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Research paper

Label-free microarray-based detection of autoantibodies in human serum

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ABSTRACT

Multiplex assays for autoantibodies have shown utility both in research towards understanding the basic biology of autoimmune disease, and as tools for clinical diagnosis. New label-free multiplex analysis methods have the potential to streamline both the process of assay development and assay workflow. We report fabrication and testing of a 5-plex autoantigen microarray using the Arrayed Imaging Reflectometry (AIR) platform. This label-free technology provides rapid, sensitive, and quantitative detection of an arbitrary number of analytes in a standard multiwell format. In this work, we demonstrate that AIR is able to detect antibodies to Ro60, La/SSB, Scl-70, BicD2, and Ro52 in single-donor human serum samples with multiplex results comparable to singleplex ELISA or Luminex assays.

1. Introduction

Diagnostic tests and basic research in autoimmune disease, including diagnostics for Sjögrens Disease and Systemic Lupus Erythematosus (SLE), rely on the availability of technologies for the sensitive detection of autoantibodies to antigens in human serum. The current “gold standard” method is indirect immunofluorescence using HEp-2 Cells as a substrate for autoantibody binding (Kumar et al., 2009; Meroni and Schur, 2010). Immunofluorescence and other traditional methods including ELISA require secondary antibodies and other labeling reagents to reveal autoantigen – antibody interactions. Multiplex tests, able to detect and quantify antibodies to more than one antigen, have proven useful (Tozzoli et al., 2013; Hanly et al., 2010; Schulte-Pelkum et al., 2005; Martins et al., 2004), but are challenging due to workflow complexity and the possibility of secondary antibody cross-reactivity. Antigen microarrays employing labeled (fluorescence or luminescence) detection have been used previously in discovery efforts in a broad range of autoimmune diseases (Hecker et al., 2016; Yeste and Quintana, 2013; Zhu et al., 2015; Carlsson et al., 2011), and there are some examples of commercial antigen array products in existence, including the Zenit AmiDot (A Menarini Diagnostics), and Blue Diver Quantrix (D-tek). These assays detect antibodies to up to 25 different antigens, and have been described as an alternative to line immunodot assays, in some cases with the possible complication of low sensitivity (Dillaerts et al., 2017). While these and bead-based (Luminex xMAP) assays have proven valuable (Rausch et al., 2016; Bruner et al., 2012; Hanly et al., 2010), alternative, label-free assay formats are attractive as a means towards simplifying workflows and allowing greater flexibility in antigen panel design. Efforts in this area have included the development of a piezoelectric sensor for antibodies to the autoantigens TRIM21 and TROVE2 (Do Nascimento et al., 2017). Optical methods have potential advantages of sensitivity, multiplex capability, and throughput. To that end, we report here the development and preliminary testing of an antigen array fabricated on the label-free Arrayed Imaging Reflectometry (AIR) platform.

Arrayed Imaging Reflectometry is a label-free, multiplex sensing technology based on the creation, and then target binding-induced perturbation, of an antireflective coating on the surface of a microarray. Described in detail elsewhere (Mace et al., 2006), AIR relies on an interference condition created when s-polarized light from a helium-neon laser is incident on a silicon chip with surface chemistry (silicon oxide, a protein adhesion layer, and spotted capture molecules such as antigens or antibodies) tuned to an appropriate optical thickness. Binding of a target molecule to a cognate probe spot causes that spot to no longer fulfill the antireflective criteria, and reflected light may be observed of intensity that is both predictable and a quantitative measure of the amount of material bound. Importantly, the detection system is simple, with no moving parts required for chip imaging (a CCD camera is employed rather than scanning). Additionally, no temperature control is required, unlike other label-free techniques such as surface

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plasmon resonance imaging (Smith et al., 2003) or ring resonators (Mudumba et al., 2017). To date, AIR has been used for detecting peptides and small proteins diagnostic for enteropathogenic *E. coli* (Horner et al., 2006), cytokines in simple backgrounds and in human serum (Mace et al., 2008a; Carter et al., 2011), small molecule pollutants (Carter et al., 2016), protein-RNA interactions (Yadav et al., 2014), and antibodies to panels of influenza antigens (Bucukovski et al., 2015). The influenza antigen panel experiments in particular suggested to us that other antigen panels might be useful. To assess this possibility, we conducted a preliminary test of an AIR microarray carrying five autoantigens (Ro60, La/SSB, Scl-70, Ro52, and BicD2). Antigens were chosen from different autoimmune diseases to highlight the potential application range of the sensor.

2. Methods

2.1. Sources of materials

Ro60, La/SSB, Scl-70, and Ro52 antigens were obtained from Diarect AG (distributed by SurModics IVD in the United States). BicD2 was prepared by Protagen using a procedure previously described (Schulte-Pelkum et al., 2016; Schulte-Pelkum et al., 2015). Amine-reactive substrates for AIR arrays were prepared in-house as described elsewhere (Carter et al., 2011; Carter et al., 2016). PBS-ET was prepared as phosphate buffer (10 mM monobasic sodium phosphate, 10 mM dibasic sodium phosphate, 150 mM NaCl) with 0.02% w/v Tween-20 and 5 mM EDTA.

2.2. Antigen formulation

Antigens were concentrated and dialyzed into phosphate buffer at pH 5.8 and pH 7.4 prior to use. During development, several printing concentrations and/or solution pH values of each antigen were tested, as well as various additives including dimethyl sulfoxide (DMSO) and sugars in order to optimize spot morphology (homogeneity) and initial probe thickness (Mace et al., 2008b). Antigen concentrations and pH values used in the final arrays to generate all data in this work are shown in Table 1.

2.3. Array fabrication

Arrays were printed using a Scienion S3 piezoelectric microarrayer (Scienion, A.G.) with spot volumes ranging from 200 to 400 pL, depending on the observed wetting properties of the antigen probe (as the probe formulation considerably impacts spot spreading). Six spots were printed for each antigen, the final layout of which is shown in Fig. 2. It should be noted that the number of spots arrayed was not critical to good analytical performance or statistical analysis. Each spot consists of approximately 100 pixels when imaged by the CCD in an AIR chip reader, with each pixel constituting a separate, discrete interrogation of a unique probe surface region. Thus, averaging these pixel values together produces an intrinsically robust measure of probe response. Additionally, non-reactive spots (dilutions of polyclonal anti-fluorescein, Rockland Inc.), and highly reactive spots (anti-human IgG, Rockland Inc.), were included as negative and positive intra-array controls, respectively. After printing, chips were mounted onto adhesive strips at appropriate spacing for 96-well plates, and then placed into 50 mM sodium acetate buffer (pH 5). Next, a 1.5% BSA solution was added to each well resulting in a final BSA concentration of 0.5% to passivate the remaining amine-reactive surface functionality. After blocking for 20 min, the chips were rinsed briefly in PBS-ET and transferred to new wells containing 10% porcine serum (Innovative Research) in PBS-ET as a secondary block, and incubated for 40 min. This step was found to be critical as it helped to counteract nonspecific binding from porcine and human serum downstream. The chips were then rinsed briefly (5 min) in new wells containing PBS-ET, then transferred to wells containing Microarray Stabilizer Solution (Surmodics IVD) where they were incubated for 20 min. Finally, the chips were dried at 40 °C in an oven for 5 min. This last step renders the sensors shelf-stable, until use in assays conducted later.

2.4. Assays

A sample diluent consisting of BSA (0.5% w/v, Rockland Inc.), lysine (5 mM), PBS, EDTA (5 mM), Tween-80 (0.02% w/v) and in some cases (discussed later) porcine serum (5% w/v, Lampire Biological Laboratories) was used to dilute polyclonal antibodies and donor human serum samples to appropriate assay concentrations. Fabricated arrays were placed into target solution wells for overnight incubation at 4 °C for 12 h with orbital agitation (500 RPM) on a microtiter plate shaker. Chips were removed after target exposure and rinsed *via* transfer to wells containing PBS-ET for 5 min. This was repeated once with fresh PBS-ET. After washing, chips were rinsed under a stream of 18-MΩ water and dried under a stream of nitrogen. Finally, the substrates were imaged using a prototype AIR Reader and internally developed imaging and assay analysis software at several integration times with dark field subtraction. Once processed, Limits of Detection for all assays were calculated by established CLSI practices (EP17-A2). Reference ELISA and Luminex experiments were performed using 1% serum samples with literature standard methods.

3. Results

3.1. Validation of antigens on the AIR platform

Prior to assessing the ability of AIR arrays to detect endogenous autoantibodies in human serum, the relative activity of each printed antigen was tested using commercial polyclonal antibodies diluted into serum-free assay buffer (150 mM phosphate buffered saline, 0.02% w/v Tween-80, 0.5% w/v BSA, 5 mM lysine, 5 mM EDTA). All five antigens showed the expected activity on the array, providing detection of their respective antibodies over a broad concentration range (Fig. 1) and

Table 1
Antigens used in this study and printing conditions.

Array ID	Name	Conc (mg/mL)	pH	Additive(s)
1	TROVE2/Ro60	0.650	5.8	3% trehalose; 0.005% SDS
2	TROVE2/Ro60	0.650	7.4	3% trehalose; 3% DMSO
3	TRIM21/Ro52/SSA1	0.300	5.8	1.5% trehalose; 1.5% DMSO
4	TRIM21/Ro52/SSA1	0.200	5.8	1.5% trehalose; 3% DMSO
5	La/SSB	0.480	5.8	1.5% trehalose; 0.8% DMSO
6	La/SSB	0.380	5.8	1.5% trehalose; 2.5% DMSO
7	DNA Top1 (Scl-70)	0.230	5.8	1.5% trehalose; 1% DMSO
8	DNA Top1 (Scl-70)	0.200	5.8	1.5% trehalose; 2.1% DMSO
9	BICD2	0.500	5.8	1.5% trehalose; 1.4% DMSO
10	BICD2	0.500	7.4	1.5% trehalose; 1.4% DMSO