Screening Autoantibody Profiles in Systemic Rheumatic Disease with a Diagnostic Protein Microarray That Uses a Filtration-Assisted Nanodot Array Luminometric Immunoassay (NALIA)

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**BACKGROUND:** We developed a cost-efficient modular system for multiplex analysis of the multiple autoantibodies that characterize systemic rheumatoid diseases.

**METHODS:** The nanodot array luminometric immunoassay (NALIA) system consists of conventional 96-well membrane-bottomed plates in which antigens or antibodies are adsorbed onto the underside of the membrane. Current arrays use a 5×5 format (25 dots/well), which allows 10 analytes to be measured in duplicate: double-stranded DNA (dsDNA), centromere protein B (CENP-B), PCNA, Sm, Sm ribonucleoprotein (Sm-RNP), U1-snRNP, Scl70, SSA/Ro, SSB/La, Jo-1, and controls. The test fluid, control sera, and subsequent reagents are drawn through the membrane. The captured analytes are quantified by monitoring chemiluminescence with a charge-coupled device (CCD) and analyzed with commercial array software.

**RESULTS:** The assay can detect <20×10^3 IU/L of anti-dsDNA. The interwell CV was 10%–14%. There was an 83% concordance (κ = 0.56) between the NALIA results obtained for anti-dsDNA assayed by β-testing in a routine immunology diagnostic laboratory and the results obtained with a conventional ELISA reagent set. The concordance values for Ro, La, Sm, and RNP were 98% (κ, 0.92), 93% (κ, 0.41), 97% (κ, 0.62), and 97% (κ, 0.73), respectively.

**CONCLUSION:** The NALIA approach promises to provide a highly economical platform for a wide range of applications that require assays of multiple analytes. The results also agreed with those obtained with conventional methods, which agreed with each other. A sample of serum from a finger stick provides a volume sufficient to perform the array assay.

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Systemic rheumatic diseases such as systemic lupus erythematosus (SLE),5 Sjögren syndrome, systemic sclerosis, mixed connective tissue diseases, dermatomyositis, and polymyositis affect 3%–5% of the population (1). A common feature of these diseases is the presence of autoantibodies, particularly antinuclear antibodies (ANAs), which are useful markers for identification and diagnosis. Positive results for both ANAs and the presence of antibodies against double-stranded DNA (dsDNA) or Sm constitute 2 of the 11 criteria of the American College of Rheumatology for the diagnosis of SLE. Increased concentrations of autoantibodies are often present many years before the onset of clinical disease and diagnosis (2). Because the worldwide economic outlay for diagnosing and monitoring rheumatic diseases is increasing (3), there is a growing demand for new medium- to high-throughput analytical procedures for cost-efficient detection of these diseases.

Traditionally, ANA profiles (4) are obtained by an indirect immunofluorescence test (IIT) on eukaryotic cells such as Hep-2. This process is time consuming, and the subjective nature of the IIT patterns requires skilled interpretation. IITs are difficult to standardize and automate, and these assays often do not define or

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5 Nonstandard abbreviations: SLE, systemic lupus erythematosus; ANA, antinuclear antibodies; IIT, indirect immunofluorescence test; NALIA, nanodot array luminometric immunoassay; snRNP, small nuclear ribonucleoprotein; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; CCD, charge-coupled device.
always detect the specific antigens whose presence prompt particular diagnoses and treatments. ELISAs are usually used to confirm ANA specificities (5). Although replacing IITs with ELISAs would be straightforward, the process would be labor intensive and relatively expensive, because each specific autoantibody is measured in separate assays. Automation of the ELISA (or the IIT) can help, but the process would benefit enormously from combining the separate ELISA tests on the same sample in one “multiplex” assay, which would both increase throughput and presumably reduce the costs. Furthermore, as autoantibody specificities become more clearly defined and new specificities with promising diagnostic implications are discovered, a multiplex assay platform would readily accommodate such additions.

Most multiplex assays require expensive equipment and tie the laboratory to a particular supplier for consumables. Our aim was to develop a cost-efficient, generic multiplex assay that had features familiar to most end users and used a modular approach flexible enough to be applied to other areas requiring medium- to high-throughput screening. To this end, we developed the nanodot array luminometric immunoassay (NALIA) system to provide an economical platform that can be configured to various numbers and types of assays, has moderate to high sample throughput, and is adaptable to existing equipment and/or robotic workstations.

Many array solid phases use glass (6) or membrane-coated glass, and traditional microtiter plates are plastic. The NALIA system, however, uses a flow-through membrane-bottomed 96-well format that avoids diffusion constraints and facilitates washing. The deposition of probes on the underside of the membrane prevents the walls of the vessel from interfering with the spotting and imaging processes.

Materials and Methods

ANTIBODIES, ANTIGENS, AND REAGENTS
We evaluated 10 autoantigens. Calf thymus dsDNA (Sigma-Aldrich) was twice precipitated with ethanol and treated with nuclease S1 (Roche Diagnostics) to remove single-stranded DNA. CENP-B (centromere protein B), U1-snRNP (small nuclear ribonucleoprotein U1) and PCNA (proliferating cell nuclear antigen) were bacterial recombinant proteins purchased from DiaRect. Sm (Smith antigen), Sm-RNP, Scl70 (DNA topoisomerase-1), SSA/Ro, SSB/La, and Jo-1 (histidyl-tRNA synthetase) were purchased from AroTec. Biotin-N-hydroxysuccinimide ester (Sigma-Aldrich) was used to label human albumin (also from Sigma-Aldrich). We purchased horseradish peroxidase (HRP)-conjugated sheep antihuman IgG from GE Healthcare UK, HRP-conjugated monoclonal antihuman IgM from Hybridoma Reagent Laboratory, and chemiluminescent substrate from BioFX Laboratories. EUROIMMUN ELISA reagent sets were used to compare results with NALIA assays.

Serum samples were collected from patients at the local hospital rheumatology clinic after we had obtained informed consent.

ANTIGEN ARRAY PRODUCTION
We used MultiScreen-HA 96-well plates, which feature a nitrocellulose/cellulose acetate membrane that forms the bottom of each well (Millipore; Fig. 1). We removed the underdrain of each microplate and allowed the plate to stand in the room atmosphere for 1 h before array printing. Antigens were diluted in phosphate-buffered saline (PBS) with the exception of dsDNA, which was diluted with 3× sodium chloride/sodium citrate buffer (i.e., 0.45 mol/L sodium chloride, 0.045 mol/L trisodium citrate, pH 7); all antigen dilutions contained 25 mL/L glycerol and 50 mg/L bromphenol blue. Antigens were deposited as 12-nL nanodots via noncontact printing with a BioDot AD3200 equipped with a microsolenoid ink jet and a 100-μm ceramic tip. The 5 × 5 antigen array in each well included nanodots of dsDNA, Sm, Sm-RNP (RNP A and C polypeptides), U1-snRNP (68 kDa), Scl70, CENP-B, SSA/Ro, SSB/La, Jo-1, and PCNA, which were deposited vertically in duplicate (Fig. 2). Biotinylated human albumin and the buffer, which were spotted in duplicate as an internal positive control and in triplicate as a background control, respectively, were located horizontally along the central axis.

Spotted plates were stored at 4 °C in a vacuum pack. Prior to screening, we brought the plates to room
temperature and then replaced the underdrain very firmly with a custom-made clamping press. The plates were then placed on a MultiScreen reduced-pressure manifold station (Millipore).

ARRAY SCREENING
Plates were first washed with degassed PBS containing 0.5 mL/L polysorbate 20 (Tween 20) by drawing the buffer through the membrane with a low reduced pressure (600–700 Pa negative pressure). The plates were then treated with 10 mL/L chicken serum in PBS as a blocking agent to minimize the nonspecific background signal. We rewashed the plates with PBS/Tween 20 before and after we passed through patient and control sera (diluted with 100 volumes of the PBS/Tween 20 solution). After additional washing steps, we visualized bound autoantibodies and biotinylated control spots by passing 1/4 000 HRP-conjugated antihuman IgG, 1/2 000 HRP-conjugated antihuman IgM, and 1/20 000 HRP-conjugated streptavidin through the membrane, washing, and adding the chemiluminescent substrate. We detached the underdrain, removed the excess liquid by lightly blotting the plate on absorbent paper, and immediately imaged the plate with a charge-coupled device (CCD) camera. Images were acquired within 2 min with 2 × 2 pixel binning and were corrected for flat field and stray light. The total assay time from sample addition to result was 95 min.

WHOLE-PLATE IMAGING LUMINOMETER
A CCD-based luminometer has been specially designed and manufactured at University College London for the NALIA program (Fig. 3). This instrument, which is not commercially available, permits whole-plate imaging from beneath the plate. Its design is an adaptation of a luminometer (7) that had previously been developed for above-plate imaging. The plate to be analyzed is positioned in a plate holder within a light-tight housing. A commercially available f/1.4 35-mm lens focuses the plate image on a computer-controlled CCD camera, and an exposure is made at an aperture of f/4.5 to maximize image quality. Two commercially available CCD cameras have successfully
been used within the instrument. The first, NALIA System NDS-V1, was specifically developed for the NALIA program by Photonic Science Limited; software control was with Image-Pro Plus (MediaCybernetics). The second camera system, NALIA System NDS-V2, consisted of the SXV-M25 camera available from Starlight Xpress and software control with MaxIm DL (Diffraction Limited). We used 2 × 2 pixel binning for both cameras as a standard for acquiring images at the best-available resolution (i.e., for the NDS-V1 camera, the detector format was 2016 × 1344 pixels with a 18-µm pixel size; the format for the NDS-V2 camera was 1512 × 1008 pixels with a 15.2-µm pixel size); pixel binning reduced the file size of saved images. We incorporated the NDS-V2 camera into the instrument because this camera permits color imaging, which may be advantageous for future applications with the luminometer. A typical exposure time for a plate was 10 s. Each acquired image file was transferred to a personal computer for analysis.

DATA ANALYSIS
We used Array-Pro (MediaCybernetics) for data analysis and subtracted local within-well background signals by using the buffer-control spots to correct for minor differences in membrane permeability. To normalize values, we used the positive-control spots of biotinylated albumin for comparison with the rest of the plate, because the signals are slightly affected by the unique serum sample loaded into each well. This step is necessary because any antigen signal is compared by ratio to 5 serum calibrators located elsewhere in the plate, which together provide at least 2 different concentrations of each autoantibody, enabling the sample to be classified as positive, borderline, or negative with respect to each antibody. We conducted prior tests with approximately 200 random samples of test sera from patients with suspected rheumatologic disorders to calibrate these serum reference samples. This testing established a cutoff point for each antigen that gave an optimal correlation with the local classification of ELISA results. To be consistent with the range of ELISA borderline values defined by the local University Hospital clinicians, we set the borderline limits at 20% above and below these cutoff points.

Results

FUNCTIONING OF THE ARRAY
Fig. 2 is a whole-plate image of a typical assay. The nanodots on the underside of the membrane-bottomed wells show a good morphology, with a within-plate CV for all of the biotinylated positive control spots of 10%–14%. The first and fourth horizontal rows in each well are duplicates, and the images are very similar. The same holds true for the duplicate second and fifth rows. The 2 positive controls in the third row are conspicuous. The use of a CCD chip with a high pixel density that incorporates a 16-bit fusion driver in the final reader (NALIA-DS2; not shown) with suitable optics and imaging from beneath the plate enabled visualization of an entire 96-well plate at a resolution of 40 × 40 pixels for each dot. Wells containing sera with known autoantibody profiles provide positive calibrators for calculating the cutoff value.

The arrays readily distinguish the various autoantibody profiles demonstrated with serum samples from another group of patients (Fig. 4).

The sensitivity (defined as the mean of 4 negative serum samples plus 3 SDs) was 17.6 × 10³ IU/L, with a clinically accepted cutoff for positivity of 50 × 10³ IU/L. The results of a NALIA array analysis of a non-pathologic serum sample spiked with different amounts of calibrator anti-dsDNA serum correlated well with the expected results (Fig. 5). Similar results were obtained in analyses of serum samples spiked with the other 9 antigens (data not shown).

AGREEMENT WITH CONVENTIONAL ELISA
To relate the performance of the NALIA system with that of conventional ELISA methods, technical staff from the Clinical Immunology Laboratory used the NALIA arrays on several occasions to test serum samples that had been received by the University College London Hospitals NHS Trust rheumatology clinic for routine autoantibody analysis. We compared the NALIA results with values for individual antibodies routinely obtained with the commercial ELISA reagent sets (Table 1). Agreement between the 2 tests for Ro antibodies was high at 98% with an excellent κ (SD) value of 0.92 (0.09). We obtained comparable concordances with antibodies to La, Sm, and RNP with κ values of 0.41 (0.06), 0.62 (0.07), and 0.73 (0.09), respectively. These κ values were likely reduced because of the low prevalence of positive results and the dependence of κ on marginal rates. The concordance between methods for anti-dsDNA was 83% with a κ value of 0.56 (0.07). Discordance was not unidirectional: some serum samples that were positive in the NALIA test were negative by ELISA, and vice versa. The laboratory did not routinely test serum samples for the other antigens, so such comparisons cannot be made.

Discussion
The combination of multiple tests in a single multiplex assay based on the concept of ligand-binding multipot spot arrays was introduced by Ekins and Chu (8, 9), and this approach may be invaluable in helping to satisfy the need for high-throughput measurement of a variety of
molecules in blood and other tissues (10). We expect the NALIA approach to be economical. We estimate the cost of each test for the production of a relatively small number of plates to be ≤10% of the costs of the corresponding commercial ELISA assays. The familiar 96-well microtiter plate format is amenable to both high throughput and automation, and so the application of arrays within this configuration (i.e., multiple tests performed on an individual sample within each well) is particularly relevant. Mendoza et al. (6) first described the feasibility of an automated system that allows microarray-based ELISAs and used a glass solid phase with 96 wells separated by a Teflon mask. Sample incubation, washing, and fluorescence-based detection steps were carried out with a robotic pipettor. A CCD camera was used for quantitative imaging. Robinson et al. (11) used a larger assay involving 386 probes to profile the autoantibodies in serum samples from patients with rheumatic disease.

We have described the development of a high-throughput NALIA multiplexing system in which autoantigen arrays were constructed by ink-jet spotting onto commercially available mixed cellulose ester–bottomed (approximately 85% nitrocellulose) 96-well plates and have investigated the application of this system to a more targeted diagnosis of autoimmune disorders. The membranes permit patient samples and wash fluids to interact with the underplate probes through the creation of a continuous or semicontinuous flow system that eliminates diffusion constraints and hence the need for shaking. This design also avoids
the aspiration of fluids between the sample, washing, and reagent steps.

Rather than depositing the nanodots within the wells, as is conventionally done, the NALIA system (UK Patent Application GB2401942) uses the innovation of fabricating the array on the undersurface of each well, which avoids interference from well walls and is more convenient for spotting machines by providing a larger surface area for the array. This design also allowed the development of a compact economical underplate system for reading the chemiluminescent spot signals via whole-plate imaging with a tailored CCD. Results are quantified with standard array software.

The minimum spot volume that could reliably be dispensed by the BioDot machine was 12 nL. To increase our confidence in the results, we constructed the current arrays with 10 different autoantigens in duplicate. Control of the assay was further ensured by including 2 spots of biotinylated human serum albumin, which were used to normalize the data. We also validated the method by assaying several serum samples with known autoantibody profiles in separate wells, and we added bromphenol blue to the spotting solutions to monitor for any failure in probe deposition. Future work will use a piezoelectric appliance that can accurately dispense picoliter volumes of probes at precise coordinates. We expect this modification to produce a more dependable array and permit an increase in the number of probes in the array. We tested serum samples at 1:100 dilution, and because the sample volume required is 50–100 µL, only a finger stick is required to obtain a sufficient sample. It will be interesting to see whether whole blood collected in a heparin-containing capillary tube can be analyzed without separating the cells, because the cells will be filtered out by the upper surface of the membrane during the assay procedure.

In preliminary studies with polyvalent anti-IgG, 1 serum sample tested positive for IgM anti-Jo-1 but negative for IgG anti-Jo-1. In studies with a limited number of samples, investigators demonstrated that IgM autoantibodies predominated over IgG in patients with incomplete lupus erythematosus (at least 1 but less than 4 of the criteria for SLE (12)). IgG usually predominates in SLE, however (13). It will be of interest to use NALIA arrays to evaluate this observation by detecting IgM and IgG autoantibodies separately rather than simultaneously, which was the approach we used.

The high degree of agreement with the ELISA results with respect to the specificities for the extractable nuclear antigens we analyzed justifies our confidence in the basis for the NALIA system. The lower concordance for anti-dsDNA was not entirely unexpected given the commonly reported discrepancies between the commercial reagent sets (14–19). The discrepancies we observed with the results obtained with the EUROIMMUN ELISA reagent sets were not due to different sensitivities, because some of the discordant serum samples were positive in NALIA testing and negative by ELISA, and vice versa. The conformational nature of the antigen, the effect of binding to the solid phase on the antigen’s physical structure, and the degree of contamination with single-stranded DNA, if any, will be contributing factors. The observed differences in results between the methods used for measuring anti-dsDNA antibodies (ELISA, the IIT, and the Farr assay) have been suggested to be attributable to each assay detecting only part of the spectrum of anti-dsDNA antibodies present in serum (20). ELISA is considered the most sensitive assay, but the Farr assay is the most specific for SLE. The latter method detects only antibodies of high avidity, however, and mild forms of SLE, in which patients have lower-avidity anti-dsDNA, might easily be missed. It is possible that the antibody spectrum detected by NALIA lies somewhere in between. Clinically, high-avidity anti-dsDNA is associated with more frequent occurrence of nephritis, whereas low-avidity anti-dsDNA antibodies are more

### Table 1. Concordance of NALIA system results with those for conventional single ELISAs with EUROIMMUN reagent sets.

<table>
<thead>
<tr>
<th>Autoantigen probe</th>
<th>dsDNA</th>
<th>Ro</th>
<th>La</th>
<th>Sm</th>
<th>RNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>182</td>
<td>123</td>
<td>128</td>
<td>126</td>
<td>123</td>
</tr>
<tr>
<td>Patients positive in NALIA, %&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24</td>
<td>19</td>
<td>2.3</td>
<td>4.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Agreement of NALIA results with commercial ELISA, %</td>
<td>83</td>
<td>98</td>
<td>93</td>
<td>97</td>
<td>97</td>
</tr>
<tr>
<td>κ (SE)</td>
<td>0.56 (0.07)</td>
<td>0.92 (0.09)</td>
<td>0.41 (0.06)</td>
<td>0.62 (0.07)</td>
<td>0.73 (0.09)</td>
</tr>
</tbody>
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<sup>a</sup> Percentage of the results in excess of borderline values, calculated as 20% above the cutoff for that antibody.
often found in patients with central nervous system involvement. It will be interesting to follow up the NALIA and ELISA results in this respect.

The current assay readout provides quantitative data about the amounts of serum autoantibodies, which are of value for monitoring lupus patients. Indeed, there is a close correlation between anti-dsDNA concentration and disease activity, especially in lupus nephritis patients, who have increases in anti-dsDNA values that precede by weeks flares in the disease (21).

Investigators have suggested that more than 100 autoantibodies may be present in SLE patients (22). Although identification of a specific antibody is often associated with a particular disease subset, the presence of such an antibody may not be totally specific. More helpful to the clinician is the autoantibody pattern or profile, which multiplex arrays such as NALIA can readily and economically provide. As mentioned above, the number of autoantigens in an array can be increased via the use of a piezoelectric dispenser to deposit smaller spots. Finally, it may be possible with this type of analysis to furnish clinicians with multiplex array data at the point of care to facilitate a more rapid diagnosis.

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