PSI-SR-1297

Development of A First-Responder Fluorescence Reader for Microarray Cytokine Assay of Human Immune Response to Disease

D.B. Fenner
D.I. Rosen
A.A. Ferrante
A.E. Stevens
C.E. Bigelow
S.J. Davis

presented at Materials Research Society 2007 Spring Meeting (San Francisco, CA) edited by E Stutz, I. Wilke, K. Kreischer, Q. Hu,, *Mater. Res. Soc. Symp. Proc.* **1016E** (paper 1016-CC07-03) (9-13 April 2007).

Mater. Res. Soc. Symp. Proc. Vol. 1004 © 2007 Materials Research Society 1004-P03-26

Development of A First-Responder Fluorescence Reader for Microarray Cytokine Assay of Human Immune Response to Disease

David B Fenner, David I Rosen, Anthony A Ferrante, Amy E Stevens, Chad E Bigelow, and Steven J Davis

Photonics, BioMed and Bio Sciences, Physical Sciences Inc., 20 New England Business Center, Andover, MA, 01810

ABSTRACT

Development of field portable apparatus and methods for cytokine assay of human saliva by fluorescent-reporting microarray plates is described. Multiplexed assay of 12 cytokines for minimally-processed saliva is read with a CCD-based imager under LED excitation. Immune responsive cytokines are measured at levels significant for indication of human disease state. The motivational context for the new apparatus development, the general optical design issues, saliva protocol, and image analysis are described.

INTRODUCTION

The practical utility of technologies for early detection of disease has been limited in many cases by the absence of non-invasive procedures and instruments suitable for first responders and the field hospital. Biomarkers that are in the disease pathway and deployable apparatus are needed to assess adverse health effects in the field. For early stage treatment, detection of disease or injury need not provide specific diagnoses. Hence the potential usefulness of ascertaining immune system imbalance as indicated by endogenous proteins in bodily fluids. Cytokines are a large number of small proteins released by cells into blood and saliva. Some cytokines signal or trigger inflammation in response to infectious challenge, while others indicate stress. A recent study of monkeys infected with the 1918 flu reported greatly elevated blood levels of selected cytokines beginning soon after infection [1]. Saliva is considerably more practical than blood for biomarker analysis and a growing body of evidence supports correlation of patterns in cytokine profiles with various disease, stress and injury states [2,3]. It would appear that cytokine profiles are distinct between viral infection, oral cancer, HIV, and physiological stress in humans [4-9].

Current methods for cytokine assay require full laboratory facilities, such as bead assay with flow cytometry or ELISA with fluorescence microscopy. Bioanalysis with stationary-site sandwich recognition is a technique whereby fluorescent labels report antibody-antigen bound pairs at microarray spots and are scanned or imaged for quantitative assay. Microarrays on a plate with multiple array replicas, each in its own well, provide highly multiplexed assay of a large number of human cytokines. Analysis of blood is the common method but saliva is more readily accessible and able to provide quick indication of immunological challenge. However, protocols for saliva need development [10] and cross-correlation study against blood assay. New methods utilizing saliva and field portable instruments are needed for point-of-care use.

We report development of a highly portable system configured with microarray cytokine capture set, dye reporter excited by a filtered LED, and fluorescence imaging by 16-bit CCD camera exported to PC. Commercial microarray plates for 12 multiplexed cytokines are utilized. Saliva methods and reader design are described. A small, minimal-risk clinical study of healthy and flu-vaccine challenged individuals is underway to assay cytokine profiles in this manner.

EXPERIMENTAL METHODS

Commercial kits for human cytokine assay by the microarray method are available from a number of vendors. For this work we have utilized the Novagen ProteoPlex kit [11] which provides assay of 12 cytokines in 16 identical wells on a single plate (glass slide). Protocol for the plate was developed by the manufacturer for blood plasma or serum, and entails a total of just under 4 hours duration [11]. Kit components are used to prepare cytokine standards (spikes), and process wash with PBST, incubate with the sample (one per well), wash, incubate with detection antibody, wash, incubate with the dye, wash, and dry. We have developed a tentative protocol for saliva preparation with an emphasis on minimizing the processing and the expectation that a modification for field use can be developed. The protocol is as follows:

1. Rinse mouth with water, discard, then also discard the first saliva.
2. Collect ~500 µl of raw saliva.
3. Centrifuge once and then repeat using a filter tube (filter alone may be sufficient).
4. Use ~100 µl of clear liquid in each well of microarray plate.

At present, the plates in use have 12 cytokines in the array with quadruplicate spots for each, and additional spots for alignment during image analysis. The individual wells are configured with areal dimensions and well-to-well spacing of industry-standard, 96-well plates. Thus, the microarray imaging instrument is well suited to potential applications with many other well-based fluorescence bioassays, a number of which are on the market or in development.

Dye in the microarray spots is excited with a Luxeon III Star LED from Lumileds, emitting at ~590 nm (amber) and driven at ~3 V and 1.4 A on an active heat sink. Two illumination geometries were investigated. A simple arrangement with direct, oblique irradiation of the array well was found to be superior in this application to the more conventional method of epi-fluorescence microscopes [12]. The LED is fitted with a collimating reflector optic, emission filter, diffuser plate, and half-dome lens. Together this yields good intensity uniformity over an individual microarray area of ~4 x 4 mm square, as illustrated in Figure 1 (a).

These plates utilize a very stable, strongly emitting dye with peak emission at ~660 nm. A bandpass filter is used with the LED and a longwave pass filter in the imaging optical train. The filter pair have a combined blocking optical density of nearly 10^6. The fluorescence from an array of spots in a well is imaged through a long working-distance (~70 mm) objective lens. Since the area of the square spot array is approximately that of the CCD chip active area, a unity-magnification relay lens system was chosen. The lens system consists of two pairs of achromatic

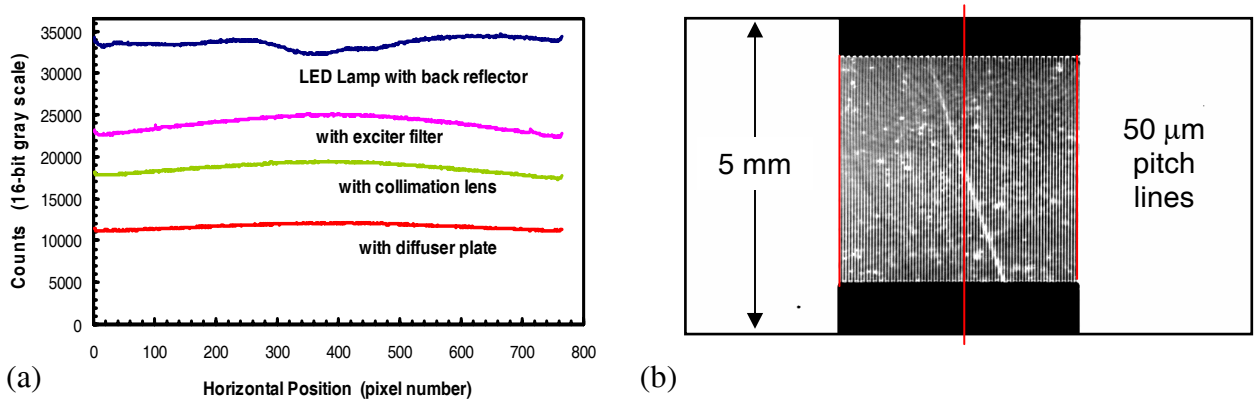


Figure 1. (a) Intensity profile by the LED system. (b) Image fidelity of a fine line ruling.

doublets. Image distortion across the field is no more than ~10 μm as in Figure 1 (b). The fluorescent spots on the plate are ~175 μm in diameter while the CCD imager has 9 x 9 μm pixels giving sufficient spatial resolution. Figure 2 shows two views of the breadboard reader apparatus and a glass slide both illuminated and imaged from below. Image data is immediately transferred into a PC for data reduction.

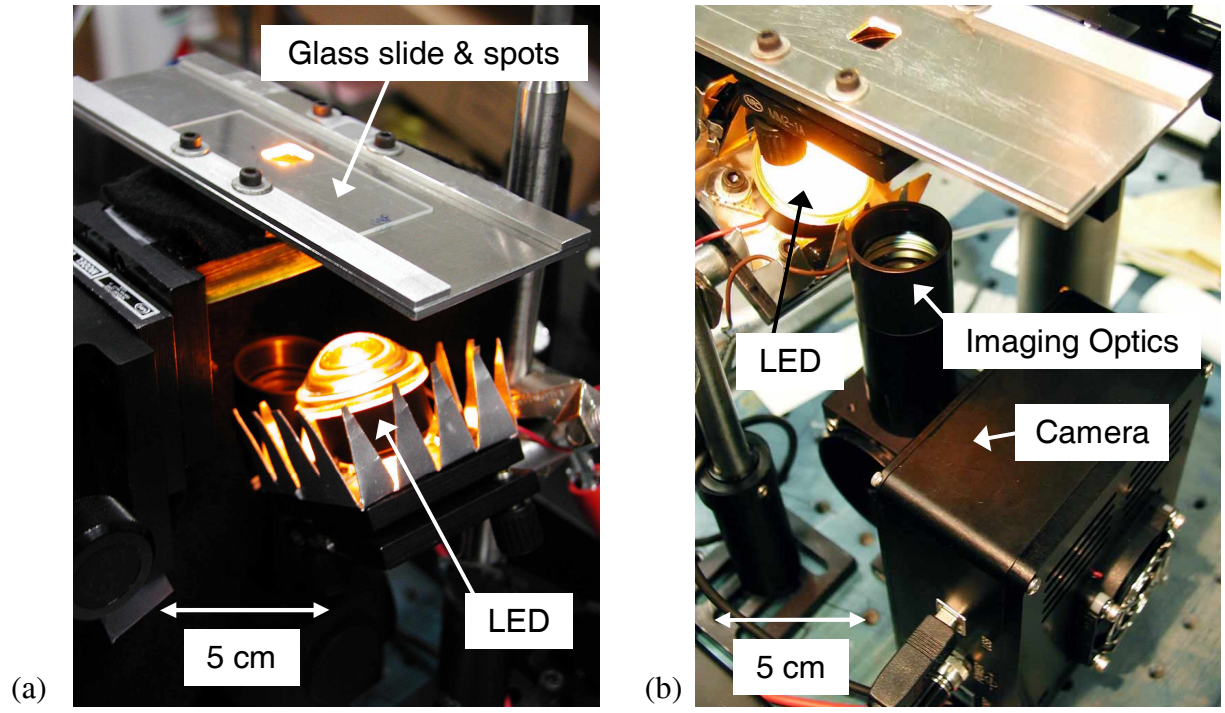


Figure 2. The breadboard apparatus for imaging fluorescent microarrays. In (a) the glass slide at the top has the spot arrays on its upper surface. In (b) the relay lens tube is also seen.

RESULTS AND DISCUSSION

Figure 3 shows an image of a microarray from a well spiked with a cocktail of the indicated cytokines at 800 pg/ml concentration each. The image was obtained with a 10 sec CCD exposure which results in the brightest fluorescent spots nearly reaching the saturation level of the instrument. Excellent grayscale range is obtained under these conditions. Residue and particles left on the plate (e.g., after the last wet wash step) are found to also fluoresce and appear in the image. Some residue can be seen in Figure 3 as faint smears and circles. The latter are found to be mainly small particles on the opposite side of the slide (bottom in Figure 2) and thus out of focus. The confocal methods used in commercial scanners are able to discriminate these undesired features but the optical complexity required precludes rugged and portable instruments [12]. From Figure 3 it can be clearly seen that although all of the cytokines in the spike cocktail have the same concentration, the binding capture efficiency varies considerable among this cytokine set: IL-8 being one of the strongest and IL-6 less so.

Data reduction to obtain analyte concentrations from an image, such as Figure 3, requires measurement of the total intensity of the spots, averaging the four in each set, subtraction of

background (blank spots, as in Figure 3), and conversion to quantitative concentration. At present, each slide has six wells devoted to calibration (out of the 16), a process based on the fluorescence observed upon spiking the six wells with known cytokine cocktails, including one well with no spike (or sample). The unspiked well serves to estimate nonspecific binding and fluorescent mislabeling, which contribute to background.

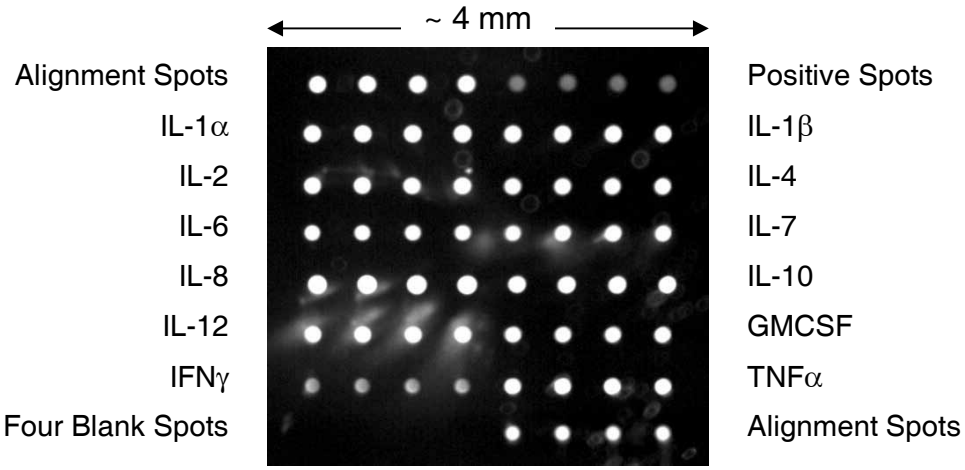


Figure 3. Image of fluorescent spot array obtained with apparatus of Figure 2. The identity of each set of four spots is indicated in the sidebars [11].

Figure 4 shows calibration curves obtained for the six spiked wells on one plate, of which Figure 3 is one such well. The blank spots in the six wells did not show any change in measured background fluorescence emission from those wells. Both IL-6 and IL-8 are seen to increase linearly in fluorescence intensity across the range from zero to 800 pg/ml of spike concentration. The linearity of response and cytokine-specific response slopes over that concentration range were verified by comparison with measurements obtained by a commercial scanner for the same slide [11,13]. Agreement was found within the uncertainties for each technique and spot quality.

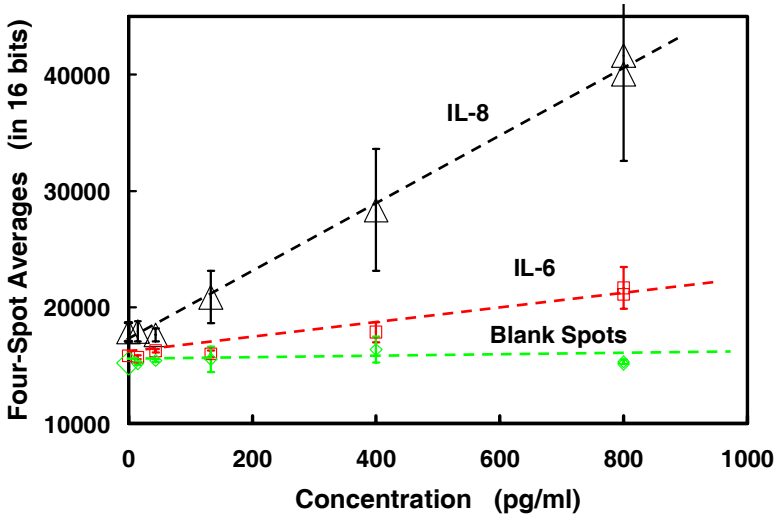
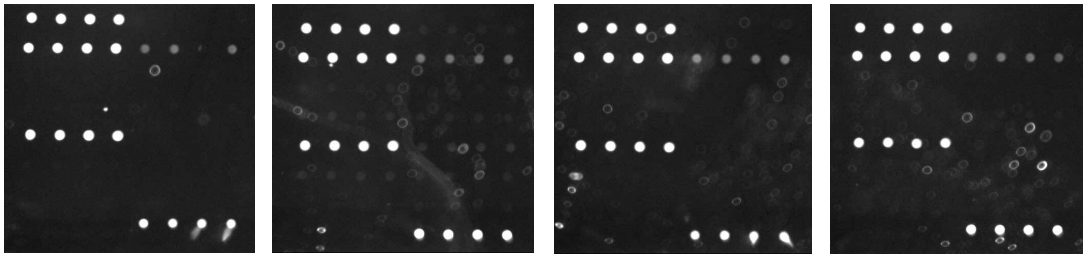


Figure 4. Calibration curves obtained from the IL-6, IL-8 and blank spots in six wells spiked with cytokine concentrations 0, 15, 44, 133, 400, and 800 pg/ml, as indicated.

Measurement uncertainties are indicated in Figure 4 both by error bars that reflect the standard deviation among the four, nominally identical spots for each cytokine in each well, and by the scatter between the two points at the highest concentration shown which represent multiple images made intentionally under similar but reproduced laboratory conditions. The standard deviation of the slope of a linear regression to the IL-6 and IL-8 points were ~13% and ~4%, respectively. Assay with this microarray reader provides cytokine concentrations over at least two orders of magnitude and measured to as little as ~10 pg/ml, similar to the limit for laboratory scanner instruments, bead cytometry assay, and within clinically significant levels.

As a model for a mucosal immune response expected to be reflected in cytokine profiles of saliva, a pilot clinical study is being carried out. In the fall of 2006, fifteen otherwise healthy adults who elected to receive an inoculation of FluMist™ nasal spray [14], gave blood and saliva samples over about one week's time span [15]. These samples are being assayed for cytokine profiles by both bead and microarray methods for cross correlation analysis and as a search for patterns in the cytokine profiles [16]. An example of assays of the four saliva samples given by a single individual in the study are shown in Figure 5. The spots representing IL-2, IL-6, and IL-12 are noticeably brighter in Figure 5 (b), one day after vaccination, than on the other sample days. IL-1α, IL-1β, and IL-8, however do not change distinctly. A more complete analysis of images from all study participants will be used to establish statistical response patterns [16].



(a) (b) (c) (d)

Figure 5. Microarray images from four saliva cytokine samples volunteered by one individual in the FluMist trial. The sample in image (a) was immediately prior to administering the vaccine, while (b) was one day later, (c) was three days later, and (d) was seven days later. Cytokine identities are the same as those indicated in Figure 3.

CONCLUSIONS

New methods have been explored for assay of cytokines as biomarkers in the disease pathway, and apparatus suited to use by first responders. We have utilized commercial microarray proteomics for multiplexed assay of human cytokine profiles. The technique has been extended to assay of saliva because it is noninvasive and readily available. The microarrays are imaged by portable apparatus developed around amber LED illumination, 1:1 imaging optics, and 16-bit CCD camera. Good fluorescent spot image contrast is possible with this simplified apparatus although it is more sensitive to contamination residue on the slide than are commercial confocal laser scanners. Calibration of the method and apparatus is illustrated with 12 cytokine spikes within the clinically significant range of 10-1000 pg/ml. A linear fluorescence intensity response by the imaging apparatus was observed and verified by comparison with measurements by commercial scanner. A small clinical study is underway to seek statistical response patterns.

ACKNOWLEDGMENTS

This work is supported by AFRL-HE, Wright-Patterson AFB, Dr. Nicholas DelRaso, through SBIR contract FA8650-06-C-6647. CEB present address Novartis, Cambridge, MA.

REFERENCES

1. D. Kobasa, et al, "Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus," Nature **445**, 319 (2007).
2. "Doctor says 'spit please'," Nature, news, 22 March 2007, and references therein.
3. F. Chiappelli, F.J. Iribarren and P. Prolo, "Salivary biomarkers in psychobiological medicine," Bioinformation **1**, 331 (2006).
4. F.G. Hayden, et al, "Local and systematic cytokine responses during experimental human influenza A virus infection," J. Clinical Invest. **101**, 643 (1998).
5. D.P. Skoner, et al, "Evidence for cytokine mediation of disease expression in adults experimentally infected with influenza A virus," J. Infect. Diseases **180**, 10 (1999).
6. R.B. Turner, et al, "Association between interlukin-8 concentrations in nasal secretions and severity of symptoms of experimental rhinovirus colds," Clinical Infect. Diseases **26**, 840 (1998).
7. N.L. Rhodus, et al, "The feasibility of monitoring NF-κB associated cytokines: TNF-α, IL-1α, IL-6, and IL-8 in whole saliva for the malignant transformation of oral lichen planus," Molecular Carcinogenesis **44**, 77 (2005).
8. E.A. Lilly, et al, "Tissue-associated cytokine expression in HIV-positive persons with oropharyngeal candidiasis," J. Infect. Diseases **190**, 605 (2004).
9. D. Lucini, et al, "Hemodynamic and autonomic adjustments to real life stress conditions in humans," Hypertension **39**, 184 (2002).
10. K.L. Wozniak, "Inhibitory effects of whole and parotid saliva on immunomodulators," Oral Microbiology Immunology **17**, 100 (2002).
11. Novagen ProteoPlex™ 16-well human cytokine array kit, supplied by EMD Biosciences Inc., Madison, WI. Information at: www.emdbiosciences.com/novagen
12. G. Mazzini et al, "Improvements in fluorescence microscopy allowed by high power light emitting diodes," *Cur. Issues Multidisciplinary Microscopy Res. Ed.,* ed. A. Mendez-Vilas and L. Labajos-Broncano (Formatex, 2004) pp. 181-189.
13. GenePix 4000 scanner with laser illumination, and measured courtesy of Novagen.
14. FluMist™ is a live influenza virus, approved by the FDA for intranasal vaccination, and distributed by MedImmune Vaccines, Inc., Gaithersburg, MD.
15. The human use research study was approved by IRB at AFRL, Wright-Patterson AFB.
16. In preparation.