A new two-color Fab labeling method for autoantigen protein microarrays

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Antigen microarrays hold great promise for profiling the humoral immune response in the settings of autoimmunity, allergy and cancer. This approach involves immobilizing antigens on a slide surface and then exposing the array to biological fluids containing immunoglobulins. Although these arrays have proven extremely useful as research tools, they suffer from several sources of variability. To address these issues, we have developed a new two-color Fab labeling method that allows two samples to be applied simultaneously to the same array. This straightforward labeling approach improves reproducibility and reliably detects changes in autoantibody concentrations. Using this technique we profiled serum from a mouse model of systemic lupus erythematosus (SLE) and detected both expected and previously unrecognized reactivities. The improved labeling and detection method described here overcomes several problems that have hindered antigen microarrays and should facilitate translation to the clinical setting.

Antibodies that are reactive against specific self antigens are characteristic of many autoimmune diseases[1]. These antigens include a diverse group of cell-surface, cytoplasmic and nuclear antigens. We have described the use of planar protein microarrays for profiling autoantibodies against a large panel of potential autoantigens in a variety of autoimmune diseases[2]. Other groups have used antigen microarrays to guide antigen-specific tolerizing therapy in mouse models of disease[3], to profile antibody specificities in macaques vaccinated and challenged with simian-human immunodeficiency virus (SHIV)[4], to identify clinical subtypes of rheumatoid arthritis with respect to autoreactivities and disease severity[5], and for identification of autoreactivities in sera from lupus patients that correlate positively or negatively with disease severity[6]. Variations of this technology have also been used to profile the antibody repertoire in patients with prostate cancer[7] and in patients suffering from allergies[8]. Although antigen microarrays function well in multiplexed antibody profiling studies, they require improvements in reproducibility and sample normalization to become a common clinical tool.

There are two methods for detecting autoantibodies bound to autoantigen microarrays. The single-color method is the most practical and involves probing an array with unlabeled serum followed by detection with a secondary antibody conjugated to a fluorophore[2] (**Fig. 1a**). This approach has the advantages of simplicity and standardization with respect to fluorophore, but it suffers from variability among array features, arrays, samples and laboratories. The two-color approach is an attractive alternative that can control for some of these sources of variability. Several reports have described two-color protein microarrays[2,9,10], but these techniques suffer from inherent limitations of *N*-hydroxysuccinimidyl (NHS) ester chemical coupling procedures. The drawbacks of this strategy include expense, labor, highly variable modification efficiency resulting from hydrolytic side reactions, and potentially reduced binding owing to modification of primary amines[2].

To develop a simple and reproducible two-color approach for probing autoantigen microarrays, we explored the possibility of using monovalent secondary Fab fragments conjugated to spectrally resolvable fluorescent dyes (**Fig. 1b**). Fab fragments, or Fabs, are fragments of an antibody produced by digestion with papain that retain one antigen binding site. We preincubated these reagents with serum to allow the monovalent Fabs to bind and indirectly label the serum immunoglobulins. We then used the mixture as if it were directly labeled. Although Fab labeling has been previously described for labeling monoclonal or polyclonal antibodies[11,12], it has not been described for labeling serum samples or for probing protein microarrays. Further, two-color analysis has not been systematically investigated for autoantigen microarray analysis.

Microarray technologies in general face a variety of problems that require high standards in reproducibility and reliability to become mainstream clinical tools[13–15]. Using the two-color Fab labeling method, we found that we could improve intraslide and interslide reproducibility and reliably detect changes in autoreactivity. To test the two-color Fab labeling method in a disease setting, we profiled the autoantibody response in a mouse model of SLE. We detected autoantibodies against expected autoantigens, but we also detected an unanticipated autoreactivity to the ribosomal phosphoprotein P0 (Ribo P). Ribo P is a prominent autoantigen previously associated with central nervous system manifestations in SLE, although this association is controversial[16]. Overall, the new two-color Fab-labeling method addresses some of the difficulties that have plagued autoantigen microarrays and is an important advance toward applying this platform in the clinical setting.

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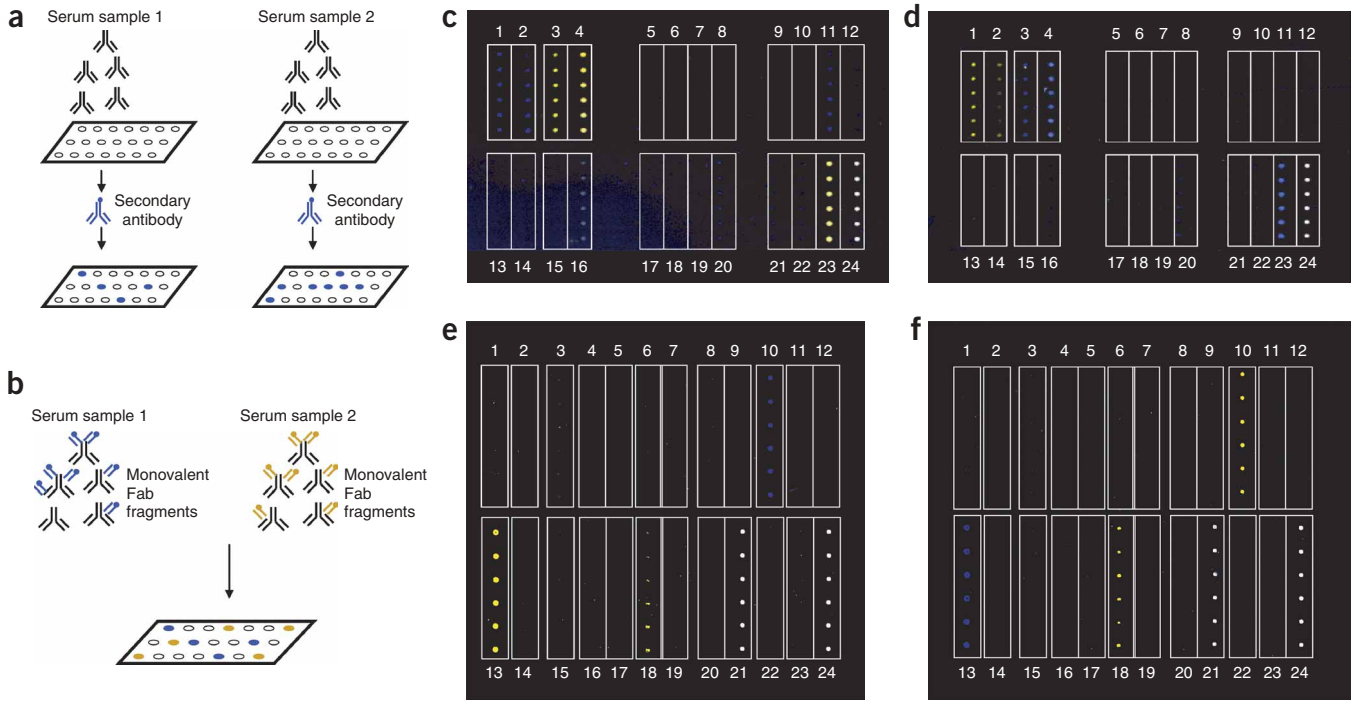

Figure 1 | Two-color Fab labeling for probing one array with different serum samples. (**a**) Single-color method schematic of probing two arrays with two different samples using a fluorescent secondary antibody. (**b**) Two-color Fab method schematic of probing one array with two samples. Samples are preincubated with fluorescent secondary monovalent Fabs and mixed before probing. (**c**,**d**) Scanned images of antigen arrays probed with two different Fab-labeled mouse serum samples. We spiked mouse monoclonal anti-MPO (2 μg) or monoclonal anti-PR3 (2 μg) into normal mouse serum (2 μl containing 20 μg total IgG). Samples were labeled with 30 μg of either Cy3 or Cy5-labeled GAM Fabs for a molar ratio of 4.5:1 Fab:IgG. Scanned image of the array probed with anti-MPO serum (Cy3) and anti-PR3 serum (Cy5) (**c**), and with anti-MPO serum (Cy5) and anti-PR3 serum (Cy3) (**d**). Antigens: sector 1, MPO-2; 2, MPO-1; 11, MPO-3; 3, PR3-2; 4, PR3-1; 23, PR3-3; 24, anti-IgG. The number after each antigen indicates a different commercial source (**Supplementary Table 3**). (**e**,**f**) Scanned images of antigen arrays probed with two different Fab-labeled human serum samples. We spiked human anti-Ro/SSA (1 μg) and anti-La/SSB (1 μg) into normal human serum (2 μl containing 40 μg of total IgG). Samples were labeled with 40 μg Alexa555– or Alexa647–goat anti-human Fabs for a molar ratio of 3:1 Fab:IgG. Scanned image of the array with anti-Ro/SSA serum (Alexa647) and anti-La/SSB serum (Alexa555) (**e**), and anti-Ro/SSA serum (Alexa555) and anti-La/SSB serum (Alexa647) (**f**). Antigens: sector 10, La/SSB; 13, Ro/SSA; 24, anti-IgG; 18, U1A. Emission at 532 nm (Cy3 or Alexa555) is pseudocolored blue, emission at 635 nm (Cy5 or Alexa647) is pseudocolored yellow and emission of equal intensity in both channels is pseudocolored white.

RESULTS

Two-color Fab labeling for probing autoantigen microarrays

To test whether two-color Fab labeling could be used to differentiate serum samples on the same array, we spiked mouse monoclonal antibodies to myeloperoxidase (anti-MPO) or to proteinase 3 (anti-PR3) into normal mouse serum. We preincubated these spiked samples with cyanine-3 (Cy3)- or cyanine-5 (Cy5)-labeled goat anti-mouse (GAM) monovalent Fabs, respectively. To remove free Fabs, we passed the mixture over mouse immunoglobulin G–coated agarose beads in a 0.5-ml spin column. We then mixed the two samples and applied them to an autoantigen microarray. The autoantigen arrays used for these experiments had been developed to study a variety of autoimmune disorders, including antineutrophil cytoplasmic antibody (ANCA)-positive vasculitides. They were composed of a diverse panel of antigens, including three preparations each of MPO (MPO-1, MPO-2 and MPO-3) and PR3 (PR3-1, PR3-2 and PR3-3). Autoantibodies with perinuclear (pANCA) and cytoplasmic (cANCA) staining patterns recognize primarily MPO and PR3, respectively[17]. The scanned images demonstrate that the two-color Fab method qualitatively differentiates the MPO- and PR3-reactive sera based on the dominant fluorescence emission at MPO or PR3 features (**Fig. 1c**). To further validate the technique, we performed a dye-swap experiment in which we preincubated each sample with the alternative fluorophore (**Fig. 1d**). The reactivities of the two serum samples reflect which fluorophore is used in the labeling reaction. One of the MPO antigens, MPO-3, did not yield as robust a fluorescent signal as the others, perhaps owing to purity or concentration. We quantified differences by calculating the $\log_2$ of the ratios averaged across both dye-swap experiments[18] and observed changes greater than twofold for relevant antigens (**Supplementary Fig. 1** online).

We validated the method for human samples using human anti-Ro and anti-La control sera spiked into normal human serum (**Fig. 1e**,**f**). Ro and La are nuclear antigens targeted in SLE and Sjögren's syndrome. For these experiments we used goat anti-human monovalent Fabs conjugated to Alexa Fluor dyes (Alexa647 is a Cy5 equivalent and Alexa555 is a Cy3 equivalent). This experiment showed that the method could be generalized to human studies. One potential drawback of two-color methods is the potential for systematic dye bias, which we did observe in our human studies for antigens such as U1A (**Fig. 1e**,**f** and **Supplementary Fig. 2** online). U1A is a component of the U1 small nuclear ribonucleoprotein that is directly targeted by antibodies found in the serum of patients with SLE and mixed connective

Table 1 | Intraslide and interslide variability

		Interslide				Intraslide			
		Single-color		Two-color		Single-color		Two-color	
Replicates	Antigen	MFI – B	c.v.	MR	c.v.*	MFI – B	c.v.	MR	c.v.**
12	PR3-1	2,477	19%	0.88	8%	2,399	15%	0.94	4%
	PR3-2	3,263	18%	0.89	7%	2,805	19%	0.94	5%
	PR3-3	1,840	13%	0.91	4%	1,643	13%	0.94	3%
6	PR3-1	2,432	20%	0.88	8%	2,442	12%	0.94	4%
	PR3-2	3,278	18%	0.89	7%	2,851	16%	0.95	7%
	PR3-3	1,832	13%	0.91	4%	1,628	17%	0.95	3%
3	PR3-1	2,214	30%	0.88	7%	2,178	20%	0.92	6%
	PR3-2	2,946	17%	0.88	7%	2,483	11%	0.92	6%
	PR3-3	1,825	12%	0.90	6%	1,661	21%	0.95	2%

We spiked mouse monoclonal anti-PR3 antibody (0.2 μg) into normal mouse serum (2 μl containing 20 μg total IgG) for "self-self" comparisons by the single-color or two-color Fab method. The MFI – B at 532 nm emission is reported for single-color data, and the median of ratios (MR) is reported for two-color data, both normalized to IgG. We analyzed twelve, six and three replicates of each antigen on the arrays. *$P < 0.0001$, single-color versus two-color interslide c.v., paired t test. **$P < 0.0001$, single-color versus two-color intraslide c.v., paired t test.

tissue disease. Notably, by averaging data from both dye-swap experiments, we were able to identify and greatly reduce this type of artifact during statistical analysis[18] (**Supplementary Fig. 1**). Using the Cy3 and Cy5 dyes, which are similar structurally, we observed substantially reduced bias for these antigens (**Supplementary Table 1** online). A mock labeling experiment performed without serum showed no fluorescent signal at any of the array features (data not shown). These data demonstrate that the two-color Fab-labeling method permits direct comparison of autoantibody profiles on autoantigen microarrays.

One potential concern with this approach is the possibility of cross-labeling. If Fabs dissociated from one sample and associated with the other, then this method would not reliably reflect differences in the serum samples. We determined that cross-labeling occurred at rates of less than 5% at room temperature (21–23 °C) and less than 1% at 4 °C, as normal mouse serum exhibited minimal PR3 reactivity when probed on an array with near-saturating amounts of anti-PR3 (**Supplementary Fig. 2**). We obtained similar results using two different human serum samples with anti-Ro and anti-La reactivity (data not shown). Moreover, a time-course experiment demonstrated stable ratios and fluorescent intensities when we incubated different samples on arrays for up to 2 h at room temperature or overnight at 4 °C (**Supplementary Fig. 2**).

Reproducibility of single-color and two-color approaches

We hypothesized that two-color data would be subject to less interslide and intraslide variability than single-color data because the former method helps control for spot-to-spot and array-to-array variability. We spiked mouse monoclonal anti-PR3 into normal mouse serum and aliquoted it into two separate pools for 'self-self' comparisons[19]. Although the median of ratios and the median fluorescent intensity (MFI) minus background (B) are entirely different measurements, the coefficient of variation (c.v.) allows the two to be compared with respect to variability (**Table 1**). In aggregate, the interslide and intraslide c.v. values for the two-color Fab method were significantly lower than the c.v. values for the conventional single-color method when using as few as three replicate features or as many as 12 replicate features (**Table 1**). Despite equivalently high variability in MFI – B for the two-color method (data not shown), the median of ratios exhibited a low c.v. (**Table 1**). Additionally, the two-color Fab method allowed for reliable detection of threefold changes in relative autoantibody concentrations (**Supplementary Table 2** online), indicating that even relatively subtle differences can be reproducibly measured using the two-color Fab method.

Signal intensity, sensitivity and dynamic range

Autoantigen arrays have previously proven to be similar to conventional enzyme-linked immunosorbent assay (ELISA) with respect to sensitivity, specificity and dynamic range[2]. The fluorescent signal from antibodies labeled with fluorescently-tagged Fabs, however, seems generally to be weaker in intensity than that observed using secondary reagents for detection[11,12]. To determine the signal intensity and dynamic range of the two labeling approaches, we spiked monoclonal anti-La into normal mouse serum in serial tenfold dilutions (**Fig. 2a**). At the highest concentration of anti-La (10%: 2 μg of anti-La in 2 μl of serum containing 20 μg of total immunoglobulin; IgG), the fluorescence signal was paradoxically low using both methods, as previously described for saturated antibody assays[20]. Both methods detected anti-La reactivity at 0.1% and 0.01% of the serum IgG, with both overall MFI and more than half of the pixels at least two standard deviations above background (**Fig. 2a**). The two methods were comparable with respect to dynamic range. Notably, MFI – B seemed to have the largest error when the signal was also the largest (1% anti-La in serum), whereas the error at more dilute concentrations was smaller (0.1% anti-La in serum; **Fig. 2b**). Although the c.v. of MFI – B seemed to depend dramatically on concentration, signal intensity or both, the c.v. of the ratio was similar at all anti-La concentrations tested (**Fig. 2b**). Additionally, the c.v. of the ratio was lower than the c.v. of MFI – B at all anti-La concentrations tested.

To compare the sensitivity, specificity and dynamic range of single-color and two-color Fab methods for measuring changes in autoantibody concentrations, we spiked antibodies to MPO and PR3 into serum at serial threefold dilutions from approximately 10% of serum IgG down to approximately 0.01% of serum IgG, representing a 3^6 (729)-fold change in concentration. We designed the seven serum samples with the gradients of anti-MPO and

anti-PR3 reactivities in opposing directions, so that the sample with the highest anti-MPO reactivity had the lowest anti-PR3 reactivity and vice versa. We calculated the $\log_3$ change relative to the middle value (anti-MPO and anti-PR3 at approximately 0.3% of total serum IgG) and fit the data by linear regression for each antigen (**Fig. 2c,d**). Although there was no statistically significant difference in the slopes between two-color and single-color methods, the two-color method had a significantly higher regression coefficient (R^2) value than the single-color method (**Table 2**). We also compared both the single-color and two-color data with those from conventional ELISA performed on the same samples and determined that the two-color method had better correlation with ELISA than the single-color method (**Table 2** and **Supplementary Fig. 3** online). Although both methods underestimated changes in autoreactivity, the two-color method provided data that were significantly more linear and better correlated with ELISA than the single-color method.

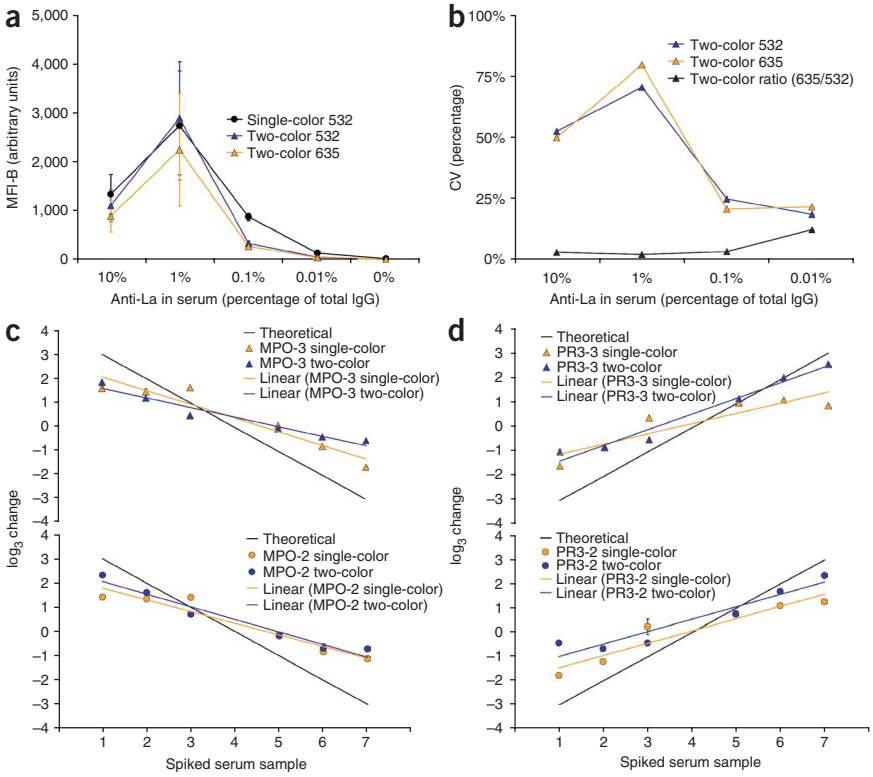

Figure 2 | Signal intensity, sensitivity and dynamic range. (**a**,**b**) Graphs of signal intensity and coefficient of variation. We spiked monoclonal anti-La/SSB into normal mouse serum (20 μg IgG) at five concentrations: 10% (2 μg), 1% (200 ng), 0.1% (20 ng), 0.01% (2 ng) and 0% (0 ng). Alexa dyes with an MSR of 1.5 dye molecules/Fab were used at a molar ratio of 6:1 Fab:IgG. The two-color data are from a self-self array; the single color data from one array. (**a**) MFI – B of the La/SSB features on the autoantigen arrays plotted against anti-La concentration. Error bars represent 95% confidence intervals, $n = 12$. (**b**) Intraslide c.v. of the La/SSB features on the two-color autoantigen arrays plotted against anti-La concentration. (**c**,**d**) $\log_3$ change in signal for spiked serum samples. We spiked monoclonal antibodies directed against PR3 and MPO into normal mouse serum at serial threefold dilutions in opposing gradients such that the highest anti-MPO reactive sample had the lowest anti-PR3 reactivity and vice versa. We calculated the $\log_3$ change relative to a middle value (anti-MPO and anti-PR3 at approximately 0.3% of total serum IgG) to monitor up- and downregulation of autoreactivity. Error bars, 95% confidence intervals, $n = 3$.

Ribo P reactivity in the SLE model

To validate the two-color Fab labeling method in a disease model, we analyzed serum samples from the pristane mouse model of lupus. The arrays contained 468 features with a redundancy of 12 replicates per antigen, including both common and uncommon autoantigens for a variety of autoimmune diseases, as well as several features used for standardization and quality control. The full list of antigens and corresponding vendors is available in **Supplementary Table 3** online. Serum from a pristane-treated BALB/c mouse 20 weeks after induction (pristane-post) was labeled with Alexa647-Fabs and compared with Alexa555-Fab labeled serum from the same mouse obtained immediately before induction (pristane-pre; **Fig. 3a**). As a negative control, serum from a phosphate-buffered saline (PBS)-treated BALB/c mouse 20 weeks after mock induction (PBS-post, Alexa647-Fab) was compared with serum from the same mouse obtained immediately before mock induction (PBS-pre, Alexa555-Fab; **Fig. 3a**). We repeatedly observed reactivity to autoantigens known to be targeted in the pristane model, such as U1A, U1C and dsDNA, (**Fig. 3a** and data not shown). We also reproducibly detected strong reactivity to Ribo P, which we did not anticipate (**Fig. 3a**).

Most of the autoantibodies that we detected using the two-color autoantigen arrays have been previously reported in pristane-treated BALB/c mice, but reactivity to Ribo P had not previously been detected[21–23]. Autoantibodies to the ribosomal P phosphoproteins are characteristic of SLE and are typically directed against three proteins, P0, P1 and P2 (35 kDa, 19 kDa and 17 kDa, respectively). Previous studies suggested that these autoantibodies target a conserved 22–amino acid sequence at the carboxyl terminus that is shared by all three proteins[24,25], but the reactivity may involve other epitopes[26]. Serum from one pristane-treated BALB/c mouse demonstrated strong, reproducible reactivity to a recombinant Ribo P0 that was used on the arrays (**Fig. 3a** and data not shown). Subsequent single-color array analysis also identified reactivity to Ribo P in pristane-treated BALB/c mice (data not shown). Although these data were from a single mouse, this unexpected reactivity encouraged us to investigate a larger panel

Table 2 | Artificial antibody up- and downregulation measured by single-color and two-color Fab methods

	Single-color	Two-color
Slope	0.56 ± 0.04	0.51 ± 0.04*
R^2	0.84 ± 0.02	0.95 ± 0.01**
Spearman *r* with ELISA	0.88 ± 0.02	0.97 ± 0.02***

Slope, regression coefficient and nonparametric correlation (Spearman *r*) with conventional ELISA for single-color and two-color Fab methods. Error reported as s.e.m., $n = 3$. *Not significant, paired *t*-test. **$P = 0.01$, paired *t* test. ***$P = 0.02$, Wilcoxon matched pairs test.

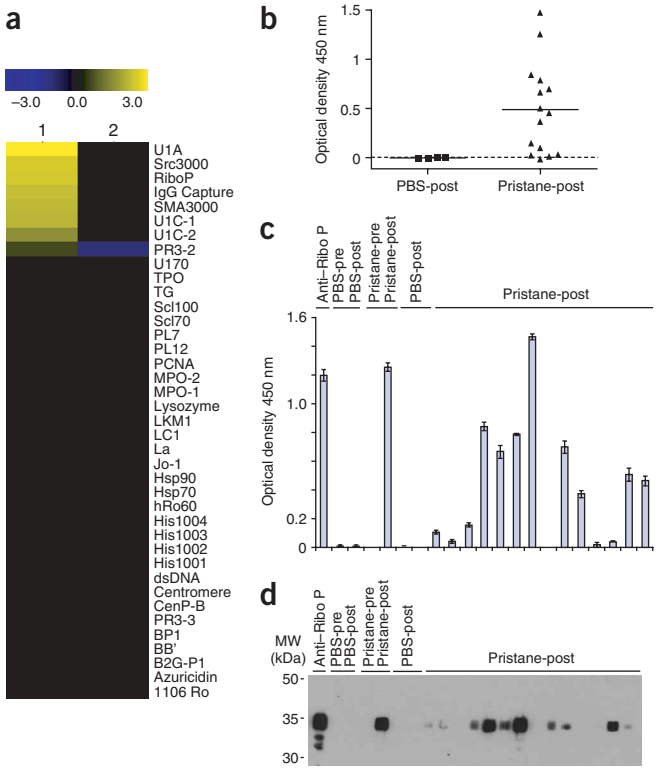

Figure 3 | Autoantibody profiling of mouse serum and Ribo P autoreactivity in the pristane model of lupus. (**a**) Heat map representations of $\log_2$ of 635 nm/532 nm ratios for each antigen. We tested mouse serum from the pristane group before treatment (pristane-pre), pristane group 20 weeks after treatment (pristane-post), PBS group before treatment (PBS-pre) and PBS group 20 weeks after treatment (PBS-post). Column 1, pristane-post (Alexa647-Fab) with pristane-pre (Alexa555-Fab); column 2, PBS-post (Alexa647-Fab) with PBS-pre (Alexa555-Fab). Positive $\log_2$ values (ratios > 1) are pseudocolored yellow and negative $\log_2$ values (ratios < 1) are pseudocolored blue. (**b**,**c**) Plots of data demonstrating autoantibodies to whole recombinant P0, obtained by ELISA using serum from pristane-treated and PBS-treated mice. (**b**) Scatter plot of ELISA results for PBS and pristane-treated mice. Horizontal bars show the mean optical density values for each group and the broken line represents mean of data for PBS treated mice plus 3 s.d. (**c**) ELISA results for individual PBS- and pristane-treated mice. Error bars represent 95% confidence intervals, $n = 3$. (**d**) Immunoblot of recombinant P0 fractionated by SDS-PAGE and probed with sera using a slot blot device. Lanes are probed with human Ribo-P–reactive serum (anti-Ribo P) and sera from mice in the following groups: PBS-pre, PBS-post, pristane-pre and pristane-post. Full-length blot presented online (**Supplementary Fig. 4**). Order of samples in **c** and **d** is the same.

of pristane-treated BALB/c mice. By conventional ELISA 9 of 15 mice (60%) exhibited strong reactivity to recombinant P0, and 5 of 15 (33%) exhibited lower reactivity (**Fig. 3b**,**c**). Mice from the PBS-treated group lacked such autoantibodies (**Fig. 3b**,**c**). To rule out contamination as a cause of this reactivity, the P0 antigen used for the ELISA and the arrays was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and probed using serum from each pristane-treated or PBS-treated mouse (**Fig. 3d**). A band at 35 kDa, corresponding to the molecular weight of P0, was detectable in all of the mice that tested positive by ELISA, arguing that this reactivity was indeed specific for P0 (**Fig. 3d**). BALB/c mice primed with pristane had previously seemed negative for Ribo P reactivity by immunoprecipitation of radiolabeled extract and ELISA using the C-terminal 22–amino acid peptide[21–23]. Consistent with these previous studies, we did not observe reactivity to Ribo P when we immunoprecipitated radiolabeled EL4 mouse lymphoma cell extract with serum from pristane-treated BALB/c mice (**Supplementary Fig. 4** online). The antibodies to Ribo P0 induced in BALB/c mice primed with pristane, although positive by ELISA and western blot, do not immunoprecipitate the protein.

DISCUSSION

Although there has been extensive effort in the field of transcript profiling to examine sources of error and variability[27–30], these issues have yet to be addressed in a systematic manner for protein arrays, particularly autoantigen microarrays. It is true that some popular transcript profiling platforms use single-color labeling methods, but these platforms often have rigorous quality control in fabrication and design that minimizes variability. Antigen arrays, however, are being developed for vastly different macromolecular species (lipids, proteins, carbohydrates and nucleic acids), widely variable molecular sizes (peptides to protein complexes), variable sample complexity (recombinant or affinity-purified proteins) and variable sample storage buffer (glycerol, PBS or other buffers), which complicate array production. Optimization of the single-color method with respect to slide surface, reagents and printing is one way to improve the reproducibility and reliability of large-scale autoantigen microarrays. In contrast, two-color methods control for many sources of variability by allowing two samples to bind the same feature on the same array. We found that our rapid two-color labeling method using Fabs improved reproducibility and linearity over a wide range of antibody concentration changes. Using the two-color Fab labeling method, we profiled autoantibodies and detected a previously unreported reactivity to Ribo P0 in the pristane model of SLE in BALB/c mice. This finding validated the technology for profiling humoral immune response changes during disease onset. We believe that ultimately the two-color Fab labeling approach will facilitate the study of more subtle changes in autoantibody profiles, such as monitoring the response to therapy over time in an individual.

One advantage of using our secondary Fab labeling approach is that we can reproducibly label very small amounts of serum samples by simply preincubating the serum-Fab mixture for several minutes at room temperature. Moreover, the primary antibodies are not chemically modified and should therefore better retain antigen-binding ability. Finally, these inexpensive labeling reagents can be generated in large amounts to improve consistency.

There are some potential drawbacks to the two-color Fab-labeling method. One is that the fluorophore-Fabs are not covalently attached to the sample, allowing for the possibility of mobility during the experiment. This did not seem to be a problem with our protocol (**Supplementary Fig. 2**). A second potential problem is systematic dye bias, which is a universal concern of two-color labeling approaches. This bias can be minimized by averaging dye-swap experiments, using cyanine instead of Alexa dyes, or using a constant reference. Finally, our data indicate that for monitoring low-abundance or weak reactivities, or for detecting more dramatic differences, a single-color method may be appropriate. On the other hand, when the goal is to detect more subtle changes over a wide range of concentrations, a two-color

method may be preferable. Taken as a whole, the improvement in reproducibility of this two-color Fab-labeling method addresses some of the problems facing autoantigen microarray technology and should help improve the reliability of autoantibody profiling for clinical studies.

METHODS

Probing and scanning autoantigen arrays. We blocked the autoantigen arrays with 3% fetal calf serum (FCS) and 0.05% Tween-20 (Sigma) in PBS (GIBCO) either for 1 h at room temperature or overnight at 4 °C. We then probed these blocked slides by either the single-color or two-color Fab methods. Single-color arrays were probed as previously described[2]. Briefly, we incubated the arrays for 1 h at 4 °C with 2 µl of serum diluted in 1 ml of 0.05% Tween-20 in PBS (PBST) containing 3% FCS. We then washed the slides twice for twenty minutes in 3% FCS in PBST. We incubated the slides with a Cy3-conjugated donkey anti-human or goat anti-mouse secondary antibody (Jackson ImmunoResearch) at a dilution of 1:1,000 for 1 h at 4 °C. After incubation, we washed the slides twice for 30 min in 3% FCS in PBST and then twice for 20 min in PBS, rinsed them for 10 s in double-distilled deionized water (ddH_2O), centrifuged them to dryness at room temperature for 5 min, and scanned them. For the two-color Fab labeling method, we first preincubated the serum and Fabs for 10–30 min at room temperature. Unless otherwise stated, we labeled the serum at an Fab:IgG molar ratio of 4.5:1 during preincubation. We added 150–350 µl of whole-IgG–coupled agarose beads (Jackson ImmunoResearch) to empty 0.5 ml Zeba spin columns (Pierce). We added the serum-Fab mixture to the column and incubated at room temperature for 5–10 min before centrifugation for 1 min at 10,000*g*. Alternatively, we precentrifuged the beads in spin columns to remove the aqueous phase and then added the serum-Fab mixture to the packed beads. We placed the flow-through from two labeling reactions on ice and diluted to a final volume of 1 ml of 3% FCS in PBST. We then applied this mixture to the slides for an incubation period of 45 min at 4 °C unless otherwise indicated. After incubation, we washed the slides three times for 5 min in 3% FCS in PBST and then for 5 min in PBS, rinsed them for 10 s in ddH_2O, centrifuged them to dryness at room-temperature for 5 min, and then scanned them.

Data analysis. We used a GenePix 4000 scanner to scan the arrays and GenePix Pro Version 5.0 software (Molecular Devices) to analyze the images. For analysis, we used either the MFI – B or the median of 635 nm/532 nm ratios, as indicated. We applied a low-intensity cutoff filter during data analysis to exclude any spots where the intensity in more than half of the pixels was less than two standard deviations above background for both the 635 nm and the 532 nm channels. We normalized the ratios at each feature to the ratio of total IgG between the two samples. To determine the ratio of IgG concentrations we used the Easy-Titer IgG Assay kit (Pierce) or, in self-self experiments, assumed a ratio of 1.0. We multiplied all ratios by the correction factor ($IgG_{ratio\_total}/IgG_{635\ nm/532\ nm}$), where IgG_{ratio_total} is the ratio of total IgG for the two samples determined before probing the microarray and $IgG_{635\ nm/532\ nm}$ is the ratio observed at the anti-IgG capture antibody feature. For single-color data, MFI – B for each feature was normalized to the MFI – B for anti-IgG. After filtering out low-intensity data and normalizing to the ratio of total IgG, the mean of the ratios or the $\log_2$ of the ratios was calculated.

Additional methods. Detailed descriptions of antigen microarray production, sources of all reagents, the treatment of mice used in these experiments, as well as our protocols for metabolic labeling, cell lysis, immunoprecipitation and western blot analysis, and conventional ELISAs are available in **Supplementary Methods** online. We obtained approval for experiments with animals from the Institutional Animal Care and Use Committee.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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