An Integrated Digital Imaging System and Microarray Mapping Software for Rapid Multiplexed Quantitation of Protein Microarray Immunoassays

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Abstract
We describe a digital imaging system and image analysis software suitable for proteomic microarrays in basic research, epidemiological studies, and clinical diagnosis. Our goal was to develop a simplified, economical system for protein microarray immunoassays that combines automated image acquisition and data reduction while preserving the sensitivity and resolution of a conventional laser scanner. Recent advances in CMOS technology have made available digital cameras designed for low light applications with performance rivaling CCD cameras, introducing the possibility of a sensitive, low-cost CMOS-based imager with simplified design and compact size. This concept is enabled in part through the use of microporous nitrocellulose film-slide reactive surfaces. These surfaces exhibit protein binding capacity orders of magnitude greater than functionalized glass and amplify fluorescent light through Mie scattering. When combined with quantum nanocrystal emitters as fluorescent labels, film-slide surfaces greatly enhance fluorescent signals, producing microarray spot intensities that are readily detected by a CMOS sensor. Using a high-power violet diode laser to excite the quantum nanocrystal labels in an epi-fluorescence optical configuration, the system has the capability to image protein microarrays with sensitivity equivalent to a laser scanner. The 2560x1920, 1/2" format sensor enables the optical system to produce a 25x75mm 16-bit grayscale image with 10µm resolution in less than one minute. Guided by an innovative image-recognition template, an automated software algorithm based on spot-intensity gradient identifies spot boundaries and quantitates the microarray with no need for manual corrections. Due to the discrete nature of quantum nanocrystal emission lines, the system has an inherent wavelength-multiplexing capability to detect multiple analytes within the same spot. A benchtop version of the platform is ideal for portable and remote imaging applications and is compatible with centralized post-processing of data. Due to the imager’s simplified optics, the camera assembly can be miniaturized for hand-held, robotic imaging, and integration with fluid handling systems.
Introduction

Immunofluorescence detection has become an accepted method to identify and quantitate low-concentration proteins in lysate and serological samples. Immunofluorescence can provide equivalent sensitivity to enzyme-linked immunosorbent assay (ELISA) with greater dynamic range and higher throughput [Tonkinson and Stillman, 2002; Garfin and Ahuja, 2005; Cheng and Kricka, 2001]. When applied to a microarray format, fluorescence provides an additional opportunity for internal signal standardization by multiplexing technology [Chang, 1983; Hamelinck, et al., 2005]. In traditional microarray laser scanning fluorescence detection, light from a highly coherent laser is focused to a micron-sized area on the microarray surface where fluorescent label has been bound by ligand-probe interaction. The laser light excites the luminescent label; the resulting fluorescent emission is gathered by a lens system and detected [Tsien, et al., 2005]. With the assumptions that label concentration is proportional to analyte concentration, and that fluorescent signal is proportional to label concentration, the amount of fluorescence can be correlated to the concentration of analyte [Chang, 1983]. Because the concentration of analyte can be very dilute, however, the amount of fluorescent light requires a very sensitive measurement method. Photomultiplier is the detector of choice due to its low noise characteristics and high signal amplification of 100 to 1000 times [Hamamatsu, 2007]. The microarray substrate, typically a surface-modified glass microscope slide, is moved relative to the focused spot of light in order to expose all areas of the target to excitation light. This enables the entire area of the microarray to be sequentially scanned as a series of pixels, in which greater fluorescent label concentration results in greater pixel intensity. Concatenating the pixels in a two-dimensional format creates an image of the target that is processed with purpose-built software to extract the location and concentration of protein in the microarray. Instruments for microarray fluorescence detection and their accompanying software are generally known as laser scanners. Immunofluorescence detection by laser scanning has gained wide acceptance because it is recognized as a sensitive, high-dynamic range method that provides reliable and reproducible results [Espina, et al., 2004].

Microarray spots are typically a few hundred microns in diameter and are spatially separated by a pitch of similar distance. A full microarray image with this degree of registration requires the scanner to have very precise mechanical tolerances and enough resolution for accurate digital processing. Additionally, due to the relatively broad emission and absorption bands of organic dyes, separate excitation and corresponding emission filtering is required for each label wavelength [Tsien, 2005]. An instrument with the required mechanical tolerances, the requirement of more than one coherent laser for wavelength multiplexing, the highly sensitive
detection system, precision optical components, and high-speed electronic control and processing can be extremely expensive--exceeding $100,000 in initial cost.

Laser scanners are typically large, complex instruments that, despite their size, can be delicate and require frequent service to maintain precise operation, even in a controlled laboratory setting. Consequently, the instrument economics and upkeep can be a barrier to small laboratories, research in remote areas, and applications such as clinical diagnostics. When laser scanning is used with robotic systems, the scanner must be equipped with automatic loaders, an expensive and fragile solution for handling large batches of array slides or plates. Recognizing the need for an immunofluorescence detection system that is economical, portable, simpler to use, provides faster imaging with improved data analysis, and requires less maintenance, we developed an alternative to traditional laser scanning that obviates the need for complex electromechanical design.

Recent advances in several technology areas suggest a pathway to obtain sensitive and high-resolution protein microarray images by combining commercially-available components into a system that relies on digital imaging rather than laser scanning. In the past, several imagers have been developed based on CCD imaging technology [Hamilton, et al., 2006; Che et al., 2001; Sukumaran, et al., 2009], yet the systems did not fully resolve optical component complexity. By contrast, our aim has been to determine whether a microarray imager could be based on CMOS camera technology, thereby simplifying the design and reducing cost. In the past, CMOS devices have seen limited use in high-sensitivity applications because this technology could not achieve the electronic sensitivity of CCD. Driven by requirements in the digital camera market, the sensitivity of CMOS imaging devices has improved; modern CMOS imaging devices now provide high dynamic range and low-light sensitivities that rival CCD devices. But to our knowledge, no imager has yet been developed that provides the speed and economy of CMOS imaging technology while preserving the sensitivity and limit of detection (LOD) of laser scanning. We achieved this endpoint through the use of thin nitrocellulose-based film as the protein binding surface and by circumventing several limitations of the conventional method, namely: (1) the weak emission properties of organic dyes, (2) the expense and complexity of laser scanning optics, and (3) the inherently slow process of digital scanning.

Beginning with the printing surface, three-dimensional binding materials such as microporous nitrocellulose film capture as much as 500 times more protein analyte per unit area compared to two-dimensional functionalized glass [Shultz et al., 2013]. When cast as a thin film onto a prepared glass substrate, nitrocellulose is a superior printing substrate for protein microarrays because of its ability to preserve conformation, its large binding capacity, and the amplification of light through Mie scattering from its porous matrix [Bell and Shultz, 2014]. In the present
design, nitrocellulose becomes a key component enabling the use of a CMOS imager since the need for a photomultiplier detector is offset by increased fluorescence signal.

Quantum nanocrystals (QNCs) provide the second vital component that enables the system performance by increasing the brightness of fluorescent light. Substituting QNCs in the place of organic dyes provides 20-80 times increase in brightness without the disadvantages of bleaching and quenching [Resch-Genger, 2008; Yu, et al., 2003; Invitrogen, 2008]. Furthermore, all QNCs can be activated with a single excitation laser, eliminating the need for multiple, high-coherence solid-state lasers for detecting more than one fluorophore wavelength.

Digital imaging offers the advantage of eliminating inefficient optical components, as only one wide-field, high-resolution lens is needed. Digital imaging greatly improves the speed of detection because the camera captures light from a large area of the target in one frame, compared to the pixel-by-pixel detection required in the case of laser scanning. Greater acquisition speed offers an additional advantage: multiple images with different laser power, exposure time, and electronic gain can be mathematically combined to increase dynamic range and reduce random noise without a large sacrifice in acquisition time.

We describe in this article ArrayCAM™, a new platform for immunoassay detection which includes these alternative components. It improves speed, portability, cost, functionality, and can be easily integrated into automated applications. A robust software algorithm provides improved spot finding and quantitation and reduces the need for human intervention in post-processing tasks. In the current work, we describe the use of this platform to image and analyze high-density protein microarrays and demonstrate that it provides equivalent results to digital laser scanning with reduced imaging time.

**Methods and materials**

In this section, we describe in detail the four essential elements that combine to form the overall imaging system from a materials, hardware, and software perspective: (1) porous nitrocellulose-based film slides as the printing surfaces, (2) the use of quantum nanocrystals as an alternative to organic fluorophores, (3) the electromechanical design of the imaging system, and (4) the software algorithm for spot location and quantitation. Detailed methods for the use and operation of ArrayCAM instrument and software are published elsewhere [Yeon, et al., 2015]. Descriptions of the assay methods are provided in the Supporting Information section.
Essential elements

Printing surface
As an immobilizing surface in molecular biology, nitrocellulose has been used in its various forms for many years [Tonkinson and Stillman, 2002]. Commercial nitrocellulose film for use as a protein binding surface is manufactured by casting a thin (~10µm) layer of semi-porous nitrocellulose onto a standard microscope slide (film-slide) or plate glass (microtiter plate). In the following, we shall refer to nitrocellulose-coated glass surfaces as “film-slides.” Nitrocellulose film prepared in this way consists of a three-dimensional scaffold containing micropores with a distribution of diameters in the hundreds of nanometers. In general, nitrocellulose film-slides have several advantages relative to functionalized glass and hydrogel surfaces. These include the ability to bind orders of magnitude greater protein per unit area than 2D surfaces [Shultz et al., 2013; Tonkinson and Stillman, 2002]. It is believed that nitrocellulose film binds protein by weak intermolecular forces rather than covalent attachment [Van Oss, et al., 1987; Tang, et al., 2003; Kingsmore, 2006], thereby preserving the authentic conformation of proteins and retaining access to binding sites [Shultz et al., 2012]. Film-slide surfaces yield excellent spot morphology in developed immunoassays [Mujawar, et al., 2013]. In the present work, we used the NOVA™ formulation of ONCYTE® nitrocellulose film (Grace Bio-Labs, Bend, OR, USA). Nitrocellulose film exhibits autofluorescence at visible wavelengths, but this effect is minimized at detection wavelengths in the near infrared (NIR) [Waggoner, et al., 2006] and is nearly as low as that of glass surfaces for wavelengths exceeding 700nm [Bell and Shultz, 2013 (2)]. Thus, by substituting NOVA film surfaces for glass and hydrogel surfaces in NIR immunofluorescence assays, many times more fluorescence signal can be achieved with only a modest penalty in autofluorescence background (Figure 1).

Another advantageous property of NOVA film differentiates it from glass and hydrogel immobilization surfaces. Mie scattering within the semi-crystalline, porous structure of the NOVA network scatters the excitation light, increasing the probability of absorption by fluorophores. Additionally, the NOVA network scatters the emission component of fluorescence, thereby increasing the flux of fluorescent light reaching the detection system. These two effects combine to provide an augmentation of fluorescence intensity [Bell and Shultz, 2013 (1)]. The property of Mie scattering is a key attribute of NOVA; when substituted for glass it helps to provide the additional fluorescence signal strength needed to permit the use of a CMOS digital imager instead of a photomultiplier detector.
Quantum nanocrystals (QNC) are inorganic spheroidal particles that exhibit electronic quantum states confined to concentric layers of the materials that comprise the particle. Typically, commercially-available QNCs are composed from II/VI and II/V semiconductor alloys that are surface-functionalized to enhance bonding to bio-molecules such as biotin, proteins, and streptavidin [Resch-Genger, et al., 2008]. Currently, QNC probes are available in a variety of conjugates including streptavidin and primary and secondary antibodies [Invitrogen, 2008]. QNCs are attractive alternatives to molecular dyes because of their improved optical stability, greater fluorescence brightness, and narrow emission bands.

The use of QNCs as fluorescence labels and contrasts between QNC fluorescence labels and organic dye labels have been described by several authors [Tsien, et al., 2005; Chan, et al., 2002; Bruchez, 2005; Makrides, et al., 2005; Waggoner, et al., 2006; Nichkova, et al., 2007]. One of the primary differences is the increased brightness of QNCs relative to molecular dyes when excited by short-wavelength laser sources. Table 1 provides a list of molar extinction coefficients for three commercially-available QNC fluorophores and their molecular dye counterparts [Life Technologies, 2015]. Over the visible to NIR emission range, QNCs are from 20 to 80 times brighter than corresponding molecular-dyes. Moreover, the ability to use a single short-
wavelength diode laser as the excitation source for all QNC labels greatly reduces the cost and complexity of instrumentation; this has been an additional motivating factor for our use of QNC labels as a component in our microarray imager.

Table 1. Molar extinction coefficients for selected QNC labels and corresponding organic fluorophore labels.
Extinction coefficients are provided for 405nm excitation in the case of Qdots and the recommended excitation wavelengths for molecular dyes. Note: QNC fluorophores are denoted for their emission band wavelength while molecular dyes are denoted for their approximate absorption band wavelength.

<table>
<thead>
<tr>
<th>Qdot Label</th>
<th>Absorption Wavelength (nm)</th>
<th>Emission Wavelength (nm)</th>
<th>Molar Extinction Coefficient (cm$^{-1}$ M$^{-1}$)</th>
<th>Molecular Dye Label</th>
<th>Absorption Wavelength (nm)</th>
<th>Emission Wavelength (nm)</th>
<th>Molar Extinction Coefficient (cm$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qdot 585</td>
<td>405</td>
<td>585</td>
<td>2,800,000</td>
<td>Alexa Fluor 555</td>
<td>555</td>
<td>565</td>
<td>150,000</td>
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<tr>
<td>Qdot 655</td>
<td>405</td>
<td>655</td>
<td>10,800,000</td>
<td>Alexa Fluor 635</td>
<td>633</td>
<td>647</td>
<td>140,000</td>
</tr>
<tr>
<td>Qdot 800</td>
<td>405</td>
<td>800</td>
<td>10,900,000</td>
<td>Alexa Fluor 790</td>
<td>784</td>
<td>814</td>
<td>270,000</td>
</tr>
</tbody>
</table>

Optical fading (photobleaching), reduces the number of times a molecular-dye-labeled immunoassay can be scanned without significant photobleaching [Eggeling, et al., 2008; Diaspro, et al., 2008]. However, photobleaching in QNCs is far less compared to organic fluorophores [Wilfried et al., 2001]. In commercial QNC formulations, Qdots are coated with polymeric shells to reduce photo-induced oxidation [Arnspang, et al., 2012] and chemical decomposition [Invitrogen, 2008; Shao, 2011]. Photobleaching is generally not observed when QNCs are used for immunofluorescence labels and imaging applications. In our experience, flux values of less than 50mW/cm$^2$ and exposure timeframes of less than 15 seconds are sufficient to permit repeated imaging of the microarray, yet do not result in measurable photobleaching.

QNCs exhibit other beneficial characteristics making them attractive for use as fluorophore labels. Emission spectra of QNCs are narrow, Gaussian-shaped curves with spectral widths that increase from 20nm for emission at 585nm to approximately 100nm for 800nm emission [Invitrogen, 2008] (Figure 2). Additionally, QNCs have optical absorption edges located primarily in the blue-to-violet range and whose magnitudes increase with decreasing wavelength [Resch-Genger et al., 2008]. Due to the large separation between the absorption edge and emission peak of individual QNCs, emission filters can have wide band pass characteristics, permitting the collection of more fluorescent light, thereby increasing signal-to-noise.

Separate, narrow emission bands are particularly useful for wavelength-multiplexed applications because individual interrogation molecules can be tagged with QNC labels having separate emission bands. By using a single laser source to excite all emitters, all labels can be activated
simultaneously and individual emission spectra can be demultiplexed with the use of individual band pass filters [Chan, et al., 2002; Goldman, et al., 2004; Nichkova et al., 2007; Che, et al., 2001; Chen, et al., 2002; Makrides, et al., 2005; Hamilton, et al., 2006]. Fluorescence from individual labels can be measured by sequentially interposing each fluorophore’s corresponding band pass filter between the target and camera while repeating the excitation and imaging for each label.

![Figure 2. Absorption edges and emission bands of selected QNC fluorophores.](image)

Three quantum nanoparticle species are shown: Q585, Q655, and Q800. All QNC species share similar absorption edges, enabling a single excitation source in the range 350-450nm to be used for all emitters with no quenching due to re-absorption of emitted light. The three wavelengths shown are an ideal combination for multiplexing as the skirt of each emission profile does not significantly overlap the peak of its neighbors.

**Optomechanical**

The basic elements of the optical system are illustrated in Figure 3. The optical configuration is a typical epi-fluorescence design with coincident laser excitation and visible to NIR emission [Webb and Brown, 2013]. Fluorescent QNC labels can be activated by any violet or blue excitation source whose wavelength is in the range of 400nm to 450nm. Although light-emitting diodes (LEDs) and discharge lamps may be used as excitation sources, their optical power is distributed over a wide wavelength range, requiring the use of excitation filters to reduce excitation light in the emission channel. Diode lasers are preferred excitation sources due to their ability to produce intense optical power within a wavelength band of 15nm, eliminating the need for excitation filters in the present design. Typical commercially-available diode lasers suitable for this application are 405, 425, and 445nm. In the present design we used a 405nm violet laser (Nichia NDL7112, Tokushima, Japan) having an output power exceeding 500mW. The
laser diode is mounted in a standard 11-mm copper heat sink to prevent overheating while it operates in a continuous wave mode. With a working distance to the target of approximately 110cm, the measured optical flux at the surface of the microarray was 50mW/cm². A hard-coated dichroic mirror (Semrock FF458-Di02-25x36, Rochester, NY) is provided at a working angle of 45 degrees to provide nearly 100% reflection of the excitation beam while permitting transmission of the emission light toward the camera.

Emission filtering consists of a hard-coated bandpass filter centered on the emission profile peak. The bandpass filter is paired with a glass blocking filter whose absorption edge is approximately 100nm lower than the emission peak. This helps to reduce scattered excitation light reaching the camera lens from surfaces near the optical path. Because of the large separation between the absorption edge and narrow emission band of each QNC, the emission filter can be wide enough to capture nearly all the light within the emission band. In the case of 800nm fluorescence, emission filtering consists of an 809nm bandpass filter of width 80nm (Semrock FF02-809/81-25) to isolate the fluorescent light and a glass blocking filter (Thor Labs FGL695, Newton, NJ) to remove specularly-reflected excitation light. For color-multiplexed assays, more than one QNC label was used, each individually isolated by interposing its corresponding matching filter in the emission beam. At 655nm and 585nm, we used a Semrock FF01-655/40-25 bandpass filter with a Thor Labs FGL590 blocking glass filter, and a Semrock FF01-585/40-25 bandpass filter with a Thor Labs FGL495 blocking filter, respectively. All filters are mounted in a custom selectable rotating filter wheel driven by a linear actuator in one design and a programmable servo in another.
Imaging can be accomplished with a variety of commercially-available high-megapixel CMOS cameras that support the resolution requirements. For the present design, we used an IDS Imaging UI-1580LE camera (Obersulm, Germany) with a 2560 horizontal by 1920 vertical pixel format. We mapped this to a 25.6mm horizontal by 19.2mm vertical field of view, so a vertically-oriented 25x75mm microscope slide image can be concatenated from four individual images. Our choice of a 1/2” format 5MP image sensor with a physical size of 5.7x4.3mm resulted in a magnification of -0.22, providing 10µm +/-1um resolution referred to the object. For a microarray deposition spot diameter of 200µm, a resolution of 10µm provides over 300 pixels per spot, sufficient for analysis of spot intensity median, mean, and volume [Hamilton et al., 2006]. Finer resolution could be achieved with the same 5MP camera and lens by imaging a
smaller area of the target. For example, a 12x12mm target area can be imaged with 6µm resolution with no resulting penalty in acquisition speed or image brightness.

A wide choice of commercial fixed-focal-length megapixel lenses are available that can support the degree of resolution in the current instrument design. We chose a Fujinon HF35SA 35mm fixed focal length lens (Fujifilm North America Corp., Edison, NJ) with a selectable f-stop set at f/4. Optical path lengths in the present design are: camera sensor to lens (image distance), 44mm; lens to target (object distance), 170mm; total path length, 214mm. The camera/lens combination was focused for QNC emission at 800nm as the primary working wavelength. To improve image quality for QNC emission at 655nm and 585nm, a custom plano-concave lens of focal length -2500mm was inserted in the objective beam together with the emission filters, located at a distance of 100mm from the target.

A custom-designed servo-activated protective shutter is provided to cover the optics when the film slide is being loaded, preventing dust and moisture from reaching the optical elements. The film-slide is held in place by a slide carriage mounted on a custom rack-and-pinion system. The stepper-motor-driven slide carriage positions the film slide for imaging by directly driving the pinion gear. To reduce noise, the pinion gear is of the split type with an internal spring to eliminate gear backlash.

The digital camera is controlled by a personal computer with a standard USB interface. After transfer of the images to the PC, an additive stacking algorithm is used to combine 8-16 individual image frames, thereby reducing random pixel noise (camera read noise). Prior to image stacking, a dark frame is obtained by imaging with the excitation source turned off. The dark frame is subtracted from all subsequent signal frames. All images are provided in 16-bit grayscale TIFF format.

Software
Following acquisition, the microarray images must be reduced by post-processing to render useful protein-specific data [Akbani, et al., 2014; Espina, et al., 2004]. This process includes both spot finding (spot location) and quantification of the spot intensities relative to either local or global backgrounds. Additionally, to reduce assay variations and to obtain real-world concentrations, spot intensities may be calibrated by normalizing to intra-array controls. Recognizing that data reduction can be the most labor-intensive task of the overall proteomic microarray protocol, we designed our software to provide accurate and precise spot evaluation with minimal human intervention. Facilitating the efficiency of our method are two key attributes: (1) the use of an image-recognition template and (2) a spot finding algorithm based
on a combination of parameters directly obtainable from each image, including the spot moment and the gradient of the spot intensity.

Figure 4 illustrates the spot location algorithm by way of a flow chart. The process begins by loading a print configuration file, commonly known as a “.gal” file. The gal file is a standard format containing a header and a list of all spots in the microarray. The header provides information on the spot size, vertical and horizontal print pitch, and the number of rows and columns of spots. The listing or body of the gal file contains a spot-by-spot delimited list of each spot’s column and row placement, its name, ID, and its block location if the microarray is divided into individual print blocks. From the gal file, the software calculates a series of field points where each spot is expected to be located, based on the location of the upper left spot (the origin), once that point is known.

Figure 4. Flow chart of spot location algorithm.

The algorithm next proceeds to the stage of individual spot location, where a series of measurements are performed to identify the actual location of each spot. Using the calculated
field points, the software searches for a centroid of intensity located in a region surrounding each field point. The centroid is equivalent to the first moment of the spot region, defined as:

\[ C = \frac{\sum_{p=1}^{N} I_p (\bar{X}_p + \bar{Y}_p)}{\sum_{p=1}^{N} I_p} \]

Where \( I_p \) is the pixel intensity at the pixel \( p \), \( \bar{X}_p \) and \( \bar{Y}_p \) are the distance vectors to the pixel \( p \) relative to an arbitrary reference point, and \( N \) is the total number of pixels in the region.

Once the centroid is located, the field point is re-centered on the spot centroid. With this new location as an origin, the software searches for inflection points in the gradient of intensity along vertical and horizontal lines through the region. These points will define the lines of steepest descent in the gradient of the signal, points that are directly related to the spot boundaries. At these inflection points, the gradient will be maximized and the second derivative of the intensity with respect to the radial coordinate will be zero:

\[ \frac{\partial^2}{\partial r^2} I(r, \phi) = 0 \]

Averaging the two inflection point locations along each axis provides the coordinates of the actual spot center. With this point as the new center, the algorithm searches for a circle whose signal intensity is bounded by lower and upper limits relative to the local background. These user-adjustable limits define the sensitivity of the detection relative to the background. A single sensitivity setting can be used for all images produced by a particular combination of spot diameter, print pitch, and assay protocol. As the software proceeds through the microarray spot location routine, it keeps a running inventory of the calculated spot centers and the actual spot centers and uses these values to adjust the projected field points for the remainder of the spots. This process compensates for possible image rotation and drifts during the printing and eliminates the need to rotate or stretch a “grid” over the microarray to account for deviations in spot locations from their projected centers (field points).

Identification of the array origin is accomplished using an image recognition method. A set of spots is printed in the first row of the microarray (Figure 5) to serve as an image-recognition and alignment feature. The alignment spots can be a pattern of spots separated by blanks, a dilution series, or any other features that fluoresce after assay. The software provides the ability to create an image of the first row of spots and save that portion of the image as a recognition template. This template can be re-used for any microarray that has been printed with the same pattern appearing in the first row. With this template as a guide, the software automatically locates the corresponding pattern in each image and uses that subset of the image to identify the spot.
located at the origin, which is Row 1, Column 1 of the microarray. Once the origin is identified, the projected field points for all spots are calculated based on their coordinates derived from the gal file. The method is powerful because it is not affected by rotations or offsets in the microarray positioning from image to image. This obviates the need for manual adjustment of a spot-location grid on each image to be processed.

![Figure 5. Microarray image and image recognition template.](image)

This graphic represents a microarray image containing a unique spot pattern as the top row. The identification row is typically arranged as an irregular pattern of spots separated by blanks. The software creates the template by cropping out the top row of spots and saving as a recognition image. Later, the software loads this file prior to performing the spot location process and subsequently identifies the top row of the microarray by matching it to the template. If no recognition spots have been printed on the microarray, the first row of spots can serve as the surrogate recognition template for its own microarray image.

**Results**

The platform described in this article is composed of electromechanical and software aspects, each of which contribute to the utility and performance of the overall system. In the following, we describe the results of experiments to examine four aspects of the system performance: (1) acquisition speed, (2) software accuracy, (3) electronic sensitivity, and (4) three-color
multiplexing capability. Performance of the system when imaging and analyzing a microarray for infectious disease detection is ongoing and initial results are published elsewhere [Liang, et al., 2015].

**Acquisition speed**
An important performance advantage of a digital imaging system relative to a laser scanner is its ability to acquire microarray images of identical size and resolution in less time. To examine this aspect, we measured the speed at which the ArrayCAM imager acquired both a full image of a 25x75mm microscope film-slide and equally-divided sub-sections of the slide. The same measurements were performed using a GenePix 4400A (Molecular Devices, Sunnyvale, CA), a commonly used microarray laser scanner. Because the ArrayCAM imager is capable of imaging one-fourth of a 25x75mm microscope slide at once, we used both instruments to sequentially capture images of four sub-sections of the entire microscope slide.

Due to the sequential sampling nature of the laser scanner, acquisition times for that instrument require a minimum of 65 seconds per 1/4 sub-section of the film slide (Figure 6). By contrast, the equivalent image can be obtained on the ArrayCAM in approximately 11 seconds. As the target area increases, the acquisition time accumulates: the laser scanner requires 252 seconds for four sub-sections while the imager requires only 29 seconds.

To produce a contiguous image of the entire film slide, the imager first acquires individual sub-section images and the software merges these images to form a single 2560x7680 image. The entire full-slide image acquisition and merging process requires about 46 seconds to acquire and merge, compared to 252 seconds for the laser scanner (see Figure 6).
Figure 6. Acquisition times for a laser scanner and the ArrayCAM system. Images were acquired sequentially in 1/4 increments of the entire 25x75mm film slide. In all cases, the resolution was fixed at 10µm/pixel. For ArrayCAM, the acquisition parameters were set at 200ms exposure, 2 seconds acquisition time, and gain of 50. For the laser scanner, the laser power was set at 100% and the photomultiplier gain at 600. The single data point ( ) is the time required to acquire and merge five sub-images, 46 seconds.

Software accuracy

We explored three aspects of software accuracy: (1) spot location fidelity, or the ability of the software to correctly locate spots in both a homogeneous microarray and a heterogeneous microarray; (2) spot-by-spot median intensity correlation with a commercially-available software application; and (3) the detection sensitivity of the software spot-location algorithm.

Spot location fidelity

Performance of the automated software component was evaluated by its ability to determine spot locations and spot intensities on a uniformly-printed and stained microarray (Figure 7). An array of 16 columns x 40 rows of 650µm diameter spots with a pitch of 1250µm was imaged and subsequently processed by the algorithm to locate and mark spot boundaries. Following the automated analysis, the boundaries were inspected visually for variances between automatically located spot centers and manually-located spot centers. When offsets were present, we
calculated the differences in median spot intensity based on the automatically-located spot centers and the manually-located spot centers. Table 2 lists the results. Fifty-three (8 per cent) of the automatically-located spot centers differed from the manually-located spot centers by a detectable amount; the mean difference was 4.9 microns or 0.75% of the nominal spot diameter. Median spot intensities were on average 0.4% greater when the spot centers were offset from manually-located centers. Spot boundaries were, in some cases offset due to irregularly-shaped spot morphology. Irregular peaks in the spot intensity distribution tend to cause shifts in the boundary, increasing the enclosed signal intensity.

Figure 7. Spot finding in a homogeneous control assay. A negative control array is used to verify homogeneity of printed content, and displays minimal but measurable signal from cross reactivity of anti-mouse Qdot655 conjugate to printed human cell lysate. Panel A shows ArrayCAM digital image of microarray (details below); panel B shows overlay of detected boundaries corresponding to individual spots on the microarray. The microarray consists of a 16 x 40 grid of cell culture lysate spots from A549 lung cancer cell line (ATCC CCL-185) printed with a concentration of 1mg/mL onto AVID porous nitrocellulose microarray film slide (Grace Bio-Labs, USA) using a solid-pin contact robotic arrayer (Aushon Biosystems, USA). The array was probed with Qdot 655 conjugated to Goat anti Mouse IgG (Lifetech, USA).
Table 2. Results of auto vs. manual spot location methods on a homogeneous microarray.

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of spots</td>
<td>640</td>
</tr>
<tr>
<td>Spot size</td>
<td>650 microns</td>
</tr>
<tr>
<td>Number of centers differing from actual</td>
<td>53 (8.3%)</td>
</tr>
<tr>
<td>spot centers</td>
<td></td>
</tr>
<tr>
<td>Average offset of non-centered spots</td>
<td>4.9 microns</td>
</tr>
<tr>
<td></td>
<td>(0.75% of diameter)</td>
</tr>
<tr>
<td>Mean difference in median spot intensities</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

The previous exercise evaluated the ability of the spot location algorithm to perform reliable spot boundary determinations on a uniformly-printed microarray of uniform spot intensities. In practice, however, microarrays may have features with irregular shapes, intensities, and locations as well as background artifacts that can interfere with algorithm accuracy. To illustrate the performance of the algorithm in the presence of these irregularities, we tested the spot finding algorithm against a microarray with significant spot placement defects (Figure 8). In this array we printed ten different recombinant antigens specific to autoimmune disorders, each in replicates of four at randomly generated positions on the array; one antigen was printed offset from its intended location by a distance equal to one-half spot diameter. As with the previous experiment, we allowed the algorithm to perform the automatic spot identifications and compared the results to manual spot identification. We also included results obtained by processing the same image with the commercially-available microarray analysis application, GenePix Pro 7 (Molecular Devices, Sunnyvale, CA). Table 3 lists the results. All of the spots that had been printed with offsets were identified with both software applications. However, each software application incorrectly placed some spot boundaries in regions of background artifacts. The average location offsets were 58.6µm in the case of GenePix software and 15µm in the case of ArrayCAM software. For the spots whose automatically-located centers differed from manually-located centers, mean differences in median spot intensities were less than 0.2% for both software applications, however.
Figure 8. Spot finding in a heterogeneous microarray. A 13x13 array was printed with each content feature represented in quadruplicate (see supplemental information section for array content and assay method). One of the antigens printed with an erroneous offset of approximately 200µm from its intended location, as shown by red circles. Additionally, areas of the microarray contained background artifacts that reduced the signal-to-background for individual spots. Both ArrayCAM and GenePix software algorithms were able to locate and correctly quantitate the offset features.

Table 3. Spot location performance on a heterogeneous microarray.

<table>
<thead>
<tr>
<th>Aspect</th>
<th>ArrayCAM Software</th>
<th>GenePix Software</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of spots</td>
<td>169</td>
<td>169</td>
</tr>
<tr>
<td>Number of offset spots incorrectly identified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of centers differing from actual spot centers</td>
<td>2 (1%)</td>
<td>7 (4%)</td>
</tr>
<tr>
<td>Average offset of non-centered spots</td>
<td>15µm</td>
<td>58.6µm</td>
</tr>
<tr>
<td>Mean difference in median spot intensities</td>
<td>0.14%</td>
<td>0.19%</td>
</tr>
</tbody>
</table>
Spot-by-spot median intensity correlation

The goal here was to explore the spot-quantitation performance of ArrayCAM software relative to two commercially-available microarray analysis software applications: GenePix Pro 7 and ScanArray (PerkinElmer, Waltham, MA). For this investigation, we used microarray images acquired from an antigen-down assay whose print configuration was 17 rows by 17 columns, printed spot size 260µm, print pitch 400µm, labeled with 800nm QNC. Using a 200ms exposure time, the spot intensities spanned a range of 0 to 30,000RFU (Figure 9). To increase statistical power, we analyzed each of three 17x17 microarray images separately and aggregated the results for a total of 867 spots per set. We compared the background-subtracted median spot intensities obtained from the three quantitation algorithms without manual adjustments of spot boundaries or locations.

The ArrayCAM and ScanArray quantitation results were well correlated, with a slope of 0.983 and an R² value of 0.999. By contrast, the overall median spot intensities obtained from ArrayCAM software were 14% greater than those evaluated from GenePix software (slope=1.14, R²=0.98).

Figure 9. Microarray with 17x17 grid of 260µm diameter spots on 400µm centers. Individual pixel values in this 16-bit image span the range of 0-30,000RFU. The description of this microarray and assay is given in the supplemental section.
Figure 10. Background-subtracted signals for ArrayCAM software, ScanArray Software, and GenePix software. Automated spot-location algorithms were used to identify all 289 spots on each of three microarrays and data were aggregated for a total of 867 spots, reporting background-subtracted median spot intensity. We compared the three data sets on a spot-by-spot basis. Intensity values are from the respective quantitation algorithms without manual adjustments. Left panel: background-subtracted median values, ArrayCAM vs. ScanArray. Right panel: background-subtracted median values, ArrayCAM vs. GenePix.

**Algorithm detection sensitivity**

The algorithm sensitivity is determined by the ability of the software to evaluate individual spot intensities whose values are approximately equal to their local background intensities. Using the microarrays described in Figure 9, we calculated the ratio of local-background-subtracted median spot signal to local-background signal:

\[
R = \frac{(S - B)}{B}
\]

Figure 11 shows the results for the lower intensity signals. The inflection point of each curve at R=1 signifies the ratio below which the software can no longer distinguish spot signals from background signals. The three software algorithms provide nearly equivalent detection sensitivity for these microarray images; the number of spots whose ratio R<1 is about 350 for each algorithm.
Figure 11. Signal-to-background values for the higher sensitivity region. Data were concatenated from three images of 17 row x 17 column microarrays as in Figure 9 to form an ensemble of 867 total points. We calculated the ratio of background-subtracted intensity to background intensity and sorted the data by lowest-to-highest value.

Camera sensitivity

The overall detection sensitivity of the system is primarily influenced by two noise contributions that limit the ability of the system to discriminate between signal and noise. These are: (1) electronic camera noise and (2) assay background, including the natural autofluorescence of nitrocellulose.

Noise in a CMOS camera is composed of both dark noise and read noise [Koklu et al., 2012]. In the present system, dark noise is minimized in signal frames by subtracting out frames obtained when the excitation source is turned off (dark frames). Read noise, however, is random, cannot be subtracted out, and represents an ultimate noise floor limit of the detector. It is not practical to theoretically predict whether read noise will limit the signal-to-noise of a CMOS-imaged microarray because CMOS sensor noise parameters are generally not available. But it is possible to empirically determine if camera read noise is less than a relative signal level in a practical application. For instance, in an assay that utilizes nitrocellulose film as the printing surface, camera noise will be a negligible influence on the measurement sensitivity provided the camera
read noise is below the nitrocellulose autofluorescence background. To explore this, we printed a dilution series of streptavidin-conjugated QNC-800 emitters on a glass slide partially coated with NOVA, beginning with an initial concentration of 5nM, diluting approximately 2x per step (Figure 12). As detected with our imager, the fluorescence signal reached the natural autofluorescence background of the nitrocellulose film when the dilution was 3 QNC fluorophores per square micron. On areas of glass where no film was present, the average signal level was 10 RFU, suggesting that the camera read-noise level was about 1 fluorophore per square micron, lower than the practical detection limit for the film.

![Graph](Image)

**Figure 12. Empirical estimation of LOD for QNC fluorophores printed on NFC film.** Streptavidin-conjugated Q-800 emitters (Life Technologies) were printed in a 2x dilution series on NFC film cast on transparent glass. Signal from the QNC emitters reached the NFC film autofluorescence background at three fluorophores per square micron. Signal intensities on areas of bare glass were on average 10 RFU, indicative of the camera noise level. Extrapolation of the QNC signal to the glass signal suggests that the absolute detection limit of the system is approximately one fluorophore per square micron.

**Three-color QNC multiplex detection**

For a color-multiplexed biological detection assay, it is important to detect fluorescent light from a specific label of interest while rejecting light from other labels within the same multiplex
group. This is accomplished by selecting multiplex labels whose emission profiles have minimal wavelength overlap and by using bandpass filters whose out-of-band transmission is typically five orders of magnitude lower than the in-band transmission. Second, detection reagents must be highly specific to their intended targets so that non-specific binding does not significantly contribute to false positive signal. The resulting uncertainty from these two sources will determine the dynamic range with which individually-labeled analytes can be separately detected in a color-multiplexed scheme.

We explored this capability in the present system by designing a simple assay whose purpose was to separately detect three immunoglobulin isotypes on the same microarray using color discrimination. Purified Rabbit IgG, Mouse IgG, and Goat IgG were printed to separate spots on an array within a reaction well. The array was probed with a mixture of three different cognate anti-species-IgG antibodies conjugated to respective QNC labels: anti-rabbit-IgG/Q800, anti-mouse-IgG/Q655, and anti-goat-IgG/Q585. Three individual band pass filters were used to collect the signal from the 800nm, 655nm, and 585nm channels (809nm center/81nm width, 655nm center/40nm width, and 585nm center/40nm width, respectively). Signal intensities from each label were recorded from all spots, including the cognate features and the non-cognate features. Non-cognate signal between channels (Figure 13, Table 4) ranged from 0.59% to 1.6% relative to the positive cognate signals.

![Signal intensities from three fluorescent labels detected simultaneously.](image)

Figure 13. Signals from three fluorescent labels detected simultaneously. With NOVA as the printing surface, arrays were printed with purified Rabbit IgG, Mouse IgG and Goat IgG on separate spots within the same reaction well.
The well was simultaneously probed with a cocktail containing three different cognate anti-species-IgG antibodies conjugated to respective QNC labels: donkey anti-rabbit-IgG/Q800, donkey-anti-mouse-IgG/Q655, and donkey anti-goat-IgG/Q585. (See supplemental section for microarray and assay description.)

Table 4. Cross-talk intensities resulting from adjacent color-multiplexed emission labels.

<table>
<thead>
<tr>
<th>Emission Channel</th>
<th>Arrayed Target</th>
<th>Labeled Detector</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit IgG</td>
<td>Mouse IgG</td>
</tr>
<tr>
<td>800nm</td>
<td>100%</td>
<td>1.6%</td>
</tr>
<tr>
<td>655nm</td>
<td>0.59%</td>
<td>100%</td>
</tr>
<tr>
<td>585nm</td>
<td>1.1%</td>
<td>1.2%</td>
</tr>
</tbody>
</table>
Discussion

Observing the need for an efficient, economical, and simplified fluorescence-detection platform for protein microarrays, we developed a new system based on imaging rather than scanning and achieved equivalent results. This was made possible through the unique combination of nitrocellulose film-slides as the printing surface and quantum nanocrystal labels. NOVA film is an ideal microarray printing surface due to its substantially larger protein binding capacity compared to glass and its ability to amplify fluorescent light through Mie scattering. Mie scattering of excitation and emission light is due to the porosity of the film; sub-micron size pores act as scattering centers, whose scattering efficiency is maximized when pore diameter is equal to the wavelength of light [Goldberg, 1953]. The NOVA formulation used here was cast to contain distributions of pore sizes peaked near the excitation (405nm) and emission (800nm) wavelengths, respectively. Combining the advantages of NOVA and the brighter intensity of quantum nanoparticles provided the signal gain needed to enable a CMOS-based imaging device to detect immunofluorescence signals. The fact that all quantum nanoparticles can be excited with a single-wavelength laser greatly simplified the electromechanical and optoelectronic system design.

There are three factors that contribute to the image-acquisition time of the system. First, a minimal exposure time is required to obtain an image with good signal quality, which can be from 10 to 100ms depending on the intensity of expressed analytes in the microarray image. Second, a minimum number of frames is required to reduce camera read noise below the fluorescence background of the nitrocellulose film, normally about 8-16 frames. Third, each frame must be transmitted to the PC by way of the USB bus and this rate is typically limited by timing within the computer operating system and the application software. When combined, these three factors limited the acquisition time to a minimum of 11 seconds for a 25.6x19.2mm target area. Previous CCD-based imaging platforms demonstrated read times of 18s [Sumitra, et al., 2009], 2-40s [Hamilton, et al., 2006], and 5-20s [Che, et al., 2001]. Thus, imaging speed in the present ArrayCAM system results in greater than 5x advantage over a laser scanner and equivalent performance to a CCD-based imager when producing an entire image of a 25x75mm format microscope slide.

The use of CMOS imaging technology has the potential to greatly improve imaging speed for large batch sizes. To image large numbers of film slides, for example twenty-five to fifty slides per batch, current practice is to use an automated slide loader in conjunction with a laser scanner. Due to its small size and simplified optics, the major components of ArrayCAM can be combined within a small acquisition assembly or “head” that can be mounted to a robotic arm. Film slides can be arranged as a matrix or rectangular array on a platen placed within the range...
of motion of the robotic arm. With an acquisition time of about forty-five seconds per slide, the ArrayCAM could then image 100 slides in just over one hour while a laser scanner would require more than seven hours.

Even greater improvements in acquisition speed can be achieved by the use of an Ethernet or USB 3.0 camera, whose transfer rates can be ten times the rate of our USB 2.0 camera. With the use of a higher speed camera, the transfer time per frame would be limited by the exposure time required to obtain the microarray image, which can be as low as 10ms. Thus, by using a high-transfer-rate camera with short exposure times, the system described here has the potential to image a 25x20mm target in less than one second, or 100 full-slide images in five minutes.

The automated software spot-detection and analysis algorithms used in ArrayCAM provide essentially equivalent results to two commercially-available software applications, ScanArray and GenePix Pro 7. On average, ArrayCAM-derived and ScanArray-derived median intensities were consistently 14% greater than corresponding intensities derived from GenePix software. This is likely due to the inclusion of zero values by the GenePix median algorithm while the ArrayCAM quantitation algorithm excludes zeros in the calculation. However, despite the differences in median spot intensities, differences in spot detection and spot boundary locations were small among the three software algorithms and the overall correlations were excellent.

In conventional microarray image analysis, semi-automated software applications utilize a grid-based spot-location algorithm. The grid, which must be manually aligned on each image, serves as a guide to the algorithm in identifying each spot boundary. The software described here eliminates this manual step by using an image-location template derived from a uniquely-printed spot pattern within the image. The automated spot-finding algorithm searches for the spot pattern within the microarray image, and uses the identified pattern as the navigational guide to locate the remaining spots. With this method, the spot-finding algorithm is able to correctly locate spots when the image is rotated over any angle between 0 and 360 degrees. It can also identify a reversed or inverted image and transform the image to its correct orientation prior to analysis. These capabilities enable analysis of microarray images in automated applications where a single image recognition template may be applied to process large sets of images without human intervention.

Electronic detection sensitivity is a fundamental parameter that sets the absolute lower limit of detection for laser scanners and imaging systems [Tsien, et al., 2005; Lynt, et al., 2004]. Laser scanners typically have an electronic detection sensitivity of about 0.1 fluorophores/µm² while microarray imaging devices based on CCD technology provide about 1 fluorophore/µm² [Tsien,
et al., 2005; Hamilton, et al., 2006; Koklu, et al., 2012]. In the present work, the primary question was whether a CMOS detector could provide enough sensitivity for fluorescence microarrays printed on nitrocellulose film. Our results demonstrated that the binding capacity of NOVA together with its ability to amplify fluorescent signals via Mie scattering provided the required increase in signal brightness so that a CMOS detector could be used. The sensitivity limit of about three fluorophores per square micron was determined by the nitrocellulose fluorescence background, not by the noise characteristics of the camera. For the microarray assays described here, the benefits of the NOVA film’s binding and light-scattering properties outweigh its autofluorescence, especially for NIR emission. Thus, the greater electronic sensitivity provided by laser scanners or imagers based on CCD technology provide no additional benefit over the use of the more economical, less complex CMOS-based detector.

We demonstrated the capability to detect wavelength-multiplexed signals from three individually-labeled probe antibodies with out-of-band signals ranging from 0.59% to 1.6%. These uncertainties are a combination of the overlap of label emission profiles in the wavelength domain as well as non-specific binding of anti-species to non-cognate antibodies. Wavelength overlap is expected to be greater for QNC emission profiles that are adjacent in the wavelength domain, but we did not see any trends in the results that were indicative of that. This suggests that the major contribution to out-of-band signal was primarily non-specific binding, which limited the dynamic range available for wavelength multiplexing to about 100:1 in the present work. Although the multiplexing demonstration was performed on separate spots within the same microarray, results of preliminary experiments in our laboratory suggest that the method applies equally well to multiplexing within the same spot [Shultz, 2014].

For the data presented here, all the experiments were performed with nitrocellulose as the binding surface and with the use of QNCs as the fluorescent label. Because the system is an imager, it may also be used with colorimetric microarray immunoassays printed on nitrocellulose film-slides [Espina et al., 2004]. For colorimetric assays we have provisioned a version of the present imager with a white-light source. Alternatively, the on-board violet laser is an excellent illumination source as well. Because the system is equipped with a selectable filter wheel, a green bandpass filter such as a CIE-Y or similar can be added to serve as the emission filter for visible-light detection.

An additional question is whether the current imager could be used for fluorescent immunoassays printed on transparent glass microarray surfaces. Signal intensity on transparent glass is reduced due to lower protein binding capacity and the absence of Mie scattering at the glass surface. Under these circumstances, organic fluorophores have not provided the brightness required to permit the use of CMOS detection. However, preliminary data from our
lab suggest that it would be worthwhile to extend the study of CMOS detection to the combination of QNC labels and functionalized glass printing surfaces, and these studies are ongoing. It is quite likely that CMOS detection may provide the sensitivity for this combination but modifications in assay conditions may be required to increase brightness, for example by enzymatic signal amplification [J. Ness, et al., 2003]. Because glass does not autofluoresce in the visible region, we expect the system sensitivity in that case to be determined by the electronic noise of the camera, which is 3x lower than the nitrocellulose autofluorescence background at 800nm. For applications requiring less resolution, electronic sensitivity can be increased by using a lower megapixel camera, or by pixel binning. In these ways, the speed and simplification benefits of using CMOS technology could extend beyond nitrocellulose film to glass and other transparent surfaces.

**Conclusion**

We have developed a fluorescence-based microarray imaging system by combining a microporous nitrocellulose printing surface with quantum nanocrystal labels, enabling the use of a CMOS imaging camera. The resulting system provides resolution and sensitivity equivalent to a laser scanner but with imaging speed that is four times faster than a laser scanner. The platform is ideal for reverse-phase and serological applications with nitrocellulose as the printing surface. The system supports multiplexing applications where several analytes can be labeled with individual emitters and detected within the same microarray or even within the same microarray spot. A number of applications would benefit from the use of a small, economical, less-complex and automated microarray imaging platform. Because of the system’s inherent speed advantage, it has the potential for use in high-throughput applications. For example, the camera assembly can be attached to the movable gantry of a liquid-handling robot, the resulting combination representing a complete microarray processing system, from wet lab to data analysis on one platform. Through reduced complexity, versions of the system become truly portable as well as being robust enough to withstand more rigorous environments. This should enable disengagement of microarray imaging from the central laboratory toward wider deployment into smaller laboratories, remote field laboratories, and in clinical diagnostic applications. The simplicity and economy of the platform argues for a variety of cost-sensitive uses such as in education, training, and use by clinicians and basic researchers in developing countries.
Acknowledgements

The authors would like to thank Virginia Espina (George Mason University) for providing microarray images for spot location fidelity evaluation and her numerous suggestions to improve and add functionality to the software. Jacob Wells (Grace Bio-Labs) provided graphics for the article. We thank Stephen Moody (Orca Photonics) for a thorough review of the optical assembly design and his many suggestions for design optimization.
References


Supporting Information

Microarray Construction

Materials and Reagents
NOVA nitrocellulose filmslides, SuperG+ blocking buffer, microarray print buffer and ProPlate modules were sourced from Grace Bio-Labs (Bend, OR, USA). Antigens were purchased from selected vendors (Table X), and were aliquoted, flash frozen, and stored at -80°C until use. Goat anti-Human IgG was purchased from Vector Labs (Burlingame, CA, USA), Rabbit anti-human IgM from ABR Affinity Reagents (Golden, CO, USA), and Mouse anti-human IgA from ThermoFisher (Waltham, MA, USA). Quantum nanocrystal conjugates Donkey anti Mouse QDot585, Donkey anti Goat QDot800, and Donkey anti Rabbit QDot655 were purchased from ThermoFisher Scientific (Waltham, MA, USA). SeaBlock was purchased from ThermoFisher; all other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Microarray Fabrication
Microarrays were printed using a non-contact inkjet printing system (Scienion S3, Scienion AG, Berlin DE). The printing chamber was pre-equilibrated to 55% relative humidity, and 16-pad NOVA nitrocellulose filmslides were incubated in the humidified print chamber for 1 hour prior to printrun initiation. The 16-pad format of the NOVA filmslides allows production of 16 arrays per slide; the corresponding 16 well ProPlate modules allow each array to be isolated and individually processed, either by itself or in parallel with the other arrays on the slide. Antigens were diluted to optimal concentrations in 20 microliters final volume of printing buffer and transferred to appropriate wells of a 384-well microtiter plate (Greiner Bio One, Monroe, NC, USA). Microarrays were printed with columns and rows separated by 400um. Each spot of the microarray received 3 sequential depositions of approximately 300pL resulting in spot sizes of 260um. After printing, the microarrays were incubated within the humidified chamber for 8-12 hours, then transferred to boxes and stored desiccated at 4°C until post print processing.

Microarray Post Print Processing
Slides with printed microarrays were assembled to ProPlate 16-well chambers and blocked by incubation with 200uL SuperG+ blocking buffer diluted 1:50 in 1XPBS for 30minutes with gentle rotational agitation. The blocking buffer was removed and the arrays were centrifuged in an inverted position for 5 minutes at 500rpm to remove all remaining buffer, then air dried before placing in a desiccated storage box at 4°C. Arrays were stable at least 3 months in these conditions.
Microarray Assay process

Anti-Nuclear Antigen Serology Assay

The basic steps of a serology microarray assay include incubation with diluted serum, incubation with primary antibody, and incubation with labeled secondary antibody. Each step requires washing protocols before and after to remove excess reactants.

The detailed protocol for 16-well microarray serology assay follows:

1) Pre-incubate arrays with 150uL of 1X PBS for 5 minutes with agitation on rotating shaker at 200rpm.
2) Prepare serum samples by diluting 1:150 in Seablock. Prepare 150uL for each microarray.
3) Incubate each array with 150uL of diluted serum solution, 30 min at 200rpm.
4) Remove serum solutions, wash wells 3X with 1X PBS, 5 minutes each wash at 200rpm.
5) Incubate each array with 150uL of Goat anti human IgG (Vector Labs) at 1:2000 dilution in 1X PBS + 1% BSA for 30 minutes at 200rpm.
6) Remove primary antibody solution, wash wells 3X with 1X PBS, 5 minutes each wash at 200rpm.
7) Incubate each array with 150uL of Donkey anti Goat QDot800 at 1:250 dilution in 50mM Tris pH7.6 +1% PEG20K + 2% BSA for 30 minutes at 200rpm.
8) Remove secondary antibody label solution, wash wells 3X with 1X PBS, 5 minutes each wash at 200rpm.
9) Rinse each well with 150uL of water; dry the arrays by inverted centrifugation at 500 rpm for 5 minutes.
10) Scan microarrays on ArrayCAM system.

Software Comparison Microarray

To compare the quantitative data collection of the ArrayCAM software with other commercial software programs, a test microarray was constructed with intentional signal distribution from low to high. The test microarray was constructed using human IgG and IgM as antigens, with each at three dilutions in the print run. The array was printed in a 17x17 grid, with randomly generated spot placement location so that each concentration of each immunoglobulin antigen was present in at least 26 locations across the array. A set of fiducial markers (biotinylated goat IgG) was placed in the first row for automated spot finding.

The microarray was processed as described above, except the assay procedure was truncated such that the serum incubation step was eliminated, instead proceeding directly to the primary
antibody incubation step. All other steps remained the same as the ANA assay. For the experiment described in the text, goat anti human IgG was used as the primary antibody, and donkey anti goat IgG Q800 was used for the fluorescent label.
### Table 5. Microarray Content and Vendor information for Anti-Nuclear Antigen Microarray assay

<table>
<thead>
<tr>
<th></th>
<th>Antigen</th>
<th>Vendor</th>
<th>Part Number</th>
<th>Spotting Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong></td>
<td>Sm (Bov)</td>
<td>Immunovision</td>
<td>SMA-3000</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>Sm (Rec)</td>
<td>Diarect</td>
<td>11701</td>
<td>0.1</td>
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<tr>
<td><strong>3</strong></td>
<td>SmRNP (Bov)</td>
<td>Arotec</td>
<td>ATR01-10</td>
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<tr>
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<td>SmRNP (Rec)</td>
<td>Diarect</td>
<td>11600</td>
<td>0.1</td>
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<td>17401</td>
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<td><strong>12</strong></td>
<td>Scl-70 (Rec)</td>
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<td>ATJ01-02</td>
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<td>12901</td>
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<td>Immunovision</td>
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<td>15633-019</td>
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<td>GBL</td>
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<td>1x</td>
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<tr>
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<td>SeaBlock</td>
<td>Thermo</td>
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Figure 14. Layout of Anti-Nuclear Antigen microarray.