MICROFLUIDIC ELISA FOR OCULAR DIAGNOSTICS
James V. Green,1 Dawei Sun,2,4 Ali Hafezi-Moghadam,2,4 Kameran Lashkari3 and Shashi K. Murthy1*

1Northeastern University, USA and
2Massachusetts Eye and Ear Infirmary, USA and
3Schepens Eye Research Institute, USA and
4Harvard Medical School, USA

ABSTRACT
Uveitis and primary intraocular lymphoma (PIOL) are diseases associated with the invasion of lymphocytes into various regions of the eye, accompanied by expression of inflammatory cytokines. While these diseases are very different in terms of survivability and treatment options they have similar symptoms that make accurate diagnosis challenging. Furthermore, the diagnostic yield with state-of-the-art techniques for cell and cytokine analysis of vitreous and aqueous humor samples is under 20% due to inadequate sensitivity. This paper describes a simple enzyme linked immunosorbant assay (ELISA) microfluidic device that is capable of identifying important analytes in ocular biopsies as a potential alternative to current diagnostic approaches.

KEYWORDS: ELISA, Microfluidics, Ocular

INTRODUCTION
Both uveitis and PIOL are diseases associated with the invasion of lymphocytes into various regions of the eye and diseases outside the eye or within ocular tissue may cause both conditions. Approximately 2.3 million Americans suffer from uveitis, and vision-loss due to uveitis accounts for 10-15% of blindness in the U.S. [1]. PIOL is manifested by the presence of malignant B-lymphocytes which may indicate tumor formation in the brain, retina, or other areas of the central nervous system. Although PIOL is an uncommon disease, patients of PIOL have a low likelihood of recovery because of the lack of early detection techniques.

The vitreous biopsy, a 20 mL sample, is an important part of diagnosing uveitis and PIOL because a detailed analysis of cell immunophenotypes present in vitreous humor allows: (a) distinction between PIOL and uveitis (b) detection of PIOL conclusively and early enough so that chemo- and radio-therapy have a greater likelihood of success, and (c) treatment of the underlying cause of uveitis with targeted therapeutic drugs instead of untargeted immunosuppressive agents that could harm the patient.

The current approach to analyzing the vitreous biopsy product consists of analysis by a skilled pathologist and flow cytometry. The current approach is limited in its efficacy for two reasons: (1) a large number of cells must typically be present in the sample for both the pathologic and flow cytometry analysis, and (2) the cells typically present in the biopsy sample are fragile and are often damaged in transit between the clinic and analytical laboratories. Indeed, the ‘diagnostic yield,’ which is the proportion of samples from which a conclusive diagnosis can be made, is only around 15% for flow cytometry, as reported in a recent study [2]. Recent work has shown that cytokines may play a role in distinguishing uveitis from PIOL but no clear correlation between cytokine presence and disease type has been identified [3]. This can be attributed to the lack of sensitivity in conventional ELISA and limited availability of PIOL samples.

Conventional ELISA systems do not have the capability to analyze large sample sizes such as the vitreous biopsy. Traditional 96-well plate ELISA is limited to the volume of a single well. Microfluidic systems have the unique ability to conduct immunoassays for both large (vitreous humor) and small (aqueous humor) samples. Several microfluidic ELISA devices have been reported in literature; however there systems are typically either too complex for point of care (POC) diagnostics or do not provide sufficiently low detection limits [4]. By contrast, the device described in the present work, shown in Figure 1, is not only simple and user-friendly but also able to surpass conventional detection limits by virtue of its design and operational protocol.

EXPERIMENTAL
A microfluidic device with two rows of vertical oval pillars, shown in Figure 1, was designed in order to achieve maximum sensitivity while performing sandwich-ELISAs. The design and fabrication of the poly(dimethylsiloxane) (PDMS) microfluidic devices followed previously described soft lithography techniques [5-6]. PDMS slabs were rendered hydrophilic through a simple two-step extraction/oxidation process that has been previously reported [7]. Capture antibody was chemically attached to the PDMS pillars and glass surface via silane chemistry and the same capture antibody attachment protocol was employed for all device types [5].

Figure 1: Schematic of the microfluidic ELISA device.
Endotoxin-induced uveitis (EIU) was induced in rats by injecting 100 µg of lipopolysaccharide (LPS) extracted from salmonella typhimurium diluted in 0.1 ml sterile saline into one hind footpad of each animal. The aqueous humor samples were drawn 24 h following LPS injection by anterior chamber puncture using a 30 G needle while the animals were under anesthesia. Aqueous humor samples from EIU rats were analyzed using microfluidic ELISA devices and purchased conventional ELISA kits (Pierce, Rockford, IL). IL-6, IL-10, TNFα, and IFNγ were measured using the protocol developed in the laboratory for the microfluidic ELISA device and by following the instructions provided by the manufacturer of the conventional ELISA kits.

Fourteen EIU Lewis rat aqueous humor samples were collected, pooled and diluted 25x with PBS in an effort to supply enough sample for multiple readings. The diluted sample was then run through a 0.2 µm sterile syringe filter to remove cellular debris and then 200 µL was loaded into a syringe. The syringe was then connected to the microfluidic device via tygon tubing and 100 µL of the sample was run through the device at a flow rate of 10 µL/min using a syringe pump over a 10 min period. The sample was then incubated in the device for a period of 15 min at room temperature. Following incubation, another 100 µL of sample was run through the device and incubated as previously described. The next sample was rinsed off the device by injecting 500 µL of PBS manually. 200 µL of the corresponding biotinylated detection antibody at a concentration of 0.02 mg/mL was then injected into the device and incubated at room temperature for a period of 1 h. The device was then flushed with 500 µL of PBS and injected with 200 µL of fluorescent NeutrAvidin biotin-binding protein and finally flushed with 500 µL of PBS after a 30 min incubation. The NeutrAvidin solution was stored as a stock solution containing 1 mg NeutrAvidin in 1 mL PBS; this stock solution was diluted with PBS to create a 0.004% (v/v NeutrAvidin stock/PBS) working solution.

Human aqueous humor samples (~50 to 200 µL) were collected from patients prior to cataract surgery. Aqueous humor samples of cataract patients were analyzed using the microfluidic ELISA device created. IL-5, IL-10, TNFα, and IFNγ were measured using a protocol similar to that described above. The only change was with respect to sample incubation within the device. For the human sample experiments, the incubation step was replaced with a continuous flow step in order to utilize the larger volume of human samples (50-200 µL) and to limit the time required to run the assay. The 50 to 200 µL human aqueous humor sample was diluted to a total volume of either 1 mL, run through a 0.2 µm nylon sterile syringe filter. 1 mL was then loaded into a syringe and then run through the device at a flow rate of 10 µL/min. In this set of experiments, four capture devices, for IL-5, IL-10, TNFα, and IFNγ, were linked in series by tygon tubing. After the sample was run through the devices, a syringe with 0.4 mL of PBS was run through the devices and incubated as previously described. The next sample was run through the devices, a syringe with 0.4 mL of PBS was run through the devices at a flow rate of 10 µL/min to ensure the entire sample was run through each device. Following this step, the devices were exposed to their appropriate detection antibodies and then fluorescent NeutrAvidin according to the protocol previously described.

Cytokine concentration measurements were made by measuring the average fluorescence intensity at the center point of each device using a Nikon Eclipse TE2000 inverted microscope. Fluorescent intensity readings were then converted to cytokine concentrations using standard calibration curves created for each type of cytokine tested. Controls that consisted of PBS were run in parallel with each experiment and this background noise was subtracted from each reading.

**RESULTS AND DISCUSSION**

The microfluidic ELISA device was equally capable of identifying and quantifying cytokines in the aqueous humor of EIU rats when compared to conventional ELISA kits; the comparison of results obtained from each ELISA platform are shown in Table 1. The elevated levels of IL-6 and TNFα measured 24 h after LPS injection agree with previous reports [8-9]. Overall, the EIU rat cytokine measurements proved the integrity of the microfluidic ELISA device as the measurements correlated well with conventional ELISA techniques. However, detection limits were hindered by the large dilution (25x) required to obtain enough sample to run multiple experiments and it was evident further optimization was required.

Larger sample sizes provided by humans allowed for increased flexibility in the method the sample was run through the microchannel and lower detection limits were achieved. For the EIU rat samples, 200 µL was loaded into a syringe and flowed through the device and incubated. Therefore the antibody-antigen interactions took place in both a dynamic and static environment. In the case of human samples, 1 mL was loaded into a syringe and flowed through the device with no incubation. This allowed for the capture antibody within the microchannel to be constantly exposed to “fresh” sample and in turn detection limits in the tens of fg/mL range were obtained. The calibration curves for human cytokines had detection ranges of 148 to 1.48×10⁶, 90 to 900,000, 80 to 800,000 and 88 to 875,000 fg/mL for IL-5, IL-10, TNFα, and IFNγ respectively. Devices were also linked in series so multiple cytokines could be tested for in one 1 mL sample.

Table 1: Detection of aqueous humor cytokines of EIU rats by the ELISA kit and microfluidic ELISA device presented. Cytokine concentrations are in pg/mL and representative of three replicates.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>ELISA Kit</th>
<th>Microfluidic Device</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>1417 ± 29</td>
<td>1269 ± 448</td>
</tr>
<tr>
<td>IL-10</td>
<td>&lt; 400</td>
<td>&lt; 463</td>
</tr>
<tr>
<td>TNFα</td>
<td>7170 ± 119</td>
<td>6913 ± 275</td>
</tr>
<tr>
<td>IFNγ</td>
<td>&lt; 200</td>
<td>&lt; 500</td>
</tr>
</tbody>
</table>
Using this approach, human cataract samples obtained from patients undergoing routine cataract surgery were tested for IL-5, IL-10, TNFα and IFNγ. These cytokines were selected, as previous literature reported no detection of IL-5 and IL-10, while concentrations of TNFα and IFNγ were obtained [10]. Readings obtained from all patients, results shown in Table 2, correlated well with literature except for the IL-10 reading of patient 3. IL-10 is an anti-inflammatory cytokine therefore it is quite possible that patient 3 had some sort of infection prior to surgery.

Using the microfluidic ELISA device measurements of IL-10 and TNFα were made below detection limits in literature [10]. However, for all patients, IL-5 was not detected. It may be possible that IL-5 is present in lower concentrations however; the dilutions on the order of 10x caused our detection limits to be raised by a factor of 10. This caveat will not be an issue when larger samples, such as the vitreous biopsy, are tested using the microfluidic device presented. As stated before, when no dilution is necessary, and a 1 mL sample is available, detection limits are in the tens of fg/mL.

CONCLUSIONS

Analysis of the vitreous fluids is necessary to make a proper diagnosis but a thorough analysis of the complex networks of cytokine present in the vitreous with a more sensitive ELISA technique may uncover an unknown correlation between cytokine presence and disease type. Furthermore, the correlation between cytokine presence in both the vitreous and serum with disease type should be investigated using this microfluidic platform, as both samples are correlated well with literature except for the IL-10 reading of patient 3. IL-10 is an anti-inflammatory cytokine therefore it is quite possible that patient 3 had some sort of infection prior to surgery.

Using the microfluidic ELISA approach can be readily adapted for the analysis of larger volume samples by multiplexing, i.e. running large numbers of channels in parallel. Multiplexing, when combined with automation of sample staining and fluorescent intensity measurement, would then provide the ability to efficiently analyze large volume samples while taking advantage of the femtomolar-level sensitivity of the microfluidic system.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from the National Science Foundation through grant CBET-0827868.

REFERENCES


CONTACT

*Shashi K. Murthy, tel: +1-617-373-4017; smurthy@coe.neu.edu