

SINGLE CELL ANALYSIS OF THE PROINFLAMMATORY RESPONSES OF MAST CELL BY A REAL TIME SECRETION ASSAY

Y. Shirasaki^{1,2*}, A. Nakahara², N. Shimura¹, N. Suzuki¹, M. Yamagishi¹, J. Mizuno², S. Shoji² and O. Ohara^{1,3}

¹Research Center for Allergy and Immunology, RIKEN, JAPAN,

²Major in Nano-science and Nano-engineering, Waseda University, JAPAN and

³KAZUSA DNA research institute, JAPAN

ABSTRACT

A real-time secretion monitoring assay from living single-cells was realized by total internal reflection fluorescence imaging with an optically optimized microwell array chip fabricated of an amorphous fluoropolymer, CYTOP on the basis of a fluorescence sandwich immunoassay. After individual mast cells were stimulated with lipopolysaccharide in the microwell array, their proinflammatory responses were monitored as cytokine secretion along time. mRNA levels of cytokines by single-cell quantitative PCR was also measured after the secretion assay. The results showed that each of stimulated mast cells is quantitatively and qualitatively distinguishable from each other in terms of cytokine secretion and their mRNA profile.

KEYWORDS: secretion monitoring assay, single-cell, CYTOP, proinflammatory response

INTRODUCTION

Single-cell analysis attracts a lot of attention from biologists since it enables us to address many concerns about cell-to-cell variations and/or fluctuations of biological events, which are considered as the key features to generate dynamics of biological processes while they have been totally missed in bulk biological measurements [1]. One of the most major methods for single-cell analysis is flow cytometry, which can quantify some specific molecules on the cell surface or within the cells of individual cells in a high-throughput manner by labeling the molecules fluorescently. However, flow cytometry cannot measure the amount of secreted proteins that frequently exert some significant function especially in cell-cell communication. Furthermore, although flow cytometry reveals phenotypic varieties among individual cells, they could not obtain information about changes of the phenotype of a single-cell along time, which might give a clue to understanding the origin of the cellular varieties. Recently, some researches have addressed this issue using Lab-on-a-chip technology and successfully measured the quantity of cytokines secreted from a single-cell [2]. However, it is still impossible to monitor the dynamics of secretion activity of the cells in real time. In this context, we developed a method to monitor secretion of cytokines from single-cells both in population and along time.

THEORY

Our system is based on sandwich immunoassay that uses a set of specific antibodies to capture and detect an antigen. While Surface Plasmon Resonance (SPR) and Optical Waveguide Grating are already reported as a real-time monitoring method of molecular binding, either method provides little information regarding whether the binding is specific or not. In contrast, the sandwich fluoroimmunoassay (FIA) is known to have high specificity and can be integrated with total internal reflection fluorescence (TIRF) imaging, which enables us to quantify only the specific binding onto the solid phase by local excitation with evanescent field in real time [3].

In this paper, we developed a real-time single-cell secretion monitoring assay by sandwich FIA with a micro-fabricated well array. The scheme of measurement principle is shown in Figure 1. The columnar microwell was formed in a micro fabricated corn shaped structure. Capture antibody was immobilized on the surface of bottom glass of the well. A cell was introduced into a microwell in solution containing fluorescently labeled detection antibody. Secreted proteins of interest from the cell were trapped and simultaneously formed specific antigen-antibody complexes. An amount of the antigen-antibody complexes was quantified by TIRF imaging.

A technical problem for TIRF imaging in this application was a difference of the refractive indices between the microwell structure and water. Because most of the materials used in micro fabricated bio chips have higher refractive indices, the inci-

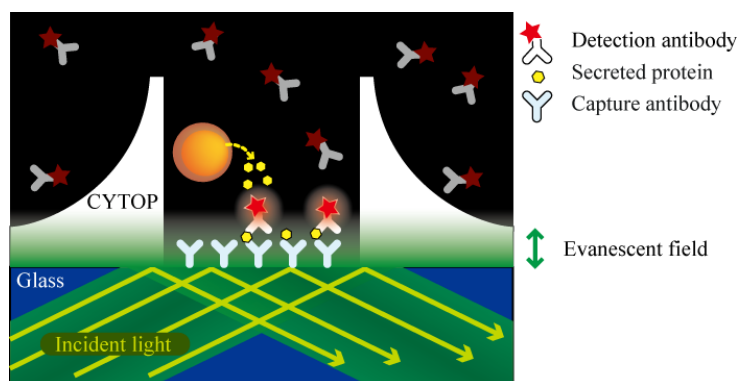


Figure 1: Scheme of single-cell real time secretion assay

dent light with critical angle for water/glass interface was partially refracted at boundary surface of the micro structure into the well making high background signal. To overcome this problem, we utilized an amorphous fluoropolymer, CYTOP, of which refractive index is almost the same as that of water, and thereby realized wide-field TIRF imaging with microstructures.

EXPERIMENTAL

Fabrication of microwell array chip

The fabrication process of CYTOP microwell array chip was described previously [5]. Briefly, inverted SU-8 pillar structures used as sacrificial layer were fabricated by standard photolithography on a glass substrate. After amination of glass, CYTOP 809A/809S was spin-coated and baked at 180°C. Then the excess CYTOP layer formed upon the SU-8 structures were etched by O₂ plasma. SU-8 structures were removed with a mixture of sulfuric peroxide. Figure 2 shows SEM image of the fabricated microwell array chip. Because CYTOP shrank during baking, the structure became conically shape. A fabricated microwell array chip was cleaned by O₂ plasma and the bottom glass was aminated.

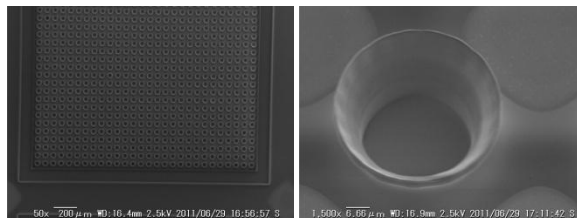


Figure 2: SEM images of fabricated CYTOP microwell array chip

Reagents and cell culture

Capture antibodies for mouse Interleukin (IL) 6 and mouse Tumor Necrosis Factor (TNF) α were covalently immobilized on the bottom of the microwell with dimethyl pimelimidate. Remaining free amino groups on the bottom of the microwell were blocked using monoethanolamine. Then, the bottom surface was treated with 2-methacryloyloxy ethyl phosphocholine (Lipidure BL-802, NOF Co., Japan) to prevent nonspecific binding. A detection antibody was prepared by incubation of a biotinylated IL-6 antibody or TNF α antibody with Quantum dot (QD) 705 streptavidin or QD625, respectively. After the incubation, unoccupied biotin binding sites on QDs were blocked with PEG₁₂-biotin.

A mast cell line, MC/9 cells were used for the real-time secretion monitoring assay. MC/9 cells were cultured in a Dulbecco's Modified Eagle Medium (D-MEM) supplemented with recombinant mouse IL-3, 30 mM HEPES buffer, 0.1 mM pyruvate sodium, and 2.5% (w/v) bovine serum albumin. The cells were randomly introduced into microwells and the excess cells outside of the microwells were washed out with culture medium containing detection antibodies. To activate MC/9 cells, the culture medium was replaced with that containing detection antibodies and Lipopolysaccharide (LPS) as a proinflammation inducer at time 0 hr.

TIRF microscopy

TIRF microscopy was performed on an automated inverted microscope (Eclipse Ti-E, Nikon, Japan) with a perfect focusing system with a 60 \times oil immersion objective (Apo TIRF, 60 \times , NA= 1.49). The excitation laser with wavelength of 532 nm was chosen to decrease the phototoxicity to cells.

Single-cell quantitative PCR analysis

After the secretion assay, some of the single-cells were picked up from microwells and mounted on AmpliGrid slide (Beckman Coulter, Inc., USA). Reverse transcription and pre-amplification of specific target transcripts were directly performed for each cells on the slide according to manufacturer's instruction. The pre-amplified products were subjected to quantitative PCR on a BioMark HD system (Fluidigm Corp., USA).

RESULTS AND DISCUSSION

In our system, 800 sections of the microwell array chip, corresponding to 7,200 microwells, could be scanned every 1 hr. Figure 3 shows an example of obtained microscopic images from a section of the chip at 4.5 hr after LPS stimulation. The time courses of secretion of IL-6 and TNF α widely varied from single-cell to single-cell, which were analyzed by a clustering method in Figure 4. Some of the cells secreted each of cytokines before LPS stimulation, but most of cells started to secrete it 1 hr after LPS stimulation. About a half of cells did not secreted TNF α , which suggested that IL-6 and TNF α secretions were under independent regulation though both are known to be induced via the same NF- κ B signaling pathway by stimulation of Toll-like receptor 4.

We then checked the amounts of IL-6 and TNF α mRNA levels. Figure 5a shows relative amounts of accumulated cytokines during 4 hr secreted from the collected cells, and Figure 5b shows amounts of the corresponding mRNAs after the secretion monitoring assay. We found there was no correlation between cytokine secretion level and mRNA expression level. Because we required for a couple of hours to collect individual cells after the secretion monitoring assay, the amounts of mRNAs might be changed during such a long period.

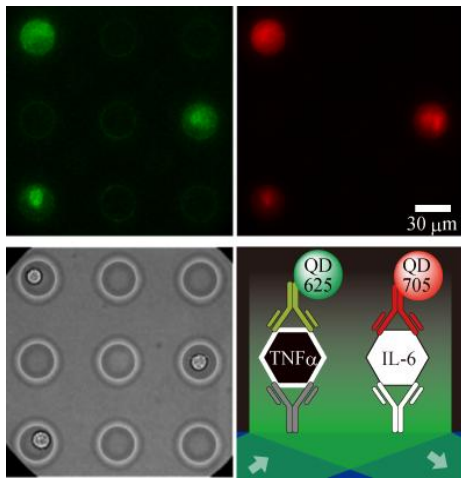


Figure 3: Microscopic images of the assay and schemas of sandwich immunocomplexes.

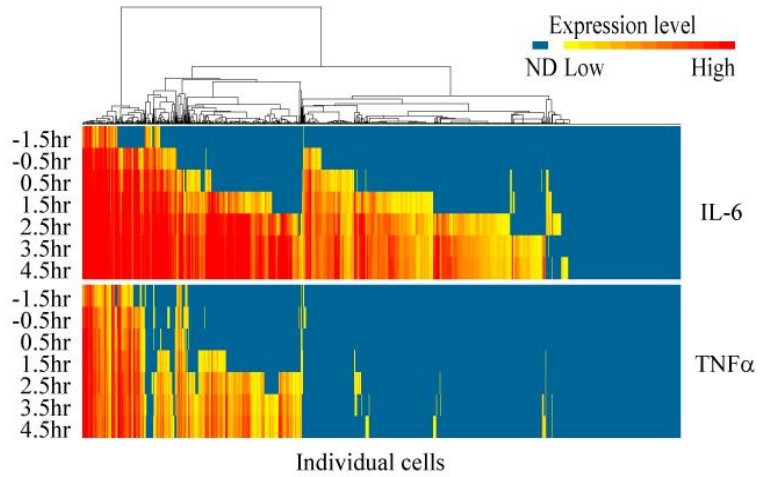


Figure 4: The expression pattern of time change secretion activity of IL6 and TNFα from single-cells. The values were expressed on logarithmic scale.

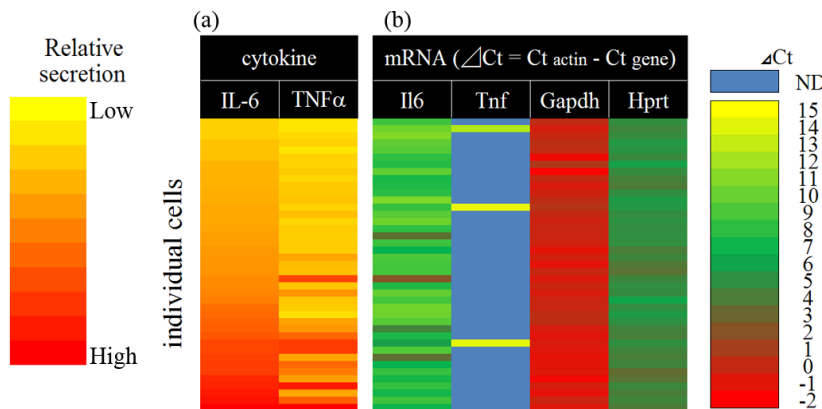


Figure 5: The correlation heat maps between secretion levels of proteins (a) and expression levels of intracellular transcripts (b) of each cytokine. The secretion values were expressed on logarithmic scale and normalized by max value of each cytokines.

CONCLUSION

We successfully developed a protein secretion monitoring system from single-cells in real time. To our knowledge, The system enabled us to compare the secretion levels with their mRNA levels in the same single-cells for the first time.

ACKNOWLEDGEMENTS

This work was supported by the Special Postdoctoral Researchers Program from RIKEN and Grant-in-Aid for Young Scientists (B) No. 23770189 from MEXT, Japan. This work was also supported by Waseda University Global COE Program” International Research and Education Center for Ambient SoC” and Grant-in-Aid for Scientific Basic Research (S) No. 23226010 and (C)No. 22560730from MEXT, Japan.

The authors thank for Nanotechnology Support Project of Waseda University for their technical advices.

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CONTACT

*Y. Shirasaki, tel: +81-45-503-9696; sirasaki@rcai.riken.jp