

Increasing robustness and sensitivity of protein microarrays through microagitation and automation

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Abstract

Assay systems that employ protein microarrays for the analysis of complex samples are powerful tools to generate a high amount of data from a limiting amount of sample. Due to miniaturization, these systems are susceptible to fluctuations during signal generation and the use of uniform conditions for sample incubation and during the assay procedure is required to get reproducible results. Diffusion limits may prevent constant conditions on all parts of the array and can lead to the decrease of the sensitivity of the array. Therefore, we set-up an automated assay system integrating a novel microagitation device using surface acoustic wave (SAW) technology. Multiplexed assays for the detection of autoantibodies from human serum and sandwich immunoassay for the detection of matrix metalloproteases (MMPs) were used to evaluate the system. Diffusion-rate limited solid phase reactions were enhanced by microagitation using the SAW technology resulting in up to three-fold higher signals.

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1. Introduction

Protein microarrays have evolved into powerful tools for detecting large numbers of analytes from minute amount of sample within one experiment [1–3]. The most prominent applications are protein profiling approaches that allow the identification and quantification of target proteins of interest. A variety of systems have been developed and they can be grouped into two major assay formats, forward-phase assays and reverse phase assays. In a forward phase protein array specific capture molecules are immobilized in a microarray on the solid support, and samples are incubated on such an array. Target molecules present in the sample bind to specific capture molecules and are visualized and quantified using reporter systems [4,5]. In a reverse phase protein array, a complex mixture of proteins originating from tissue, cell lysate or from sample fractions are immobilized on the arraying substrate. These sample arrays are probed with antibodies and information on the expression of a

specific analyte obtained [6–8]. Other applications of protein microarrays include functional studies like the parallel interaction analysis between using a large set of immobilized proteins that is probed with differing types of binding molecules such as proteins, nucleic acids, peptides, low molecular weight compounds or oligosaccharides [9,10].

Due to the miniaturization and parallelization, protein microarrays allow a tremendous increase in throughput and output of biological experiments. The generation of protein arrays within a microtiter plate is of interest for all those applications that focus on a small and clear defined set of analytes, which is often the case in diagnostic systems. The detection of autoimmune antibodies in human serum or the testing for antibodies directed against parasitic and viral antigens or allergens [11–17] are typical diagnostic applications for focused protein microarrays. Protein arrays within a microtiter plate format can also be used to perform miniaturized parallelized sandwich immunoassays to quantify several parameters from one single sample. Several laboratories developed sandwich immunoassays in the protein microarray format and commercially available kits are already on the market. The microtiter plate format allows an easy automation and first approaches showed that

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miniaturized and parallelized sandwich immunoassays can be set-up. A fully-automated, microarray-based approach for high-throughput, enzyme-linked immunosorbent assays adapted to the microtiter plate format was described by Mendoza et al. [18]. This system consisted of an optically flat glass plate with 96 wells separated by a Teflon mask. A microarray containing more than a hundred different types of capture molecules could be immobilized in each well. Sample incubation, washing and fluorescence-based detection was performed with an automated liquid pipettor. The microarrays were quantitatively imaged with a scanning charge-coupled device detector. Thus, the feasibility of multiplex detection of arrayed antigens in a high-throughput fashion using marker antigens could be successfully demonstrated. Nielsen et al. [19] described an antibody microarray integrated in 96-well microtiter plates with which they could quantify amount and phosphorylation state of ErbB receptors in cell lysates. However, these approaches have not found their way into the research laboratories, probably due to the fact that most of the current microarray readers are using the microscope-slide format. Therefore, systems that are capable of adapting the slide format to the easy to automate format of the microtiter plate have been developed. Shao et al. [20] established miniaturized and multiplexed sandwich immunoassays, allowing the quantification of 150 different cytokines and other serum components. The assay was carried out on microscopic slides, using 16 subarrays.

Several companies have started to offer adaptors from microscope-slides to microtiter plates. Here we use a grid corresponding to two columns of a 96-well microtiter plate that is placed onto a single microscope slide containing up to 16 identical arrays. Four slides can be used to fill a frame of a standard microtiter plate format. This set-up allows a parallel processing of a large number of microarrays using standard pipetting systems. An increase in throughput can be anticipated by processing several microtiter plates of microarrays. After the assay, the grid is removed and the microscopic slides can be imaged in a standard biochip reader. This set-up allows individual research facilities to increase throughput substantially [4]. Other multiplex technologies that are capable of running similar assays have been developed. Bead-based systems present an interesting alternative to low complexity planar microarrays and for those automation is not problematic, as these bead-based systems are already adapted to the microtiter plate format [4].

Here we describe the integration of a microagitation device into an automated system for processing microarrays in the microtiter plate format. It employs surface acoustic waves generated by a piezoelectric device for the mixing of the fluid sample incubated on a protein microarray (Fig. 1). The energy generated by the SAW chip is coupled through the glass slide into the sample and is an efficient source of agitation. This microagitation is capable of increasing signal intensity and homogeneity during microarray-based assays [21]. We show that the integration of microagitation into an automated system taking over of all the liquid handling steps improves assay reproducibility and has the capability to increase throughput.

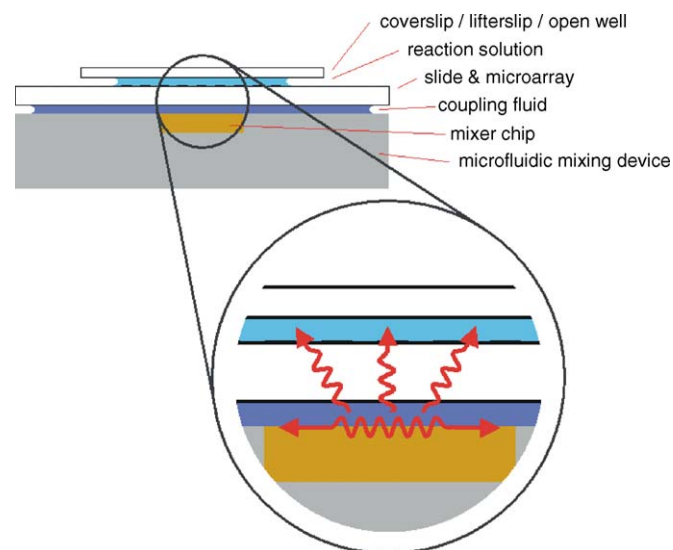


Fig. 1. Surface acoustic waves induced on the mixer chip are coupled through the glass slide into the reaction fluid. Thereby effective mixing of the reaction volume is induced, overcoming the diffusion limitations of most biological reactions.

2. Experimental

2.1. Microarray production

For the generation of autoimmune microarrays, five human recombinant autoantigens Ro 52 kDa (67.5 $\mu\text{g/mL}$), Scl 70 (1000 U/mL), Ro 60 kDa (1000 U/mL), SmB (1000 U/mL) and Pm-Scl 100 (745 $\mu\text{g/mL}$) (kindly provided by Pharmacia Diagnostics, Freiburg, Germany) were printed onto aldehyde-coated glass slides (CEL Associates, Pearland, TX) with a noncontact spotting system (BioChip Arrayer, Perkin-Elmer, Wellesley, MA) (Fig. 2a and b) under clean room conditions at 20 °C and 60% relative humidity. Three hundred and thirty picolitre of each antigen solution was spotted resulting in a microspot with a diameter of 180 μm . Additionally, each autoimmune array contains PBS spots as negative controls, goat anti-human IgG spots (Jackson ImmunoResearch Laboratories, West Grove, PA) for the quantification IgG in serum samples and marker spots containing Cy5 labeled BSA (10 μg BSA-Cy5/mL PBS) for orientation. The autoimmune array contains 28 replicates of each analyte and covers an area of 4.7 mm $\times$ 4.7 mm. Each array consists of four subarrays separated by marker spots. Twelve arrays were printed on a glass slide within the standard microtiter plate pitch of 9 mm.

For sandwich immunoassays a different array layout was used. Here seven MMP specific capture IgGs, solved in PBS at concentrations between 0.2 and 0.5 mg/mL, (MMP1, 2, 3, 7, 8, 10, 13; R&D, Minneapolis, MN, USA; Neomarkers, Newmarket, UK) were immobilized in quadruplicates in a microarray format.

2.2. Blocking

After printing, slides were incubated in a humid chamber at room temperature over night allowing bond formation between

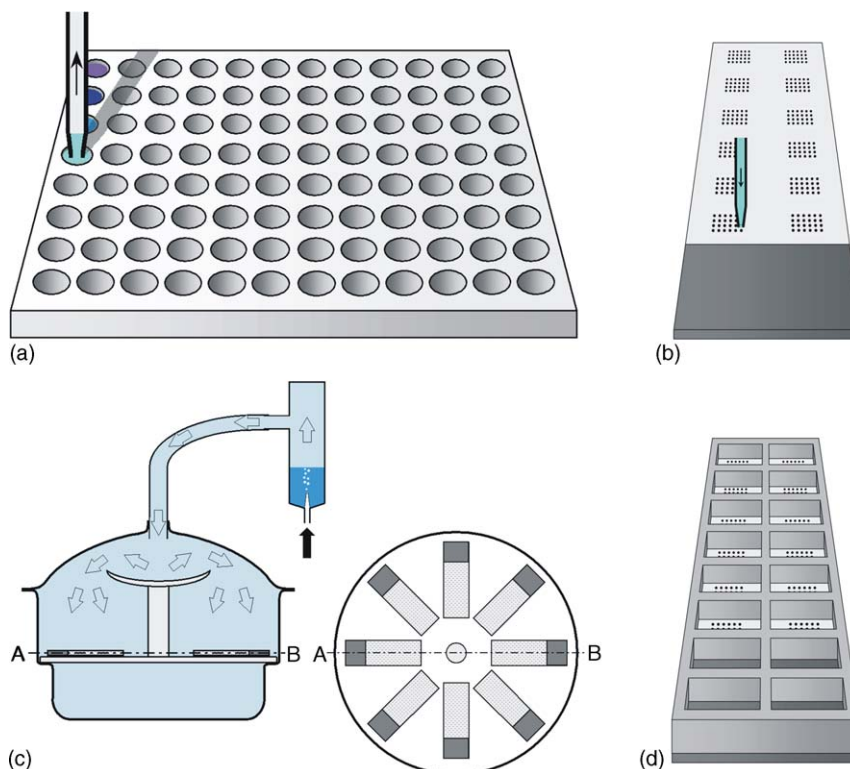


Fig. 2. For the microarray generation autoantigens or capture antibodies were provided in a standard 96- or 384-well microtiter plate (a) and arrayed onto aldehyde-coated glass slides with a noncontact spotting system (b). After printing slides were covered with a thin blocking solution layer using a nebulizer (c) for neutralizing unspecific binding sites. For performing the assay in an automated system standard robotic format chambers were mounted to the glass slides (d).

free amino groups of capture reagents and the aldehyde-coated slide surface. To block the remaining reactive groups on the arraying substrate, slides were incubated in an aerosol of blocking buffer (0.1% Tween 20; 1.5%, w/v BSA; 5%, w/v low fat powdered milk in PBS) for 30 min in a closed container (Fig. 2c). The aerosol was generated by dispersing the blocking buffer into small droplets using the Pari Boy nebulizer (PARI GmbH, Starnberg, Germany). After blocking, the slides were allowed to dry at room temperature for 1 h. Dried slides were stored dry at 4 °C and can be stored under such conditions for several months without significant loss of activity of the immobilized proteins.

2.3. Assay

2.3.1. Automation

Prior the assay, the glass slides with the microarrays were mounted into the ProPlate™ Multiarray Slide adapter (Grace Bio-Labs, Bend, OR). This system adds a reaction chamber to each of the microarrays on the glass slide with a well to well distance of 9 mm (Fig. 2d); by placing four slides inside a frame with the size of a standard microtiter Plate 64 individual microarrays can be processed in parallel.

All liquid handling steps were performed with the Cartesian pipetting robot BioRobot 3000 (Qiagen, Hilden, Germany; Fig. 3 left). This robot is capable of aspirating and dispensing fluids in a range of 10 µL to several millilitres and to move objects with

the shape of microtiter plate. During the incubation times, the ProPlate™ Multiarray Slide adapter was covered by the robot with a lid.

2.3.2. Integration of the mixing device

A prototype of the SAW mixing device (Advalytix AG, Brunenthal, Germany) was integrated into the robotic platform (Fig. 3 right). For the evaluation of the effect of SAW mediated microagitation, one slide was placed in the SAW mixing device. Without direct contact between the sample solution and the mixer chip, the energy generated by the SAW transducer is coupled into the immunoassay and agitates the sample. The SAW mixing device was set to maximum mixing power and interval mixing, meaning 1 s on and 2 s off for 30 min.

2.3.3. Sample preparation and rehydration of slides

Samples, diluted buffers containing the detection molecules and wash buffer were provided in plates or troughs on the deck of the robot. Arrays were rehydrated by rinsing each of the reaction chambers five times with 100 µL of wash buffer (PBS containing 0.1% Tween 20). Both for the autoimmune assay and for the MMP assay, the wash buffer was removed prior samples were applied.

2.3.4. Autoimmune assay

For the analysis of human serum (kindly provided by Pharmacia Diagnostics) the autoimmune microarrays were used. Serum

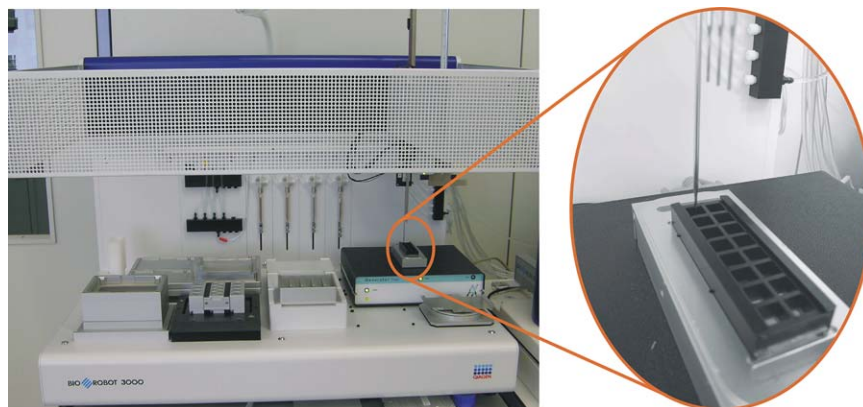


Fig. 3. Assays were performed automatically with a Cartesian pipetting robot. A stirrer less microfluidic mixing device (Advantix, Brunnthal, Germany) based on SAW technology was integrated into the robotic platform to enhance the assay performance. The glass slide with the mounted chamber system was placed on the mixer chip on top of the mixing device (magnification) and can be accessed by the pipetting robot.

had been characterized by Pharmacia with the Varelista test and results were confirmed by data gained from microarrays. Serum was diluted in the range 1:400–1:6400 in assay dilution buffer (0.1% Tween 20; 1.5%, w/v BSA and 2.5%, w/v low fat powdered milk in PBS). The diluted serum samples were incubated for different times on different arrays. After washing the array five times with wash buffer Cy5 goat anti-human IgG detection antibody (3 $\mu\text{g/mL}$ in assay dilution buffer; Jackson ImmunoResearch Laboratories, West Grove, PA) was added and the arrays were incubated for 30 min (Fig. 4a). A kinetic of the mixing was obtained by applying identical samples to different wells at different time points.

2.3.5. MMP assay

The sandwich immunoassay was performed using the following conditions: (1) 30 min sample incubation; (2) 30 min

incubation with biotinylated detection antibody mix; (3) 10 min incubation with streptavidine-Cy5 (Fig. 4b). For this study a mixture of recombinant MMPs (7.8 ng/mL to 1.9 $\mu\text{g/mL}$) (R&D, Minneapolis) was used. The detection antibody solution contained the different MMP specific antibodies (R&D, Minneapolis; Neomarkers, Newmarket) at a concentration of 1.0–3.0 $\mu\text{g/mL}$ and streptavidine-Cy5 (Amersham Biosciences Europe GmbH, Freiburg, Germany) was diluted to 5 $\mu\text{g/mL}$. MMPs, detection antibodies and streptavidine-Cy5 were diluted in assay dilution buffer.

2.4. Detection and quantification of signals

The ProPlateTM Multiarray Slide System was removed from the slides and the slides were washed once manually with wash buffer and then rinsed briefly with cold tap water. Finally,

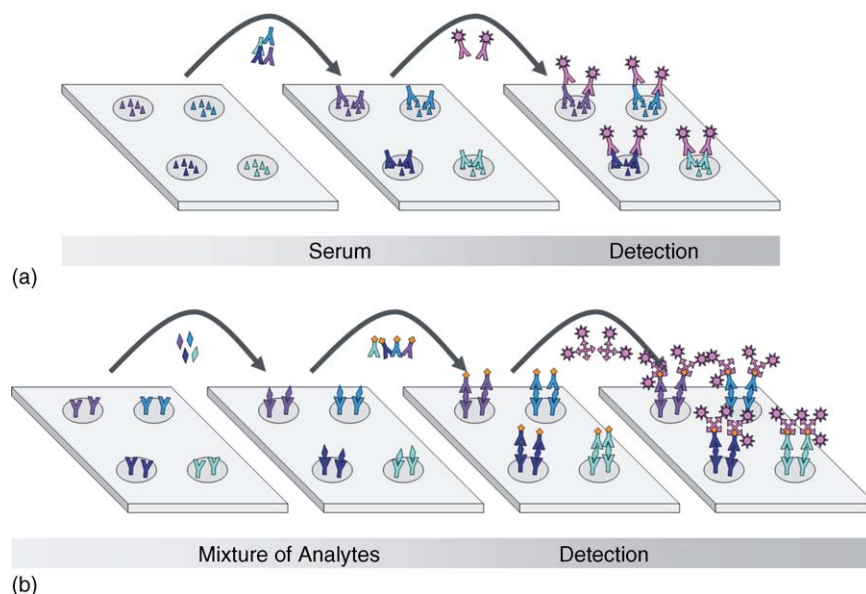


Fig. 4. Two types of assays were used: (a) antigen–antibody format; arrayed autoantigens were incubated with a serum sample of a patient suffering from autoimmune disease. Detection of bound autoantibodies was achieved using a fluorescently labeled human IgG specific detection antibody and (b) sandwich immunoassay format; capture antibodies were arrayed to capture their specific antigen out of a mixture of different MMPs. Bound MMPs were visualized using biotinylated detection antibodies and fluorescently labeled streptavidin as the reporter molecule.

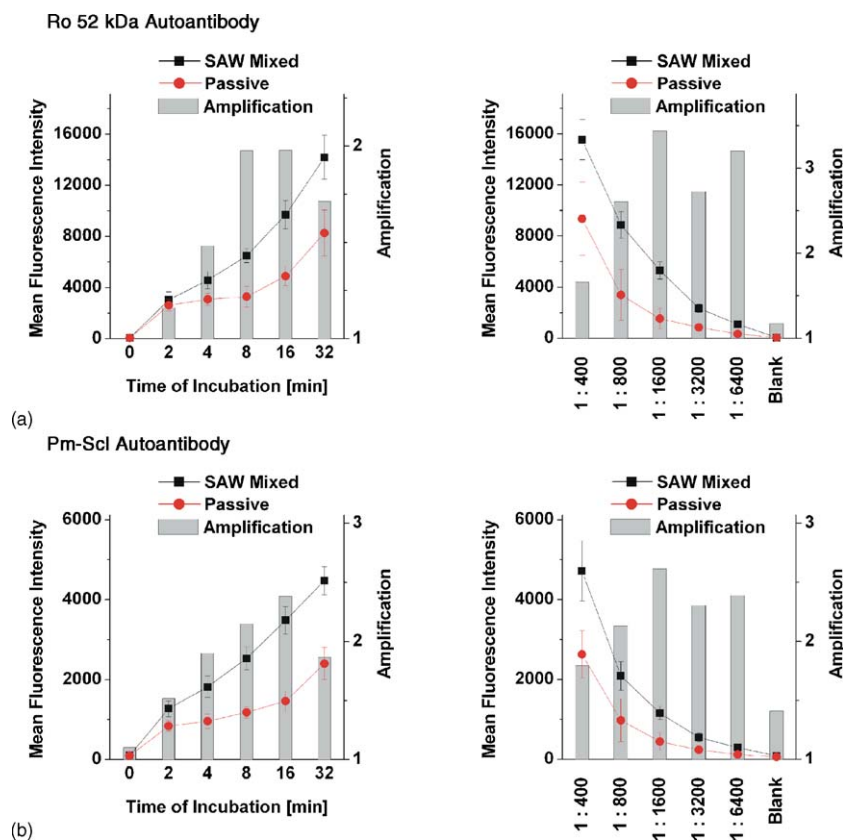


Fig. 5. Analysis of human serum was added to protein microarrays consisting of different recombinant autoantigens. Signal intensities on the antigen spots containing Ro 52 kDa (a) and Pm-Scl (b) are shown. Serum was diluted from 1:400 to 1:6400 and incubated for 30 min. For the time course between 0 and 30 min, serum was diluted 1:400. The bars represent standard deviations of 28 replicates and columns show the ratio of SAW mixed vs. passive incubation and can be interpreted as an amplification factor.

remaining traces of buffer were washed away with Millipore grade water and the slides were dried in a stream of nitrogen.

Bound fluorescent reporter molecules were visualized with a confocal scanner. Slides were scanned with a GenePix 4000B microarray scanner (Molecular Devices Corporation, Union City, CA). The laser wavelength has been adjusted to Cy5 dye and photomultiplier gain was adjusted to avoid saturation of signals. The resolution of resulting 16 bit TIFF images was set to 10 μm . Picture analysis was done with ImageJ 4.0 (BioDiscovery, El Segundo, CA, USA).

3. Results and discussion

An integrated system that uses standard liquid handling robotics in combination with a novel integrated microagitation device was set-up to increase throughput and reproducibility of protein microarray-based assays.

Antigen antibody interactions and miniaturized sandwich immunoassay formats were used to study the effect of microagitation using the surface acoustic wave technology. The reaction of antibodies from an autoimmune serum with different immobilized autoantigens was used to analyze the influence of SAW mediated microagitation on the binding to the immobilized autoantigens. The serum has a known reactivity for the autoantigens Ro 52 kDa, Ro 60 kDa, and Pm-Scl. Arrays con-

sisting of 28 replicates of autoantigens (Ro 52 kDa, Scl 70, Ro 60 kDa, SmB, Pm-Scl 100) and control spots (BSA, spotting buffer) were used to analyze the sample at different dilutions and for differing sample incubation times. A dilution series of serum ranging from 1:400 to 1:6400 is shown in Fig. 5 on the left; a time series with incubation times varying from 2 to 32 min is seen in Fig. 5 on the right.

With the dilution series the specific signals for Ro 52 kDa and PmScl100 specific autoantibodies were enhanced by a factor of 1.6 up to 3.4 when mixed with the SAW-based mixer and signal increase is more significant at lower autoantibody concentrations. These results are in accordance with observation that show that analyte depletion in solid phase assays is responsible for a loss of sensitivity since the concentration of analyte in the vicinity of microspots is decreased [22]. The resulting gradient of analyte concentration is compensated by SAW mixing. The use of surface acoustic wave mixing lead only to slight increase in background (data not shown).

For time series the serum was diluted 1:400 in assay dilution buffer and assay times were varied from 2 to 32 min. At first glance the kinetic seems to be accelerated by SAW mixing but as mentioned above the reaction turns from a mass transport controlled reaction towards a real kinetic. Hereby the highest amplifications are gained at 8 and 16 min. In theory, the most obvious depletion of analyte is observed at short incubation

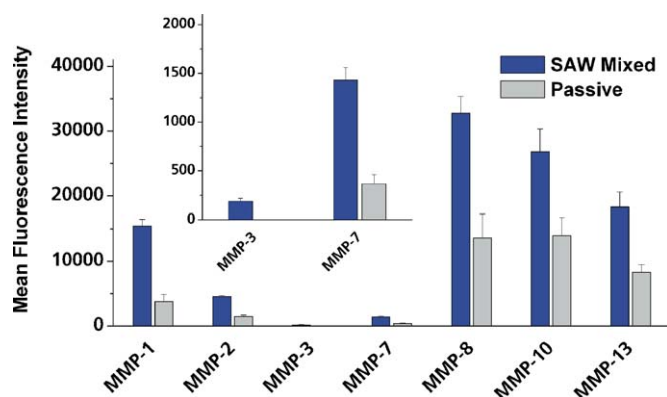


Fig. 6. Simultaneous detection of different recombinant MMPs in a mixture. Assays enhanced by microfluidic mixing are confronted with passive incubation. Each column represents quadruplicates.

times, here the effect of mixing is seen later. Due to an unavoidable delay in the working protocol of the robot, the incubation time of the passively incubated slide took 10 s longer than the mixed one. While the increase of 10 s for the passively incubated slide is small at long incubation times, it does contribute significantly to the generated signal when the incubation time is short.

A miniaturized sandwich immunoassay for the analysis of seven different MMPs was used as the second model assay. A similar increase in signal intensity up to a factor of three could be achieved with this system. Again, the effect of the SAW mixing was more prominent at low analyte concentrations. As shown in Fig. 6, the analyte MMP3, which was used at very low concentration, was only detectable when using SAW microagitation.

As shown in Tables 1–3 both, the autoimmune assay as well as the sandwich immunoassays result in significant lower standard deviations when using the SAW based microagitation. While the automation alone gave a significant increase in throughput the integration of the SAW based microagitation also results in an increase in sensitivity. An enhancement of the measured signals up to a factor of three was observed while false positive signals

Table 1
Analytical results and relative standard deviation (R.S.D.) for dilution series of serum of an autoimmune patient

Analyte	Dilution	Method			
		SAW mixed		Passive	
		Mean (MFI)	R.S.D. (%)	Mean (MFI)	R.S.D. (%)
Ro 52	1:400	15547 ± 1564	10.1	9365 ± 2854	30.5
	1:800	8864 ± 1037	11.7	3402 ± 1984	58.3
	1:1600	5329 ± 664	12.5	1550 ± 805	51.9
	1:3200	2355 ± 317	13.5	864 ± 131	15.1
	1:6400	1113 ± 199	17.9	348 ± 150	43.2
	Blank	69 ± 15	21.5	59 ± 14	23.3
PmScl	1:400	4716 ± 754	16.0	2630 ± 595	22.6
	1:800	2089 ± 359	17.2	981 ± 536	54.6
	1:1600	1162 ± 164	14.1	444 ± 227	51.1
	1:3200	548 ± 103	18.8	238 ± 53	22.1
	1:6400	299 ± 50	16.9	125 ± 38	30.1
	Blank	89 ± 5	5.9	63 ± 16	25.9

Table 2

Analytical results and statistical data for kinetic of serum of an autoimmune patient

Analyte	Time (min)	Method			
		SAW mixed		Passive	
		Mean (MFI)	R.S.D. (%)	Mean (MFI)	R.S.D. (%)
Ro 52	32	14172 ± 1716	12.1	8269 ± 1787	21.6
	16	9690 ± 1103	11.4	4898 ± 756	15.4
	8	6494 ± 538	8.3	3287 ± 806	24.5
	4	4582 ± 684	14.9	3090 ± 466	15.1
	2	3049 ± 619	20.3	2627 ± 462	17.6
	0	55 ± 9	16.8	57 ± 19	32.4
PmScl	32	4479 ± 353	7.9	2403 ± 398	16.6
	16	3485 ± 339	9.7	1463 ± 265	18.1
	8	2533 ± 285	11.3	1180 ± 142	12.0
	4	1821 ± 265	14.5	959 ± 184	19.1
	2	1274 ± 198	15.6	840 ± 129	15.4
	0	103 ± 39	37.9	93 ± 35	37.3

and background noise were not significantly enhanced. In independent experiments, it could be confirmed that the increase in signal intensity was not caused by an increase in temperature. For these experiments, the assay temperature was raised in steps of 1 °C from 37 to 45 °C. The maximum signal intensity (without SAW mixing) was reached at 41 °C. The signal was about 25% above the signal at 37 °C, i.e. well below the signal intensity boost observed in the SAW experiments. Without SAW microagitation no amplification was observed.

As described in Section 2, we employed a novel approach to apply the blocking buffer to the microarrays. By using a commercially available nebulizer an aerosol was generated and left to settle on the surface of the glass slides containing the printed capture proteins. This treatment results in a homogenous layer of blocking proteins (Fig. 7a) that efficiently suppress unspecific binding while a uniform spot morphology is preserved. Often the amount of capture molecules spotted to the surface exceeds the binding capacity of the substrate. When the blocking buffer is applied into the chamber and is flushed over the microspots, excess capture molecules are washed away and may bound onto the substrate, resulting in a tail of the spot that exalts the local background (Fig. 7b and c). In addition the spraying of the blocking buffer on the surface leads to a very thin and defined blocking

Table 3

Analytical results and statistical for measurement of different MMPs out of a mixture within a single assay

Sample	Method			
	SAW mixed		Passive	
	Mean (MFI)	R.S.D. (%)	Mean (MFI)	R.S.D. (%)
MMP-1	15443 ± 979	6.3	3794 ± 1092	28.8
MMP-2	4542 ± 179	3.9	1481 ± 201	13.6
MMP-3	189 ± 29	15.3	0.38 ± 0.75	197.4
MMP-7	1430 ± 127	8.9	365 ± 98	26.8
MMP-8	32787 ± 2568	7.8	13575 ± 3676	27.1
MMP-10	26821 ± 3503	13.1	13975 ± 2745	19.6
MMP-13	18305 ± 2288	12.5	8314 ± 1138	13.7

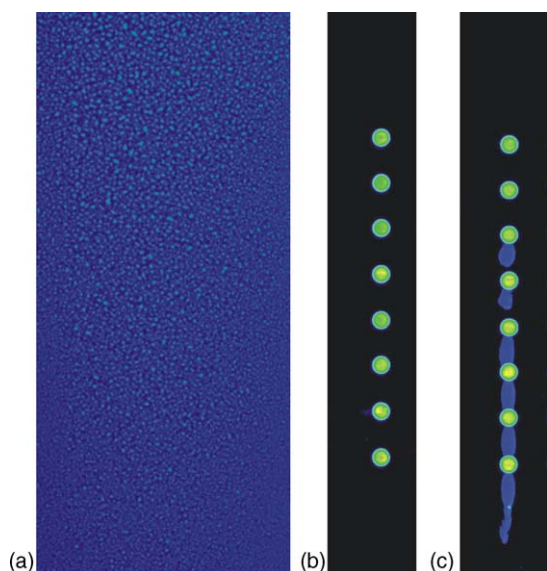


Fig. 7. Slides are blocked with a nebulizer resulting in a thin and homogenous layer of blocking proteins (a). Additionally, the uniform spot morphology is preserved even if excess capture molecules have not bound to the surface (b). Often excess capture molecules are washed away resulting in a tail of the spot when blocking buffer is flushed onto the microspots (c).

layer that is easily removed during the rehydration step prior the assay.

4. Conclusions

Protein microarray assays, like all solid phase assay systems, are driven by diffusion. While the miniaturization helps to overcome analyte depletion effects [23] and equilibrium conditions are reached faster as in macro systems, diffusion limitation still can account for a loss in sensitivity. Therefore, we tested the surface acoustic wave technology as a possibility to mix samples incubated on a protein microarray. The technology is capable of mixing very small volumes. In principle SAW are not more effective than mechanical mixing but it is still feasible in small volumes where mechanical shaking or stirring is not possible any more. Moreover, there is no need for moveable components that may cause troubles like ageing of the components or shaking the periphery of the mixer and the whole system.

SAW microagitation results in a more homogeneous distribution of the analytes in the sample, and increases sensitivity or shortens incubation times by up to a factor of three. SAW microagitation turns the reaction from a mass transport controlled reaction towards a kinetic reaction. SAW microagitation can easily be integrated into conventional and existing processes. Mixing occurs without any direct contact to the analytical solution and no major changes during an assay workflow have to be introduced. We tested our system using a multiplexed autoimmune assay and a parallelized sandwich immunoassay. These types of reactions were chosen since they resemble the most commonly used assay employed in diagnostic testing. Therefore, the approach of using automated microarray-based assays for diagnostic purposes is feasible.

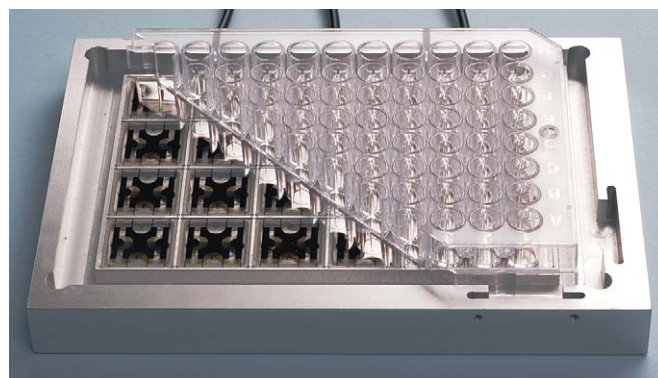


Fig. 8. SAW mixer for 96-well microplates enabling effective agitation of fluid in each single well without moving the microplate. The mixer is planned to be extended to plates with higher well and can be also extended to mixing slides in parallel.

Nevertheless, before multiplexed systems will be able to enter the highly robust screening or diagnostic area, sound validations of miniaturized assay systems are required. Any new assay format intended for use in screening or diagnostic applications has to compete with the current highly robust technology not only with regard to its performance but also with regard to its costs. Microarrays are not competitive if only a few additional parameters have to be analyzed from a sample. It would be much more economic to increase the throughput of the currently highly robust and automated analyzers. However, with a continuously growing number of parameters to be analyzed and with the increasing knowledge in system biology and cellular networks, multiplexing analyses will become a very demanding issue. Planar microarrays or bead-based systems offer promising solutions. Therefore, the integration of microagitation devices using surface acoustic waves can be of high interest for DNA, Oligonucleotide, protein, and tissue microarrays since it is capable of improving signal-to-noise ratios and can lead to improvements in the homogeneity of the assay. There are already solutions of SAW microagitation systems available for 96-well microplates enabling effective agitation of fluid in each single well without moving the microplate (Fig. 8). The SAW technology of Advantix is completely compatible to the microtiter-plate-world and all kinds of automation. Mixers for plates with higher well numbers are in progress and the technology is easily extendable for mixing slides in parallel.

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