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Measuring Interleukin-6 in Human Serum from Children with a Pediatric Concussion Using a Single Molecule Counting Platform

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Dr. Ronald Myers
Scientific Editor
Journal of Visualized Experiments
Cambridge, MA, USA

Re: Submission to Journal of Visualized Experiments

Dear Dr. Ronald Myers,

Thank you for the opportunity to revise our manuscript entitled "A New Single Molecule Counting Platform for Protein Quantification: Interleukin-6 in Human Serum in Pediatric Traumatic Brain Injury as an Exemplar" for your consideration to publish in your esteemed journal, *Journal of Visualized Experiments* (JoVE). We have addressed the changes proposed by the editorial staff, specifically by referring less to the commercial supplier unless absolutely relevant.

We are confident that JoVE is the ideal place to publish these novel methods as the unique video format will highlight the user-friendly workflow. We strongly feel that the methods described in our manuscript will be relevant to your readership. Single molecule counting offers an unprecedented opportunity to better understand low-abundance biomarkers in the brain injury population and beyond.

The open-access fees associated with this article would be industry-sponsored by MilliporeSigma, a company that one of the authors (Adam S. Venable) works for. We strove and continue to strive to ensure our article is rigorous and free of commercial bias and are happy to provide any additional information regarding sponsorship as needed. Thank you for considering our manuscript for publication. If you require any additional information at this time, please do not hesitate to contact us using the contact information for the corresponding author provided below the signature line.

Sincerely,

Nicole Osier

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TITLE:

Measuring Interleukin-6 in Human Serum from Children with a Pediatric Concussion Using a Single Molecule Counting Platform

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KEYWORDS:

Biological markers, biomarkers, protein, interleukin-6, serum, ultrasensitive, quantification, pediatric traumatic brain injury, biomarker platform, assay, new instrument, single molecule counting

SUMMARY:

Methods are described for ultrasensitive quantification of biomarker proteins using an immunoassay system capable of single molecule counting. The platform can be used to quantify a wide array of proteins in a variety of tissues from several species. This exemplar assays interleukin-6 in human serum from pediatric concussion patients.

ABSTRACT:

Efforts to expand the precision science knowledge base and promote translation of findings to clinical care remain an important area of ongoing inquiry. Part of this effort includes quantification of proteins that can be used as biological markers (i.e., biomarkers) of pathophysiological processes and other important cellular and tissue activities. Potential applications for biological markers include disease or injury diagnosis, symptom prognosis, and therapy selection/evaluation. The increased understanding of the current and future utility of protein biomarkers, combined with the realization that many biomarkers normally exist in very low levels, has prompted efforts to develop new protein quantification systems with enhanced sensitivity, improved workflows, and shorter read times. Here, we provide an overview of an ultrasensitive immunoassay system and compatible interleukin-6 (IL-6) assays. This assay platform is bead-based, like many other commercially available systems; however, rather than quantifying the fluorescent signal in the spectral address of bead regions, the ultrasensitive

immunoassay system quantifies free-floating fluorochromes using a rotating laser, charge-coupled device (CCD) camera, and avalanche photodiode (APD). This high-performance system can be used to quantify a myriad of protein biomarkers, in a variety of biological specimens collected from many species. In this article, quantification of IL-6 in human serum obtained from pediatric brain injury patients will be used as an exemplar.

INTRODUCTION:

The benefits of precision care initiatives are becoming increasingly appreciated, and efforts to expand current clinical applications are underway. This increased interest is fueling inquiry into protein biomarkers that have the potential to provide important insights into physiological processes inside the body that can inform clinical care. Current applications for protein biomarkers include diagnosing conditions[1], selecting appropriate therapies[2], and tracking response to treatment[2]. These and other precision care successes have led to an expansion in the number and nature of biomarkers being explored. Many putative biomarkers of interest naturally exist at levels below the limit of detection of standard methods for assaying proteins. Likewise, the relatively slow translation of biomarkers to clinical care and large number of candidate biomarkers in the validation pipeline highlights the need for improved throughput of technologies used for biomarker analysis[3]. These deficiencies have motivated efforts to develop new methodologies that offer ultra-sensitivity, improved workflow, and other advantages over traditional methods. The purpose of this article is to provide a protocol for using a bead-based single molecule counting technology to measure IL-6 in serum.

Traditional protein quantification methods such as western blot, enzyme-linked immunosorbent assay (ELISA), liquid chromatography, mass spectroscopy, and proprietary polymerase chain reaction (PCR) assays , are characterized by sensitivities in the nanogram (ng; 10^{-9}) or picogram (pg; 10^{-12}) per milliliter (mL) range, restricting detection of proteins at or below the femtogram (fg; 10^{-15}) per mL range[4–8]. However, many proteins that may provide useful windows into important physiological processes (e.g., cytokine signaling) are low-abundance with endogenous levels falling below the detection limit of conventional protein analysis techniques[9,10]. Emerging new platforms are improving sensitivity through the development of single molecule counting technologies[11–13.] Ultrasensitive systems facilitate reduced signal-to-noise ratios and, most importantly, detection of concentrations as low as the fg/mL range. The platform is unique from other systems with respect to how it counts the single molecules. During the proprietary elution step, the fluorescent dye-labeled detection antibodies are dissociated from the immunocomplex, resulting in free-floating (i.e., in suspension) fluorochromes. A rotating laser is focused through a high-numeric aperture objective to a diameter less than 4 µm. Emitted light is captured by the same objective then passed through a series of positioned mirrors and a long-pass dichroic filter. The image is captured using a charge-coupled device (CCD) camera and individual photons are counted by an Avalanche Photodiode (APD). All digital events, defined as six standard deviations above a background threshold, are counted using a digital event algorithm across a spectrum of time series for a given standard curve; the ultimate result is highly sensitive and reproducible biomarker quantification. Other technologies exist that that allow for ultrasensitive protein quantification. The first ultrasensitive system on the market was the predecessor to the system described in this manuscript; it is a flow cytometry-based instrument that detects Alex 647-

labeled detection antibody-analyte complexes in solution. The solution is aspirated into the instrument and is irradiated by a red laser that is focused through a confocal lens. Read times are more than 11 hours per 384-well plate, due to the time-consuming nature of the aspiration/detection method. Another company then launched fully automated system capable of detecting in the fg/mL. Despite the automation and perceived ease-of-use, the monthly cost of maintenance and initial procurement cost may be prohibitive, especially in academic laboratories.

The digital algorithm described above also addresses the limitation of many instruments with respect to dynamic range, especially for high concentrations[14,15]. A consequence of the narrow dynamic range characteristic of conventional ELISA and other common protein quantification platforms is the reduced utility for certain clinical populations and/or a need for special preparation (e.g., multiple sample dilutions) for cases and controls. Eliminating variable sample dilutions both reduces employee workload and minimizes the effects of dilution as a potential source of error[16–18].

The device itself is relatively small (16" high x 14" wide x 17.5" deep / ~55 pounds) and does not require fluidics; these features make the system portable and easy to maintain. The system's workflow is designed to improve the speed of data acquisition through automation, options to save templates, and faster sample reads via use of early-termination technology. The typical time to prepare the assay (including incubations) is approximately 3.5 h for the IL-6 kit, but may be longer for other proteins if overnight incubation is recommended. The read time is approximately 1-3 h for a 384 well plate (vs. up to 13 h for other platforms); the exact time of the assay depends on the concentration of the analyte of interest with faster reads occurring at higher concentrations. The system is able to run both bead-based and plate-based assays and sample volume requirements are as low as 5 µL (average of 10-25 µL for pre-clinical samples and 10-100µL for clinical samples). If desired, the plates can be re-assayed, because the dye that is eluted off the immunocomplex is stable if stored at 4 °C. Moreover, the system offers the option to use an optimized serum matrix to produce a standard curve that more closely simulates native levels of the analyte in serum/plasma. The inclusion of optimized serum matrix is unique and is in agreement with guidelines for immunoassays outlined by the American Association of Pharmaceutical Sciences[19].

The system's primary limitation is that debris/bubbles in the sample or scratches/damage to the underside of the plate can interfere with signal acquisition and adversely affect results, due to the ultrasensitive nature of the instrument. However, when a clean workspace is maintained and care in preparation of samples and handling of plates is exercised, extremely low levels of proteins can be precisely, accurately, and reproducibly quantified. Likewise, other manageable limitations common to all protein quantification methods (e.g., effects of pre-analysis storage[20,21]) apply; best laboratory practices and manufacturer instructions should be strictly adhered to.

This article is the first to demonstrate this single molecule counting system. A large and growing number of high-quality off-the-shelf and custom kits are available for this platform. Rigorous

quality control testing ensures assays are consistent with standards set forth by the World Health Organization. Because this method can be used for a wide variety of sample types and sources (e.g. species), the methods described below are applicable to a number of other clinical populations and animal models. The protocol assays interleukin-6 (IL-6) in human serum collected from a sample of pediatric concussion patients.

PROTOCOL:

All the procedures including human subjects described in this protocol have been approved by the Institutional Review Board (IRB) at the University of Texas at Austin. This protocol is specifically adjusted for targeted assay of IL-6 in serum; adjustments to the protocol should be made when assaying other protein biomarkers, consistent with manufacturer's instructions.

1. Collect, process, and store samples

1.1. Collect blood and process the biological samples for serum according to the manufacturer's protocol.

1.2. Store serum aliquots at -80 °C until processing for laboratory analysis.

2. Prepare the workspace and reagents

2.1. Use 70% isopropanol to wipe down the bench and pipettes.

2.2. Allow all reagents to reach room temperature (20-25 °C), including the single molecule counting High Sensitivity Human IL-6 Immunoassay Kit, Standard Analyte, Quality Controls, and 10x System Wash Buffer.

2.2.1. Keep the kit, especially the fluorescently conjugated Detection Antibody, away from light until the time of use.

2.2.2. Dilute the 10x wash buffer to 1x with deionized water and mix thoroughly.

2.2.3. Resuspend IL-6 Antibody-Coated Paramagnetic Beads using a rotisserie spin rotator or manual inversion for 10-20 min.

3. Prepare samples and quality controls

3.1. Prepare samples and quality controls immediately prior to use.

3.2. Centrifuge samples and quality controls at >13,000 x *g* for 10 min; alternatively, use a plate filtration method, which eliminates the need to transfer samples into a new tube.

3.3. Pipette the supernatant fluid into a new microcentrifuge tube, using care to avoid particulates.

4. Prepare the initial standard stock and perform the serial dilution

4.1. Prepare the standard stock solution.

4.1.1. Use a benchtop minicentrifuge to quickly spin the standard vial; and then mix via pipetting.

4.1.2. Refer to the Certificate of Analysis for the initial concentration and use a three-step dilution to achieve a final concentration of 50 pg/mL for Standard 1; ensure that each transfer has a volume ≥ 10 µL to reduce error associated with low-volume micropipetting.

4.2. Serially dilute the remaining standards in a 12-channel reservoir using 1:3 dilutions for Standard 2 through Standard 5, then 1:2 dilutions for Standard 6 through Standard 11). Ensure Standard 12 is left as a blank containing only 500 µL of Standard Diluent.

4.2.1. For the IL-6 assay, use concentrations of 16.67, 5.56, 1.85, 0.62, 0.31, 0.15, 0.08, 0.04, 0.02, 0.01 pg/mL for Standards 2-11, respectively.

4.2.2. Use a 12-channel pipette to promote consistency when transferring volumes from the sample plate after serial dilution.

5. Prepare target capture and incubate in a 96-well plate

5.1. Pipette 75 µL of standards and samples into a 96-well polypropylene plate.

5.2. Gently invert the bottle of Antibody-Coated Paramagnetic Beads to resuspend.

5.2.1. Aliquot 9 mL of the Assay Buffer into a 15 mL tube and add the entire 500 µL vial of the Antibody-Coated Beads.

5.2.2. Ensure that all Beads have been transferred, by rinsing the bead vial with 500 µL of Assay Buffer then transfer that volume into the already diluted Antibody-Coated Beads. Repeat this step two more times to bring the total volume of diluted Beads up to 11 mL.

5.2.3. Mix the diluted Beads using gentle inversion.

5.3. Transfer 100 µL of the diluted Antibody-Coated Beads into each well.

5.4. Cover the plate with plate sealing film and incubate at 25 °C while shaking for 2 h (use a speed setting of ~1000 rpm on the plate shaker).

5.5. Prepare the detection antibody when there is approximately 10 min left in the incubation.

5.5.1. Pipette 10 µL of Detection Antibody into 90 µL of Assay Buffer.

5.5.2. Add 75 µL of diluted Detection Antibody into 2925 µL of Assay Buffer.

5.5.3. Filter the diluted Detection Antibody using a 0.2 µm filter and place in a clean tube.

6. Post-Capture wash, detection, and post-detection wash

6.1. Wash the plate manually or use an automated system (following the manufacturer's protocol).

6.1.1. Use the Post Capture Wash setting on the automated system.

6.2. Hold the 96-well plate on a magnet and add 20 µL of Detection Antibody to each well, taking care to neither disturb the pellet nor touch the sides of the polypropylene plate.

6.2.1. Use a single set of pipette tips for the whole plate if proper technique is maintained and there is no evidence of contamination.

6.3. Apply sealing film to the 96-well plate using firm pressure to prevent cross-contamination and spills.

6.4. Incubate at 25 °C on a microplate incubator/shaker for 1 h on a speed setting of ~1000 rpm.

6.5. Remove the sealing film carefully, and wash manually, or use an automated system (following the manufacturer's protocol).

6.5.1. Use the 4 cycle Pre-Transfer wash setting on the automated system.

6.6. Remove the plate from the magnet or automated system and shake for 1 minute on a speed setting of ~750 rpm.

6.7. Return to the magnet or automated system and complete the final aspirate.

7. Elution

7.1. Add 10 µL of Elution Buffer B to the 96 well plate containing the microparticles using a 12-channel pipette.

7.2. Cover the plate with plate sealing film and incubate for 10 min at 25 °C on a microplate incubator/shaker on a speed setting of ~1000 rpm.

7.3. While the plate is incubating, add Buffer D to the appropriate wells of a new 384-well polypropylene plate, keeping the underside protected with the cover provided.

7.4. Place the original 96-well plate on the magnet for at least 2 min, until the Beads form a dense pellet.

7.5. Transfer 10 µL of eluate to each well in the 384-well plate, by row, using a 12-channel pipette with new tips for each transfer and exercising care to avoid disturbing the pelleted Beads.

7.6. Cover the top of the plate with a hard universal plate cover and spin the plate at 1,000 x *g* for 1 min.

7.7. Seal the 384-well plate tightly using an adhesive aluminum foil seal.

8. Run the assay on a biomarker quantification system

8.1. Set up the test template or open a saved template that designates which wells contain standards, controls, and test samples of unknown concentrations, as well as specifies the number of replicates and the dilution type/factor. If desired, users can define unique identifiers for their samples as appropriate.

8.2. Insert the 384-well plate into the device, and run according to the manufacturer's instructions.

8.3. Wait for the run to complete.

8.4. Remove and discard or store the plate at 2-8 °C.

9. Complete data processing and analysis

9.1. Create a new file of test results that contains the raw data along with information about the standard curve group and fit. Adjust the standard curve and/or remove outliers as appropriate.

9.2. Export data as a comma separated value (CSV) file for further analysis in the statistical software of choice.

REPRESENTATIVE RESULTS:

Using a Five Parameter Logistic Curve Fit (5PL) for the standard curve generated, the Lower Limit of Quantification (LLoQ) was 1.852 pg/mL and the Limit of Detection (LoD) was 0.011 pg/mL with a 0.996 R squared coefficient. Representative data from the ultrasensitive immunoassay system exported CSV file on the n=17 samples ran can be seen in **Supplemental Table 1**. For this run, the mean concentrations and coefficient of variation were utilized for direct comparison as seen in **Table 2**. **Figure 1** was created to visualize the concentrations that were detected in each unknown sample.

FIGURE AND TABLE LEGENDS:

Figure 1: Sample Concentrations of IL-6. Error bars show ± 1.96 times the standard error of the mean to represent a 95% confidence interval. This figure is used to visualize the range of concentrations determined using this system. It also shows that even with a 95% confidence interval, the assay is still accurate within a pg/mL for many samples despite the %CV.

Supplemental Table 1: Standard Curve and Sample Concentrations with Percent Coefficient of Variation. This table shows each of three replicates as well as the average concentrations and their associated %CV for the standard curve replicates and the patients' samples. This demonstrates the assay's capability of detecting a range of concentrations from 0.025 to 48.893 pg/mL in the standard curve samples and then using that curve to determine the concentrations of the unknown samples with a range of 0.227 to 4.717 pg/mL. The %CV for the standard curves varied from 4.10% to 47.50% and 0.50% to 15.60% for the patients' samples.

Supplemental Table 2: Overview of Demographic Characteristics for Study Participants. The sample was predominantly male (88.2%), white (70.6%), Hispanic (70.6%), and had an average age of 11.5 years.

DISCUSSION:

The use of several specific types of equipment can serve to further simplify the protocol and improve results. A rotisserie rotator can be used to promote adequate suspension of the beads and to save time and energy associated with manual inversion. By using a filtration plate over a standard 96 well plate during centrifugation of the samples, the number of plate transfers can be reduced, further saving time. When performing serial dilution of the standards, a 12-channel pipette and 12-well trough could be used to transfer the standards to the plate to limit time pipetting. The use of an automated plate shaker/incubator facilitates each incubation step in the protocol to further reduce the number of plate transfers and automated plate washers. Although these various instruments are not necessary for the use of the single molecule counting system, they help to reduce time during preparation and to save lab materials, like 96-well plates. To further automate the protocol, a robotic liquid handling system can be incorporated.

As with all laboratory techniques, troubleshooting efforts may be needed to address common issues, such as high background, high variability of samples, a LLOQ that is higher than expected, as well as bead-related issues (e.g., failure to resuspend; low bead counts). If the background is too high, a likely reason is that incubation with the detection antibody for longer than 1 hour occurred; a timer should be set to ensure that incubation is not prolonged. Another possible cause of high background would be well-to-well cross contamination due to failure to adequately seal the plate during incubation or poor pipetting practices; care should be taken to pipette without touching the existing reagents in the plate and change tips whenever possible contamination is suspected. Likewise, contamination of reagents in the bottles or poor technique when making the standard serial dilution can lead to contamination that would increase the background. Finally, if none of the abovementioned sources of error are likely, cleaning the

instrument following the manufacturer guidelines may reduce background. If the coefficient of variation (CV) is high within individual samples, an early trouble-shooting step is ensuring proper pipetting technique and other efforts to minimize cross-well contamination such as those described above (proper application of plate seals, changing pipette tips when warranted, etc.). Likewise, researchers should ensure that the pipettes are in proper working order and have been calibrated and validated within the last 6 months. Another possible explanation is that high concentrations of particulates or contaminates in the sample contributed to the high CVs; pre-assay filtration or centrifugation of samples minimizes this variability. Sufficient agitation during the incubation steps also minimizes sample variability; a speed that results in constant motion within the wells without leading to splashing is recommended (e.g., 700-1000 rpm). Bead-related issues include failure to adequately resuspend the beads and loss of beads during the experiment. Loss of beads may be due to the use of the wrong type of magnet when washing, or a failure to prime the lines of an automated plate washer with buffer resulting in the beads coming into contact with water. Alternatively, loss of beads may indicate a need to clean the plate washer according to the manufacturer's protocol. It is important to resuspend the beads for at least 10-20 minutes and ensure that they are never frozen as this will interfere with bead resuspension. Finally, if the LLOQ is higher than anticipated it could be that beads were lost or that the standard was not properly prepared. It is important to verify that the Certificate of Analysis is matched to the specific lot number on the kit used in the experiment when preparing the stock standard. Alternatively, if proper technique was used when preparing the standard, it could be that too much time elapsed between when the standard was prepared and when the capture incubation was started. A maximum time of no more than 10 minutes is recommended for best results.

The primary limitation of the techniques outlined in this protocol is used for singleplexing only. Researchers interested in exploring multiple protein targets within a single biological specimen may trade-off sensitivity for multiplexing. A secondary limitation of the methods is the higher cost of data acquisition when compared to lower-sensitivity assays. For research populations where proteins of interest are known to exist in high abundance, less expensive, analytical methods may be preferred. A third and final limitation is the potential for small defects on the plate to drastically impact the results due to the manner in which the plate is read from below and ultrasensitivity of the data captured. Strategies for reducing the impact of plate defects are described below.

Single molecule counting technology offers several improvements over traditional protein quantification systems including enhanced sensitivity, improved workflow, and quicker read times. The presently described method and instrument addresses several shortcomings in existing methods and technologies. First, assay read times have been drastically reduced when compared to the ultrasensitive immunoassay platform's predecessor as well as other commercial systems. Often, assay read times were prohibitive, which restricts the number of data points that could be generated in a single workday. By cutting the assay read time to less than half of what was previously required, researchers can generate more data in less time. Second, due to the fluidics-free design, the ultrasensitive immunoassay platform requires no daily, weekly, or monthly maintenance. Instrument maintenance in fluidics-based instruments require regular

attention so that tubing and moving components are maintained in working order. Moreover, most immunoassay targets can be adapted into an assay that is compatible with the ultrasensitive immunoassay platform. The flexibility of the assay and instrument provides ease-of-use for the researcher wishing to create novel targets for protein quantification.

The single molecule counting system presented here allows for the detection of many analytes in the fg/mL range. In the representative data, this can be seen through the standard curve's low LLoQ and LoD, as well as Sample 477's mean concentration of 0.364 pg/mL. Although no control, non-concussed patient samples were included in the outlined protocol, a previous study[22] has reported of IL-6 from 1.6-9.2 pg/mL using an ELISA assay for a sample of 79 children aged 0 to 5 and a mean concentration of 1.0-1.3 pg/mL for a group of adolescents from age 12 to 14. This improved detection capabilities mean that analytes could be measured where they were not previously thought to be, which may reveal undetected relationships between biomarkers and health conditions. Single molecule counting technology allows for the IL-6 concentration of pediatric concussion patients to be detected at low serum concentrations in this experiment. With IL-6's existing applications as a diagnostic tool[1] and for tracking treatment response[2], the ability to detect changes in low concentration analytes in the serum allows for researchers to investigate if small changes to serum concentrations could prove significant in predicting health outcomes. With the growing amount of kits available for use with the single molecule counting system, many different analytes could be detected using very similar protocols to the one described here.

Future applications for researchers studying concussions include replication of these findings in larger sample sizes and from additional geographic areas. Corroboration of biomarker data with short and long-term symptom and critical data should also be pursued. With respect to biomarker research as a whole, additional evidence is needed to understand how subtle fluctuations in biomarker levels may be relevant for diagnosis and prognosis of concussion as well as other conditions. Evaluation of biomarkers in multiple biospecimen types should also be pursued.

Care during preparation of the workspace and reagents prior to beginning the assay is important to obtain the best results. This includes proper processing and storage of samples prior to beginning the assay, maintenance of a clean workspace, and preparation of all reagents according to the guidelines provided in the detection kits. The single molecule counting detection antibodies are a critical component of these kits, so following manufacturer guidelines and protecting the detection antibodies from light as much as possible during preparation is important for ensuring low-noise results. Following the outlined procedures help to produce the highly sensitive results as shown above.

Also worth noting is that the ultrasensitive capabilities of this instrument mean small imperfections can affect the results. Smudges, debris, particulates, and scratches on and within the wells can increase the noise during detection. Fortunately, these limitations are easy to overcome by using precautions to avoid compromising the glass-bottom plates such as using base plates while handling them during preparation. As mentioned above, maintaining a clean workspace, as well as adhering to proper techniques for pipetting and handling of

reagents/samples will further reduce errors during detection.

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DISCLOSURES:

The author Adam S. Venable is an employee of Millipore Sigma that produces the instrument and reagents used in this Article. The other authors have nothing to disclose.

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Figure

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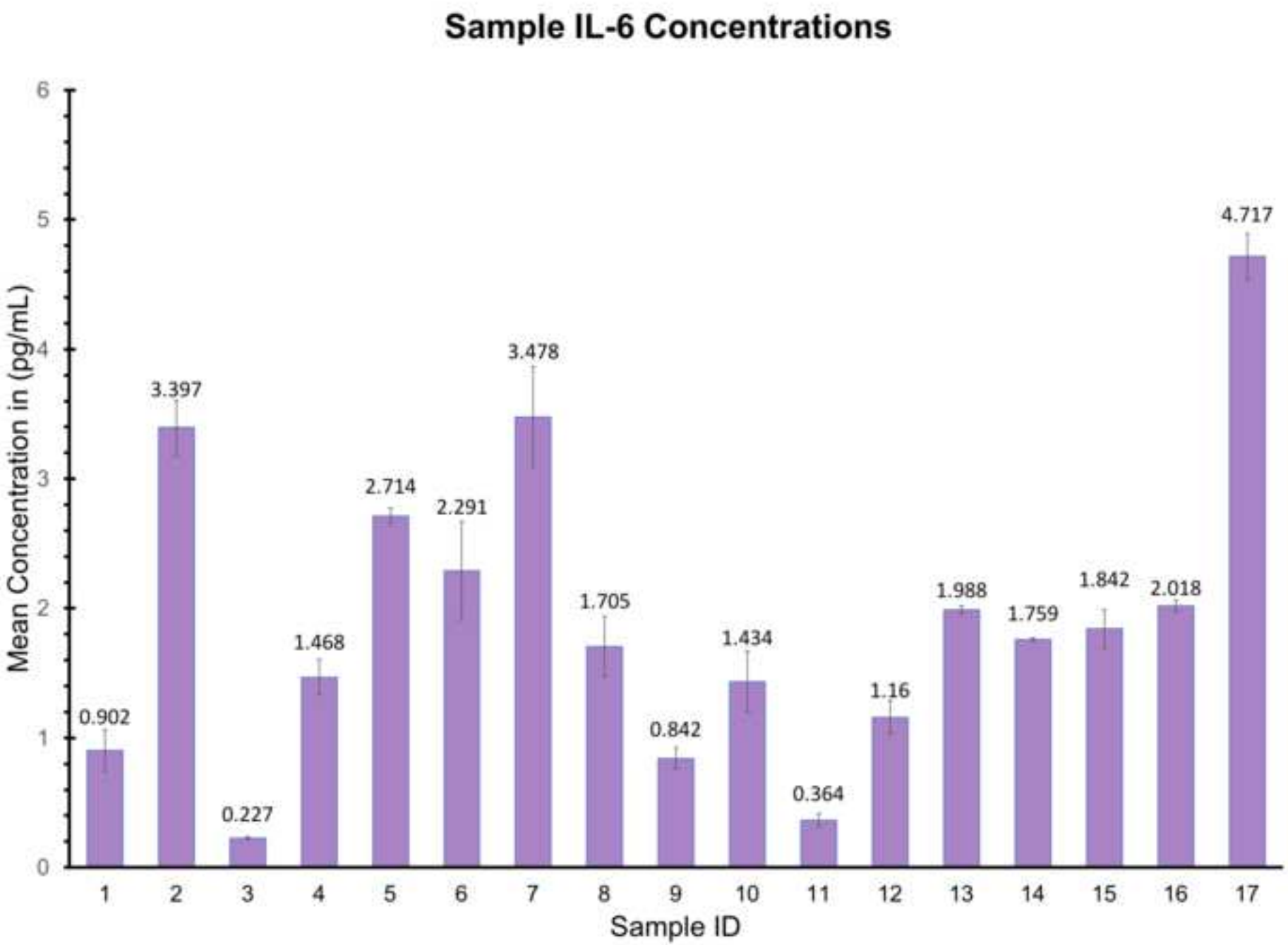

Table 1

Click here to access/download;Table;JOVE_Supplementary Table 1.xlsx

Standard Curve			Sa	
Replicates	Average Conc. (pg/mL)	%CV	ID	Replicates
46.885	48.893	4.10%	1	0.895
50.890				0.765
48.903				1.047
15.480	15.576	4.40%	2	3.607
16.305				3.251
14.943				3.335
5.564	6.16	15.90%	3	0.232
7.291				0.327
5.624				0.222
2.134	1.914	19.80%	4	1.574
2.132				1.339
1.477				1.492
0.567	0.863	47.50%	5	1.999
1.332				2.745
0.691				2.683
0.274	0.289	11.90%	6	2.647
0.328				1.979
0.264				2.246
0.152	0.164	17.20%	7	3.112
0.197				3.534
0.144				3.789
0.075	0.076	8.70%	8	1.908
0.070				1.494
0.083				1.714
0.033	0.034	9.20%	9	0.764
0.031				0.854
0.037				0.909
0.019	0.025	19.40%	10	1.650
0.026				1.238
0.028				1.415
			11	0.390
				0.532
				0.338
			12	1.290
				1.078
				1.114
			13	2.021
				1.970
				1.973
			14	1.765
				1.753

				1.265
			15	1.460
				1.766
				1.917
			16	2.013
				2.060
				1.979
			17	4.621
				4.898
				4.634

mples	
Average Conc. (pg/mL)	%CV
0.902	15.60%
3.397	5.50%
0.227	3.10%
1.468	8.10%
2.714	1.60%
2.291	14.70%
3.478	9.80%
1.705	12.20%
0.842	8.70%
1.434	14.40%
0.364	10.10%
1.16	9.80%
1.988	1.40%
1.759	0.50%

1.842	5.80%
2.018	2.00%
4.717	3.30%

Table 2

Click here to access/download;Table;JoVE_Supplemental Table 2.xlsx

Characteristic	Statistic
Male Sex	n=15 (88.2%)
Age	Mean = 11.5 years (range = 7-16)
Hispanic Ethnicity	n=12 (70.6%)
(*note: all Other identified	n=12 (70.6%)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1L Stericup Filter (0.22 µm; polyethersulfone)	MilliporeSigma	SCGPU11RE	Optional and not used in this study; used to sterilize
384-well SensoPlate	Griener BioScience		
500 mL graduated cylinder	Any	Varies	
5 mL syringe	Any	Varies	
96-well V-bottom polypropylene plate (500 µL)	Axygen	781892	
Centrifuge with plate rotator	Any	Varies	Must be capable of reaching a speed of 1,100 x g
Container capable of holding 500 mL	Any	Varies	
De-ionized or disitilled water	N/A	N/A	
Erenna 10x System/Wash Buffer	MilliporeSigma	02-0111-00	
Jitterbug Plate Shaker	Boekel Scientific	70-0009-00	A suitable alternative can also be used.
Microcentrifuge	Any	Varies	
Microcentrifuge tubes	Any	Varies	
Modified microplate washer	BioTek	95-0004-05	The microplate washer is modified to include a sphe
MultiScreenHTS BV 96-well Filter Plate	EMD millipore	P-96-450V-C	
Personal protection equipment	Any	Varies	Gloves, labcoat, closed-toed shoes
Reservoirs for 12-channel pipettors	Any	Varies	
Rotisserie Rotator	Any	Varies	
Sealing tape	Any	Varies	
Single channel pipettes	Any	Varies	Must have pipettes capable of transferring 10-250 µ
SMC High Sensitivity Human IL-6 Immunoassay Kit	MilliporeSigma	03-0089-01	Human assay optimized for use with serum and EDT
SMCxPROTM Complete System	MilliporeSigma	95-0100-00	This is the device used to run the assays and quantif
Syringe filter (0.2 µm)	Any	Varies	
Titer Plate Shaker	VWR	12620-926	Optional and not used in this study; used when incu
Universal plate cover	Any	Varies	

e remaining 1x wash buffer for storage (up to 1 month).

ere mag-plate assembly. A suitable alternative (automated or manual) can be used provided it can be modified to include the mag-plate assembly and alsc

ıL
TA plasma samples. The kit contains the following 9 items (1) Assay Buffer; (2) Beads; (3) Standard Diluent; (4) Detection Antibody; (5) Standard; (6) Contrc
fy the biomarker; the complete kit comes with the device and the associated computer and software.

ıbating overnight

› has a vacuum regulator as well as a dispense/waste system including a vacuum pump

›l 1; (7) Control 2; (8) Control 3; (9) 10X Wash Buffer; (10) Buffer D; (11) Elution Buffer B.

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Title of Article:	A New Single Molecule Counting Platform for Protein Quantification: Interleukin-6 in Human Serum in Pediatric Traumatic Brain Injury as an Exemplar
Author(s):	Nicole D. Osier, David Hutto, Claire Slote, & Adam S. Venable

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CORRESPONDING AUTHOR

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Signature:	Nicole Osier
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Editorial Comments:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

- After editing, we proofread the entire manuscript to ensure there are no spelling or grammatical errors

Please avoid punctuation marks in the title (e.g., colon, hyphen), and edit the title to avoid split titles.

- The title has been edited to avoid the use of punctuation and to add clarity.

Protocol Language:

1) Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

- We edited the entire document to minimize the use of notes.

Examples NOT in imperative voice: 2.2.1, 2.2.2, 2.2.3, 4.2.1, 4.3 etc.

- We changed the protocol section to imperative voice in all descriptions including, but not limited to sections 2.2.1, 2.2.2, 2.2.3, 4.2.1, and 4.3.

2) Please use complete sentences throughout each step (e.g. 2.2.2)

- We edited step 2.2.2 to create a complete sentence and we searched the document to change any other similar errors in syntax
- The original 2.2.2 was deleted and we inserted a new step 2.2.2
- 2.2.3 was revised to be a complete sentence

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:

1) Please provide all settings and parameters in generic units.

- We edited the protocol language and added specific wording in order to provide more detailed directions for the viewers, including settings and parameters in generic units.

Protocol Numbering: Please add a one-line space between each protocol step.

- We added a one-line space between each protocol step

Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

- Any steps that are highlighted include all relevant details necessary to complete that step

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

- We read through the highlighted steps to ensure that there is a logical flow in a narrative format

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

- We made sure to highlight complete sentences and we confirmed that our total highlighted text is approximately 2.5 pages

4) Notes cannot be filmed and should be excluded from highlighting.

- We did not highlight any notes.

Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

- Thank you for bringing this to our attention. We have added subheadings to the discussion and reorganized the existing discussion per the outline provided. We have also added additional content in order to populate the Limitations of the Technique and Future Applications section.

Figure/Table Legends: Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

- We have updated the Table and Figure Titles and Captions to provide a better, general description of each.

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generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

- We located and replaced all commercial sounding language with generic names and reference the table of materials when necessary

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

- We removed the registered trademark symbols TM/R from the table of reagents/materials

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- None of the figures have been previously published.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
Good review and method for emerging single molecular counting method for blood-based biomarker measurement

Major Concerns:
none

Minor Concerns:
Since SigmaMillipore SMCxPROTM Complete System is used for this manuscript - that should be mentioned in the abstract.

- Per request of the editor we have restricted the use of commercial language consistent with JoVE guidelines. The specific immunoassay system is referred to in the table of materials which is cited in text.

In the introduction, other single molecular counting platform (such as Quanterix) and perhaps Singulex platform should be discussed and compared.

- We discussed other single molecular counting platforms in the introduction, being careful not to use commercial language.

The current TABLE 1 - is too detailed- it should be included in supplementary data only.

- The original Table 1 has been moved to the supplementary data section.

Table 1 only shows one standard curve concentration. Since this is a new assay format - standard curve concentrations of IL-6 vs. readout values (replicates) should be plotted out or tabulated. For all concentration data point, the individual intraassay %CV should be listed also. A new table or figure should be used to show such representative standard curve data.

- The new Table 1 has been updated to show the replicates for both the standards and the samples. The %CV is also listed.
- With the addition of this new data, we did not feel it was necessary to add an additional figure based on the other reviewer and editorial comments.

Commented [1]: davidhutto@utexas.edu we need the replicates as well

Commented [2]: https://docs.google.com/spreadsheets/d/1lYrqAZIRUsYe8TlAFUBR_wUn0Jv03EA4CEJiGSjYF8w/edit#gid=808965976

Ideally, the authors should give interassay %CV also, based on multi-day experiments.

- All data was generated on a single day, and interassay %CV data based on multi-day experiments are not available.

On the pediatric TBI samples - it will be very helpful if there are baseline levels for injured controls to see if the pTBI levels are higher. Are such samples or values available. If not, perhaps even adult healthy controls levels should be included or cited as a reference point.

- We agree that this information would be germane; however, the data is not available. We did review the literature and found that IL-6 concentrations in healthy children depends on a number of factors including age and exercise status as outlined in the systematic review: https://doi.org/10.3389/fped.2017.00255. Moreover, considering the editors comment to restructure the discussion to focus more on certain aspects of the methods per the scope of the journal, we were unsure where to best place the inclusion of normative data. We are open to additional input if you think that this would enhance the readers' experience and is consistent with the scope of the journal.

Reviewer #2:

Manuscript Summary:

The manuscript describes a single molecule counting based detection of proteins using a new device platform. The authors have used pediatric brain injury serum samples to test the sensitivity of IL-6 after the injury. The method is described sufficiently in detail. There are few concerns which should be addressed before publication.

Major Concerns:

1. The authors have only used TBI serum samples for measuring the IL-6. They have not used any control samples to compare the relative concentrations of IL-6. The controls should have much less IL-6 detection with this system.

- Please see our response to Reviewer 1's last comment on the pediatric TBI samples.

2. The main aim of the manuscript is to show that the system being used is better than the existing platform available, however, the authors have not tested the same samples in any other platforms to show that this method is indeed superior.

- We agree that this is a limitation of the paper. However, as a new laboratory space, no equipment was available for less sensitive analysis. We have tried to provide objective and balanced discussion surrounding how this method compares to conventional approaches, citing relative literature. Other JoVE publications that we have accessed did not directly compare methods.

3. The percent Cv between the samples varies quite a bit. Can the authors explain why such a variation is noted between the samples in a group analyzed together.

- Per this comment and the editors suggestions, we have revamped the discussion section in order to better discuss factors that can impact variability, including limitations of the technique and protocol steps. Additionally, the data originally submitted as part of this manuscript was one of our laboratories first application of this new technology which may have contributed to any variability.

4. There is no information/demographics for the serum sample which are used for this study. Please include a table with the demographic details of the samples.

- Thank you for bringing this to our attention. We have generated a new Supplemental Table 2 with select demographic data for participants and added placed this table with other supplemental data.

Minor Concerns:

1. Please include some more information of the technology/platform in the long abstract. Also, there is not enough detail in the introduction section about the platform and the device.

- We added more information about the technology/platform in the long abstract and added more details in the introduction section.

2. There is no comparison with the other more sensitive protein detection platform. Please describe the major advantages over the currently available single molecule detection methods.

- In our discussion section, we added details comparing other protein detection platforms, including the advantages of the current system in use. Please refer to the Significance with Respect to Existing Methods paragraph in the Discussion section.

3. In the figure 1, it will be better to use samples 1-17 instead of the sample codes which are currently used.

- We edited Figure 1 using samples 1-17 instead of the sample codes.