioanalyzer that allow flow cytometric analysis of protein expression using a minimum number of fluorescently stained primary cells.

For the assays described, the staining reactions and the detection can be performed on-chip; the analysis is done without washing steps. We have successfully applied the assays to detect several protein targets on multiple cell lines. Results obtained with the Agilent 2100 bioanalyzer show good correlation with data obtained using a standard flow cytometer, but demonstrate new dimensions in low reagent usage and cell consumption.

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Experimental

Antibodies, reagents and chemicals

APC-conjugated anti-human CD3, CyChrome-conjugated anti-human E-selectin (CD63E) and IL-1β were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Phorbol-12-myristate-13-acetate (PMA) was obtained from Calbiochem (San Diego, CA, USA). Calcein-AM was purchased from Molecular Probes (Eugene, OR, USA). Cell Buffer, an isobuoyant buffer, is a component of the Cell Fluorescence LabChip Kit (Agilent Technologies GmbH, Waldbronn, Germany).

Primary cell culture

Human umbilical vein endothelial cells (HUVEC), culture media, and trypsin/EDTA solution were obtained from Cambrex Corp./Clonetics (East Rutherford, NJ, USA). HUVECs were maintained in EGM-2 medium.

Primary lymphocyte preparation

Buffy coat from normal donors was obtained from Stanford Blood Center (Stanford, CA, USA). Lymphocytes were isolated from buffy coat by differential centrifugation through Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) according to the supplier's protocol. The cells were suspended in RPMI medium supplemented with 20%FCS, Pen/Strep, 20 µM 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate (all Irvine Scientific, Santa Ana, CA, USA) and cultured in a CO_2 incubator at 37 °C overnight under cell culture conditions.

Activation of human peripheral blood lymphocytes

Human peripheral blood lympocytes were subcultured at 2 x 10^5 cells/mL in 12-well culture plates in the presence of phorbol-12-myristate-13-acetate (PMA) for 5 hours. Cells were harvested after counting by centrifugation at 200x g for 5 min and resuspended in Cell Buffer at 3 x 10^3 cells/µL by gentle pipetting.

IL-1β treatment of HUVECs

HUVECs grown in 12-well culture plates were treated with IL-1β for 4 hours. Cells were harvested by treatment with trypsin-EDTA solution as described by the supplier. After counting and centrifugation, the cells were resuspended in Cell Buffer at 3 x 10^3 cells/µL.

On-chip staining protocol for the Agilent 2100 bioanalyzer

1. Harvest cells and adjust cell density to 3 x 10^6 cells/mL in Cell Buffer (CB). Adherent or sticky cells are previously trypsinized as described in supplier's protocol.
2. Prime chip with 10 µL priming solution (PS) and wait for 1 min.
3. Add 10 µL focusing dye (FD) to the dye well.
4. Place 30 µL CB in the buffer wells.
5. Place 10 µL cell suspension into each sample well.
6. Add 2 µL of a 1:50 dilution of calcein in CB (final calcein conc. = 1.4 µM).
7. Add 2 µL of the diluted antibody (CD3: predilute 1:5 in CB; CD62E 1:30 in CB) to the sample wells.
8. Vortex chip for 1 min at 1000 rpm (place speed selector to the 12:00 position of the vortexer supplied with the 2100 bioanalyzer.)
9. Incubate for 25 min at room temperature* in a humidified chamber. Alternatively chips may be stacked using an old or unused chip on top to prevent excessive evaporation.
10. Vortex 1 min at 1000 rpm.
11. Load and run chip in the 2100 bioanalyzer.

*At 4 °C if antigen is shed or internalized upon antibody binding.

Conventional flow cytometry

Cells were stained and analyzed on a FACSCalibur flow cytometer (BD Biosciences), according to standard procedures. Sample volume was 0.5 mL at a cell density of $5x10^5$ cells/mL. Comparable wavelengths settings as those of the 2100 bioanalyzer were used for the experiments (FL1, FL4). Acquisition was set to 10,000 events for each sample.

Results and Discussion

On-chip evaluation of IL-1β induced expression of E-selectin (CD62E) on endothelial cells

IL-1β has been shown to upregulate E-selectin expression in endothelial cells [2]. Control HUVECs and cells that had been treated with IL-1β for four hours were added to the wells of a cell chip and stained directly with calcein and CyChrome conjugated anti E-selectin antibody. For data analysis, live cell events, as indicated by strong calcein staining intensity [3], were selected; appropriate regions and gating markers were set to include only E-selectin expressing cells within the live cell population.

Figure 1A shows the dot plots of the individual samples from a single chip run. E-selectin expression was not detectable in control HUVEC (Well #1). An increase in the level of E-selectin expression was observed when HUVEC were treated with increasing doses of IL-1β for four hours. The increase in E-selectin expression was dose dependent from 0.0033 ng/mL to 0.033 ng/mL and saturated at 0.1 ng/mL. The response of E-selectin expression in HUVEC to increasing doses of IL-1β is summarized in Figure 1B. When the same cells samples were stained using conventional methods and analyzed on a conventional flow cytometer, similar results were obtained.

Stimulation of T lymphocytes and induction of CD3

Treatment with phorbol 12-myristate 13-acetate (PMA) has been shown to increase the synthesis and assembly of the T cell receptor CD3 (4) in the human leukemic T cell line Jurkat. We performed a similar experiment using human peripheral blood lymphocytes and demonstrated that the dose response effect of PMA on the cells could be evaluated in a single chip run. In this case, a control and five treated lymphocyte samples that had been incubated with different doses of PMA for 5 hours were added to the sample wells of a cell chip and stained with calcein and APC-conjugated anti-CD3 antibody on-chip as described. The chip was analyzed with the Agilent 2100 bioanalyzer and the results are shown in Figure 2.

Figure 2A shows the CD3 histograms of the calcein positive cell population in a representative chip run. Figure 2B summarizes the CD3 upregulation as seen in 3 experiments. In the control lymphocyte sample (Well #1), 58% of the cells were CD3 positive. Treatment of the cells with PMA (3.3 pg/mL to 1 ng/mL at half-log concentrations) increased the percentage of CD3 positive cells up to 86% (Well #2 to Well #5). An increase in PMA concentration to 1 ng/mL did not appear to have a further effect on CD3 expression (Well #5).

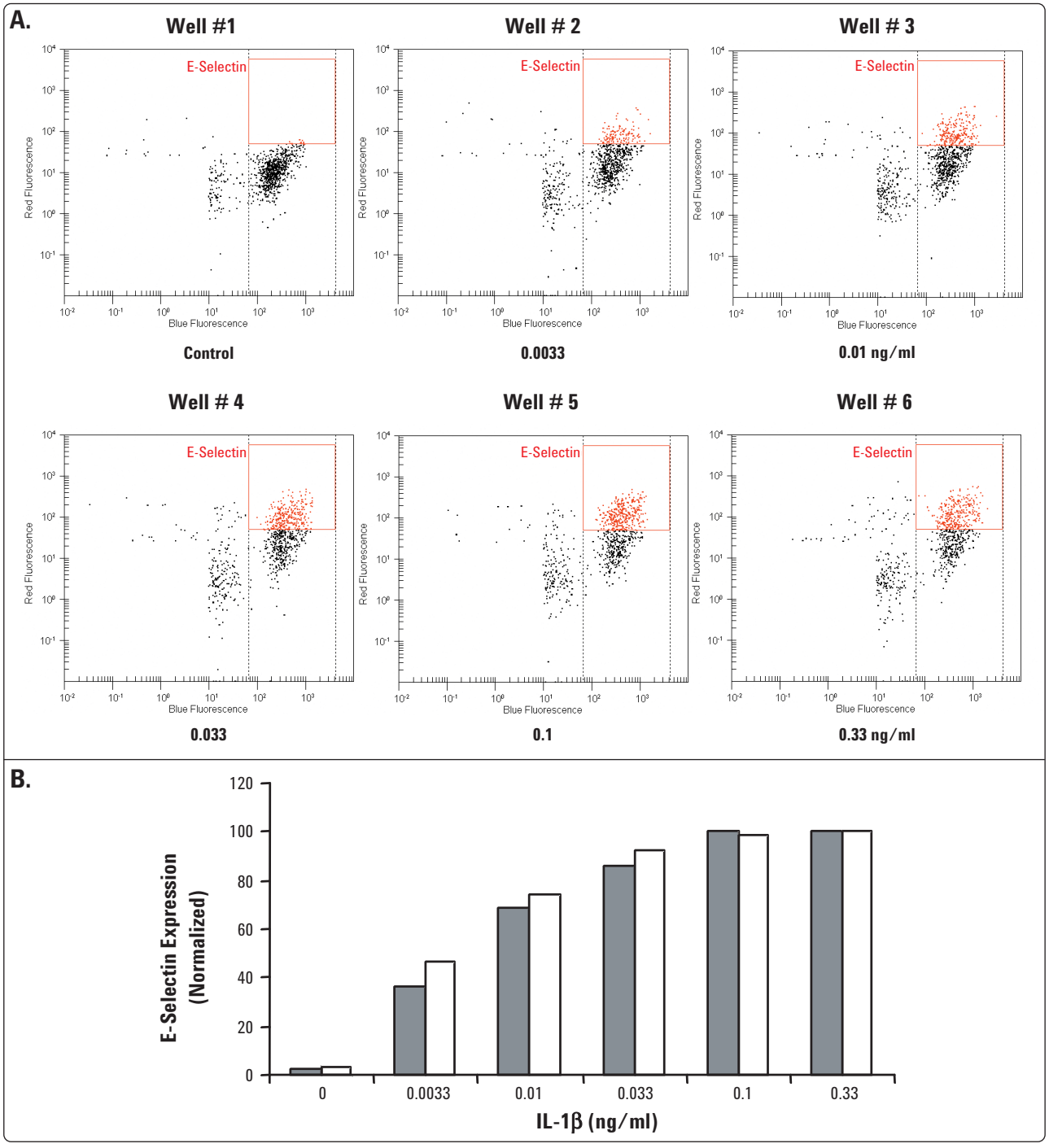

Figure 1. IL-1β induced expression of E-selectin (CD62E) on HUVECs. HUVECs were treated with 3.3 x 10^{-3}, 1 x 10^{-2}, 3.3 x 10^{-2}, 0.1, or 0.33 ng/mL IL-1β for 4 hours. Cells were harvested and stained on-chip with CyChrome-conjugated anti-human E-selectin antibodies and calcein. Untreated control cells were loaded in sample Well #1, and treated cells were loaded in sample Wells #2 to #6 of the cell chip. Data are shown as dot plots of red (y-axis) against blue (x-axis) fluorescence (A). The same cell samples were stained and analyzed on a conventional flow cytometer. A comparison of the results obtained from on-chip assay (white bars) and conventional flow cytometry (greyed bars) is shown in B.

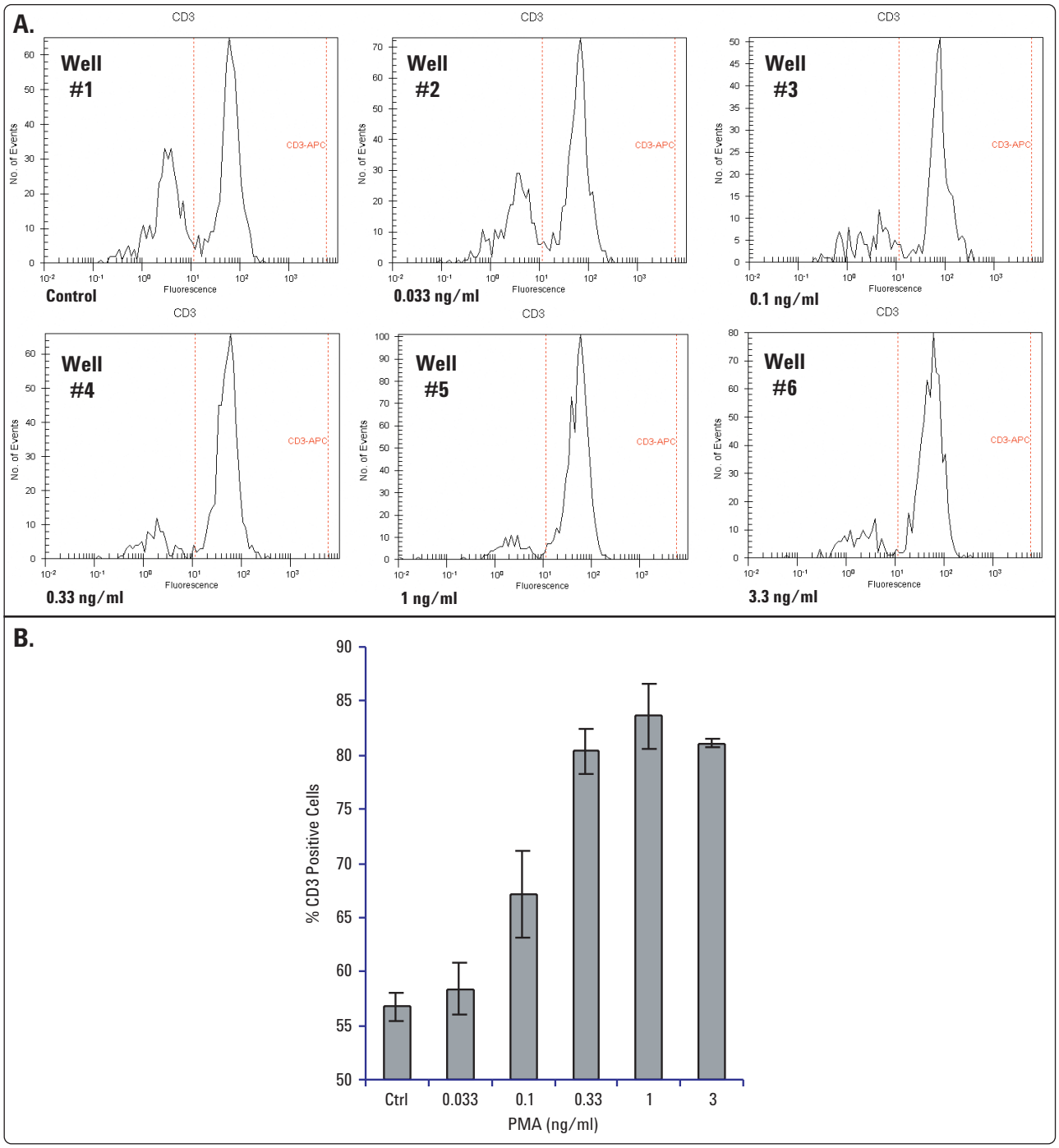

Figure 2. Activation of peripheral blood lymphocytes by phorbol-12-myristate-13-acetate (PMA). Lymphocytes were treated with 3.3 x 10^{-2}, 1 x 10^{-1}, 3.3 x 10^{-1}, 1, or 3.3 ng/mL of PMA for 5 hours. Cells were harvested and stained on-chip with APC-conjugated anti-human CD3(y-axis) and calcein(x-axis) and measured on the microfluidic system. Control cells were loaded into sample Well #1, and treated cells were loaded into sample Wells #2–#6 of the cell chip. CD3 histograms of the calcein-positive cell population from a representative experiment are shown in Figure 2A and the mean ± SD from 3 experiments are shown in Figure 2B.

Conclusion

Flow cytometric analysis of primary cells can present a challenge for researchers due to limited availability and life span of primary cells. A microfluidic chip-based technology lends itself perfectly for the analysis of very few cells per sample. The 2100 bioanalyzer requires approximately 30,000 cells per sample. Data quality is comparable to conventional flow cytometry. On-chip staining protocols further underline this feature by simplifying sample preparation while in addition requiring lower starting cell concentrations. Fewer cells are required due to the deletion of wash steps, where cells are frequently lost. Because the sample is handled on the chip less reagents are required. The Agilent 2100 bioanalyzer with its ease of use, simple protocols and fast analysis time is an excellent tool for the analysis of primary cells.

References

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