Validation of an electrochemiluminescent-based assay for α-Synuclein detection in cerebrospinal fluid

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Abstract

Background: Abnormal α-synuclein aggregation and deposition is the pathological hallmark of Parkinson's disease and dementia with Lewy bodies, but is also found in Alzheimer's disease. Therefore, there is a gaining interest in α-Synuclein in cerebrospinal fluid (CSF) as potential biomarker for these neurodegenerative diseases.

Objectives: To broaden the available choices of α-Synuclein measurement in CSF, we developed and validated a new, independent assay for detecting total α-Synuclein.

Methods: This novel ELISA uses commercially available antibodies and is based on electrochemiluminescence technology. The assay protocol is straightforward with short and simple incubation steps and requires only small amounts of CSF. We validated this assay for precision, parallelism, dilution linearity, specificity, and spike recovery. We further compared it to the newly validated α-Synuclein assay from BioLegend by analyzing a set of 50 CSF samples with both assays.

Results: The new assay quantifies α-Synuclein in CSF with a lower limit of detection of 0.0363 ng/ml and shows no cross-reactivity with human β- and γ-Synuclein. Results of dilution linearity, parallelism, spike recovery and precision classify this assay as well suited for α-Synuclein detection in human CSF samples.

Conclusion: We present a novel assay based on freely available components to quantify total α-Synuclein in CSF as an additional method for α-Synuclein as a biomarker in neurodegenerative diseases. The assay convinces with its simple and convenient protocol paired with high sensitivity.

Introduction

There is a frequent overlap of symptoms between Parkinson's disease (PD), dementia with Lewy bodies (DLB) and other parkinsonian disorders as well as between DLB, Alzheimer's disease (AD) and other dementias. This results in diagnostic difficulties that can result in faulty or delayed diagnosis. This is of critical importance in the treatment of these diseases and highlights the need of diagnostic and prognostic biomarkers.

α-Synuclein plays a prominent role in the pathogenesis of several severe neurodegenerative diseases, and PD and DLB in particular.[1,2] It is the main component of Lewy bodies,[3] which are intraneuronal deposits of insoluble protein, a feature that is also present in up to 60% of patients with sporadic AD.[4]

Aggregation and deposition of α-Synuclein is most likely caused by an imbalance in the equilibrium between synthesis, aggregation and clearance.[2] The extent of oligomerisation and fibrillation in the brain could influence the amount of total α-Synuclein present in cerebrospinal fluid (CSF), just like the reduction in β-amyloid (Aβ42 and the Aβ42/Aβ42) in CSF likely is due to retention of the peptide as aggregates in the brain in AD (Blennow, 2014). Thus, findings in CSF might reflect the pathologic events in the brains of patients with abnormal α-Synuclein.

Accordingly, α-Synuclein in CSF has been proposed as biomarker for PD,[5-8] ENREF_5 DLB,[9] and AD.[10] Both encouraging and negative findings have been reported. In PD, α-Synuclein levels have been repeatedly reported to be decreased as compared to controls although some studies could not confirm this.[6] Further, a meta-analysis including 7 studies indicates that α-Synuclein distinguishes DLB from AD.[9]

The majority of studies employ modifications of an in-house assay first described by Tokuda et al.[11] or an in-house assay developed by Mollenhauer at al.[12], the successor of which just

recently became commercially available and validated. [13,14,15] To uncover α-Synuclein's biomarker potential, further independent, accessible and well validated methods are needed. As a new approach to quantify total α-Synuclein in CSF we therefore developed an electrochemiluminescence detection ELISA test entirely based on freely available components and validated it according to the newest guidelines.[16]

Materials and Methods

Developed detection method

Plate preparations: Wells of a 96-well high binding capacity plate (#L15XB, Meso Scale Discovery) were spot-coated with 5µl of 15µg/ml anti-α-Synuclein antibody (#610787, BD Transduction Laboratories) in DPBS (#SH30028, HyClone) and allowed to adsorb for 24hrs at room temperature (uncovered). The wells were then directly blocked with 150µl of 1% skim milk (#1.15363, Merck KGaA) and 1% BSA (#A7030, Sigma-Aldrich) in PBS-Tween 20 (0.05%, Merck KGaA) pH 7.4 (PBS-T) overnight at +4°C for use the following day. Blocking solution was freshly prepared, agitated for 15min to completely dissolve BSA and centrifuged at 2000×g for 10min prior to use.

Assay procedure: CSF samples were diluted 1:2 with PBS-Tween 20 (0.1%) pH 7.4 to achieve a final concentration of 0.05% Tween 20. A 4-fold serial dilution of recombinant α-Synuclein (#S-1001-1, rPeptide) in PBS-T (0.05%) ranging from 0.0098ng/ml to 160ng/ml served as calibrator. All steps were performed at room temperature and plates were sealed during incubation.

Blocking solution was aspirated and wells were washed 3x with 150µl PBS-T (0.05%). 25µl of controls, calibrators, and diluted samples were added to the wells and incubated for 1h with gentle shaking (400rpm). Plates were washed 4x with 150µl PBS-T (0.05%) and incubated with 25µl of 2µg/ml anti-α/β/γ-Synuclein (FL-140, #sc-10717, Santa Cruz Biotechnology), diluted in blocking solution for 1h (400rpm). Wells were washed 4x with 150µl PBS-T (0.05%) and incubated with 25µl of 1µg/ml goat anti-rabbit antibody with Sulfo-tag (#R32AB-5, Meso Scale Discovery) in blocking solution for 1h in the dark (400rpm). Plates were washed 4x with 150µl PBS-T (0.05%) and read on a Sector Imager 2400 using 150µl

2X Read buffer (#R92TC-1, Meso Scale Discovery, diluted from 4X with deionized water) per well. All samples were run in duplicate.

CSF samples

The CSF samples were leftover aliquots from clinical routine, following a procedure approved by the Ethics Committee of University of Gothenburg. All samples were also de-identified in accordance with the Swedish biobanking law. All CSF samples were centrifuged at 2,000 x g for 10 minutes before frozen in polypropylene tubes at -80°C. Aliquots of 500 µl were stored in 1.7 ml and aliquots of 20 µl for precision tests were stored in 0.5 ml tubes. Samples were subjected to one freeze-thaw event for aliquotation purposes prior to analysis

Validation of detection method

The chosen design of the assay validation experiments was based on recently published guidelines and methods. [14,16] Both pools of CSF and CSF from single donors were used in the validation experiments. CSF numbering is consistent throughout the validation experiments.

Lower Limit of Quantification (LLOQ): 16 replicates of the blank, the calibrator diluent (0.05% PBS-T), were analyzed and the LLOQ was determined as the concentration corresponding to the signal of the mean of these 16 replicates plus 10 times the SD.

Specificity for a-Synuclein: The detection antibody FL-140 recognizes α-, β-, and γ-Synuclein. Therefore, serial dilutions of recombinant β- and γ-Synuclein (#SNB2001, #SNG2001, ATGen) in PBS-T (0.05%) ranging from 0.0156 to 40 ng/ml were tested to prove specificity to α-Synuclein.

Parallelism: Four CSF samples were diluted 1:2 in PBS-Tween 20 (0.1%, final concentration in diluted sample: 0.05% Tween 20) and then further diluted in 1:2 steps in PBS-T (0.05%) until a final dilution of 1:16. All dilutions and the undiluted CSF samples were analyzed in duplicates.

Dilution Linearity: Four CSF samples were spiked with calibrator to a final concentration approx. 100-times higher than the highest α-Synuclein concentration of the CSF used in the validation procedures and serial diluted 1:2 in PBS-T (0.05%). The first dilution was again made with PBS-T (0.1%). All dilutions and the undiluted, spiked CSF samples were analyzed in duplicates.

Spike-recovery: Five samples of 1:2 diluted CSF (PBS-T, final conc. 0.05% Tween 20) were spiked with calibrator to a final concentration of 10, 0.625, and 0.039 ng/ml in addition to the endogenous levels by adding 1 volume of calibrator to 15 volumes of CSF. Endogenous α-Synuclein concentration was determined by analyzing CSF spiked with calibrator diluent in the same manner. Endogenous α-Synuclein concentration was subtracted from concentration in samples with spike before calculation of recovery.

Precision: Six CSF samples were divided into aliquots in small volume polypropylene tubes and frozen at -80 °C. On five different occasions, two to ten aliquots were analyzed each, in total 25 for each CSF. Precision replicates had been frozen individually and were diluted independently on the assay day. For the precision experiments different lots of Sulfotag-antibody and assay plates were used.

<u>CSF measurements</u>

Hemoglobin measurement: Samples were analyzed for hemoglobin content using a commercial kit (#E88-135, Bethyl Laboratories). Samples with hemoglobin levels higher than 300 ng/ml were excluded from analysis. Samples with available erythrocyte count (collected in Sweden) were not analyzed for hemoglobin. All of these samples had a count of below 100 erythrocytes per μL.

α-Synuclein measurement with the here described assay: 50 CSF samples were analyzed using the protocol described above. Samples were diluted by adding 1 volume of sample to 1 volume PBS-T (0.1%) in polypropylene predilution plates (#P-96-450R-C, Axygen),

centrifuged at 300 x g for 1 min, and mixed by shaking at 600 rpm for 5 mins. All samples were run in duplicate on the same day and reagents from the same lots were used.

Comparison with the α-Synuclein assay from BioLegend

An identical set of sample aliquots was analyzed with the commercial assay from BioLegend (#844101, BioLegend) at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Sweden, according to the manufacturer's instructions.

Antibodies

We tested different combinations of antibodies 211 (Santa Cruz Biotechnology, #sc-12767), FL-140 (Santa Cruz Biotechnology, #sc-10717), anti-α-Synuclein clone 42 (#610787, BD Transduction Laboratories), MJFR1 (#ab138501, Abcam), anti-α-Synuclein (#SAB1300799, Sigma Aldrich), and anti-α-Synuclein (#NBP1-67696, Novus Biologicals).

Data analysis

Standards and samples were analyzed in duplicates. Standard curve fitting (4 parameter logistic fit), plotting of calibrator curve and calculation of concentrations were performed using the Mesoscale Discovery Workbench software. All other calculations were performed in Microsoft Excel. Coefficients of variation (CV) < 20% for repetitive measurements and < 15% for intra-assay measurements were considered as acceptable. For dilution linearity and parallelism, absolute values were normalized to values of the assay-recommended 1:2 dilution for better comparability. Intra- and inter-assay CVs for precision samples were calculated according to ISO 5725-2 using the Excel sheet provided by U. Andreasson as supplementary file to [16].

Results

Validation of the assay for the detection of α-Synuclein in human CSF

To establish this assay, we first searched for antibody combinations that work well to detect recombinant α-Synuclein. The combination of antibodies anti-α-Synuclein clone 42 and FL-140 gave the best results. Thus we optimized this assay further and validated it for the following characteristics:

Lower limit of quantification

The novel assay detects α-Synuclein with a LLOQ of 0.0363 ng/ml in CSF. We did not determine the upper limit of quantification because α-Synuclein levels in CSF were generally below 3 ng/ml, which is far below the end of the linear range of the assay ($\leq$ 160 ng/ml, $r^2 >$ 0.99, n = 6, Fig. 1). Extending the calibrator to 640 ng/ml resulted in a plateauing of the signal above 160 ng/ml (not shown). The mean signal of 16 replicates of calibrator diluent was 164.3 $\pm$ 4.29 units (CV = 2.6%) and significantly below the lowest signal of CSF samples (317 units).

Specificity for α-Synuclein

β- and γ-Synuclein are structurally related to α-Synuclein, and the detection antibody (FL-140, St. Cruz Biotechnologies) recognizes both α-, β- and γ-Synuclein. However, this assay showed no cross-reactivity with recombinant human β- and γ-Synuclein (Fig. 1), which proves specificity to α-Synuclein.

Parallelism

Parallelism was within accepted criteria (CV < 15%) for all tested dilutions in all four CSF samples. α-Synuclein concentrations were normalized to the standard assay dilution of 1:2 (Fig. 2a).

Dilution linearity

After spiking with high calibrator of approximately 100 times of typical CSF α-Synuclein concentrations, CV values were well within accepted criteria (< 15%) for all tested dilutions in all four CSF samples. Values are normalized to the standard assay dilution of 1:2 (Fig. 2b).

Spike recovery

Four CSF samples were spiked with three different concentrations of calibrator ranging from below, within and above the range of typical CSF concentrations (0.0391, 0.625, and 10 ng/ml). After subtraction of the endogenous α-Synuclein concentrations, the recovery rate was between 80.4% and 81.7%. Only the CV value for the recovery of the lowest spike, which was 0.0391 ng/ml, was outside the accepted criteria (25.5%) (Table 2).

Precision

Intra-assay CVs (repeatability) for the six CSF tested were between 9.9% and 15.8%. The inter-assay CVs (intermediate precision) were between 14% and 21.2% (Table 1). Intra-assay CV improved with history of performed assays.

Comparison with commercial assay from BioLegend

Recently, the validation of a previously academic assay that now is commercially available (#844101, BioLegend) was published.[14,15] We therefore analyzed an identical set of 50 samples on both assays. Samples were randomly distributed over 5 assay plates. The CV values of the three implemented quality control samples were lower than 6% between plates. We found a strong correlation between the two assays as assessed by Spearman *rho* (r = 0.718, n = 50, *P* < 0.001, Fig. 3).

Discussion

Over the past years, quantification of α-Synuclein in CSF and its potential as biomarker in neurodegenerative diseases has gained a growing interest. To explore the suitability of biomarker candidates, validated and accessible detection techniques are essential. For α-Synuclein, several detection methods, both academic and commercial, are described, but to our knowledge, only the assay from BioLegend has been comprehensively validated.[14] We therefore developed and validated an electrochemiluminescence detection ELISA based entirely on freely available components with a simple and convenient protocol. Then we compared the assay to the commercially available, validated α-Synuclein ELISA from BioLegend using a set of 50 CSF samples.

The chosen design of validation experiments was based on recently published guidelines,[16] and the recent validation of the BioLegend assay.[14] We decided to include determination of LLOQ, specificity, parallelism, dilution linearity, spike recovery, and precision. A comprehensive set of pre-analytical confounders has been already tested by Kruse et al.,[14] who demonstrated that α-Synuclein CSF levels are not affected by delay of freezing, centrifugation regime, and a diverse range of storage conditions, concluding that α-Synuclein is a very stable protein.[14] These tests were therefore not repeated. Due to lack of certified reference material (CRM), Trueness, i.e. concordance between the reference value of the CRM and actual measurements, could not be addressed.

The LLOQ of 0.0363 ng/ml was significantly lower than the amounts of α-Synuclein observed in all tested samples and CSF pools used in validation experiments. Also, the antibody tandem showed no cross reactivity with β- or γ-Synuclein. This means that this assay is well suitable for the detection of α-Synuclein in CSF.

For α-Synuclein several isoforms exist. However, the capture antibody has been successfully used by us and others[17] to detect specifically α-Synuclein-140 from various human samples and tissues,[18] thus we conclude that this assay detects only the 140 aa form of α-Synuclein.

The assay passed the criteria for parallelism and dilution linearity. The CV value for the spike recovery for the lowest spike was 25.5%. This may be explained by the very low addition of exogenous α-Synuclein to the endogenous levels of α-Synuclein. Nevertheless, spike recovery was about 80-82% for all spikes and thus within the accepted criteria[16] of 80-120%. CV values of the moderate and high spike were below 10%. Precision inter- and intra-assay CV values were within accepted criteria with two exceptions where limits were slightly exceeded (cf. Table 1). We observed that precision improved with history of performed assays and therefore recommend training of staff with the assay-specific sample handling prior to analyze clinical samples in addition to the general recommendation of run control application. Accordingly, the plate-to-plate precision of the five plates used for the comparison with the BioLegend assay was much improved and below 6%.

The high correlation between our assay and the BioLegend assay may indicate that the two methods detect similar alterations in CSF levels of α-Synuclein. The observed correlation of > 0.7 (Spearman rho) is in the same magnitude as previously seen for two commercial assays to detect Aβ42 in human CSF samples.[19]

Given the excellent assay performance, we will employ this assay with clinical cohorts to further determine the possibility of using α-Synuclein as a diagnostic or prognostic biomarker. We also intend to adapt our assay for the detection of phosphorylated (predominant species in Lewy bodies [20]) and oligomeric forms (assumed to be the main toxic form [21]) of α-Synuclein as those forms, alone or combined with total α-Synuclein, may further enhance the diagnostic and prognostic performance of α-Synuclein measures in CSF.

Conclusion

We validated a novel assay, which reliably detects α-Synuclein in human CSF. The assay has several strengths including a convenient protocol and high sensitivity paired with need of only small amounts of CSF.

Table 1

Repeatability and Intermediate precision

Name	Mean conc. (ng/ml)	Repeatability (%CV$_r$)	Intermediate precision (%CV$_{Rw}$)
CSF 1	0.312	10.0	19.8
CSF 2	0.220	11.5	16.5
CSF 3	0.308	11.3	14.0
CSF 4	0.388	15.8	18.2
CSF 5	0.469	12.8	17.8
CSF 6	0.521	9.9	21.2
Mean	**-**	**11.9**	**17.9**

Table 2

Spike Recovery

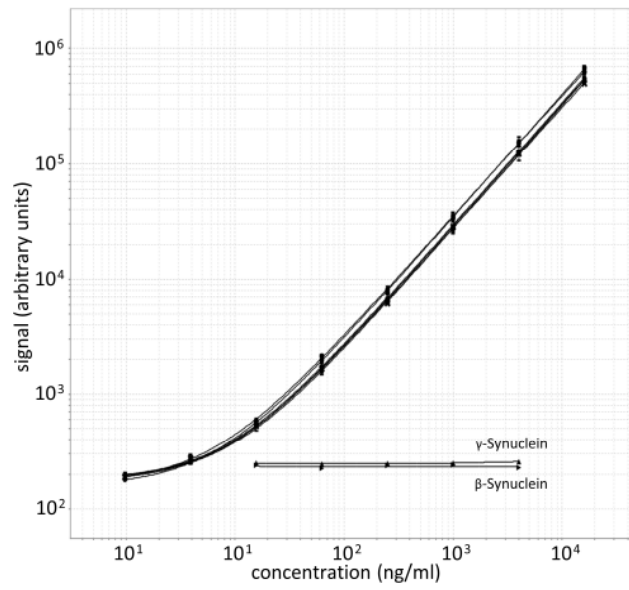|	Spike (ng/ml)		
	0.0391	**0.625**	**10**
Name	**Recovery (%)**		
CSF 1	108,1	77,6	79
CSF 2	79,4	93,2	82,6
CSF 3	94,9	74,9	81,4
CSF 5	59,4	80,7	86,2
CSF 6	63,4	75,5	79,4
Mean	**81,04**	**80,38**	**81,72**
SD	20,65	7,52	2,90
CV	25,49	9,35	3,55

Fig 1

Figure 1: Superimposition of six calibrator curves that were used in the validation experiments. Two representative curves for β- and γ-Synuclein, respectively, were included.
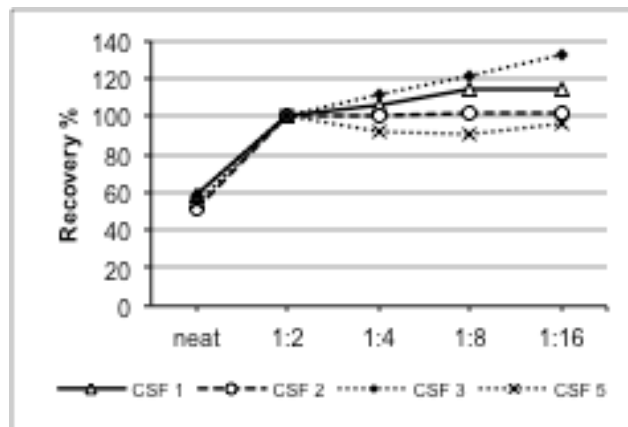
Fig. 2a
Parallelism

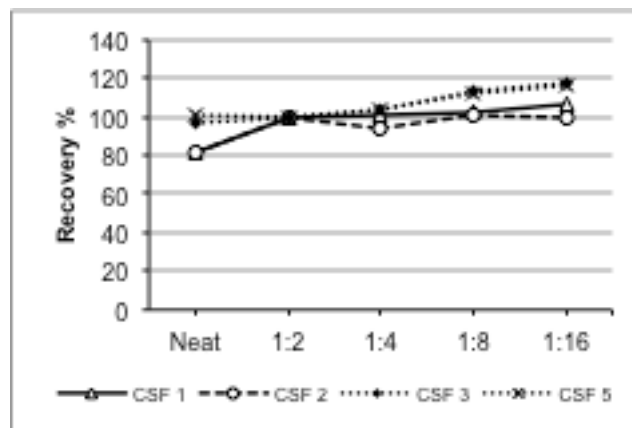
Fig. 2b
Dilution linearity

Figure 2. A): Parallelism of serially diluted CSF samples. B) Dilution linearity of serially diluted CSF samples after spiking with high concentration of α-Synuclein.
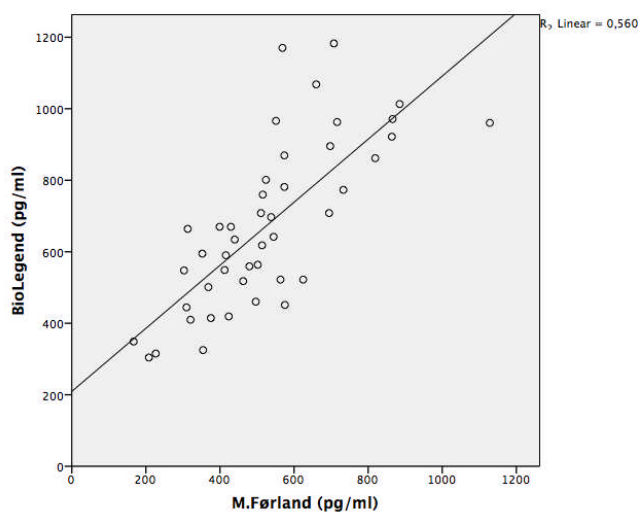
Fig. 3
Correlation of a-Synuclein measurements

Figure 3: Correlation of α-Synuclein levels determined by the presented and the BioLegend assay.

Acknowledgments

Author roles

Marthe Gurine Førland:	Conception of experiments, Execution of experimental work, Design and Execution of Statistical Analysis, Review and Critique of the Manuscript
Annika Öhrfelt	Organization of Research Project, Review and Critique of the Manuscript
Linn Silje Oftedal:	Execution of experimental work, Review and Critique of the Manuscript
Ole-Bjørn Tysnes:	Organization of Research Project, Review and Critique of the Manuscript
Jan Petter Larsen:	Organization of Research Project, Review and Critique of the Manuscript
Kaj Blennow:	Organization of Research Project, Review and Critique of the Manuscript
Henrik Zetterberg:	Organization of Research Project, Review and Critique of the Manuscript
Guido Alves:	Organization of Research Project, Review and Critique of Statistical Analysis and the Manuscript
Johannes Lange:	Conception of experiments, Execution of experimental work, Design and Execution of Statistical Analysis, wrote the first draft, Review and Critique of the Manuscript

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Marthe Gurine Førland received a personal grant from the Western Norway Regional Health Authority.

Annika Öhrfelt has nothing to report.... .

Linn Silje Oftedal has nothing to report.

Ole-Bjørn Tysnes has been invited speaker for GSK, Orion Pharma, Pfizer, UCB, and Lundbeck. He has participated in an advisory board for Lundbeck.

Kaj Blennow has served at Advisory Boards for IBL International, Roche Diagnostics, Eli Lilly and Alzheon, and is co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at University of Gothenburg.

Henrik Zetterberg is co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. Apart from this, he reports no disclosures.

Jan Petter Larsen has served as a member of an advisory board for Lundbeck and received a personal grant from the Michael J. Fox Foundation.

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Johannes Lange has received a personal grant from Stavanger University Hospital.

Figure captions

Figure 1: Superimposition of six calibrator curves that were used in the validation experiments. Two representative curves for β- and γ-Synuclein, respectively, were included.

Figure 2a: Parallelism of serially diluted CSF samples.

Figure 2b: Dilution linearity of serially diluted CSF samples after spiking with high concentration of α-Synuclein.

Figure 3: Correlation of α-Synuclein levels determined by the presented and the BioLegend assay.

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