

RESEARCH ARTICLE

Detection of allergen specific immunoglobulins by microarrays coupled to microfluidics

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Allergen microarrays are under development for a component-resolved diagnosis of Type I (IgE-mediated) allergies. Here we report an improved microarray coupled to microfluidics for the detection of allergen specific immunoglobulin E (IgE). The signal intensity for IgE detection in serum has been improved by using glass slides coated with a novel poly[DMA-co-NAS] brush copolymer which is able to immobilize allergens in their native conformation and by carrying out the incubation step in dynamic conditions. The assay, fully automated, was performed in a microcell, using a software-controlled fluidic processor, to bring assay reagents on the surface of the array. Microfluidics turns the binding between serum immunoglobulins and immobilized allergens from a diffusion-limited to a kinetic-limited process by ensuring an efficient mixing of serum samples on the surface of the microarray. As a result of this, the binding of high affinity IgE antibodies is enhanced whereas that of low affinity IgG antibodies, which are present at higher concentration, is impaired paving the way to more accurate and sensitive results.

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1 Introduction

Clinical manifestation of an allergic disease requires several sequential steps: exposure to an allergen, allergen specific IgE synthesis, binding of allergen-specific IgE onto the IgE-receptor on the surface of mast cells and basophils, re-exposure to the same or homologous allergen, cross-linking of membrane-bound IgE, cell activation and mediator secretion, with subsequent effects on end-organs such as blood

vessels, bronchial smooth muscles, the gastrointestinal tract or the skin [1]. Accordingly, allergen-specific IgE is the molecular entity which determines the patient's allergy and thus it is a major cornerstone of a specific allergy diagnosis.

Various immunoassays using crude allergen extracts such as ELISA, Radio Allergo Sorbent Test (RAST) and high-capacity solid phase test, e.g. the ImmunoCAP[®]-system, are currently in use for serological *in vitro* testing for specific IgE detection [2]. However, these tests are time-consuming and expensive, they require large quantities of serum sample and often use poorly characterized preparations of allergen extracts. Moreover, these conventional assays do not provide the throughput to screen for all the known allergens which are estimated to exceed 300 [2]. Due to low serum volume consumption, high-throughput capacity and flexibility, protein microarrays are becoming important tools for the determination of allergen-specific IgE [3]. The principle of the allergen microarrays is an immunoassay consisting of recombinant allergens spotted and immobilized on functionalized microarray slides as the capture agents. The

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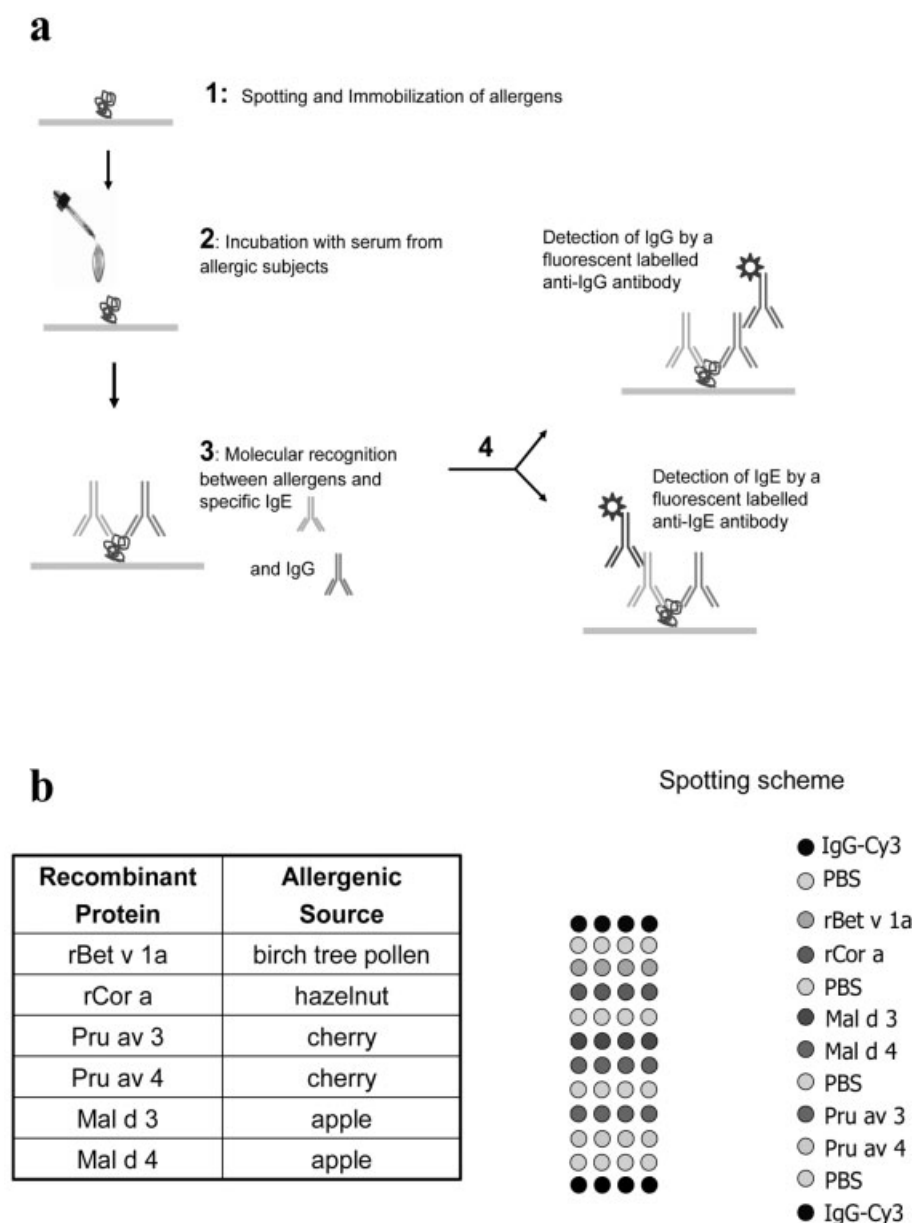
Abbreviations: AP, alkaline phosphatase; DMA, *N,N*-dimethylacrylamide; MPS, (3-mercaptopropyl)trimethoxy silane; NAS, *N,N*-acryloyloxysuccinimide; PDMS, polydimethylsiloxane; RT, room temperature

serum sample is screened in a single test for allergen-specific IgE by labelled anti-IgE (Scheme 1a), thus, a multitude of single allergens can be simultaneously probed for IgE binding allowing the identification of molecules the patients are sensitized and therefore the target molecules for a putative specific immunotherapy [4]. Several examples of allergen microarrays have been published in recent years [2, 5, 6]. Moreover, several studies have been reported using microarrays produced by VBC–Genomics (Vienna, Austria) [4, 7, 8].

Measuring IgE levels in serum by microarrays is a challenging task for several reasons. First of all IgE is typically the least abundant antibody isotype in blood serum (for example IgE levels in a normal individual are

~75 ng/mL, compared to 10 mg/mL for IgG). Moreover, most of the IgE-binding epitopes are conformational epitopes, therefore the native structure of allergens must be kept during the assay to be properly recognized by the specific IgE. A microarray surface for allergen specific IgE detection must therefore provide high allergen binding capacity and low nonspecific background. In addition, allergens must maintain their native conformation upon surface immobilization.

A further difficulty originates from the fact that in serum, both allergen specific IgE and IgG are present. During the assay on microarrays (which is a solid phase micro-immunoassay), IgE and IgG compete for the binding on the same immobilized allergens. IgE, though provided with high



Scheme 1. (a) Scheme of the microarray assay to detect allergen specific immunoglobulins. Allergens are spotted and immobilized on a coated microscope glass slide; antibodies from the patient's serum recognize the immobilized proteins on the array surface. A secondary antibody, labelled by Cy3, allows the detection of the target molecules. An anti-IgE secondary antibody is used for the detection of allergen specific IgEs, an anti-IgG is used to study the pattern of allergen specific IgGs in the serum samples. (b) A set of six recombinant allergens produced as described in Section 2.2 (rBet v 1, rCor a 1, rPru av 3, rPru av 4, rMal d 3, rMal d 4) were spotted at a concentration of 1 mg/mL in PBS buffer according to the spotting scheme reported.

affinity [9] are present in much lower concentration than IgG. Therefore, IgG binding to surface immobilized allergens can hinder the detection of IgE.

Measurements of the kinetics of antigen–antibody reactions at solid surfaces are often hampered by the effects of mass transport limitation that influence the reaction at plane surfaces. The mass transport limitation is due to depletion of antibodies in the reaction zone close to the surface (for microarrays in the vicinity of spots) [10]. The analyte depletion in the vicinity of microspots is often responsible for a significant loss of sensitivity.

Hartmann *et al.* [11] increased the robustness and sensitivity of protein microarrays through microagitation.

The mixing can therefore compensate the gradient of analyte concentration in the vicinity of spots and can turn the interaction between probes, immobilized on the surface, and antibodies, in the solution, from a mass transport-controlled reaction towards a kinetic-controlled one. Importantly, these authors have reported a more significant increase of the specific signals at lower antibody concentration.

To overcome the mass transport limitation typical of solid phase technology, a liquid phase assay for specific IgE taking advantage of the ADVIA Centaur technology platform, was developed [12]. In this test, the detection of specific IgE, is based on their affinity to specific allergens. However, this technology requires the labelling of allergens and the use of 25 μ L of serum per each measurement.

In this work we demonstrate a significant enhancement in the signal intensity of allergen specific IgE on microarrays through the optimization of two distinct assay parameters, surface chemistry and binding kinetics. Improvements were achieved:

(i) by enhancing allergens binding capacity through the use of glass slides coated with a new brush polymeric coating based on a copolymer of *N,N*-dimethylacrylamide (DMA) and *N,N*-acryloyloxysuccinimide (NAS) and

(ii) by integrating the allergen microarray in a microflow cell that allows to perform the serum incubation in a dynamic mode thus favouring specific IgE binding and dis-favouring that of IgG.

2 Materials and methods

2.1 Reagents and equipments

Tris, BSA, Triton X-100, PBS tablets, DMA and [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) were purchased from Sigma (St. Louis, MO). As the secondary antibodies, goat anti-human IgG labelled with Cy3 from Jackson Immuno Research and monoclonal anti-human IgE from Pharmingen (clone G7-26) in house labelled by Cy3 were used. NAS was from Polysciences (Warrington, PA). Sylgard 184 (PDMS elastomer kit) was from Dow Corning (Midland, MI). Polydimethylsiloxane (PDMS) etched structures for the microfluidic cell were moulded using custom designed sili-

con masters and moulding stations from GeSim (Groß-erkmannsdorf, Germany). The fluidic processor and its control software were also purchased from GeSim.

2.2 Allergens

As model allergens, recombinant allergens from different allergenic sources were used: Pathogenesis-related proteins of the class 10 (PR-10) from birch (Bet v 1; GenBank accession no. X 15877) and hazelnut (Cor a 1.04; GenBank accession no. AF 136945), profilins from apple (Mal d 4; GenBank accession no. AF 129428) and cherry (Pru av 4; GenBank accession no. AF 129425), as well as the Lipid-Transfer proteins (pathogenesis-related proteins of the class 14 (PR-14)) from apple (Mal d 3; GenBank accession no. AF 221502) and cherry (Pru av 3; GenBank accession no. AF 221501). Different expression systems and purification methods were used: For PR-10 proteins and profilins *Escherichia coli* was used whereas Lipid-Transfer proteins were over-expressed in *Pichia pastoris*.

2.2.1 PR-10 proteins

Expression and subsequent purification of the two PR-10 proteins were performed as previously described for rCor a 1.04 [13]. Briefly, both proteins were expressed as fusion proteins in *E. coli* and purified by a two-step FPLC method (IMAC, SEC). Finally, the target proteins containing fractions were pooled, dialysed against PBS and stored at 4°C.

2.2.2 Profilins

Expression of rMal d 4 and rPru av 4 was carried out as described before for the PR-10 proteins with some modifications. The cell pellet of the culture was reconstituted in 20 mL lysis buffer (50 mM Na_2HPO_4 , 500 mM NaCl, 2 mM imidazole, pH 8.0 + 375 U = 15 μ L benzonase) and frozen at –80°C. Frozen cells were subjected to two cycles of freezing and thawing in liquid nitrogen. The lysate was incubated with 0.5% Triton X-100 for 30 min at 4°C and centrifuged (15 000 $\times$ g, 30 min, 4°C). For further purification the supernatant was applied to a Poly-L-Proline (PLP)–Sephacrose (Poly-L-Proline: Sigma-Aldrich, Taufkirchen, Germany; CNBr-activated; Sepharose™4B: GE-Healthcare, Uppsala, Sweden) and incubated overnight at 4°C. PLP–Sephacrose was washed six times with lysis buffer and packed into a column. Bound profilin was eluted with 6 M urea in lysis buffer. Profilin containing fractions were pooled and dialysed stepwise against PBS for refolding (4 M urea to 0 M urea in PBS). The final product was stored at 4°C.

2.2.3 Lipid-transfer proteins

The expression and purification of recombinant Mal d 3 and Pru av 3 was similarly performed as described for the yeast

expression of the hazelnut lipid-transfer protein Cor a 8 [13]. Briefly, proteins were expressed as nonfusion-proteins in *P. pastoris* and purified by a two-step FPLC method (IEC, SEC). Finally, target protein containing fractions were pooled, dialysed against PBS and stored at 4°C.

2.2.4 Electrophoresis and immunoblotting

The purity and quality of each of the produced allergen was analysed by SDS-PAGE (17.5%) under nonreducing conditions and by IgE-antibody-reactivity. For immunoblotting, proteins were transferred to NC membranes (0.45 µm) by electroblotting (400 mA, 50 min). Membranes were blocked in TBS with 0.3% Tween 20 for 1 h at room temperature (RT) and incubated with 1:10 diluted patients' sera overnight at RT. Bound IgE antibodies were detected with mouse-anti-human-IgE coupled with alkaline phosphatase (AP; 1:750) (Pharmingen, Hamburg, Germany) and visualized with NBT/BCIP as substrate in 0.1 M TBS, pH 9.5 according to the manufacturers' instructions (Biorad, Munich, Germany).

2.2.5 Biotinylation of allergens and coupling to Streptavidin-ImmunoCAPs

For measurement of specific IgE-binding to purified recombinant allergens using the commercial ImmunoCAP®-system (Phadia GmbH, Freiburg, Germany) as reference all allergens had to be biotinylated and subsequently coupled on Streptavidin-ImmunoCAPs. For biotinylation the concentration of the allergens was normalized to 0.1 mg/mL. A 200 µL aqueous biotin-X-NHS solution (1 mg/mL Calbiochem, San Diego, USA) and 5 mL of the allergen (0.1 mg/mL), was mixed to obtain a ratio of 1:2.5 (biotin:allergen, w/w). After incubation for 5 h at RT under gentle shaking, the reaction was stopped by the addition of 300 µL 0.1 M NH₄Cl and then incubated for 30 min at RT under gentle shaking. Unbound biotin was removed by dialysis against MOPS buffer (20 mM MOPS, 500 mM NaCl, pH 7.6). For verification of biotinylation the dot-blot technique was applied. Therefore, 20 µL of biotinylated allergen (corresponding to approx. 2 µg) was applied to a 0.2 µm NC membrane. A dialysed biotin solu-

tion was provided as buffer control and nonbiotinylated allergen as negative control. Allergen bound NC membranes were dried and subsequently incubated in blocking buffer (0.3% Tween 20 in TBS) for 1 h at RT. Two different detection methods were performed:

(i) Detection with streptavidin-AP: After washing with TBST buffer (0.05% Tween 20 in TBS) 4 × 5 min, the membrane was incubated with streptavidin-AP for 30 min (RT). Detection was performed with the AP conjugate substrate kit (BioRad).

(ii) Detection with patient serum: After washing with TBST buffer (4 × 5 min) membrane was incubated with patient serum (1:10 diluted) overnight (RT). Unbound Ig was washed out with TBST buffer (4 × 5 min) and the membrane was incubated with a monoclonal AP-labelled anti-human-IgE antibody (Pharmingen) for 4 h at RT. Detection was performed with the AP conjugate substrate kit (BioRad).

The coupling of biotinylated recombinant allergens to Streptavidin-ImmunoCAPs (Phadia) was performed using a UniCAP® 100 (Phadia). Streptavidin-ImmunoCAPs were prewashed according to the standard procedure. After washing Streptavidin-ImmunoCAPs were transferred to a 96-well plate and 50 µL (approx. 4.5 µg) of the biotinylated allergen was applied. After incubation for 30 min at RT the reaction was stopped by washing the Streptavidin-ImmunoCAPs using the UniCAP® 100 system. Coupled ImmunoCAPs were stored until measurement at 4°C.

2.3 Serum samples

To compare the specific IgE antibody reactivity with the purified proteins by immunoblotting, the ImmunoCAP®-system and the microarray four-reference sera for each allergen were used. These patients (nos. 1–4) were allergic to birch, hazelnut, apple or cherry, had a positive case history (oral allergy syndrome, rhinitis, conjunctivitis, breathlessness and swelling of the throat) and CAP class 1 or higher to at least one of the allergic sources. Four sera of nonallergic subjects were used as negative controls (NC 1–4). The IgE-sensitization pattern of the patients is shown in Table 1.

The serum samples used in this work are part of the patient's sera recruited for the NANOSPAD project, EC con-

Table 1. Serum reactivity determined by IgE-immunoblotting

Serum	Bet v 1	Cor a 1	Pru av 3	Pru av 4	Mal d 3	Mal d 4
1	+	+	+	+	+	+
2	+	+	–	–	–	–
3	+	+	–	+	–	+
4	+	+	–	–	–	–
NC 1	–	–	–	–	–	–
NC 2	–	–	–	–	–	–
NC 3	–	–	–	–	–	–
NC 4	–	–	–	–	–	–

tract number 016610 (NANOSPAD) FP6. All individuals enrolled in this study gave informed consent as approved by the local ethic committee.

Serum IgG removal was performed by ProMax Serum IgG Removal Kit from Polysciences (Warrington, PA) according to manufacturer's instructions.

2.3.1 Determination of specific immunoglobulin E

Quantification of specific IgE-binding to purified recombinant allergens were performed using the UniCAP®-system (Phadia). Streptavidin-ImmunoCAPs coupled with recombinant allergens (4.5 µg ImmunoCAP) were prepared as described in Section 2.2. Results of the CAP measurements are shown in Table 2.

2.4 Microarray slides coating by MPS-poly[DMA-co-NAS]

Microscope glass slides were immersed in a solution of water, H₂O₂ (30% in water) and NH₄OH (28–30% in water) in the ratio 5:1:1 v/v/v respectively, for 2 min and heated at 60°C. Then they were washed in milliQ water and dried in a vacuum oven at 80°C for 20 min. The slides were then dipped in 1 M NaOH for 30 min, washed in milliQ water and dried in vacuum oven for 30 min at 80°C.

The pretreated slides were covered with a 1% w/v (3-mercaptopropyl)trimethoxy silane (MPS) solution in dry toluene and left in this solution for 4 h at RT. After the reac-

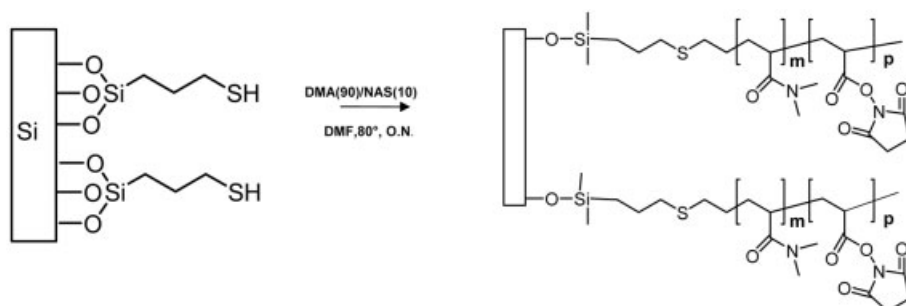
tion was completed, the slides were washed with toluene, THF and then dried under vacuum in oven at RT for 30 min. The slides were then immersed in a solution of DMA (0.9 M), NAS (0.1 M), 2,2'-Azobisisobutyronitrile (AIBN) (1 mM) in degassed DMF. The reaction mixture was allowed to react under Argon atmosphere, overnight at 80°C (Scheme 2). After this period, the slides were rinsed in dry DMF, washed in anhydrous THF and dried under nitrogen flow and then in a vacuum oven at room temperature for 30 min.

2.5 Microarray experiments

An array of rabbit IgG labelled with Cy3 (reference), PBS (negative control) and six recombinant allergens dissolved in PBS at a concentration of 1 mg/mL was patterned using a SciFlexArray spotter from Scienion (Berlin, Germany) according to Scheme 1b. In the experimental conditions used, the volume of the spotted drops is 400 pL. Printed slides were placed in a humid chamber and incubated at RT overnight. The slides were then blocked by 2% w/v BSA in PBS for 1 h, washed with water and dried by centrifugation. In the optimized static conventional experiments, arrayed slides were incubated for 2 h with serum samples (25 µL) diluted 1:2 in the incubation buffer (0.05 M Tris/HCl pH 7.6, 0.15 M NaCl, Tween 20 0.02% v/v, BSA 1% w/v), washed by the washing buffer (0.05 M Tris/HCl pH 9, 0.25 M NaCl, Tween 20 0.05% v/v) for 10 min under stirring, rinsed with water and incubated with the labelled secondary antibody 0.01 mg/mL in the incubation buffer for 1 h. Slides were washed with PBS (10 min) and water (10 min).

Table 2. CAP values (U/mL) of the analysed serum samples. CAP values > 0.70 U/mL (CAP classes > 2) are reported in bold. 1 KU = 2.4 µg of IgE

Serum	rBet v 1	rCor a 1	rPru av 3	rPru av 4	rMal d 3	rMal d 4
1	29.4	10.1	18.1	10.2	18.5	10.1
2	45.4	30.7	0.16	0.04	0.14	0.07
3	20.1	13.2	0.16	4.14	0.26	3.98
4	43.8	25.0	0.20	0.06	0.50	0.18
NC 1	0	0	0	0	0	0
NC 2	0	0	0	0	0	0
NC 3	0	0	0	0	0	0
NC 4	0	0	0	0	0	0



Scheme 2. MPS-poly[DMA-co-NAS] coating for microarrays. A grafting from polymerization has been used to generate a copolymer of dimethylacrylamide (DMA) and *N,N*-acryloyloxysuccinimide (NAS) starting from (3-mercaptopropyl)trimethoxy silane (MPS) immobilized on the glass slides.

For the experiments performed under microfluidic control, an optimized protocol was developed as follows: incubation with serum (25 μ L diluted to 50 μ L in the incubation buffer) for 10 min under a forward and backward flow of 1 μ L/s, washing with washing buffer under flow of 5 μ L/s for 5 min, incubation with the secondary antibody 0.01 mg/mL in the incubation buffer for 5 min, washing with PBS flow of 5 μ L/sec for 5 min.

Scanning for fluorescence evaluation was performed by a Scan Array Express scanner from Packard Bioscience (Boston, MA) using 90% PMT (Photomultiplier) and 90% laser power for IgE detection and 50% PMT and 50% laser power for the detection of IgGs. Fluorescence intensities of four replicated spots were averaged.

3 Results and discussion

3.1 Allergen microarrays on MPS-poly[DMA-co-NAS] coated slides

Here we report on the development of an allergen array for the diagnosis of type I allergy by the detection of allergen specific IgEs in serum samples. The scheme of the microarray assay is depicted in Scheme 1a: allergens are spotted and immobilized on a coated microscope glass slide; antibodies from the patient's serum recognize the immobilized proteins on the array surface. A secondary antibody, labelled with Cy3, allows to detect and quantify the target molecules. An anti-IgE secondary antibody is used for the detection of allergen specific IgEs, which are relevant for allergy diagnosis, whereas an anti-IgG is used to study the pattern of allergen specific IgGs in the serum samples.

An optimized surface for protein (allergen) microarrays should possess the following characteristics:

- (i) high capacity to bind allergens in a native conformation allowing them to selectively interact with their specific antibodies,
- (ii) high quality spot morphology which leads to accurate data output and
- (iii) low background noise which prevents false negative results and low sensitivity, especially when using serum samples.

The afore mentioned surface characteristics contribute to the overall results of a microarray experiment by improving signal sensitivity and specificity.

In this work, *grafting from* polymerization was used to generate a novel macromolecular coating for microarrays made of a copolymer of DMA and NAS tethered to the solid surface by one end (Scheme 2). A two-step procedure for attaching reactive polymers to a glass surface was devised. In the first step, chemical groups that act as chain transfer agents (CTAs) during the course of free radical polymerization reactions were linked to the substrate surface by silanization with MPS. Thiols, well known CTAs, readily enter into free radical polymerization reactions due to their high reac-

tivity with radical species. Since MPS is immobilized on the glass surface, upon polymerization, a brush-like coating with 3-D structure is formed. The high density of the functional groups (NAS) in the copolymer, which can be tailored according to specific needs, confers to the substrate a very high binding capacity. The allergens bind to the polymeric coating by their accessible primary amine groups.

A set of six recombinant allergens (rBet v 1, rCor a 1.04, rPru av 3, rPru av 4, rMal d 3 and rMal d 4) produced as described in Section 2.2 were spotted on MPS-poly[DMA-co-NAS] coated slides at a concentration of 1 mg/mL in PBS buffer according to the array reported in Scheme 1b.

The MPS-poly[DMA-co-NAS] coated slides were used to perform microarray assays of four allergic patients' sera (nos. 1–4) and four negative controls (NC 1–4). The serum samples used in this work have been characterized for allergen specific IgE antibody reactivity by immunoblotting (Table 1) and by the ImmunoCAP®-System from Phadia (Table 2) as described in Section 2.3.

3.2 Allergen specific immunoglobulin detection on micorarrays

In the first set of experiments (data not shown) incubation was carried out in static conditions by covering the surface of the array with different sera for variable amounts of time. An increase of IgE signals was obtained using an incubation time of 2 h. Not only the intensity but also the pattern of allergen specific IgE was affected by the incubation time, revealing higher signals for longer incubation times. These results demonstrated that in conventional (static) incubation conditions, relatively long times are required to achieve the maximum sensitivity in the detection of allergen specific IgEs. Therefore an optimized protocol was set for microarray experiments as reported in Section 2.5.

In order to accelerate the diffusion of targets onto their binding site, the assay was performed inside a microcell with the aid of an optimized microfluidic protocol. The microcell, used for this purpose, is depicted in Fig. 1a and b. An etched PDMS substrate (same size of a microscope glass slide) was moulded on a silicon master with a central incubation chamber ($9 \times 3 \times 0.1$ mm³) connected by two channels (500 μ m wide, 100 μ m deep) to an inlet and an outlet. The etched microcell was placed on the surface of a flat MPS-poly[DMA-co-NAS] coated slide previously arrayed with allergens according to Scheme 1b. A micro incubation chamber was formed by clamping the etched PDMS substrate and the glass slide in a poly-carbonate chip-holder connected to a selector valve and to a syringe pump. A software controlled fluid processor, by switching among four reservoirs, allows to sequentially introduce, in the microcell, the reagents needed to carry out the assay (sample, washing solutions *etc.*). Liquid volumes and flow rates inside the microcell are precisely defined thanks to the software that controls the flow processor. In Fig. 1c a picture of the microcell is shown. The area of the incubation cell has been mag-

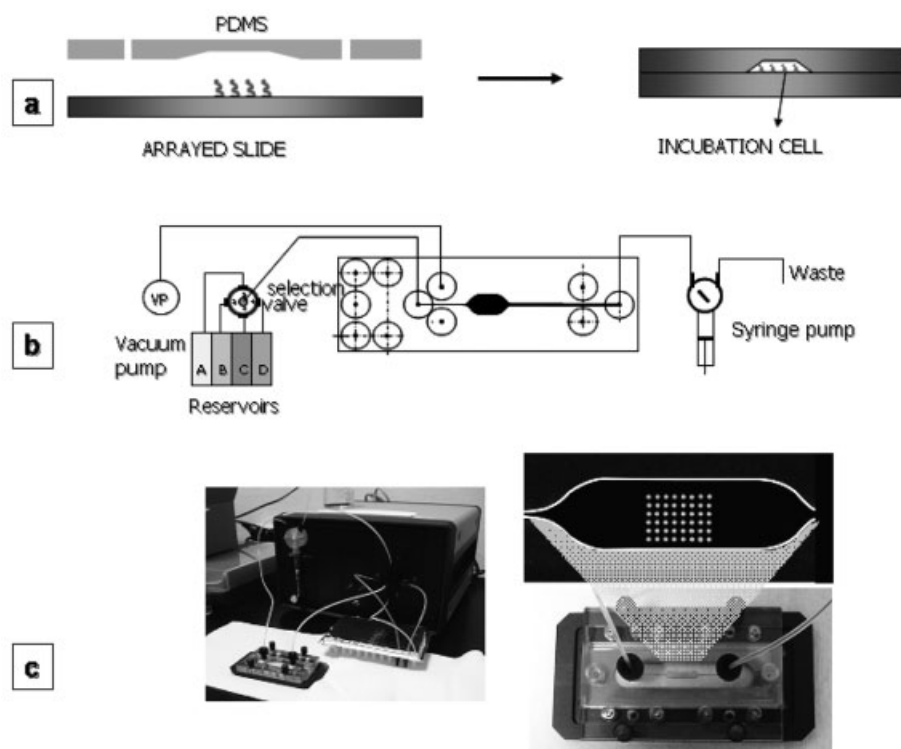


Figure 1. (a) Structure of the microcell. An etched polydimethylsiloxane (PDMS) structure (same dimensions of a microscope glass slide) has been moulded based on a silicon master with a central incubation chamber ($9 \times 3 \times 0.1 \text{ mm}^3$) connected by two channels ($500 \text{ }\mu\text{m}$ wide, $100 \text{ }\mu\text{m}$ deep) to an inlet and an outlet. The etched microcell is placed on the surface of a flat MPS-poly[DMA-co-NAS] coated slide which has been previously arrayed by allergens positioned in the same area of the incubation chamber. (b) Fluidic connections to a selector valve and to a syringe pump. The pump is under a software controlled fluid processor which sequentially suck reagents, switching over four available reservoirs. (c) Picture of the microfluidics set up and of the microcell. The area of the incubation cell has been magnified and a microarray is shown.

nified and a typical output of microarray experiment is shown. Complete microarray assays, from sample introduction to detection, were conducted in the microfluidic cell in only 25 min (Section 2.5). The introduction inside the microcell of the various assay reagents was software controlled and did not require any manual intervention. The most important step in the protocol, the incubation with the serum sample, was performed under a forward and backward flow of diluted serum $1 \text{ }\mu\text{L}/\text{sec}$ for 10 min. The alternating flow allowed us to reduce sample consumption ($25 \text{ }\mu\text{L}$ diluted to $50 \text{ }\mu\text{L}$ with the incubation buffer) without compromising the efficient mixing of the sample on the surface of microarray.

By combining the fields of microfluidics and microarrays, the advantages of both fields can be exploited simultaneously [14]. Microfluidics allows for automated delivery of controlled reagent volumes. Furthermore, the circulation and mixing of sample volume achievable by the use of microflows during incubation can improve the results of the molecular interactions on the microarray surface that are generally hindered by the slow diffusion kinetics [11].

Figure 2 a–d reports a comparison between the allergen specific IgE fluorescence signals detected by the conventional protocol (2 h static incubation) and by the microfluidic assay (10 min dynamic incubation) for the serum samples 1 (a), 2 (b), 3 (c) and 4 (d). The IgE signals are generally increased using microfluidics. They are in good agreement with those detected on the serum samples by immunoblotting (Table 1) and by the ImmunoCAP®-System (Table 2).

The negative controls didn't show any nonspecific signals neither with the conventional nor with the dynamic protocol.

A comparison of the fluorescence signals relative to allergen specific IgGs detected in static and dynamic incubation conditions is reported in Fig. 3 a–d. Generally, in each of the tested sera the signals of allergen specific IgGs were decreased when using the flow microcell.

These data suggest that a short and dynamic incubation can increase the signal intensity of allergen specific IgE on microarrays turning the binding reaction between the immobilized allergens and the Igs in sera from a diffusion controlled reaction towards a real kinetic one. This condition favours the binding of the low concentration antibodies with higher affinity (the IgEs) and impairs the binding of the IgGs. IgG are present in a large amount in serum but they have lower affinity to allergens. They are not relevant for Type I allergy diagnosis and are detrimental for IgE detection since they can block the allergen interaction sites thus hindering the binding of IgEs [15]. Additional advantages provided by microfluidics are assay automation and analysis time reduction. The assay was carried out in only 25 min whereas the various steps of conventional one require 2 h of serum incubation, 1 h of secondary antibody incubation and 30 min of washing. The results of the specific IgE analysis performed on MPS-poly[DMA-co-NAS] coated microarray slides for the serum samples from all patients using the microfluidic protocol are shown in Fig. 4. The allergen microarrays show clear fluorescence signals in accordance with the serum samples reactivity characterized by immunoblotting (Table 1) and

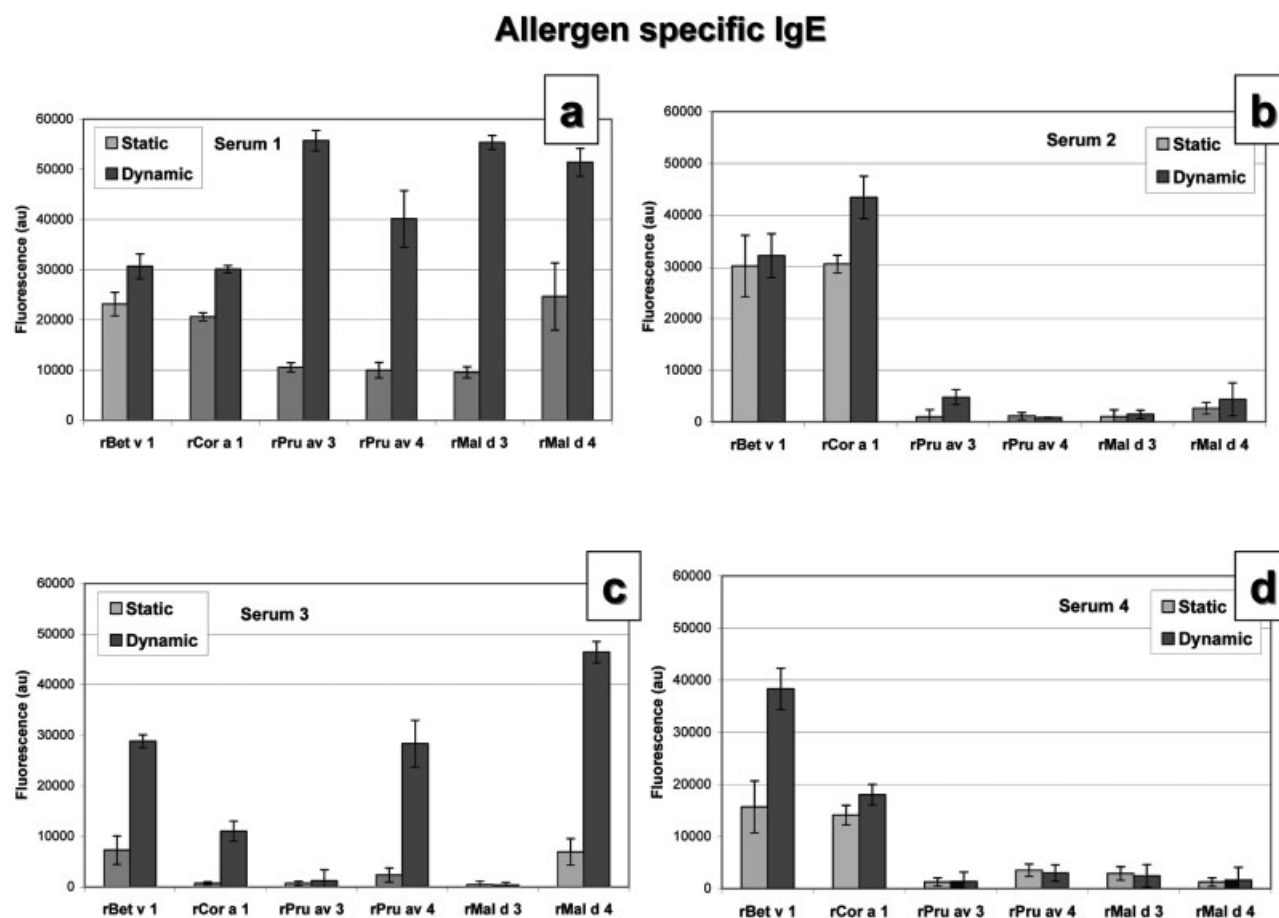


Figure 2. Comparison between the allergen specific IgE fluorescence signals detected by the conventional protocol (2 h static incubation) and by the microfluidic assay (10 min dynamic incubation) for the serum samples 1–4 (a–d). 25 μ L of serum, diluted 1:2 with the incubation buffer was used for every assay. The error bars represent the SD of the fluorescence of four replicate spots. Fluorescence was detected using 90% laser power and 90% PMT. The fluorescence values are calculated by subtracting the background level round about the spots from the fluorescence intensity of each spot.

ImmunoCAP®-System (Table 2). The negative controls (NC 1–4) did not show any unspecific signal (Fig. S1 of Supporting Information). The very low fluorescence background of the MPS-poly[DMA-co-NAS] coated slides allowed us to use a high laser power (90%) and PMT (90%) thus enhancing the sensitivity of the assay. On these slides, fluorescence signals were detected for all tested sera with specific concentrations of at least 4 U/mL. The results demonstrated the usefulness of an MPS-poly[DMA-co-NAS] based allergen microarray coupled to microfluidics for the diagnosis of Type I allergy.

We investigated if sera IgG immunodepletion would give comparable improvements in IgE signal intensity in the static test to demonstrate that the competition between IgG and IgE is the parameter affected by the dynamic mode incubation. In order to perform immunodepletion with any commercial kit it is necessary to dilute the sera. This is not favourable for detecting IgEs which are already present at a very low serum concentration. In Fig. S2 (Supporting Information) the typical results of the IgG and IgE detection

assays performed on MPS-poly[DMA-co-NAS] coated microarray slides in static mode on a serum subjected to IgG depletion are reported. After IgG depletion, due to sera dilution (1:5), the IgE signals are not visible any more. These results suggest that IgG immunodepletion with commercial kits is not a workable strategy to enhance IgE signal intensity. On the other hand enhancement of specific IgE antibody detection by removal of IgG from an antiserum was successfully shown in mice immunized with peanut and shrimp allergens. Removal of IgG by 80–90% resulted in a 3–5-fold increase of IgE reactivity [15].

Recently, a microfluidic chip integrated with microarrays for chemiluminescent detection of allergen-specific antibodies has been developed [16] based on allergen immobilization on PDMS by a ‘macro-molecules to PDMS transfer’ [17] and the use of a SU-8/glass microcell. Unlike these authors, our approach is based on the use of standard microarray supports (modified microscope glass slides) and a PDMS microcell. Microarray slides, bearing the surface

Allergen specific IgG

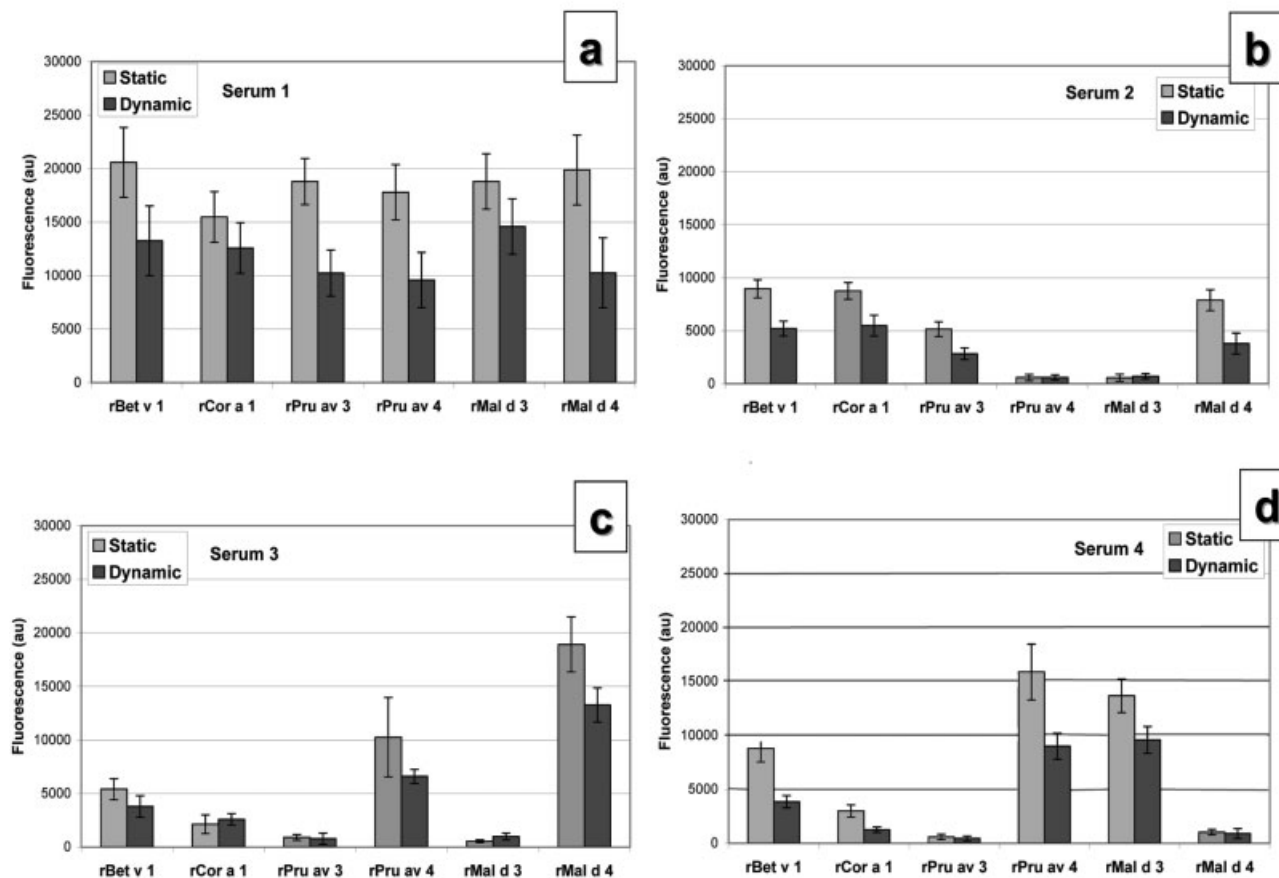


Figure 3. Comparison of the fluorescence signals for allergen specific IgGs performed with the conventional protocol (2 h static incubation) and by the microfluidic assay (10 min dynamic incubation) for the serum samples 1–4 (a–d). 25 μ L of serum diluted 1:2 with the incubation buffer was used for every assay. The error bars represent the SD of the fluorescence of four replicate spots. Fluorescence was detected using 50% laser power and 50% PMT. The fluorescence values are calculated by subtracting the background level round about the spots from the fluorescence intensity of each spot.

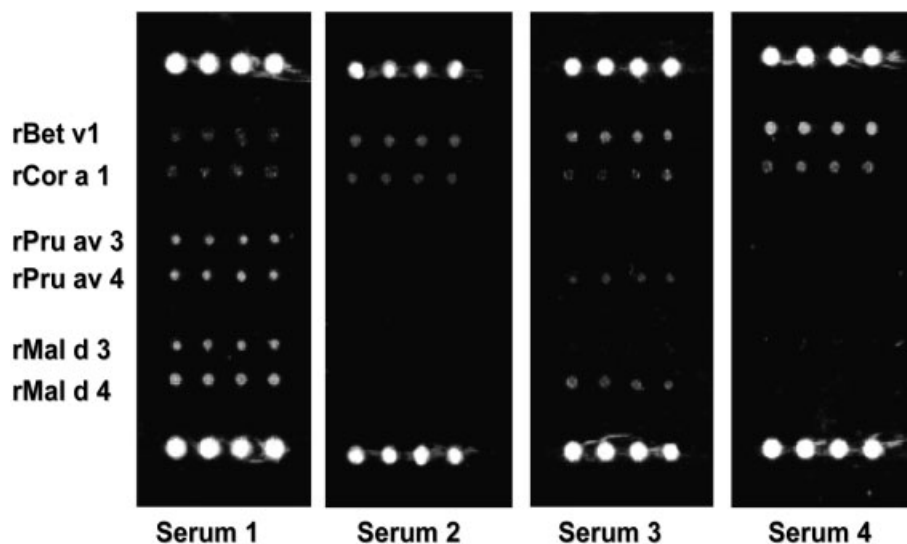


Figure 4. Fluorescence images (90% laser power and 90% PMT) of the detection of allergen specific IgE in serum samples 1–4 on MPS-poly[DMA-co-NAS] coated microarrays using the 25 min automated microfluidic protocol described in Section 2.5.

chemistry optimal for the specific user's application, can be purchased (or in house produced) and arrayed by conventional spotting facilities for microarray production, even in large quantities. The microcell is rather made of PDMS, a very popular material in microfluidics for its ease of manufacture. The PDMS microchip is produced by soft lithography (which is a cheap technique and does not require the facilities for glass and silicon microfabrication) and can be adapted to the user requirements allowing the flexibility needed to optimize microcell volume and geometry and, if necessary, the multiplexing of inlets and incubation chambers.

4 Conclusion

In this work, a microarray for allergy diagnosis coupled to microfluidics has been reported. The signal intensity of allergen specific IgEs in serum has been improved by the use of a novel MPS-poly[DMA-co-NAS] brush-like coating for microarrays able to immobilize allergens in their native conformation and by the use of dynamic conditions during the incubation step. Microfluidics allowed us to obtain an efficient mixing of the serum samples on the surface of the array thus turning the binding of serum Igs with immobilized allergens from a diffusion limited towards a kinetic reaction. In this way, the binding of high affinity antibodies (IgE) is enhanced and the binding of low affinity, highly concentrated antibodies (IgG) is impaired.

Through the use of a software controlled fluidic processor, a complete microarray assay, without any manual intervention can be performed in just 25 min.

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The authors have declared no conflict of interest.

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PROTEOMICS

Supporting Information for Proteomics

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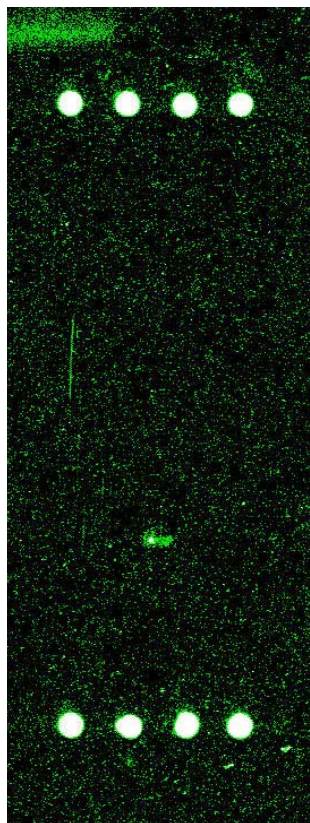
Marina Cretich, Gabriele Di Carlo, Cinzia Giudici, Sven Pokoj, Iris Lauer,
Stephan Scheurer and Marcella Chiari

**Detection of allergen specific immunoglobulin
by microarrays coupled to microfluidics**

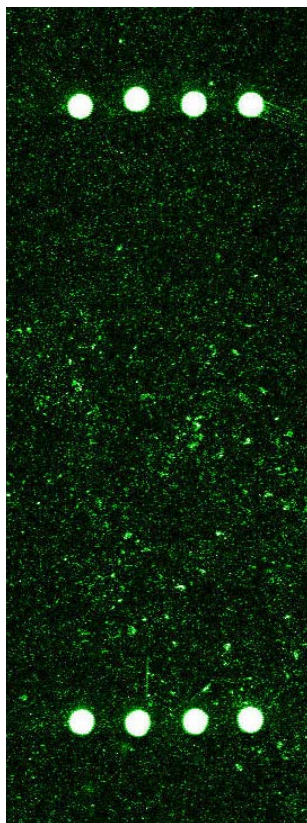
Figure S1: fluorescence images (90% laser power and 90% PMT) of the detection of allergen specific IgE in the negative control samples (NC 1-4) on MPS-poly[DMA *-co-* NAS] coated microarrays using the 25 minutes automated microfluidic protocol described in section 2.5. No fluorescence signals (except for the fluorescent positive controls) were detected.

Figure S2: fluorescence images (90% laser power and 90% PMT for IgE detection and 50% laser power and 50% PMT for IgG detection) of allergen specific IgE and IgG in serum sample 3 on MPS-poly[DMA *-co-* NAS] coated microarrays using the optimized static conventional incubation described in section 2.5. Panel **A** shows the fluorescence signals obtained analyzing untreated serum (dilution 1:2), panel **B** shows the results of the same analysis performed on the serum subjected to IgG depletion, panel **C** reports the results of the analysis performed on untreated serum subjected to dilution 1:5 as in the immunodepletion procedure.

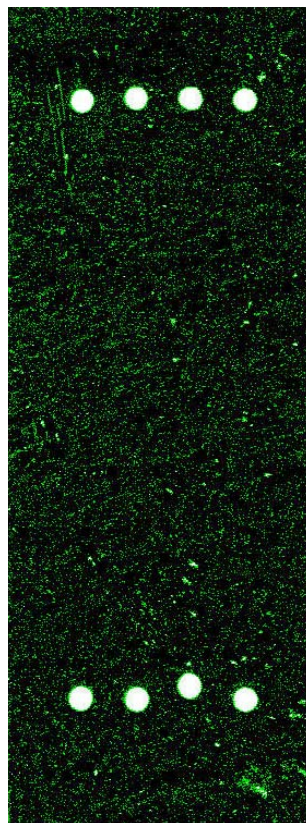
The IgG immunodepletion is efficient in subtracting the total amount of IgG in the serum as shown by the reduced fluorescence background and spot intensity in panel **B**. Unfortunately, the dilution in the specific buffer, necessary to perform this procedure, is unsuited when detecting low concentration analytes as the IgEs. Therefore, the IgE fluorescence signals visible in panel **A** (untreated serum diluted 1:2) are no more visible neither in panel **B** nor in panel **C** due to the sample dilution. As a consequence, the IgG depletion with the used commercial kit is not a workable strategy to demonstrate an enhanced IgE signal intensity.



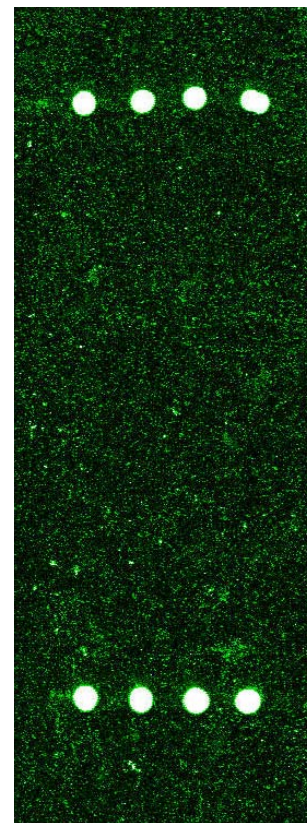
NC1



NC2



NC3



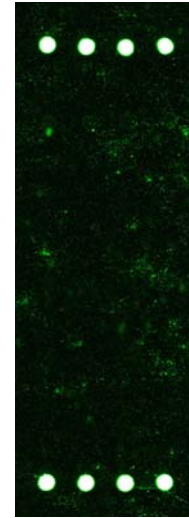
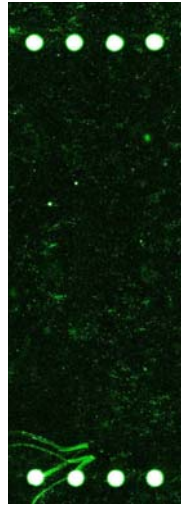
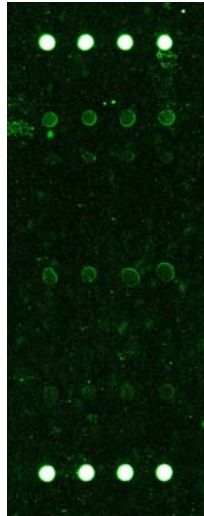
NC4

**A: Serum
1:2 dilution**

**B: Serum after
immunodepletion
(1:5 dilution)**

**C: Serum
1:5 dilution**

IgE detection



IgG detection

