

Mast-Cell-Based Fluorescence Biosensor for Rapid Detection of Major Fish Allergen Parvalbumin

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S Supporting Information

ABSTRACT: In this study, we developed a rat basophilic leukemia cell (RBL-2H3) fluorescence sensor to detect and identify the major fish allergen parvalbumin (PV). We constructed and transfected a CD63-enhanced green fluorescent protein (EGFP) plasmid into RBL cells through a highly efficient, lipid-mediated, DNA-transfection procedure. Stable transfectant RBL cells were then obtained for a cell fluorescence assay with confocal laser scanning microscopy. Results show that the cell surface expression of CD63 reflects degranulation, indicating that a fluorescence assay with these cells could efficiently measure the activation of antigen-stimulated transfectant cells and detect antigens with a nanogram level. Therefore, this cell-based fluorescence biosensor technique for detecting fish PV exhibits promise for quantifying fish PV after anti-PV immunoglobulin E (IgE) stimulation. Results show that fluorescence intensities increased with purified PV concentrations from 1 to 100 ng/mL, with a detection limit of 0.35 ng/mL [relative standard deviation (RSD) of 4.5%], confirmed by β -hexosaminidase assays. These rat basophilic leukemia (RBL) mast cells transfected with the CD63–EGFP gene and responded to PV only when they were sensitized with the specific IgE antibody. This demonstrates the utility of this highly sensitive biosensor for food allergen detection and prediction.

KEYWORDS: fluorescence sensor, RBL-2H3 mast cells, fish parvalbumin, CD63, green fluorescent protein, confocal laser scanning microscopy

INTRODUCTION

Fish represent an important constituent of the human diet. However, some species may cause severe allergic reactions even in trace quantities that make fish allergies among the most common food allergies.¹ Nowadays, with the mass consumption of fish in all parts of the world, the growth of the population infected with fish hypersensitivity has accelerated dramatically.² A recent U.S. survey reported that ~0.4% of the general population have fish allergies, in which nearly 40% of children are sensitized to fish.³

Stimulation with fish allergens can trigger severe immunoglobulin E (IgE)-mediated allergic diseases, such as urticaria, diarrhea, and dyspnea.⁴ Fish allergenicity is mainly related to the expression concentration of parvalbumin (PV), the major allergen in fish.⁵ This ~12 kDa protein has been commonly found in all fish sarcoplasmic proteins with characteristics of high water solubility, well stability, and an isoelectric point of 4. Moreover, PVs are believed to be the only major allergens for 95% of the patients affected with IgE-mediated fish hypersensitivity.⁶

Currently, a number of technical approaches have been developed to identify fish PV in food, including chromatography,⁷ mass spectrometry,⁸ real-time polymerase chain reaction (PCR),⁹ protein chip,¹⁰ and enzyme-linked immunosorbent assay (ELISA).^{11,12,1} However, none of these detection methods is perfect, and each method has its own drawbacks, such as a long detection time, complex sample pretreatment, and complicated interpretation of results. Hence, there is a need for a rapid and visible tool to accurately quantify fish PVs as a new attempt and supplement.

Recently, living-cell-based sensors have provided a new strategy for *in vitro* tests. Sensors for detecting target antigens are developed by a specific biological recognition element, and the identification process can be converted into a recordable and quantitative signal.¹³ Therefore, cell-based biosensors have been widely employed in many fields, including drug screening, environmental test, medical diagnosis, and national security.^{14–17} Using the natural ability of mast cells to identify antigens is a stable and accurate strategy that mimics physiological conditions,^{18,19} because there are abundant high-affinity receptors (Fc ϵ RI) on the surface of rat basophilic leukemia (RBL) cells, to which IgE antibodies can tightly bind. Subsequent cross-linking of Fc ϵ RI/IgE complexes causes cell degranulation and release of intracellular inflammatory mediators that initiate the antigen-recognition ability of these pre-sensitized cells. Hence, such a cell sensor can respond to very low antigen concentrations with a rapid response.²⁰

However, traditional methods for measuring the release of cell inflammatory mediators are time-consuming and require complex sample pretreatment procedures, and the entire reaction process of cell sensitization cannot be directly observed.²¹ To address these issues, this study constructed and transfected a plasmid for the expression of CD63–enhanced green fluorescent protein (EGFP) fusion protein into RBL mast cell nuclei. This gave these cells the ability to express

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fluorescence spontaneously when exposed to an allergen antigen. CD63 glycoprotein can be easily found on basophilic granular membranes in many cells, such as basophils, mast cells, and platelets.^{22,23} The integration of cytoplasmic granules with the plasma membrane occurs when the basophils or mast cells are activated along with the successive release of inflammatory mediators, such as histamine,^{24,25} although CD63 antigen expression on mast cell surfaces has always been detected by flow cytometric analysis.²⁶ However, the real-time expression of CD63 in individual cells cannot be observed.²⁷ Therefore, this study employed confocal fluorescence microscopy to examine CD63 surface expression on RBL mast cells stimulated with antigen, resulting in a rapid quantification method for fish PVs.

This study developed a novel, mast-cell-based, fluorescent sensor to quantify fish PV and evaluate IgE-mediated hypersensitivity. We constructed and transfected CD63–EGFP plasmid into RBL cells, to obtain stable genetically fluorescent cell lines. We also explored the use of the real-time surface expression of CD63–EGFP in anti-2,4-dinitrophenyl (DNP) IgE pre-sensitized RBL-2H3 cells exposed to DNP–bovine serum albumin (BSA) as a model for fluorescence detection by confocal laser scanning microscopy (CLSM). We measured changes in fluorescence intensity induced by the fish PV on mast cells and analyzed the relationship between different allergen concentrations and impedance signals. These RBL cell sensors successfully detected and identified target allergens within minutes, and the allergic reaction process was monitored in real time by CLSM.

■ EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Mouse monoclonal anti-DNP IgE antibody, DNP–BSA, sulforhodamine B (SRB), bromochloroindolyl phosphate, nitro blue tetrazolium, and trichloroacetic acid were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Purified fish PV and mouse monoclonal anti-fish PV IgE antibody were purchased from Novus Biologicals (Littleton, CO). RBL cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium and fetal bovine serum (FBS) was obtained from Gibco Laboratories (Gaithersburg, MD). Glass-bottomed dishes (35 mm) were purchased from Shengyou Biotechnology Co., Inc. (Hangzhou, China). Other conventional reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All solutions were prepared with deionized water, and all reagents were of analytical grade.

Instrumentation. Fluorescence signals were recorded by CLSM (LSM 710, Carl Zeiss Microscopy GmbH, Göttingen, Germany). A FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) was used to evaluate cell apoptosis. RBL cells were incubated in a CO₂ incubator (Thermo Scientific Forma Series II Water Jacket, Thermo Fisher Scientific, Inc., Waltham, MA).

Construction of a CD63–EGFP Prokaryotic Expression System. The complete coding sequence of mouse CD63 (NM_001042580) was retrieved from the National Center for Biotechnology Information (NCBI) GenBank database and amplified from mouse liver cDNA. The primers employed were mCD63, 5′-cgc gtc gac atg gcg gtg gaa gga g-3′ (forward) and 5′-gcg gga tcc cat tac ttc ata gcc ac-3′ (reverse). The mCD63 PCR products and apEGFP-N1 vector were digested with *Bam*HI and *Sall*, and the resulting fragments were purified and ligated with T4 DNA ligase. The ligated product was transformed into DH5a competent cells and an isolated positive clone named pEGFP-N1-mCD63.

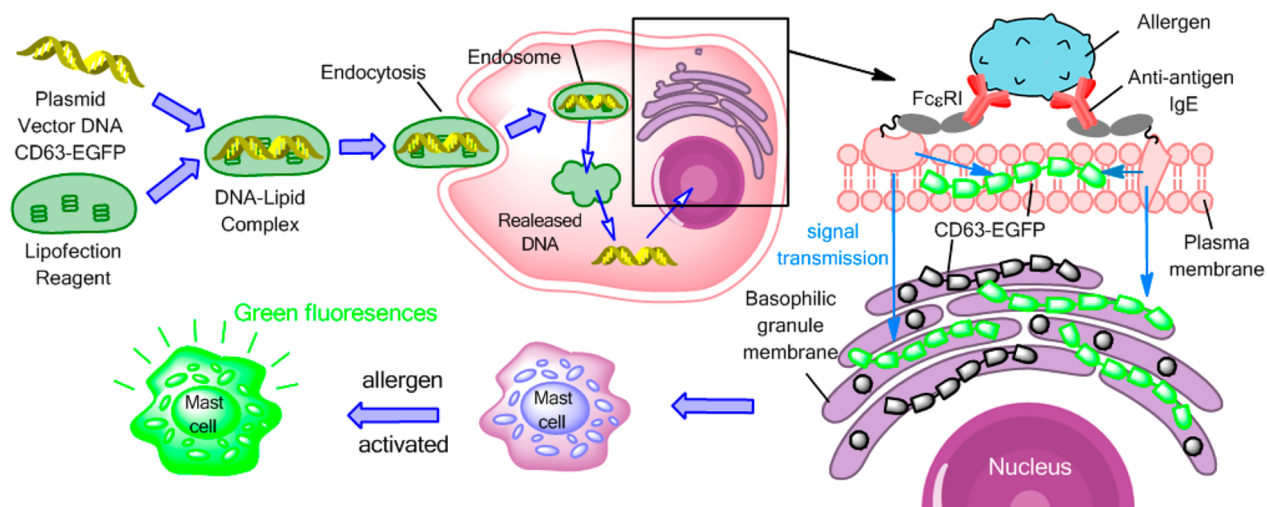
Transfection of Plasmid DNAs. At 1 day before transfection, plates were prepared containing 2×10^5 RBL mast cells in 500 μ L of growth medium without antibiotics, such that the cells were 90–95% confluent at the time of transfection. The ratio of cationic carrier/DNA was optimized on the basis of the instructions of the

manufacturer. Briefly, 4 μ g of DNA were diluted in 50 μ L of Opti-MEM I reduced serum medium without serum (Gibco Invitrogen, Life Technologies) and mixed gently. A total of 10 μ L of Lipofectamine2000 (Invitrogen, Life Technologies, Grand Island, NY) was mixed gently before use and added to 50 μ L of Opti-MEM I medium. After a 5 min incubation at room temperature, diluted DNA was combined with the diluted Lipofectamine 2000 (total volume of 100 μ L) and incubated for 20 min at room temperature. Then, the mixture was added to RBL cell plates. After 4 h for DNA transfection, the transfection medium was replaced with fresh culture medium and these cells with CD63–GFP plasmid DNA were cultured in 35 mm dishes for a few days. Then, stable transfectants were obtained by selection with the antibiotic G418 (Gibco Invitrogen) and a stable transfectant RBL cell line with EGFP selected.

Western Blot Analysis for CD63–EGFP Expression. To each tube of harvested cells (in 20 μ L), 180 μ L of radio-immunoprecipitation assay (RIPA) lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) was added and incubated for 10 min at room temperature (RT). Equal amounts of proteins were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after boiling for 5 min in 4 $\times$ loading buffer and transferred onto Immobilon-P membranes (Millipore Corp., Billerica, MA). The membranes were then incubated with anti-EGFP antibody and a second antibody immunoglobulin G (IgG horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactivity was detected using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The target protein bands were quantified by scanning densitometry using ImageJ processing software and normalized to the signal intensity of GAPDH.

In Vitro Cell Proliferation and Apoptosis Assay. RBL cells seeded on a 96-well plate at 2×10^5 cells/well were infected with CD63–EGFP using the procedures described above or received only changes with fresh media, as controls. At 1, 2, or 3 days post-infection, SRB assays were performed as described by Cung et al.²⁸ In brief, cells were fixed at 4 °C and in 3.3% (w/v) trichloroacetic acid for 1 h without removing the culture media. After removal of the fixation solution by tapping until the plates were completely dry, each well of cells was incubated with 100 μ L of 0.057% (w/v) SRB at RT for 30 min. The wells were then rinsed 4 times with 1% (v/v) aqueous acetic acid and dried, and the stained cells were dissolved in 200 μ L of 10 mM Tris base (pH 10.5) with shaking for 5 min. Finally, the 510 nm absorbance was measured on a microplate reader (BioTek Instruments, Inc., Winooski, VT). The influence of EGFP gene transfer on cell survival was examined by evaluating apoptosis via fluorescence-activated cell-sorting analysis, including an annexin-V–EGFP apoptosis detection kit (Nanjing KeyGen Biotech Co., Ltd., Shanghai, China). Briefly, RBL cells on 12-well plates were harvested by trypsinization at 3 day after infection with CD63–EGFP plasmid. After neutralization by washing with culture media containing FBS and phosphate-buffered saline, the cells were resuspended in 100 μ L of annexin-V binding buffer and stained in the dark with 4 μ L of annexin-V–EGFP (an apoptosis marker) and 4 μ L of propidium iodide (PI, a necrosis marker) for 15 min at room temperature as a control. After 400 μ L of annexin-V binding buffer was added, 10 000 cells per sample were analyzed by flow cytometry and CellQuest software. Fluorescent signals from EGFP and PI were distinguished by three color detectors.

PV Purification. The PV from crucian carp, bream, and silver carp was extracted as described previously.²⁹ Briefly, 2 g of white muscle of each sample was extracted with 10 mL of 20 mM Tris–HCl (pH 7.5), using a homogenizer (NS1001 L2K, Niro Soavi, Italy). Supernatants were obtained as PV crude extracts after centrifugation at 8000g for 30 min. To achieve a clear solution for test, crude extracts were purified by taking advantage of their thermostability, heating at 70 °C for 5 min, and then immediately cooled in ice water, followed by centrifugation at 4000g for 30 min. Supernatants composed mainly of PV were analyzed by SDS–PAGE. Then, the extract solutions were subjected to anti-PV antibody pre-sensitized RBL cells, which were monitored by a confocal laser scanning (CLS) microscope for fluorescence analysis.

Scheme 1. Schematic Illustration of the Working Principle of a RBL Cell Sensor^a

^aFusion plasmid CD63–EGFP was introduced into RBL cells to obtain stable transfectant fluorescent RBL cells. When allergen antigen bound to the antibody pre-sensitized mast cells, cytoplasmic granules would be fused with the plasma membrane and trigger the expression of CD63 and fluorescent protein on the cell surface. Therefore, allergen antigen would be detected by the elevation of intracellular fluorescence emitted by EGFP expression in RBL cells.

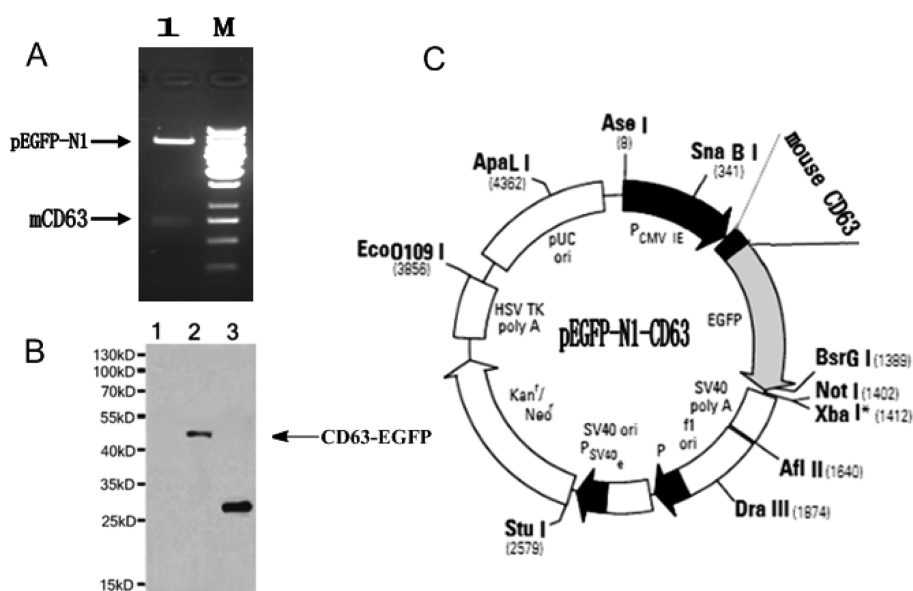


Figure 1. Construction of the pEGFP-N1-mCD63 vector and expression of the CD63–EGFP protein. (A) pEGFP-N1-mCD63 plasmid double-digested results, where M represents DNA markers (250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 45000, 5000, 6000, and 7000 bp) and 1 represents the pEGFP-N1-mCD63 plasmid double digested with *Bam*HI and *Sal*I. (B) Western blotting of lysates prepared from RBL cells at 24 and 48 h after transfection with the pEGFP-N1-mCD63 vector with anti-EGFP antibodies: 1, negative control (non-transfection cell); 2, CD63–EGFP fusion protein; 3, positive control (cell transfected with pEGFP-N1). (C) Map of the pEGFP-N1-CD63 plasmid. Mouse CD63 gene (not including the stop codon) was introduced at the *Sal*I and *Bam*HI restriction sites of the multiple cloning site (MCS) region of the pEGFP-N1 (Clontech, PT3027-5) vector.

RBL Cell Sensor Assay. RBL-2H3 cells were cultured in a flask in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin ($100 \mu\text{g mL}^{-1}$), and streptomycin ($100 \mu\text{g mL}^{-1}$) at 37°C in a humidified atmosphere containing 5% CO_2 . At the growth retardation stage after 3 days, cells reached the logarithmic growth phase, after which they were plated onto 35 mm glass-bottomed dishes (2×10^5 cells/dish) to form a monolayer overnight. Cells were then washed with Tyrode's buffer (Maishi BioTech, Suzhou, China) and pre-sensitized by incubation with Tyrode's buffer containing 10 mg/mL antibodies and 2.5 mM probenecid at 37°C for 30 min. The resulting cells were then washed and maintained in Tyrode's buffer. A volume of $50 \mu\text{L}$ of serially diluted samples containing DNP–BSA or

fish PV antigen was prepared in Tyrode's buffer and subjected to the prepared RBL sensor cells. The cell responses were monitored using a LSM 710 CLS microscope (Carl Zeiss Microscopy GmbH). Time-lapse sequences of RBL cells, recorded every 30 s, were collected, and the total fluorescence intensity for each image was calculated using Scion Image software (Scion Corp., Frederick MD). Cells not exposed to DNP–BSA (or fish PV antigen) served as controls.

β -Hexosaminidase Assay for Measurements of Cell Degranulation. A β -hexosaminidase assay was performed to validate the result of fluorescence analysis by IgE-mediated allergic response.³⁰ Cells growing in 96-well plates were washed, incubated in Tyrode's buffer, and stimulated with medium containing different allergen

concentrations. After 1 h, the supernatant was removed and the cell monolayer was lysed in Tyrode's buffer containing 0.5% TX-100 to obtain the total cellular β -hexosaminidase activity. This activity was measured in both supernatant and lysates by adding 50 μ L of substrate *p*-nitrophenyl-*N*-acetyl- β -glucopyranoside (1 mg/mL) to each well. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 150 μ L of 0.4 M glycine at pH 10.7 and the 405 nm absorbance was read in an ELISA reader (BioTek Instruments, Inc.). The ratio of β -hexosaminidase release was calculated using the following formula: β -hexosaminidase release (%) = $100 \times [(\text{supernatant}/\text{blank supernatant})/(\text{supernatant}/\text{blank supernatant}) + (\text{total cell lysate}/\text{blank total cell lysate})]$. Cells incubated in medium without allergen served as blanks.

RESULTS AND DISCUSSION

Working Principles of RBL Cell Sensors. Scheme 1 illustrates the working principle by which a RBL cell sensor

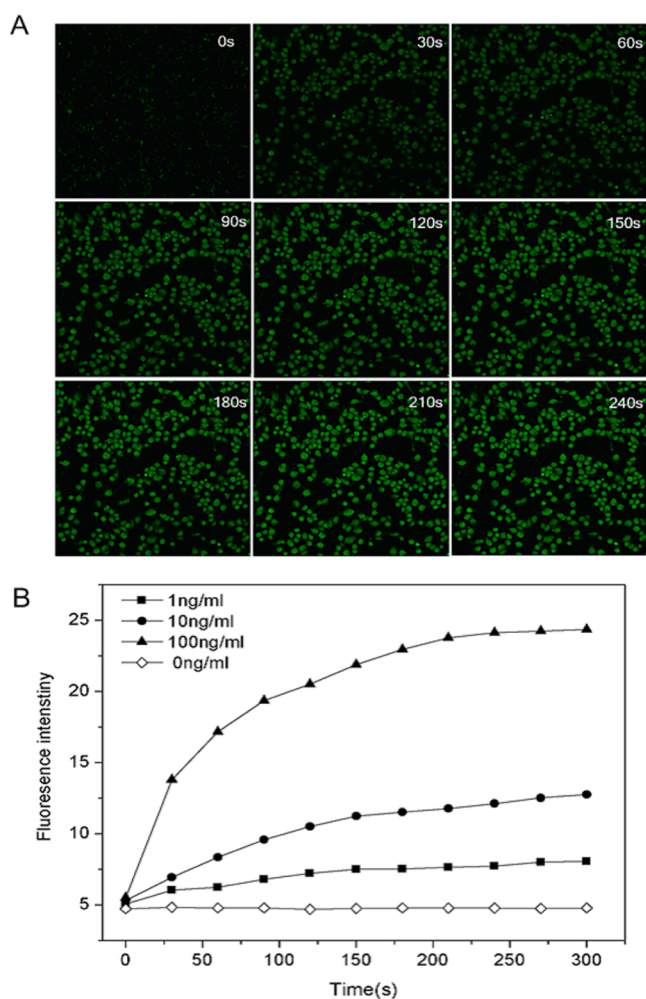


Figure 2. Fluorescence intensity changes of mast cells exposed to various concentrations of DNP-BSA. (A) Time-dependent images of cell fluorescence intensity responses. DNP-BSA (100 ng/mL) was employed to stimulate anti-DNP IgE pre-sensitized RBL cells. The images were recorded every 5 s and displayed every 30 s. (B) Quantification of fluorescence intensity in RBL cells after sensitization with 10-fold serial dilutions of DNP-BSA.

recognizes and quantifies a target antigen. In this study, we fused EGFP to mouse CD63, in which the C terminus of CD63 was conjugated with EGFP. Then, we introduced the fusion plasmid CD63-EGFP into RBL cells through a highly efficient,

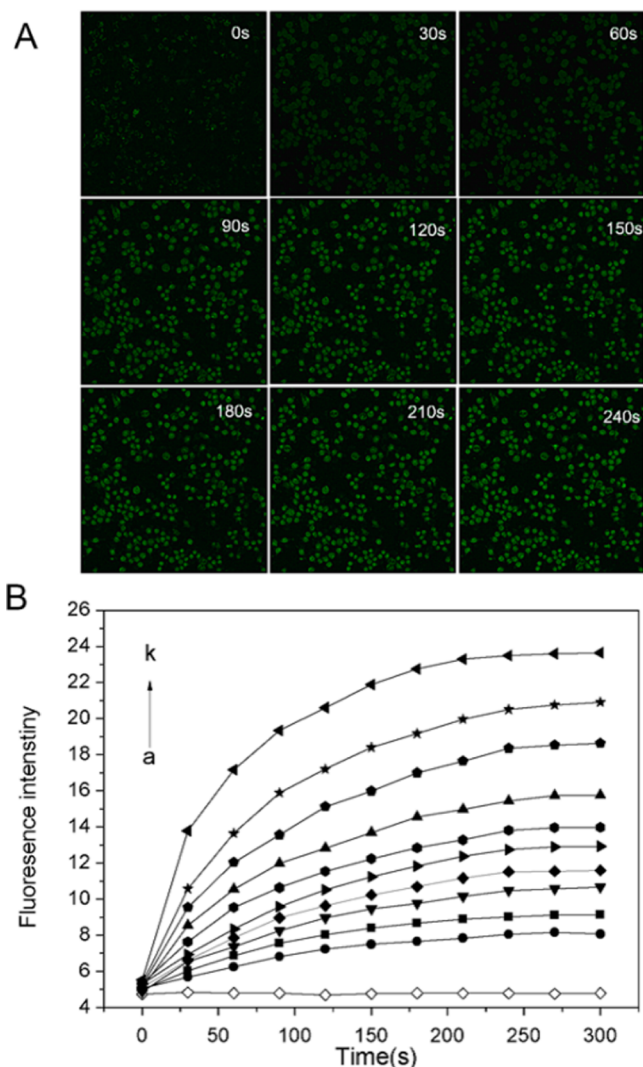


Figure 3. Fluorescence intensity changes of RBL cells with different concentrations of fish PV. (A) Time-dependent images of cell fluorescence intensity variation. PV (100 ng/mL) was used to stimulate RBL cells that pre-sensitized with anti-PV antibodies. The images were recorded at an interval of 5 s and exhibited every 30 s. (B) Quantification of fluorescence intensity in RBL cells exposed to 10 different concentrations of PV (from a to k: 0, 1, 2.5, 5, 10, 20, 40, 60, 80, and 100 ng/mL).

lipid-mediated, DNA-transfection procedure. DNA-lipid complexes were fused with the membrane by endocytosis, and the fluorescent protein gene was diffused throughout the intracellular membranes, thus entering the nucleus. Next, RBL cells were selected with the antibiotic G418 to obtain stable transfectant cells. Results show that CD63 expression on cell surfaces increased, along with the successive expression of fluorescent protein upon antibody activation in RBL mast cells pre-sensitized with allergen antigen (see the enlarged drawing on the right of Scheme 1). In this way, we monitored cell responses and quantified the allergen antigen through intracellular fluorescence changes observed under a CLS microscope.

Successful Construction and Expression of CD63-EGFP Chimera Proteins. We amplified the CD63 gene from cDNA of mouse liver tissue with oligonucleotides, so that *SalI* and *BamHI* restriction sites were introduced at the 5' and 3'

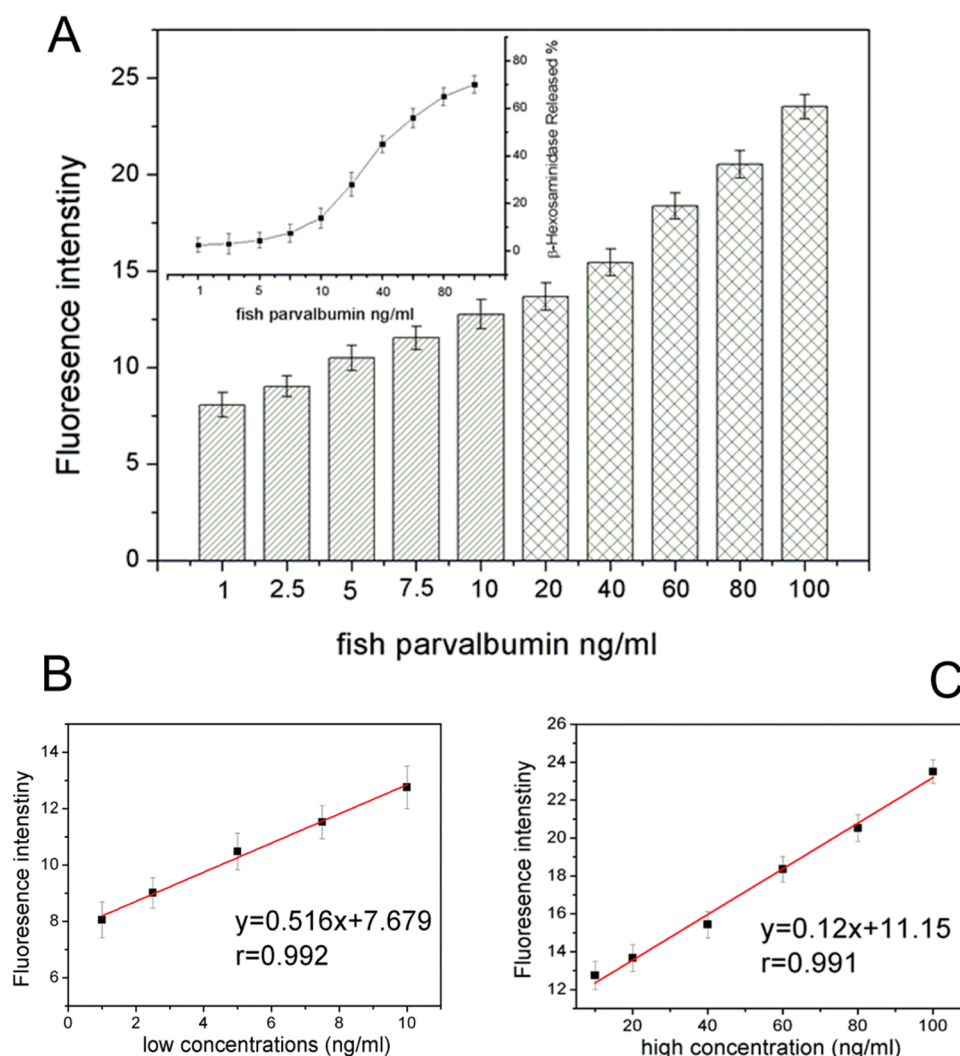


Figure 4. Quantification of fish allergen PV by RBL-2H3-cell-based sensor. (A) Fluorescence intensity spectroscopy for RBL-2H3 cells treated with different doses of fish allergen PV: 1, 2.5, 5, 7.5, 10, 20, 40, 60, 80, and 100 ng/mL. (Inset) β -Hexosaminidase assay for confirmation of the cell activated by fish allergen PV. (B) Linear regression of fluorescence intensity versus low concentrations of PV (1–10 ng/mL). (C) Linear regression of fluorescence intensity versus high concentrations of PV (10–100 ng/mL). Data represent the mean $\pm$ SE of three different experiments under similar conditions.

ends, respectively. The PCR products and pEGFP-N1 plasmids were also double-digested with *Bam*HI and *Sal*I, ligated with T4 DNA ligase, and transfected into DH5a competent cells (Figure 1A). The recombinant plasmids were double-digested with *Bam*HI and *Sal*I, and the positive clone was named pEGFP-N1-mCD63 (Figure 1C). The anti-EGFP antibody was used to detect CD63–EGFP fusion protein expression. One major band, ~46 kDa (the CD63 and EGFP fusion protein), was observed (Figure 1B), which was in good agreement with the sum of the molecular sizes of CD63 and EGFP. The RBL cells infected with CD63–EGFP showed strong fluorescence signals from EGFP expression at day 3 after antigen stimulation, indicating a high transfection efficiency of about $83.27 \pm 1.45\%$ through fluorescent observation and flow analysis (see Figure S1A of the Supporting Information).

SRB-based proliferation assays were performed to investigate potential adverse effects during the transfection process. Results show no difference in RBL cell numbers at 1, 2, and 3 days between the CD63–EGFP-infected and control groups (see Figure S1B of the Supporting Information). Flow cytometric analyses were conducted at day 3 for the CD63–EGFP-infected

and control groups, using annexin V–EGFP and PI as markers for apoptosis and necrosis, respectively. Results show similar low rates of early apoptosis (0.86 ± 0.21 versus $0.72 \pm 0.18\%$, respectively) and necrosis (0.17 ± 0.13 versus $0.23 \pm 0.15\%$, respectively; see Figure S1C of the Supporting Information).

Quantification of Fish Allergen PV by RBL Cell Sensors. Naive RBL cells cannot recognize any antigens. They can only recognize antigens when anti-antigen IgE antibodies bind tightly to the high-affinity receptors (Fc ϵ RI) on their cell surfaces. This binding allows them to be stimulated by cross-linking with protein allergens to initiate a sequence of biochemical events, including cellular degranulation and release of inflammatory molecules.³¹ We verified the use of cell sensors to recognize allergen antigen recognition with DNP–BSA, a multivalent model antigen, to stimulate RBL cells pre-sensitized with anti-DNP IgE.

We also used time-lapse imaging of cell responses after stimulation with DNP–BSA (100 ng/mL) and measured fluorescence intensity changes over time to determine the amplitude and speed of cell responses. Results show that both declined with a decreased DNP–BSA concentration (panels A

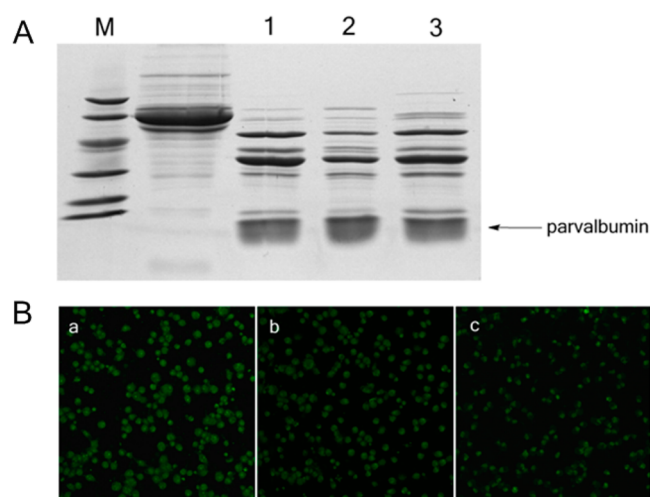


Figure 5. (A) SDS–PAGE analysis for PV from three kinds of different fish purifications: M, protein marker (97, 66, 44, 29, 20, and 14 kDa); 1, crucian carp; 2, silver carp; and 3, bream. (B) Quantification of three kinds of fish PV by a RBL-2H3-cell-based sensor: (a) crucian carp, (b) silver carp, and (c) bream.

Table 1. Concentration and Content of PV in Three Kinds of Fish^a

sample	crucian carp	silver carp	bream
fluorescence intensity (mean $\pm$ SD)	18.84 $\pm$ 0.08	14.88 $\pm$ 0.14	13.68 $\pm$ 0.16
PV concentration (ng/mL $\pm$ SD)	64.08 $\pm$ 0.22	31.08 $\pm$ 0.35	21.08 $\pm$ 0.33
PV content (mg/g $\pm$ SD)	1.92 $\pm$ 0.23	0.93 $\pm$ 0.28	0.63 $\pm$ 0.26

^aSD = standard deviation ($n = 5$).

and B of Figure 2, respectively). These results are in accordance with previous reports. They indicate the presence of target antigens and demonstrate that RBL cell sensor responses can be monitored in real time with CLSM.¹⁹

Next, fish PV standards were applied to evaluate the feasibility of RBL cell sensors. We prepared cell sensors using pre-sensitized RBL cells with anti-fish PV antibodies. We monitored the resulting cell fluorescence changes with CLSM in real time. Time-lapse images of RBL cell sensors responding to fish PV (100 ng/mL) reveal that activating mast cells with antigens led to a rapid and robust increase in cell fluorescence intensity (Figure 3A). We also measured the response of sensors to different PV concentrations in terms of the fluorescence intensity versus time (Figure 3B). PV samples were added after a stable fluorescence signal was obtained. At low PV concentrations (1–100 ng/mL), from the time of addition (at 0 s), RBL cells showed a slow increase in fluorescence intensity. This indicates the initiation of CD63–EGFP chimera protein expression on cytoplasmic granular and plasma membranes. This, in turn, created a non-uniform distribution of fluorescence on the cell surface and in the cytoplasm, leading to a lower fluorescence intensity.

At high PV concentrations, cell fluorescence intensity rose rapidly, reaching a plateau of sustained saturation within 60 s after stimulation. Further investigation of the allergic responses of these cells reveals that high activation of mast cells induced fusion of cytoplasmic granular membranes with plasma membranes. This resulted in the degranulation of secretory vesicles and release of inflammatory molecules, such as

histamine and β -hexosaminidase.³² We also examined fluorescence changes on the surface of single cells, and the result is in accordance with previous results (see Figure S2 of the Supporting Information).

A histogram of the linear relationship between the fish PV concentrations and fluorescence intensity clearly shows that fluorescence signals follow the same trend as that of DNP–BSA-activated cells (Figure 4A). Fluorescence intensity was proportional to PV, ranging from 1 to 100 ng/mL. A β -hexosaminidase assay was performed to confirm the fluorescence analysis results (inset of Figure 4A). This indicates that the β -hexosaminidase release rate increased gradually at low PV concentrations, while it grew rapidly at high concentrations. These data agreed well with the dose–response curve obtained from fluorescence measurements.

At both low and high PV concentrations, fluorescence intensities exhibited good linear relationships with fish PV concentrations, with correlation coefficients of 0.992 and 0.991 (low and high PV concentrations in panels B and C of Figure 4, respectively). The limit of detection (LOD) calculated from Figure 4B was 0.35 ng/mL, according to the formula: LOD = $3s/m$, where s represents the blank sample standard deviation ($n = 3$) and m represents the slope of the related PV calibration curve. These measurements were performed in triplicate to assess the reproducibility and precision of freshly fabricated cell sensors. The relative standard deviation (RSD) of the detection results were all <5%, showing acceptable performance.

Real Sample Measurements. To assess the utility of these sensors for rapidly recognizing target allergens from the complex food matrix, we used crucian carp, bream, and silver carp extra to test the performance of this sensor. As shown in Figure 5A, purified PV was applied to the gel and SDS–PAGE was performed. There was a major protein band at 12 kDa, which was identified by Coomassie Brilliant Blue staining (arrow in Figure 5A). This indicated substantial purification on PV. Then, the activation of cell sensors with three kinds of fish extracts also led to a rapid and robust increase in cell fluorescence intensity (Figure 5B). The concentrations and contents of PV in three fish samples calculated from the standard curve of the fluorescence intensity versus PV standards in Figure 4B are shown in Table 1. The results obtained using the RBL sensors were similar to those reported methods, which confirms that this biosensor can be applied effectively for the detection and prediction in real allergen samples.

Investigation of RBL Cell Sensor Specificity and Reproducibility. We also assessed the effectiveness of this new sensor for recognition of fish PV by challenging RBL sensor cells that were pre-sensitized with anti-fish PV antibodies, fish PV, shrimp allergen Pen a 1, and peanut allergen Ara h 1 (prepared in-house). Non-specific interactions between cells and antigens were excluded by challenging naive, unsensitized RBL sensor cells in parallel with pre-sensitized cells. Shrimp allergen Pen a 1 or peanut allergen Ara h 1 produced few observable responses (see Figure S3 of the Supporting Information). Therefore, it can be assumed that contamination with shrimp and peanut allergens would not influence sensor recognition of fish PV in heterogeneous mixed-food samples containing various allergens.

To demonstrate the reproducibility of this cell-based sensor, measurements were performed in triplicate with the same prepared RBL cells to assess reproducibility and precision (SD = 0.446 ± 0.04). Moreover, RBL cells were repeatedly activated

by anti-PV IgE and PV antigens within a 24 h interval. Results of fluorescence analysis show that reactivated RBL cells in response to PV levels retained 80% of the original value (as seen in Figure S6 of the Supporting Information), which well illustrates the reproducible characteristics of the sensor.

■ ASSOCIATED CONTENT

● Supporting Information

Efficiency of CD63–EGFP transfection and influence on cell proliferation and survival (Figure S1), time-lapse imaging of single cell fluorescence intensity change challenged with 100 ng/mL PV (Figure S2), specific detection of fish PV by RBL cell sensors (Figure S3), time-dependent antigen-stimulated increases in cytoplasmic Ca^{2+} detected by Fluo-4 fluorescence (Figure S4), time-dependent increases in stimulated production of reactive oxygen detected by DCDHF cytoplasmic fluorescence (Figure S5), and reproducibility of cell-based sensors activated with 100 ng/mL PV (Figure S6). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Mast cell-based fluorescence biosensor for rapid detection of major fish allergen parvalbumin

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Figure S1. Efficiency of CD63-EGFP transfection and influence on cell proliferation and survival. (A) a: Morphology and density of RBL after transfection (stimulated with 100ng/ml DNP-BSA); b: Fluorescent detection of expression rates of EGFP at the same region (stimulated with 100ng/ml DNP-BSA); c: 0.24% of CD63-EGFP expression in non-transfection cells by flow analysis; d: 83.27% of CD63-EGFP expression in the transfection cells by flow analysis. (B) Optical densities (OD) at 510 nm from sulforhodamine B assays show no difference in cell number at 1 to 3 days between transfected and control RBL cells. (C) FACS analysis of apoptosis in control (left) and transfection RBL cells (right). The lower left quadrant contains annexin V-EGFP (-) and PI (-) viable cells; the lower right quadrant, annexin V-EGFP (+) and PI (-) early apoptotic cells; the upper right quadrant, annexin V-EGFP (+) and PI (+) late apoptotic or necrotic cells; the upper left quadrant, annexin V-EGFP (-) and PI (+) necrotic cells. Bars indicate % of apoptotic or necrotic cells in each group expressed as mean $\pm$ SD of data obtained from three independent experiments.

Figure S2. Time lapse imaging of single cell fluorescence intensity change challenged with 100ng/ml Parvalbumin.

Figure S3. Specific detection of fish parvalbumin by RBL cell sensors. Quantification of the cell responses to stimulation with parvalbumin shrimp, shrimp allergen Pen a 1 and peanut allergen Ara h 1. Response of naive RBL cells to fish parvalbumin was recorded in parallel.

Figure S4. Time-dependent antigen-stimulated increases in cytoplasmic Ca²⁺ detected by Fluo-4 fluorescence.

Figure S5. Time-dependent increases in stimulated production of reactive oxy-gen detected by DCDHF cytoplasmic fluorescence.

Figure S6. The reproducibility of this cell-based sensors activated with 100ng/mL of Parvalbumin.

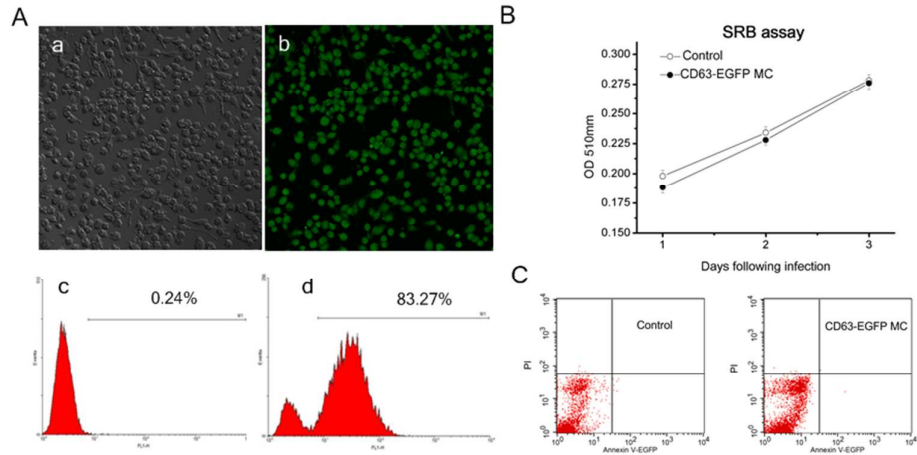


Figure S1. Efficiency of CD63-EGFP transfection and influence on cell proliferation and survival. (A) a:Morphology and density of RBL after transfection; b:Fluorescent detection of expression rates of EGFP at the same region; c:0.24% in non-transfection group by flow analysis; d:83.27% in the optimal transfection group by flow analysis.(B) Optical densities (OD) at 510 nm from sulforhodamine B assays show no difference in cell number at 1 to 3 days between transfected and control RBL cells. (C) FACS analysis of apoptosis in control (left) and transfection RBL cells (right). The lower left quadrant contains annexin V-EGFP (-) and PI (-) viable cells; the lower right quadrant, annexin V-EGFP (+) and PI (-) early apoptotic cells; the upper right quadrant, annexin V-EGFP (+) and PI (+) late apoptotic or necrotic cells; the upper left quadrant, annexin V-EGFP (-) and PI (+) necrotic cells. Bars indicate % of apoptotic or necrotic cells in each group expressed as mean $\pm$ SD of data obtained from three independent experiments.

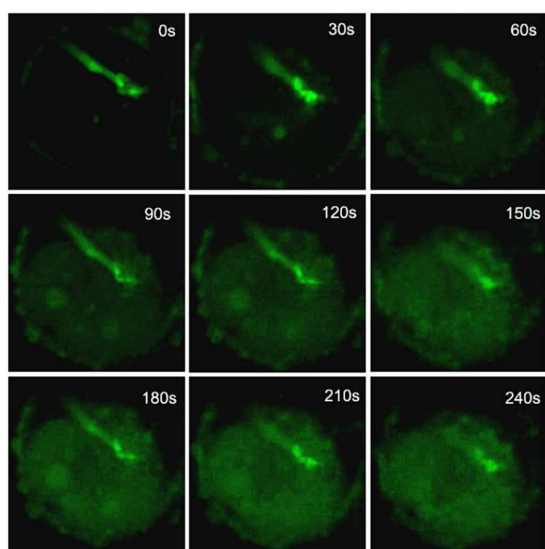


Figure S2. Time lapse imaging of single cell fluorescence intensity change challenged with 100ng/ml Parvalbumin.

As you can see that CD63 was located on the endoplasmic reticulum membrane and basophilic granular membranes at the beginning of activation, with the time lapse, the fluorescence began to spread to the plasma membrane which meant the fusion of cytoplasmic granules with the plasma membrane. Finally, nearly all the surface of RBL cell turned green which indicated the sufficiently expression of cd63 protein and actually occurrence of allergic reaction.

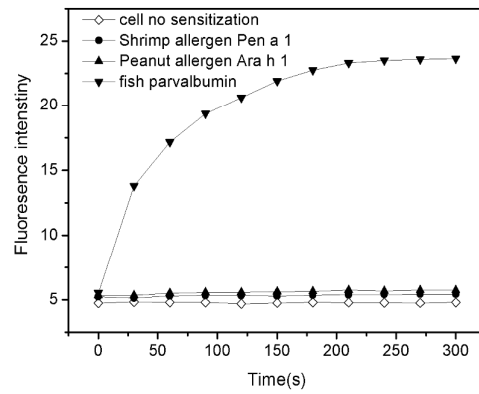


Figure S3. Specific detection of fish parvalbumin by RBL cell sensors. Quantification of the cell responses to stimulation with parvalbumin shrimp, shrimp allergen Pen a 1 and peanut allergen Ara h 1. Response of naive RBL cells to fish parvalbumin was recorded in parallel.

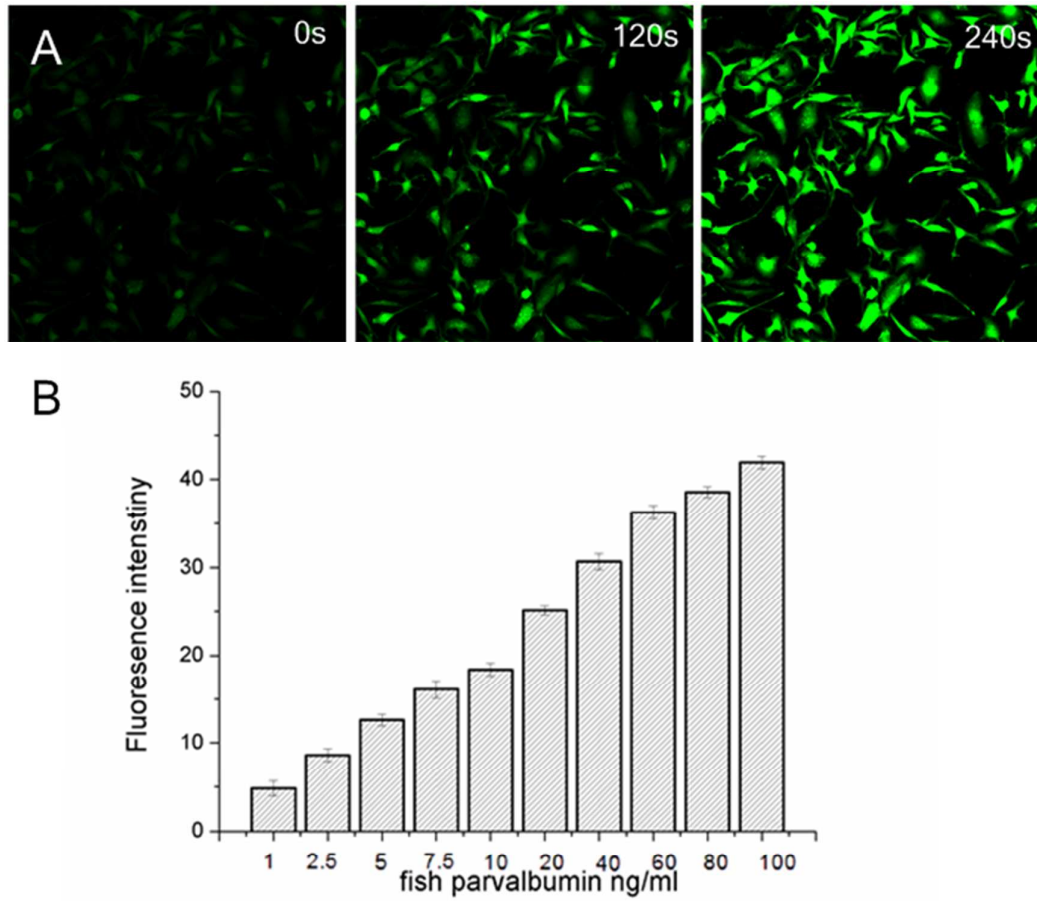


Figure S4. Time-dependent antigen-stimulated increases in cytoplasmic Ca^{2+} detected by Fluo-4 fluorescence. (A) Time lapse imaging of cell fluorescence intensity change. RBL cells were stimulated with 100ng/mL of Parvalbumin. (B) Fluorescence intensity spectroscopy for RBL-2H3 cells treated with different doses of fish allergen parvalbumin.

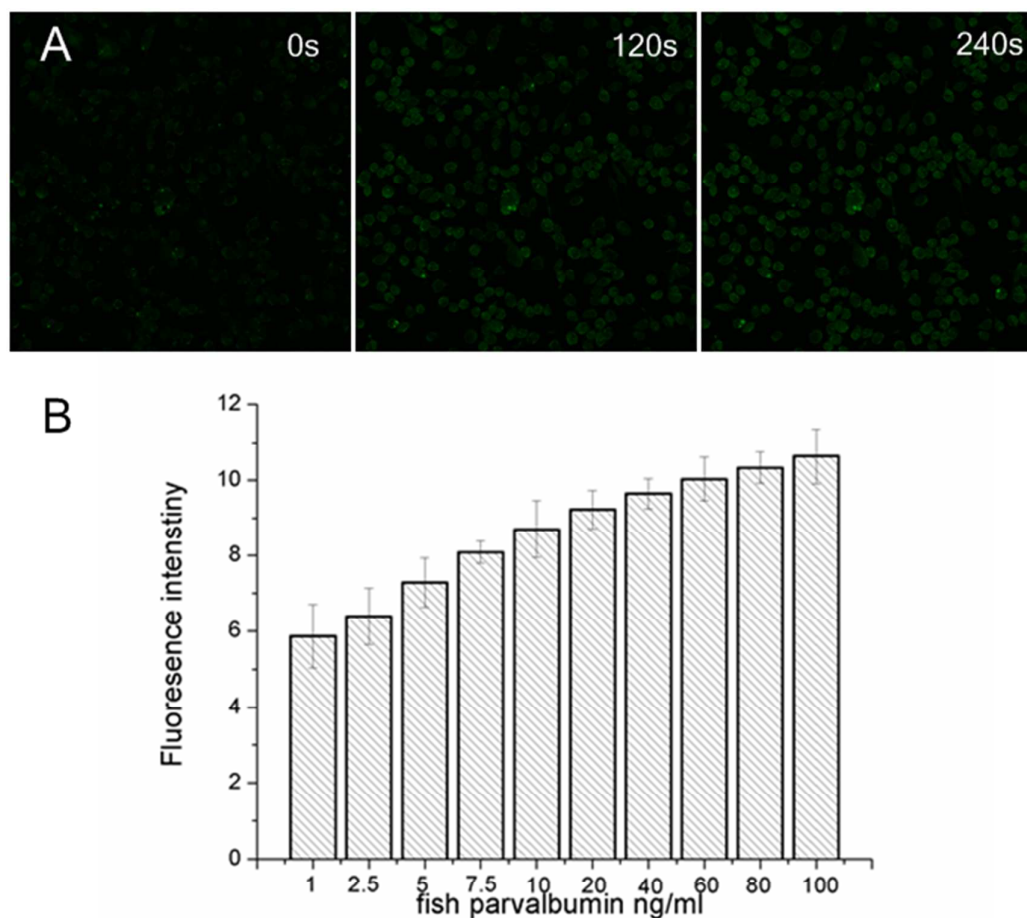


Figure S5. Time-dependent increases in stimulated production of reactive oxy-gen detected by DCDHF cytoplasmic fluorescence.(A) Time lapse imaging of cell fluorescence intensity change. RBL cells were stimulated with 100ng/mL of Parvalbumin. (B) Fluorescence intensity spectroscopy for RBL-2H3 cells treated with different doses of fish allergen parvalbumin

As seen in Fig.S5, It can be seen from the images that Fluo-4 fluorescence increases strongly in response to PV antigen stimulation which can achieve a maximum of 41. While, in Fig.S6, cells treated with PV antigen steadily increase their green fluorescence in the range of 5.8-11. Therefore, the results of Fluo-4 and ROS assay exhibited a linear relationships with fish PV concentration which well illustrate the feasibility of RBL cells for identifying PV allergen sample

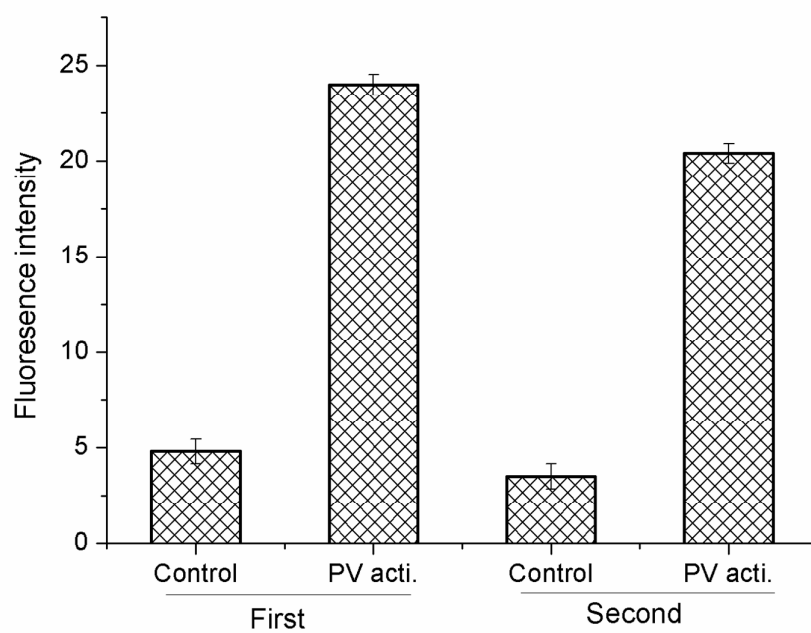


Figure S6. The reproducibility of this cell-based sensors activated with 100ng/mL of Parvalbumin.