

IgE and IgG4 Epitope Mapping of Food Allergens with a Peptide Microarray Immunoassay

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Abstract

Peptide microarrays are a powerful tool to identify linear epitopes of food allergens in a high-throughput manner. The main advantages of the microarray-based immunoassay are the possibility to assay thousands of targets simultaneously, the requirement of a low volume of serum, the more robust statistical analysis, and the possibility to test simultaneously several immunoglobulin subclasses. Among them, the last one has a special interest in the field of food allergy, because the development of tolerance to food allergens has been associated with a decrease in IgE and an increase in IgG4 levels against linear epitopes. However, the main limitation to the clinical use of microarray is the automated analysis of the data. Recent studies mapping the linear epitopes of food allergens with peptide microarray immunoassays have identified peptide biomarkers that can be used for early diagnosis of food allergies and to predict their severity or the self-development of tolerance. Using this approach, we have worked on epitope mapping of the two most important food allergens in the Spanish population, cow's milk and chicken eggs. The final aim of these studies is to define subsets of peptides that could be used as biomarkers to improve the diagnosis and prognosis of food allergies. This chapter describes the protocol to produce microarrays using a library of overlapping peptides corresponding to the primary sequences of food allergens and data acquisition and analysis of IgE- and IgG4-binding epitopes.

Key words Peptide microarray, Epitope mapping, Food allergy, Linear epitope, Biomarkers

1 Introduction

Food allergy has become an important health problem in industrialized countries in recent decades. It is generally accepted that food allergy affects 1–2 % of individuals in these populations and 6–8 % of children under 3 years of age [1, 2]. The most common food allergies in children are cow's milk, hen's eggs, peanuts, and fish.

The diagnosis of food allergies is based on a detailed medical history (family history, diet, symptoms), the presence of specific immunoglobulin E (IgE) antibodies directed against proteins in the offending food using a skin prick test or radioallergosorbent test (RAST), and a double-blind placebo-controlled food challenge test (DBPCFC), which demonstrates the relationship

between symptoms and the intake of the offending food. The DBPCFC is the gold standard test for confirming a diagnosis. Once the food allergy diagnosis is established, the management of the patient includes the prescription of an offending-food-free diet and regular follow-up visits to reevaluate the patient's allergic status [2]. Patients and their families must be aware that all forms of the offending food must be avoided at all meals, every day, and must be trained to administer emergency treatment in cases of accidental ingestion, including antihistamines, corticosteroids, and autoinjectable adrenaline for anaphylaxis. In most cases, IgE-mediated cow milk and hen egg allergies are transient and young children outgrow these allergies by 3–5 years of age [3–6]. However, children with peanut or fish allergy do not as readily outgrow their allergies [1].

Although the only current treatment option for persistent food allergy is strict avoidance of the allergen, promising immunotherapeutic treatments have been developed in recent years [7]. Among these, oral immunotherapy (OIT) has been shown to be highly effective in desensitizing individuals to cow's milk, eggs, and peanuts [8–11], although the treatment is not risk-free [9, 12]. OIT involves the administration of gradually increasing quantities of the offending food, starting with very low doses and gradually increasing up to an amount equivalent to the usual daily intake.

IgE plays a central role in the development of immunotolerance, and food-specific IgE antibodies can be used as a marker of persistent allergy and the development of tolerance. High food-specific IgE levels predict clinical reactivity and the persistence of allergy [3, 13], whereas decreasing food-specific IgE levels over time predict developing tolerance [14]. Other specific antibody classes, such as IgG4, have also been associated with the development of tolerance, and high food-specific IgG4 levels during infancy are associated with the earlier development and maintenance of tolerance [13, 15, 16].

Food allergen epitopes are usually classified as either linear or conformational epitopes, based on the recognition of its primary structure (linear sequence of amino acids) or the three-dimensional tertiary structure of the proteins, respectively [17]. In recent years, different food allergens have been identified and the importance of sequential epitopes in food allergies has been established. Sensitization to linear epitopes is much more common in individuals with persistent allergies than in those who have overcome milk [18–23], peanut [24–26], or egg allergy [27, 28]. Linear epitopes are also associated with more-severe reactions to milk [22, 23], peanut [24, 25], lentils [29], fish [30, 31], shrimp [32], and wheat [33]. The development of tolerance to milk is associated with reduced IgE levels and increased IgG4 levels against linear epitopes [22, 34].

Different methods have been used to map the linear epitopes of food allergens (enzyme-linked immunosorbent assay [ELISA], immunoblotting, immunodot blotting, RAST, SPOT® membrane), but peptide microarrays have recently become a powerful tool for identifying linear epitopes in a high-throughput manner. Microarray-based immunoassays allow thousands of peptides to be assayed simultaneously and several immunoglobulin subclasses to be tested simultaneously and require only small volumes of patient samples. However, a major limitation to the clinical use of microarray is the automated analysis of the data [35].

Recent studies mapping the linear epitopes of food allergens with peptide microarray immunoassays have identified peptide biomarkers for the diagnosis and prognosis of food allergies. Savilahti et al. developed a panel of 15 peptides that bind IgE and IgG4 to distinguish at the time of diagnosis those patients with persistent cow's milk allergy from those who will recover early [22]. Lin et al. proposed a decision tree built to classify peanut-allergic and peanut-tolerant individuals based on three peptides [26]. Perez-Gordo et al. identified a 15-amino-acid antigenic region in the Gad m 1 allergen from the Atlantic cod for use as a biomarker of the severity of fish allergy [31]. More recently, Savilahti et al. and de la Hoz et al. proposed two possible approaches to predicting the outcome of OIT for cow's milk allergy with a peptide microarray immunoassay [36, 37].

For the last decade, using the advantages of the microarray technology, we have been involved in epitope mapping the two most important food allergens in the Spanish population, cow's milk and chicken eggs. In this chapter, we describe the methods used for microarray fabrication, sample hybridization, and data analysis.

2 Materials

2.1 Microarray Printing

1. SpotArray 72 spotter (PerkinElmer, Waltham, MA, USA) with SMP6 split pins (Arrayit Corporation, Sunnyvale, CA, USA).
2. Dendron-modified slides, functionalized with *N*-hydroxyl succinimidyl (NSB27 NHS) (NanoSurface Biosciences POSTECH, Seoul, Korea) (*see Note 1*).
3. A library of overlapping peptides corresponding to the primary sequences of the food allergen of interest is synthesized commercially, with a purity >70 %, and analyzed with high-performance liquid chromatography and mass spectrometry (GenScript Corporation, Piscataway, NJ, USA) (*see Note 2*). The custom-made peptides are usually supplied as lyophilized powder, resuspended in phosphate-buffered saline (PBS; bio-Mérieux, Marcy l'Etoile, France) as stock concentrations of 20 mg/mL, and stored at -80 °C until use.

4. Anti-CREB-1 antibody blocking peptide (Santa Cruz Biotechnology, Dallas, TX, USA).
5. Ovomucoid and bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA).
6. Protein printing buffer (PPB; Arrayit Corporation).
7. V-bottomed 384-well plates (Greiner Bio-One, Monroe, NC, USA).
8. Aluminum seal tape (Nalge Nunc International, Rochester, NY, USA).

2.2 Microarray Hybridization

1. microBOX™ incubation chamber for glass slides (Quantifoil Instruments GmbH, Jena, Germany) with a slide holder for four glass slides.
2. MixMate orbital shaker (Eppendorf AG, Hamburg, Germany).
3. Hydrophobic Super Pap Pen (Daido Sangyo Co. Ltd., Tokyo, Japan).
4. PBS containing 0.1 % Tween 20 (PBS-T).
5. BSA (Sigma-Aldrich).
6. BlockIt™ blocking buffer (Arrayit Corporation).
7. Secondary antibodies: monoclonal mouse antihuman IgE antibody (clone G7-26) and monoclonal mouse IgG4 antibody (clone G17-4; BD-PharMingen, San Diego, CA, USA).
8. Spike-in control antibody: Alexa Fluor® 647-conjugated anti-CREB-1 rabbit polyclonal antibody (240; Santa Cruz Biotechnology).
9. Alexa Fluor® 546 Monoclonal Antibody Labeling Kit (Invitrogen, Molecular Probes, Eugene, OR, USA).
10. Alexa Fluor® 647 Monoclonal Antibody Labeling Kit (Invitrogen, Molecular Probes).
11. Centricon-30 concentrator (Amicon, Beverly, MA, USA).
12. Centrifuge with a JA14 rotor (Beckman Coulter, Inc., Fullerton, CA).

2.3 Data Acquisition and Analysis

1. ScanArray® Express microarray scanner (PerkinElmer) with two lasers (543 and 633 nm) and the associated software.
2. Arrayit microarray air jet (Arrayit Corporation).
3. Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and TIGR MultiExperiment Viewer (MeV v3.1) (<http://www.tm4.org/>) software.

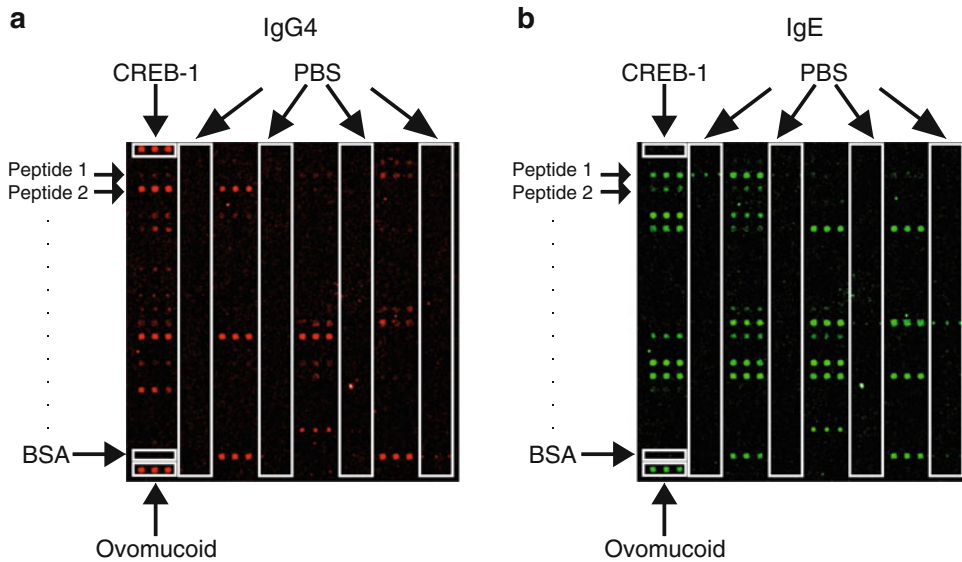


Fig. 1 Example of a peptide microarray. The serum of an egg-allergic patient was incubated with a microarray containing a set of overlapping 20-amino-acid peptides corresponding to the primary sequence of ovomucoid and labeled with Alexa Fluor® 647-conjugated antihuman IgG4 and Alexa Fluor®-546-conjugated antihuman IgE secondary antibodies. CREB-1 blocking peptide was printed as the spike-in control. Ovomucoid and BSA were printed as the positive and negative controls, respectively. PBS was printed to minimized any cross-contamination and for background normalization. Each feature (peptide, proteins, or PBS) was printed in triplicate. The microarray was scanned with a red laser to detect Alexa Fluor® 647 (a) and with a green laser to detect Alexa Fluor® 546 (b)

3 Methods

3.1 Peptide Microarray Printing

1. To prepare the printing plate, stock solutions of peptides (20 mg/mL) are diluted 1:10 with PBS and transferred (10 μ L/well) to V-bottomed 384-well plates, according to the printing protocol (Fig. 1). As the spike-in control, a blocking peptide for the anti-CREB-1 antibody is printed at the beginning of the microarray (100 μ g of peptide in 0.5 mL of PBS containing <0.1 % sodium azide and 0.2 % BSA). Ovomucoid and BSA are also printed as the positive and negative controls, respectively. One well containing 10 μ L of PBS is included between each peptide or protein to minimize cross-contamination and for background normalization (PBS spots). Once the plate is complete, 10 μ L of protein printing buffer (Arrayit Corporation) is added to each well. The final concentration of each peptide is 1 mg/mL. The printing plates are sealed with aluminum seal tape and stored at -80°C .

2. For printing, a SpotArray 72 spotter is used with SMP6 split pins. Each feature (peptide, protein, or PBS) is usually printed in triplicate. The humidity for printing is adjusted to 60 %. To minimize the cost of the slides and to increase the number of samples that can be processed simultaneously, two microarrays are printed on each slide. After the printing procedure is complete, the microarrays are left overnight in the deck of the printer, and the printer kept closed, to allow the slow equilibration of the humidity with the ambient conditions and the spots to dry slowly. The slides produced are labeled with barcode tags, stored in a box, and sealed in a plastic bag with a desiccant bag (silica gel packet).

3.2 Microarray Hybridization

3.2.1 Antibody Labeling

1. Antihuman IgE secondary antibody is labeled with the Alexa Fluor® 546 Monoclonal Antibody Labeling Kit and antihuman IgG4 secondary antibody is labeled with the Alexa Fluor® 647 Monoclonal Antibody Labeling Kit. Both antibodies are supplied at concentrations of 0.5 mg/mL in 1 mL of aqueous buffered solution containing ≤ 0.09 % sodium azide. The optimal concentration for labeling with the monoclonal antibody labeling kits is 1 mg/mL and these kits are optimized to label 100 μ g of antibody per reaction. The presence of low concentrations of sodium azide (≤ 3 mM) does not interfere with the conjugation reaction. Therefore, in the first step, the secondary antibodies are concentrated about twofold with a Centricon-30 concentrator, with centrifugation in a JA14 rotor for 15 min at 4 °C.
2. The antibody solution is recovered and 90 μ L of antibody solution containing 10 μ L of 1 M sodium bicarbonate buffer is transferred to a vial of reactive dye. The vial is capped and gently inverted several times to fully dissolve the dye.
3. The solution is incubated for 1 h at room temperature, with gentle inversion every 10–15 min to mix the two reactants and increase the labeling efficiency.
4. An aliquot (100 μ L) of the reaction volume is loaded dropwise into the center of the resin at the top of the spin column and centrifuged for 5 min at $1100 \times g$.
5. The labeled antibody is eluted in approximately 100 μ L of PBS (pH 7.2) containing 2 mM sodium azide and stored at 4 °C, protected from light.

3.2.2 Blocking

1. A round area that includes the printed microarray is drawn using the hydrophobic Super Pap Pen (*see Note 3*). The slides are placed in a microBOX™ incubation chamber.
2. To establish constant humidity in the microBOX™ incubation chamber, gauze moistened with deionized water is placed in a basin within the chamber.

3. The slides are loaded into the slide holder and a 1:1 solution of BlockIt™ blocking buffer and PBS-T containing 2 % BSA is placed onto each microarray to block nonspecific binding.
4. The slide holder is loaded into the microBOX™, which is placed on a MixMate orbital shaker (Eppendorf AG) for gentle agitation.
5. The microarrays are incubated for 1 h at room temperature.
6. The blocking solution is removed by aspiration and the samples washed twice with PBS. Using one microBOX™ with two slide holders and four glass slides, 16 samples can be processed simultaneously.

3.2.3 Serum Incubation

1. The patient's serum (50 µL), diluted 1:1 with PBS-T containing 2 % BSA, is placed onto each microarray and then into the microBOX™.
2. The microarrays are incubated overnight on an orbital shaker at 4 °C with gentle agitation.
3. The slides are removed from the microBOX™ and placed into 50 mL tubes (two slides per tube) for washing. The slides must be placed with the nonprinted faces in contact and the printed areas facing outward.
4. The microarrays are washed twice (3 min each) with 40 mL of PBS-T at room temperature on an orbital shaker with gentle agitation.

3.2.4 IgE and IgG4 Detection

1. The microarrays are loaded into the microBOX™ and incubated for 2 h at room temperature with a mixture of antibodies diluted in PBS-T containing 2 % BSA: Alexa Fluor® 647-conjugated anti-CREB-1 rabbit polyclonal antibody, diluted 1:500, antihuman IgE antibody covalently tagged with Alexa® 546 and diluted 1:1000, and antihuman IgG4 antibody covalently tagged with Alexa® 647 and diluted 1:1000 (*see Note 4*).
2. The slides are placed into a 50 mL tube and washed twice (3 min each) with 40 mL of PBS-T at room temperature on an orbital shaker with gentle agitation.

3.3 Data Acquisition and Analysis

3.3.1 Microarray Scanning

1. Before scanning, the slides are washed quickly with deionized water and dried under a particle-free air stream produced with the Arrayit microarray air jet.
2. The slides are scanned with ScanArray Express. The Alexa® 546 signal is scanned with a green laser (543 nm) and the Alexa® 647 signal with a red laser (633 nm). The scanner produces green Alexa® 546 and red Alexa® 647 16-bit TIFF image files.

3.3.2 Gridding, Spot Finding, and Quantitation

1. The Alexa® 546 and Alexa® 647 Tiff images are loaded into the ScanArray Express software.
2. The grid information from the gal file generated by the SpotArray 72 spotter software is loaded, and the positive controls at the beginning and end of the microarray (CREB-1 and ovomucoid, respectively) are used for grid alignment (Fig. 1).
3. The adaptive circle algorithm is used for spot finding and quantitation.
4. Before the quantitative data are saved, visual inspection of the spots is required to confirm that all the spots are correctly detected and have the appropriate shape and size.
5. PBS spots with carry-over contamination are removed.
6. After visual inspection, any unreliable spots are flagged and removed from further analysis.
7. The quantitation results are saved in the comma-separated value (CSV) file format.

3.3.3 Data Analysis and Visualization

1. The microarray analysis is performed according to the method of Lin et al. [38, 39]. This method is based on the calculation of a Z-score, which is a measure of the standardized fluorescence intensity for IgE and IgG4 recognition. The Z-score is calculated for each peptide spot (Z_i) according to the formula, $Z_i = [S_i - S_{\text{PBS}}] / \text{MAD}(S_{\text{PBS}})$, where S for each peptide spot (S_i) or PBS (S_{PBS}) is the median fluorescent signal of the spot divided by the local background and log2 transformed. $\text{MAD}(S_{\text{PBS}})$ is the median absolute deviation of all the readouts for PBS spots. The total Z-value for each peptide is the median of the Z-scores for the three replicate spots within the same microarray. Finally, to accommodate overlapping peptides, the weighted average of Z-values is calculated according to the formula: $Z = (0.25 \times Z_{-1}) + (0.5 \times Z_0) + (0.25 \times Z_{+1})$. An individual peptide sample is considered positive if the standardized fluorescence intensity (represented as the weighted average Z-score) exceeds 3. Major specific IgE (sIgE) epitopes are identified based on the IgE/IgG4 Z-score ratio, i.e., when the ratio is >1 in more than 20 % of the patient sera. The Z-score calculation for each data set is performed in Microsoft Excel software using *pivot tables* and links to the original csv data files.
2. Next, a tab-delimited text file containing the weighted average Z-score for each data set (IgE, IgG4, or IgE/IgG4 ratio) is generated in Microsoft Excel. The weighted average Z-scores for each peptide are in rows and the samples in columns. Peptide annotation labels are displayed in the row headers and sample annotation labels are displayed in the column headers (Fig. 2).

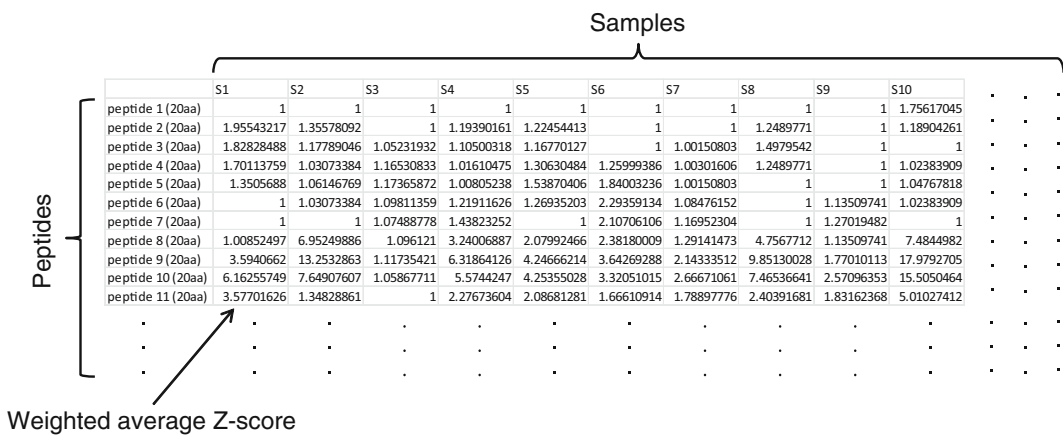


Fig. 2 Example of a tab-delimited text file containing the weighted average Z-score for each data set used for visualization and clustering analysis with the MeV v3.1 software

3. Data visualization and clustering are performed with the TIGR MultiExperiment Viewer (MeV v3.1) software (<http://www.tm4.org/>). MeV is an open-source software tool used to analyze and visualize processed data from microarray experiments. Although MeV has been developed for gene expression microarrays, many of its features are useful in epitope-mapping peptide microarray analyses (Fig. 3). The display color scheme can be adjusted from a single gradient to a double gradient. A single gradient is used to represent IgE recognition (black to green gradient) or IgG4 recognition (black to red gradient). A double gradient (red–black–green) is used to represent the IgE/IgG4 recognition ratio. Increasing green intensity denotes predominant IgE recognition, increasing red intensity denotes predominant IgG4 recognition, and black represents unrecognized spots or spots that do not differ in their IgE/IgG4 recognition. To visualize the IgE/IgG4 Z-score ratio, the data are usually log2 transformed. Log2 (ratio) measures allow data representation, stabilize the variance, compress the range of the data, and increase the normal distribution of the data, which allow the data to be analyzed statistically. The MeV software includes different methods of clustering. A hierarchical clustering analysis can be used to identify differential recognition patterns between allergic patients. It can also be used in several statistical analyses, such as the *t*-test or one-way ANOVA, to identify peptides that are differentially recognized in groups of patients. More complex analyses can be performed using the R programming language, including linear and nonlinear modeling, time-series analysis, classification, clustering, data mining, machine learning analysis, etc. (<http://www.r-project.org>).

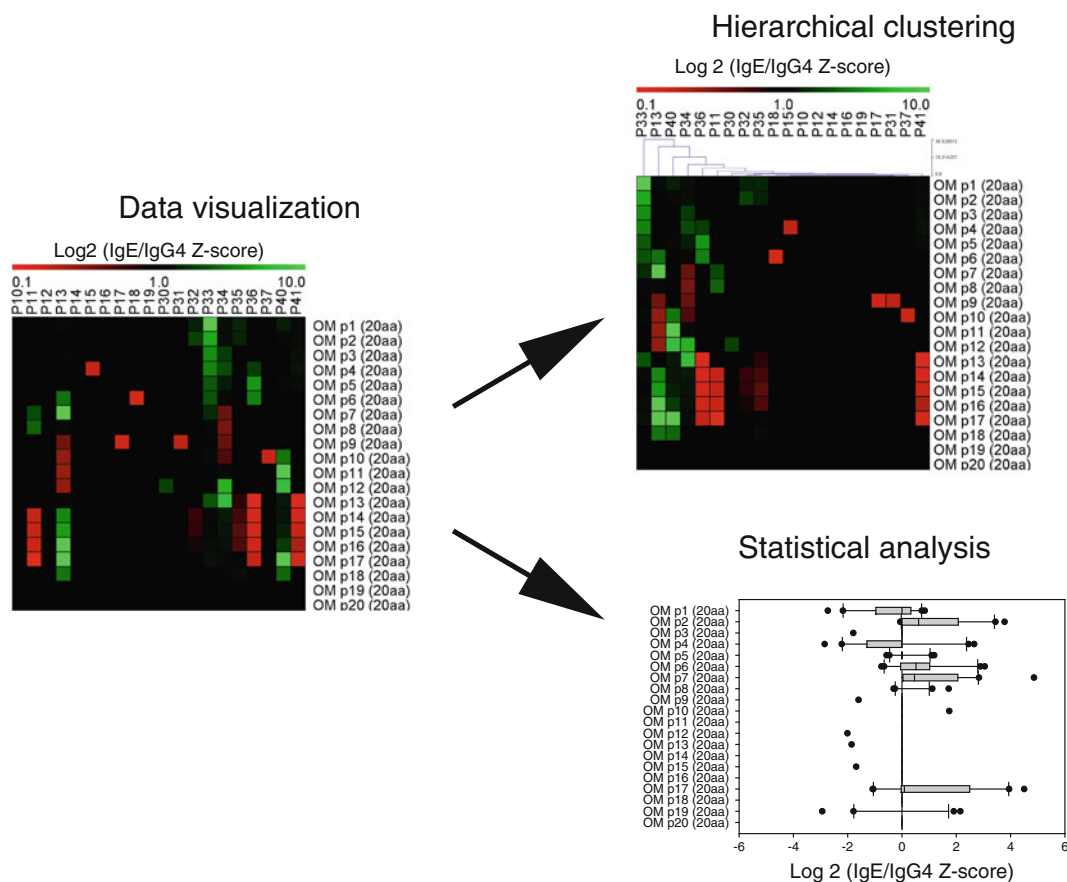


Fig. 3 Visualization and data analysis using the MeV v3.1 software

4 Notes

1. We have tested dendron-modified slide functionalized with *N*-hydroxyl succinimidyl (NSB27 NHS; NanoSurface Biosciences POSTECH) and several slides coated with epoxide groups, and in our experience, the dendron-modified slide gives the best results (Fig. 4). NSB27 NHS slides are coated uniformly with a cone-shaped organic compound, called nanocone, functionalized with *N*-hydroxyl succinimidyl. Coating the slides with nanocone ensures controlled regular spacing between the proteins or peptides, minimizes the steric hindrance, and enhances the binding kinetics [40]. When the slide surface is coated with this monodispersed molecule, branches of the nanocone form stable chemical bonds with the surface of the substrate, and the functional group at the cone's apex is utilized for the immobilization of a bioactive molecule (Fig. 5). The average spacing on NSB27 NHS slides is 6–7 nm, which is

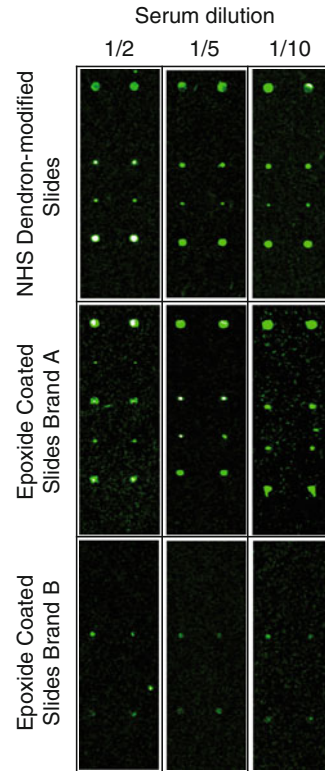


Fig. 4 Performance test for different microarray slides. Different dilutions of serum from an egg-allergic patient were incubated with three different types of slides and labeled with Alexa Fluor® 546-conjugated antihuman IgE secondary antibody

recommended for protein microarrays. NHS-microarray slides contain a highly reactive NHS ester surface, which forms avid covalent bonds with primary amine groups. The NHS slides offer a high loading capacity and extremely low background for the immobilization of proteins (antibodies and antigens).

2. Several peptide lengths have been reported in the literature and the signal intensity generally increases for both IgE and IgG4 as the peptide length increases [41]. In our experience, the best performance is achieved with 20-amino-acid peptides [27]. To design a library of overlapping peptides, it is important to exclude the sequences corresponding to the signal peptide because is not present in the mature protein and is therefore not antigenic.
3. Good performance is also achieved with LifterSlips™ Cover Slips (24x24l-2-4709; Erie Scientific Company, Portsmouth, NH, USA) or CoverWell perfusion chambers (PC1R-0.5; Grace Bio-Labs, Bend, OR, USA).

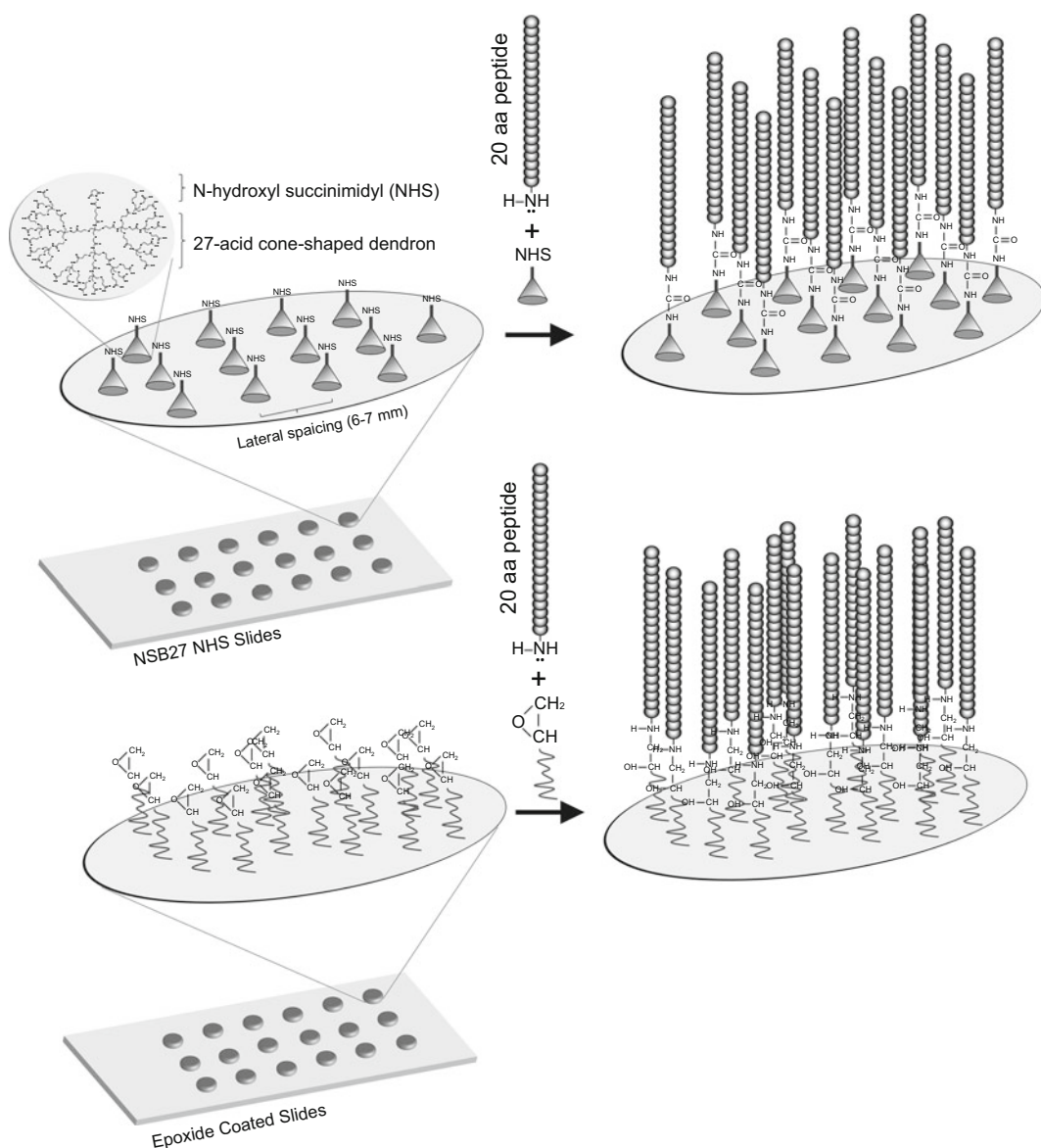


Fig. 5 Schematic illustration of dendron-modified slides functionalized with *N*-hydroxyl succinimidyl (NSB27 NHS slide) and epoxy-coated slides. The chemical structure of the 27-acid cone-shaped dendron is shown. After immobilization and deprotection, the NH₂ group at the cone apex is activated and generates NHS groups, which form avid covalent bonds with the primary amine groups of peptides. The highly reactive epoxide groups on the epoxide-coated slides react specifically with the primary amines of peptides, resulting in covalent bonds

4. The working concentration of each batch of secondary antibody may vary, depending on the labeling efficiency, and must be tested after each labeling reaction using serial dilutions. To improve the sensitivity of IgE recognition, a biotin mouse anti-human IgE (clone G7-26; BD-PharMingen) can be used in combination with CyTM3-streptavidin (PA43001; Amersham GE Healthcare, Little Chalfont, UK).

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