Molecular Vision 2016; 22:177-188 <http://www.molvis.org/molvis/v22/177>
Received 3 February 2015 | Accepted 20 February 2016 | Published 23 February 2016

Analysis of tear inflammatory mediators: A comparison between the microarray and Luminex methods

Karen Dionne, Rachel L. Redfern, Jason J. Nichols, Kelly K. Nichols

The Ocular Surface Institute, College of Optometry, University of Houston, Houston, TX

Purpose: Inflammatory mediators have been shown to modulate dry eye (DE) disease and may correlate with disease severity, yet the methods used and the associated findings vary significantly in the literature. The goal of this research was to compare two methods, the quantitative microarray and the magnetic bead assay, for detecting cytokine levels in extracted tear samples across three subject groups.

Methods: Tears were collected from Schirmer strips of the right and left eyes of 20 soft contact lens wearers (CL), 20 normal non-contact lens wearers (NOR), and 20 DE subjects and stored at −80 °C. Tear proteins were eluted and precipitated using ammonium bicarbonate and acetone. The right and left eye samples were combined for each subject. Following the Bradford protein quantitation method, 10 µg of total protein was used for each of the two analyses, Quantibody® Human Inflammation Array 3 (RayBiotech) and High Sensitivity Human Cytokine Magnetic Bead Kit (Millipore). The assays were run using the GenePix® 4000B Scanner (Molecular Devices) or the Luminex MagPix® plate reader (Luminex), respectively. The data were then compared between the two instruments and the three subject groups

Results: Of the 40 proteins on the Quantibody® microarray, seven had average expression levels above the lower limit of detection: ICAM-1, MCP-1, MIG, MCSF, TIMP-1, TIMP-2, and TNF-RI. Significant differences in expression levels ($p<0.05$) were detected between the CL and DE groups for MCSF, TIMP-1, and TNF R1, between the NOR and DE groups for ICAM-1, and between the CL and NOR groups for ICAM-1, MCP-1, MCSF, TIMP-1, TIMP-2, and TNF-R1 when using the Student *t* test. Of the 13 proteins tested with Luminex, IL-1β, IL-4, IL-6, IL-7, and IL-8 had expression levels above the minimum detectable level, and these were most often detected using the Luminex assay compared to the Quantibody® microarray. Contrarily, IL-2, IL-12, IL-13, INF-g, and GM-CSF were detected more frequently using the Quantibody® microarray than the Luminex assay. Significant differences in expression levels ($p<0.05$) were only detected between the CL and DE groups for IL-7 and IL-8 and between the CL and NOR subjects for IL-8.

Conclusions: In addition to detecting more significant differences between the subject groups, the Quantibody® microarray detected more inflammatory cytokines in total within the range of detection than the Luminex assay. Differences were also noted in the types of cytokines each assay could detect from the limited protein samples. Both methods offer advantages and disadvantages; therefore, these factors should be considered when determining the appropriate assay for analyzing tear protein samples.

Tear samples can provide valuable insights into certain diseases of the ocular surface, such as dry eye (DE) disease [1]. The composition of the tears can reflect the state of inflammation or ocular surface damage involved, and proteins such as inflammatory mediators are thought to modulate DE disease and correlate with disease severity [2,3]. Several studies have shown that proteins such as matrix metalloproteinases, cytokines, and chemokines are present in human tears [4-7] although the extent of the expression levels is not fully understood [8]. It is important to analyze the tear film, both basal and reflexive, to better understand the underlying ocular surface health.

Tear analysis can be performed using a variety of techniques, including mass spectrometry, western blotting [9,10], enzyme-linked immunosorbant assays (ELISA) [11,12], microarrays [13], and multiplex assays such as Luminex [14-20]; however, tear collection and analysis has historically been challenging due to limited sample quantities, most notably from DE patients. DE is associated with tear deficiency or excessive evaporation [1], resulting in reduced tear volume and difficulty collecting tears using microcapillary or Schirmer strips. Furthermore, no two collection procedures are identical, even from the same patient, where reflexive tearing can increase or decrease with a proportional effect on tear protein concentrations [21]. In addition to the potentially wide biologic variation in the human tear proteome, factors such as time of day or year as well as inter-clinician techniques may affect the proteomic profile of an individual [22]. The need exists to optimize the results from single collected samples and thus to understand the limitations of the various assays.

Correspondence to: Rachel L. Redfern, The Ocular Surface Institute, College of Optometry, University of Houston, 505 J. Davis Armistead, Houston, TX 77204; Phone: 713-743-1943 FAX:713-743-2053 email: rredfern@central.uh.edu

A commonly used method for analyzing tear samples is the Luminex multiplex immunobead assay [14-20]. One of the advantages of this method is that it is possible to assay several proteins with one sample, thereby increasing the exposure of limited starting material. Appendix 1 outlines the types of inflammatory mediators seen in tears from the most recent results of Luminex assays and compares those to the cytokines and chemokines examined in this study. The most common proteins investigated in DE studies usually include IL-1β, IL-6, IL-8, INFγ, and TNFα. In this study, 13 inflammatory mediators were evaluated using the Luminex assay, including those most commonly associated with DE disease.

Another recent method for examining proteins in tear film, which allows for greater coverage of analytes per sample than the Luminex method, is the quantitative microarray. Similar patterns in expression levels of inflammatory mediators have been noted when compared to the Luminex method, such as increased TNFα and IL-6 in DE groups compared to normal eyes [13]. It is also possible to use a small sample volume, which, when combined with the increased number of analytes available for analysis, makes this technique suitable for a broad protein analysis of the tear film. In this study, we were able to analyze 40 different inflammatory mediators using a single tear sample with a quantitative microarray. The purpose of this study was to compare these two methods for detecting levels of inflammatory mediators in human tear samples.

METHODS

Human subjects: This study was approved by the Institutional Review Board of the University of Houston, following the tenets of the Declaration of Helsinki, and informed consent was obtained from all the subjects before the study commenced. Sixty subjects were enrolled in the study: 20 soft contact lens wearers (CL), 20 non-contact lens wearers (NOR), and 20 DE disease subjects, with each of the three groups conforming to specific inclusion criteria. Group 1: This group comprised contact lens wearers who were of normal ocular health, with the exception of unaided visual acuity, and were current hydrogel contact lens wearers with no change in contact lens type or care solutions for the duration of the study. Group 2: These normal, non-contact lens subjects were of normal ocular health, with the exception of unaided visual acuity. Group 3: These ocular surface disease subjects, categorized as DE for the purpose of this study, were clinically diagnosed using the International Classification of Disease codes (ICD-9) with DE disease (e.g., ICD-9 375.15) or blepharitis (ICD-9 373.0) with a duration of more than 6 months. Basal tear samples were collected on Schirmer strips during one visit from the right and left eyes of each subject. The wetting length was recorded, and the samples were stored at −80 °C until processed.

Tear protein elution and quantitation from Schirmer strips: The protein was eluted from each Schirmer strip in an equal volume of 100 mM ammonium bicarbonate at room temperature for 1 h, precipitated in acetone at −20 °C overnight, and centrifuged at 16,000 G for 10 min [23]. The protein pellet was resuspended in PBS. The right and left eye samples were combined for each subject, and the protein was quantitated using the Pierce™ Coomassie Bradford Assay (Thermo Fisher Scientific, Waltham, MA) [24]. Ten micrograms of total protein was used in a normalized volume per the manufacturers' instructions for each assay and method: Quantibody® Human Inflammation Array 3 (RayBiotech, Inc., Norcross, GA) using the GenePix® 4000B microarray scanner (Molecular Devices, Sunnyvale, CA) and the High Sensitivity Human Cytokine Magnetic Bead Kit (EMD Millipore, Billerica, MA) using the Luminex MagPix® magnetic bead plate reader (Luminex, Austin, TX).

The GenePix® and MagPix® instruments both report results as a function of fluorescence intensity. The mean fluorescence intensity (MFI) takes into account the number of fluorescing pixels within the scan area, such as a specific area on a microarray slide or an individual bead on the Luminex assay, and reports the mean of those accumulated values.

Quantibody® human inflammation array 3: For the Raybiotech Quantibody® Array, each 40-panel array was spotted 16 times on a standard slide. Within the array, each individual cytokine was represented four times, which allowed for an analysis of standard deviation. The array-specific cytokine standards, with predetermined concentrations, were reconstituted per the manufacturer's protocol, and serial 1:3 dilutions were made for the working concentrations, which are accounted for in the data analysis software provided by the manufacturer. Slides were blocked with a sample buffer for 1 h before use. The tear samples and standards were incubated on the array overnight at 4 °C. The slides were washed with a proprietary buffer and then incubated with a detection antibody at room temperature for 2 h. The slides were then washed and incubated with Cy3 equivalent dye-conjugated streptavidin at room temperature for 1 h. Thereafter, the slides were washed a final time and then dried thoroughly before scanning with the GenePix® 4000B scanner using the Cy3 excitation profile. The MFI was then compared to a standard curve using the material provided with the kit by the vendor to calculate the cytokine concentration in pg/ml. Each standard curve was then individually analyzed for outliers and adjusted as necessary to achieve linearity ($R^2 \geq 0.8$).

High sensitivity human cytokine magnetic bead kit: Plates were prepared as per the manufacturer's instructions. Briefly, each plate was blocked with wash buffer for 10 min before use. The mixed beads were dispensed into each well and washed twice. The standard curve was generated by reconstituting the high sensitivity human cytokine standard, per the manufacturer's protocol, with serial 1:5 dilutions for a working concentration range of 0.13–400 pg/ml. The Millipore kits also provided quality control (QC) material to confirm the accuracy of the assay. Each analyte in the QC sample was intended to fall within a designated range, and if these values deviated from this range, the data was considered invalid.

The samples and standards were incubated with the mixed beads overnight at 4 °C while shaking. The beads were washed and then incubated with a detection antibody at room temperature for 1 h and with streptavidin for an additional 30 min. The beads were washed twice, resuspended in Luminex MagPix® drive fluid, and the plate was subsequently analyzed on the Luminex MagPix® plate reader.

The MFI was then compared to the standard curve, as previously described, to calculate the cytokine concentration in pg/ml. Each standard curve was then individually analyzed for outliers and adjusted as necessary to achieve linearity ($R^2 \geq 0.8$).

For both methods, the MFI values were adjusted for the background, and all replicates, where applicable, were averaged. This value was then plotted on the linear range of the standard curve. None of the values reported in our experiments were outside the confines of the linear standard curve range. The limit of detection (LOD), which is the lowest analyte concentration that can be reliably detected by a method, was calculated using the manufacturer's data analysis software for each set of standards included with the microarrays although the LOD was provided by the kit manufacturer of the Luminex assay. Only those values above the LOD were reported in this work. Statistical analyses were performed using a two-tailed Student *t* test, assuming unequal variance, and Pearson's correlation coefficient in Microsoft Excel (Microsoft, Redmond, WA). Significant differences ($p<0.05$) and positive correlations ($r>0.5$, $p<0.05$) were reported.

For the purposes of this manuscript, the term "Luminex" will be defined as the Luminex MagPix® plate reader and "Luminex method" as the Millipore High Sensitivity Human Cytokine Magnetic Bead kit. The term "Microarray" will be defined as the GenePix 4000B scanner and "Microarray method"as the Raybiotech Quantibody® Human Inflammation Array 3, unless otherwise noted.

RESULTS

Schirmer strip wetting length and tear protein quantitation correlation: The wetting length and total protein from the Schirmer tear collection were recorded for each group classification (Table 1). There was a positive correlation ($r = 0.61$, $p<0.001$) between the wetting length (mm) and the total protein extracted (µg/µl) with the NOR group yielding the strongest correlation ($r = 0.77$, $p<0.001$) and the DE group also yielding a correlation ($r = 0.56$, $p = 0.004$). The CL group had the highest total wetting length (sum of OD and OS) and total protein, while the DE subjects had the lowest yield for both parameters. The overall range of total protein for all 60 subjects was 29–208 µg; however, due to the quantitation assay and the pipetting process, all the samples were normalized to the lowest remaining protein concentration of 20 µg, then split evenly between the two assays, thus allowing an equal total protein amount of 10 µg per subject to be used for both the Luminex method and the Microarray method.

Cytokine comparison between kits: The Luminex method is a kit comprising 13 cytokines, all of which were represented on the 40-panel Microarray (Appendix 1). In general, the types of detectable inflammatory mediators differed depending on the method used. The Genepix® detected cytokines that were involved with lymphocytic activation and macrophage recruitment, chemokine activity, tissue inhibitors, and cytotoxicity (Figure 1A). The Luminex detected immunomodulatory cytokines (Figure 1B). Despite the fact that all the cytokines and chemokines present in the Luminex magnetic bead kit were also present in the Microarray, none of the overlapping mediators showed expression in both the methods when the samples were averaged within each group.

CL subjects: For the Microarray method, eight of the 40 inflammatory mediators had expression levels greater than the LOD of the assay (Figure 1A). For the Luminex method however, four of the 13 had expression levels greater than the LOD, and of these, only IL-7 was detected in all the subjects in the CL group (Figure 1B).

DE subjects: Of the inflammatory mediators represented using the Microarray method, eight of the 40 inflammatory mediators had expression levels greater than the LOD (Figure 1A), while for the Luminex method, four of the 13 had expression levels greater than the LOD of the assay (Figure 1B). Of these four analytes in the Luminex method, only IL-8 was expressed in all the subjects in the group, while IL-1β expression occurred the least, in only two subjects.

NOR subjects: For the non-contact lens wearers, six of the 40 inflammatory mediators in the Microarray method had expression levels greater than the LOD (Figure 1A), while in

Table 1. Schirmer strip wetting length and total protein+ concentrations.

Subject	Measure	OD (mm)	OS (mm)	(OD+OS; mm)	Protein (ug/ul)	Correlation (Sum (OD+OS), Protein)
ALL	Average	20.60±11.75	18.33±11.23	38.93±21.08	83.93±37.01	r=0.61, p=0.0000001
	Range	(1, 35)	(0, 35)	(1, 70)	(29, 208)	
	P value		0.282391			
DE	Average	14.95±12.61	11.45±8.45	26.4±18.43	75.64525±30.97	r=0.56, p=0.004
	Range	(1, 35)	(0, 35)	(1, 70)	(35, 142)	
	P value		0.309808			
CL	Average	25.70±8.26	23.90±8.94	49.60±15.10	98.94±35.67	r=0.33, p=0.08
	Range	(14, 35)	(9, 35)	(24, 70)	(58, 208)	
	P value		0.512334			
NOR	Average	21.15±11.87	19.65±12.49	40.80±22.87	77.19±40.78	r=0.77, p=0.00004
	Range	(2, 35)	(2, 35)	(4, 70)	(29, 159)*	
	P value		0.699171			

*indicates the lowest value of protein concentration to which all other samples were normalized +Total protein concentrations are before aliquot taken for quantitation

the Luminex method, four of the 13 mediators had expression levels greater than the LOD (Figure 1B). Of the four in the Luminex method, only IL-7 and IL-8 were present in all the subjects in this group, while IL-4 expression occurred the least, in only four subjects.

Subject group comparison: When comparing the groups, the CL group showed elevated expression levels compared to the DE group for some mediators specific to macrophage recruitment, such as MCP-1. The NOR group showed elevated expression levels of MCP-1 when compared to both the DE and CL groups. Of the 13 cytokines present in both assays, only three showed expression levels greater than the LOD in all three subject groups using the Luminex method (IL-6, IL-7, and IL-8). None of the 13 overlapping analytes showed expression levels greater than the LOD in any of the subject groups for the Microarray method, indicating that the results from the two assays could not be compared in terms of the protein amount tested (Table 2).

The Microarray method, however, showed significance between the groups for a greater number of analytes than the Luminex method ($p<0.05$, Table 3). This occurred despite the averaged expression levels not reaching the LOD because in some cases, individual subjects within the group showed expression levels above the LOD. A comparison of the CL group versus the NOR group showed the greatest number of statistically significant differences, for 19 of the 40 analytes. The DE versus NOR comparison showed the lowest number of significant differences, in only two cases, those of G-CSF and ICAM-1. For the Luminex method, only five (IL-5, IL-6, IL-7, IL-8, and TNFα) of the 13 cytokines showed a statistical significance (Table 3) between the groups. The CL versus DE comparison revealed the most significant differences, for all but IL-6, and the CL versus NOR comparison had the lowest number of significant differences, only for IL-8.

The same significance between the subject groups was not seen for both methods. For example, IL-6 was determined to be significant between the CL and NOR subjects for the Microarray method but not the Luminex method.

DISCUSSION

From our data, it can be concluded that both the Microarray and Luminex methods are challenged by low yield samples, such as the tears obtained from DE patients. The inability to obtain sufficient tear samples from individuals suffering with such tear film deficiency has been a limitation in many analysis techniques, often producing sub-optimal or results

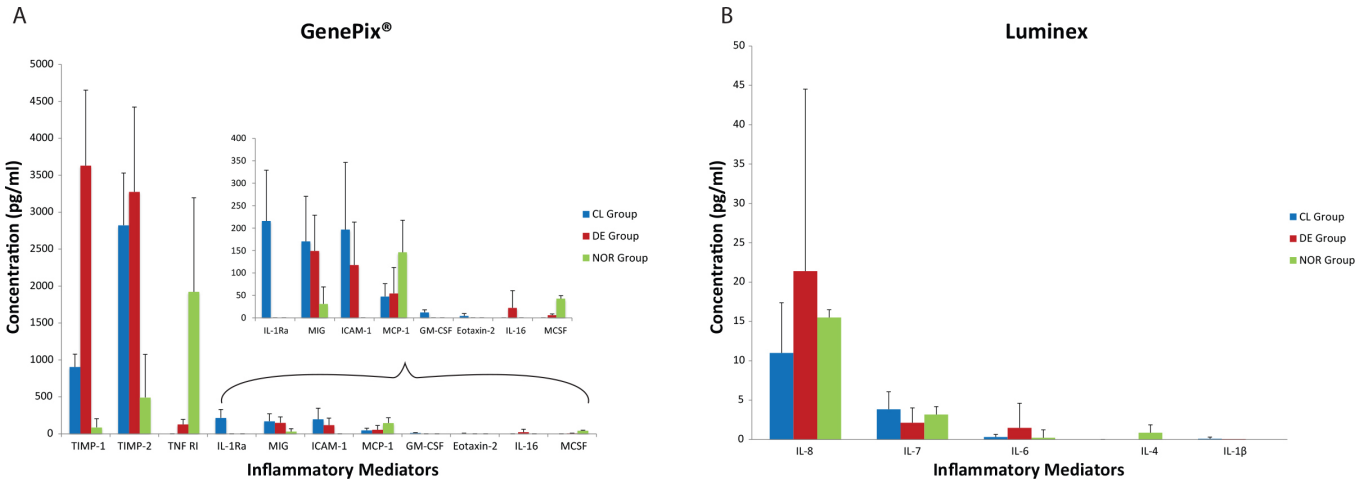

Figure 1. Average inflammatory mediator expression levels for each method and group. **A**: Contact lens (CL) group: For the microarray, eight inflammatory mediators had expression levels greater than the minimum detectable level (MDL) of the assay for the contact lens wearers: ICAM-1, MCP-1, MIG, GM-CSF, Eotaxin-2, IL-1ra, TIMP-1, and TIMP-2. Dry eye (DE) group: Of the inflammatory mediators represented in the microarray, eight had expression levels greater than the MDL: ICAM-1, IL-16, MCP-1, MCSF, MIG, TIMP-1, TIMP-2, and TNF-RI. Non-contact lens (NOR) group: For the non-contact lens wearers, six inflammatory mediators included in the microarray showed expression levels greater than the MDL: MCP-1, MCSF, MIG, TIMP-1, TIMP-2, and TNF-RI. **B**: CL group: Among the 12 inflammatory cytokines and one chemokine present in the Luminex magnetic bead kit, only four had expression levels greater than the MDL: IL-1β, IL-6, IL-7, and IL-8. Of these, only IL-7 was shown to express in all the subjects in the group. DE group: Of the 13 inflammatory mediators present in the Luminex magnetic bead kit, four had expression levels greater than the MDL of the assay: IL-1β, IL-6, IL-7, and IL-8. Of these four analytes, only IL-8 was present in all the subjects in the group, with IL-1β expression occurring the least, in only two subjects. NOR group: For the Luminex magnetic bead kit, four mediators showed detectable levels of expression: IL-4, IL-6, IL-7, and IL-8. Of these, only IL-7 and IL-8 were present in all the subjects in this group, with IL-4 occurring the least, expressing in only four subjects.

Table 2. Analytes and the limits of detection (LOD) included in each assay

Analytes	Raybiotech Quantibody Human Inflammation Array 3 (pg/ml)	Millipore High Sensitivity Human Cytokine Magnetic Bead Kit (pg/ml)
IL-1β	4.49	0.06
IL-2	23.42	0.26
IL-4	12.35	0.42
IL-5	19.99	0.04
IL-6	11.47	0.2
IL-7	9.38	0.2
IL-8	9.47	0.05
IL-10	15.24	0.48
IL-12p70	1.98	0.34
IL-13	5.98	0.18
IFNγ	43.89	0.18
GM-CSF	12.06	0.15
TNFα	25.25	0.07
BLC	9.37	
Eotaxin	8.58	
Eotaxin-2	3.11	
G-CSF	23.99	
I-309	12.65	
ICAM-1	95.82	
IL-1α	14.56	
IL-1ra	253.45	
IL-6sR	24.99	
IL-11	78.10	
IL-12p40	39.48	
IL-15	77.23	
IL-16	17.93	
IL-17	24.83	
MCP-1	12.05	
MCSF	4.23	
MIG	74.91	
MIP-1a	4.23	
MIP-1b	0.50	
MIP-1d	6.47	
PDGF-BB	9.16	
RANTES	6.60	
TIMP-1	21.46	
TIMP-2	51.22	
TNFβ	83.58	
TNF RI	81.68	
TNF RII	34.51	

Table 3. Statistical significance between subject groups as detected by both methods.

Kit	Molecule	DE versus CL	Increased expression	DE versus NOR	Increased expression	CL versus NOR	Increased expression
Microarray Inflammatory Mediators	Eotaxin-2	p=0.034	CL	p=0.367	n/a	p=0.002	CL
	G-CSF	p=0.012	DE	p=0.039	DE	p=0.641	n/a
	GM-CSF	p<0.001	CL	p=0.455	n/a	p<0.001	CL
	I-309	p=0.114	n/a	p=0.411	n/a	p=0.006	CL
	ICAM-1	**p=0.055**	**CL**	p=0.039	DE	p=0.001	CL
	IFNg	p=0.037	DE	p=0.897	n/a	p=0.003	NOR
	IL-2	p=0.023	DE	p=0.535	n/a	p<0.001	NOR
	IL-6	**p=0.053**	**DE**	p=0.634	n/a	p<0.001	NOR
	IL-6sR	p<0.001	DE	p=0.807	n/a	p<0.001	NOR
	IL-7	p=0.111	n/a	p=0.772	n/a	p=0.041	NOR
	IL-12p40	p=0.047	DE	p=0.931	n/a	p=0.008	NOR
	IL-13	p=0.831	n/a	p=0.109	n/a	p=0.025	NOR
	MCP-1	p=0.637	n/a	p=0.095	n/a	p=0.045	CL
	MCSF	p<0.001	DE	p=0.552	n/a	p<0.001	NOR
	MIP-1a	p<0.001	DE	p=0.587	n/a	p=0.001	NOR
	TIMP-1	p<0.001	DE	p=0.551	n/a	p<0.001	NOR
	TIMP-2	p=0.143	n/a	p=0.083	n/a	p=0.001	NOR
	TNFb	p=0.012	DE	p=0.227	n/a	p<0.001	NOR
	TNF RI	p<0.001	DE	p=0.567	n/a	p<0.001	NOR
	TNF RII	p=0.006	DE	p=0.472	n/a	p=0.006	NOR

Red, significant Bold, borderline significant Black, not significant n/a, not applicable, there were not two data sets to compare

that are difficult to compare. While the samples in this study were normalized to the lowest yielding total protein sample, which was from the DE group, the overall average total protein was not statistically significant between the DE and CL groups, which was unexpected (Table 1). This could be due to the nature of the subject inclusion criteria, which only required self-reporting of DE symptoms. Furthermore, the type of DE was not further categorized as aqueous deficient versus inflammatory although aqueous deficient would, by definition, be expected to challenge tear collection techniques and therefore yield lower total protein.

A disparity in detection levels between the two assays was also seen and could have been influenced by many key factors, such as the starting sample, the methods of collection and storage, or the amount of protein required for detection versus recommended for detection. In this study, 10 µg of total protein from Schirmer strips was used for both assays, while similar studies utilized different collection methods, constant volumes, or dilutions of collected tears [19,20]. Tear fluid, however, can have differing amounts of protein due to the collection time of day, patient variability in reflex versus basal tears, or differences in clinician collection techniques. To mitigate these variables, the samples in this study were normalized to total protein concentration.

The recommended protein amount for use in the Microarray is a minimum of 5 µg, whereas 10 µg of total protein per sample was used for this experiment. The Luminex method does not have a minimum protein recommendation. While the results indicated that 10 µg of protein is sufficient for the detection of some cytokines, an increased amount could be necessary to reach the minimum detection level required for both methods for all the cytokines being expressed because total protein does not reflect the amount of each cytokine in a sample. The detectable cytokines using the Luminex method, with its lower LOD, indicated that expression levels may be detected by the Microarray method if more starting material is available. The detection levels could also be affected by the approach utilized by each assay. The Luminex method combines flow cytometry with microspheres where the captured antibody is covalently immobilized on the bead surface and a reporter antibody must bind to each captured bead. The beads themselves are dense and tend to quickly settle out of any mixture, and the magnetic field can become disrupted during the washing and decanting steps leading to a loss of material. The kit used for the Luminex method also requires additional protein to run duplicates to obtain the standard deviation of the analytes within a single sample. While not necessary for viable results, determining the variation that can occur within a sample provides a more complete picture of the protein expression levels at any given time, which can be compromised by the diluted samples in the assay for the Luminex method. The manufacturer does provide dual quality controls, QC1 and QC2, to better assess the reliability of results; however, when these quality controls fail, as QC2 did for 73% of the proteins in our experiment, the result can be considered unreliable despite the LOD having been met or exceeded in line with the manufacturer's specifications. For this reason, only QC1 was evaluated for the results of the Luminex method. The LOD is also reported by the manufacturer, not measured or calculated, and does not take into account any processing or user errors. The standard curves, which are necessary to quantitate the amount of each protein expression in the sample, require adjustment for linearity, which was harder to achieve with the Luminex method; all 13 cytokines had to be adjusted for outliers, the implication being that considerable optimization is necessary.

In contrast, the Microarray method requires each cytokine to be spotted in duplicate onto a glass slide and uses binding affinity to capture the protein present in the sample. This allows for better retention of material during processing. Since duplicates are incorporated into each array, this could lead to a lower detectable expression level, especially when using a diluted starting material, and standard deviation can easily be determined for a single sample without the use of additional material. The Microarray scanner can be adjusted to obtain information from weak signals in the event of low binding due to weak antibody interaction, low protein, or excess washes, for example. For the Microarrays used in this study, the standard curves were also more reliable than the Luminex method. Slightly less than one-half of the inflammatory mediators (19 out of 40) needed to be adjusted for outliers to achieve linearity. The areas for analysis can also be indicated or defined by the user to optimize the fluorescent data captured by aligning the cytokine map provided by the manufacturer with the fluorescent map generated by the instrument, something which cannot be done when using the Luminex method. Most importantly, a larger number of analytes can be assessed using the Microarray whereas additional protein is necessary to increase analyte exposure with the Luminex. A summary of the key factors differentiating the methods, which could have influenced the results of this study, can be found in Table 4. While no method proved better than the other overall, the preferences for each factor are indicated.

The LOD was possibly the most relevant factor to consider for each method (Table 2). This is the threshold (in pg/ml) for both the kits used in this study, below which no results are reliable, according to the manufacturers. This

Table 4. Comparison of key factors between methods.

Factors	Luminex	GenePix
Limit of Detection	**Lower limit of detection which is constant and set by the manufacturer.**	Limit of detection is calculated for each slide as a function of background intensity.
Reliability	**Quality Control checks, QC1 and QC2, provided by the manufacturer. However QC2 failed for 11/13 cytokines.**	**Each array includes material to assist in optimization.**
Standard Deviation	**Must load duplicates to determine standard deviation.**	**Standard deviation, n=4, accounted for within the array.**
Standard Curve	**Linearity was harder to achieve. All 13 cytokines had to be adjusted for outliers.**	**Only 19/40 cytokines required adjustment for outliers to achieve linearity.**
Binding Affinity	**Antibody affinity and magnetic interactions must be maintained.**	**Only antibody affinity must be maintained.**
Sample Prep	**Requires more sample manipulations.**	**Requires less manipulations but longer wash steps.**
Ease of Use	**User interface is straightforward. However, no optimization can be done to the scan.**	Scanning can be optimized for more reliable results.

Normal font indicates the preferable method for the associated factor, bold font indicates no preference

limit determines the sensitivity of each method. For the Microarray, this threshold is calculated with every slide scanned as a function of the background pixels surrounding each fluorescing spot and can presumably be optimized. The Luminex, however, has a constant LOD set by the manufacturer, which does not take into account sample processing or user error. Despite the LOD being theoretically lower for the Luminex kits, the ability to detect the overlapping mediators in individual subjects using this method was less than expected when compared to the Microarray. For example, the average LOD of IL-2 was 0.26 pg/ml for the Luminex and 23.4 pg/ml for the Microarray. Despite this difference, IL-2 was not detected in any single sample using the Luminex, whereas it was detected in 20% of the CL subjects, 35% of the DE subjects, and 50% of the NOR subjects when using the Microarray (Table 5). The manufacturer-reported LOD for the Luminex was clearly not achieved. This finding was interesting and requires examination beyond the scope of this study. It further emphasizes the consideration that is needed when choosing between the two methods and instruments.

The method of tear analysis most often cited in publications is the Luminex method. Previously reported results using the Luminex method, as described here, have shown similar expression levels to those reported in this study for IL-8 only, the most sensitive chemokine on the panel [2]. Most methods used larger volumes of tear samples [17,18,20], fewer subject numbers [15], or different patient populations [14]. There were also disparities in the protein extraction methods, where applicable, although previous studies have shown ammonia bicarbonate to be an effective reagent [23].

Microarray analysis has a wider variety of protocols, which are discussed in the literature. These methods tend to utilize other materials, such as saliva [25], and different methods or kits encompassing similar, though not exact, inflammatory mediators [13], making direct comparisons difficult. Despite this, the inflammatory markers most reported in the DE group in our study, such as IL-6, IL-8 and TNFα, were not seen in our overall results with the Microarray method using 10 µg of extracted protein, although we did see expression in individual subjects within this group, as noted above (Table 5).

There was also a notable difference in statistical significance within the subject group comparisons. The DE subjects showed increased inflammatory cytokines in both assays when compared to the CL subjects but not when compared to the NOR subjects. This result was unexpected in that the NOR subjects were presumed to have normal expression levels, yet this NOR group also had expression levels of many inflammatory mediators commonly associated with DE, such as IL-6 and IL-8 (Table 3). The CL versus NOR comparisons showed the greatest number of statistically significant differences using the Microarray, with the NOR group exhibiting increased expression, whereas the DE versus NOR comparisons were expected to show more significant differences. The statistical significance between each assay differed as well. For example, the increased expression of IL-6 in the NOR

Table 5. Percentage of subjects in each group with analyte* expression levels above the LOD for both methods.

Analytes	CL		DE		NOR	
	Luminex	Microarray	Luminex	Microarray	Luminex	Microarray
IL-1b	30%	5%	15%	15%	20%	5%
IL-2	0%	20%	0%	35%	0%	50%
IL-4	30%	30%	20%	5%	15%	10%
IL-5	0%	0%	0%	5%	0%	0%
IL-6	55%	10%	80%	25%	45%	35%
IL-7	100%	0%	90%	25%	100%	20%
IL-8	100%	20%	100%	25%	100%	25%
IL-10	0%	0%	0%	0%	5%	0%
IL-12(p70)	0%	5%	0%	15%	0%	20%
IL-13	20%	10%	15%	25%	20%	35%
IFNy	0%	0%	0%	20%	0%	10%
GM-CSF	0%	40%	0%	5%	0%	0%
TNFα	0%	15%	5%	0%	0%	0%

*Of analytes present in both methods. The analytes are only those present both methods in both the Luminex High Sensitivity Human Cytokine Magnetic Bead Kit and the Raybiotech Quanitbody® Human Inflammation Array 3.

subjects compared to the CL subjects was determined to be significant when analyzed using the Microarray, but the same subject group comparison using the Luminex was not found to be significant (Table 3).

These deviations from the expected outcomes could be due to undiagnosed or incorrectly attributed symptoms that may occur in CL and NOR populations and would otherwise be characterized as DE. This study could possibly benefit from more examination-based or stringent inclusion and exclusion criteria. It is also known that contact lenses can adsorb components of the tear film, thus potentially reducing the presence of inflammatory markers in the CL group [26]. Inter-method differences can be attributed to variations in kit sensitivity, as seen in this study.

Overall, using an equivalent amount of Schirmer strip-extracted tear protein, the Microarray method detected a greater number of individual cytokines due to the larger analyte panel and also revealed more significant differences between some subject groups. The Luminex method, however, had a higher sensitivity to certain analytes that are commonly associated with DE, such as IL-8. When analyzing tear protein samples, these factors should be taken into consideration before selecting the method of analysis.

APPENDIX 1. INFLAMMATORY MEDIATORS PREVIOUSLY INVESTIGATED AS COMPARED TO THIS STUDY

• Inflammatory mediators present on the Raybiotech Quanitbody® Human Inflammation Array 3, ◦ Inflammatory mediators present on the Luminex High Sensitivity Human Cytokine Magnetic Bead Kit, * Indicates previously reported microarray data Highlighted mediators in gray were compared between methods. To access the data, click or select the words "Appendix 1."

REFERENCES

1. Research in dry eye: report of the Research Subcommittee of the International Dry Eye WorkShop (2007). Ocul Surf 2007; 5:179-93. [PMID: 17508121].
2. Huang JF, Zhang Y, Rittenhouse KD, Pickering EH, McDowell MT. Evaluations of tear protein markers in dry eye disease: repeatability of measurement and correlation with disease. Invest Ophthalmol Vis Sci 2012; 53:4556-64. [PMID: 22695964].
3. von Thun Und Hohenstein-Blaul NF. S.; Grus, FH. Tears as a source of biomarkers for ocular and systemic diseases. Exp Eye Res 2013; 4835:213-3. .
4. Fodor M, Kolozsvari BL, Petrovski G, Kettesy BA, Gogolak P, Rajnavolgyi E, Ujhelyi B, Modis L, Petrovski BE, Szima GZ, Berta A, Facsko A. Effect of contact lens wear on the release of tear mediators in keratoconus. Eye Contact Lens 2013; 39:147-52. [PMID: 23411991].
5. Hori S, Shoji J, Inada N, Sawa M. Evaluation of chemokines in tears of patients with infectious keratitis. Nippon Ganka Gakkai Zasshi 2013; 117:117-25. [PMID: 23534256].
6. Huang D, Xu N, Song Y, Wang P, Yang H. Inflammatory cytokine profiles in the tears of thyroid-associated ophthalmopathy. Graefes Arch Clin Exp Ophthalmol 2012; 250:619-25. [PMID: 22124787].
7. Poyraz C, Irkec M, Mocan MC. Elevated tear interleukin-6 and interleukin-8 levels associated with silicone hydrogel and conventional hydrogel contact lens wear. Eye Contact Lens 2012; 38:146-9. [PMID: 22415150].
8. Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR, Beuerman RW. In-depth analysis of the human tear proteome. J Proteomics 2012; 75:3877-85. [PMID: 22634083].
9. Bjerrum KB, Prause JU. Collection and concentration of tear proteins studied by SDS gel electrophoresis. Presentation of a new method with special reference to dry eye patients. Graefes Arch Clin Exp Ophthalmol 1994; 232:402-5. [PMID: 7926870].
10. Saijyothi AV, Angayarkanni N, Syama C, Utpal T, Shweta A, Bhaskar S, Geetha IK, Vinay PS, Thennarasu M, Sivakumar RM, Prema P. Two dimensional electrophoretic analysis of human tears: collection method in dry eye syndrome. Electrophoresis 2010; 31:3420-7. [PMID: 20882555].
11. Karns K, Herr AE. Human tear protein analysis enabled by an alkaline microfluidic homogeneous immunoassay. Anal Chem 2011; 83:8115-22. [PMID: 21910436].
12. You J, Hodge C, Wen L, McAvoy JW, Madigan MC, Sutton G. Tear levels of SFRP1 are significantly reduced in keratoconus patients. Mol Vis 2013; 19:509-15. [PMID: 23441124].
13. Boehm N, Riechardt AI, Wiegand M, Pfeiffer N, Grus FH. Proinflammatory cytokine profiling of tears from dry eye patients by means of antibody microarrays. Invest Ophthalmol Vis Sci 2011; 52:7725-30. [PMID: 21775656].
14. Carreño E, Enriquez-de-Salamanca A, Teson M, Garcia-Vazquez C, Stern ME, Whitcup SM, Calonge M. Cytokine and chemokine levels in tears from healthy subjects. Acta Ophthalmol (Copenh) 2010; 88:250-8. .
15. Enríquez-de-Salamanca A, Castellanos E, Stern ME, Fernandez I, Carreño E, Garcia-Vazquez C, Herreras JM, Calonge M. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. Mol Vis 2010; 16:862-73. [PMID: 20508732].
16. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear cytokine profiles in dysfunctional tear syndrome. Am J Ophthalmol 2009; 147:198-205. [PMID: 18992869].
17. Lee SY, Han SJ, Nam SM, Yoon SC, Ahn JM, Kim TI, Kim EK, Seo KY. Analysis of tear cytokines and clinical correlations in Sjogren syndrome dry eye patients and non-Sjogren

syndrome dry eye patients. Am J Ophthalmol 2013; 156:247-53. [PMID: 23752063].

18. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. Cornea 2009; 28:1023-7. [PMID: 19724208].

19. Na KS, Mok JW, Kim JY, Rho CR, Joo CK. Correlations between tear cytokines, chemokines, and soluble receptors and clinical severity of dry eye disease. Invest Ophthalmol Vis Sci 2012; 53:5443-50. [PMID: 22789923].

20. VanDerMeid KR, Su SP, Ward KW, Zhang JZ. Correlation of tear inflammatory cytokines and matrix metalloproteinases with four dry eye diagnostic tests. Invest Ophthalmol Vis Sci 2012; 53:1512-8. [PMID: 22323462].

21. Posa A, Brauer L, Schicht M, Garreis F, Beileke S, Paulsen F. Schirmer strip vs. capillary tube method: non-invasive methods of obtaining proteins from tear fluid. Ann Anat 2013; 195:137-42. [PMID: 23357333].

22. González N, Iloro I, Duran JA, Elortza F, Suarez T. Evaluation of inter-day and inter-individual variability of tear peptide/protein profiles by MALDI-TOF MS analyses. Mol Vis 2012; 18:1572-82. [PMID: 22736947].

23. Green-Church KB, Nichols KK, Kleinholz NM, Zhang L, Nichols JJ. Investigation of the human tear film proteome using multiple proteomic approaches. Mol Vis 2008; 14:456-70. [PMID: 18334958].

24. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54. [PMID: 942051].

25. Greenwell-Wild T, Moutsopoulos NM, Gliozzi M, Kapsogeorgou E, Rangel Z, Munson PJ, Moutsopoulos HM, Wahl SM. Chitinases in the salivary glands and circulation of patients with Sjogren's syndrome: macrophage harbingers of disease severity. Arthritis Rheum 2011; 63:3103-15. [PMID: 21618203].

26. Mann A, Tighe B. Contact lens interactions with the tear film. Exp Eye Res 2013; 117:88-98. [PMID: 23886658].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 23 February 2016. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.