Title

Living cell positioning on the surface of gold film for SPR analysis

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Abstract

Living cell reactions are detected as changes of the angle of resonance (AR) for surface plasmon resonance (SPR). Since SPR reflects the events in the field of evanescence, cells need to be fixed on the sensor chip. In this study, we developed methods to fix living cells on a gold surface and to recover adherent cells from the culture dish, preserving their functions to be analyzed by SPR. Human basophils and B-cells were fixed to the sensor chip by a biocompatible anchor for cell membranes (α-succinimidyloxysuccinyl ω-oleyloxy polyoxyethylene), aminoalkanethiol (cyteamine, 8-amino octanethiol) or an amino-reactive cross-linker (Dithiobis [succinimidylpropionate]). They showed an increase of AR in response to various stimuli. RBL-2H3 cells, which firmly adhered to the culture dish, were cultured/recovered with HydroCell^{TM}/simple pipetting, with RepCell^{TM} /pipetting at 4 ^{o}C, or on normal plastic culture dishes with trypsinization or by scraping at 4 ^{o}C, respectively. The exocytosis of RBL-2H3 cells was largely impaired by scraping, but only slightly by the treatment with pipetting on HydroCell^{TM}, on RepCell^{TM}, or with trypsin. The membrane ruffling of the cells prepared by the last three treatments induced by antigens appeared the same. However, the change of AR with cells prepared by trypsin and those by scraping at 4^{o}C were lower than those by HydroCell^{TM} or RepCell^{TM}, suggesting that trypsin may harm molecules involved in cellular reactions. Thus, the methods of cell fixation and removal with HydroCell^{TM} or RepCell^{TM} should enable us to analyze various reactions in either adherent or non-adherent cells by SPR.

Key words:

Biosensor, surface plasmon resonance, non-adherent cells, cell fixation, recovery.

1. Introduction

There is a continuously increasing demand for biosensors that are able to detect living-cell activation (Ziegler et al., 1998). Surface plasmon resonance (SPR) sensors are capable of characterizing the binding of detectants in the field of resonance on a sensor chip in real-time without any labeling (Karlsson et al., 2004; Cooper 2003; Szabo et al., 1995). They provide a useful means to study the interactions of any biological molecules from proteins, oligonucleotides, and lipids to small molecules, such as phages, viral particles and cells (Rich et al., 2000). We previously reported that SPR sensors can detect unexpectedly large changes of angle of resonance (AR), when RBL-2H3 mast cells were cultured on a sensor chip and activated by antigen (Hide et al., 2002). We also reported that SPR sensors can detect changes other than the area of adhesion and the overall constructions in living cells observed using an ordinary light microscope (Yanase et al., 2007). For such analyses, cells are required to be fixed on the sensor chip, so as to be placed in the field of evanescence. Therefore, our previous report was performed using adherent cells cultured on the sensor chip overnight. In this study, we investigated methods to fix non-adherent cells on a gold surface and those to recover adherent cells from culture conditions, preserving their functions to be

analyzed by SPR.

To fix non-adherent cells, we examined three types of methods which made use of a gold surface : 1) the biocompatible anchor for cell membranes (BAM: SUNBRIGHT OE-040CS) which is inserted into cell membranes without causing any damage to cells (Kato et al., 2003); 2) amino-alkanethiol (cysteamine or 8-aminoalkanethiol) that have an amino group (positive charge) with electrical affinity to connect cell membranes (negative charge); 3) Dithiobis[succinimydylpropionate] (DSP) that reacts with primary amines and forms covalent amide bonds (Grubor et al., 2004).

For the analysis of adherent cells, we studied the methods to recover cells from a culture dish and to rapidly fix them to the sensor chip: 1) culture on super-hydrophile property polymer-treated dishes (HydroCell™), where adherent cells are cultured in a floating state (Moriguchi et al., 2006), 2) culture on a temperature-responsive polymer (RepCell™), which becomes liquid at 32 ℃ or at lower temperatures (Gordon et al., 2006), 3) culture and trypsinization on standard cell-culture dishes, 4) culture and vigorous pipetting at 4 ℃ on standard cell-culture dishes.

The results of these experiments have demonstrated that SPR sensors can detect reactions of a wide variety of living cells with pretreatment of sensor chips for the attachment of non-adherent cells and methods of cell culture for rapid recovery of adherent cells

2. Materials and Methods

2.1. Reagents

The chemicals used were obtained from the following sources: bovine serum albumin (BSA), dinitro-phenol-conjugated human serum albumin (DNP-HSA), DNP-specific rat monoclonal IgE, cysteamine from Sigma-Aldrich Japan (Tokyo, Japan); human IgE antibody from BETHYL (Montgomery, Texas); human basophil, B Cell Isolation kits from Miltenyi Biotec (GmbH, Bergisch Gladbach, Germany); DSP from PIERCE (Rockford, IL); goat $F(ab')_2$ anti-human IgM from Southern Biotech (Birmingham, AL); SUNBRIGHT (BAM) from NOF CORPORATION (Tokyo, Japan); 8-aminooctanethiol from DOJINDO (Kumamoto, Japan); 4-{alpha}-pholbol 12-myristate 13 acetate (PMA), ionomycin from Calbiochem (San Diego, CA).

2.2. Cell culture

RBL-2H3 cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin as described previously (Yanase et al., 2007). The day before experiments, cells were harvested using trypsin. They were then cultured (4×10^5 cell/ml) in the presence of 0.05 μg/ml anti-DNP IgE in normal culture dishes, HydroCell^{TM}

dishes, RepCell^{TM} dishes or on SPR biosensor chips. Recovered cells firmly adhered within 20 min at room temperature without there being any pretreatment of the sensor chips, 96 cell plates or glass based dishes. Ramos cells were cultured in a floating condition with RPMI-1640 medium supplemented with 10 % FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin as described by Yamada et al (2002).

2.3. SPR sensor chip

SPR sensor chips provided by a manufacturer (Moritex, Tokyo, Japan) were cleaned by sonication in acetone for 5 min followed by rinsing with ethanol. The layer of aminoalkanethiol was prepared by immersing the sensor chips, cleaned as above, in 1 mM aminoalkanethiol (cysteamine, 8-aminooctanethiol) in ethanol for 1 hr, and rinsing it with ethanol for three times. To form the layer of DSP, the sensor chips were immersed in 1 mM DSP solution in DMSO for 1 hr, followed by rinsing with ethanol for three times. The layer of SUNBRIGHT was prepared by immersing the cysteamine-coated sensor chips in 1 mM SUNBRIGHT, 10% DMSO in PBS for 30 min, followed by rinsing with ethanol three times.

2.4. Preparation of human blood cells

Human peripheral blood basophils and B lymphocytes were isolated from fresh heparinized

blood of healthy donors using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient separation followed by magnetic depletion of non-basophils or non-B cells respectively (MACS basophil isolation kit, MACS B cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)).

2.5. Fixation of cells on the sensor chip

Non-adherent cells suspended in HEPES buffer (0.8% NaCl, 0.02% KCl, 0.52mM Na_2PO_4, 10mM HEPES, 0.1% Glucose, 2mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4) containing 0.03% HSA were placed at two appropriate spots on the aminoalkanethiol- or SUNBRIGHT-treated sensor chip and were left for 20 min at room temperature. Non-adherent cells suspended in HEPES buffer without HSA were placed at two appropriate spots on the DSP-treated chip and were left for 20 min at room temperature. RBL-2H3 cells were placed at two appropriate spots on the normal sensor chip for 20 min at room temperature.

2.6. SPR analysis

The changes of AR in response to various stimuli to the cells were detected using SPR apparatus, SPR-CELLIA (Moritex, Tokyo, Japan), as described previously (Hide et al., 2002). A 0.1 change of AR detected by SPR-CELLIA is equivalent to 1,000 RU detected by Biacore sensors,

as in other universal sensors of SPR (Alfthan K. 1998). The cells were perfused with HEPES buffer containing 0.03% HSA at a flow rate of 10 μl/min for basophils or B-cells, or with PIPES buffer containing 119 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 5.6 mM glucose, 25 mM piperazine-N- N'-bis (2-ethanesulfonic acid) (Pipes) and 1 mg/ml bovine serum albumin (BSA), pH7.2 (Hide et al., 2002) at a flow rate 10 μl/min for RBL-2H3 cells. All SPR analyses were performed at 37 ℃, except for that performed at 4 ℃ in the experiment shown in Fig. 2a.

2.7. β-hexosaminidase assay and histamine release test

The degree of exocytosis of RBL-2H3 cells was estimated by measuring the release of a granular marker, β-hexosaminidase, as described previously (Hide et al., 2002). Histamine-release tests with human basophils were performed as described previously with a goat anti-IgE antibody (670 ng/ml) as a positive control (Koro et al., 1999). Histamine was extracted and measured by reverse-phase HPLC.

2.8. Actin cytoskeleton staining

RBL-2H3 cells were placed on glass-bottomed dishes, and then were stimulated by 50 ng/ml DNP-HSA for the indicated minutes and fixed with 4% paraformaldehyde for 10 min. After two washes with phosphate buffered saline (8 mg/ml NaCl, 0.2 mg/ml KCl, 1.15 mg/ml Na_2HPO_4 0.2 mg

/ml KH_2PO_4) at pH7.4, the cells were treated with PBS containing tetramethylrodamin B isothiocyanate (TRITC)-phalloidin for actin staining for 20 min. After the fourth wash with PBS, the fluorescence was observed with a confocal laser scanning fluorescent microscope. The localization of TRITC-phalloidin in the upper regions of individual cells was captured by taking laser confocal slices (<0.5 μm).

3. Results

3.1. Fixation of floating cells onto the surface of a SPR sensor chip

When basophils were isolated from human blood using AutoMACS ™, and incubated in a floating condition, they specifically released histamine in response to anti-IgE antibody (23% of the total cellular histamine). On the other hand, no or only marginal increase of AR was observed when they were located on the sensor chip of SPR without any treatment, although some of them were observed by microscopy to have adhered to the sensor chip (data not shown). To determine the most suitable method of fixing floating cells on the sensor chip and to measure SPR signals, we examined three types of molecules to anchor cells, namely amino-alkanethiol (Cysteamine and 8-amino-octanethiol), DSP and BAM. Basophils were firmly fixed to the chips pretreated with any of these molecules. Moreover, when they were exposed to anti-IgE antibodies, a rapid increase of AR was observed as shown in Fig. 1a-d. The increase of AR continued after the removal of anti-IgE

antibodies from the perfusion buffer. The reaction time (time of maximal increase was 10-30 min) and the degree of AR increase (0.2 - 0.4 angle) was variable among donors (more than three different donors). However, regardless of the method of fixation, the overall shapes of the reaction curves were the same and similar to those observed when RBL-2H3 mast cells were stimulated on a ordinary SPR sensor chip. Since fixation of cells using amino-alkanethiols was the easiest, we used this method for the following experiments.

Fig.1

3.2. AR reflects the reaction of human basophils and human B cells fixed on the aminoalkanethiol-coated sensor chip in response to various stimuli

Human basophils were fixed on the cysteamine-coated sensor chips and exposed to various concentrations of anti-IgE or other stimuli. Anti-IgE caused the increase of AR at 37 ℃ in a concentration-dependent manner (Fig. 2a). However, no change of AR was induced when cells were stimulated at 4 ℃ even by the optimal concentration of anti-IgE (Fig. 2a). Moreover, the exposure of human basophils to ionomycin (calcium ionophore 1 μM) or PMA (phorbol ester 50 nM) caused the increase of AR, as observed with RBL-2H3 cells (Fig. 2b). However, unlike RBL-2H3 cells, human basophils caused no apparent increase of AR in response to NECA (agonist for adenosine A3 receptor 100 μM) (Fig. 2b). To explore the feasibility of this method for other non-adhering cells, we fixed Ramos cells, a human B cell line, and human peripheral B cells on the cysteamine-coated sensor chips and stimulated them with anti-IgM (20 μg/ml). Unexpectedly, the exposure of human B

Fig.2

cells to anti-IgM resulted in a decrease of AR during the stimulation. The AR after the stimulation gradually increased above the base line (Fig. 3a). The exposure of Ramos cells to anti-IgM also caused a decrease of AR. Degrees of increase, however, after the stimulation were not as large for B cells prepared from peripheral blood (Fig. 3b).

Fig.3

3.3. Methods of prompt recovery of adherent cells for SPR analysis

To recover adherent cells with minimal damage of their structures and functions for the analysis of SPR, we examined four methods as described in the Material and Methods section. The changes of AR with cells prepared by trypsinisation or pipetting at 4 ℃ ware substantially smaller than those with normally cultured cells (Fig. 4). The maximum increase of AR with cells prepared by these methods were 0.44 ± 0.11, 0.30 ± 0.036, 0.78 ± 0.050 (average ± standard error of the mean (SEM), n=3), respectively. However, there was no difference between changes of AR with cells recovered from the culture on RepCell and HydroCell and those with normally cultured cells (Fig. 4). The maximum increase of AR with cells prepared by these methods were 0.77 ± 0.11, 0.82 ± 0.049 (average ± SEM, n=3). Likewise, the exocytosis of RBL-2H3 cells was partially impaired by the pipetting at 4 ℃, but not by the culture and pipetting on RepCell, by those on HydroCell, or by the treatment with trypsin (Fig. 5a). Cells prepared by the last three treatments, showed a similar change of the structure in response to DNP-HSA (50 ng/ml), to that of the cells prepared by normal culture (Fig. 5b).

Fig.4

4. Discussion

In this study, we have demonstrated that SPR sensors can detect not only reactions of adherent cells, but also those of non-adherent cells, by locating them on the surface of a SPR sensor chip. Three types of anchoring molecules, BAM, aminoalkanethiol (cysteamine, 8-aminooctanethiol) and animo-reactive cross-linker (DSP) were all capable of fixing cells on the sensor chip of SPR sensor. All basophils fixed by these methods showed a change of AR which was large enough to be analyzed for the study of cell activation or to distinguish them from non activated cells. However, to fix cells with DSP, all proteins must be removed from the buffer solution at the time of cell fixation, since DSP nonspecifically binds proteins in buffer solutions via its NHS group. NHS groups also bind cell surface molecules, such as cell surface receptors that may activate or inactivate cells. The treatment of a sensor chip with BAM is cumbersome, since it requires presetting of the chip with amino groups. The binding of cells to aminoalkanethiols by solely an electric charge may be weak, but has been proved to be sufficient to hold cells against the flow of reaction mixtures to stimulate cells.

We therefore employed cysteamine, an aminoalkanethiol, to fix cells to SPR sensor chips and analyzed reactions of human basophils and B cells. When human peripheral blood basophils were stimulated with anti-IgE antibodies, PMA or ionomycin at 37 ^{o}C, they caused the increase of AR,

which was similar to that observed with RBL-2H3 cells stimulated with an antigen. However, unlike RBL-2H3 cells, human basophils showed no detectable change of AR when exposed to NECA, which induces the exocytosis of RBL-2H3 cells, but exerts no effect or rather inhibits the antigen-induced histamine release of human basophils. In the case of B cells, both Ramos cells, human peripheral B cell line, and human peripheral B cells showed a decrease of AR in response to anti-IgM antibodies, which activate B cells. Such a decrease of AR was also observed by SPR, when PAM212 cells were stimulated with EGF (Yanase et al., 2007). Recently, Fang, et al. showed the decrease of the refractive index of A431 cells stimulated by EGF, using resonant waveguide grating biosensors (Fang et al., 2006). These results reinforce our previous finding that AR reflects intracellular signal transductions of living cells, regardless of the direction of changes. The change of membrane conditions induced by B cell antigen receptors and that of EGF receptors may be similar in terms of electrophysics in the near field of plasma membranes.

The specific molecules that contribute to the change of AR are a matter for discussion. When cells were stimulated by extracellular stimuli via receptors, they initiated various intracellular events, such as the phosphorylation or dephosphorylation of membrane associated proteins, translocation of cytosolic proteins to the plasma membrane and/or other organelles, release of membrane proteins into the cytosol, and changes in pH, temperature and membrane potentials. In mast cells and basophils, the binding of antigen to IgE bound to the high-affinity IgE receptor (FcεRI) on the cell

surface cross-links FcεRI, resulting in the phosphorylation, usually by Lyn, of the tyrosine residues in the immunoreceptor tyrosine based activation motif (ITAM). The tyrosine phosphorylated ITAMs then acts as a scaffold for the binding of additional cytoplasmic signaling molecules with Src homology domain 2 (SH2) domains, such as the cytoplasmic protein tyrosine kinase Syk. Activated Syk then either directly or indirectly phosphorylates tyrosines of several proteins, including the linker for the activation of T cells (LAT), the SH2-containing leukocyte-specific protein 76 kDa (SLP-76), Vav and phospholipase C γ . Tyrosine-phosphorylated PLC γ catalyzes the hydrolysis of PIP_2, resulting in the generation of inositol-1-4-5-triphosphate (IP_3) and 1,2-diacylglycerol, second messengers that release Ca^{2+} from internal stores and activate protein kinase C (PKC), respectively. These interactions result in the release of preformed mediators from granules, the generation of newly synthesized mediators, such as arachidonic acid metabolites, cytokines and the changes in membrane structure (Shiraganian et al., 2003). We previously reported that RBL-2H3 cells cause a substantial increase of AR in response to antigen, even when their morphological changes and mediator release were abolished by specific reagents respectively (Yanase et al. 2007).

In this study, we have demonstrated that an SPR sensor readily detects reactions of spheroid cells with diameters of 8-10 μm, such as basophils and B cells, as well as those of adhering cells. Taking into account that evanescence waves may penetrate to only a few hundred nanometers from a gold film surface, cellular events detected by the SPR sensor should be those on and/or just above

the plasma membrane (approximately 10 nm thickness), rather than those a few hundred nanometers away from the membrane. Further studies of these receptors with amino acid mutations with gain- and/or loss-functions should clarify the precise relationship between SPR signals and intracellular signaling.

As well as methods to connect plasma membranes to a sensor chip, methods for rapid isolation of adherent cells without functional impairment are essential for the wide application of SPR biosensors for living cells. Among four methods tested in this study, both RepCell and HydroCell were equally suitable for this purpose. The degree of exocytosis of RBL-2H3 cells collected from a culture on these materials were somewhat small, but degrees of both the increase of AR and their membrane ruffling were similar to those cultured on a sensor chip under ordinary conditions. These methods should be useful not only for functional analysis of cells that may not be cultured directly on a sensor chip, but also for cells that may proliferate during the culture on a sensor chip.

In conclusion, we have demonstrated that SPR sensors can detect the reactions of non-adherent cells, including human basophils and lymphocytes, as well as adherent cells, by fixing them to the sensor chip. SPR sensors may be a useful tool for the analysis of clinically important cell functions, such as basophil histamine-release, and lymphocyte stimulation.

Figure legends

Fig.1. **Human basophils cause the increase of AR in response to anti-IgE antibody on the sensor chip treated by molecules which can anchor cells.**

An SPR sensor chip was treated with cysteamine (a), 8-amino-octanethiol (b), DSP (c) or BAM (d) before cell attachment. Human basophils were then placed on sensor chip and perfused with or without anti-IgE antibodies (670 ng/ml). Horizontal bars show the period of anti-IgE perfusion. Inlets show the surface of sensor chips at the beginning of the perfusion.

Fig. 2. Change of AR with human basophils, fixed by cysteamine on sensor chips and stimulated under various conditions.

Human basophils were exposed to anti-IgE antibody (670 or 200 ng/ml) (a,b), PMA (50 nM) (b), ionomycin (1 μM) (b) or NECA (100 μM) (b) at 37 ^{o}C (a,b) or 4 ^{o}C (a). Horizontal bars show the periods of stimulation.

Fig. 3. Change of AR with human B lymphocytes and Ramos cells, fixed on sensor chips and stimulated with anti-IgM antibody.

Human B lymphocytes (a) and Ramos cells (b) on cysteamine-treated sensor chips were

stimulated with anti-IgM antibody (20 μg/ml) at 37 ^{o}C. Horizontal bars show the periods of stimulation.

Fig. 4. Change of AR with RBL-2H3 cells prepared by methods for rapid isolation and placement.

RBL-2H3 cells were cultured and recovered as described in the "Materials and Methods" section. They were placed on SPR sensor chips and stimulated with DNP-HSA (50 ng/ml) at 37 ^{o}C. The period of stimulation was depicted as a horizontal bar. The maximum change of AR with cells prepared in each condition was as follows: normal culture (0.78 ± 0.05); Repcell, (0.77 ± 0.11); Hydrocell (0.82 ± 0.05); trypsin, (0.44 ± 0.11); 4 ^{o}C scrape, (0.30 ± 0.04) (average ± standard error of the mean, n=3), respectively.

Fig. 5. Exocytosis and morphological changes of RBL-2H3 cells prepared by methods for rapid isolation and placement.

RBL-2H3 cells were cultured and recovered as described in the "Materials and Methods" section. They were placed on 96 well culture plates (a) or glass-bottom culture dishes (b) and stimulated with DNP-HSA (50 ng/ml) at 37 ^{o}C. The release of β-hexosaminidase was measured in three independent experiments (average ± standard error of the mean). Morphological changes of cells 5 min after the

stimuli were observed by a confocal microsope. The difference between exocytosis in each condition was tested using one-way ANOVA followed by Turkey-Kramer test and considered significant with hazard ratio (P) < 0.01 (**).

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Fig. 1

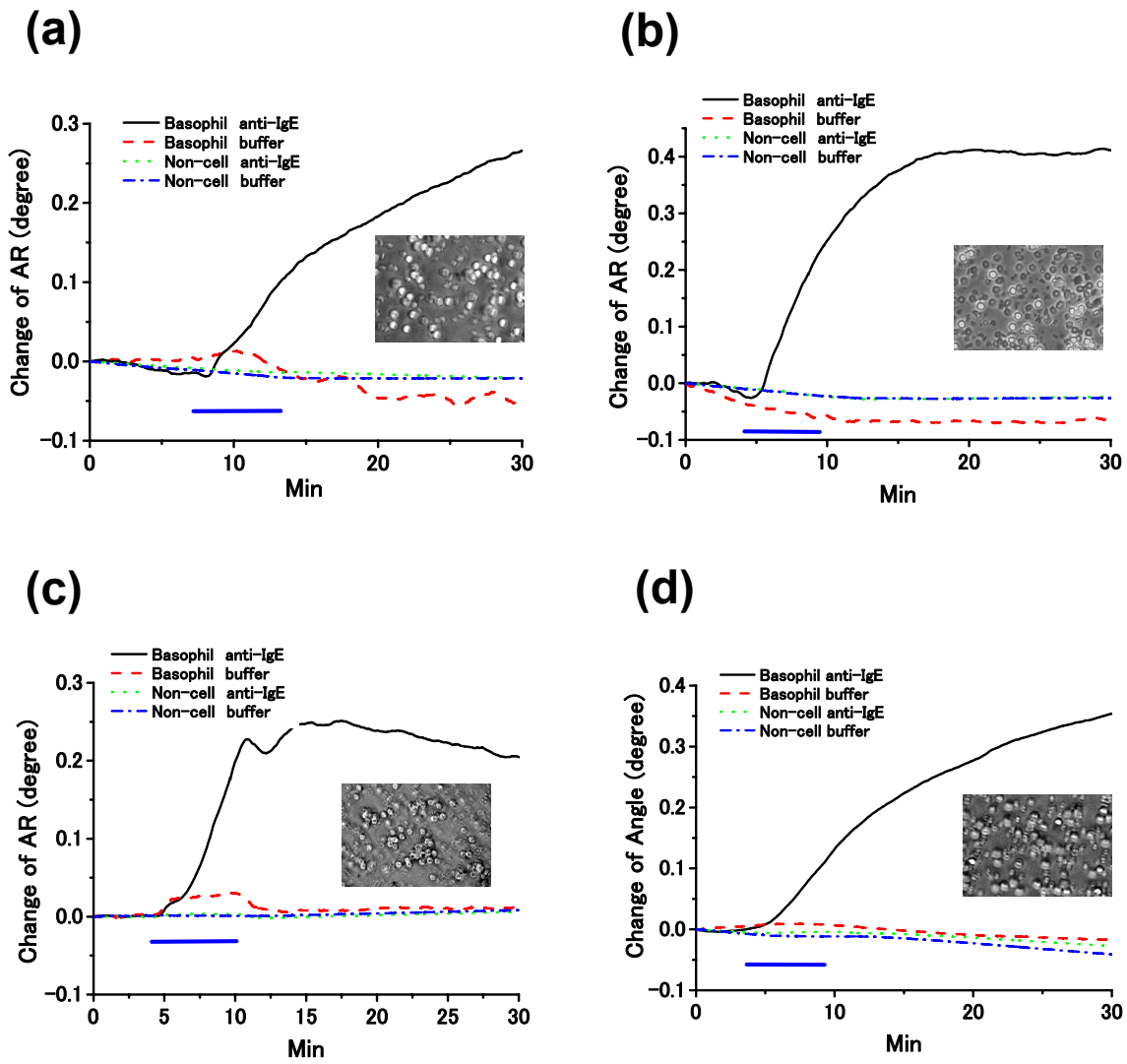
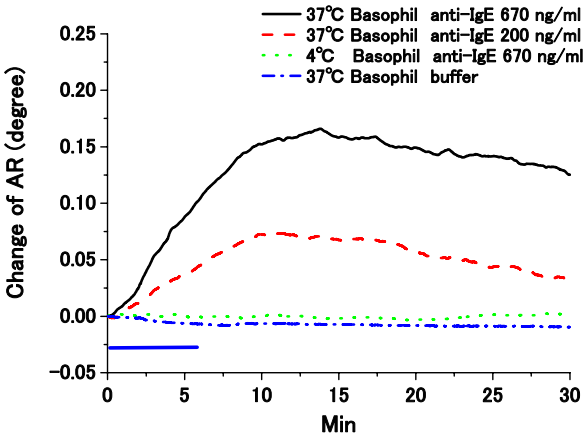

(a)
Basophil anti-IgE
Basophil buffer
Non-cell anti-IgE
Non-cell buffer
Change of AR (degree)
Min
(b)
Basophil anti-IgE
Basophil buffer
Non-cell anti-IgE
Non-cell buffer
Change of AR (degree)
Min
(c)
Basophil anti-IgE
Basophil buffer
Non-cell anti-IgE
Non-cell buffer
Change of AR (degree)
Min
(d)
Basophil anti-IgE
Basophil buffer
Non-cell anti-IgE
Non-cell buffer
Change of Angle (degree)
Min

Fig. 2

(a)


37℃ Basophil anti-IgE 670 ng/ml
37℃ Basophil anti-IgE 200 ng/ml
4℃ Basophil anti-IgE 670 ng/ml
37℃ Basophil buffer
Change of AR (degree)
0.25
0.20
0.15
0.10
0.05
0.00
−0.05
0
5
10
15
20
25
30
Min

(b)

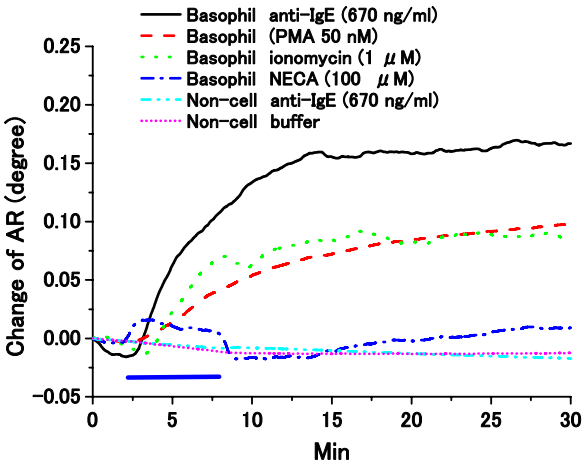
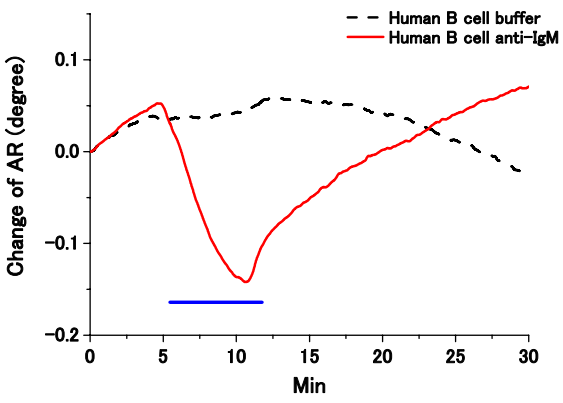

Basophil anti-IgE (670 ng/ml)
Basophil (PMA 50 nM)
Basophil ionomycin (1 μM)
Basophil NECA (100 μM)
Non-cell anti-IgE (670 ng/ml)
Non-cell buffer
Change of AR (degree)
0.25
0.20
0.15
0.10
0.05
0.00
−0.05
0
5
10
15
20
25
30
Min

Fig. 3

(a)


Human B cell buffer
Human B cell anti-IgM
Change of AR (degree)
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0.0
−0.1
−0.2
0
5
10
15
20
25
30
Min

(b)

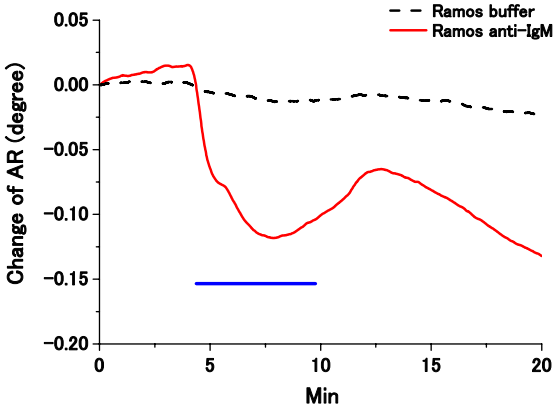

Ramos buffer
Ramos anti-IgM
Change of AR (degree)
0.05
0.00
−0.05
−0.10
−0.15
−0.20
0
5
10
15
20
Min

Fig. 4

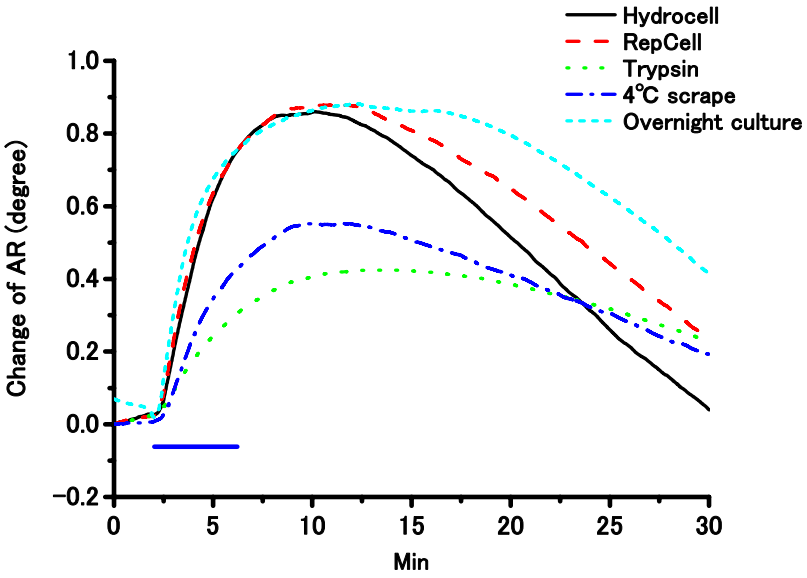
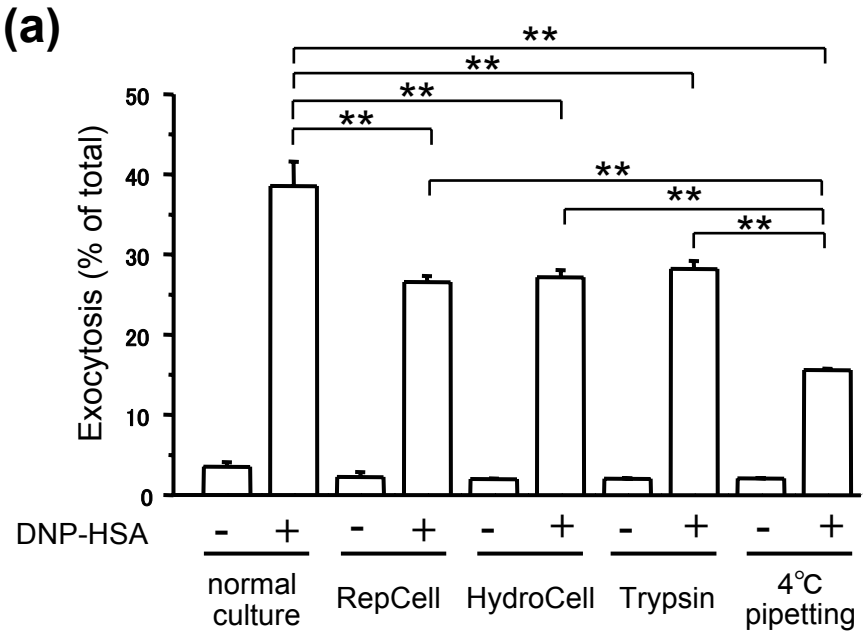
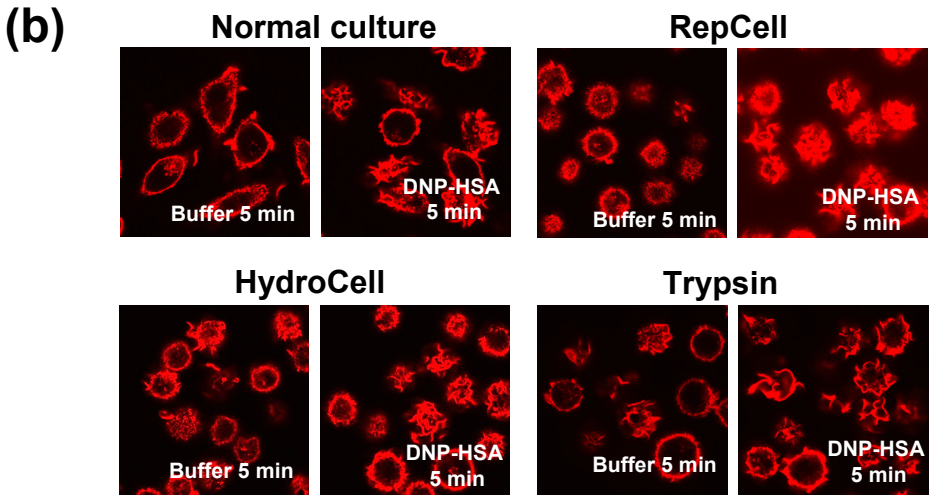

Hydrocell
RepCell
Trypsin
4℃ scrape
Overnight culture
Change of AR (degree)
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
0
5
10
15
20
25
30
Min

Fig. 5


(a)
Exocytosis (% of total)
50
40
30
20
10
0
**
**
**
**
**
**
**
DNP-HSA
-
+
-
+
-
+
-
+
-
+
normal culture
RepCell
HydroCell
Trypsin
4℃ pipetting


(b)
Normal culture
Buffer 5 min
DNP-HSA 5 min
RepCell
Buffer 5 min
DNP-HSA 5 min
HydroCell
Buffer 5 min
DNP-HSA 5 min
Trypsin
Buffer 5 min
DNP-HSA 5 min