ARTICLE

TECHNOLOGY

Aqueous two-phase systems enable multiplexing of homogeneous immunoassays

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Quantitative measurement of protein biomarkers is critical for biomarker validation and early disease detection. Current multiplex immunoassays are time consuming costly and can suffer from low accuracy. For example, multiplex ELISAs require multiple, tedious, washing and blocking steps. Moreover, they suffer from nonspecific antibody cross-reactions, leading to high background and false-positive signals. Here, we show that co-localizing antibody-bead pairs in an aqueous two-phase system (ATPS) enables multiplexing of sensitive, no-wash, homogeneous assays, while preventing nonspecific antibody cross-reactions. Our cross-reaction-free, multiplex assay can simultaneously detect picomolar concentrations of four protein biomarkers ((C-X-C motif) ligand 10 (CXCL10), CXCL9, interleukin (IL)-8 and IL-6) in cell supernatants using a single assay well. The potential clinical utility of the assay is demonstrated by detecting diagnostic biomarkers (CXCL10 and CXCL9) in plasma from 88 patients at the onset of the clinical symptoms of chronic graft-versus-host disease (GVHD).

INNOVATION

Sensitive and robust no-wash homogeneous immunoassays, that are typically available only as singleplex assays, are multiplexed using aqueous two-phase systems (ATPS). ATPS microdroplets co-localize complementary antibody reagent pairs to prevent non-complementary antibody reactions. The solution phase-separation not only enables patterning of microarrays of homogeneous immunoassays, but also prevents unwanted antibody cross-reactions, enabling more accurate multiplexed analysis of diagnostic protein biomarkers.

INTRODUCTION

Protein analysis is clinically important for patient stratification[1], early disease detection[2] and signal transduction research[3]. The ideal protein assay should be quantitative with high sensitivity and specificity, rapid, inexpensive in terms of reagents and sample consumption and compatible with standard laboratory equipment. Although immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) and the amplified luminescent proximity homogeneous assay (AlphaLISA^{TM}), have been used for single protein detection, there is a drive to develop robust methods for multiplexing these immunoassays[4,5]. Multiplex immunoassays are beneficial because they enable the simultaneous detection of multiple biomarkers from minute biological samples, reducing hands-on time and cost, while providing valuable information about complex biological pathways[6].

Multiplexing ELISAs is difficult because cross-reactions among mismatched pairs of antibodies and target proteins can produce false-positive readouts, resulting in the improper diagnosis and treatment of patients. This issue becomes more problematic as the number of antibody pairs in the assay increases, as the likelihood of nonspecific interactions will increase exponentially[7]. To mitigate risks of cross-reactions, tedious systematic evaluations of nonspecific interactions between each antibody and each measured protein must be performed to validate these assays[7–10]. Alternatively, matched capture and detection antibody pairs can also be co-localized[11,12]. Multiplexing AlphaLISA^{TM} is even more challenging because single-color signals, generated by homogeneously distributed antibody-bead reagents, cannot be spatially localized within one well or spectrally resolved using conventional methods. Thus, even though AlphaLISA^{TM} eliminates wash steps, reduces the overall assay incubation time and increases dynamic range, its use has been limited by an inability to perform multiplexed analysis[5].

Here we enable multiplexing of homogeneous immunoassays, such as AlphaLISA^{TM}. Our approach makes use of micropatterned aqueous two-phase systems (ATPSs), which form between the phase-separating polymers, polyethylene glycol (PEG) and dextran (DEX)[13–15]. We exploit the ability of ATPS to effectively partition antibody/bead reagents stably in the DEX phase, thereby preventing cross-reactions between mismatched antibody reagents, while allowing discrete readouts from multiple luminescent signals patterned within one well (**Fig. 1**). In our multiplex AlphaLISA^{TM}, minute samples of either human plasma or cell supernatants are mixed into the PEG phase. The target proteins then simultaneously diffuse from the PEG phase into the DEX microdroplets, where they interact with partitioned antibody reagents, which are retained in the DEX phase. Since the complementary pairs of AlphaLISA^{TM} bead/antibody reagents are co-localized and retained within the DEX microdroplets, nonspecific interactions between mismatched antibody pairs do not occur. Based on this localization strategy, the target proteins become sandwiched between the antibody-bead pairs. A multiplexed readout is obtained when

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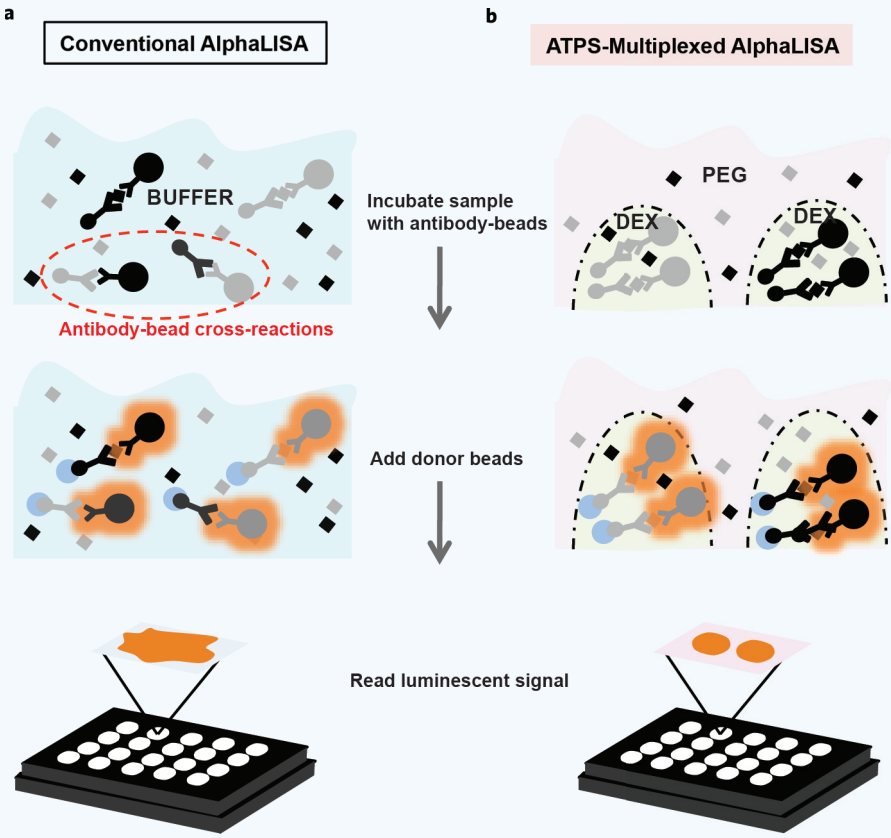

Figure 1 Schematic representation of ATPS-enabled multiplexed AlphaLISA. (**a**) Conventional AlphaLISA assays can perform no-wash singleplex antigen detection with wide dynamic ranges but cannot be multiplexed because antibody-bead complexes freely diffuse and circulate in the assay wells, making it impossible to resolve single-color luminescent signals from multiple biomarkers. Also the antibody-bead complexes may cross-react (red circle), resulting in false positive detection. (**b**) In ATPS-multiplexed AlphaLISA, antibody-bead complexes are spatially confined in DEX droplets and biomarker specific readouts are spatially resolved, while eliminating antibody cross-reactions (PEG, polyethylene glycol; DEX, dextran).

photosensitizing donor beads are mixed with a second DEX mixture, added to the previously patterned DEX droplets and excited at 680 nm to elicit amplified luminescent signals at 615 nm from the acceptor beads. The discrete, single-color, luminescent signals obtained from each DEX droplet can be read using commercially available microplate readers, allowing our homogeneous ATPS-multiplexed assays to be easily adopted by life scientists in the biomedical and biotechnology disciplines.

RESULTS

Identification of the optimum assay conditions for the no-wash, multiplex assay

Unlike conventional multiplex assays, where a cocktail of detection antibodies are applied as a mixture to all of the capture antibody spots (**Fig. 1a**), our method localizes detection antibody reagents only to DEX droplets containing complementary capture antibody reagents (**Fig. 1b**). This reagent co-localization makes it possible to create spatially addressable homogeneous immunoassay microarrays where amplified luminescent signals from different antigens are spatially resolved. This strategy also prevents unwanted cross-reactions between mismatched antibody pairs.

To enable high throughput, homogeneous, multiplex protein detection from biological samples, our assays must be compatible with standard lab equipment such as liquid handlers and plate readers. Therefore, we designed custom 96-well microplates (**Fig. 2a**). Each of the 96 wells contained four micro-basins for spatial patterning of 2 µl DEX assay droplets. For compatibility with standard 384-well plate readers, the center-to-center spacing of the DEX micro-basins was 4.5 mm (**Supplementary Fig. 1**). These microbasins were also necessary to prevent neighboring DEX droplets from moving because each DEX droplet is dispensed into a unique microbasin.

In the ATPS-multiplexed homogeneous assays, ATPSs composed of 18 wt% PEG (Mw. 35 kDa) and 18 wt% DEX (Mw. 10 kDa) were used because these polymer concentrations favored antigen and antibody-bead reagent partitioning to the DEX phase. This ensured maximum antigen transport into the DEX droplets, while maintaining high retention of the patterned antibody-bead reagents in the DEX phase.

We applied our ATPS-multiplexed AlphaLISA approach for the detection of four protein biomarkers (chemokine (C-X-C motif) ligand 10 (CXCL10), CXCL9, interleukin (IL)-8 and IL-6). These cytokines and chemokines are proinflammatory biomarkers, which are typically elevated in inflammatory diseases such as pulmonary diseases and chronic graft-versus-host disease (GVHD). The distribution of biomolecules, such as cytokines, chemokines and antibodies, between the two immiscible polymer phases of the ATPS is described by the partition coefficient (K_{part}), which is defined as the reagent concentration in the PEG phase divided by the reagent concentration in the DEX phase. For example, a K_{part} of 1.0 means that reagents partition equally between both phases. Factors governing protein partitioning include the size and hydrophobicity of both the polymers and the biomolecules[15]. **Supplementary Table 1** displays the calculated partition coefficients of the detection antibodies, beads and target protein biomarkers for different molecular weight dextrans (DEXT10 and DEXT500). Regardless of antibody type (monoclonal or polyclonal) or antibody species (goat, mouse or rat), the antibodies partitioned more preferably to DEXT10 than DEXT500 in the PEG/DEX two-phase system. Streptavidin-coated donor and antibody-conjugated acceptor beads partitioned preferably to DEX because of their dextran coatings. Stable partitioning of antibody-bead reagents in DEX made it possible to localize one type of antibody in one DEX-rich phase droplet. Low molecular weight chemokines and cytokines, as well as antibodies and 250 nm dextran-coated assay beads, partitioned more preferably to the smaller molecular weight DEX. Thus, we chose 18 wt% DEXT10 to ensure maximum antigen transport into and retention of reagents within the DEX droplets over the course of the assay.

Of our target antigens, IL-6, had the highest molecular weight. By definition of the diffusion coefficient, IL-6 should diffuse the slowest of any of the antigens from PEG into DEX. Therefore, we optimized our assay incubation time based on data from singleplex ATPS experiments using IL-6. We chose an incubation time of 2 h based on DEX droplet stability, measurement of the 10.3 pg/mL limit of detection (LoD) of IL-6 and the steep gradient of the standard curve (**Supplementary Fig. 2**).

Next, we assessed the effect of ATPS on the amplified luminescent signals generated when the target protein biomarkers become sandwiched

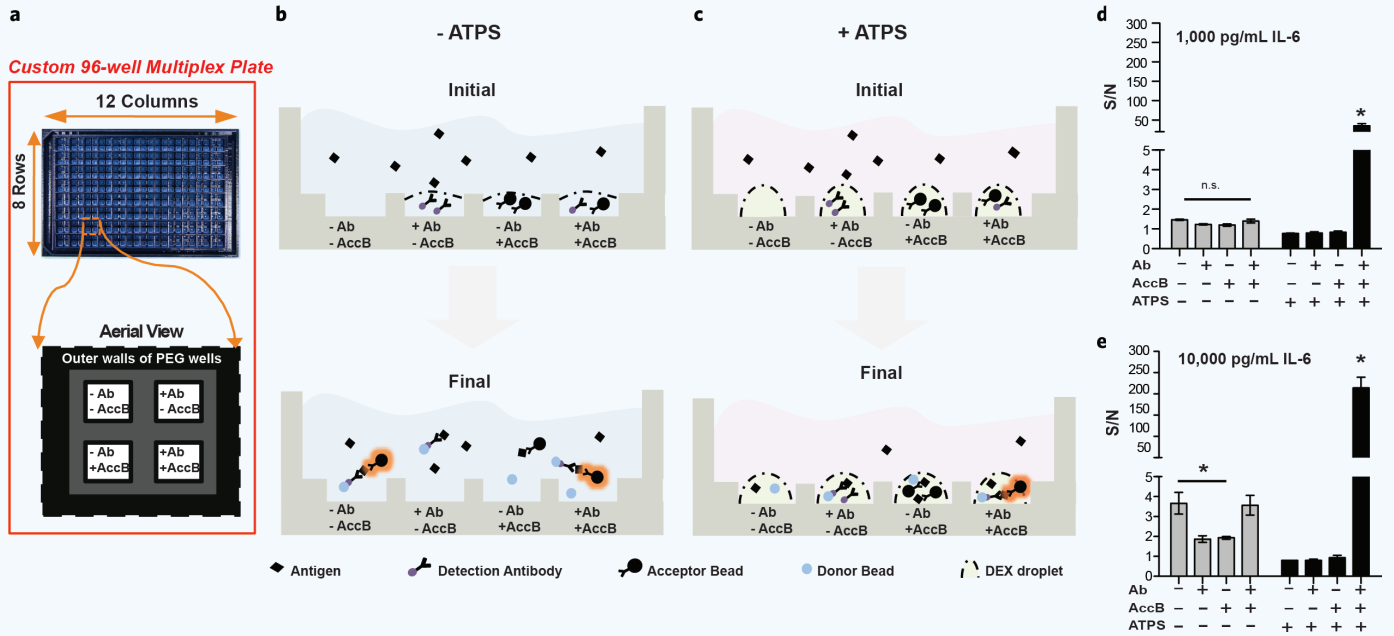

Figure 2 ATPS-multiplexed AlphaLISAs confine antibody-bead reagents and prevent reagent cross-reactions. (**a**) Custom 96-well microplates for multiplex ATPS-AlphaLISAs. (**b**) In the absence of ATPS, detection antibodies (Ab), acceptor beads (AccB), and donor beads freely circulate in assay buffer. (**c**) In the presence of ATPS, Ab, AccB, and donor beads remain localized in DEX droplets while antigens diffuse from the bulk PEG phase into DEX droplets. (**d**) In the absence of ATPS, droplets containing Ab only, AccB only or no reagent generated luminescent signals similar to the signal detected when both Ab and AccB were dispensed into the same microbasin (grey bars) (n.s. by one-way ANOVA and Tukey's post-test). However, spotting Ab, AccB, and donor beads in DEX, confined the reagents at the correct working concentrations in the micro-basins, producing the expected level of luminescent signal for 1000 pg/mL IL-6, without any enhancement of signal for the Ab only, AccB only or the Empty cases (black bars) (*$P < 0.05$ by one-way ANOVA and Tukey's post-test). (**e**) In the presence of ATPS, background levels were low, no cross-reactions were observed, and maximum luminescent signal was detected only when both Ab & AccB were dispensed into the same microbasin (black bars). But in the absence of ATPS, background levels were high and significant cross-reactions occurred (grey bars) (*$P < 0.05$ by one-way ANOVA and Tukey's post-test). AlphaLISA signal was normalized to control experiments where the buffer or DEX respectively did not contain spiked IL-6. Error bars represent mean values ± SEM, over 3 replicates.

between pairs of proximity antibody-beads. In **Supplementary Fig. 3**, we show that standard curves for CXCL10, CXCL9, IL-8 and IL-6 are similar in terms of dynamic range (DR), LoD and limit of quantification (LoQ) for 2 µL ATPS-singleplex AlphaLISA™ and 20 µL conventional singleplex AlphaLISA™. Moreover, the signal-to-noise ratios were comparable between assay formats for all tested biomarkers, even though the ATPS format consumed ten times less antibody and bead reagents. The LoD, LoQ and DR data, along with the low statistical variation between the measured signals, indicated that the ATPS did not interfere with the generation of the amplified luminescent signals.

Confinement of matched antibody-bead pairs in polymer microdroplets prevents antibody cross-reactions in multiplex assays

ATPSs were required to localize antibody-bead reagents and generate reliable multiplex signals, as demonstrated in **Fig. 2**. In the absence of an ATPS, locally dispensed reagents were subject to diffusive dispersion and convective circulation, leading to a loss of observable signal for 1,000 pg/mL of IL-6. However, when ATPS reagent localization was used, the DEX droplets containing both the acceptor beads (AccBs) and the biotinylated detection antibodies bound to streptavidin-coated donor beads (Abs) generated strong signals. On the other hand, the DEX droplets containing only Abs, only AccBs or no reagents at all did not produce signals. When the concentration of IL-6 was increased to 10,000 pg/mL, weak background signals were detected everywhere due to reagent cross-reactions in the absence of an ATPS. In contrast, the ATPS assay generated strong signals only in droplets with all of the required reagents.

We generated multiplex standard curves of the four target protein biomarkers in our ATPS-multiplexed homogeneous assays (**Fig. 3a**). The data were fit using a 4-parameter logistic nonlinear regression model, as is typical for sandwich immunoassays[16]. We observed inter-assay percent coefficient of variation (%CV) below 20% for all of the tested biomarkers, indicating the high precision of our multiplex assay (**Fig. 3b**). In fact, having %CVs less than 25% is an important consideration of immunoassays[17]. In some conventional multiplex ELISAs, the assay sensitivity decreases by as much as 2- to 5-fold compared to singleplex assays because of antibody cross-reactions and high background signals[18]. However, the sensitivity values for our multiplex platform were similar to the sensitivity values obtained for singleplex ATPS assays in side-by-side experiments in custom microplates. The singleplex ATPS assays had detection limits of 74 pg/mL for CXCL10, 41 pg/mL for CXCL9, 13.3 pg/mL for IL-8 and 8 pg/mL for IL-6. Similarly, the multiplex ATPS assays had detection limits of 91.4 pg/mL for CXCL10, 68.4 pg/mL for CXCL9, 14.6 pg/mL for IL-8 and 10.3 pg/mL for IL-6. Differences in the sensitivity values can be attributed to antigen and antibody partitioning, as well as diffusion of antigens across the PEG-DEX interface. We suspect that differences in sensitivity between singleplex assays in this experiment (**Fig. 3b**) and singleplex assays in commercially available 384-microwell plates (**Supplementary Fig. 3**) can be explained by differences in plate material. First, both plates have similar dimensions; however, the commercially available plates are all-white polypropylene to maximize detection of luminescent signal, whereas our custom plates have white microbasins embedded in an opaque black matrix. The special design of the plates was critical for eliminating microbasin-to-microbasin optical

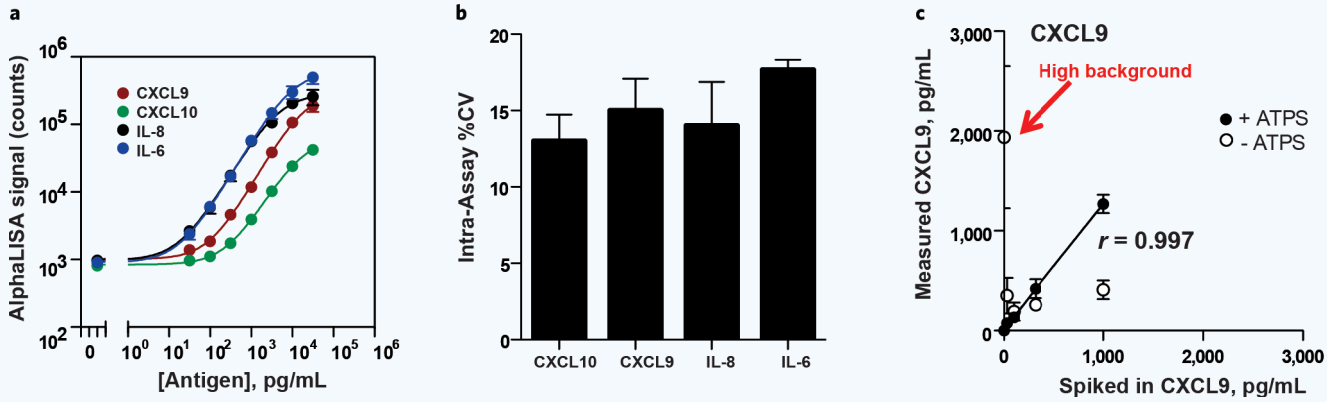

Figure 3 Multiplexed cytokine and chemokine detection by ATPS-multiplexed AlphaLISA. (**a**) Standard curves generated by ATPS-multiplexed AlphaLISA. (**b**) Coefficient of variation of ATPS-multiplexed AlphaLISA. (**c**) Comparison between CXCL9 measurement by ATPS-multiplexed AlphaLISA in the presence and absence of ATPS.

crosstalk, which was observed in custom all-white microplates. Second, since the same two-phase formulations were used in the singleplex and multiplex ATPS assays, the partitioning of the antigens, and by extension the detection limits, must be the same for both assay formats. Third, when ATPS-singleplex and ATPS-multiplex assays are performed side-by-side in our custom black-white plates, the detection limits between the two assays are similar (data not shown). We postulate that further optimization of our custom black and white microplates can achieve improved sensitivities.

We further illustrate the critical importance of using an ATPS to confine assay reagents, and thereby prevent antibody cross-reactions in **Fig. 3c**. We analyzed assay buffer samples spiked with varying concentrations of CXCL9, CXCL10, IL-8 and IL-6 for CXCL9 content by multiplex AlphaLISA in the absence or presence of an ATPS. As expected, in the absence of an ATPS, the measured CXCL9 did not correlate with the spiked-in CXCL9. Specifically, assay wells that contained 0 pg/mL CXCL9 also contained 0 pg/mL IL-6, 10,000 pg/mL CXCL10 and 10,000 pg/mL IL-8. High background levels were recorded due to reagent cross-reactions in the assay buffer. We postulate that what the reader detected as high CXCL9 background may actually be from luminescent IL-8 and CXCL10 signals, since the beads for all of the antigens were freely circulating in the system. In contrast, when ATPS reagent localization was used, the measured CXCL9 strongly correlated with the spiked-in CXCL9, as indicated by the Pearson correlation coefficient value (0.997).

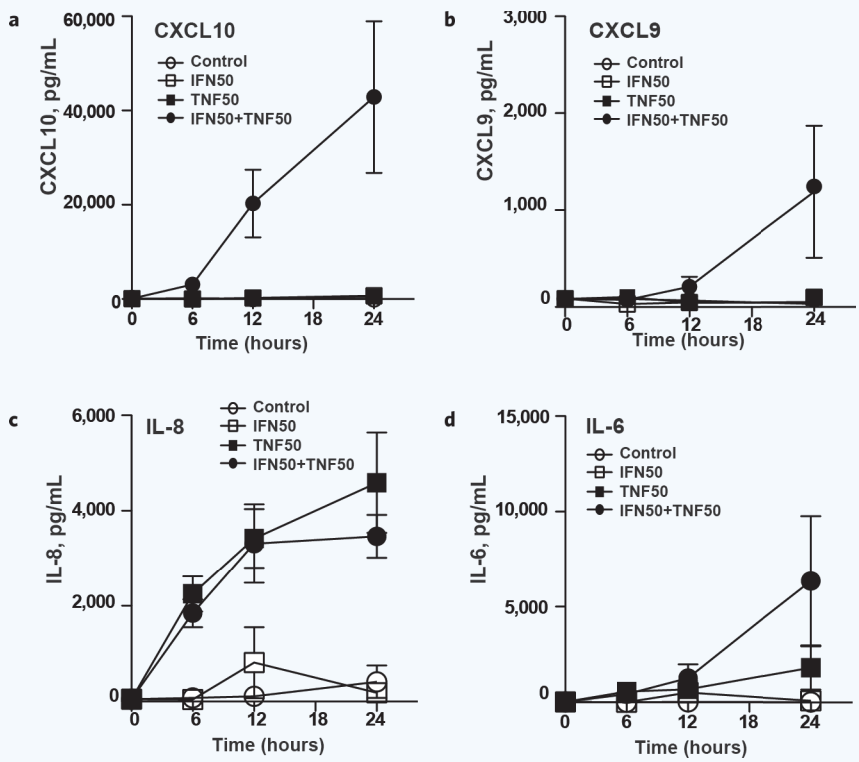

Figure 4 Dose and time-dependent effects of TNF-α and IFN-γ stimulated A549 cells on the release of proinflammatory cytokines: CXCL10, CXCL9, IL-8 and IL-6. Cells were stimulated at the indicated times with 50 ng/mL TNF-α (TNF50) alone, 50 ng/mL IFN-γ (IFN50) alone, or 50 ng/mL IFN-γ and 50 ng/mL TNF-α (IFN50 + TNF50). Protein levels measured via ATPS-multiplexed AlphaLISA. Error bars represent means ± SEM.

ATPS-AlphaLISA enables multiplexed protein detection in cell culture supernatants

One application of our multiplex ATPS assay is monitoring cytokine cascades in cytokine-stimulated cells. Tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) play important roles in pulmonary disease[19,20]. We validated our assay for cell culture supernatants by measuring 4 proinflammatory cytokines (CXCL10, CXCL9, IL-6 and IL-8) in supernatants from A549 human pulmonary type II alveolar epithelial cells stimulated with TNF-α and IFN-γ alone or in combination (**Fig. 4**). As expected, stimulation with TNF-α or IFN-γ alone did not

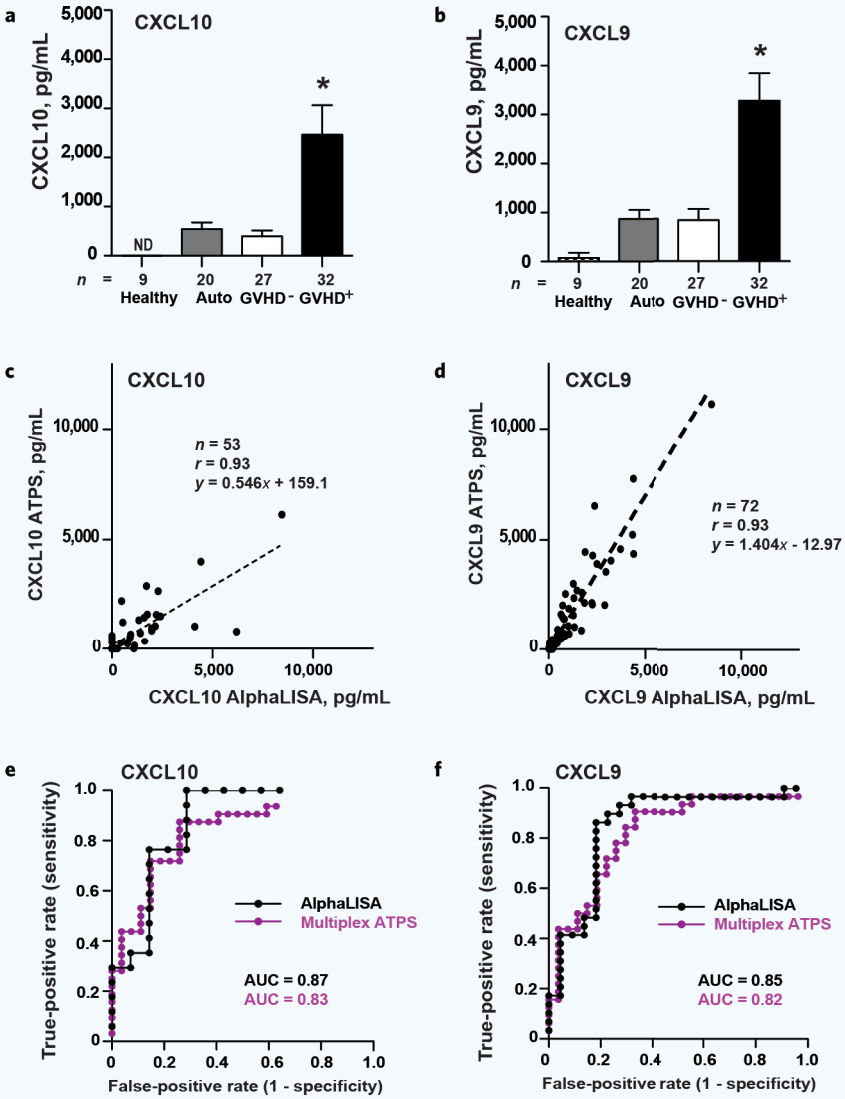

Figure 5 Multiplex protein measurements in plasma of patients who had undergone bone marrow transplantation. (**a** and **b**) Mean values of CXCL10 and CXCL9 concentrations from 88 healthy, autologous, no GVHD and chronic GVHD patients measured by ATPS-Multiplexed AlphaLISA assays. Samples were measured in triplicate on one plate and in 3 independent experiments. Both CXCL10 and CXCL9 were significantly elevated in GVHD+ patients compared to all other patient groups (*$P < 0.05$ by one-way ANOVA and Tukey's post test). (**c** and **d**) Correlation of measured values between the multiplex ATPS assay and conventional AlphaLISA for plasma samples. Correlation of protein levels in clinical patient plasma for CXCL9 and CXCL10 are reported using the Pearson correlation coefficient. (**e** and **f**) Receiver operating characteristic (ROC) curves comparing CXCL10 and CXCL9 concentrations in patients without GVHD and patients with GVHD. ROC curves for ATPS-Multiplexed AlphaLISAs are compared to ROC curves obtained from singleplex AlphaLISA. All error bars represent means ± SEM. ND = Not Detectable.

result in a pronounced release of CXCL10 or CXCL9[21,22]. In agreement with other studies, our ATPS-multiplexed assay showed that IFN-γ alone was not sufficient to mediate IL-8 or IL-6 release in A549 cells[23], but that TNF-α alone or in combination with IFN-γ could mediate both IL-8 and IL-6 release[24,25]. In contrast, stimulation with combinations of TNF-α and IFN-γ caused dramatic increases in CXCL10, CXCL9, IL-8 and IL-6, although the level for IL-8 was similar to TNF-α stimulation alone. ATPS-multiplexed assays correlated strongly with singleplex AlphaLISA™ ($n > 33$), as indicated by Pearson's coefficients greater than 0.7 (data not shown). IL-8 had the weakest correlation (0.7) between the ATPS-multiplexed assay and the conventional singleplex AlphaLISA™, likely because as compared to the other biomarkers, IL-8 had the highest partition coefficient, indicating that it partitioned poorly to DEX.

ATPS-multiplexed AlphaLISA™ enables sensitive, simultaneous detection of GVHD biomarkers in human plasma

We further validated our assay for use with clinical samples by measuring two biomarkers in plasma from patients who received autologous and allogeneic bone marrow transplantation. Patients who receive allogeneic bone marrow transplantation may become afflicted with chronic graft-versus-host disease (GVHD), a multi-organ disorder beginning 100 days after transplantation. In accordance with previous reports, both CXCL9 and CXCL10 were significantly elevated in GVHD+ patients compared to GVHD– patients ($P < 0.05$ by ANOVA), demonstrating the potential of these two cytokines as diagnostic GVHD biomarkers (**Fig. 5a,b**)[26–29]. Specifically, GVHD+ patients had mean CXCL9 values of 3226 pg/mL, while GVHD– patients had mean CXCL9 values of 843.3 pg/mL. Similarly, GVHD+ patients had mean CXCL10 of 2463.4 pg/mL, while GVHD– patients had mean CXCL10 values of 398 pg/mL. Moreover, ATPS-multiplex assay measurements correlated with singleplex AlphaLISA™, with Pearson correlation coefficient values of 0.93 for both CXCL10 and CXCL9. Again, the ATPS-multiplexed assay data correlated well with singleplex AlphaLISA™ (**Fig. 5c,d**). We also compared the individual area under the curve (AUC) values for CXCL10 and CXCL9 between assay platforms (**Fig. 5e,f**). For both CXCL9 and CXCL10, AUC values were greater than 0.8, demonstrating that CXCL10 and CXCL9 distinguish between patient groups in our multiplex assay.

DISCUSSION

The technological specifications for an ideal multiplex protein assay are extensive. Assays should be sensitive enough to detect antigens within the biological dynamic range, consume only small sample volumes, have similar precision to conventional singleplex assays, demonstrate high specificity due to lack of antibody cross-reactions, and require only short processing times[6]. Current multiplex assays, however, do not meet these requirements. Cross-reactions become especially problematic as the number of antibody pairs in a multiplex immunoassay increases, since the likelihood of nonspecific antibody interactions increases exponentially[7]. Therefore, tedious systematic evaluations of nonspecific interactions between each antibody must be performed to mitigate the risks of cross-reactions in conventional multiplex assays[7,8,18,30].

We eliminated many of these problems with our no-wash ATPS-multiplexed AlphaLISA™ technology. Confinement of the antibody-conjugated beads within the DEX droplets prevented the free circulation of beads and enabled, for the first time, multiplexing of homogeneous AlphaLISAs. ATPS-AlphaLISAs are inherently designed to prevent antibody cross-reactions. Antibody-beads stably partition to DEX; therefore,

they do not diffuse from one DEX droplet to another. Because DEX is more hydrophilic and has a lower molecular weight than PEG, small cytokines and chemokines in the PEG-sample pool preferentially partition to DEX, and thus are simultaneously delivered to all DEX droplets. Although non-target antigens can also diffuse into the DEX droplets, only target antigens that recognize matched pairs of high affinity, high specificity antibodies in DEX will elicit amplified luminescent signals. We achieved detection of CXCL10 and CXCL9 at concentrations of 10.3 pg/mL – 90 pg/mL in as little as 20 µl of 25% plasma with dynamic ranges (2.5 – 3.5 log units) for the ATPS assay that were comparable to singleplex AlphaLISA™ and greater than most existing multiplex ELISA assays[31].

Compared to other multiplex bead-based assays, such as Luminex™, ATPS-multiplexed AlphaLISAs do not require any wash steps or specialized equipment other than a plate reader to detect signals from the bead complexes[32]. In addition, the ATPS-multiplexed assays are scalable because there is no worry of antibody cross-reactions or increased background that typically accompanies multiplex immunoassays that use detection antibody cocktails. Although in the present study, we demonstrated a 4-plex assay, there is no fundamental limitation for higher multiplexing using our method. For example, 16 microbasins can be added within a single sample well, and luminescent signals can be detected via a 1536-well plate format. Furthermore, in contrast to conventional sandwich ELISA or Luminex™ technology, our assay can be completed in as little as 2 h using minute volumes of biological or clinical samples.

Upon measuring concentrations in 88 different GVHD patient plasma samples, the ATPS-multiplexed AlphaLISA™ and conventional AlphaLISA™ results strongly correlated. Notably, the CXCL9 results obtained in our multiplexed analysis were very similar to recent singleplex ELISA data for CXCL9[26]. In fact, both studies show an area under the receiving operating characteristic curve of above 0.8. Our results confirm that CXCL9 and CXCL10 are elevated in chronic GVHD patients. Chronic GVHD is the leading cause of mortality and morbidity in long-term transplant survivors[29]. Moreover, the use of ATPS patterning allowed us to reduce AlphaLISA™ reagent costs by 10-fold, from $2.40/well of a 384-well plate to $0.24/assay droplet.

It is possible that poor antigen partitioning can affect biomarker detection. However, in future versions of this multiplex assay, charged dextran derivatives can be added to the DEX-rich phase droplets to enhance preferential partitioning of antigen molecules into the DEX phase[15]. Furthermore, even if the same PEG phase is used, the antigen and antibody partitioning can change once different DEX formulations are used. It is also possible to reduce the 2-hour incubation time by (i) using charged formulations of dextran to enhance biomarker partitioning to the DEX phase, (ii) reducing the dextran volume to shorten mixing times and (iii) slightly increasing the temperature to 30–37 °C instead of room temperature (25 °C) to increase the diffusion rate. Finally, while this paper focused on multiplexing of AlphaLISA reagents, the concept should be applicable to multiplexing of a variety of homogeneous immunoassays. In summary, the ATPS-multiplexed assay provides unprecedented yet practical capabilities for high throughput protein detection in research settings and potentially in clinics by enabling scalable multiplexing of previously non-multiplexable homogeneous immunoassays.

MATERIALS AND METHODS

Reagents

Antioxidant-free polyethylene glycol (PEG) (Mw. 35,000) was purchased from Fluka. Dextran (DEX) (Mw. 10,000 and 500,000) was purchased from Pharmacosmos. Rhodamine-Dextran (Mw. 10,000) was purchased from Sigma and AlphaScreen Omnibeads was purchased from Perkin Elmer.

AlphaLISA protocol

Human CXCL10 (AL259C), human CXCL9 (AL280C), human IL-8 (AL224C) and human IL-6 (AL223C) AlphaLISA immunoassay kits were purchased from Perkin Elmer and performed according to the manufacturer's protocol. For cell culture supernatant samples, the assay buffer contained 25 µM HEPES, 50 mM NaCl, 10 mM sodium EDTA, 2 mg/mL dextran (Mw. 500,000), 0.5% casein and 0.05% Tween-20, adjusted to pH 7.4. For plasma samples, the assay buffer also contained 0.1% bovine gamma globulin (G-5009, Sigma Aldrich) and 0.2 µg/mL HBR1 (3KC533, Scantibodies). Standards, biotinylated detection antibodies, and acceptor bead solutions were prepared in assay buffer. The assays were performed in 384-well white polypropylene conical-bottom microplates (4307, Thermo Scientific). Two microliters of standards, cell culture supernatant or plasma sample were incubated with 10 µg/mL acceptor beads and 1 nM biotinylated detection antibody for 1 h at room temperature. Streptavidin-coated donor beads (40 µg/mL) were added to the mixture and incubated for an additional 30 min. The plate was read on a PHERAstar FS plate reader (BMG LabTech) where the bead complex was excited at 680 nm for 1 s and emission signals were collected at 615 nm for 1 s after a 40-millisecond delay.

Multiplex ATPS homogeneous immunoassay protocol

An 18 wt% PEG solution was prepared in 25 mM HEPES buffer containing 0.1 wt% casein. Casein is a blocking agent and prevents nonspecific binding of antibody-bead reagents to the microplate. An 18 wt% DEX was prepared in assay buffer specific to either cell culture supernatants or plasma samples. PEG was mixed with standards or sample solutions in a 4:1 volume ratio and the mixture was vortexed. For singleplex ATPS assays, 10 µL of the PEG-antigen mixture was dispensed into 384-well microplates such that each well contained approximately 2 µL of standard or sample and 8 µl of PEG. Acceptor beads (25 µg/mL) and biotinylated detection antibodies (2.5 nM) were prepared in 18 wt% DEX. For multiplex ATPS assays, 100 µL of the PEG-antigen mixture were dispensed into each PEG well. DEX solutions, containing acceptor beads and detection antibodies against 4 different antigens (CXCL10, CXCL9, IL-8 and IL-6) were dispensed into each of the four DEX micro-basins located in the common PEG well. Using a multipipettor, 1 µL of the DEX/antibody mixture was dispensed in each DEX micro-basin and the mixture was incubated at room temperature for 1 h. To prevent evaporation, the plates were sealed with sealing film. An 80 µg/mL solution of donor beads were prepared in 18% DEX. At the end of the 1 h incubation, 1 µL of the DEX/donor bead solution was dispensed into each micro-basin and incubated for an additional 60 min. Since the antibody reagents remained confined in the DEX droplets, the final concentrations of the reagents were 12.5 µg/mL acceptor beads, 1.25 nM detection antibody and 40 µg/mL donor beads. The assays were read on a PheraSTAR FS Plus plate reader after bead excitation for 1 s.

For the experiment illustrated in **Fig. 3c**, the assay buffer was spiked with CXCL9, IL-6, CXCL10 and IL8. While CXCL9 and IL-6 linearly increased in concentration (0 pg/ml, 10 pg/ml, 31.6 pg/ml, 100 pg/ml, 316.7 pg/ml, 1000 pg/ml, 3167 pg/ml, 10,000 pg/ml), the concentrations of CXCL10 and IL8 linearly decreased (10,000 pg/ml, 3167 pg/ml, 1000 pg/ml, 316.7 pg/ml, 100 pg/ml, 31.6 pg/ml, 10 pg/ml, 0 pg/ml). As a result, one microwell contained 0 pg/ml for both CXCL9 and IL-6, but 10,000 pg/ml for both CXCL10 and IL8.

Plate fabrication

Multiplex ATPS assays were performed in custom plates fabricated from black (Objet FullCure 870 Vero Black, Proto 3000) and white (Objet FullCure 830 Vero White, Proto 3000) resins using an Objet printer. In this design, white individual DEX micro-basins (2.5 mm × 2.5 mm × 2.5 mm) were located within a solid black plate matrix (well size 8 mm × 8 mm and 11.5 mm in height). Generally, an all-white plate matrix has better reflectivity to maximize the emission signal and is therefore commonly used for luminescent-based detection. In contrast, an all-black

plate matrix absorbs light, minimizing background signals and optical crosstalk. Black plates are used primarily for fluorescent-based detection. In our custom plates, white micro-basins were designed to maximize amplified luminescent signal. Adjacent micro-basins were separated by the black plate matrix to minimize optical crosstalk. The micro-basins were arranged in a standard 384-well format (24 columns, 16 rows, 4.5 mm spacing) to comply with the custom plate specifications of the Society of Biomolecular Screening, and the plate was read using a narrow 2.0 mm aperture.

Cell culture

A549 human pulmonary type II alveolar epithelial cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% antibiotics within a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded in 24-well tissue culture plates (8×10^5 cells per well) 24 h before use. At the start of experiments, medium was replaced with complete RPMI supplemented with 50 ng/mL IFN-γ, 50 ng/mL TNF-α or 50 ng/mL IFN-γ/50 ng/mL TNF-α. Cell culture supernatants were collected at 0 h, 6 h, 12 h and 24 h. Recombinant human IFN-γ was purchased from Peprotech and recombinant human TNF-α was purchased from R&D systems.

Plasma samples

Heparinized plasma samples from 88 patients were acquired from a clinical study at the University of Michigan between 2000 and 2010. Of these 88 samples, 20 samples were collected from patients who received autologous bone marrow transplantation, 59 samples were collected from patients who received allogeneic bone marrow transplantation (GVHD– = 27, GVHD+ = 32) and 9 samples were from healthy patients. The institutional review board at University of Michigan approved plasma samples collection. For AlphaLISA and multiplex ATPS assays, plasma samples were diluted 1:4 in the appropriate assay buffer, i.e., 5 µL plasma was diluted with 15 µL assay buffer.

Statistical analysis

Each experiment was repeated in triplicate, using 3 different plates. Results were expressed as mean values +/– standard error of mean. Graph Pad Prism was used to fit standard curves with a four parameter logistic function to calculate sample values. The limit of detection (LoD) was computed as LoB + 1.645 ($SD_{\text{low concentration sample}}$), where SD is the standard deviation and LoB is the limit of blank. LoB was calculated from LoB = $\text{mean}_{\text{blank}}$ + 1.645 (SD_{blank}). The limit of quantification (LoQ) was defined as the $\text{mean}_{\text{blank}}$ + 10SD. The dynamic range (DR) was calculated as the ratio of the maximum linear response versus the LoD[33]. CVs were calculated as the SD divided by the mean. Student's t-test (two-tailed) and one-way ANOVA with the Tukey's post-test were used to assess significance between sample groups. Pearson's correlation analysis was used to test the agreement between conventional AlphaLISA and ATPS-multiplexed AlphaLISA™.

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AUTHOR CONTRIBUTIONS

A.B.S. designed and conducted all experiments, analyzed the data, created the figures and wrote the manuscript. J.F. designed some experiments and assisted with writing the paper. N.H. assisted with experiments. K.K., S.P., and S.T. conceived the approach and supervised the study.

COMPETING INTERESTS STATEMENT

A.B.S. and S.T. are shareholders of a company (PHASIQ, Inc.) working on related technology.

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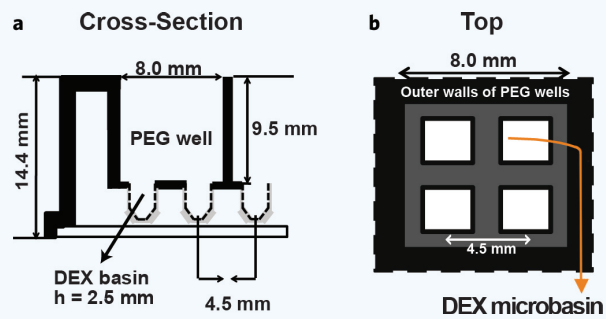
SUPPLEMENTARY INFORMATION

Protein and bead partitioning

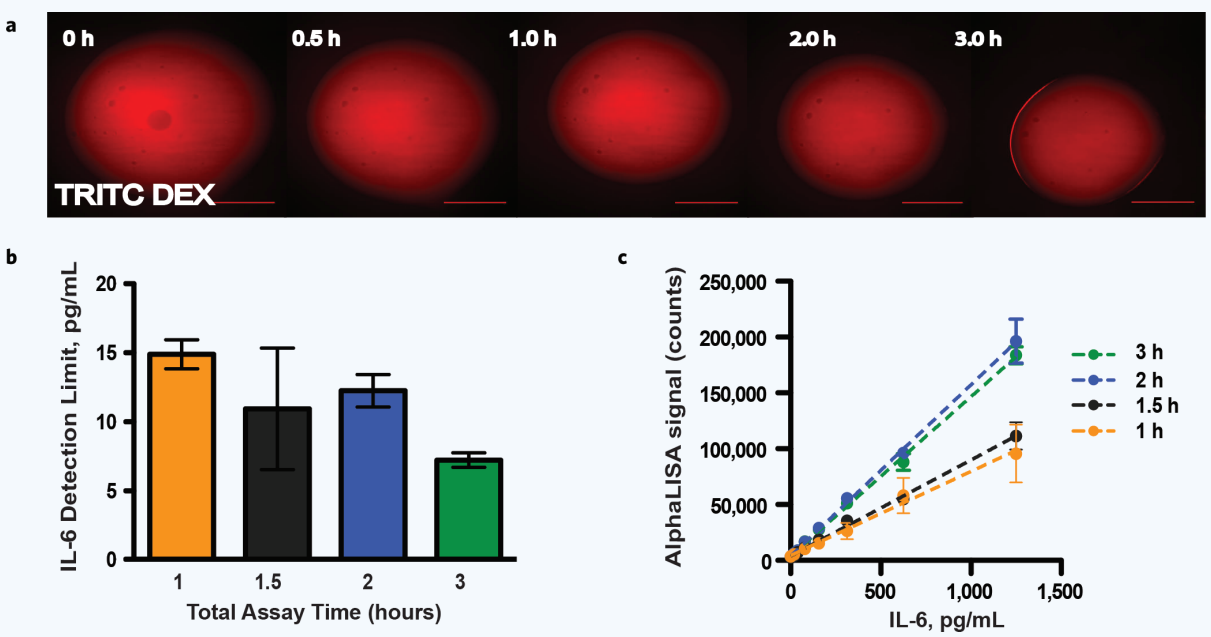
Protein partitioning was determined by AlphaLISA. Solutions containing 2500 pg/ml antigen were added to Eppendorf tubes containing 8.4 wt% PEG and 8.4 wt% DEX (total volume, 80 µl). Control tubes contained 5 µl PBS instead of the antigen. The contents of each Eppendorf tube were mixed by vortexing. The tubes were then centrifuged at 400 rcf at 4 °C for 15 min. After centrifugation, the PEG and DEX phases were separated. Two microliters of the PEG phase or the DEX phase were assayed as described above. The partition coefficient was defined as the protein concentration in the PEG phase divided by the protein concentration in the DEX phase. For partitioning of Omnibeads, 5 µl of the separated PEG and DEX phases was directly dispensed into 384-well plates. Since the beads contain all of the chemical components needed to generate luminescent signals, samples can be directly excited at 680 nm for 1 s.

Detection antibody partitioning

Partitioning of the biotinylated detection antibodies was determined by dot blot. Five microliters of 500 nM detection antibodies was added to Eppendorf tubes containing 8.4 wt% PEG and 8.4 wt% DEX (total volume of contents, 80 µl). Control tubes contained 5 µl PBS instead of the detection antibody sample. The contents of each Eppendorf tube were mixed by vortexing. The tubes were then centrifuged at 400 rcf at 4 °C for 15 min. After centrifugation, the PEG and DEX phases were separated. Polyvinylidene fluoride (PVDF) membranes (Bio-Rad) were soaked in methanol for 15 s, water for 2 min, and finally PBS for 5 min. The PEG and DEX phases (0.3 µl of each) were dispensed onto the PVDF membranes, and membranes were blocked in 1% BSA for 1 h and washed 4 times with PBS containing 0.05% Tween-20. The membranes were then incubated with streptavidin-conjugated horseradish peroxidase for 1 h, washed 4 times and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (37074, Thermo Scientific). Chemiluminescent signal was detected using a FluorChem M (Protein Simple) imaging system. The partition coefficient was defined as the reagent concentration in the PEG phase divided by the reagent concentration in the DEX phase.



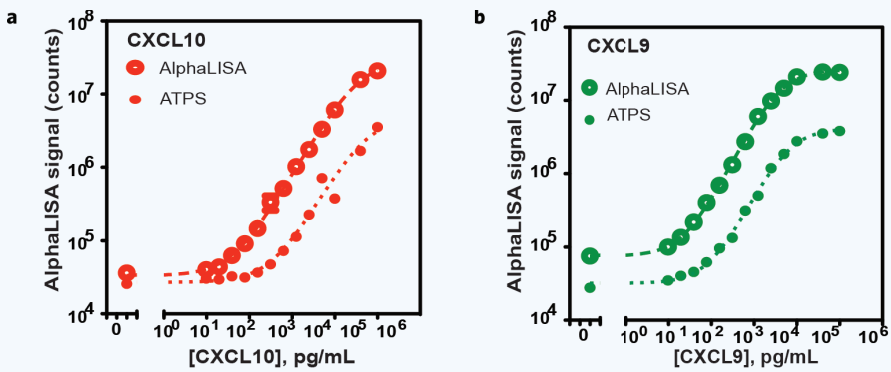
Supplementary Figure 1 Schematics of custom microplate. (**a**) A cross-sectional diagram of the custom plate showing micro-basins for DEX droplet stabilization. (**b**) A top-view the custom plate showing micro-basins for DEX droplet stabilization.



Supplementary Figure 2 Optimizing ATPS assay parameters. (**a**) Time lapse showing DEX droplet stability in the custom plates over the course of 3 h at room temperature. Scale bar, 500 mm. (**b**) Mean limit of detection values for singleplex IL-6 ATPS assays as a function of total assay time. The means were not significant upon analysis by one-way ANOVA and Tukey's post-test. (**c**) Standard curves for singlplex IL-6 ATPS assays indicate that 2-hour assay times have greatest AlphaLISA signal counts. Error bars represent means ± SEM.

Supplementary Table 1 Partition coefficients of antigens and antibody-bead reagents in ATPS. Antigens and antibody-bead reagents partition to DEX in our ATPS assays. The partition coefficient (K_{part}) was defined as the concentration of reagent in PEG divided by the concentration of reagent in DEXT10 or DEXT500.

	Detection antibody type	Detection antibody, K_{part}	Antigen, K_{part}	Bead, K_{part}
CXCL10	Goat polyclonal	0.23	0.09	—
CXCL9	Goat polyclonal	0.30	0.28 (DEXT10) 0.95 (DEXT500)	—
IL-8	Mouse monoclonal Protein A purified	0.07	0.64	—
IL-6	Rat monoclonal	0.18	0.28 (DEXT10) 1.33 (DEXT500)	—
Omnibeads		—	—	0.01



e Detection Limits and Dynamic Range (DR)

		CXCL10	CXCL9	IL-8	IL-6
Singleplex ATPS	LoD	15.0 pg/mL	27.9 pg/mL	5.4 pg/mL	7.0 pg/mL
	LoQ	201.4 pg/mL	76.4 pg/mL	27.4 pg/mL	7.0 pg/mL
	DR	3.3 logs	3.0 logs	3.7 logs	3.6 logs
AlphaLISA	LoD	11.8 pg/mL	20.1 pg/mL	20.6 pg/mL	6.2 pg/mL
	LoQ	11.8 pg/mL	26.5 pg/mL	199.8 pg/mL	19.9 pg/mL
	DR	3.4 logs	3.1 logs	3.2 logs	3.7 logs

Supplementary Figure 3 Comparison of limit of detection (LoD) and dynamic range (DR) data between singleplex ATPS (2 µl reagents) and conventional singleplex AlphaLISA assays (20 µl reagents) (**a–d**). (**e**) Quantification of LoDs and DRs. Error bars represent means ± SEM. The tenfold decrease in required antibody-beads accounts for the overall reduced AlphaLISA signal in the ATPS assays. Consequently ATPS standard curves are shifted downwards from the AlphaLISA curves. However, the signal-to-noise ratios are comparable between both assay formats for all tested antigens. Error bars represent means ± SEM.