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A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics

In order to quantify autoantibodies in the sera of patients with autoimmune disease, we have created a microarray-based immunoassay that allows the simultaneous analysis of 18 known autoantigens. The microarrays contain serial dilutions of the various antigens, thereby allowing accurate determination of autoantibody titer using minimal amounts of serum. The assay is very sensitive and highly specific: as little as 40 fg of a known protein standard can be detected with little or no cross-reactivity to nonspecific proteins. The signal intensities observed from serial dilutions of immobilized antigen correlate well with serial dilutions of autoimmune sera. Miniaturized and highly parallelized immunoassays like these will reduce costs by decreasing reagent consumption and improve efficiency by greatly increasing the number of assays that can be performed with a single serum sample. This system will significantly facilitate and accelerate the diagnostics of autoimmune diseases and can be adapted easily to any other kind of immunoassay.

Keywords: Immunoassay / Microarray / Autoimmune disease / Chemiluminescence / Enzyme-linked immunosorbent assay

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1 Introduction

Biochip array technology allows the simultaneous analysis of thousands of molecular parameters with a single experiment [1, 2]. Microarrays are produced either using on-chip synthesis strategies or with an arrayer based on contact printing or ink jet technology. Nucleic acids or proteins can be deposited as microspots in defined positions on the chip [3]. These capture molecules, termed ligands or probes, and are immobilized in rows and columns on the solid support. The chips are exposed to samples containing appropriate analytes, termed receptors or targets, which bind to their immobilized counterparts. The concentration of the analyte in the sample can be determined by

detecting the resulting ligand-analyte complex in the microspots of the chip. Such binding assays include hybridization of immobilized DNA probes exposed to complementary targets as well as receptor-ligand interactions and immunoassays using either immobilized antibodies or antigens. The recognition of target molecules may be detected using fluorescence or radioactive labeling, chemiluminescence, mass spectrometry or electrochemical methods. This technology has been successfully applied to the characterization of gene expression [4], the detection of single nucleotide polymorphisms [5], and to the analysis of DNA-protein interactions by double-stranded DNA arrays [6]. Unlike nucleic acids, relatively few protein-based microarray assays [7–10] have been described.

One way to detect analytes in a highly specific manner is to use antibodies as capture molecules. If very low amounts of antibody are immobilized on a tiny microspot, only a minor fraction of the analyte is captured and this fraction reflects its concentration in the sample. This type of assay is termed an ambient analyte immunoassay because the analyte concentration is measured without disturbing its total concentration [11]. Assays performed under such conditions result in a maximal fractional occupancy of the capture antibodies and, therefore, in an optimal signal – to – noise ratio. Microarrays with a spot area less than 100 μm^2 containing 10^6 – 10^8 capture molecules fall within ambient analyte assay limits [12].

In contrast to the ambient analyte theory, Sitzel *et al.* [8] developed the concept of mass sensing arrays, which absorb substantial amounts of analyte molecules from a

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Abbreviations: BM-T, Hoffmann La Roche multitag marker; FusM, fusion protein; IgG, immunoglobulin G; MPO, myeloperoxidase; tPO, thyroid peroxidase

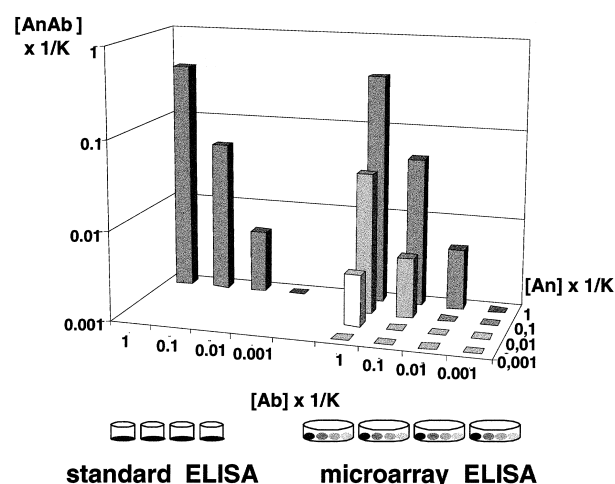


Figure 1. Theoretical prediction of antigen-antibody product formation based on the mass action law for single point assays (e.g., standard ELISA, left) and for microarray assays (right). Decreasing concentrations of both antigen and antibody result in a lower amount of antigen-antibody product. Single point assays are usually performed at one antigen concentration by stepwise dilution of antibody concentration. Microarray assays are usually performed in one experiment at one antibody concentration with the dilution series of antigens immobilized on the array. The resulting amount of antigen-antibody product is identical in both cases. Concentrations are given in units of 1/K.

sample by maximizing the number of capture molecules within each microspot. These authors used microspots with a size of 30 000 μm^2 containing 10^{10} capture molecules. The high amount of capture molecules harvest nearly the entire analyte population out of the sample volume. This results in a much stronger signal in comparison to the ambient analyte assay.

Protein microarrays for gene expression profiling and antibody screening were published by Lueking *et al.* [9]. Recombinant proteins derived from a human fetal brain

cDNA expression library were arrayed and screened with different antibodies. Specific protein-target interactions with minimal cross-reactivity could be detected within the microspots. This very sensitive protein expression profiling and antibody specificity screening should provide a powerful tool for ligand-receptor interaction studies in the near future. Mendoza *et al.* [10] describe a biochip approach capable of high-throughput, enzyme-linked immunosorbent assay (ELISA) using antigen arrays in a miniaturized 96-well format on a glass substrate. With marker antigens they demonstrated the feasibility of multiplex detection of arrayed antigens.

Here we describe a microarray-based for the parallel detection of autoantibodies in human sera. Arraying known autoantigens in different concentrations within one array allows us to determine the titer of different autoantibodies from a minimal amount of serum by a single immunoassay experiment. Our array screens for autoantibodies directed against 18 different autoantigens used as serological markers in prominent autoimmune diseases like autoimmune thyroiditis (Hashimoto's thyroiditis and Graves' disease), systemic rheumatic diseases like systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome, scleroderma or polymyositis, systemic vasculitis and antiphospholipid syndrome (APS). The corresponding antigen to autoimmune thyroiditis is thyroid peroxidase (TPO), a microsomal membrane protein, and the antigen to systemic vasculitis is myeloperoxidase (MPO) [13]. Antinuclear antigens (ANAs) are important markers for systemic rheumatic diseases: dsDNA, Sm, SmB' histone, U1 snRNP A and C for SLE [14]. U1 snRNP 68 kDa for MCTD, Ro (SSA) and La (SSB) for Sjögren's syndrome, Scl 70 for scleroderma. CENP B for Crest syndrome, Jo 1 for polymyositis and PM-Scl 100 for an overlapping syndrome. In the monitoring of SLE patients, the dsDNA antibody titer is used as a marker for disease activity [15]. Antibodies against β 2-glycoprotein I are described as highly specific markers for the APS [16]. Our microarray demonstrates

Table 1. Spotting scheme of the antigens used in the evaluation assay

DNA 100%	DNA 100%	BM-T 100%	Ro 60 100%	BM-T 100%	FusM 100%	Ro 60 100%	Histone 100%	Histone 100%	FusM 100%
BM-T 30%	Ro 60 30%	Histone 100%	DNA 30%	Histone 100%	Histone 30%	Histone 30%	BM-T 30%	DNA 30%	Ro 60 30%
FusM 10%		Histone 10%			Ro 60 10%		DNA 10%	FusM 10%	Histone 10%
BM-T 10%	Ro 60 10%	DNA 10%	Histone 10%	BM-T 10%	FusM 30%	Histone 10%	FusM 30%	Histone 30%	Histone 30%

Antigens were spotted in three different concentration (100%, 30%, 10%) and in three different volumes (5, 1.5, 0.5 nL) resulting in spots of 300, 150 or 75 μm , respectively. The FusM antigen, which is recognized by the mAB9E10, is printed in bold letters.

the parallel identification and quantification of several autoimmune diseases. This system will significantly facilitate and accelerate the diagnostics of autoimmune diseases and is easily adaptable to immunoassays in general.

2 Material and methods

2.1 Antigens and antisera

The following autoantigens were produced by P&U Diagnostics (Freiburg, Germany) by recombinant *baculo virus*/Sf9 expression technique: h TPO, h U1-snRNP 68 kDa, h U1-snRNP A, h U1-snRNP-C, h Sm B' h Ro 60 kDa (SSA), h Ro 52 kDa, h La (SSB), h Scl 70, h CENP B, h Jo-1, h PM-Scl 100; using the recombinant *Escherichia coli* technique: dsDNA (plasmid DNA); by chromatography: β 2-glycoprotein I (from human serum), Sm (from calf thymus), MPO (from buffy coats of normal blood donors), RNP-Sm and histone (from HeLa cells). Concentrations of the antigens were between 0.2 and 1.2 mg/mL. Twenty-five characterized antisera of patients with autoimmune diseases and 10 sera from unmonitored blood donors were used for the microarray ELISA. The cDNA coding for six myc epitopes was excised with *Bam*HI/StuI from the pCS2+vector [17] and cloned into the pQE9 expression vector treated with *Hind*III, Klenow fill, and *Bam*HI (Qiagen, Hilden, Germany). The fusion protein was expressed and purified according to the manufacturer's protocol. The monoclonal mouse antibody 9E10 is directed against a short peptide from the c-myc protooncogene [18]. Streptavidin-peroxidase conjugate and rabbit anti-human IgG-peroxidase conjugate were purchased from Dianova (Hamburg, Germany) and Dako (Glostrup, Denmark) respectively.

2.2 Protein arrays

Either a Microdrop Autodrop system (Norderstedt, Germany) equipped with a 100 μ m capillary or a GenArray417 (Genetic Micro Systems, Woburn, MA, USA) were used for the fabrication of the arrays. For the Microdrop Autodrop system the antigens had to be dialyzed extensively against PBS to eliminate glycerol and detergents which would interfere with the Microdrop spotting procedure. After dialysis, antigens were diluted in PBS. The antigens were diluted in stabilizing buffer containing 10% glycerol, 0.1% SDS and 5 μ g/mL BSA for the GenArray417 contact printing procedure. Nitrocellulose membrane (0.4 μ m; Schleicher and Schuell, Dassel, Germany), silylated slides (TeleChem International, Sunnyvale, CA, USA) or poly-L-lysine coated slides (Sigma Aldrich, Deisenhofen, Germany) were used as biochip solid supports. Arrays with 120 spots on 0.7 cm² and 288

spots on 2 cm² were produced with the Microdrop device and the GenArray417, respectively. Prior to spotting, bromophenol blue was added to the stabilized antigen solutions to a final concentration of 0.1%. This allows monitoring of sample deposition after the arraying procedure. Images of bromophenol blue arrays were obtained with a Kodak Image Station 440CF (NEN Life Science Products, Wilmington, DE, USA). The protein arrays were stored at room temperature in the dark for up to one month without any significant loss of activity.

2.3 Assay performance and detection system

Antigens on the filters or slides were rehydrated in PBS for 5 min, incubated with blocking buffer (1.5% BSA, 5% low-fat dry milk in PBS) for 30 min and with 100 μ L antiserum (diluted 1:100 in blocking buffer, 0.1% Tween-20) for 30 min at room temperature, followed by four 5 min washes with PBS, 0.1% Tween-20 and a 30 min incubation with rabbit anti-human peroxidase-conjugated secondary antibody (0.3 μ g/mL in blocking buffer, 0.1% Tween-20). Subsequently the arrays were washed four times with PBS, 0.1% Tween-20. Freshly prepared luminol substrate (ECL Western blotting Detection Reagents RPN2109; Amersham Pharmacia Biotech, Uppsala, Sweden) was applied to the filters or slides for subsequent chemiluminescent measurements. Images of chemiluminescence were acquired with a Peltier/air-cooled (−70°C) slow scan CCD camera (NightOWL LB 981, PerkinElmer Berthold, Bad Wildbad, Germany) equipped with a Xenon objective ($f = 0.95$, 25 mm; Schneider, Bad Kreuznach, Germany). Data analysis was performed with the WinLight software.

3 Results and discussion

3.1 Theory of titer determination

The antibody titer in a serum is defined as the highest dilution of the serum at which the binding of the antibody to its antigen can still be detected in indirect immunofluorescence. Considering the mass action laws it is obvious that the titer may also be determined by dilution of the antigen (Fig. 1). Figure 1 shows the calculated amounts of an antigen-antibody complex that is obtained according to the mass action law. A concentration range for $[Ab]_0$ and $[An]_0$ from 1/K to $0.001 \times 1/K$ was chosen to simulate conditions encountered in our microspot immunoassay. These values represent a concentration range of $[Ab]_0$ and $[An]_0$ from 10^{-9} to 10^{-14} M, considering an association constant K_a ranging between 10^9 and 10^{11} M^{−1} as observed for most known antibodies. Calculations were performed under the assumption that there will be no diffusion and no mass transport limitation effects. It was also assumed that there will be no unspecific adsorption of the

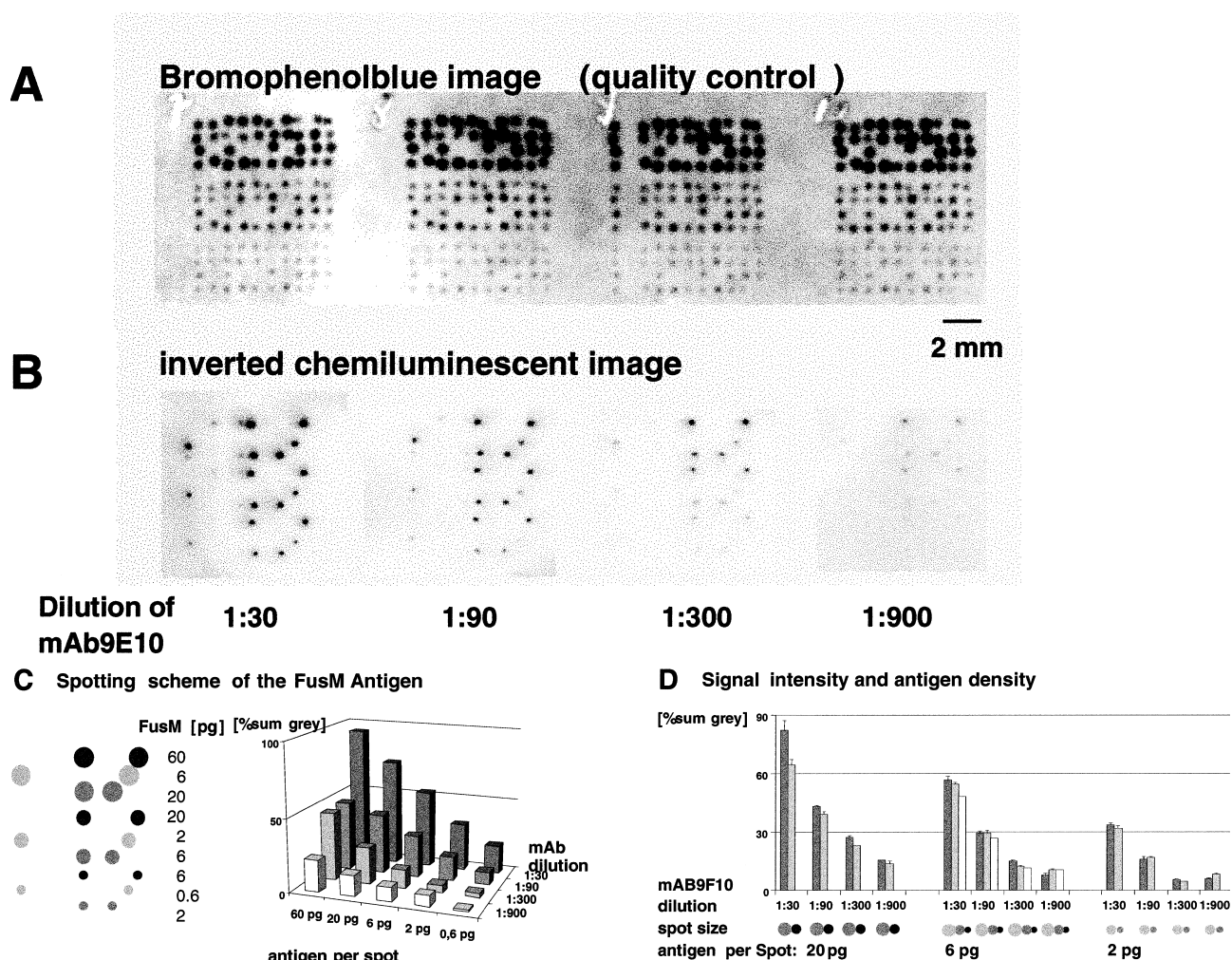


Figure 2. mAb9E10 microarray assay. (A) Images of four microarrays with 3×36 spots on nitrocellulose membrane ($0.7 \times 1 \text{ cm}^2$). Three different volumes of antigens (0.5, 1, 1.5 nL) mixed with bromophenol blue were immobilized as shown in Table 1. The integrity of the arrays was tested by analysis of the bromophenol blue pattern. (B) Inverted image of the chemiluminescent signals obtained after incubation of the array with different dilutions of mAb9E10. (C) Spotting scheme and different spot sizes of antigen FusM within the arrays. FusM antigen was immobilized in amounts from 60 pg down to 0.6 pg. Antigen densities on the membranes were different due to the different spot sizes. The diagram shows means of duplicates of the relative intensities of the chemiluminescent signals in (B). These experimental results coincide with the calculated values in Fig. 1. The measured signal intensities were integrated over the spot area and are presented as [sum grey %] of the maximum value obtained. (D) Correlation between chemiluminescent signal intensities and varying antigen densities. Signal intensities between spots with high and low antigen density decreased with increasing dilutions of mAb9E10 and decreasing absolute amounts of antigen per spot.

antibodies to the surface and no difference in antigen densities within the different microspots, which normally results in a different rebinding of dissociating antibodies [19].

Calculations were also performed for an antigen array where the antigens are immobilized in decreasing concentrations and which are incubated with a dilution of the appropriate antibody. In a standard ELISA, the antibody solely reacts with a high amount of antigen immobilized in

a single well. Dilution of the antibody minimizes the antigen-antibody product and results in a titer determination above which the product is under the detection limit. In a microarray assay, titers can be directly determined from the antigen-antibody products by using a set of microspots with different concentrations of immobilized antigen. The titer can be defined as the minimal antigen concentration at which the product can still be detected (Fig. 1). Incubation of such a microarray with a dilution series of an antibody should yield a similar result. Using

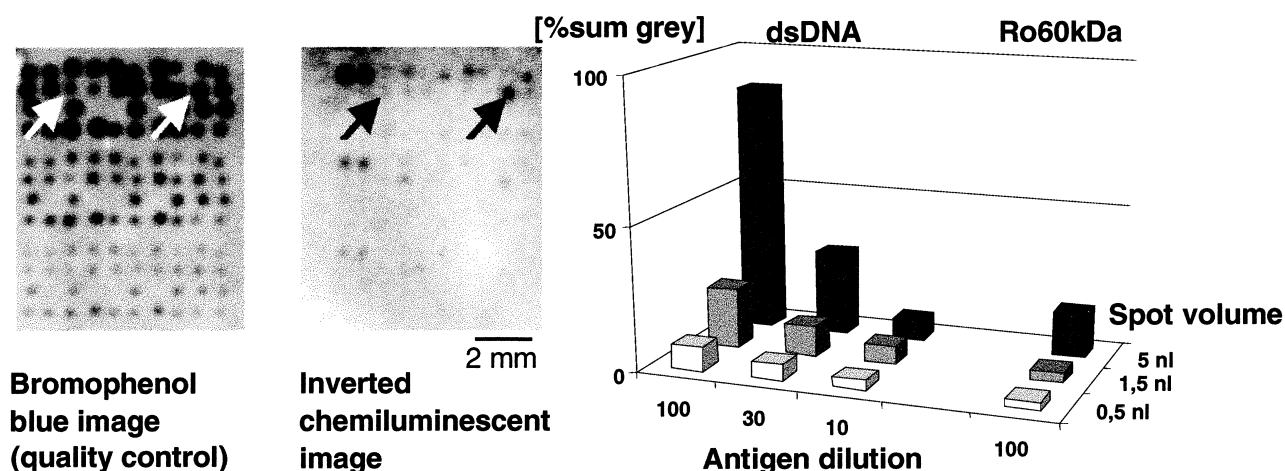


Figure 3. Microarray of dsDNA and Ro. Antigen positions and concentrations are given in Table 1. Chemiluminescent signals were detected after incubation with a serum containing autoantibodies directed against dsDNA and Ro. The intensity of the dsDNA signal in the second row (left arrow) was smaller than the intensity of the duplicate signal (right arrow). This was due to a smaller amount of antigen spotted at this location, clearly detectable in the bromophenol blue quality control (arrows, left image). The relative intensities of the chemiluminescent signals for dsDNA decreased with decreasing antigen spot volumes and antigen concentrations, resulting in a reduction of absolute amounts of immobilized antigen. Due to a significant loss of antigenicity of immobilized Ro protein the Ro signals were very weak and could only be detected at the highest Ro antigen (100%) concentration. The measured signal intensities have been integrated over the spot area and are presented as [sum grey %] of the maximum value obtained.

microarrays, a determination of the titer will depend on the array format, the spot size and the amount of antigen immobilized within the spots. For this reason, only titers originating from the same array format may be compared. In addition, the titer depends on the detection system. A more sensitive detection method will result in a higher titer, independent of whether the serum or the antigen is diluted. Titers obtained by different kinds of assays may only be compared by taking into account all these variations.

3.2 Generating and testing of antigen microarrays

First, we made microarrays to test whether real assays would give results comparable to the predicted values based on the calculations according to the mass action laws. In addition, we wanted to test whether microspot size or antigen density would influence the sensitivity or the selectivity of the assay in our test system. Antigens and control proteins were spotted in three different concentrations, each with three different volumes: 5, 1.5 and 0.5 nL yielding spot sizes of 300, 150 and 75 μm in diameter, respectively (Table 1). Every combination of concentration and volume was spotted in pairs. The following samples were arrayed as shown in Table 1: plasmid DNA, Ro 60 kDa, the Hoffmann LaRoche multitag marker (BM-T), a fusion protein with six myc-epitopes (FusM) and histone. The starting concentration of the probes ranged from 100–500 $\mu\text{g/mL}$ and were not adjusted to equal molar concentrations for the individual antigens. As

antibodies we used the mAb9E10, which recognizes the c-myc sequence EQKLISEEDL present in the fusion protein FusM and in the BM-T and patient sera with autoantibodies directed against dsDNA and Ro 60 kDa. Bromophenol blue added to the spotting solutions enabled us to control the microdrop application and the integrity of each array (Fig. 2A). Arrays were incubated with four different dilutions (1:30, 1:90, 1:300, 1:900) of mAb9E10. Bound mAb9E10 was detected by a peroxidase-labeled secondary antibody and a chemiluminescent reaction monitored by a CCD camera (NightOWL; PerkinElmer Berthold; Fig. 2B). Images were taken with an integration time of 20 s without pixel binning. The antigen dilution of two orders of magnitude could easily be fitted by the experimental dynamic range of the NightOWL camera, which is at least four orders of magnitude at a single integration time. The spot pairs of identical antigens within the arrays should produce two identical signals. The analysis of signal pairs reveals variable, irreproducible conditions on the microarray. The data obtained from the experiments with the four dilutions of the antibody and their binding to the microspots containing five different amounts of immobilized antigen are shown in Fig. 2C. As predicted by the theoretical calculations, similar antigen-antibody products were obtained either by serial dilution of the immobilized antigen FusM or the mAb9E10 with which the microarray was incubated (Figs. 1 and 2C). Only minor signals were obtained in this assay with mAb9E10 and the BM-T proteins. This was due to the low amount of BM-T proteins present on the array and, therefore, no detailed quantification was

performed for these signals. No significant cross reaction between the mAb9E10 and the other proteins on the array were observed.

Comparing the signals derived from the same antigen concentration immobilized within different spot sizes allowed us to determine whether the antigen density influenced the signal intensity and whether there is an optimal spot size for our assay system (Fig. 2D). Minimizing the spot size will minimize the diffusion constraints on the rate of antibody binding to the immobilized antigen. The smaller a spot, the more closely the reaction kinetics approximates the kinetics characterizing a homogeneous liquid-phase reaction system [7]. Our spots, from 60 000 μm^2 down to 5000 μm^2 , theoretically contain 10^{10} – 10^8 antibody binding sites. Such arrays are likely to be regarded as mass sensing assays and not as ambient analyte assays assuming spot sizes below 100 μm^2 with 10^6 – 10^8 capture molecules [1]. In our assay system, the same amount of antigen spotted in larger spot sizes resulted in stronger signals for nearly all antigen and antibody concentrations. A further dilution of the antigen or the antibody diminished the differences of signal intensities from different spot sizes with the same amount of immobilized antigen (Fig. 2D). With our test system we obtained the expected decrease in signal intensity with a falling concentration of antigen or antibody without any major influence of the size of the microspot and the density of the immobilized antigen. Even a very high antigen density did not increase unspecific binding of antibodies.

Incubation of the arrays with an autoimmune serum containing antibodies directed against dsDNA and Ro 60 kDa resulted in the expected decreasing signals with decreasing amounts of antigen or antisera (Fig. 3). The autoantibodies directed against Ro 60 kDa gave only a very weak signal. Therefore, only the highest concentrations in each row were quantified and showed the expected decrease in signal intensity. The requirement of a quality control of each array is demonstrated in Fig. 3. Comparing the dsDNA signals (arrows) it is obvious that one of the microspots in the second row did not give the expected signal intensity. Looking at the bromophenol blue image obtained for quality control of the spotting procedure, it is visible that less amount of antigen was deposited by the spotting device at this position (Fig. 3).

The overall signals for the autoantibodies were much lower than expected from the data obtained with a standard ELISA. This was not due to a loss of antigen during the dialysis step (data not shown), which was necessary to eliminate high concentrations of glycerol and detergents that would interfere with the Microdrop spotting procedure. The low signals were probably caused by a loss

of antigenicity of the antigens due to the absence of their stabilizing buffer system. To keep the antigens in their stabilized solution we switched from Ink jet to contact printing technology to create further arrays. As a support we used either nitrocellulose membranes or aldehyde-activated glass surfaces. Eighteen autoantigens and control proteins were arrayed as duplicates. Each sample was applied in six different concentrations in fivefold dilution steps from undiluted to 3125-fold dilution. As control proteins the fusion proteins FusM, BM-T, human immunoglobulin G (IgG) and biotinylated BSA were included in the array. The arrays contained 288 microspots with a diameter of 150 μm . After the spotting procedure, the arrays were controlled for integrity by analysis of the optical density stemming from the bromophenol blue added to the sample (Fig. 4). The fusion protein FusM and the BM-T served as controls to detect unspecific binding of serum antibodies. The biotinylated BSA was detected with peroxidase conjugated to streptavidin, which was included during the incubation step with the peroxidase-conjugated rabbit anti-hulGg antibody. Together with the human IgG, the biotinylated BSA served as an internal control for the chemiluminescent reaction on the array. Signals obtained from the control spots allowed us to compare the chemiluminescent reaction between different arrays and different experiments.

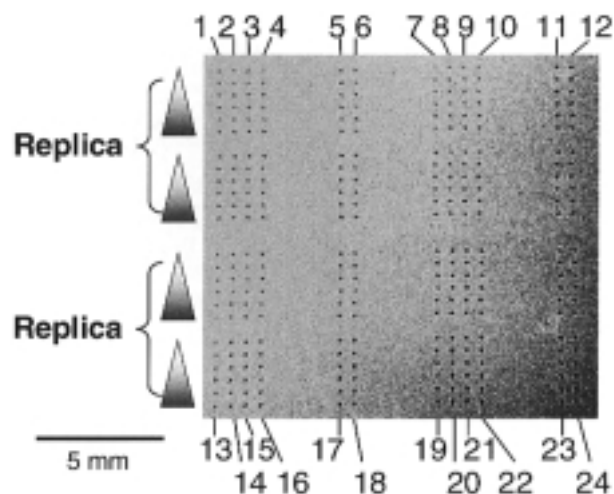


Figure 4. Protein microarray ($1.5 \times 1.5 \text{ cm}^2$) containing 18 different antigens together with control proteins (bromophenol blue image). All samples were spotted as duplicates in six different concentrations as indicated on the left by the shaded triangles. Samples were diluted in fivefold steps. Samples: (1) La; (2) dsDNA; (3) β 2-glycoprotein; (4) Jo-1; (5) BM-T; (6) buffer; (7) PM-Sci 100; (8) Sci 70; (9) CENP B; (10) TPO; (11) Fus-M; (12) hulGg; (13) histon; (14) SmB' (15) RNP A; (16) Ro 60 kDa; (17) MPO; (18) biotin-BSA; (19) Sm; (20) RNP 68 kDa; (21) U1 RNP C; (22) Ro 52 kDa; (23) RNP-SM; (24) mouse IgG.

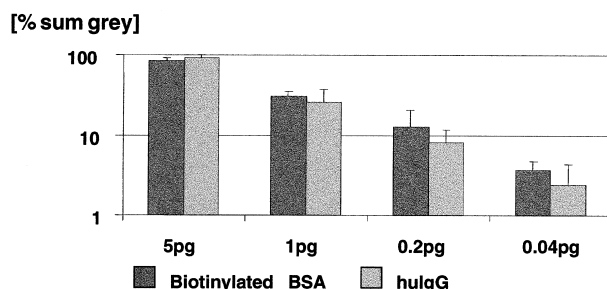


Figure 5. Interassay variation control. The protein microarray in Fig. 4 contained the control proteins biotin-BSA (left bars) and hulGg (right bars). Signals for integrated spot values are given in arbitrary units for the amounts of 5, 1, 0.2, and 0.04 pg per spot. Bars represent data from duplicates of 8 different arrays. Due to the low interassay variation of the signals of both proteins, they could be used as internal standards for comparison of data from different assays. The measured signal intensities have been integrated over the spot area and are presented as [sum grey %] of the maximum value obtained.

In general, the human IgG and the biotinylated BSA spot pairs on the array gave similar signals for each dilution series. Figure 5 shows the signals derived from biotinylated BSA and human IgG spots from eight different arrays. A standard deviation of 6.4 and 8.6% was achieved for the undiluted biotinylated BSA and the hulGg, respectively, with variation coefficient of 6.3 and 8.7%. The intensities of the human IgG signals were profoundly influenced by the presence of serum autoantibodies. Strong signals derived from autoantibodies resulted in a

decreasing human IgG signal (Fig. 7A, No. 12, dilution series of antisera). Obviously, the hulGg within the control microspots competed with the bound autoantibodies for the secondary antibody. With an increasing amount of secondary antibody, more constant human IgG signals were obtained (data not shown), but the background signals increased as well. In contrast to the hulGg, the biotinylated BSA within the arrays served as an internal control which is not influenced by the sera's antibodies.

3.3 Sensitivity of the microarray assay

The dilution steps of the hulGg were used to test the detection limit of the microarray assay. The hulGg concentrations range from 5 pg/spot in a fivefold dilution to 1.6 fg/spot. Microarrays on nitrocellulose were incubated with 0.3 µg/mL of a peroxidase-coupled anti-human IgG rabbit serum. In addition, microarrays on aldehyde-activated glass slides were incubated with four different concentrations of the secondary antibody ranging from 0.3 µg/mL to 7 ng/mL. Images were taken with an integration time of 60 s with 2×2 pixel binning. The lowest concentration that delivered specific signal pairs was the 8 fg hulGg spot on the nitrocellulose array. Therefore, the detection threshold for our assay system could be defined as 8 fg or 0.05 amol in a 0.25 nLspot (Fig. 6). This is six-fold more sensitive compared to a standard ELISA format with the lowest signal, obtained by chemiluminescent assay, reported to be 0.3 amol [20]. When using aldehyde-activated glass slides as supports for the microarrays, the lowest concentration that delivered specific sig-

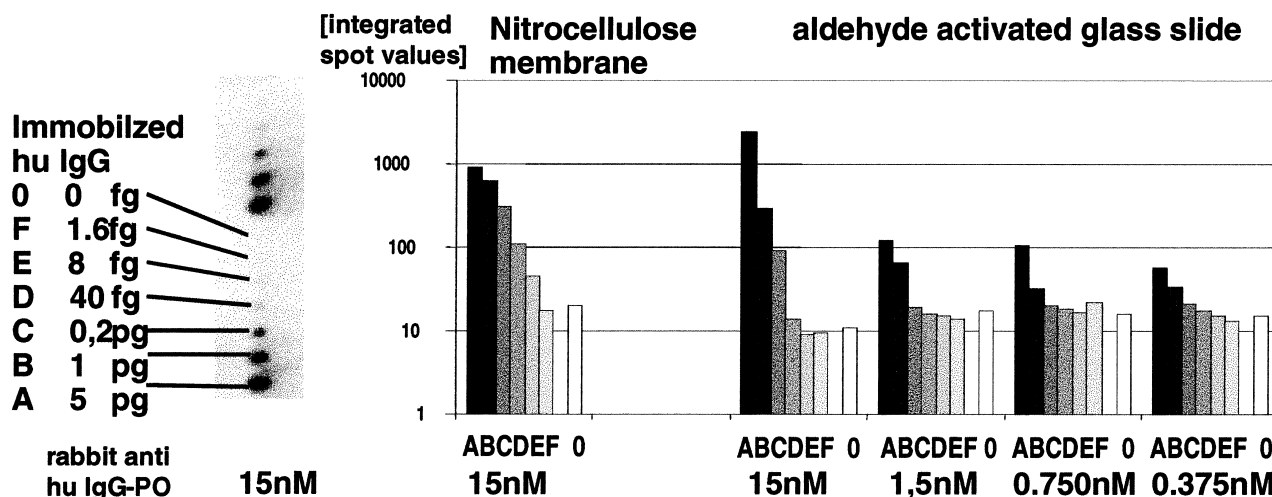


Figure 6. Sensitivity of specific chemiluminescent-based protein detection on microarrays. Inverted image of the chemiluminescent signals (left) obtained with immobilized hulGg spots on nitrocellulose membrane (1.6 fg to 5 pg, sample 12, Fig. 4). Signals in the diagram (right) are shown as integrated spot values given in arbitrary units. Arrays on glass slides were incubated with different concentrations of peroxidase-conjugated secondary antibody (15–0.385 nM) as indicated at the bottom of the diagram. On nitrocellulose microarrays the detection limit is 8 fg hulGg, on glass arrays 40 fg. Integrated spot values present signal intensity integrated over spot area in arbitrary units (counts).

nals was the 40 fg hulgG spot. Dilution of the rabbit anti-serum still gives strong signals at a concentration of 7 ng/mL (0.37 nM). Similar assay sensitivity was obtained

by Mendoza *et al.* [10]. Increasing the amount of hulgG to the values used for the antigens (up to 500 pg per spot) should further increase the assay sensitivity.

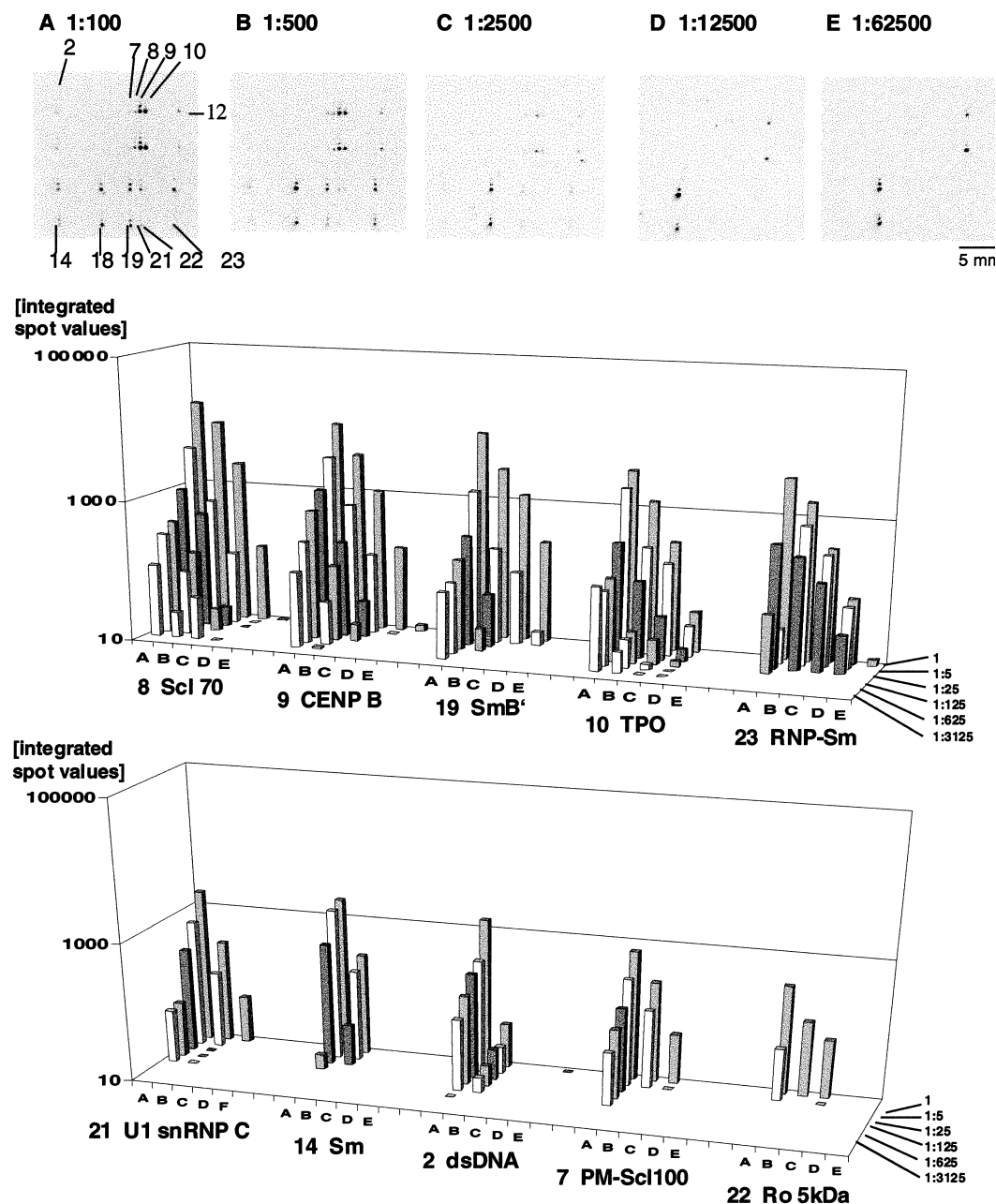


Figure 7. Microarray autoantigen ELISA tested with sera containing antibodies directed against Scl70, CENP B, Sma B' TPO, RNP-Sm, U1 RNP C, Sm, dsDNA, PM-Scl 100, and Ro 52 kDa. (A) Chemiluminescent signals obtained with different dilutions of sera. (B) Integrated spot values given in arbitrary units by means of the duplicates of the chemiluminescent signals seen in (A). Titers of different autoantibodies in one serum can be determined in parallel in one experiment using the signals from the 6 dilutions of each antigen in one microarray. The titers can also be determined by incubation of arrays with increasing dilutions of sera (A, B, C, D, E). Integrated spot values present signal intensity integrated over spot area in arbitrary units (counts).

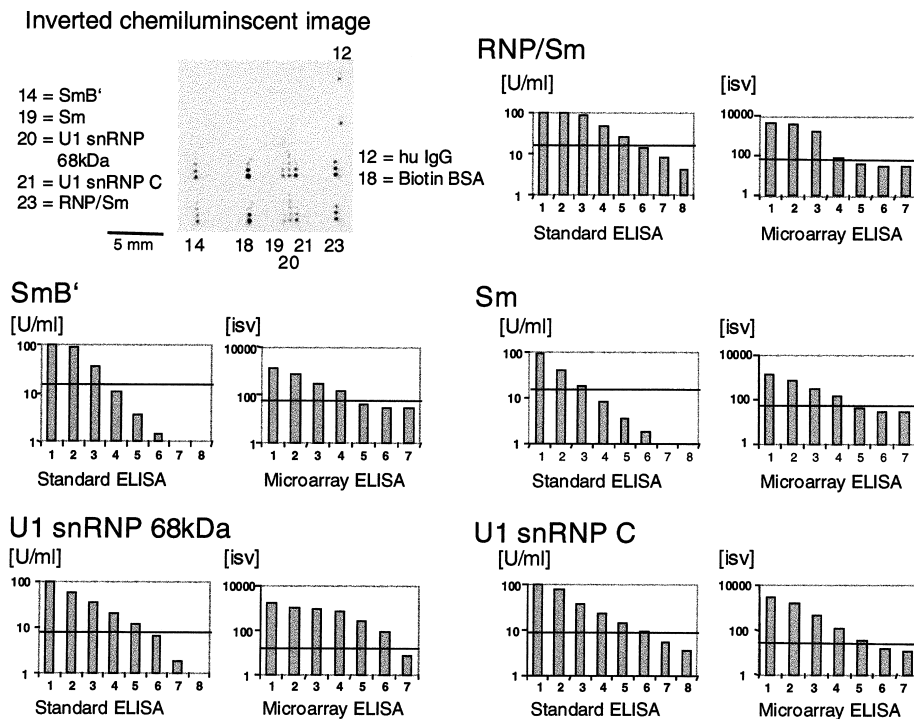


Figure 8. Comparison of standard ELISA and microarray ELISA (serum 3484). Data obtained by testing a dilution series of antisera with standard ELISA. Maximum values, obtained with a superstandard were used to define activity in units per mL [U/mL]. Detection thresholds are marked with a line. Data obtained with microarray ELISA. Signals are shown as integrated spot values (ivs) in arbitrary units. Thresholds are defined as twice the unspecific background signal and are indicated by a line. The titers found in standard ELISA corresponded with the ones found in the microarray ELISA.

3.4 Autoantigens

The microarrays were incubated with different dilutions of a mixture of three different sera containing antibodies against Scl70, CENP B, Sm, TPO, RNP/Sm, U1 RNP C, SmB', dsDNA, PmScl 100, and Ro 52 kDa. The arrays were incubated with the sera in fivefold dilution steps ranging from 1:100 to 1:62500. Bound antibodies were detected with a peroxidase-conjugated secondary antibody and a chemiluminescent reaction. Images were taken with an integration time of 60 s with 2×2 pixel binning. Mean signals of duplicates shown as integrated spot values were plotted against the dilution value of the mixed sera and the dilution value of the antigen. The graphs for all ten positive antigens are shown in Fig. 7.

Specific titers for each antigen were determined by evaluating the signals obtained through the dilution of the mixed sera or by diluting the specific antigen within the array. A high titer for Scl70, CENP B, Sm or TPO was observed. With the 1:100 serum dilution, these autoantibodies gave a significant signal even with the spots containing the lowest antigen concentration (1:3125 dilution; Fig. 7). The signals obtained by a titration of the sera readily confirmed this result. For the same four autoantibodies the sera had to be diluted to 1:62500 to fall below the detection limit. For U1 snRNP C and RNP/Sm antibodies, a serum dilution greater than 1:2500 resulted in a total loss of signal. Again, this correlated with the results obtained when using the 1:100 dilution of the mixed sera. In

this case, only five antigen dilutions on the microarray gave the expected signals. The weak signals obtained with the 1:100 dilution for Sm, dsDNA, PmScl 100, and Ro 52 kDa autoantibodies disappeared after one further dilution step of the sera (Fig. 7). Looking at the experimental data in Fig. 7 and comparing them with the data obtained from the theoretical calculations shown in Fig. 1, it is obvious that there is excellent correlation between signal intensities derived from antigen dilution and signals derived from titration of the antisera.

We compared data obtained using the microarray ELISA with data from standard ELISA. Sera were diluted in a 2-fold dilution step from 1:100 to 1:12800 for the standard ELISA (Fig. 8). The same sera were incubated with the microarrays. Signal intensities for each antigen dilution on the microarray were determined. The threshold above which signals were counted as positive is indicated by a line. Results obtained by the standard ELISA technique were comparable to the results we obtained with our microarray ELISA, looking at the antigen-antibody product formation obtained with decreasing antigen concentrations. Only in a few cases could the high titers obtained with the standard ELISA not be reproduced with the microarray approach. In two cases, this was due to a loss of antigenicity during storage of the arrays. Freshly produced arrays gave the expected signals (data not shown). So far we have tested 25 sera, well characterized with standard ELISA, and 10 sera from unmonitored blood donors. All characterized sera gave the expected results

with the microarray ELISA. One of the ten blood donor sera gave a signal for dsDNA, which was later confirmed with the standard ELISA.

4 Concluding remarks

We have created a microarray containing immobilized antigens in different concentrations that can be used to simultaneously determine titers of different autoantibodies in a single immunoassay experiment. Our microarray immunoassay is very sensitive and highly specific with little or no cross-reactivity for nonspecific proteins. We intend to adapt our system to fluorescently labeled probes and we will include standardized antibodies labeled with a second fluorophore similar to the internal standards used in DNA chip technology [2]. Including an internal standard for each antigen within one assay will allow quantitative analysis. Microarrays similar to the ones presented in this paper could be used for routine diagnostics and for screenings as in performed for Sjögren's syndrome and hepatitis C, for instance [21]. In principle, every relevant disease-dependent protein parameter may be measured using a single microarray. For this reason, microarray ELISA will reduce cost by decreasing reagent consumption and offer the chance to perform simultaneous analysis of hundreds of parameters in one experiment.

Furthermore, in the context of genome and proteome analysis, antibody arrays could allow the simultaneous measurement of hundreds of concentrations of specific cellular proteins within a single experiment. The concept of array-based proteomics was recently discussed by Albala and Humphery-Smith [22]. Even differential analysis could be performed with proteins derived from different sources. Labeling the proteins with different fluorophores [23] followed by a microarray assay could immediately reveal the expression level of proteins binding to their specific microspot. Such microarrays will provide a powerful tool for expression analysis of defined proteins or receptor ligand interactions.

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