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An integrated lab-on-a-disc for automated cell-based allergen screening bioassays

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ABSTRACT

We have utilized various valving scheme to leverage purely rotation-regulated flow control to enable comprehensive cell-based bioassays (CBBs) on centrifuge-based lab-on-a-disc (LOAD). A LOAD has been developed to examine allergic degranulation from live basophils for allergens screening for the first time, which can also be adjusted to suit a wide range of CBBs. In this system, controlled allergic reaction together with mediator separation from basophils using siphon valving and centrifugal sedimentation are realized inside microstructured network. The entire degranulation analysis process including on-demand release of samples, reaction and degranulation, allergic mediator separation and detection is executed in an automatic sequence within a single run. To validate our cell-based approach, detection of degranulation mediated by known secretagagues, ionomycin or chemotatic peptide formyl-methionine-leucine-pheylalanine (fMLP), is first demonstrated. Further experiments using real allergens house dust mite protein (Der p1) and its corresponding human serum IgE also show positive results. The overall efficiency of the assay is 80.6%, which is comparable to other conventional methods. With 4 identical units on a disc running in a parallel format, the device offers the possibility of single-step, multiplexed allergens screening. The device is capable of reporting a result within 30 min. It has many desirable merits including fast and multiplexed analysis, low cost, single-step operation, minimal sample volume, less discomfort and most importantly increased safety as patients are no longer susceptible to possible anaphylactic shock reactions induced by the common skin-prick-test. The flexibility of the flow control within the device makes it suitable to a wide range of CBBs.

1. Introduction

Live cells are the ultimate targets of all drugs and chemicals. Utilizing them as responders to show the effect of a chemical will provide a better understanding about the real response from life. Because of this, cell-based bioassay (CBB) has attracted intense interest from the public in the past ten years. In the CBBs, when cells come in direct contact with any target of interest through specific receptor binding, they will give a rapid and sensitive response after amplifying the input signal through various biochemical cascades. Therefore, CBB is now readily applied not only in laboratories, but also in clinics and industry [1,2]. Currently, high-throughput screening using live cells in microplates is a gold standard for the CBBs. However, such system is challenged by several issues such as high cost and tedious liquid handling procedures, as CBBs in microplate are usually conducted by manual loading of different reagents. Also, stepwise operation may induce artifacts that lead to false results, and often require expensive instruments such as flow cytometer, confocal microscope, etc. Furthermore, the reagents consumption is as much as hundreds of microliters [3]. In contrast, the use of microfluidics is expected to circumvent these shortcomings and in fact microfluidics set-up are designed to increase experimental throughput and assay reliability and at the same time to reduce the consumption of reagents [4–6].

The concept of automated liquid handling with minimum external driven support in the lab-on-a-chip system is also applied to the CBBs. Ideally, the centrifugal microfluidics or "Lab-on-a-Disc" (LOAD) is an integrated and miniaturized device in which complex analytical steps can be conducted in an automated manner. LOAD integrates a number of microfluidic components for liquid operation on a compact disc through the use of capillary effect and pseudo-forces such as centrifugal force and coriolis force on a

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rotating reference frame. Therefore, LOAD only requires a single motor to control complex fluid transportation and sample loading at different rotation speeds that translates complex assay procedures into a simple protocol.

Up to date, there has been much work on LOAD for biomedical applications such as blood-based processing [7,8], nucleic acid analysis [9,10] and immunoassays [11,12]. Still, cell-based applications using LOAD remain relatively unexplored [13]. In this work, we developed a highly integrated LOAD for the CBBs. As a proof of concept, we used our LOAD design to screen the IgE allergerns, which provided great convenience in terms of experimental parallelization, miniaturization and automation.

2. Material and methods

2.1. Reagents

The photoresist SU-8 2075 was purchased from MicroChem Corp. The polydimethylsiloxane (PDMS, Sylgard®184) was from Dow Corning. Dust mite allergen Der p1 (5 μg/ml) was a kind gift from Prof. CK Wong in the Hong Kong Prince of Wales Hospital (PWH). Serum containing IgE against Der p1 was obtained from patient with allergy to dust mite which was confirmed clinically in the PWH. Acridine Orange (AO) and *N*-formyl-methionine-leucine-phenylalanine (fMLP) were purchased from Sigma-Aldrich. KU812 basophils were obtained from ATCC (The American Type Culture Collection). Fetal bovine serum (FBS) was purchased from Gibco, USA. RPMI 1640 and phenol red-free RPMI 1640 (pRPMI) medium and penicillin/streptomycin (PS) were purchased from Invitrogen, USA. Ionomycin was purchased from Merck, USA.

2.2. Device fabrication

The LOAD device contains four identical units. Each can be used independently to screen allergens (Fig. 1(a)). Our LOAD consists of two layers of synthetic polymers, with the upper PDMS layer housing all microfluidic items, and the lower PMMA (polymethyl methacrylate) layer providing a flat substrate bound to upper layer for mechanical support. The upper layer features for liquid control and sample inlets, with the compartments and channels made by standard soft lithography [14] on PDMS using negative photoresist SU-8 2075. The PDMS positive relief (~1 mm thick) was assembled to the PMMA substrate (1 mm thick) by self-adhesion. LOCTITE 495 fast bonding glue was used to improve the bonding strength on the edge.

2.3. Device design

The aim of our study is to build a LOAD for a wide range of CBBs. Since cells (~10 μm in diameter) are much larger than analytes, versatile centrifugal-based valves and sedimentation were employed to manipulate liquid translocation in this work. As shown in Fig. 1(b), self-regulated liquid manipulations are performed as described [15]: (1) a reaction chamber (R1) connected with a "high speed-close (OFF)" siphon valve (V5) to control the time of reaction and separation of analytes; (2) a series of "low speed-close (OFF)" capillary stop valves (V1–V4) incorporated in the LOAD for on-demand addition and translocation of reagents; and (3) a detection chamber (D1) with a concentrating tip for analytes sensing. Combining the capillary stop valves, centrifugal sedimentation and siphon valve design, the reaction sequence can be accurately controlled in a simple spinning protocol (Fig. 1(b)). Briefly, at a certain speed (burst frequency), different materials were allowed to pass through different capillary stop valves to the reaction chamber. Keeping the rotation speed at the siphon valve OFF state, reagents and cells were mixed to allow reactions to occur. The flow inside the microfluidic channel is typically of low Reynolds number, i.e., laminar flow. Therefore we expect little mixing of the fluids except through diffusion at the boundary between the two liquids. Since the reaction chamber is designed with a volume of 30 μl with a quite wide interface, mixing between the two liquid components will take place via diffusion. Mixing is further facilitated by centrifugation.

Once the reaction was completed, cells in large size were settled stably at the bottom of chamber while analytes in small size were retained in the supernatant. The siphon valve was then open by decreasing the rotation speed to <27 rpm, and the analytes in the supernatant above the siphon inlet were translocated to D1 for subsequent analysis. This reaction series could be programmed and run automatically. The degranulation assay protocol on the LOAD is shown in Fig. 1(b).

2.4. Cell culture and degranulation assay

Human KU812 basophil cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS and 1% of PS at 37 °C, 5% CO_2. To study the IgE mediated degranulation, cells (1×10^6/ml) were first incubated with AO (0.5 μg/ml) overnight at 37 °C, 5% CO_2. During incubation, AO was actively accumulated in

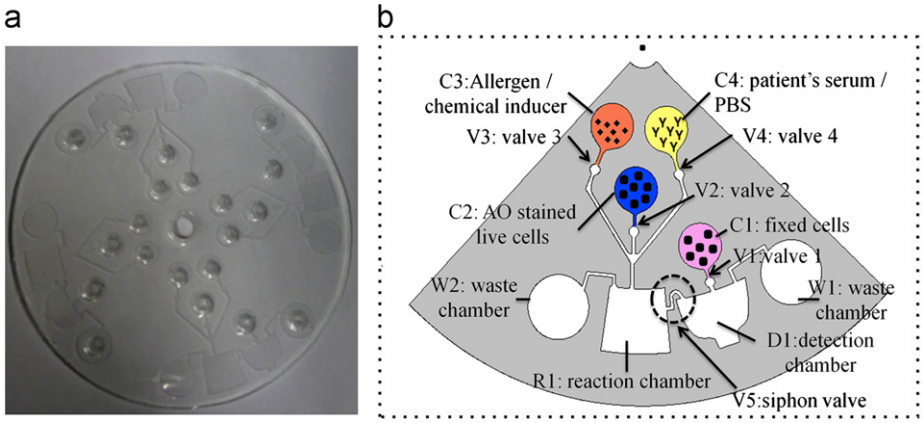

Fig. 1. (a) The LOAD design used in this study containing four identical units. The disc is 100 mm in diameter. The micro-channel is 500 μm in width and 300 μm in depth. (b) A diagram showing the detailed microfluidic layout and functions. The disc contains 4 identical units for parallel assays, each units includes reservoirs (C1–C4) for sample loading, waste chambers (W1, W2), capillary stop valves (V1–V4), siphon valve (V5), compartment for control of reaction and separation, and detection (D1).

the cytoplasmic granules, quenched each other and emitted red fluorescence upon excitation [16]. After stimulation, the AO and other allergic mediators in the cytoplasmic granules will be released into the buffer. AO will then be translocated to the D1 to label the DNA of the fixed cells. The fixed cells were prepared by treating KU812 cells (1×10^6/ml) with 1% paraformaldehyde for 30 min and then washed twice with phosphate buffered saline (PBS).

To further confirm the use of AO to report degranulation, The AO-loaded KU812 cells were challenged with ionomycin and the release of AO was determined by confocal microscopy (Nikon TE 2000-U microscope with confocal attachment, Nikon Inc., Japan). Briefly, the AO-loaded cells were seeded on a glass bottom dish (Ibidi, Germany) with pRPMI medium to reduce the noise generated from the phenol red in the normal RPMI medium. Cells were then scanned with an agron laser (488 nm) and the red and green AO fluorescence were recorded simultaneously. Fluorescence was expressed in a pseudo-color format.

3. Results

3.1. Automated liquid handling

3.1.1. On-demand release of reagents through capillary stop valve in LOAD

In this work, sequential sample loading and mixing of different reagents are realized by the use of capillary stop valves. The capillary stop valve is a microchannel with a sudden expansion in width at the junction (Fig. 2). The opening of the valve can be controlled by the rotation speed during disc spinning. This is an important design to direct the sequence of liquid flow. When the microchannel is in a form with the bottom surface hydrophilic while the other three sides are hydrophobic (Fig. 2), the capillary pressure at the junction is given by the following equation according to the work from Li et al. [17]:

$$P_c=\gamma\left[\frac{2}{h}\left(\frac{\cos^2\theta_{PMMA}-1}{\cos\theta_{\mathrm{PMMA}}}\right)-\frac{1}{w}\right] \quad (1)$$

where θ_{PMMA} is the equilibrium contact angle of PMMA, γ is the corresponding surface tension, w and h are the channel width and depth, respectively. According to the geometry of the microchannel, as well as contact angle of aqueous solution on PMMA $\sim73^\circ$, the capillary pressure P_c is found to be negative, which stops the liquid flow at the junction.

To allow the liquid to move along the microchannel, the required liquid pressure at the meniscus must overcome the capillary barrier. To this end, the centrifugal force acting on the liquid can be described as [18], $\Delta P_\omega=p\omega^2\,\bar{r}\Delta r$ where ω (rad/s) is the rotation speed of the disc, Δr is the radial extent of the fluid; $\bar{r}$ is the average radial distance of the liquid plug; ρ is the density of the liquid as shown in Fig. 2(a).

When the centrifugal force overcomes the capillary force at a certain rotation speed we call it burst frequency, the fluid will be moved through the valve. The theoretical burst frequency can be expressed by Eq. (2) as follows:

$$f=\frac{30}{\pi}\sqrt{\frac{\gamma\left[2/h\left(\frac{\cos^2\theta_{\mathrm{PMMA}}-1}{\cos\theta_{\mathrm{PMMA}}}\right)-1/w\right]}{\rho\bar{r}\Delta r}} \quad (2)$$

where f is the rotation frequency in revolution per minute (rpm). The theoretical burst frequencies for V1–V4 are 649, 760, 991 and 991 rpm, respectively. To test the calculated burst frequency, aqueous dye solutions were used. The rotation speed was first set to a nominal level according to Eq. (2), and the speed was adjusted 10 rpm stepwise until the valve became open. The

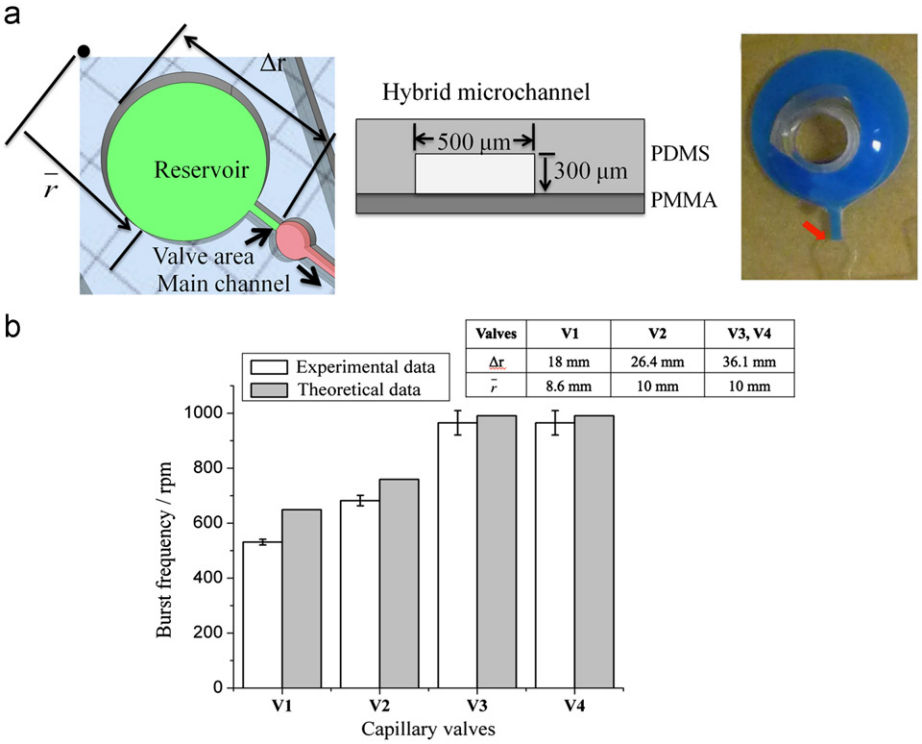

Fig. 2. Theoretical and experimental burst frequencies of capillary stop valves V1–V4 using dye solution in our LOAD design. (a) An abrupt expansion circular chamber is introduced to stop spontaneous liquid flow at the junction in a PDMS and PMMA hybrid mirochannel. A blue dye solution is stopped at the valving area. (b) Experimental and calculated value for the V1–V4 burst frequency. Results are mean ± SD (n=6 different LOADs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

average experimental burst frequencies of V1–V4 (with different radial distances r) were found to be 531, 682, 965 and 965 rpm, respectively (Fig. 2). The variation of the burst frequency among different disc is less than 5% ($n=6$). We also tested the flow using PBS, and the results were almost the same. No failure and variations among discs were noted. As shown in Fig. 2, the discrepancy between the calculated frequency and the experimental value is believed to be caused by the inaccurate estimation of θ_c used in the calculation, and the control of w and h in disc fabrication.

The capillary stop valves can be used to realize flexible liquid manipulation. The release time of liquid as well as the duration for the liquid release from different chambers can be controlled by the rotation speed.

3.1.2. Control of reaction time and separation of cells from analytes in LOAD

According to Stokes' Law, the motion of a suspended particle is counteracted by the viscous friction force in a liquid medium with viscosity η. The drift velocity of a particle in a centrifugal field in steady state can be described by Eq. (3) as described [19,20]:

$$\mu_{\text{drift}} = \frac{(\rho_p - \rho_l) \times r \times d^2 \times (\pi/30 \times f)^2}{18 \times \eta} \quad (3)$$

where ρ_p and ρ_l are mass density of particles and liquid medium, respectively, d is the diameter of the particle, r is the radial distance from the rotation center. The ratio of drift velocity between two particles can be expressed as:

$$\frac{\mu_{\text{drift}}(p1)}{\mu_{\text{drift}}(p2)} = \frac{\rho_{p1} - \rho_l}{\rho_{p1} - \rho_l} \times \frac{d_{p1}^2}{d_{p2}^2} \quad (4)$$

According to Eq. (4), upon centrifugation with a certain rotation speed, the drift velocity of a particle on a certain position depends on the particle size and mass density. Large particle like cells (for example, basophils, with a diameter around 10 μm and density between 1072 and 1076 kg/m^3 [21]) having a larger size and mass density than small molecules (for example, AO, with diameter around ~1 nm and mass density approximately to water, i.e., 998 kg/m^3, when dissolved in water), results in $\mu_{cells} \gg \mu_{small\ molecules}$, so that the cells should be settled rapidly and become packed at the bottom of the chamber during disc rotation while small molecules will be distributed uniformly in the supernatant due to the ignorable difference to medium in terms of mass density and volume size (Fig. 3(a)). Experimentally, the fixed KU812 basophils were concentrated and densely stacked at the bottom of the chamber after rotating at 1050 rpm for 3 min, with a shock-interface between the cells and PBS supernatant (Fig. 3(b)).

The time interval allowing reaction to complete and separation of analytes are essential in the CBBs. Our LOAD has its structure to control the time interval for reaction based on sedimentation and siphon valve (Fig. 3(a)). Siphon valve is the "High speed-close (OFF)" passive valve, which was first introduced by Steigert et al. in 2007 and has been used for blood assay [22]. It relies on a net force to pump liquid into a hydrophilic channel by the capillary action and repulsed by centrifugal force. At high rotation speed, the centrifugal force is stronger than the capillary force and the siphon valve is "OFF" therefore keeping the liquid in the chamber for reaction. Once the rotation speed is reduced to a threshold, the valve is turned "ON", and the liquid flows through the channel until the liquid level is hydrostatically balanced. Hence, the siphon valve serves as a switch for timed liquid manipulation, either kept in left chamber for reaction or translocated to right chamber for subsequent analysis.

As shown in Fig. 3(c), when the rotation speed was reduced to <27 rpm (ON state), the blue dye solution on the left of siphon valve flew through the valve to the right until the liquid level was

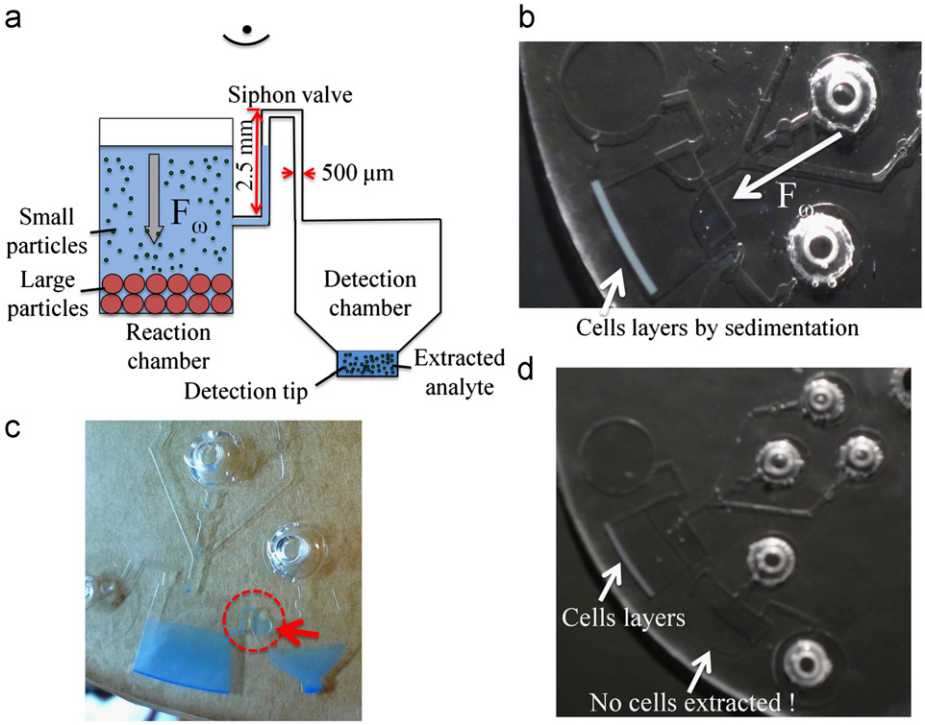

Fig. 3. (a) Diagram showing the structure with siphon valve and sedimentation chamber of different particles. (b) Photograph of fixed cell sedimentation on centrifugation at 1050 rpm. (c) Photograph showing the siphoning effect in our LOAD with a blue dye solution. (d) Liquid above the siphon inlet was extracted to the right chamber, fixed cells deposited at the outermost region rather than transfer through the siphon to the right chamber. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

balanced. In our design, 40% of the total liquid volume or 12 μl was translocated to the detection chamber as the rotation stopped (Fig. 3(c)). In our LOAD, analytes to be analyzed in the detection chamber are therefore separated from cells and selectively extracted through the siphon valve. To prove that the cells in reaction chamber will not be translocated to the detection chamber, fixed cell suspension and PBS were loaded into C2, C3 and C4, respectively while leaving C1 blank. No cells were found in the detection chamber after a single run as shown in Fig. 3(d). Our results show that the supernatant can be well separated through this structure.

3.2. Degranulation assay in LOAD

3.2.1. Using acridine orange as a degranulation reporter

To prove the concept of LOAD as a promising and competent tool in the CBBs, we here demonstrated its biomedical application for allergen screening using a human basophil cell line KU812. Basophils are one of the major cell types for the allergic response in human. Mechanistically, allergic mediators are released from the granules of basophils through the IgE-mediated process (Fig. 4(a)). For the IgE-mediated allergy, it is a process mediated by the acquired immune response with the production of antibody IgE. Basically, the allergens cross-linked the IgE molecules on the surface IgE receptors (FcεRI) in a specific manner will lead to the basophils releasing a number of mediators such as histamine and serotonin. Alternatively, there are many environmental pollutants such as the bisphenol A (BPA) plastic estrogen that can elicit the release of the allergic mediators in the absence of IgE [23].

In this study, we employed a fluorescent dye AO as a degranulation reporter. When cultured with live cells, AO is taken up and accumulated in the basophilic granules in an energy-dependent manner [17]. Upon stimulation, AO will be released from the granules together with the allergic mediators. To validate this process, we used confocal laser scanning microscopy to study the AO release from cells. As can be seen in Fig. 4(b), AO showed a strong red fluorescence in granules but a low green fluorescence in the cytoplasm in KU812 cells before stimulation. Upon activation with ionomycin (1 μM), a well-known trigger for degranulation [24], the red fluorescence intensity in the hot spots decreased and at the same time the green fluorescence increased in the cytosol (Fig. 4(b)). This observation suggests that AO was released from the granules and labeled the DNA in the cell nucleus and RNA in the cytosol.

3.2.2. Assay protocol and results from LOAD

The degranulation assay protocol on our LOAD was shown in Fig. 5. Each disc contained four identical working units for simultaneous assay. Practically, live KU812 cells preloaded with AO were challenged with allergens Der p1 and IgE, the AO released from the live KU812 cells were used to label the DNA of the fixed cells in the detection chamber D1 to increase the signal sensitivity (Fig. 5(c) and (d)). The detection tip in D1 was employed to facilitate the detection of AO with the fixed cells (Fig. 5(e)). The fixed cells here act as the signal reporters that generate fluorescence when the released AO labeled the DNA of the fixed cells at the detection tip under centrifugation. When rotation has stopped after completion of a test, we measured the fluorescence signal using an argon laser (488 nm) as the excitation source. Lock-in detection (lock-in amplifier (SR830 lock-in amplifier, Stanford Research Systems Inc., Sunnyvale, CA) was performed with modulation introduced by a chopper (SR540, Stanford Research Systems Inc., Sunnyvale, CA). A photomultiplier tube (PMT) (R316, Hamamatsu, Inc., Japan) was used to capture the fluorescence photons. Fluorescence signal from each of the four units was detected sequentially. The green fluorescence intensity recorded by the PMT (i.e., signal from the detection tip in D1 detection chamber) is therefore a direct measure of degranulation. Details of procedures are shown in Fig. 5.

As described before for the CBBs, cells amplify the input signal and enhance system sensitivity. In this work, we tested our LOAD using the secretagague ionomycin (1 μM), fMLP (0.67 μM) as well as Der p1 and IgE for the IgE-mediated allergy. As shown in Fig. 6, fMLP showed the highest degree of degranulation, ~8 fold increase in the fluorescence intensity when compared to that of the negative control (PBS) ($n=12$, $p<0.005$). Also, ionomycin showed a significant response (~5 fold increase, $n=9$, $p<0.005$). On the other hand, the dust mite allergen Der p1 and patient serum with IgE also showed the IgE-mediated degranulation. Using real allergens and samples obtained from clinics, we aimed to demonstrate the high competence and potentiality of our LOAD design for on-site allergen screening. At present, the overall efficiency (fraction of all samples that the test status matched actual status) was 80.6% from a total of 36 testing results. This was comparable to skin prick test, which was the standard allergy test for multiple allergens.

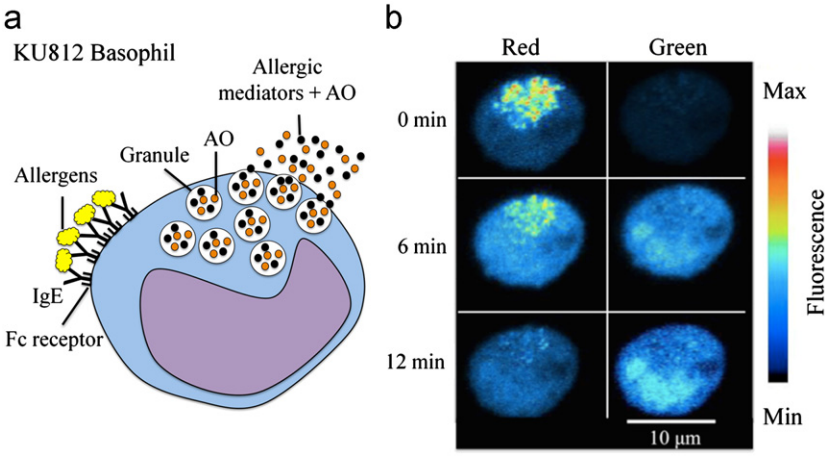

Fig. 4. (a) Mechanism of degranulation in KU-812 basophils. (b) Pseudo-color images of a single KU812 cell loaded with AO before and after treatment with ionomycin (1.0 μM) under a confocal microscope. The scale bar represents the cell dimension and the pseudo-color bar represents the green and red fluorescence intensities. This observation is a typical response of cells from at least three trials. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

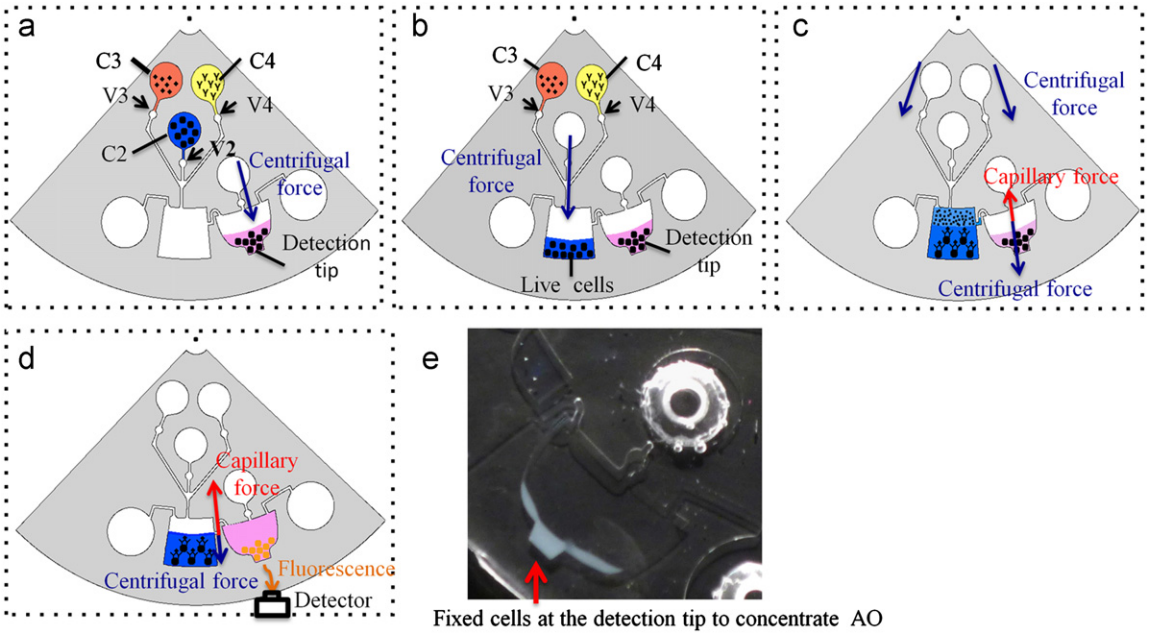

Fig. 5. ((a)–(d)) Schematic showing the steps involved in the allergy testing assay. Live KU-812 cells preloaded with AO (40 million/ml, 10 μl), allergens (10 μg/ml Der p1, 10 μl) patient's serum (IgE) (10 × dilution, 10 μl), and 10 μl of fixed cell suspension (40 million/ml) are loaded into their corresponding reservoirs before the test. (a) At the burst frequency of capillary stop valve V1 (531 rpm), the fixed cell suspension in chamber C1 is released through the valve to the detection chamber D1. (b) Upon reaching the burst frequency of capillary stop valve V2 (682 rpm), the AO stained live cell suspension in chamber C2 is released through the valve into the reaction chamber R1. (c) Upon reaching the burst frequency of the capillary stop valves V3 and V4 (965 rpm), the allergen/chemical inducer in chamber C3, as well as the patient's serum/PBS in chamber C4 are released simultaneously through the valves into the reaction chamber. Cells and reagents are kept inside the reaction chamber R1 for a duration (20 min) long enough for all reaction to complete sufficient. The required flow-stop action (i.e., "siphon valve-closed (OFF)" state) is achieved by rotating the disc at a high speed (1050 rpm). (d) After completion of the reaction, the rotation speed is reduced (< 27 rpm) to initiate the "siphon valve-open (ON)" state. The supernatants containing the released AO and allergic mediators are extracted to the detection chamber D1 through the siphon channel. In D1, DNA of the fixed cells is labeled by AO that has passed through the plasma membrane. Green fluorescence from the detection tip upon excitation by a 488 nm argon laser is a direct measure of degranulation. (e) Fixed cells are concentrated at the detection tip under centrifugation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

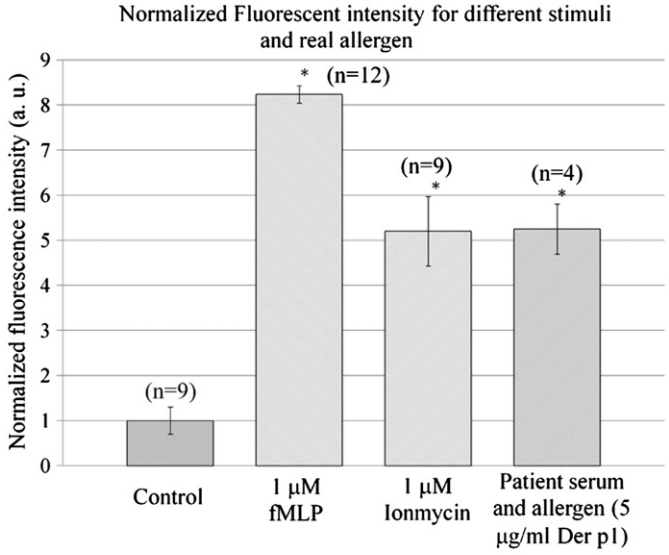

Fig. 6. Degranulation triggered by different stimuli. KU812 cells preloaded with AO were stimulated with medium alone (Control), Ionomycin (1.0 μM), fMLP (0.67 μM) or allergen (Der p1, 5 μg/ml) with patient serum (IgE) in LOAD. Signals from test groups are normalized with the control data. Results are mean ± SD for a number of determinations as indicated. *$P < 0.005$, compared with the control by Student's t test.

4. Discussion

In this study, we have introduced a highly integrated fluorescence-based LOAD for automated CBB. In particular, we have used our LOAD design and AO as the degranulation reporter to study the IgE-mediated degranulation in basophils. To the best of our knowledge, we are the first to integrate individual centrifugal microfluidic components i.e., capillary stop and siphoning valving, and centrifugal sedimentation for allergy testing. We also simplified the tedious procedures into a simple rotation process. In addition, our system provides a number of advantages over the traditional practice, including non-invasiveness, experimental parallelization, simple operation procedures, rapid experimental process and sensitive detection. Apart from using the LOAD for allergy testing, LOAD can be used for different assays such as determination of the toxicity of environmental toxicants in human cells. To ensure appropriate and accurate liquid handling in the system, we have proven the feasibility of each microfluidic component individually by experiments and compared it with the calculated parameters. In conclusion, we have demonstrated that our LOAD design can be used to study the IgE-mediated allergy. Through modification and adjustment on the microfluidic design, LOAD can be used for a variety of CBBs.

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