A Dedicated Low-Cost Fluorescence Microfluidic Device Reader for Point-of-Care Ocular Diagnostics

Noah Pestana
Department of Electrical and Computer Engineering,
Northeastern University,
Boston, MA 02115

David Walsh

Adam Hatch

Department of Chemical Engineering,
Northeastern University,
Boston, MA 02115

Paul Hahn

Glenn J. Jaffe

Department of Ophthalmology,
Duke University Eye Center,
P. O. Box 3802, Durham, NC 27710

Shashi K. Murthy
Department of Chemical Engineering,
Northeastern University,
Boston, MA 02115

Mark Niedre[1]
Department of Electrical and Computer Engineering,
Northeastern University,
Boston, MA 02115
e-mail: mniedre@ece.neu.edu

Microfluidic fluorescence assay devices show great promise as preclinical and clinical diagnostic instruments. Normally, fluorescence signals from microfluidic chips are quantified by analysis of images obtained with a commercial fluorescence microscope. This method is unnecessarily expensive, time consuming, and requires significant operator training, particularly when considering future clinical translation of the technology. In this work, we developed a dedicated low cost fluorescence microfluidic device reader (FMDR) to read sandwich immunofluorescence assay (sIFA) devices configured to detect vascular endothelial growth factor ligand concentrations in ocular fluid samples. Using a series of sIFA calibration standards and a limited set of human ocular fluid samples, we demonstrated that our FMDR reader has similar sensitivity and accuracy to a fluorescence microscope for this task, with significantly lower total cost and reduced reading time. We anticipate that the reader could be used with minor modifications for virtually any fluorescence microfluidic device.
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1 Introduction

Microfluidic devices are increasingly important diagnostic tools for preclinical and clinical applications. The advantages of microfluidic technology (e.g., rapid assay speed, low sample/reagent volumes, potential for automation, small footprint, and low cost) have driven the rapid development of point-of-care (POC) devices [1–4]. These have been used frequently for quantification of specific cell types and target biomolecules in a sample. Of particular interest for this work are microfluidic devices that utilize a fluorescent-label based antibody capture scheme for sensing and quantification of target biomolecules, more specifically, vascular endothelial growth factor (VEGF) from the eye. The Federal Drug Administration (FDA) approved anti-VEGF agents (i.e., Macugen®, Lucentis®, Avastin®, and Eylea®) have been used to treat a range of ocular diseases including neovascular age-related macular degeneration (AMD), retinal vein occlusion (RVO) and proliferativediabetic retinopathy (PDR) [5–11]. Direct measurements of VEGF in the eye may help correlate the progression of the disease with the treatment. The sandwich immunofluorescence assay (sIFA) device developed by Murthy et al. (shown in Fig. 1(*a*)) is an optimal device for VEGF measurement [12]. The device utilizes two rows of vertical oval pillars in a series of detection channels (Fig. 1(*b*)) that are coated with anti-VEGF antibodies. When samples of ocular fluid are passed through each channel, free VEGF is captured; these are subsequently detected with a biotinylated antibody which is then labeled with fluorescent avidin. The fluorescent signal from individual channels can then be "read" to determine the concentration of VEGF in the original sample.

Until now, fluorescence microscopy has been used to read sIFA microfluidic devices [12]. This technique entails a two-step approach, whereby fluorescence images are first acquired on a microscope and subsequently analyzed pixel-by-pixel using commercial analysis software. While this approach has been robust in the laboratory, in the anticipated translation to routine clinical use this method has at least three significant limitations, including: (i) the operation of a fluorescence microscope requires significant operator training as does analysis of the fluorescence images, (ii) a fluorescence microscope is an unnecessarily generalized optical instrument for the task and consequently has a high associated cost ($60,000), and, (iii) the two-step imaging and analysis process means that obtaining target biomarker concentrations is a relatively time-consuming process.

To address these limitations, in this work we have developed and tested a new low-cost dedicated fluorescence microfluidic device reader (FMDR). This instrument has a task-specific simplified optical design that allows reading of microfluidic devices with a total cost that is at least an order of magnitude lower than a typical fluorescence microscope. We first tested this design with a series of sIFA calibration standards as well as a limited set of clinical vitreous humor samples. We verified that results obtained using our dedicated reader, which had similar sensitivity and accuracy compared to those obtained using a fluorescent microscope in the known clinical range of VEGF concentrations in ocular fluid. These results were also obtained significantly more rapidly (in ~5 min per plate versus 20–30 min). As such, this design may ultimately be useful for point-of-care clinical use of the microfluidic assay.

2 Materials and Methods

Images of the sIFA microfluidic device are shown in Figs. 1(*a*) and 1(*b*). Figure 1(*a*) is a photograph of the device showing 12 independent fluidic channels and Fig. 1(*b*) shows the interior detail of one channel where VEGF has been captured and illuminated with a fluorophore. Each channel contains vertical pillar structures to enhance the surface area of immobilized capture antibodies from a sample flowing from top to bottom or vice versa in Fig. 1(*b*). Microfluidic devices were fabricated as described previously [12].

A schematic and photograph of the fluorescence microfluidic device reader (FMDR) is shown in Figs. 1(*c*) and 1(*d*),

[1]Corresponding author.

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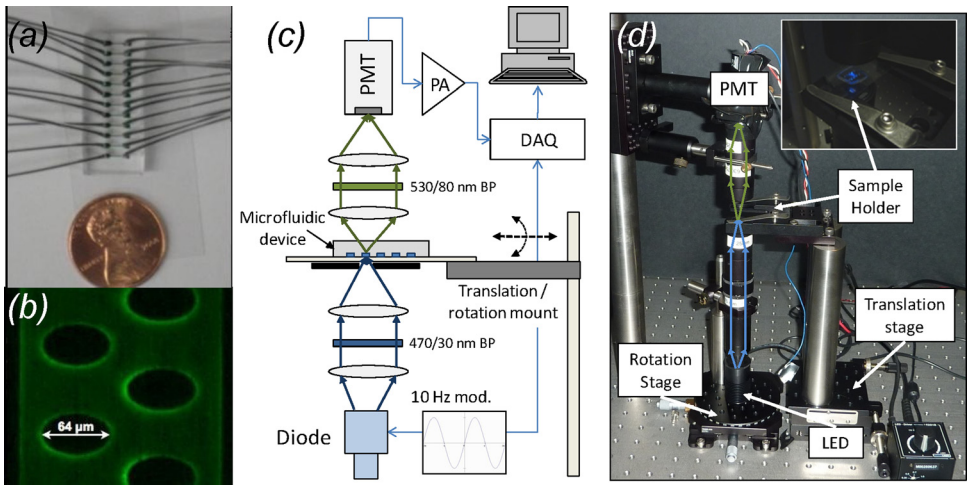

Fig. 1 (*a*) Photograph of the sandwich immune-fluorescence assay device, (*b*) an example fluorescence microscopy image, showing pillar and channel surfaces coated with a capture antibody and Oregon Green labeling. (*c*) A schematic diagram and (*d*) photograph of the dedicated fluorescence reader for the microfluidic devices. The total cost of all system components was approximately $5,000.

respectively. The light source was a modulateable 470 nm laser diode (M470L2; ThorLabs, Newton, NJ). The output light was collimated using a plano-convex lens ($f = 50$ mm; Edmund Optics, Barrington, NJ) and then passed through a 470 nm clean-up interference filter with a 30 nm bandpass (Chroma Technology Corp, Rockingham, VT). Using a second plano-convex lens ($f = 50$ mm; Edmund) the filtered light was focused and then passed through a 1 x 3 mm slit (Edmund Optics NT58-547, Barrington, NJ) so that only a single channel of the sIFA microfluidic device was illuminated at a time. The microfluidic chip was held in place using a microscope slide holder (MAX3SLH; ThorLabs) and manually aligned with respect to the excitation light slight using micrometer-driven linear X–Y translation stages (PT3A; ThorLabs). The light source and associated optics were mounted in a light-tight lens tube and then attached to the center of a rotational mount (XYR1; ThorLabs) so that the slit could be precisely aligned with the channels on the device. Emitted fluorescent light from each channel was collected and collimated using a plano-convex lens ($f = 25$ mm; Edmund) and then passed through a 530 filter with an 80 nm bandpass (Chroma) to remove the excitation light. Fluorescent light was then focused on the anode of a photomultiplier tube (PMT; H5783-04, Hamamatsu Corporation, Bridgewater, NJ). The output current from the PMT was converted to a voltage using a 1 MΩ resistor in parallel to the input analog channel of a data acquisition device (DAQ; NI-USB-6251 National Instruments, Austin, TX). The operation of instrument and data collection was controlled using custom written LABVIEW software (National Instruments).

Each microfluidic chip was positioned on the reader so that a single channel out of five (see below) was illuminated at a time. For these experiments, the LED output was modulated at 10 Hz using an analog output channel of the DAQ card. The detected fluorescence signal from the PMT was then analyzed using LABVIEW software to extract the 10 Hz component. We note that this step was not necessary but was empirically found to improve the quantitative accuracy of our results since it allowed removal of DC drift and high-frequency noise. The output signal from the PMT was averaged over 1 s for each measurement to obtain the reading for each channel. The chip was then repositioned to illuminate the next channel and the procedure was repeated. The entire reading process required less than 2 min per sIFA device.

To characterize the sensitivity and accuracy of the dedicated FMDR, we developed two sets of calibration standards. The first set utilized a microfluidic channel with a bare pillar array [14] filled with solutions of Oregon Green fluorophore (A-6374; Invitrogen, Grand Island, NY) at concentrations of either 1, 5, 50, or 100 pmol/L. These solutions were passed into each of the five channels on each microfluidic device manually with a syringe. We chose this approach since it allowed us to directly test the capabilities of our FMDR reader at known fluorophore concentrations in the absence of complicating factors such as coating of the sIFA posts with antibodies and VEGF-antibody binding efficiency (see below). A set of control chips (phosphate buffered saline only) was also fabricated so that background measurements could be obtained and subtracted from each sample. For each device, the mean and standard deviation from the five channels were computed. This was repeated four times for each concentration.

A second set of sIFA calibration standards were prepared where the posts were coated with anti-VEGF (MAB293, R&D Systems, Minneapolis, MN) capture antibodies. Solutions containing known concentrations of VEGF (293-VE, R&D Systems) were then passed through the microfluidic device using a syringe pump (PHD2000 microsyring pump, Harvard Apparatus, Holliston, MA) and captured on the coated posts. A biotinylated detection antibody (BAF293, R&D Systems) and an Avidin-Oregon Green conjugate (A-6347, Invitrogen) were then sequentially passed through each channel of the device. A series of chips was fabricated using solutions of 1, 5, 10, 50, 100, 500, and 1000 pg VEGF/mL. This range was selected since it approximately covers the expected concentration range of VEGF in clinical vitreous fluid samples. In particular, previously reported literature values are in the range of 35–7000 pg VEGF/mL [12,13]. As above, a control microfluidic device (0 pg/mL) was also prepared, and each experiment was repeated four times for each concentration.

As an initial test of the ability of our FMDR reader to quantify the VEGF concentration from clinical fluid samples, we performed a limited test on two intraocular fluid samples obtained during vitrectomy surgery. This project was conducted in compliance with all applicable Health Insurance Portability and Accountability Act regulations. Informed consent was obtained from all subjects on whom personal health identifying information was recorded in accordance with a Duke Institutional Review Board-approved protocol. These vitreous samples, diluted by balanced saline infusion fluid were obtained from eyes of patients who underwent planned vitrectomy at the Duke Eye Center. Samples were frozen and shipped to Northeastern University. Prior to analysis, these samples were concentrated using centrifugal filter devices (Amicon Ultra-15 10 k Da membrane, Millipore, Billerica,

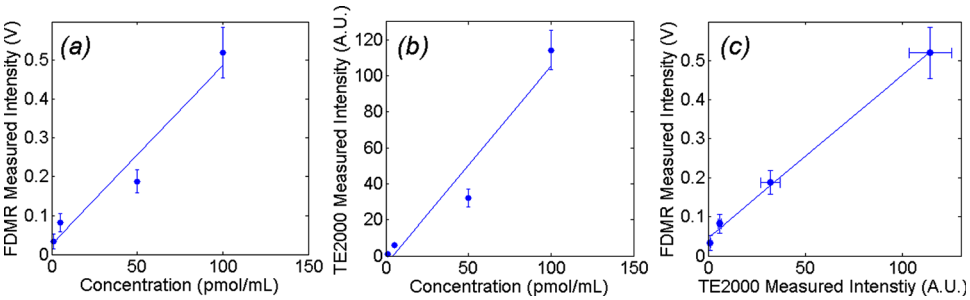

Fig. 2 Fluorescence measurements from a series of bare-pillar microfluidic device calibration standards filled with 1 to 100 pmol/L Oregon Green solutions, obtained using (*a*) the FMDR, and (*b*) fluorescence microscopy. (*c*) Comparison of readings obtained using the FMDR and fluorescence microscopy.

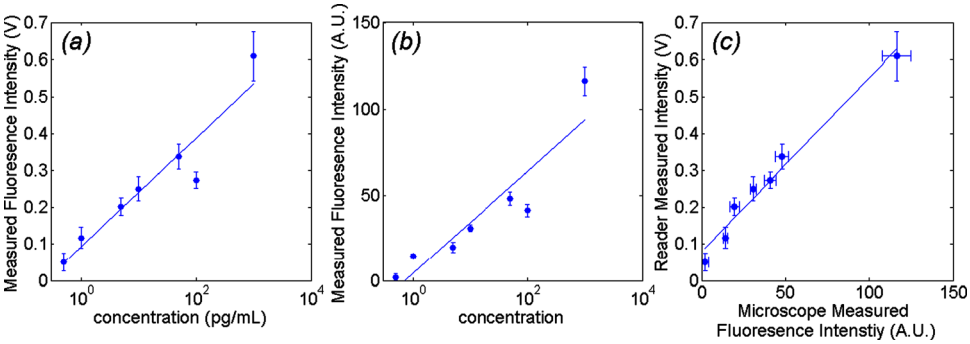

Fig. 3 Fluorescence measurements from a series of microfluidic device calibration standards from 1 to 1000 pg/mL VEGF, obtained using (*a*) the FMDR, and (*b*) fluorescence microscopy as a function sample concentration. (*c*) Comparison of readings obtained using the two methods.

MA) from 50–200 mL total volume to between 1–3 mL. A single microfluidic chip containing five channels was prepared as above for each of the clinical samples.

All sIFA microfluidic devices were also read using the previously described fluorescence microcopy method. A Nikon Eclipse TE2000 inverted microscope with a charged-coupled device camera (CCD) was used, and images were taken with a constant gain (16X) and an exposure time of 0.5 s. Image analysis software (Nikon NIS Elements 2.1) determined the average fluorescence intensity for a rectangular region in the center of each channel. This measurement was made on the same z-plane for all devices, although fluorescence measurements showed negligible variation at different z-planes [14].

3 Results

We first quantified the Oregon Green fluorescence intensity from a series of microfluidic device calibration standards (with uncoated pillars) as described above. The mean fluorescence intensity obtained using our FMDR instrument is shown in Fig. 2(*a*), along with the best fit-line. Likewise, the fluorescence intensity obtained from the same set of calibration standards using the Nikon TE2000 microscope (and image analysis) are shown in Fig. 2(*b*), along with the best-fit line. As expected, in both cases, the measured fluorescence intensity increased linearly with increasing Oregon Green concentration over the full two orders of magnitude tested. Using these fits, and assuming that the minimum reliable detectable signal level was three standard deviations of noise above the background, we determined that the minimum detectable concentration (sensitivity) for the reader was about 0.2 pmol/L of Oregon Green, and about 0.5 pmol/L for the fluorescence microscope. As such, the FMDR reader was at least as sensitive as the microscope under the conditions tested. The measured fluorescence intensities obtained with the FMDR instrument were then compared directly to those obtained with the fluorescence microscope for all concentrations as shown in Fig. 2(*c*). Here, an excellent linear correlation was observed ($r^2 = 0.98$).

We next tested our FMDR reader using a series of VEGF-sensing sIFA microfluidic devices with standard samples that contained increasing concentrations of VEGF from 1 to 1000 pg/mL, which approximately covers the concentration range anticipated clinically [12,13]. The data obtained using the FMDR reader and the fluorescence microscope are shown in Figs. 3(*a*) and 3(*b*), respectively. The measured fluorescence signal generally increased with increasing VEGF concentration as expected. A single data point (100 pg/mL) yielded unexpectedly low fluorescence readings. We attribute this outlier to minor variability in sample preparation, but it is important to note that similar values were obtained with both methods. As above, we then plotted the fluorescence measurements obtained with the FMDR directly against the fluorescence microscope, and an excellent linear correlation was again observed ($r^2 = 0.96$).

Finally, we tested the FMDR reader using a small set of clinical vitreous humor samples obtained from the Duke University Eye Center. As described above, samples were run through the VEGF sIFA microfluidic devices and then read with the FMDR and the fluorescence microscope. Here, raw fluorescence measurements from the reader and microscope were converted to absolute VEGF concentrations using the calibration curves obtained in Figs. 3(*a*)

Table 1 VEGF concentrations in pg/mL from two non-diagnostic vitrectomy samples obtained using the FMDR reader and Nikon TE2000 fluorescence microscope

	FMDR	TE2000 microscope
Sample 1 (pg VEGF/mL)	0.6 ± 0.2	0.8 ± 0.2
Sample 2 (pg VEGF/mL)	0.8 ± 0.2	0.6 ± 0.2

and 3(*b*), respectively. As shown in Table 1, the two devices yielded statistically identical readings for the clinical samples. In summary, these data demonstrate that the FMDR yielded results at least as sensitive and accurate versus fluorescence microscopy in reading sIFA microfluidic devices.

4 Discussion and Conclusions

We have developed and tested a dedicated, low cost fluorescence microfluidic device reader ultimately intended for POC clinical use. The simplified design and operation of the reader allowed fluorescence readings to be obtained significantly faster (2 versus 20–30 min) and at lower cost ($5,000 versus $60,000) with virtually identical sensitivity and accuracy compared to fluorescence microscopy. In the present study, samples were manually positioned on the FMDR reader. However, in the future, this process will be automated with computer controlled motorized translation stages to further simplify the process. We anticipate that the speed, ease of use and low cost will be particularly useful to translate the sIFA microfluidic device technology into clinical patient care. We are currently testing this technology as a POC ocular diagnostic assay using an expanded set of clinical samples. We also anticipate that, with minor modification to the optical design, our FMDR instrument would be useful as a low-cost reader for virtually any fluorescence-based microfluidic device.

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