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Clinical diagnosis of type I allergy by means of SPR imaging with less than a microliter of peripheral blood

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ABSTRACT

The identification of antigen that induces activation of mast cells and basophils by crosslinking IgE bound to the cell surface is crucial to avoid symptoms of allergic diseases. Surface plasmon resonance imaging (SPRI) possesses a great potential for clinical diagnosis of allergy, in that it reveals living cell activation following the binding of antigens to IgE, on real-time and single cell basis without artificial labeling. However, present technique of SPRI requires freshly isolated basophils of patients and cannot analyze multiple samples in parallel. To overcome such problems, we developed devices for SPRI to make a broad observation area and a multi-well SPRI sensor chip with a hydrophobic membrane. The employment of human IgE receptor-expressing mast cell lines (RBL-48 cells) sensitized with serum, collected and stored from less than a microliter of patient's blood, allowed us to detect specific reactions of RBL-48 cells in response to antigens. This technique may be a useful tool as a high throughput screening system of type I allergy not only for freshly prepared basophils but also for sera stored in clinical practices.

1. Introduction

Basophils, resident in blood, and mast cells, resident in connective and mucosal tissues, have important roles in type I allergy or IgE-associated diseases, such as food allergy, hay fever, and atopic dermatitis (AD). The binding of antigen (also called allergen in the field of allergy) to IgE bound to the high-affinity IgE receptor (FcεRI) on the surface of basophils and mast cells cross-links FcεRI, resulting in the release of preformed and newly synthesized inflammatory mediators, such as histamine, arachidonic acid metabolites and cytokines, and causes allergic symptom (Fig 1) [1–3]. Therefore, the identification of antigens that provoke mast cell and basophils-induced allergic reactions is crucial to avoid anaphylactic shock and aggravations of allergic diseases. Specific binding of antigens to the corresponding IgE may be detected by various immunological methods, such as ELISA. However, these tests cannot evaluate whether or not the antigen-binding IgE activates mast cells and basophils in patients. Therefore, the evaluating the potential of antigen-specific IgE to activate mast cells and basophils from a patient is more important than the analysis of simple IgE–antigen binding in the diagnosis of type I allergy [4–6].

Surface plasmon resonance (SPR) sensors can characterize the binding of detectants in the detection area on a SPR sensor chip coated with gold thin film (50 nm) in real time without any labeling [7,8]. They provide a useful means to study the interaction of various biological molecules, ranging from proteins, oligonucleotides, and lipids to small particles, such as phages, viral particles as well as cells [9–15]. We previously reported that SPR sensors could detect real-time large changes of refractive index (RI) in response to the activation of living cells, such as mast cells, keratinocytes, human basophils and B lymphocytes on a sensor chip without any labeling. We also revealed that SPR reflects sequential events of intracellular signaling rather than the binding of ligands on cell surface, suggesting the potential of SPR as a tool for cell analysis in basic research and diagnosis for allergy and immunology [16–22].

Moreover, we recently developed an SPR imaging (SPRI) system in order to visualize RI changes in individual cells induced by stimuli, and analyze the subcellular distribution of RI changes [23]. The SPRI sensor could visualize the activation of human basophils isolated from patient's blood in response to various antigens at single cell levels, suggesting the potential of the sensor as a tool for diagnostics of type I allergy [24,25]. However, the diagnosis technique using SPRI sensor requires fresh basophils isolated from patient's blood (within 2 day after collection) and can analyze only

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one sample at the same time (single channel). Meanwhile, we reported that an RBL-48 cell line expressing the α-subunit of FcεRI and sensitized with human IgE was activated with anti-human IgE antibody (anti-IgE) or specific antigens which induced allergic reaction in a patient (Fig. 1) [26]. Therefore, RBL-48 cells sensitized with serum IgE of patients may be used to detect specific and biologically active IgE that induces allergic reactions in response to antigens. In this study, we constructed an SPRI sensor with a broad detection area to make for multiple detection spots, and analyzed the reaction of RBL-48 cells, but not fresh human basophils, sensitized with human IgE in less than a microliter of peripheral blood in response to various antigens.

2. Materials and methods

2.1. Reagents

Chemicals used were obtained from the following sources: bovine serum albumin (BSA) from Sigma–Aldrich Japan (Tokyo, Japan). Anti-IgE from BETYL (Montgomery, TX, USA); mite extract-Df and cedar pollen extract-Cj from Cosmo Bio Co., Ltd. (Tokyo, Japan). Fetal calf serum (FCS) from Biowest (Nuaillé, France). Penicillin/streptomycin, trypsin and G418 were from Life Technologies (Carlsbad, CA, USA).

2.2. Cells lines

The RBL-48 cell line was derived from a rat basophilic leukemia (RBL)-2H3 cell line transfected with the human α-subunit of FcεRI, kindly supplied as a gift by Dr. Joko Kochan (Hoffmann-La Roche, Nutley, NJ, USA) and cultured in IMDM medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 mg/ml G418 [27]. The day before experiments, RBL-48 cells were harvested using trypsin. They were then cultured in the presence of human serum in a ratio of cells suspension (2.0×10^5/ml): serum of 30:1 (ex 30 μl and 1 μl). The volume of cell suspension on a uni-compartment and multi-well SPRI sensor chip were 60 μl and 3 μl respectively. Cells density at the SPR measurement was approximately 60% confluent.

2.3. Instrument for SPR imaging (SPRI)

A uni-compartment SPRI sensor with an objective lens (×4) for detection of single cell reactions was constructed as described previously [23]. A multi-well SPRI sensor for broad detection area was composed of a light source (640 nm LED, Ocean Optics), achromatic lens (Sigma koki Co., Ltd., Tokyo, Japan), P-polarizer (Sigma Koki Co., Ltd.), prism (S-LAL10, RI = 1.72), thermostat and telecentric lens (×0.6, Edmund, Barrington, NJ, USA) and CMOS camera (Monochrome-digital CMOS camera, 1280 × 1024 pixel 15 fps, Japan) (Fig. 1b). The SPRI sensor chips (S-LAL10, 20 mm × 20 mm × 1 mm, RI = 1.72) were coated with gold thin film (1 nm Cr layer and 49 nm gold layer) by means of vapor deposition (Osaka Vacuum Industrial Co., Ltd., Osaka, Japan). The flow chamber was composed of a cover glass, polyvinyl chloride tubes and a silicone rubber (20 mm × 20 mm × 5 mm) from which the center was cut out as a flow space (Fig. 2). Obtained images and changes of light intensity of reflected light were analyzed with Image-Pro® Plus (Media Cybernetics, Inc. Rockville, MD, USA).

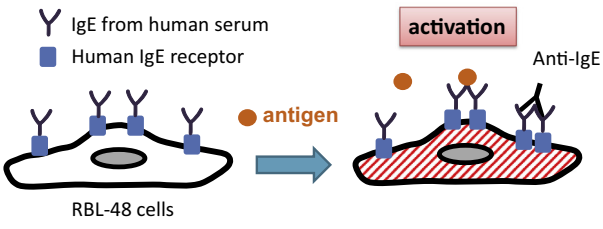

Fig. 1. Scheme of RBL-48 cells activation. IgEs in serum bind to IgE receptors expressed on the surface of the RBL-48 cell. The crosslinkage of IgEs by an anti-human IgE antibody or multivalent antigen induces the activation of RBL-48 cells.

2.4. SPRI measurement

RBL-48 cells were cultured on a uni-compartment SPRI sensor chip or multi-well SPRI sensor chip with or without human serum collected from peripheral blood. The sensor chip, on which living cells were cultured, was placed on a prism with matching fluid (RI = 1.72). The flow cell was placed on the sensor chip and then washed with 1,4-piperazinediethanesulfonic acid (PIPES) buffer at 37 °C. The stimulation was performed in the flow chamber by injection with a manual syringe at 37 °C. Two dimensional images were created from the light reflected at the interface between the gold film and glass using an objective lens and a CMOS camera. Living cells and living cell reactions were visualized by the difference of RI between buffer solution and living cells as described previously [17–19]. Images which show the distribution of reflected light intensity were taken every 10 s using the CMOS camera. SPRI sensor chips were disposed after each assay in order to obtain reliable results. The change of light intensity (reflective index changes) surrounded by area of interest (AOI), from which the intensities of the area without cells was subtracted as background at each time point, was plotted versus time (seconds) with Image-Pro.

2.5. Release of β–hexosaminidase (beta hexosaminidase assay and histamine release test)

Degranulation of RBL-48 cells was evaluated with the release of β-hexosaminidase, a granule marker, by the hydrolysis of p-nitrophenyl-N-acetyl-β-D-glucosaminide to the chromatophore, p-nitrophenol as described previously.

2.6. Subjects

Levels of total and specific serum IgE against cedar pollen antigen and mite antigen measured by Phadia's total and specific IgE test and reactions to sweat antigen of patients are shown in Table 1.

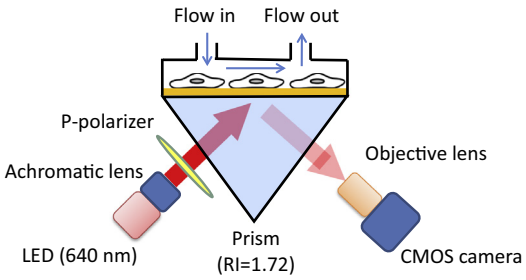

Fig. 2. Scheme of SPRI for broad area. The sensor is composed of an LED (640 nm), an achromatic lens, a P-polarizer, a S-LAL10 (RI = 1.72) glass prism, a sensor chip (S-LAL10) coated with gold film (50 nm), an objective lens (×0.6) and a CMOS camera. The SPRI sensor chip, on which RBL-48 cells were cultured with human serum, was placed on the prism with matching solution (RI = 1.72). The light was directed to the prism surface at the incident angle 56°.

Table 1
Levels of total serum IgE and antigen-specific IgE measured by Phadia's total and specific IgE test, and reactions to sweat antigen of subjects.

	Total IgE (IU/ml)	aMite-IgE (UA/ml) score (0–6)	aCedar-IgE (UA/ml) score (0–6)	Reaction to sweat antigen
Donor 1 (HF)	123	0.44 1	31.9 4	Negative
Donor 2	29.6	<0.34 0	<0.34 0	Negative
Donor 3 (AD)	13,200	>100 6	>100 6	Positive
Donor 4 (mild asthma)	579	74.1 5	41.1 4	Negative

3. Results

3.1. SPR imaging sensor could detect the activation of RBL-48 cells treated with human serum in response to anti-IgE

Multivalent antigen or anti-IgE can crosslink IgE antibody-FcεRI complex on RBL-48 cells, resulting in the activation of the cells (Fig. 1). We first investigated the histamine release (degranulation) from RBL-48 cells treated with human serum overnight in response to anti-IgE. As shown in Fig. 3a, RBL-48 cells treated with human serum, but not those without serum, were activated in response to anti-IgE antibody. We then observed RI changes in individual RBL-48 cells in response to anti-IgE using an SPRI system with ×4 objective lens, as described previously [23]. As shown in Fig. 3b, reflected light intensity in individual RBL-48 cells cultured with human serum increased in the presence of anti-IgE (Smovie 1), but not in the absence of anti-IgE (Smovie 2).

In order to perform multiple spot cell analysis by SPRI sensor, we then constructed an SPRI sensor that can observe RI distribution in a broad area (20 mm × 20 mm) on a SPRI sensor chip, as described in Section 2 (Fig. 2). Using the SPRI sensor, we investigated RI changes in RBL-48 cells cultured with human serum on the SPRI sensor chip in response to anti-IgE. Fig. 4a shows images of RI of RBL-48 cells, treated with human serum and exposed to, or not exposed to anti-IgE at indicated time points. In the absence of anti-IgE, RI in RBL-48 cells was not changed (Fig. 4a upper panels, Smovie 3), but clearly increased in response to anti-IgE (Fig. 4a lower panels, Smovie 4). The changes of RI in cell areas were plotted in Fig. 4b. When RBL-48 cells treated with human serum were stimulated with anti-IgE, RI in cells began to increase within a few minutes, and lasted or continued to increase for more than 30 min (Fig. 4b). These results indicate that the sensor can visualize RI changes of RBL-48 cells cultured with human serum in response to anti-IgE in a real time manner.

3.2. Development of multi-well SPRI sensor chip

In order to perform multiple spots cell analysis for clinical diagnosis, we then developed a multi-well SPRI sensor chip. As shown in Fig. 5a, nine wells for living cell culture and analysis were constructed with a hydrophobic membrane on the SPRI sensor chip. The multi-well SPRI sensor chip allowed us to place 3 μl culture medium in each well. Thus, 3 μl of RBL-48 cells suspension containing 0.1 μl human serum were successfully cultured in each of the wells (Fig. 5b). Fig. 5c shows RBL-48 cells cultured in a well observed under a phase-contrast microscope. These results suggest that RBL-48 cells can be cultured in each well of the multi-well SPRI sensor chip with less than one microliter of serum of a patient.

3.3. Multiple spots analysis of RBL-48 cells treated with human serum from patients in response to antigen

To investigate the potential of an SPRI sensor with a multi-well sensor chip as a tool for diagnosis of type I allergy, we investigated the reaction of RBL-48 cells cultured with sera from various donors: donor 1 with pollen allergy (HF), donor 2 without allergic disease, donor 3 with atopic dermatitis (AD), donor 4 with mild asthma. Sensitivities of the donors are shown in Table 1. RBL-48 cells cultured with serum of donor 1 (pollen hypersensitivity, (Fig 6a, bottom 3 wells) increased their RI in response to cedar pollen antigen, but those cultured with serum of donor 2 (Fig 6a, no allergy, top 3 wells) and those cultured without human serum (Fig 6a, middle 3 wells) did not change their RI (Smovie 5). Average changes of the intensities in upper and lower lane wells were plotted in the bottom panel of Fig. 6a. The intensity in the middle lane (3 wells) was subtracted as background at each time points. RBL-48 cells cultured with serum of donor 3 with AD, but those cultured with serum of donor 4 without AD changed their RI in response to sweat antigen (Fig. 6b, Smovie 6) [28]. On the other hand, both

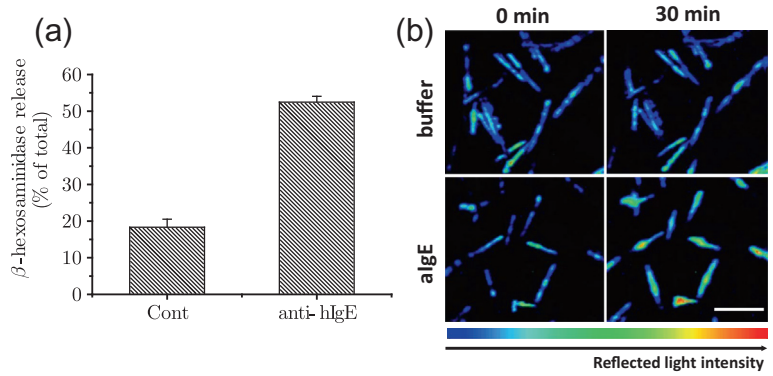

Fig. 3. Activation of RBL-48 cells treated with human serum in response to anti-IgE. (a) Beta-hexosaminidase release (degranulation) in RBL-48 cells sensitized with human serum was induced in response to anti-human IgE antibodies (anti-IgE). (b) RI changes in individual RBL-48 cells in the absence or presence of anti-IgE observed by an SPRI sensor constructed as described previously.

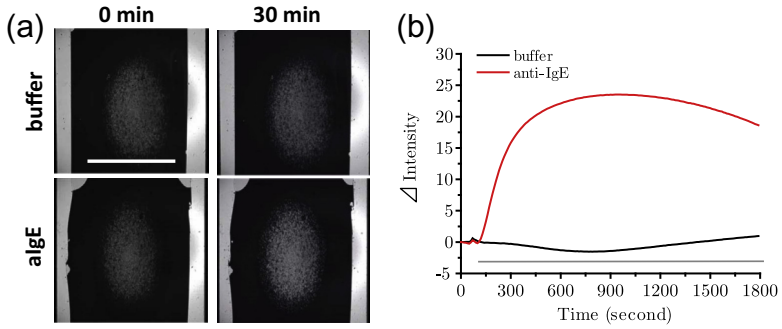

Fig. 4. RI distribution of RBL-48 cells. (a) RI distribution changes of RBL-48 cells were observed by the SPRI sensor. The areas with high RI show RBL-48 cells. White bar shows ca. 10 mm. (b) The time course of RI changes in the area with RBL-48 cells in the presence or absence of anti-IgE was plotted at every 10 s. Dotted circle shows a typical area of AOI in which the sensor detects changes of reflected light intensity.

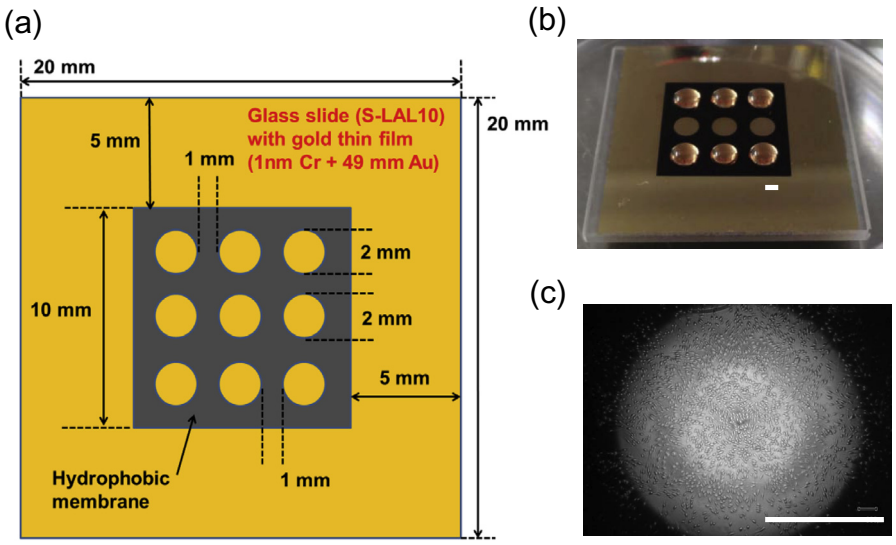

Fig. 5. Development of multi-well SPRI sensor chip for multiple spots cell culture. (a) Multi-well (9 wells) SPRI sensor chip were constructed with a hydrophobic membrane. The diameter of each well was 2 mm. (b) A cell suspension (3 μl) containing 0.1 μl human serum was dropped onto the multi-well SPRI sensor chip. The image was taken by a digital camera. White bar shows ca. 1 mm. (c) RBL-48 cells were cultured in a well overnight with human serum observed under differential interference contrast microscopy. White bar shows ca. 1 mm. Similar results were obtained from three independent experiments.

RBL-48 cells cultured with serum of donor 3 and those with serum of donor 4 changed their RI in response to mite antigen (Fig. 6c, Smovie 7). The change of intensities in RBL-48 cells cultured with serum from donor 3 or 4 in response to sweat antigen and mite antigen were plotted in the bottom panels of Fig. 6b and 6c, respectively. These results suggest that the multi-well SPRI sensor can sufficiently detect specific activation of RBL-48 cells in response to antigens by monitoring RI changes in each well.

4. Discussion

The identification of antigens that provokes mast cell and basophils activation is crucial to avoid anaphylactic shock and the aggravation of allergic and/or atopic diseases, such as food allergy, allergic rhinitis, atopic dermatitis and asthma. The detection of antigen specific IgE in serum implies hypersensitivity against the antigen. Thus, a variety of immunological methods to detect antigen specific IgE, have been developed and utilized in clinical practice [29]. However, there are often substantial discrepancies between these serological tests and clinical symptoms. In vivo tests, such as skin tests and antigen challenge tests, are more reliable in reflecting the clinical scenario. However, these tests may be physically painful for the patient, and have the potential to evoke anaphylactic shock when a patient is extremely sensitive to a particular antigen [30]. Moreover, the intradermal injection of an antigen cannot avoid the risk of sensitization of subjects who are not sensitized to the antigen as well as the risk of disease aggravations. Histamine release tests which detect histamine release from basophils and the detection of a surface activation marker of basophils, such as CD203c and CD69 in vitro are safe, sensitive and widely used as clinical examinations in the field of allergy. However, basophils of certain individuals, who show type I hypersensitivity in vivo, neither release histamine nor express an activation marker upon the activation of the IgE receptor in vitro (non-responders). Moreover, fresh peripheral blood (within 1 or 2 days after collection) is required for these tests. Therefore, more sensitive and minimally invasive tests with high reliability and without a time restriction are increasingly demanded for diagnosis of type I allergy. We previously developed a basophil activation test by means of SPRI sensor which could visualize the activation of human basophils in response to specific antigens [24,25]. That system required only a small amount of peripheral blood and exhibited good sensitivity. However, the test also requires freshly obtained peripheral blood (within 2 days after collection). In this study, we developed a system based on SPRI to perform multiple

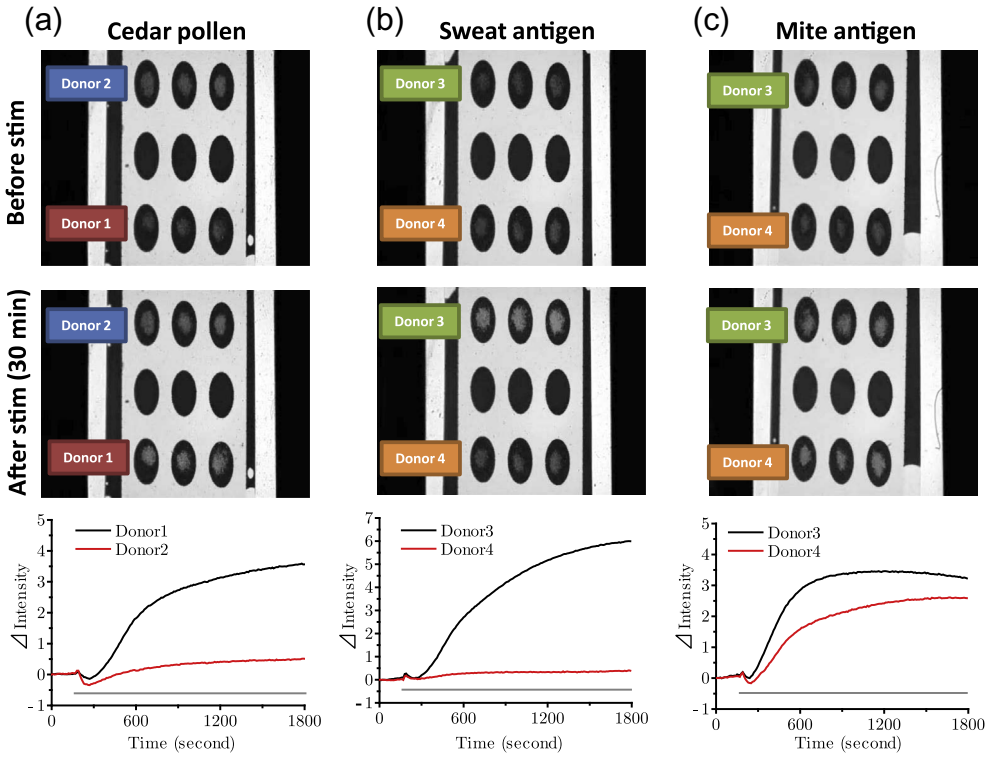

Fig. 6. Multi spots analysis of RBL-48 cell activations cultured on a multi-well SPRI sensor. (a) RBL-48 cells with serum of donor 1 (upper spots) or serum of donor 2 (bottom spots) were cultured on the SPRI sensor chip and then stimulated with cedar pollen antigen for 30 min. RI in cells sensitized with serum of donor 1, but not those with serum of donor 2, increased in response to the pollen antigen. (b) RBL-48 cells sensitized with serum of donor 3 (upper spots) or serum of donor 4 (bottom spots) were cultured on the SPRI sensor chip and then stimulated with sweat antigen for 30 min. RI in cells sensitized with serum from donor 3, but not those with serum of donor 4, increased in response to the sweat antigen. (c) RBL-48 cells sensitized with serum from donor 3 (upper spots) or serum from donor 4 (bottom spots) were cultured on the SPRI sensor chip and then stimulated with mite antigen for 30 min. Both RI in cells sensitized with sera of donor 3 and those of donor 4 increased in response to the mite antigen. Intensity changes were saturated around 30 min after stimulation and then decreased to base line within 90 min. Intensity changes due to RBL-48 cells (approximately 60% confluent) at 30 min after the stimulation with anti-IgE was approximately 3 (date not shown).

spots cell analysis. The system consists of a low-power telecentric lens (×0.6) with a diaphragm, which has a long focal depth (±1.4 mm). Therefore, the sensor developed here can detect the reaction of single cells and/or cell clusters in a broad detection area (>10 mm × 10 mm), which is suitable for multiple spots assays. Using this sensor, we were able to detect the activation of RBL-48 cells sensitized with a single microliter of patient's serum, as a surrogate of the patient's basophils, regardless of the storage time and conditions, such as freezing. Since the number of cells to detect reactions can theoretically be miniaturized down to the single cell level, this system may be used as a good example of applying a living cell reaction-based biosensor for a sensitive and specific tool for various clinical diagnosis. The depth of detection field from the surface of gold film on SPR sensor is less than 500 nm, which is much smaller than the cell height. Therefore, SPR sensors detect RI changes only near the plasma membrane sensitivity. The precise mechanism for cells to make such a large change of RI near the plasma membrane remained unclear. However, several reports demonstrated that RI change near plasma membrane reflects not only the area of cell attachment, but also the accumulation and the rearrangement of proteins in response to intracellular signal transductions.

5. Conclusion

The technique developed in this study visualized the reactions of multiple spotted RBL-48 cells sensitized with human serum in response to specific antigens. Further studies to validate this technique by clinical trials and the development of SPRI sensor chips with a larger number of wells to stimulate with different antigens should enable us to perform a sensitive and high-throughput screening for type I allergy with single microliter volumes of peripheral blood.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sbsr.2014.10.014.

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