

Click-Chemistry Based Allergen Arrays Generated by Polymer Pen Lithography for Mast Cell Activation Studies

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The profiling of allergic responses is a powerful tool in biomedical research and in judging therapeutic outcome in patients suffering from allergy. Novel insights into the signaling cascades and easier readouts can be achieved by shifting activation studies of bulk immune cells to the single cell level on patterned surfaces. The functionality of dinitrophenol (DNP) as a hapten in the induction of allergic reactions has allowed the activation process of single mast cells seeded on patterned surfaces to be studied following treatment with allergen specific Immunoglobulin E antibodies. Here, a click-chemistry approach is applied in combination with polymer pen lithography (PPL) to pattern DNP-azide on alkyne-terminated surfaces to generate arrays of allergen. The large area functionalization offered by PPL allows an easy incorporation of such arrays into microfluidic chips. In such a setup, easy handling of cell suspension, incubation process, and read-out by fluorescence microscopy will allow immune cell activation screening to be easily adapted for diagnostics and biomedical research.

1. Introduction

The study of allergic responses draws broad attention from researchers and clinicians because of their immense impact on the health and well-being of a substantial percentage of the world's population.^[1,2] The severity of allergic reactions can vary from mere discomfort to life-threatening conditions

and death depending on personal conditions. Therefore, profiling of allergic substances and cellular activation profiling without the need to actually expose the patient to potential allergens is an important option for high risk persons.^[3] Furthermore, the study of allergic cellular responses on patterned surfaces can yield novel insights into the mechanism governing the activation process itself, and consequently improve the development of novel treatment options.^[4,5]

Existing platforms for mast cell activation profiling based on dip-pen nanolithography (DPN) with phospholipids (L-DPN)^[6,7] have been shown to be effective tools for research on the mast cell activation process and its moderation by glucocorticosteroids.^[8,9] However, for cellular activation profiling to become a standard tool in biomedical research or in clinical settings, sample stability issues and the relative ease of use must be addressed. Here we present the application of polymer pen lithography (PPL)^[10] for the generation of large area, covalently bound allergen arrays that are stable and easily integrated with microfluidic chips and could be used in automated setups.

PPL can be viewed as a hybrid technique of DPN and conventional microcontactprinting (μ CP), combining the beneficial aspects of both methods. It offers large area (cm^2) patterning at high resolution with inexpensive stamp

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materials and retains full pattern flexibility. The desired pattern layout does not need to be represented already in the PPL stamp (as it is in the case of μ CP) but is generated by repeated stamping. The single dot features produced in each subsequent stamping generate the desired pattern by a raster process. PPL can be used not only to pattern small molecules like thiols, but also more delicate substances like proteins^[11,12] or larger inorganic particles^[13,14] in appropriate carriers. In addition to noncovalent deposition, click-chemistry approaches can also be employed in PPL to provide stable covalently bound patterns.^[15,16] Multiplexing (i.e., integration of different compounds next to each other or interdigitated within a sub-pattern) can be achieved either by special inking^[11,17] or writing strategies.^[12]

Click-chemistry approaches are a versatile and common approach to covalently bind two reactive molecules in a selective and reliable way, with high yields and under mild conditions.^[18–20] From the existing click-chemistry toolbox, we chose the copper (I) catalyzed cycloaddition (CuAAC) reaction between an azide and a terminal alkyne because of its reliability, specificity, biocompatibility, and broad availability of reagents.^[18,21,22] CuAAC shows high potential in solid phase chemistry^[23] and was previously shown in DPN and microchannel cantilever spotting to be compatible with protein binding and cell culture applications.^[24–27]

We have used click-chemistry to pattern allergen by PPL for mast cell studies. As a model allergen, we chose dinitrophenol (DNP), which has previously been used in mast cell activation assays.^[4,5,8,9] DNP is commercially available in azide modifications and can therefore be easily used in the CuAAC click-chemistry platform assay.

2. Results and Discussion

2.1. Fabrication of Allergen Arrays

The general scheme for the preparation of the allergen arrays is given in **Figure 1**. The PPL stamp coated with the allergen containing ink was repeatedly brought into contact with the alkyne modified sample surface with precise control of dwell time and spatial positioning enabled by the DPN setup. This allowed ink to flow from the stamp onto the substrate, generating a regular array of microdroplets. A typical outcome of such patterning process is shown in Figure 1b. Each section of 9 by 10 dot features was written by a single pen of the stamp (which contains 10 000 pens per cm^2 , resulting in the massive large area parallelization typical for PPL). Each of the microdroplets acts as a microreactor allowing the CuAAC reaction to take place and to couple the azide-bearing allergen in the ink to the alkyne groups on the substrate (inset of Figure 1b). The samples were left overnight at room temperature, to ensure a completion of the reaction. Next, the samples were washed with ethanol and DI water to remove excess ink prior to mounting the microfluidic chips, antibody staining, and cell incubation.

To validate the click-chemistry reaction and to characterize the resulting arrays, fluorescent TAMRA-azide was printed before switching to DNP-azide for antibody binding and mast cell activation experiments (**Figure 2**). Figure 2a,b shows fluorescence microscopy images of TAMRA-azide dot patterns before and after washing with ethanol and DI water. Washing removes the excess ink and unbound azide, leaving behind only a potential monolayer of bound azide compound on the sample surface. This is also reflected by the higher exposure time needed for fluorescence imaging after washing

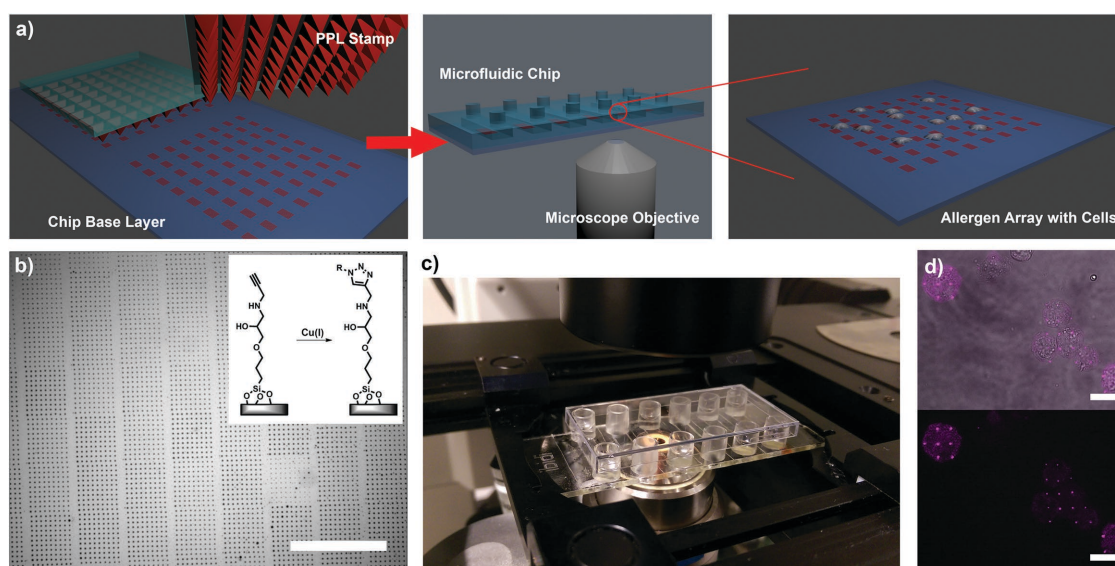


Figure 1. Scheme of chip production and experimental setup. a) The base layer of the chip is functionalized by PPL with an allergen presenting array (left). The base layer is then incorporated into a microfluidic chip allowing easy incubation and observation by fluorescence microscopy (middle). Mast cells were seeded onto the pattern and colocalization and activation was monitored (right). b) Overview of the pattern with DNP-azide containing microdroplets. Scale bar equals 200 μm . The inset exemplifies the CuAAC reaction for the immobilization of the allergen. c) A picture of a six channel microfluidic chip on microscope table. d) Combined bright field and fluorescence image (top) and fluorescence channel only (bottom) of mast cells showing co-localization of IgE receptor with the allergen array. Scale bars are 20 μm .

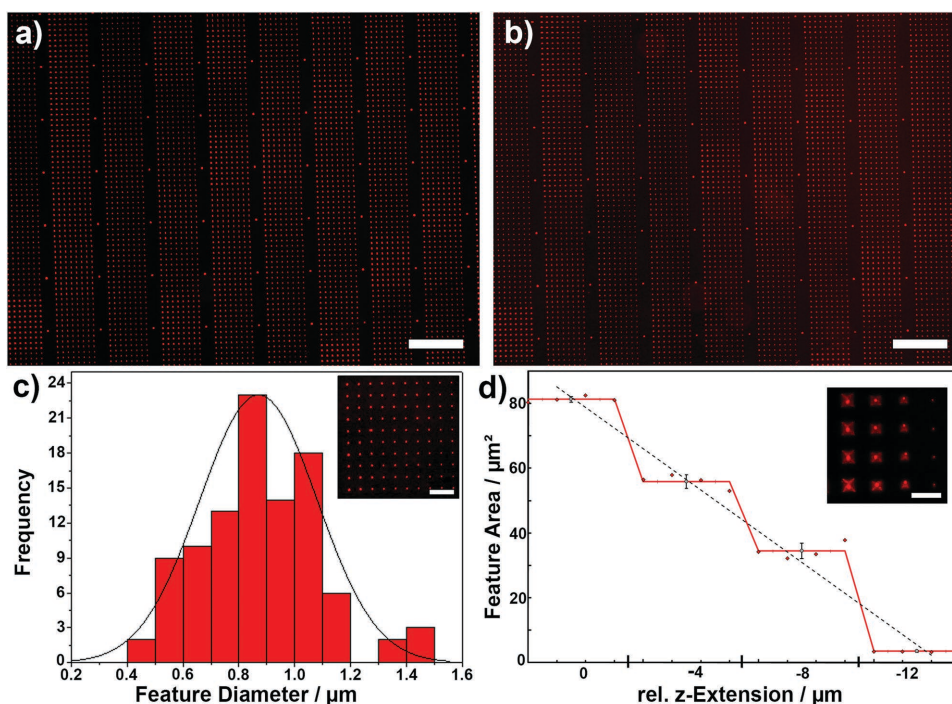


Figure 2. Fluorescence microscopy images of TAMRA-azide arrays on alkyne modified glass. a) Dot array of TAMRA-azide before and b) after washing with ethanol and DI water. Scale bars are 100 μm . Exposure time before washing was 3 s and after washing 15 s, respectively, reflecting the removal of excess ink microdroplets. c) Histogram of dot diameters in a close up of 10×10 dots (inset, scale bar is 20 μm) average diameter of the dot features is (0.87 ± 0.21) μm . d) Feature area of a 4×4 array with features written by applying varying pressures within each column (inset, scale bar is 20 μm). Solid line shows the average feature area in each column, the dashed line is a linear fit to the average feature area.

(e.g., 3 s before and 15 s after washing for the 10x low magnification overviews). A close-up of a washed 10×10 TAMRA-azide dot pattern written by a single pen of the stamp array at 60% relative humidity (RH) and with 1 s dwell time is shown as inset in Figure 2c (exposure time 300 ms). The distribution of dot size in this array shows an average dot diameter of (0.87 ± 0.21) μm .

When more pressure is applied to the PPL stamp during the writing process, square features can be achieved by (elastic) deformation of the pens apexes. This can be exploited to control feature size, as is shown as inset in Figure 2d. Here, a 4×4 square array of TAMRA-azide was written with different amount of applied pressure in each column. Each feature within a column is written with the same pressure on the stamp. For each column, the stamp was raised by an additional 4 μm , resulting in a reduced pressure on the stamp. The average area of the dot in the first column was (81.28 ± 0.85) μm^2 , in the second column (55.86 ± 2.07) μm^2 , in the third column (34.37 ± 2.40) μm^2 , and in the fourth column (3.43 ± 0.30) μm^2 .

In order to demonstrate the retained activity of the printed DNP-azide, antibody recognition was tested. Surface bound arrays of DNP-azide were printed in the same fashion as the fluorescent TAMRA dye. After washing and drying, the samples were incubated with a fluorescently labeled antibody against DNP (CF488-anti-DNP IgE). **Figure 3a** shows a typical fluorescence microscopy image of such an array after antibody binding (exposure time 300 ms). The antibody recognized the surface bound DNP and attached selectively to the pattern, demonstrating the availability of the DNP in the

pattern. The average spot size and distribution within such a field is given in Figure 3b. The average diameter of a dot feature was (2.06 ± 0.19) μm .

An alternative way of varying the feature size of the DNP-azide pattern was explored. By varying the contact time of the PPL stamp with the sample surface, feature size can be tuned due to changing amounts of ink delivered. In addition, the dot-like shape of features is preserved rather than being transformed into squares as is the case when the stamping pressure is raised to increase feature size. Figure 3c shows a bright field microscopy image of a 5×4 DNP dot pattern written with different dwell times of 20 s, 15 s, 10 s, 5 s, and 2 s, respectively, in each column, directly after lithography. The average dot diameter of the dot for 20 s dwell time was (4.38 ± 0.08) μm , (3.94 ± 0.09) μm for 15 s, (3.26 ± 0.06) μm for 10 s, (2.54 ± 0.02) μm for 5 s, and (1.91 ± 0.06) μm for 2 s. Thus the amount of ink transferred to the surface is clearly correlated with the dwell time. The graph in Figure 3e shows that the dot diameter is linearly dependent on the dwell time. After washing off the excess ink, allergen availability was examined by the binding of fluorescently labeled (CF488) anti-DNP IgE antibody (Figure 3d). The average dot diameter versus dwell time (Figure 3f) ranged from 2.21 to 0.70 μm , which is comparable in size to the areas covered by ink in the bright field images.

2.2. Test of Bioactivity

To test the bioactivity of the DNP allergen arrays, the response of mast cells sensitized with the fluorescently

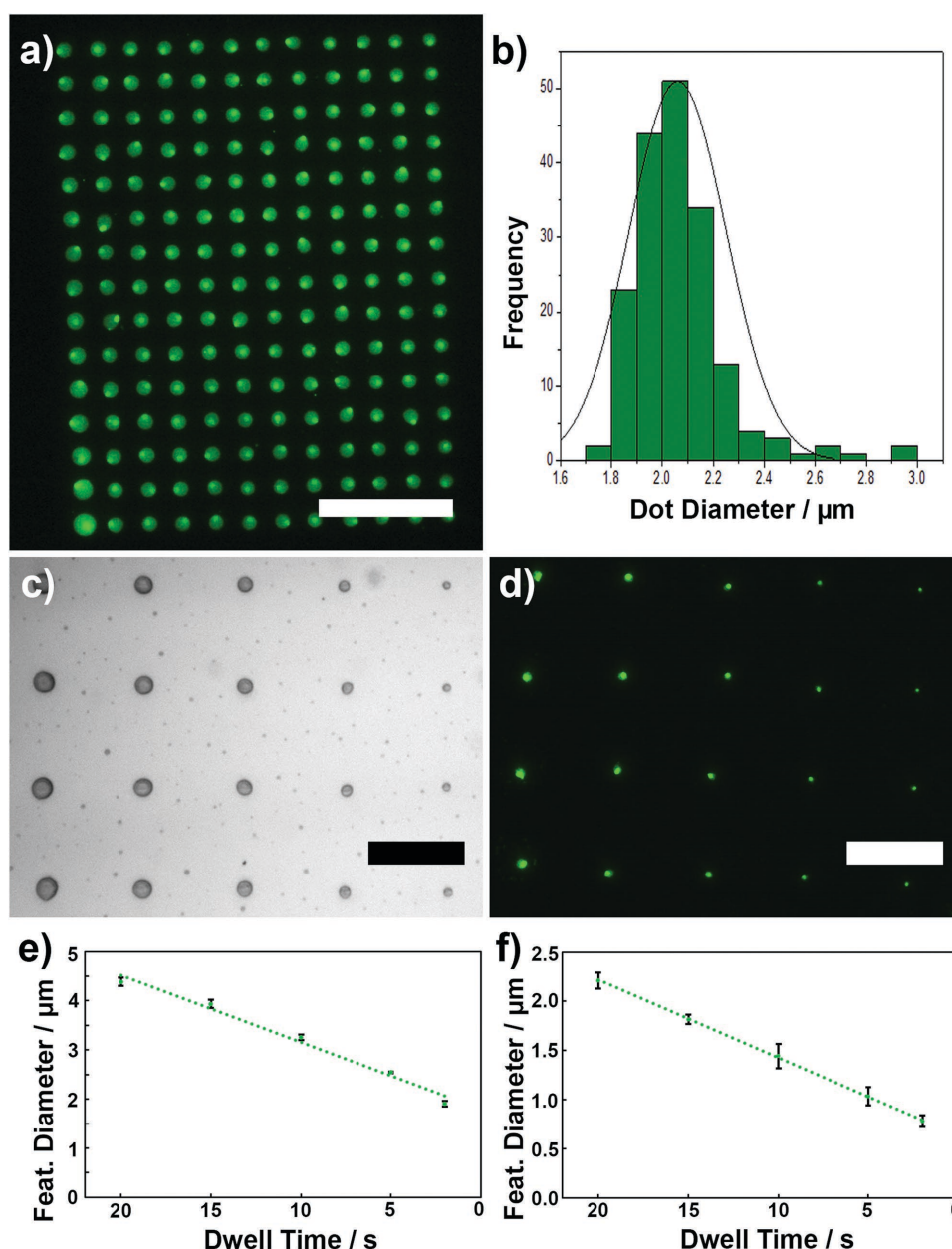


Figure 3. Fluorescence microscopy images of DNP-azide pattern on alkyne modified glass. a) A 12×15 array of DNP-azide written by a single pen of the PPL stamp array after binding of fluorescent CF488-anti-DNP IgE antibody. b) Histogram of the dot diameters in (a). The average dot diameter was $(2.06 \pm 0.19) \mu\text{m}$. c) Microscopy image of a 5×4 DNP-azide dot pattern written with different dwell times of 20 s, 15 s, 10 s, 5 s, and 2 s, respectively, in each column before washing. d) Sample after staining with CF488-anti-DNP IgE antibody. e, f) Graph of the dot diameter versus dwell time for the images in (c) and (d), respectively. All scale bars are 20 μm .

labeled antibody was analyzed. RBL-2H3 cells were sensitized for 2 h with the fluorescently labeled antibody (CF488-anti-DNP IgE) and then incubated on the arrays for 5 to 30 min. During sensitization, the IgE antibody binds to the high affinity IgE receptors (Fc ϵ RI) expressed on the surface of the cells. Crosslinking of these receptors by allergens recognized by the previously bound antibody can activate the mast cells and trigger diverse signal cascades in the cell.^[28,29] Following attachment of the cells to the pattern, a colocalization of the labeled IgE-Fc ϵ RI complex on the cells' membrane with the underlying DNP array was observed (fluorescent images in **Figure 4**). In contrast, homogeneously

fluorescent cells were observed in areas without DNP pattern (left and right sides of the images in **Figure 4a–c**). This indicates an even, unclustered distribution of the IgE-Fc ϵ RI complex on the cell surface when not in contact with the allergen pattern. **Figure 4d,e** shows the magnification of **Figure 4c** in the areas denoted by boxes and marked by I and II, respectively. Here, for easy recognition, the boundaries of flat cells attached to the allergen array have been demarcated with a red dashed line. **Figure 4d** shows that in the process of co-localization of the fluorescent IgE-Fc ϵ RI complexes, the underlying DNP pattern becomes visible only for parts of the pattern that are covered by cells. In other words, when a cell

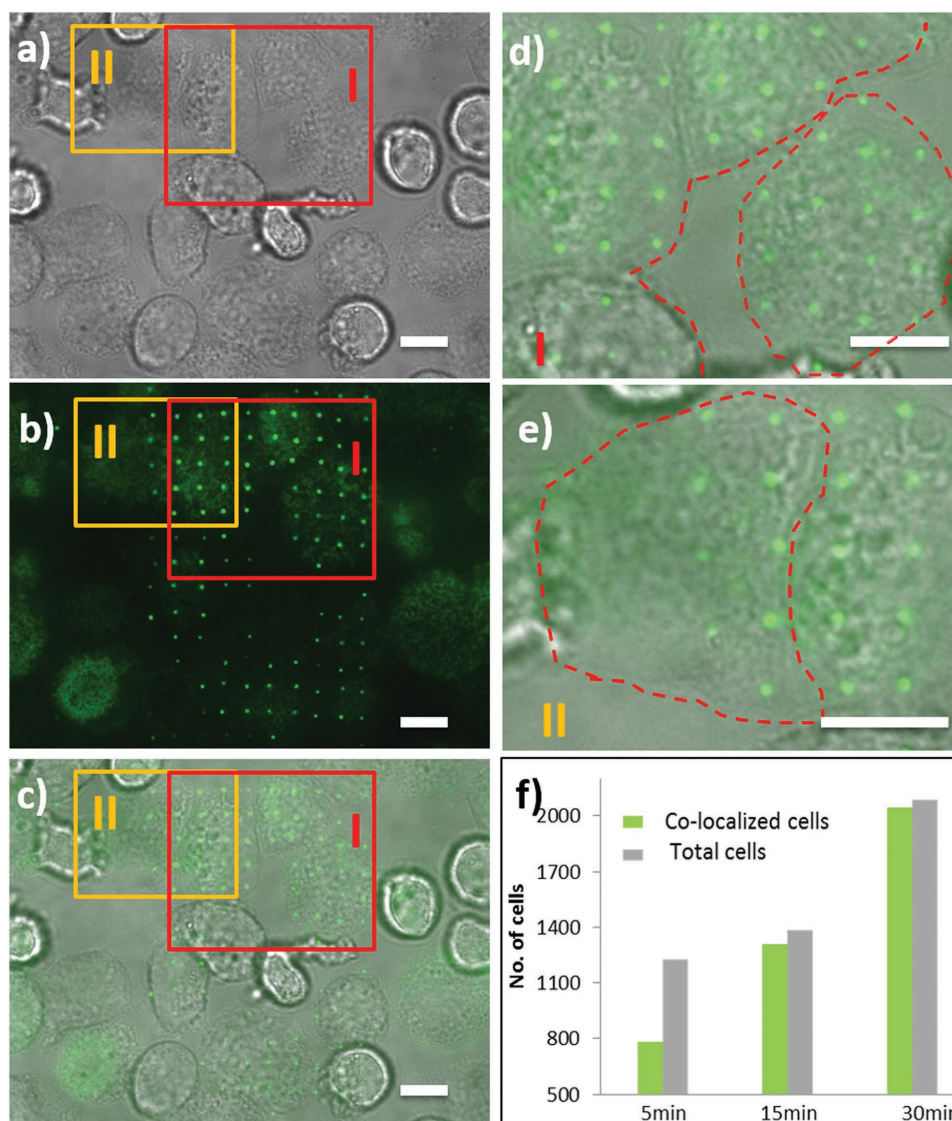


Figure 4. Fluorescence microscopy images of IgE-Fc ϵ R1 in RBL-2H3 cells colocalization on a DNP-azide array. The cells were sensitized for 2 h with fluorescently labeled CF488-anti-DNP IgE antibody and incubated on the pattern for 30 min. a) Bright field channel, b) EGFP channel, c) merged image of (a) and (b). d,e) Merged higher magnification images of area I and II of the image in (b). The contours of the cells are marked by dashed red lines. A cell that is positioned only half way on the DNP array shows colocalization only in the part that contacts the allergen array. All scale bars are 20 μ m. f) Bar chart of the number of cells showing co-localization of IgE-Fc ϵ R1 on pattern as a function of time. Cells showing co-localization (green bars) are compared with total cells (gray bars) on the DNP array (counted from 74 images per time point).

is positioned partly off the DNP array, only the part of the cell on the array shows colocalization of the receptor complex (Figure 4e). The graph in Figure 4f shows a quantification of the total number of cells and the proportion of cells showing colocalization of the IgE-Fc ϵ R1 complex with the allergen dot pattern as a function of the incubation time of the cells on the samples. It is evident that after 15 to 30 min, almost all the cells have recognized the DNP pattern.

Negative control experiments were performed incubating RBL-2H3 cells sensitized with fluorescently labeled Alexa 647-anti-DNP IgE antibody on the sample having both TAMRA- and DNP-azide arrays in alternating fashion next to each other. Colocalization was only observed on the DNP-azide patterns but not on the TAMRA patterns (see Supporting Information).

The first observable events after activation of mast cells are tyrosine phosphorylation of the Fc ϵ R1 clusters and receptor-proximal kinases.^[30] Since these can be monitored by immunofluorescence with an anti-phosphotyrosine antibody staining, the anti-DNP IgE-Fc ϵ R1 clusters were analyzed for total tyrosine phosphorylation in the present study. RBL-2H3 mast cells, sensitized for 2 h with anti-DNP IgE antibody labeled with Alexa 647, were loaded onto the array, allowed to settle on the dots for 30 min and then fixed and probed with anti-phosphotyrosine antibody. A clear phosphotyrosine staining was detected at the dots containing the DNP confirming a biological response of the mast cells at the point of contact with the allergen (Figure 5). The graph in Figure 5d shows a quantification of the total number of cells and the proportion of cells showing phosphotyrosine staining

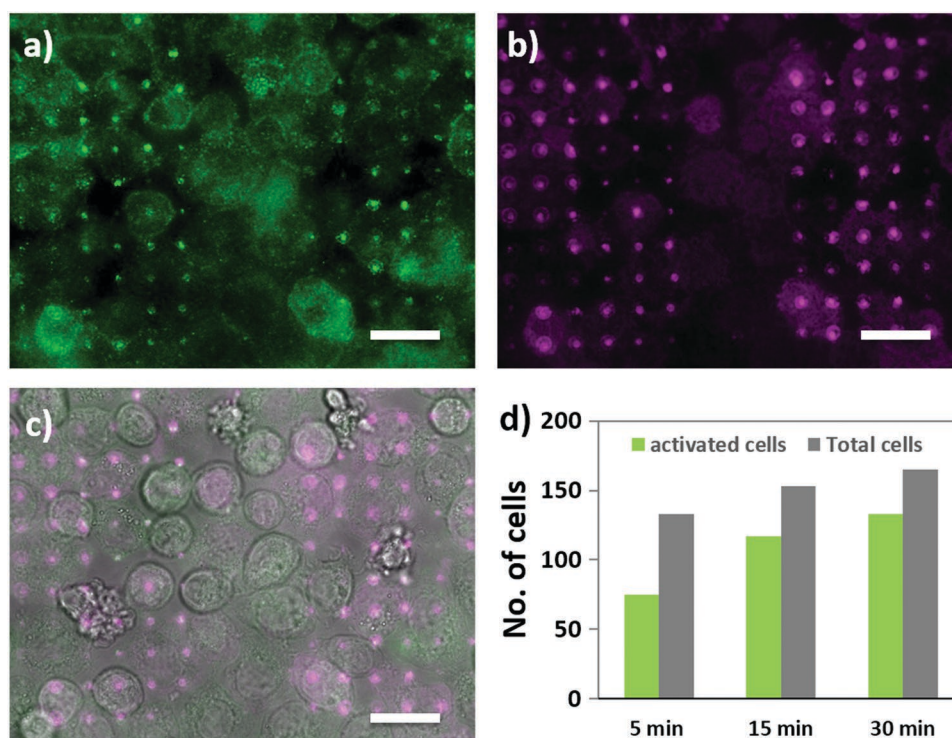


Figure 5. Activation of mast cells and colocalization of IgE-receptor with phosphorylated tyrosine. Fluorescence images showing a) activation signal (phosphotyrosine staining-green), and b) colocalization signal (IgE staining-purple). c) Bright field image merged with (a) and (b). All scale bars are 20 μm . d) Activated and total cells on pattern as a function of time (counted from 12 images per time point).

of the IgE-Fc ϵ RI clusters with the allergen dot pattern as a function of the incubation time of the cells on the samples. It is evident that with increasing incubation time, the number of activated cells has also increased on the DNP pattern.

Furthermore, this result demonstrates that the (easier to detect) colocalization of receptor with the DNP pattern can be used as proxy for the identification of molecules invoking a cellular response.

2.3. Cell Experiments in Microfluidic Chips

As a first step to turn the allergen arrays into an easy-to-use tool for biological and bio-medical research or for diagnostic purposes, we incorporated the allergen arrays into a microfluidic chip (cf. Scheme in Figure 1a). The patterns were printed on a standard $2.4 \times 5.0 \text{ cm}^2$ glass slide and mounted on a matching microfluidic chip comprising 5 mm wide and 400 μm high channel. This setup allowed easy incubation of cell suspension by simple delivery with a 3 mL syringe. Approximately 300 μL of cell suspension (10^6 cells per mL RBL-2H3 cells sensitized with fluorescently labeled Alexa 647-anti-DNP IgE antibody) were delivered into the chip and cells were allowed to settle down. The whole setup can easily be monitored in real-time on an inverted microscope in bright field and fluorescence imaging mode (Figure 1c,d).

In another set of experiments, we explored the possibility of identifying sensitized mast cells in a background of unsensitized cells in the microfluidic setup. Here, we spiked CF488-anti-DNP IgE antibody sensitized bone marrow derived mast cells (BMMCs) in different ratios into a

background of unsensitized BMMCs. After loading and incubation of the mixture of cells in a 6 channel chip carrying an allergen array in each channel, the patterns were screened for IgE-Fc ϵ RI clustering via colocalization with the allergen pattern.

As the anti-DNP IgE antibody used for sensitization of the cells was fluorescently labeled (CF488), while the unsensitized cells had the nucleus stained with a different dye (Hoechst 33342), these two could be easily distinguished on the sample. BMMCs showing IgE-Fc ϵ RI clustering and unsensitized BMMCs on the pattern were quantified for each different seeding ratio (Figure 6). Remarkably, sensitized BMMCs could not only clearly be identified in fluorescence

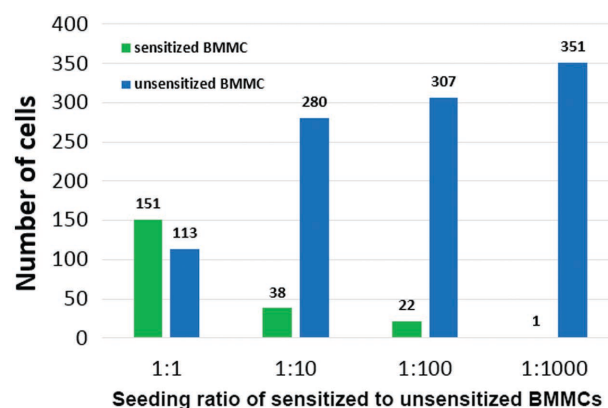


Figure 6. Cell counts on the DNP-azide arrays inside the microfluidic chip for different seeding ratios of sensitized to unsensitized BMMCs. Compared to the seeding ratios, the sensitized BMMCs are enriched on the pattern.

by the IgE-FcεRI clustering, but it was observed, that the ratio of sensitized to unsensitized BMMCs on the pattern was generally larger than the seeding ratio. This indicates that the sensitized cells are enriched on the pattern. In a similar experiment, sensitized BMMCs were mixed with (also sensitized) adherent cells from the bone marrow (non-mast cells). Again, the BMMCs were not only easily distinguished from the background adherent cells, but also enriched in relation to the seeding ratio (see Supporting Information). This relative enrichment of BMMCs occurs likely due to the specific receptor–antibody–allergen interaction that binds sensitized cells stronger to the allergen pattern (compared with the non-specific adhesion of the background cells). This gives a higher chance for the sensitized cells to remain adhered during the washing steps involved in incubating, fixing and staining. The phenomenon could be utilized for enrichment and capture of mast cells out from a background of other cell types.^[31]

3. Conclusion

Click-chemistry bound allergen arrays produced by PPL technique can be integrated in microfluidic chips and be utilized as a platform for mast cell activation studies and cell sorting applications. We have successfully patterned a model allergen by attachment of azide-functionalized DNP on alkyne modified glass, showing the potential of the PPL technique to create large-area allergen patterns ($\approx 1 \times 1 \text{ cm}^2$). The specific binding of the IgE antibody to the allergen arrays and the rapid response of the FcεRI receptor in live RBL-2H3 mast cells without any obvious toxic effects of the click-chemistry based surface modification shows the potential of this immobilization strategy in allergen arrays for diagnostics and research. The arrays can easily be incorporated in microfluidic chips, further facilitating their use in controlled and standardized setups as desired for diagnostic applications. To this end, we modeled a cell sorting application identifying sensitized BMMCs in a background of either unsensitized BMMCs or adherent cells. Here, the allergen arrays show a pronounced retention of BMMCs making this particular procedure useful for mast cell sorting and enrichment.

4. Experimental Section

Substrate Preparation: Substrates (alkyne terminated glass) for the CuAAC patterning were prepared using a procedure similar to what has previously been reported.^[25] In brief, glass cover slips (VWR, Germany) were cleaned by sonication subsequently in chloroform, ethanol and DI water for 5 min in each solvent. After these cleaning steps, the cover slips were dried with a N_2 stream and activated in oxygen plasma (0.2 mbar, 100 W, 10 sccm O_2 , 2 min, ATTO system, Diener electronics, Germany). After activation, the cover slips were silanized in a 1 vol% (3-Glycidyloxypropyl)-trimethoxysilane (GPTMS) solution in toluene for 8–12 h (usually overnight). To clean off excess silane, glass slides were sonicated in acetone for 1 min and then finally submerged into a solution of 2 vol% propargylamine in acetonitrile for 8 h at 50 °C. After the alkynization, the cover slips were sonicated in ethanol for 30 s,

dried with N_2 and stored in a glass petri dish until use. All chemicals for this process were purchased from Sigma-Aldrich, Germany.

Ink Solutions: Patterning was performed with inks of varying ratio of catalyst and functional azide solution (ranging from 51×10^{-6} to $658 \times 10^{-6} \text{ M}$). While DNP-azide (Jena Bioscience, Germany) was used for the allergen arrays, TAMRA-azide (Life Technologies, Germany) was used for fluorescent visualization of the pattern. To avoid premature drying of the ink, up to 20% (v/v) of glycerol (87% in DI water) was added to the ink solutions. Typically, ink solutions used in the present work were a mixture of 5 μL CuSO_4 ($10 \times 10^{-3} \text{ M}$ in DI water), 5 μL sodium ascorbate ($20 \times 10^{-3} \text{ M}$ in DI water) (both Jena Bioscience, Germany), 1 μL of either TAMRA-azide solution (0.5 mg mL^{-1} in DMSO) or 1 μL of DNP-azide solution (0.5 mg mL^{-1} in DMSO), respectively, and an added 2.75 μL of glycerol to prevent drying.

Preparation of PPL Stamps: PPL tip arrays were prepared as previously reported in the literature.^[10] The pen array was inked with 4 μL of the respective ink solutions by spin coating (3500 rpm, 3 min). Patterning was carried out on a NLP 2000 instrument (Nanoink Inc., USA), with a custom made pen array holder. The pen array was first glued with two component epoxy resin adhesive (Uhu, Germany) to a microscopic glass slide, then the whole glass slide was glued to the bar of a custom made holder mounted onto the instrument. Leveling of the stamp parallel to the substrate was achieved by an optical alignment procedure controlling the elastic tip deformation of the stamp upon tip array-surface contact on a clean sacrificial cover slip.^[32] The built-in internal camera in the instrument allows the detection of deformations and for a satisfactory homogeneous alignment over the whole sample area.

Patterning Procedure: All patterning was carried out on a NLP 2000 instrument (Nanoink Inc., USA) under humidity-controlled conditions (60% to 80% RH). Unless otherwise stated, writing schemes were arrays of 10×10 dot features with a pitch of 10 μm and dwell times of 1–5 s. With these parameters, patterning a full square centimeter (typical stamp size) with 100 000 dot features took ≈ 2.4 to 9.2 min. After lithography, samples were kept overnight at ambient conditions to allow click-reaction to occur and then washed with DI water and ethanol to remove excess and unbound ink, dried with N_2 and stored dry and dark until used for experiment.

Integration into Microfluidics: For the integration of the allergen arrays into microfluidic chips, either single channel chips (μ -Slide I 0.4 Luer, ibidi, Germany) or 6 channel chips (sticky-Slide VI 0.4, ibidi, Germany) were used. These chips have no bottom plate, but instead a sticky coating that allows to bond them to glass slides of matching size. After preparation of the allergen arrays and peeling off the protective layer on the bottom of the ibidi chips, the chips are manually aligned with the edges of the patterned substrate. The achieved alignment is usually sub-millimeter and sufficient due to the large area patterning achieved with the PPL as the patterned area is larger than the channel width.

Detection of the DNP Pattern by Antibody Binding: To ensure successful patterning, the samples were first blocked with 10% BSA in phosphate buffered saline (PBS) for 30 min, washed with PBS, and then incubated for 1.5 h at room temperature with mouse anti-DNP IgE antibody (50 μL at a concentration of 20 $\mu\text{g mL}^{-1}$ in PBS) which has previously been fluorescently labeled with a Mix-n-Stain CF 488A antibody labeling kit (both from Sigma-Aldrich, Germany). After staining, the samples were washed with PBS and imaged.

RBL-2H3 Cell Culture: RBL-2H3 mast cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Germany) supplemented with 15% fetal bovine serum (FBS, Sigma-Aldrich, Germany) under standard cell-culture conditions: 37 °C and 5% CO₂.

Sensitization and Preparation of Cells: 1×10^6 RBL-2H3 mast cells were seeded overnight in a 60 mm cell culture dish (CELLSTAR, Greiner Bio-One, Germany) and sensitized with monoclonal anti-DNP IgE (Sigma-Aldrich, Germany) $0.5 \mu\text{g mL}^{-1}$ for 2 h. The cells were then washed with PBS to remove unbound IgE, harvested in 1 mL of prewarmed (37 °C) Tyrode's buffer (20 mM HEPES, 135×10^{-3} M NaCl, 5×10^{-3} M KCl, 1×10^{-3} M MgCl₂, 1.8×10^{-3} M CaCl₂, 5.6×10^{-3} M glucose, pH 7.4) with 0.1% BSA and used for the different experiments.

Immunofluorescence Imaging: 100 μL of sensitized RBL-2H3 cells were loaded and allowed to settle on the sample arrays, fixed at the indicated time points with 3.7% paraformaldehyde, washed with PBS, blocked with 10% BSA in PBS, and incubated with anti-phosphotyrosine 4G10 antibody (1 h, $5 \mu\text{g mL}^{-1}$ in PBS, Merck Millipore, Germany). Second incubation was performed with goat anti-mouse-FITC-conjugated antibody (45 min, $5 \mu\text{g mL}^{-1}$ in PBS, Merck Millipore, Germany). Negative control experiments were conducted by incubating the samples with the secondary antibody without prior primary antibody incubation.

Culture of Bone Marrow Derived Mast Cells (BMMCs): Bone marrow derived mast cells were isolated from femurs and tibias of 7–9 weeks old C57BL/6 mice. All animal experiments were performed according to European and German statutory regulations. Upon isolation as described by Stassen et al.,^[33] every 3–4 d for 5–6 weeks nonadherent cells were collected, centrifuged, and cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum from south America origin (Gibco, ThermoFisher Scientific, Germany), 2×10^{-3} M L-glutamine (Gibco, ThermoFisher Scientific, Germany), 1×10^{-3} M pyruvate (Gibco, ThermoFisher Scientific, Germany), 1% Penicillin/Streptomycin (Gibco, ThermoFisher Scientific, Germany), 100 ng mL⁻¹ stem cell factor (kindly provided by Michael Stassen), 5 ng mL⁻¹ IL-3 (PreproTech, Rocky Hill, NJ), and 50×10^{-6} M 2-Mercaptoethanol (Gibco, ThermoFisher Scientific, Germany). This medium promoted the differentiation of nonadherent cells into BMMCs.

Experiments in Microfluidic Chip: For testing the assay in a microfluidic environment, a DNP-azide array prepared as described above on a standard $2.4 \times 5.0 \text{ cm}^2$ glass slide was mounted on a microfluidic channel (μ -Slide I 0.4 Luer, ibidi, Germany). RBL-2H3 cells (300 μL , 10^6 cells per mL) previously sensitized with fluorescently labeled Alexa 647-anti-DNP IgE antibody were delivered to the microfluidic chip by a standard 3 mL Luer lock syringe. Cells were incubated for 30 min then fixed with 3.7% paraformaldehyde (PFA) in PBS for 20 min. Incubation, fixation, and receptor colocalization were monitored on an inverted fluorescence microscope (TE2000, Nikon, Germany).

In a coculture experiment, we used unsensitized but nuclear stained (Hoechst 33342 $1 \mu\text{g mL}^{-1}$, Invitrogen, Germany) BMMCs and BMMCs sensitized for 2 h with $0.5 \mu\text{g mL}^{-1}$ fluorescently labeled CF488-anti-DNP IgE antibody. The cells were washed with PBS and suspended in 0.1% BSA in Tyrode's buffer at the concentration of 1×10^6 cells per mL. The two cell types were then mixed at different ratios and 100 μL of the respective mixture was loaded into the microfluidic chip, in this case a 6 channel version

(sticky-Slide VI 0.4, ibidi, Germany), to facilitate parallel testing. Cells were incubated for 30 min, then ≈ 70 –80 μL of liquid was removed from the chip chamber and replaced by 100 μL 3.7% PFA for 20 min to fix the cells. The cells were then washed once with PBS and were analyzed by fluorescence microscopy and counted. A similar experiment was also performed using another setup, where BMMCs sensitized with Alexa 647-anti-DNP IgE were mixed into a background of also sensitized adherent bone marrow cells coming from two weeks old BMMCs cultures.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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