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A new time-resolved fluorometric microarray detection system using core–shell-type fluorescent nanosphere and its application to allergen microarray

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Abstract We have developed a new time-resolved fluorometric (TRF) microarray detection system consisting of fluorescent NH_2 nanosphere, TRF microarray detector and gamma-irradiated polystyrene chip. Using the TRF microarray detector, we detected 500 particles of the fluorescent nanosphere in one channel. Cross-talk fluorescence from the adjacent channels was little observed in the TRF microarray detector ($<0.0004\%$). The TRF microarray detection system was further applied for serum allergen-specific immunoglobulin E (IgE) multi-analyses. As a labeled tag antibody, an anti-human IgE Fab' fragment-conjugated fluorescent nanosphere (Fab' nanosphere) was prepared as described previously. As a chip surface appropriate for allergen immobilization, the polystyrene chip surface was modified by gamma irradiation. The immunoassay reactivity using the gamma-irradiated polystyrene chip was approximately 2.5-times improved compared with that of the non-treated polystyrene chip. Non-specific adsorption of the Fab' nanosphere onto the gamma-irradiated polystyrene chip surface was very low level ($<0.0009\%$). In only 20 μl of serum, six allergen-specific IgEs could be simultaneously determined in one reaction well in fewer than 90 min. Good correlation curves were obtained between the microarray immunoassay and the

CAP RAST fluoro-enzyme immunoassay (CAP/RAST FEIA) method ($r>0.961$). Reproducibility (CVs) of the microarray immunoassay was 8.6 % to 19.0 % ($n=5$).

Keywords Fluorescent nanosphere · Microarray · Time-resolved fluorescence · Allergy-specific IgE

Introduction

A protein chip based on the established DNA microarray technology [1, 2] was launched in 1999 [3], using covalent linking of protein ligands to the activated silica surface of microscope slides [4]. The concept of using microarrays as a diagnostic test system has been applied for testing serum samples to multiple antigen probes in order to reveal antibody binding, e.g., to autoantigens [3, 5–7]. Recently, the detection of allergen-specific immunoglobulin E (IgE), which is several magnitudes lower in abundance in serum ($\sim 1\text{ ng/ml}$), has been reported to be feasible by microarrays [8–12]. Generally, the microarray-based IgE test accelerates immune diagnosis significantly by analyzing in parallel all relevant diagnostic parameters of interest. The amount of information that can be obtained from these protein arrays is significantly increased, while the sample quantity is relatively low [13]. Especially, a reduced serum volume is of relevance, e.g., in allergy diagnosis in infants and young children. Therefore, a highly sensitive microarray detection system for serum allergy-specific IgE multi-analyses is needed for allergy diagnosis in infants and young children. Recently, allergen microarrays for multi-analyses of serum allergen-specific IgEs, using fluorescent labels [10, 13] and a chemiluminescent label [14], have been reported.

In a previous paper [15], we reported on the development of a highly sensitive, solid-phase, time-resolved fluorometric (TRF) immunoassay using a core–shell-type fluorescent nanosphere incorporating a large number of chelated Eu^{3+} ions into the core of the nanosphere (fluorescent NH_2 nanosphere). The fluorescent NH_2 nanosphere had strong fluorescence, since the 6,300 chelated

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Eu^{3+} ions were incorporated into a single nanosphere. The detection limit of the fluorescent NH_2 nanosphere was 2,000 particles in 50 μl of sample volume, using a conventional TRF detector. The fluorescent NH_2 nanosphere enables one to perform a highly sensitive immunoassay by using it as a labeling reagent. Onto the fluorescent NH_2 nanosphere, Fab' fragments of antibody could be covalently conjugated after converting NH_2 groups to maleimide groups using a hetero-cross-linking agent, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). The maleimide group-introduced fluorescent nanosphere (maleimide nanosphere) was collected from a gel permeation chromatography column to remove the excess SMCC.

Recently, a two-dimensional TRF imaging detector, Signifer time-resolved fluorescence microimager (Perkin-Elmer Life Sciences, Wellesley, Mass., USA) has been reported [16, 17] and used for a model microarray experiment using biotinylated antibody spots as targets [18]. The detection limit of the Signifer microimager was almost equal to that of a standard time-resolved plate fluorometer. Luo and Diamandis have also reported on time-resolved fluorometry for protein microarray application [19].

In this paper we report on our newly developed TRF microarray detection system, consisting of the fluorescent NH_2 nanosphere, the TRF microarray detector and the gamma-irradiated polystyrene chip. The TRF microarray detection system is highly sensitive. Therefore, it is applied to multi-analyses of serum allergen-specific IgEs to milk, egg white, rice, soybean, peanut and wheat in food allergy diagnosis in infants and young children. Construction of the TRF microarray detector for the multiple TRF detections, a modification method of the polystyrene chip surface suitable for immobilization of allergen extracts, and a preparation method for the anti-human IgE Fab' fragment-conjugated fluorescent nanosphere (Fab' nanosphere) are described in detail. The TRF microarray detection system is further applied to serum allergen-specific IgE microarray immunoassay. In only 20 μl of serum, six foods allergen-specific IgEs are simultaneously determined in one reaction well with the TRF microarray detection system, and the results are compared with that of the Pharmacia CAP/RAST FEIA system.

Experimental methods

Nanosphere

The fluorescent NH_2 nanosphere was applied to the analyses of serum allergen-specific IgEs. The Fab' nanosphere was prepared as well as the anti-human AFP Fab'-conjugated fluorescent nanosphere [15, 20]. Briefly, the primary amino groups on the core-shell-type nanosphere surface, incorporating fluorescent Eu^{3+} chelates into the core of the nanosphere (fluorescent NH_2 nanosphere), were converted into maleimide groups by a hetero-cross-linking

agent, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). The maleimide group-introduced fluorescent nanosphere (maleimide nanosphere) was collected from a gel permeation chromatography column to remove the excess SMCC.

Fab' nanosphere

The Fab' fragment of anti-human IgE antibody was prepared approximately as described previously [21]. To 3.3 mg of anti-human IgE monoclonal antibody (IgG_1 , κ , Mitsubishi Kagaku Iatron, Tokyo, Japan) dissolved in 1 ml of 0.15 M NaCl was added 0.6 ml of 0.5 M citrate buffer of pH 3.5. To the antibody solution was added 5.08 μl of pepsin (5 mg/ml, Roche Diagnostics, Basle, Switzerland) solution prepared with 0.1 M citrate buffer of pH 3.5. After incubation at 37 °C for 1 h, 0.35 ml of 2 M Tris solution was added to the reaction solution. The F(ab')_2 fraction of antibody was collected by a Sephacryl S-200 HR column (1.5 cm $\times$ 90 cm, Pharmacia), with 0.05 M acetate buffer of pH 5.0 as an eluent.

The Fab' fragments were prepared by reducing the F(ab')_2 of antibody as follows. To 3.25 mg of F(ab')_2 dissolved in 2 ml of 0.05 M acetate buffer of pH 5.0, 50 μl of 0.75 M 2-mercapto-ethylamine/water solution was added, and the solution was incubated at 37 °C for 1 h. The reaction mixture was loaded on a G3000SWXL column (21.5 mm $\times$ 300 mm, Tosoh, Tokyo, Japan) with 0.05 M phosphate buffer of pH 7.0 containing 1 mM EDTA and eluted with the same buffer solution, at a flow rate of 3 ml/min. The fractions of anti-human IgE Fab' were collected.

The anti-human IgE Fab' fragments prepared were covalently conjugated onto the maleimide nanosphere as described previously [15]. To 6.5 mg of the maleimide nanosphere suspension (7.3 ml) was added 1.13 mg of anti-human IgE Fab' fragment solution (4.2 ml), and the mixture was incubated at 4 °C for 16 h in the dark. To the reaction mixture, 50 μl of 2-mercapto-ethylamine/water solution (50 mg/ml) was added and incubated at room temperature for 30 min in the dark to block the remaining maleimide groups on the nanosphere surface. After that, the nanosphere suspension was loaded into a Sepharose CL-6B column (1.5 cm $\times$ 90 cm, Pharmacia) with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.15 M NaCl, and eluted with the same buffer solution. Finally, the Fab' nanosphere fractions having strong fluorescence were collected and used for the solid-phase sandwich immunoassay of allergen-specific IgE. The Fab' nanosphere was suspended with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.2 % bovine serum albumin (BSA), 0.15 M NaCl and 0.05 % NaN_3 , and stored at 4 °C in the dark prior to use.

The excitation and emission maxima of the prepared Fab' nanosphere were 346 nm and 617 nm, respectively, which had a spectrum similar to that of the previously described nanosphere [15].

TRF microarray detector

Figure 1 shows a schematic optical block diagram of the TRF microarray detector. The detector parts, the electric circuits and the computer software for time-resolved fluorescence measurement were purchased from the Corona Electric Co., Ltd. (Ibaraki, Japan).

A combination of mechanical chopper, which served both at excitation and emission sides, and a pulsed N_2 gas laser were used for the time-resolved measurements. The mechanical chopper has independent holes for excitation and emission lights. When excitation light is passed through the hole at the excitation side of the mechanical chopper, the chopper at the emission side is closed. When emission light from the sample is detected, the chopper at the excitation side is closed. The combination of the pulsed N_2 gas laser and the mechanical chopper has advantages in that a high signal per noise ratio can be achieved because of the lower background fluorescence than the pulsed N_2 gas laser alone. The light source has high output power (photon flux), monochromaticity, good spatial definition, pulse widths of the order of nanoseconds, and excellent between-pulse stability. The gated system characteristics are as follows: laser emission wavelength 337.1 nm; laser pulse duration 4 ns; repetition rate 20 pulses/s; delay time 200 μ s; measurement time 400 μ s; recovery time after completion of measurement to next flash 52.9 ms; cycle time 54 ms; measurement time/channel 5.4 s (100 flashes). A sampling of the fluorescence signal emitted from the sample is carried out optically by the mechanical chopper. The rotation of the mechanical chopper is controlled by the circuit. The sampling gate to an optimal time position of the fluorescence decay signal can be moved. The fluorescence light passing through the mechanical chopper is detected by the PMT as a voltage signal. No filters or lenses were used between the mechanical chopper and the PMT, because the filters and lenses themselves have fluorescence (phosphorescence) and it leads to increased fluorescence background noise. Instead of lenses, aluminum toroidal mirrors were used in the optical system. After the mechanical chopper at the emission side, a UV band-cut

filter and an interference filter were inserted. The wavelength characteristics of the two filters are suitable for measuring Eu^{3+} chelate fluorescence. Nine channels on a reaction well of chip can be scanned by an X-Y chip stage. The operation and the data recording were performed by a personal computer, which was connected to the TRF microarray detector via an RS-232C cable.

Polystyrene chip

A polystyrene chip designed for the TRF microarray detection system is shown in Fig. 2. The polystyrene chip has a reaction well (12 mm $\times$ 12 mm $\times$ 4 mm) with a flat bottom for sample solutions and labeled reagents, which can be used as well as a microtitration well. The sample materials and the labeled reagents can be easily washed away by a conventional aspirator, since the well corner has a taper. The location of nine measuring channels and the channel numbers in the reaction well are shown in Fig. 2b.

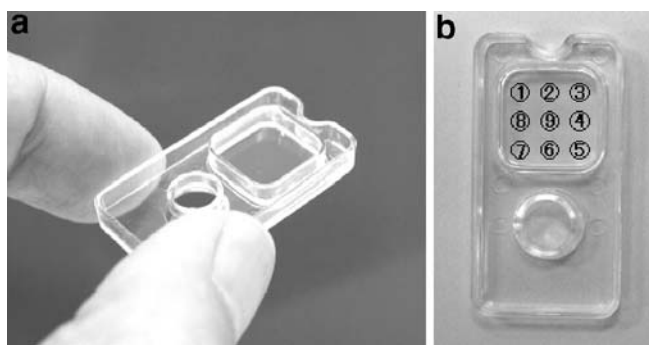
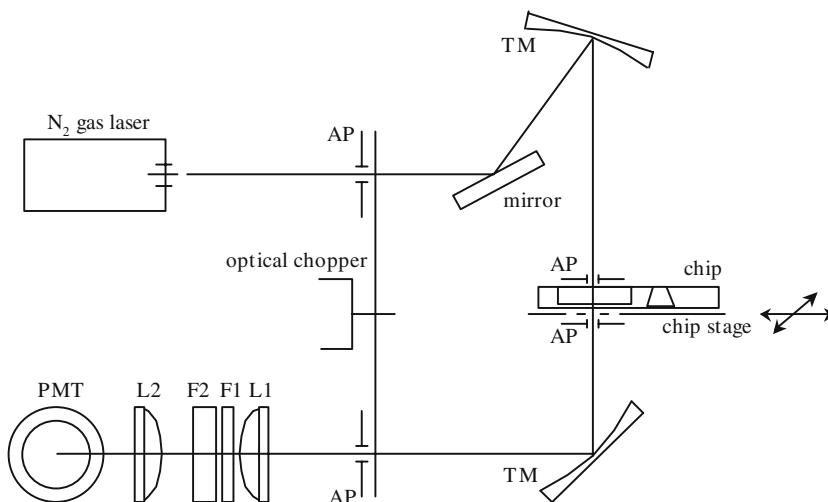


Fig. 2 **a** Polystyrene chip for TRF microarray detection system; **b** nine measuring channels in the reaction well. The time-resolved fluorescence intensities of nine channels can be measured with the TRF microarray detector

Fig. 1 Schematic optical block diagram of TRF microarray detector. *L1* and *L2* quartz lenses, *AP* aperture, *TM* toroidal mirror, *F1* UV band-cut filter, *F2* interference filter (610 nm), *PMT* photomultiplier tube



Gamma irradiation

Cobalt-60 (^{60}Co) gamma irradiation was carried out at Tokyo Metropolitan University RI Center (Hachioji, Japan). The polystyrene chip was irradiated with 6 kGy (600 krad), 15 kGy (1,500 krad), and 30 kGy (3,000 krad) at a dose rate of 2 kGy/h (200 krad/h). The gamma-irradiated polystyrene chips were stored at room temperature prior to use.

Allergen microarray

We prepared an allergen microarray by immobilizing six allergen extracts onto the respective measuring channel in the reaction well of gamma-irradiated polystyrene chip. Six allergen-specific IgEs to milk, egg white, rice, soybean, peanut and wheat were selected as markers of food allergy diagnosis in infants and young children.

Allergens Allergen extracts, including milk, egg white, rice, soybean, peanut and wheat, were obtained from Greer Laboratories, Inc. (Lenoir, N.C., USA). The allergen extracts were dissolved in 0.01 M phosphate buffer of pH 7.0, containing 0.15 M NaCl, and used for the allergen-immobilized microarray and microtitration well preparations.

Preparation of allergen microarray The allergen extracts (1 μl each) were spotted onto the respective channel of the reaction well using a PixSys 4200 (Cartesian Technologies, Inc., Irvine, Calif., USA). After incubation at 37 °C for 1 h, the allergen extracts were removed by aspiration. The reaction wells were each rinsed four times with 400 μl of 0.01 M phosphate buffer of pH 7.4, containing 0.15 M NaCl. After that, 100 μl of 0.01 M phosphate buffer of pH 7.4, containing 0.15 M NaCl and 0.25 % of Lipidure BL-B03 (NOF Co., Tokyo, Japan), was placed into the reaction wells. The above solution containing Lipidure BL-B03 was used as a blocking solution. The allergen microarray was sealed with the blocking solution and stored at 4 °C prior to use.

Immunoassay

Samples Panel sera of presumptive allergic patients (Plasma Lab International, Everett, Wash., USA) were diluted tenfold with 0.01 M phosphate buffer of pH 7.4, containing 0.15 M NaCl, and used for the allergen-specific IgE microarray immunoassay.

Immunoassay procedure Following the removal of the blocking solution, 200 μl /well of the prepared sample solution was added to the well and incubated at room temperature for 1 h. The reaction wells were each rinsed three times with 400 μl of 0.1 M Tris-HCl buffer of pH 8.5, containing 0.05 % Tween-20 and 0.15 M NaCl (buffer A) and once with 400 μl of 0.01 M phosphate buffer of pH 7.4, containing 0.15 M NaCl (buffer B). After

that, 200 μl of the Fab' nanosphere suspension (20 million fluorescence counts per test) with 0.01 M phosphate buffer of pH 7.4, containing 3 % of BSA, 0.15 M NaCl and 0.05 % NaN_3 , was added to the reaction well and incubated at room temperature for 30 min. Then, the reaction wells were each rinsed five times with 400 μl of buffer A and once with buffer B, and the time-resolved fluorescence intensity in the solid phase was measured with the TRF microarray detector.

Reagents and chemicals

SMCC reagent was obtained from Zieben Chemicals Co., Ltd. (Tokyo, Japan). BSA (RIA grade) was obtained from Sigma-Aldrich Co. (St. Louis, Mo., USA). The 96-well microtitration plate used was a FluoroNunc Modules & Plates (Maxisorb, Nalge Nunc International, N.Y., USA). All other reagents were of analytical reagent grade.

CAP/RAST FEIA

IgEs specific to six allergens, including milk, egg white, rice, soybean, peanut and wheat, were determined by Pharmacia CAP/RAST FEIA (Pharmacia Diagnostics, Uppsala Sweden; CPS/RAST) according to the standard procedure.

Instruments

Conventional time-resolved fluorometer The Arcus 1234 fluorometer was used for the measurement of time-resolved fluorescence intensity in the microtitration well assays. The measurement conditions were: delay time 0.20 ms, window time 0.40 ms, and flash rate 1.00 ms.

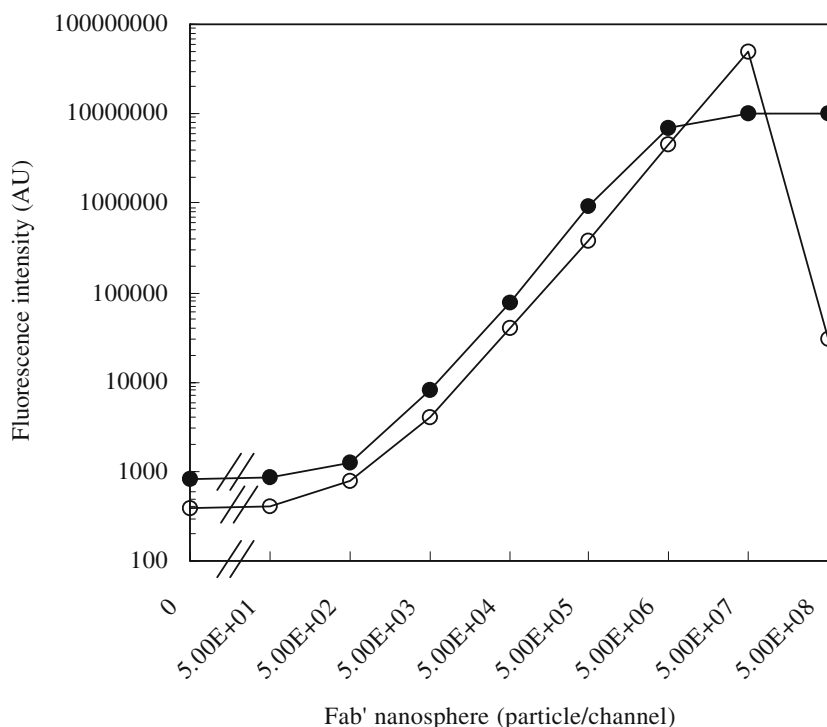
HPLC system The HPLC system consisted of a Hitachi HPLC system (Tokyo, Japan), a Hitachi L-7100 HPLC pump, a Hitachi L-7420 UV/VIS detector, a Hitachi L-7490 RI detector, and a Reodyne (Cotati, Calif., USA) model 7725I syringe-loading sample injector valve equipped with a 2 ml sample loop.

Results and discussion

Performance of TRF microarray detector

Linearity and detection sensitivity The tenfold dilution series of the Fab' nanosphere suspension were prepared with 0.05 M Tris-HCl buffer of pH 7.8, containing 0.2 % BSA, 0.15 M NaCl and 0.05 % NaN_3 , and 1 μl aliquot of the suspension was spotted onto the no. 9 measuring channel of the reaction well using the PixSys 4200 microarrayer. After drying of the suspension, the fluorescence intensity of the Fab' nanosphere was measured with the TRF microarray detector. As shown in Fig. 3, the linear

Fig. 3 Relationship between time-resolved fluorescence intensity and concentration of Fab' nanosphere. The Fab' nanosphere suspensions (1 μ l each) were spotted onto the no. 9 channel of the reaction well (*closed circle*) and the center of the microtitration well bottom (*open circle*). After drying, the time-resolved fluorescence intensity was measured with the TRF microarray detector (*closed circle*) and the Arcus 1234 fluorometer (*open circle*). (AU arbitrary units)



range covers over four orders of magnitude. The Fab' nanosphere suspension (1 μ l) was spotted onto the center of the microtitration well bottom, and the fluorescence intensity was also measured with the Arcus 1234 fluorometer after the drying of the sample. The detection sensitivity of the TRF microarray detector was almost equal to that of the Arcus 1234 fluorometer, which is a microtitration plate reader using a xenon flash lamp as a pulsed excitation light source and a PMT for the signal detection. The detection limit was found to be 500 particles of the Fab' nanosphere in one channel (1 μ l). The detection limit was defined as the number of particles corresponding to a signal 3 SDs above the mean of five replicates of the dilution buffer. The CVs ($n=5$) of the TRF microarray detector were between 5.2 % and 15.1 %. In the Arcus 1234 fluorometer the decrease in the fluorescence intensity was observed in the highest concentration of the Fab' nanosphere suspension, supposing that the signal detection of Arcus 1234 fluorometer was performed by a single photon counting method.

Cross-talk fluorescence The PMT detection method has advantages over CCD and image intensifier detection methods in detection sensitivity and cost. However, in the TRF microarray detector using a PMT, adjacent channels having strong fluorescence might affect the fluorescence value of a measuring channel. Then, the cross-talk fluorescence of the TRF microarray detector was investigated. The Fab' nanosphere (700 million fluorescence counts per 1 μ l) suspension was spotted (1 μ l) onto the no.9 measuring channel of the reaction well. After drying the suspension, we measured the fluorescence intensity of each channel (no.1 to no.9) of the reaction well with the TRF microarray detector.

The increase of fluorescence intensity at the no. 1 to no. 8 channels of the reaction well was less than 0.0004 % when 700 million fluorescence counts (per 1 μ l) of the Fab' nanosphere suspension were spotted onto the no. 9 channel of the reaction well. When 700 million fluorescence counts (per 1 μ l) of the Fab' nanosphere suspension were spotted onto the no. 1 to no. 8 channels of the reaction well, the increase in fluorescence intensity at the no. 9 channel was

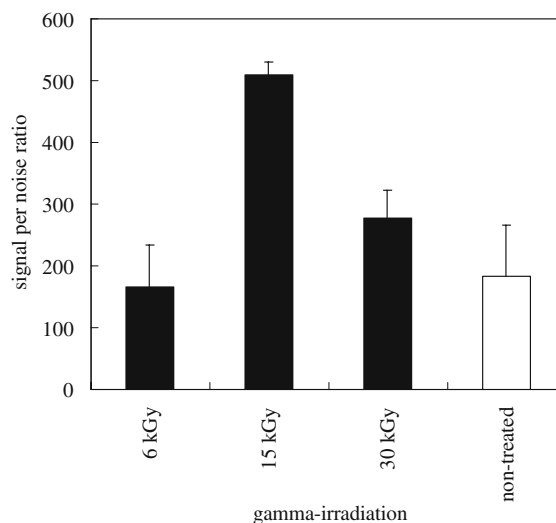


Fig. 4 Allergen-specific IgE immunoassay using gamma-irradiated polystyrene chip and non-treated polystyrene chip. The polystyrene chip was irradiated with 6 kGy, 15 kGy and 30 kGy of gamma ray, and the chip surface was modified. Using the gamma-irradiated and non-treated polystyrene chip, we analyzed serum milk-specific IgE as described in the [Experimental methods](#) section. *Open column* gamma-irradiated polystyrene chip, *closed column* non-treated polystyrene chip. Signal-per-noise ratio, fluorescence value of the positive serum/fluorescence value of the negative serum

Table 1 Classification of specific IgE levels of Pharmacia CAP System and microarray immunoassay (AU arbitrary units)

Class	CAP/RAST Specific IgE (U/ml)	Microarray* Fluorescence intensity (AU)
0	0.35	3,500
1	0.35–0.70	3,500–7,000
2	0.70–3.5	7,000–35,000
3	3.5–17.5	35,000–175,000
4	17.5–50	175,000–500,000
5	50–100	500,000–1,000,000
6	100	1,000,000

*Provisional assignment of fluorescence counts to IgE classes

<0.0013 %. The fluorescence value of the measuring channel was little affected by the adjacent channels with the strong fluorescence. Therefore, it was suggested that the cross-talk fluorescence in the TRF microarray detector could be neglected, since the Fab' nanosphere reagent (20 million fluorescence counts per test) was used in the following microarray immunoassay.

Gamma irradiation of the polystyrene chip

In general, allergen extracts are mixtures of different allergenic and non-allergenic proteins [22]. Carbohydrate components, e.g., from glycoproteins, are also known to be of relevance in this context [14]. Gel electrophoresis, in combination with immunoblotting techniques, allowed the determination and purification of the major allergenic compounds of several allergen mixtures [22].

Polystyrene is used as a material for protein immobilization. Proteins are immobilized onto polystyrene surface by hydrophobic interaction. However, adsorption of glycoproteins onto polystyrene surfaces is relatively low. The amount of protein adsorption onto polystyrene surfaces can be increased by the use of gamma-irradiated polystyrene [23]. It is well known that cleavage of C–C and C–H bonds of polystyrene, production of radicals and ions on the

surface, and the cross-linking reactions are brought about by gamma irradiation. Therefore, in this study, the polystyrene chip was irradiated by gamma-ray to increase the protein adsorption onto the chip surface.

The polystyrene chip was irradiated with 6 kGy (600 krad), 15 kGy (1,500 krad), and 30 kGy (3,000 krad) at the dose rate of 2 kGy/h (200 krad/h). The milk allergen extract (1 µl) was immobilized onto the no. 9 channel of the reaction well. As a reference polystyrene chip, the milk allergen-immobilized reaction well, using a non-treated polystyrene chip, was also prepared. The milk-specific IgE positive and negative panel sera (200 µl each) were added to the reaction wells, and the immunoassay was carried out as described in the [Experimental methods](#) section. The results are shown in Fig. 4. When the polystyrene chip irradiated with 15 kGy of gamma-ray was used in the assay, the signal-per-noise ratio (fluorescence value of the positive serum/fluorescence value of the negative serum) was approximately 2.5 times improvement compared to that of the non-treated polystyrene chip. On the other hand, the fluorescence value of the positive serum was decreased when the polystyrene chip irradiated with 30 kGy of gamma-ray was used in the assay. The excessive irradiation (30 kGy) by gamma ray resulted in the decrease of signal-per-noise ratio. The reproducibility of the immunoassay, compared with that of the non-treated polystyrene chip, was also improved when irradiation was with 15 kGy. Therefore, the polystyrene chip irradiated with 15 kGy of gamma ray was used for the following serum allergy-specific IgE microarray immunoassays. The gamma-ray irradiation of the polystyrene surface is important for immobilization of proteins, especially those such as allergenic compounds.

Allergen-specific IgE microarray immunoassay

The TRF microarray detection system consisting of the Fab' nanosphere, the TRF microarray detector and the gamma-irradiated polystyrene chip was applied for serum allergen-specific IgE multi-analyses.

Table 2 Reproducibility of allergen microarray testing of food allergen-specific IgE

Chip	Allergens immobilized onto chip												
	Milk		Egg white		Rice		Soybean		Peanut		Wheat		None
	Intensity ^a	Class ^b	Intensity	Class	Intensity	Class	Intensity	Class	Intensity	Class	Intensity	Class	Intensity
1	327,270	4	1,303,784	6	4,439	1	9,497	2	1481	0	1,092,974	6	918
2	388,154	4	1,672,670	6	5,195	1	9,368	2	1495	0	992,549	5	1,026
3	384,320	4	1,266,190	6	3,778	1	8,266	2	1545	0	871,997	5	916
4	278,776	4	1,289,031	6	3,603	1	7,068	2	1860	0	1,066,953	6	1,379
5	249,572	4	1,446,244	6	3,620	1	7,296	2	1189	0	991,272	5	861
Mean	325,618		1,395,584		4,127		8,299		1514		1,003,149		1,020
SD	61,920		170,196		688		1,129		239		86,014		209
CV (%)	19.0		12.2		16.7		13.6		15.9		8.6		20.5

^aRaw fluorescence intensity

^bIgE levels classified into 0 to 6 as listed in Table 1

Reproducibility and non-specific adsorption The measurements obtained with the CAP/RAST FEIA method are expressed in units/milliliter (U/ml) or in specific IgE classes (0–6) [24]. The CAP/RAST FEIA method is based on a two-step sandwich fluoro-enzyme immunoassay, which uses 50 μ l of sample volume per single allergen-specific IgE assay. Total assay time is approximately

200 min. The assignment of units to IgE classes [25] is shown in Table 1. In the microarray immunoassay, the provisional assignment of fluorescence counts to IgE classes is also listed in Table 1.

The reproducibility of the microarray immunoassay was studied in the measurement of serum samples from presumptive allergic patients ($n=5$). The obtained fluorescence

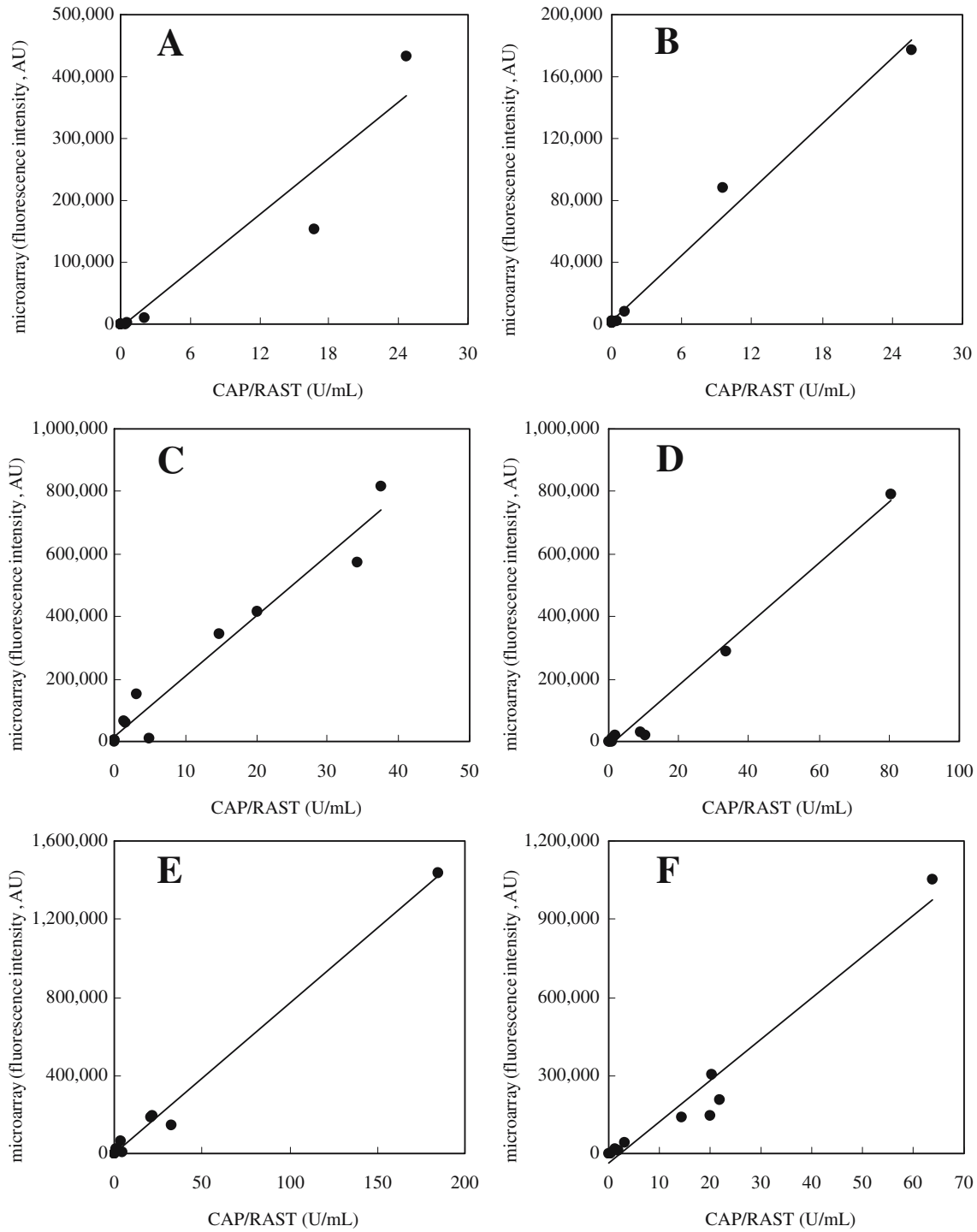


Fig. 5 Correlation between test results of serum allergen-specific IgE obtained with microarray immunoassay and CAP/RAST FEIA method. The sera of ten presumptive allergic patients were screened for IgE reactivity. **a** Milk; **b** egg white; **c** rice; **d** soybean; **e** peanut; **f** wheat-specific IgE. (AU arbitrary units)

intensity and the IgE classes in the microarray immunoassay are listed in Table 2. Good reproducibility was obtained in the allergen-specific IgE microarray immunoassay.

The fluorescence intensity of the allergen-free channel was 1,020 counts. The Fab' nanosphere (20 million fluo-

rescence counts per test) was used in the microarray immunoassay. The fluorescence adsorbed non-specifically onto the chip surface was 180 counts, since the background fluorescence intensity of chip was 840 counts. Therefore, the non-specific adsorption of the Fab' nanosphere onto the

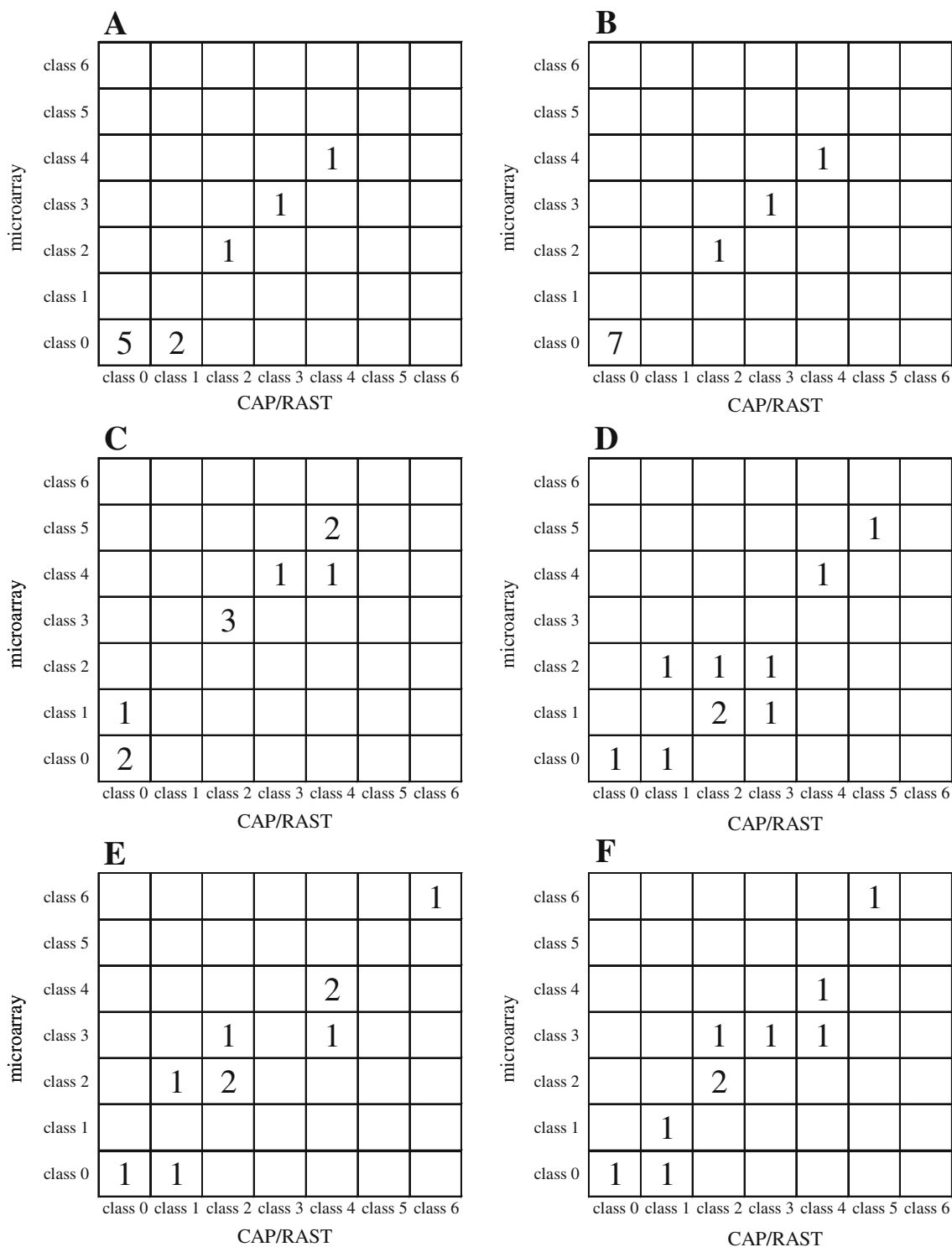


Fig. 6 Correlation between classes of serum allergen-specific IgE levels by microarray immunoassay and those by the CAP/RAST FEIA method. The test results were categorized into seven classes, from 0 to 6, as listed in Table 1. **a** Milk; **b** egg white; **c** rice; **d** soybean; **e** peanut; **f** wheat-specific IgE

reaction well surface was 0.0009 %, which was almost equal to that of the previously described Fab' nanosphere onto the microtitration well surface (Maxisorb, Nunc) [15].

Comparison of allergen-specific IgE detection between microarray immunoassay and CAP/RAST FEIA method
The experimental and clinical relevance of the quantitative measurement of the food allergen-specific IgE using the microarray was evaluated in ten sera. The results were compared with those measured by the CAP/RAST FEIA method. As shown in Fig. 5, good correlation curves were obtained between the fluorescence intensity of the allergen-specific IgEs microarray immunoassay and the IgE units of the CAP/RAST FEIA method: $r=0.961$ for milk; $r=0.993$ for egg white; $r=0.977$ for rice; $r=0.993$ for soybean; $r=0.996$ for peanut; $r=0.977$ for wheat.

Although, in this study, the fluorescence intensity should be converted to IgE concentration by a purified human IgE calibrator, the fluorescence intensities of the ten sera obtained by the microarray immunoassay were provisionally categorized into seven classes, from 0 to 6, as listed in Table 1. Figure 6 shows the correlation between classes of serum allergen-specific IgE levels by the allergen-specific microarray immunoassay and those by the CAP/RAST FEIA method. The numbers in Fig. 6 are the numbers of samples categorized into each class. Classification of the ten sera in the allergen-specific IgE microarray immunoassay corresponded well to those of the CAP/RAST method; $r=0.961$ for milk; $r=1.000$ for egg white; $r=0.970$ for rice; $r=0.900$ for soybean; $r=0.934$ for peanut; $r=0.935$ for wheat.

We selected six allergy markers, including milk, egg white, rice, soybean, peanut and wheat, as food allergen-specific IgE diagnosis in infants and young children. In spite of the reduced serum volume (20 μ l), which is one-fifteenth of that of the CAP/RAST FEIA method in the six allergen-specific IgEs determination, the fluorescence intensity and the classes of IgE levels by the microarray immunoassay were well correlated with those of the CAP/RAST FEIA method. The detection limits of the microarray immunoassay were less than 0.35 U/ml in the allergen markers tested, which were comparable to those of the CAP/RAST FEIA.

Thus, the new TRF microarray detection system could be applicable to serum allergen-specific IgE multi-analyses, using only 20 μ l of serum in fewer than 90 min. It could be expected that the system would become a useful tool for allergy diagnosis in infants and young children.

Conclusions

The new TRF microarray detection system consists of a Fab' nanosphere, a TRF microarray detector and a gamma-irradiated polystyrene chip and has been developed for serum allergen-specific IgE multi-analyses. Using the TRF microarray detection system, we could detect 500 particles of the Fab' nanosphere in one channel, without the cross-

talk fluorescence from the adjacent measuring channels. The polystyrene chip surface was irradiated with gamma rays of ^{60}Co , and the sensitivity of allergen-specific IgE immunoassay was improved approximately 2.5 times compared with that of a non-treated polystyrene chip.

The TRF microarray detection system was used for serum food allergy-specific IgE multi-analyses. The classification of ten sera in the allergen-specific IgE microarray immunoassay corresponded well to those of the CAP/RAST FEIA method. The serum volume used in the microarray immunoassay was one-twelfth of that used in the CAP/RAST FEIA method in the allergen-specific IgE multi-analyses. The TRF microarray detection system would be a suitable tool for diagnosis in infants and young children.

Using the polystyrene chip with a 12 mm $\times$ 12 mm reaction well, one could measure the signals from nine channels in one chip, without the cross-talk fluorescence from the adjacent channels. Channels can be easily added by increasing the area of the reaction well. When a chip with 24 mm $\times$ 24 mm of reaction area is used, 49 individual channels in one chip would be measured. If a chip with 33 mm $\times$ 33 mm of reaction area were used, 100 individual channels in one chip would be measured. The measuring channels can be added by minor changes to the system, with no loss of detection sensitivity.

Thus, the TRF microarray detection system would be applicable not only to serum allergy-specific IgE multi-analyses but also to other biomedical and diagnostic multi-analyses.

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