MICROFLUIDIC BARCODE ASSAY FOR MULTIPLEXED CLINICAL DIAGNOSTICS

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ABSTRACT

Here we present a novel method for creating three-dimensional, photocaptured protein patterns that can be completed with no additional linker molecules required. We utilized the photopatterned proteins to selectively capture antibodies and implemented a microfluidic sandwich assay. We demonstrate Hepatitis C virus (HCV) diagnostic device based on the technology and show that the device is capable of assaying human sera and can positively identify HCV+ human patient serum samples in 60 minutes. We also demonstrate a barcode assay using lectins to detect differences in prostate specific antigen (PSA) glycan structure. Additionally, we image the HCV diagnostic chip using a low-resource setting compatible imaging system – the fluorescence CellScope.

INTRODUCTION

Near-patient clinical diagnostics require simple-to-use assays. A powerful canonical format is the ‘dipstick’ lateral flow assay (e.g., home pregnancy test, HIV oral fluid screening test). These low or no power rapid dipstick tests provide only “yes/no” readouts for limited biomarkers. Nevertheless, to address 21st century challenges spanning from global health to skyrocketing costs in the developed world, new fast and simple – yet multiplexed and quantitative – tests are needed. Consequently, cell phones are emerging as feasible for diagnostic questions requiring sophisticated (beyond binary) readouts.¹⁻³ To yield a sophisticated diagnostic compatible with cell phone imaging, we introduce a simple and rapid microfluidic “barcode” immunoassay format, demonstrated for confirmatory diagnosis of Hepatitis C. The 60 minute, low-power (1mW) barcode assay advances telemedicine by eliminating the need for a centralized clinical laboratory.

In tandem with use of mobile technology in telemedicine,⁴⁻⁷ healthcare delivery would benefit from reduced dependence on centralized laboratory facilities. For hepatitis C virus (HCV) confirmatory diagnosis after a positive rapid screening result, semi-quantitative measurement of reactivity against specific antigens or test of viral RNA presence comprises the gold-standard confirmation of infection. Current HCV confirmatory assays require a clinical lab facility. Taken together, we see assays that require no sophisticated hardware – e.g. pumps/valves – and mobile devices as potentially central to advancing towards new solutions to global health challenges.

The microfluidic format is uniquely suited to work with mobile imaging modalities. First, the small footprint of microfluidic devices along with the reduced bulk of smartphone-based imaging techniques lends to being adapted to the point-of-care setting. Second, materials used to fabricate microfluidic devices are usually optically clear – elastomers, glass and plastics – and are therefore compatible with the optical detection mechanism used for phone-based diagnostic systems. Lastly, the inherent small volumes leveraged in the microfluidic format are often difficult to be visualized with the naked eye. The ability to be combined with a low-cost mobile reader device greatly increases the utility of microfluidics in near-patient settings. A variety of microfluidic assays have been demonstrated to work with a mobile imaging device.⁸⁻¹¹

The ability to define distinct regions in microfluidic devices

serves as the basis for the creation of multiplexed analytical platforms. Of the more commonly employed methodologies, photopatterning has emerged as perhaps the most prominent techniques in creating complex patterns inside microfluidic devices.¹² Immobilization on bare channel surfaces suffers from the disadvantage of requiring grafting of reactive groups onto the surface and/or the use of linkers to attach proteins to the surface. Additionally, only a low percentage (as low as 0.01%) of proteins from the bulk solution can be captured onto the surface.¹³ The low immobilization efficiency onto microchannel surfaces is largely due the extremely low surface area to volume ratio while operating in the microscale. Polymer monoliths occupy part of channel volume and provide increased surface area for the immobilization of proteins. However, the use of polymer monoliths still requires linker groups to be attached to the monoliths. Lastly, hydrogel matrices have been used as an immobilization matrix as well. However, as opposed to covalently immobilizing the proteins, the dense gel matrix physically traps the proteins.¹⁴⁻¹⁷ In a recent implementation of immobilization inside hydrogel matrices, streptavidin-functionalized hydrogel was utilized. However, the technique involves a blocking step that lead to long processing times when creating complex patterns.¹⁸

In this manuscript we demonstrate a heterogeneous barcode immunoassay implemented in a microfluidic format (Figure 1a) that is capable of multiplexed detection against up to 5 distinct targets. We leverage a photopatternable gel substrate - light-activated volume-accessible gel (LAVAgel)¹⁹ as the functional matrix. A multiplexed Hepatitis C virus (HCV) barcode assay on a microfluidic chip is used to test human serum samples to distinguish patients with and without HCV infection. We also demonstrate a barcode assay using lectins to detect differences in prostate specific antigen (PSA) glycan structure. Additionally, the HCV diagnostic chip can be imaged directly using the fluorescence CellScope (Figure 1b) – a low-resource setting imaging system based on a mobile phone.^{7,20} Taken together, we believe the microfluidic barcode assay and fluorescence CellScope offers a robust and facile platform for implementing multiplexed heterogeneous immunoassays designed for point-of-care applications.

Figure 1 Microfluidic assay and imaging platform. a) Photograph of microfluidic chip with 4 (devices) well-pairs each connected by 3 microfluidic channels. The channels are filled with dye to facilitate visualization. b) Photograph of microfluidic chip being placed into the fluorescence CellScope.

MATERIALS AND METHODS

Chemicals and Reagents

Aqueous solution of 30% (w/v) (29:1) acrylamide/bis-acrylamide, glacial acetic acid, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), methanol and 3-(trimethoxysilyl)-propyl methacrylate and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO). N-[3-[(4-benzoylphenyl) formamido-[propyl]-methacrylamide (BPMAC) was custom synthesized by PharmAgra Labs (Brevard, NC). AlexaFluor 488 (AF488) and AlexaFluor 555 (AF555) labeled Ovalbumin (OVA) and bovine serum albumin (BSA) were purchased from Life Technologies (Carlsbad, CA). FITC-labelled anti-BSA antibody was purchased from MyBiosource Inc. (San Diego, CA). Hepatitis-C Virus (HCV) positive serum, AF488 conjugated HCV Core(c22p), NS3(c33c) and NS4(c100p) antigens were provided by Norvartis Diagnostics (Emeryville, CA). HCV negative human sera were purchased from SeraCare Life Sciences (Oceanside, CA). AlexaFluor 568 (AF568) conjugated secondary goat anti-human antibody was purchased from Life Technologies (Carlsbad, CA). All antigen, antibody and serum samples were diluted into 1X Tris-Glycine buffer before introduction into the chip. 1X Tris-Glycine buffer was purchased at 10X concentration from Bio-Rad (Hercules, CA).

Image Acquisition, Control and Patterning Instrumentation

Fluorescence images were acquired on an Olympus IX-50 inverted microscope (Olympus USA, Center Valley, PA) with fluorescence illumination provided by X-cite exacte illumination system from Lumen Dynamics (Mississauga, Canada). Images were acquired through a 10X/0.3 NA objective (Olympus USA, Center Valley, PA) with filter cubes for GFP – Omega XF100-3 and Texas Red – XF102-2 (Omega Optical, Brattleboro, VT). Image analysis and stitching of multiple adjacent microscope images was performed using ImageJ from NIH (Bethesda, MD). Custom-built programmable high-voltage power supply (HVPS) was used for electrophoretic control with platinum electrodes directly inserted into the sample reservoir wells. UV for photopatterning was provided by a Hamamatsu Lightningcure LC5 unit (Bridgewater, NJ) through a Lumatec series 380 liquid light guide (Deisenhofen, Germany). Photomasks designs were created in-house and laser cut from 50 μ m thick stainless steel sheet using Universal Laser PLS6MW with a 30W fiber laser cartridge (Scottsdale, AZ).

Microfluidic chip preparation

Chip designs were performed in-house and fabrication was performed by Caliper Life Sciences (Hopkinton, MA). Standard wet etching and drilling methods were used followed by thermal bonding. Each device consists of three parallel microfluidic channel of 1.2mm length, 90 μ m width and 20 μ m depth connecting wells of 2mm in diameter and 1mm deep that serves as the sample reservoirs. Fig. 1a shows a photograph of a single device. One of the reagent wells is filled with dye to assist with visualization of the three microfluidic channels. Each chip contains 4 devices with 3 channels each.

Prior to introduction of polyacrylamide gel precursor solutions the glass channel surfaces were functionalized with acrylate-terminated self-assembled monolayer as previously described. Precursor solution with 1X TG, 4% wt/vol total acrylamide (4%T) with 2.6% of the total as cross-linker bisacrylamide (2.6%C) and 1.6nM BPMAC were mixed and degassed with sonication and vacuum. The BPMAC imparts photo-activatable capture capability to the polyacrylamide gel. Immediately prior to introduction into the device 0.08% (wt/vol) of

APS and 0.08%(vol/vol) of TEMED were added to the precursor solution to initiate polymerization.

Barcode assay fabrication

Photopatterning of proteins bands (barcodes) inside the microfluidic channels are performed after completion of chip preparation. Briefly, proteins to be immobilized (labeled or unlabeled) are diluted into 1X TG buffer prior to pipetting into the sample wells (~6 μ L). The samples are then electrophoretically loaded into the microfluidic channel by applying a 200V electrical bias between the sample and sink wells. After loading, the photomask was placed on top of the channels and the light guide is placed directly on top of the opening and in contact with the mask. Subsequently, UV illumination is applied for five seconds at 20% diaphragm opening controlled through the instrument interface. After illumination the wells were each rinsed three times with 1X TG buffer and a reverse bias of 500V was applied for five minutes to electrophoretically wash out non-immobilized proteins. Successful protein band patterning was confirmed using fluorescence imaging.

Mobile Microscope Design

The CellScope fluorescence microscope used in this study consisted of a transmission light path capable of fluorescence and bright-field illumination and a finite conjugates imaging pathway built into a 3D printed casing designed to couple to an iPhone 4S. The collection optics included a 10x/0.25 NA objective (Edmund Optics, Barrington, NJ, #36-132) spaced 160mm from a 20x widefield eyepiece (#39-696) with a pair of silver turning mirrors (Thorlabs, Newton, NJ, CM1-P01) to make the design more compact. The casing, stage, and phone adapters were printed using thermally extruded ABS plastic utilizing a desktop 3D printer (Stratasys, Eden Prairie, MN, uPrint Plus). Illumination for the simultaneous dual-color fluorescence was provided by a mounted RebelNeutral White (4100K) LED purchased from Luxeon Star(MR-WN090-20S, Brantford, ON, CA) attached to a heat sink (Digikey, Thief River Falls, NY, 294-1111-ND). An aspheric lens (Newport, KPA031) with a diameter of 25 mm and 17 mm effective focal length was separated from the LED by its back focal length and used as a collector lens with a second asphere of the same type used as a condenser lens. The dual band-pass excitation filter (59022x, Chroma Technology) was placed after the collector to select two excitation bands centered at 470 nm and 570 nm from the collimated source. After the excitation filter, a coverslip set at 45° is used to couple in light from a standard 5mm green LED for bright-field imaging of the chip. An emission filter (59022m, Chroma Technology) is placed as close as practical to the back focal plane of the objective to select emission bands centered at 520 nm and 630 nm. A separate modular enclosure contains a rechargeable lithium ion battery (Astro Pro External Battery, Anker), a 700 BuckPuck DC Driver (3023-D-E-700, Luxeon Star), and a switch for selecting between fluorescence and bright-field illumination. Final separation of spatially overlaid fluorescent probes was done through the on-board Bayer Filter on the phone which, in combination with an on-board demosaicing algorithm, assigns a red, green, and blue value to each pixel. Imaging was done using the default camera application on the iPhone 4S with the exposure time and focus locked on a dark field to maximize sensitivity.

RESULTS AND DISCUSSION

HCV Diagnostic with Human Serum Sample

Figure 2 Microfluidic barcode assay testing biological samples. a) Fluorescence readout of Hepatitis C virus (HCV) assay examining human serum samples using an epi-fluorescence microscope with stitched images. HCV+ sample shows clear bands at 2 of the 3 HCV antigen locations. HCV- Sample shows only signal at the positive control location. b) Profile plots of the antigen patterns and blotted serum signals. c) Lectin-based barcode assay can be used to test for differences in prostate specific antigen glycan structure.

In order to demonstrate the platform's ability to make biologically relevant measurements, we move to implement a microfluidic barcode Hepatitis C virus (HCV) diagnostic assay. For the HCV diagnostic device a five band pattern was created (Fig. 6a) including three AF488-labeled HCV antigens, one negative control (UV only) and one positive control band patterned with protein L. Protein L is a bacterial protein that binds to human immunoglobulins and is used in the assay as positive control to verify the successful loading of serum samples. The first photograph – “Pattern” – in Fig. 2a shows a typical fluorescence image of the patterned proteins after patterning of all five bands. The three HCV antigens can be clearly visualized as three green bars on the left side of the microchannel, while the negative control band has no proteins immobilized and the positive control (Protein L) is unlabelled and shows no fluorescence.

To test the device's ability to identify HCV infection, serum samples are diluted 1:40 into 1X Tris-Glycine buffer and electrophoretically loaded into the HCV diagnostic device. The volume of human serum consumed per triplicate assay is approximately 150nl. After antibody washout the device is imaged using an inverted fluorescence microscope, the results are shown in Fig. 2a. The HCV+ serum sample shows secondary antibody signal at the c100p and c33c antigen locations in addition to the protein L band, suggesting the serum sample contains human antibodies that react with the patterned viral proteins – indicative of HCV

infection. The HCV- sample shows only a single fluorescence band at the protein L location suggesting successful sample loading but no reactivity at any of the patterned antigen sites. Fig. 2b shows the electropherograms – patterned antigen in green line and secondary blotting results in red – for the HCV+ and HCV- sample. In the HCV+ case the blotting result peaks align perfectly with the patterned antigens at c100p and c33c, suggesting specific interactions of human antibodies with those antigens. The region with only UV illumination shows no signal indicating no nonspecific interaction with the activated gel. The HCV- sample device traces shows comparable levels of patterned antigens compared to the HCV+ sample device but no probed signal, suggesting the serum contains no HCV-reactive human IgGs. As non-infectious diseases, such as cancer become increasingly urgent in global settings, we also demonstrate a lectin-based barcode assay to assess differences in prostate specific antigen (PSA) glycan structure to potentially differentiate healthy vs. cancer-correlated PSA (Fig. 2c).

To further demonstrate the platform's potential to create diagnostic tools that are compatible with the point-of-care setting we imaged the HCV diagnostic device using a customized fluorescence-capable CellScope. It provides high resolution imaging in a portable form factor that takes advantage of the recent improvements in LED illumination and phone camera sensitivity.

Figure 3 a) Photograph of the customized fluorescence CellScope. The light path used for imaging the microfluidic chip and the schematic of the optical components used is overlaid and labeled. b) Readout from microfluidic barcode assay device obtained using the fluorescence CellScope. Images were obtained by splitting the RGB image acquired from the smartphone into 3 separate channels/images. Only the green and red channel was used in the figure as no signal was present in the blue channel.

In the customized CellScope shown in Fig. 3a, a white light LED illumination source was paired with a dual band-pass filter set and the built-in Bayer Filter Array of the mobile phone to collect two channels of fluorescence data. Each photopatterned protein band in the barcode assay was imaged simultaneously in the green and red channels, enabling the correlation of the position of the antigen and captured antibodies. By utilizing this approach, there is no need to switch filters or illumination source during imaging of a given protein band location. This avoids the cost and complexity of the additional optics, electronics, and mechanical components that are used in multi-color fluorescence imaging with a scientific monochrome camera. Fig. 3b shows the set of images acquired using the CellScope that are analogous to those from Fig. 2a. The system was designed to capture the full field of view of the microscope, yielding a circular field of view inscribed on the rectangular sensor of the mobile phone. The first row shows each of the patterned protein bands with the second and third row showing the HCV- and HCV+ immunoblotting results respectively. As in Fig. 2a, the HCV+ and HCV- assays were performed in different devices with the identical pattern. In this case the pattern images were taken after the assay was complete. This allowed the images of the antigen pattern and the HCV+ probed results to be acquired simultaneously without the need to move the chip; resulting in perfect alignment of the immunoblotted band and the patterned antigen.

CONCLUSION

We presented here a novel methodology for photopatterning complex patterns in microfluidic devices using a functionalized polyacrylamide gel matrix (LAVAgel). We then implemented a multiplexed microfluidic HCV diagnostic tool that is capable of detecting human anti-HCV antibodies from dilute human patient sera within 60 minutes. The microfluidic diagnostic assay was interfaced with a custom fluorescence CellScope and together demonstrates the platforms' potential to be used in a point-of-care setting. Taken together, we believe the microfluidic barcode assay and fluorescence CellScope offers a robust and facile platform for implementing multiplexed immunoassays for point-of-care applications.

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