Layered Peptide Arrays

High-Throughput Antibody Screening of Clinical Samples

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High-throughput methods to detect and quantify antibodies in sera and other patient specimens have use for many clinical and laboratory studies, including those associated with cancer detection, microbial exposures, and autoimmune diseases. We developed a new technique, termed layered peptide array (LPA), to serve as a screening tool to detect antibodies in a highly multiplexed format. We demonstrate here that a prototype LPA was capable of producing approximately 5000 measurements per experiment and appeared to be scalable to higher throughput levels. Sera and saliva from Sjögren's syndrome patients served as a test set to examine antibody titers in clinical samples. The LPA platform exhibited both a high sensitivity (100%) and high specificity (94%) for correctly identifying SSB antigen-positive samples. The multiplex capability of the platform was also confirmed when serum and saliva samples were analyzed for antibody reactivity to several peptides, including Sjögren's syndrome antigens A and B. The data indicate that LPA analysis will be a useful method for a number of screening applications. (*J Mol Diagn* 2005, 7:427–436)

Antibodies play a major role in the adaptive immune response due to high-affinity binding to specific epitopes on target antigens.¹ Human sera contain approximately 10 million different antibodies with activity against a wide-range of potential pathogens.² In clinical medicine, sera from patients are frequently analyzed for the presence or

absence of a few specific antibodies as a guide to diagnosis and therapy, for example, in the case of infectious or autoimmune diseases. More recently, it has been suggested that high-throughput antibody screening might have additional uses in the clinic and laboratory.² For example, detection of autoantibodies that recognize tumor antigens may become an effective screening tool for cancer. In this approach, patient sera would be tested for the presence of any one of a relatively large panel of antibodies against unique antigens expressed by neoplastic cells. Applied successfully, it would allow physicians to screen whole populations (or specific at-risk populations) for the presence or recurrence of a tumor as an adjunctive tool to current diagnostic techniques. Similarly, screening sera samples for a panel of antibodies directed against toxic or infectious agents could be useful for monitoring exposures in a population. In the laboratory, multiplex antibody screening may facilitate research efforts, for example, by allowing investigators to rapidly and inexpensively identify hybridoma clones that produce antibodies with a well-characterized antigen binding profile.³

In the current genomic era, high-throughput analysis tools have found widespread popularity and have facilitated a number of laboratory operations, ranging from large-scale DNA sequencing strategies, to high-density expression microarrays, to production and analysis of complex proteomic datasets. In each case, the work was made possible because of technical advancements permitting highly parallel analyses to be performed at relatively low cost. Such a new technology is needed to advance the field of antibody screening. Ideally, the assay system would allow sizeable numbers of samples to be tested for relatively large panels of antibodies, perhaps on the order of 25 or more depending on the rationale for the examination. In the present study, we evaluated the ability of a layered peptide array (LPA) platform to detect and quantify antibodies. Throughput capability, sensitivity, and specificity of the assay were evaluated using purified antibodies or antibody combinations under a variety of experimental conditions. To evaluate the clin-

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ical effectiveness of the assay, serum and saliva samples from Sjögren's syndrome (SS) patients, an autoimmune connective tissue disorder with characteristic autoantibodies,⁴ were analyzed, and the data were compared with that derived from matching enzyme linked immunosorbent assays (ELISAs).

Materials and Methods

Antibodies and Serum Samples

Serum samples were collected from 35 SS patients who were diagnosed at the National Institutes of Health (NIH) Salivary Gland Dysfunction Clinic and from eight healthy normal volunteers (NV). All individuals signed consent to participate in a clinical research study that was approved by the Institutional Review Board (study numbers 84-D-0056 and 94-D-0018). Patients were grouped to early, moderate, or severe disease activity according to Greenspan grading of the minor salivary glands.⁴ Sera were tested on the day of collection at the NIH clinical center for the presence or absence of anti-SS antigen A (anti-SSA) and anti-SS antigen B (anti-SSB) as determined by ELISA (Hemagen Diagnostics, Columbia, MD). Antibodies and peptides used in the study are shown in Table 1. All dilutions were performed in phosphate-buffered saline, pH 7.4 (Invitrogen Corporation, Carlsbad, CA). Detection of antibodies on membranes was done using secondary rabbit anti-goat-fluorescein isothiocyanate (FITC), goat anti-human IgG-FITC or mouse anti-rabbit-FITC in a dilution of 1:400 (catalog numbers sc-2777, sc-2456, and sc-2359, respectively; Santa Cruz Technology, Santa Cruz, CA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples were evaluated for anti-SSB using an ELISA kit (Hemagen Diagnostics) according to the manufacturer's recommendation.

Layered Peptide Array-Coated Membranes

P-FILM Smart Antibody Affinity membranes were used in the study (20/20 GeneSystems, Inc., Rockville, MD; www.2020gene.com). The membranes were cut to appropriate size to fit the gel or the 96-well plate.

Layered Peptide Array

Prototype 1

Membranes were equilibrated in transfer buffer (6.07 g of 50 mmol/L Tris, 380 mmol/L glycine, 28.54 g in 1 L of deionized water). A 2% agarose gel (Gibco-BRL, NY) was prepared according to the manufacturer's recommendation in a B-1 casting booth (OWL Separation Systems, NH), and then a 14-well comb was inserted.

The wells were loaded with antibodies or serum samples diluted in PBS as shown in Table 1. The gel

was supported on a gel blot paper GB004 (Schleicher & Schuell, NH), and a multilayered system for capillary transfer was prepared as follows (from bottom to top layers): 1) large tray (14 × 20 cm); 2) inverted B1 casting booth; 3) gel supported by blot paper; 4) P-FILM Smart Antibody Affinity membranes; 5) 20 blot papers (5 × 8 cm); 6) weight (7 to 10 g/cm²); and 7) sealing wrap.

Transfers were done overnight, followed by washing of membranes three times in Tris-buffered saline Tween 20 (TBST) (50 mmol/L Tris HCl, 150 mmol/L NaCl, and 150 mmol/L Tween 20) for 5 minutes each, and then incubation with second antibody for 30 minutes at room temperature with shaking, followed by another wash in TBST. Membranes were dried on a filter paper (Whatman, NJ) and scanned on a Typhoon scanner with 520 BP40 filter (Typhoon 9410; Amersham Biosciences, NJ).

Prototype 2

P-FILM Smart Antibody Affinity membranes were placed within a vacuum plate (Bio-Rad, Hercules, CA). Antibodies were applied to the 96 wells in the plate and incubated for 5 minutes. Vacuum was applied for 5 minutes followed by washing of the membranes in TBST, application of secondary antibodies, and scanning as described above.

Thirty-two SS and eight NV serum or saliva samples were placed in duplicates in the 96-well plate and P-FILM membranes coated with SSA, SSB, major outer membrane protein (MOMP), Cocksackie and adenovirus receptor (CAR), CagA, muscarinic acetylcholine receptor 3, Fas and caspase 3 were placed in duplicates between the samples and the vacuum. For every patient/NV, 1 μ l of serum or 5 μ l of saliva was used per experiment. The vacuum was performed for 5 minutes followed by disassembling the plate, washing the membranes, and reacting with secondary, fluorescein-conjugated antibody following the protocol for prototype 1. Each experiment was repeated four times, and the mean membrane signal intensity was calculated for eight total membranes for each peptide/antigen (four experiments, two membranes per experiment for every antibody/peptide set) for each patient.

Normalization with a lactoperoxidase antibody-antigen pair was performed in each well. The patient and NV samples were placed in an arbitrary order in the plate, and the calculation of the signal was done in a blinded manner. Unmasking of the groups was done after the signals were averaged and the SD calculated. Background activity was calculated as the signal of the non-relevant positive control antibodies for the other membranes in the stack. For example: for the SSA and SSB membranes, the background was the mean signal from the positive control antibodies for CAR, M3, caspase 3, caspase 1, aquaporin 5 (AQP5), cytokeratin 7, and PIM1 in the same experiment.

Table 1. Antibodies and Antigens

Antibody	Antigen	Catalog number	Company	Dilutions
Cytokeratin 7 goat anti-human	Cytokeratin 7 peptide	sc-17116	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
AQP5 goat anti-human		sc-17116p	Santa Cruz Technology	2 μg/ml
Pim-1 goat anti-human	AQP5 peptide	sc-9890	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
	Pim-1 peptide	sc-9890p	Santa Cruz Technology	2 μg/ml
Muscarinic acetylcholine receptor M3 goat anti-human		sc-7856	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
	sc-7856p	Santa Cruz Technology	2 μg/ml	
Lactoperoxidase rabbit anti-bovine	Muscarinic acetylcholine receptor M3 peptide	sc-7474	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
	Lactoperoxidase antigen	sc-7474p	Santa Cruz Technology	2 μg/ml
FAS rabbit anti-human		RAB/LPO	Nordic Immunology (Tilburg, The Netherlands)	1:100, 1:200, 1:400, 1:600
<i>Helicobacter pylori</i> (CagA) goat anti- <i>H. pylori</i>	FAS peptide	L8257	Sigma-Aldrich (St. Louis, MO)	25 μg/ml
	<i>Helicobacter pylori</i> (CagA) peptide	sc-715	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
Chlamydia, MOMP goat anti- <i>C. pneumoniae</i>		sc-715p	Santa Cruz Technology	2 μg/ml
	Caspase 3 goat anti-human	sc-17450	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
sc-17450p		Santa Cruz Technology	2 μg/ml	
Coxsackie and adenovirus receptor goat anti-human	Chlamydia, MOMP peptide	sc-17376	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
	Human autoantibody against SSA antigen	sc-17376p	Santa Cruz Technology	2 μg/ml
Human autoantibody against SSB antigen		Caspase 3 peptide	sc-1225	Santa Cruz Technology
	SSA antigen	sc-1225p	Santa Cruz Technology	2 μg/ml
SSB antigen		sc-10314	Santa Cruz Technology	1:50 (4 ng/μl), 1:100, 1:200, 1:400
	SSB antigen	sc-10314p	Santa Cruz Technology	2 μg/ml
SSA antigen		HSA-0100	Immunovision (Springdale, AR)	1:200
	SSB antigen	SSA-3000	Immunovision	7.5 μg/ml
SSB antigen		HSB-0100	Immunovision	1:200
	SSB antigen	SSB-3000	Immunovision	7.8 μg/ml

A list of the antibodies, peptides, and antigens used in the study.

Data Analysis

Density Measurements

Images of the membranes were imported to the ImagePro 4.5 analysis software (MediaCybernetics, MD) for analysis. Each membrane included slots according to the amount of wells (in prototype 1) or 96 dots (in prototype 2). The optical density was calculated by the program for each well in the membrane by marking a rectangle around each slot (in prototype 1) or a circle containing each dot (in prototype 2). The optical density was defined according to the following formula:

$$OD = -\log_{10}(x/256)$$

where 256 represents the total number of gray levels in the image and x represents the individual level of gray of each object (each slot of the total slots per membrane in prototype 1 or each well of the 96 wells for each membrane in prototype 2). The measurements were repeated four times, while changing the position of the different

coated membranes in the stack. Thus we generated a dataset of average optical densities for each well in all of the membranes. The data were imported to Microsoft Excel, and mean $\pm$ SD values were calculated.

Comparison of LPA with ELISA Results

To compare the results of the ELISA, which are represented in arbitrary units, and the LPA values, which are represented in intensity of signal, we had to normalize both sets to transfer them to one common set of values between 0 and 1. Thus we divided each set by the maximal result in this set.

Statistical Analysis Software

One-way analysis of variance was applied to the data using PartekPro (Partek Inc., St. Charles, MO). The principal component analysis (PCA) module of the PartekPro software package (Partek) was used to analyze the re-

Figure 1. Schematic of LPA system. Each membrane is coated with a different peptide or antigen containing an epitope for the antibody of interest. Antibodies move from the multiwell grid and through the membranes where they are captured by their corresponding antigens. Labeled secondary antibodies are used for detection.

sults. The data were imported from Microsoft Excel to PartekPro 5.1, and a PCA scatterplot graph was generated for the two different groups (SS patients and NV) with 520 measurements (mean values of 40 serum samples multiplied by 8 antibody groups = 320, plus mean values of 40 saliva samples multiplied by 5 antibodies = 200).

Results

Use of the LPA platform is shown schematically in Figure 1. Membranes are coated with peptides specific to the antibodies of interest. Test samples are placed in a multiwell grid and passed through the analysis layers while maintaining their two-dimensional positions within the network. If present in a sample, antibodies are specifically captured by their target peptide as they pass through the layers and subsequently detected using standard secondary antibody-based methods. The LPA platform can be described in terms of three dimensions: the x-y plane of the life science platform (the array of samples in this case) and the z-dimension representing the stack of capture membranes. In the present study, we demonstrate measurement of 96 samples across 50 membranes, producing 4800 measurements in each experiment.

The initial evaluations of the LPA platform for antibody detection were performed using a first-prototype system

containing 10 layers and 5 different antibodies (against cytokeratin, lactoperoxidase, caspase1, PIM1, and AQP5). To test reproducibility, peptides corresponding to each antibody were coated onto two different membranes within the stack (z-dimension), for example, cytokeratin peptide was coated onto membranes 1 and 6, lactoperoxidase peptide was coated onto membranes 2 and 7, and so on. The sample set (x-y plane) was composed of six samples in individual wells, including each antibody in purified form (wells 1 to 5, respectively), and a sixth well that contained a mixture of the five antibodies together. The samples were passed slowly through the membrane stack overnight by capillary transfer, and the antibody capture was assessed on day 2 using a FITC-labeled secondary antibody. The results shown in Figure 2 indicate that each antibody was captured on its corresponding peptide-coated membrane, with little or no non-specific background signal. Additionally, the capture occurred efficiently for single antibodies as well as with the antibody mixture.

We next moved to the evaluation of the assay using clinical samples. In these experiments, we measured the levels of two characteristic autoantibodies used in an established classification criteria set for the autoimmune disease Sjögren's syndrome. The assay format and experimental conditions were similar to those used in the experiment described above, except that proteins corresponding to SSA and SSB were used in place of the

Figure 2. LPA prototype 1: antibody control experiment. Demonstration of successful antibody capture using a 10-layer LPA system.

lactoperoxidase and caspase1 peptides. Figure 3A shows the order of the coatings among the five membranes (z-dimension), and Figure 3B shows the data in bar graph form. The samples included: sera from three SS patients seen in a clinical study at the NIH known to be positive for SSA and/or SSB; positive control sera for SSA and SSB; normal human serum as a negative control; and purified antibodies corresponding to each of the five antigens coatings. The data shown in Figure 3B indicate that the assay system was capable of reliably detecting the SSA and SSB antibodies in the clinical serum samples, similar to the corresponding purified antibodies. Serum from patient 1 was positive for SSA antibody only. Antibodies against SSB, cytokeratin, AQP5, and PIM1 were not detectable. Serum from patient 2 was positive for SSA and slightly positive for SSB. Serum from patient 3 was positive for SSA and SSB. The positive and negative control samples performed as expected. The panel represents a summary of four different experiments with each sample run in triplicate.

To further evaluate the data, we compared the LPA results with those derived from standard ELISAs performed at the NIH Clinical Center for the three SS patients. Figure 4A, left, shows that all three were positive for SSA, with patients 2 and 3 showing SSA titers higher than patient 1. Similar results were seen on the LPA

Figure 3. LPA prototype 1: serum samples. **A:** Schematic drawing of LPA system. Each color designates a different peptide coating. **B:** Bar graph showing LPA analysis of patient samples and controls.

platform, as shown in Figure 4A, right. For SSB antigen, we performed an ELISA in the laboratory side-by-side with the LPA analysis. As seen in Figure 4B, the two approaches produced similar results, with patient 1 being negative for SSB by both methods, patient 2 positive for SSB, and patient 3 strongly positive for SSB.

Having established the basic experimental parameters for the assay, we next moved to a second-generation prototype system composed of a 96-well vacuum plate and 50 membranes, capable of producing 4800 measurements per experiment (Figure 5A). We initially tested the system using purified antibodies as shown in Figure 5B. The experiment used 10 antibody-antigen pairs, with each peptide coated on every 10th membrane, to produce 5 “identical” experiments. For example, cytokeratin peptide was coated on membranes 1, 11, 21, 31, and 41; lactoperoxidase peptide was coated on membranes 2, 12, 22, 32, and 42; and so on. The vacuum-based prototype system offered the advantage of well-controlled movement of the samples through the membrane stack, thus we were able to examine transit times and determine that antibody capture occurred efficiently in as short as 5 minutes. The 96-well grid contained each of the antibodies at four separate dilutions, as well as a mixture of the antibodies together. Figure 5B illustrates the layout of the x-y grid along with the first set of seven membranes that showed a positive signal (z axis). Membrane 1 was coated with cytokeratin peptide; membrane 2, lactoperoxidase antigen; membrane 3, PIM1 peptide; membrane 4, AQP5 peptide; membrane 6, CAR peptide; membrane 8, M3 peptide; and membrane 9, caspase 3 peptide.

The positive results were highly reproducible among the five replicate membranes for each antibody-peptide/antigen, and little or no cross-reaction was observed among the antibodies and nontarget membranes. This is illustrated in Figure 5C showing capture of the CAR antibody by its peptide on membranes 6, 16, 26, 36, and 46. Three antibody-peptide pairs (MOMP, CagA, and FAS)

Figure 4. Comparison of LPA prototype 1 with standard ELISA. **A:** Comparison of SSA antibody titer. **B:** Comparison of SSB antibody titer.

were completely negative by LPA and reflected what was observed with these antibody preparations in other experiments in the laboratory, that they do not efficiently hybridize to peptides immobilized on a surface (data not shown). A simple rule of thumb for LPAs is that any commercially available antibody (or antibody in a patient sample) that recognizes its antigen on a blot (immuno-blot, dot-blot) will work effectively in the system. Figure 5D shows a dilution curve for each of the positive antibody-antigen pairs, indicating that LPAs are capable of measuring twofold changes in antibody titer.

The next step in examining LPA prototype 2 was to analyze serum samples from the clinic. Thirty-two sera from SS patients and 8 normal volunteer controls were assayed using 10 peptide-coated membranes. One of the membranes was coated with SSB antigen, and the results from the LPA system correlated with standard ELISA measurements performed previously as part of the clinical protocol at the NIH. The results are shown in Figure 6A. LPA analysis shows a sensitivity of 100% (correctly identified 22 of 22 positive cases) compared with ELISA, and an LPA specificity of 94% (correctly identified 17 of 18 negative cases). To investigate the single patient in whom a discrepancy was observed, we performed a new ELISA test of this sample. The assay showed that this symptomatic SS patient classified as positive by LPA had low levels of SSB antigen as determined by ELISA, although it was below the Clinical Center threshold for assignment as a positive result. Figure 6B shows the average signal for the SS patients and

normal volunteers for both ELISA and LPA analysis. In each assay system, the average intrapatient reproducibility was similar: a SD of 0.1 for LPA, and a SD of 0.14 for ELISA.

We next used the LPA platform to analyze 80 clinical samples for SSA and SSB, as well as for several autoantibodies previously detected in autoimmune disorders.⁵⁻⁹ In these experiments, the test set included serum samples from 32 SS patients and 8 healthy control subjects and saliva samples from the same 32 SS patients and control group. The analysis membranes were coated with the following peptides: SSA, SSB, MOMP, CAR, CagA, M3, Fas, and caspase 3, as shown in Figure 7. Each sample was run in duplicate and repeated four times. As expected, SSA and SSB were statistically elevated in the patients' sera compared with controls (Figure 7A). The other autoimmune-related antibodies were also elevated in the patients, although the increase was less pronounced and was statistically significant for only two of the comparisons (Figure 7B). The multiplex capability of the LPA platform enabled us to determine the overall difference in serum and saliva immunoreactivity between the patient and control groups. By combining all of the data together, the SS patients and normal volunteers segregated from each other at a *P* value of 0.0000427 for sera and 0.000798544 in saliva. This effect is shown graphically in Figure 8 using a PCA clustering. Moreover, the multiplex dataset allowed us to look for a relationship between the titer of each antibody, or various antibody combinations, and the pathology of the patients. No statistically significant correlation was observed among the early, moderate, or advanced disease categories (data not shown).

Discussion

Rapid, high-throughput screening of antibodies in biological samples will facilitate both clinical and laboratory efforts and may eventually be useful for patient diagnosis or prognosis. In the present study, we used LPAs, a new application of layered expression scanning,¹⁰ as a screening tool to detect antibodies in human sera. LPAs offer increased capacity for antibody measurements, thus allowing investigators to examine patient populations at relatively low cost. Similar to other aspects of genomics and proteomics in which analysis of molecular profiles and patterns is revealing novel insights into pathobiology, analysis of multiplex autoantibody datasets likely will provide value beyond the information provided by a single analyte. For example, Zhang et al¹¹ studied antibodies in cancer patient sera and found that a profile of seven different autoantibodies raised the cancer detection rate significantly compared with analyzing a single antibody. Discovery of new serum autoantibodies against human cancers is currently an area of active investigation in many laboratories, with nearly 200 candidates identified to date.^{12,13} Thus, cancer autoantibody screening may become a useful adjuvant screening tool for oncologists in the future.

Figure 5. LPA prototype 2: antibody control experiment. **A:** Schematic drawing of the system. **B:** Table showing organization of samples, and images of membranes showing successful capture of antibodies. **C:** CAR antibody capture on membranes 6, 16, 26, 36, and 46 (from top to bottom). **D:** Quantitative measurement of signal intensity on each membrane showing twofold dilution of samples.

High-throughput antibody screening may also facilitate serology testing of patient samples for infectious diseases, screening of donated blood products, and monitoring of the immune response to vaccinations, both qualitatively (epitope recognition and response timing) and quantitatively (serum titers). Moreover, the ability to inexpensively, rapidly, and efficiently screen animals for antibodies against diseases such as viruses and other agents is critically important in protecting the food supply.

The on-going development of new technologies for high-throughput antibody screening is a testament to the potential future importance of the field.^{14–22} For example, Robinson et al¹⁴ introduced a microarray for multiplex characterization of autoantibody response. They designed a chip that contained 196 biomolecules and applied serum samples from patients with autoimmune diseases on the chips. Alternatively, Noya et al¹ designed a test strip with 28 different antigens that could be used to evaluate serum samples. Antibody microarray detection

A

Patient number	Patient/Normal Volunteer	SSB ELISA Clinical center ELISA	LPA for SSB antigen
1	P	0	+
2	P	+	+
3	P	+	+
4	P	0	0
5	P	+	+
6	P	+	+
7	P	+	+
8	P	0	0
9	P	+	+
10	P	0	0
11	P	+	+
12	P	+	+
13	P	+	+
14	P	0	0
15	P	+	+
16	P	0	0
17	P	+	+
18	P	+	+
19	P	0	0
20	P	0	0
21	P	+	+
22	P	+	+
23	P	+	+
24	P	+	+
25	P	+	+
26	P	+	+
27	P	0	0
28	P	+	+
29	P	0	0
30	P	+	+
31	P	+	+
32	P	+	+
33	NV	0	0
34	NV	0	0
35	NV	0	0
36	NV	0	0
37	NV	0	0
38	NV	0	0
39	NV	0	0
40	NV	0	0

Figure 6. LPA prototype 2: serum samples. Comparison with standard ELISA. **A:** Table showing comparison of the LPA system and a standard ELISA for SSB antibody titer. Thirty-two Sjögren's syndrome patients and eight healthy volunteers were analyzed. **B:** Quantitative measurements of SSB antibody analysis using an ELISA or the LPA system.

systems are also being developed.^{15,23–25} As an example, Zhou et al¹⁵ developed an antibody microarray detection system for a sensitive two-color measurement

Figure 7. Specific antibody detection in sera and saliva. **A:** Measurement of antibodies in sera and saliva for SS patients (P) or controls (NV). **B:** Analysis of variance values for SS patients and normal volunteers in serum and saliva samples.

using dual label-based detection of antibodies on an array-based platform.

A particular strength of LPAs is the simultaneous assay of multiple patients (or other biological) samples for a relatively large number of analytes as is represented in Figures 7 and 8. The prototype system that we present here is capable of 4800 measurements per experiment; however, the platform is scalable in both the x-y and z dimensions. Based on tissue section analyses, the x-y spatial resolution is less than 100 μm , thus LPAs can be adapted to standard 96-, 384-, or 1536-well plate formats, or even a denser array if necessary. This will permit a high volume of samples to be tested in each experiment and allow for variations in assay design (for example, dilution curves and peptide competition experiments),

Figure 8. PCA clustering for patients (P) and controls (N). PCA mapping of serum and saliva antibodies for 32 SS patients (P) and 8 controls (N).

depending on the needs of the investigator. For the z-dimension, 50 membranes do not appear to be an upper limit for the system. We have previously shown that tissue sections and purified nucleic acid preparations can traverse as many as 100 membrane layers with minimal loss of x-y architecture or decreased capture of biomolecules.¹⁰ Thus, the measurement capacity of LPAs can be extended significantly beyond what is presented here.

To assess the sensitivity and specificity of LPAs, we studied patient serum samples and compared the LPA results with those of ELISAs that were carried out as part of a clinical trial. The two methods performed similarly with respect to identifying positive cases of SS, and the detection sensitivities of LPAs and ELISAs were nearly identical. However, a significant advantage of LPAs over standard ELISA kits is that approximately 10-fold less sample is required to perform the assay. Thus, if a 10-layer system is used, a 100-fold increase in the number of measurements per sample volume is achieved. This is particularly useful for limiting clinical specimens such as the saliva samples in the present study. The relative ability of LPAs and ELISAs to precisely measure antibody levels in clinical sample has yet to be determined. In the future, it is possible that each method will serve a distinct purpose, for example, high-throughput sample screening using LPAs, followed by more quantitative studies of positive samples by ELISA.

A large number of autoantibodies have been reported in primary Sjögren's syndrome.^{26–28} In some instances, the presence of the antibodies is related to the extent and severity of the patient's disease. The LPA system detected increased levels of several of these antibodies in SS patient serum and saliva samples, including anti-SSA and anti-SSB, the diagnostically most important autoantibodies in primary Sjögren's syndrome.²⁶ Additionally, the LPA detected a statistically significant increase in antibodies against the muscarinic M3 receptor, which is expressed in salivary and lacrimal glands. This finding validates a recent report describing this potential importance of anti-M3 receptor autoantibodies in the pathogenesis and impaired glandular function in Sjögren's syndrome.²⁷

The next phase in development of the LPA platform is to create a fully automated system. We have shown previously that the entire assay process (primary and secondary antibody hybridization steps and all related wash steps) can be performed while maintaining the analysis membranes as an intact stack, thus negating the need to individually process each membrane in a staining dish. The final automation step will require engineering of a processing device that delivers each membrane individually to a detector for analysis. Used in conjunction with currently available fluid robotics for multiwell assay plates, LPAs likely will be capable of serving as an effective, high-throughput tool for antibody screening applications.

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