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# High-Throughput Screening of Single-Chain Antibodies Using Multiplexed Flow Cytometry

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We have developed a screening method that has the potential to streamline the high-throughput analysis of affinity reagents for proteomic projects. By using multiplexed flow cytometry, we can simultaneously determine the relative expression levels, the identification of nonspecific binding, and the discrimination of fine specificities to generate a complete functional profile for each clone. The quality and quantity of data, combined with significant reductions in analysis time and antigen consumption, provide notable advantages over standard ELISA methods and yield much information in the primary screen which is usually only obtained in later screens. By combining high-throughput screening capabilities with multiplex technology, we have redefined the parameters for the initial identification of affinity reagents recovered from combinatorial libraries and removed a significant bottleneck in the generation of affinity reagents on a proteomic scale.

**Keywords:** antibodies • single-chain antibodies • high-throughput screening • flow cytometry

## Introduction

Phage display has been widely used for many different purposes, including the selection of peptides binding to antibody epitopes,[1−3] the selection of specific antibodies from large naïve[4−10] or immune[11−18] libraries, and the optimization of the binding affinities of many other proteins.[19−22] Of these, the selection of antibodies recognizing specific antigens is arguably the most successful use of phage display, and antibodies with affinities comparable to those obtained using traditional hybridoma technology can be selected from large naïve antibody libraries. When the selected antibodies are used as the basis for subsequent libraries and selection, affinities can be further increased to levels unobtainable in natural immune systems.[23−27] In general, the affinity of antibodies selected from naïve libraries is proportional to the size of the library, with $K_d$ values ranging from $10^{-6}$ to $10^{-7}$ for the smaller libraries[28,29] to $10^{-9}$ for the larger ones,[6−9,30] a finding in line with theoretical considerations.[31]

The development of high-throughput screening technologies has become essential for the initial identification of novel affinity reagents from phage combinatorial libraries. Preliminary screening of phage antibody selections has traditionally been carried out by ELISA,[29] a procedure which has been automated.[32] However, while it is relatively straightforward to carry out automated ELISAs with a few microtiter plates, the assay is intrinsically complex and technically laborious. By the elimination of liquid handling, more recent array-based methods using filters[33] or microscope slides[34,35] have significantly increased the number of antibody clones that can be screened at one time. Although these formats are very effective for screening large numbers of antibodies against a single target, or single antibodies against many targets, with one exception,[35] they cannot be easily adapted to the screening of hundreds of antibodies in multiplex, that is, where individual antibody clones are analyzed against many targets in a single analytical step. The inability to multiplex is a significant bottleneck for high-throughput screening, especially when contemplating generation and characterization of antibodies against all proteins encoded by a genome.

Flow cytometry methods accommodate multiplexing by the use of microsphere-beads that are distinguished either by variations in size[36] or intrinsic fluorescence,[37,38] with the latest bead sets comprising 100 individually distinguishable microspheres encoded by two fluorescent dyes.[39] Targets of interest are conjugated to individual microsphere sets, and the analytes are labeled with a different fluorescent dye. Side scatter signals trigger event detection, and the fluorescence is simultaneously measured for both microsphere and reporter dye fluorescence. This allows simultaneous identification of the coupled target, and the amount of bound analyte (using appropriate calibration approaches), at a throughput of 10 000−20 000 microspheres per second.

In a direct comparison between microarray-based systems and multiplexed flow cytometry to detect bacterial or viral proteins, flow cytometry has been shown to have lower limits of detection and increased dynamic range.[40] Similarly, comparisons between Western blotting, ELISA, indirect immunofluorescence, microagglutination, and multiplexed flow cytometry, in the serological diagnosis of tularemia, have shown flow cytometry to be as good as the best diagnostic measures.[41]

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Although not previously used for the screening of phage antibody libraries, multiplexed flow cytometry has been used to identify mAbs recognizing different epitopes,[42,43] to screen serum for binding to peptide vaccines,[44] infectious agents,[45−47] including specific neutralizing epitopes,[48] and to evaluate serum auto-antibodies levels.[49] Multiplexing using a cell-based approach has more recently been described for high-throughput drug screening and signaling profiling.[50]

We have developed multiplexed flow cytometry for high-throughput analysis of individual scFvs selected from a large phage antibody library.[7] For this purpose, an eight-microsphere multiplex set was created that would generate a complete binding profile for each scFv, including analysis of scFv protein expression levels, the level of nonspecific binding, and the identification of antibodies with selective and specific binding properties. As we reasoned that it should be possible to identify scFvs with the ability to discriminate between closely related antigens, we carried out selections on chicken lysozyme and screened the selection outputs against three additional lysozyme antigens differing from chicken lysozyme by one (quail), five (turkey), and 19 (duck) surface amino acids. By applying the multiplex capabilities of flow cytometry technology toward the analysis of selected antibodies, we have significantly expanded the experimental boundaries of high-throughput screening.

## Methods

**Preparation of the Biotinylated Antigens.** Lysozymes were purified from the egg whites of chicken, quail, turkey, and duck using ionic exchange chromatography (Ayriss et. al, in preparation). Briefly, egg whites were sonicated into 50 mM glycine buffer pH = 9.2, followed by centrifugation at 21 000*g* and filtration through 0.45 $\mu$m syringe filter. The extract was loaded onto HighS Sepharose column (Bio-Rad) and eluted with NaCl gradient. The purified proteins were quantified using the BCA Protein Assay kit (Pierce no. 23227) and tested for activity using the EnzChek Lysozyme Assay Kit (Molecular Probes no. E22013). Each purified lysozyme protein, alcohol dehydrogenase (ADH; Sigma no. A9589,) and ubiquitin (Sigma no. U6253) was conjugated with Biotin (Pierce no. 21343) and the level of biotinylation quantified using the EZ Biotin Quantification Kit (Pierce no. 28005). The four lysozymes, ADH, and ubiquitin were biotinylated so that each molecule was conjugated to 1−2 biotins.

**Preparation of the Microsphere-Bead Multiplex.** Eight different color-coded, carboxylated microsphere sets were purchased from Luminex (xMAP 101, 103, 122, 130, 139, 149, 159, 170). Each individual microsphere set was coupled with Neutravidin as described by Yan et al.[57] The level of neutravidin coupling to each bead set was quantified using biotinylated monomeric azami green (Mag) fluorescent protein (Supporting Information Figure 1). A total of 200 $\mu$L of each color-coded microsphere set, containing $2 \times 10^6$ microspheres, was incubated with 40 nM biotinylated antigen in a final volume of 2.5 mL. Following incubation at room temperature for 1 h, the microspheres were washed twice with PBS and 0.05% Tween 20 and re-suspended in 200 $\mu$L of PBS ($10^4$ microspheres/$\mu$L). The antigen-coupled microspheres were combined to create the multiplex (1600 $\mu$L) for analysis of 100 scFv samples, as 16 $\mu$L of the multiplex ($1.6 \times 10^5$ microspheres) was used for the analysis of one ScFv sample.

**Preparation of Fluorescently Labeled scFv's.** Phage selections using the scFv phage display library[7] were carried out against biotinylated chicken lysozyme (Sigma) prepared as described above. The selection process was automated using the Kingfisher magnetic bead system (Thermo Lab Systems), and panning was carried out in solution with biotinylated antigens captured onto streptavidin magnetic beads (Dynabeads, M-280). The stringency of the selection protocol was increased through three subsequent rounds of panning. The antigen amount was decreased 10-fold (from 600 to 60 nM) from the first to second round, and wash times were increased (1, 5, and 15 min) with each subsequent round. DNA recovered from the third round selection output was cloned into the pEP-E-coil vector (Pavlik et al., in preparation). The DNA was digested with BssHII and NheI, purified, and ligated into the pEP-E-coil vector. The ligation reaction was transformed into BL21 Gold Electrocompetent cells, and positive clones were selected on kanamycin (50 $\mu$g/mL final) agar plates. Each scFv clone was expressed as an E-coil fusion in 1 mL of kanamycin selective, auto-induction media[54] in a 96 deep well plate (Thomson no. A252596) covered with a sheet of AirPore (Qiagen no. 19571). Following incubation with shaking (900 rpm) at 18 °C for 36 h, the expressed scFv protein was recovered, within the media supernatant, by centrifugation at 4000 rpm for 30 min. No further protein purification was required. The scFvE-coil fusion proteins were fluorescently labeled using synthetic K-coil conjugated to Alexa488 (Pavlik et al., in preparation). The K-coil peptide (KVSALKEKVSALKEKVSALKEKVSALKEKVSALKEC) was synthesized in-house using solid-phase chemistry and labeled at Cys residue using sulfhydryl-reactive Alexa Fluor 488 maleimide reagent (Invitrogen A-10254). In total, 100 $\mu$L of each expressed scFv-E-coil fusion was fluorescently labeled with 10 nM K-coilA488 (final concentration) for 1 h at room temperature and then mixed with 16 $\mu$L ($1.6 \times 10^5$ microspheres) of the multiplex. Following incubation of labeled scFv with the multiplex for 1 h at room temperature, the sample volume was increased to 300 $\mu$L with PBS and directly analyzed using the HTS feature of LSRII; no further washing was required prior to analysis.

**Multiplexed Analysis of Binding Ligands Using the LSRII Flow Cytometer.** The mean fluorescence data of each bead set, within the multiplex, was collected using the high-throughput analysis feature of the Becton Dickinson LSRII Flow Cytometer and analyzed by DIVA software. The bead multiplex was separated into gates by excitation using 633 nm laser through APC-Cy7 (780/60BP) and APC (660/20BP) detectors. The mean fluorescent value of each gate was recorded following excitation using 488 nm laser through FITC (530/80BP) detectors. The samples were analyzed using the following Log parameter settings: FSC 313, SSC 177, FITC 650, APC-CY7 381, and APC 266. A total of 25 $\mu$L of each sample was injected at a rate of 0.5 $\mu$L/s, and the mean fluorescence of 5000 scattered, gated microspheres was recorded.

**Flow Cytometetric Data Analysis.** The mean fluorescence data was collected for each microsphere set within the multiplex. The mean fluorescence was divided by the corresponding SV5 value and converted to a percentage of the original SV5 value. This was done to standardize the results to the corresponding SV5 value to account for differences in expression levels between the scFv's. Both expressed proteins and positive binders were identified if the mean fluorescent value was 3 times or more above average background (the average of the mean fluorescent value coming from ADH, ubiquitin, and neutravidin microspheres).

**Surface Plasmon Resonance Analysis of scFv's Q4F4 and Q2E5.** Surface plasmon resonance analysis was carried out

using the Biacore 2000 (Biacore). The fourth, third, and second flow cells of a Biacore Streptavidin chip were saturated, in the order described, with Biotinylated chicken, quail, and turkey lysozyme, respectively. The first flow cell remained free of coupled antigen as a negative control. The Q2E5 and Q4F4 scFv proteins were purified by Ni-NTA chromatography and diluted to a final concentration of 200 nM and 1 $\mu$M in HBP-S buffer, respectively. A total of 50 $\mu$L of each purified protein was injected over the chip surface, in triplicate, at a rate of 30 $\mu$L/min. The chip surface was regenerated with 25 $\mu$L of 10 mM glycine, injected at a rate of 10 $\mu$L/min. Regeneration successfully removed all bound analyte and did not affect the stability of the ligand (lysozyme) coupled with the chip surface.

**ELISA Analysis of scFv Binding Ligands.** The ELISA was automated using a Tecan genesis 2000 workstation (Tecan). Each well of a 384 Maxisorp plate (NUNC no. 464718) was precoated with 50 $\mu$L of 10 $\mu$g/mL neutravidin (Pierce no. 31000), washed with PBS-LT (0.01%,v/v, Tween 20), and blocked with 100 $\mu$L of 1% (w/v) BSA (Sigma no. A7906) for 1 h at room temperature. A total of 50 $\mu$L of each biotinylated-antigen (2 $\mu$g/mL) was captured, in replicate, onto the neutravidin surface. Following washing with PBS-T (0.1%, v/v, Tween 20), 50 $\mu$L of each expressed scFv antibody was added and incubated at room temperature for 1 h. Following washing, 50 $\mu$L of anti-SV5 mouse monoclonal antibody (5 $\mu$g/mL) was added and incubated at room temperature for 1 h. The plate was washed (3 $\times$ 200 $\mu$L) with PBS-T (0.1% Tween 20) and (3 $\times$ 200 $\mu$L) with PBS-LT prior to adding 50 $\mu$L of 5 $\mu$g/mL secondary Anti-Mouse IgG-Alkaline Phosphatase (AP) conjugate (goat polyclonal, Dakocytomation no. D0486). Following incubation at room temperature for 1 h, the plate was washed (3 $\times$ 200 $\mu$L) with PBS-T, PBS-LT, and PBS. Binding events were detected following treatment with 80 $\mu$L of AP substrate (NBT/BCIP, Pierce no. 34042) and analyzed by absorbance at 405 nm using the SPECTRAfluor Plus spectrofluorometer.

## Results

**The Technical Strategy for Flow Cytometry Multiplexing.** An 8-microsphere multiplex was prepared by capturing a panel of biotinylated antigens onto individual colored microsphere sets coated with neutravidin. This approach was used to overcome problems associated with the inefficient and variable coating we obtained when attempting to coat microspheres with antigen directly and, thus, provided quantitative uniformity between the bead sets. The amount of neutravidin coupled with each bead set was quantified using a biotinylated fluorescent protein and shown to be comparable. Biotinylated fluorescent protein, rather than, for example, biotinylated fluorescein, was used to simulate binding of biotinylated protein antigens used in screening experiment. The antigen was added in excess, resulting in similar amounts of antigen coupled per microsphere set (Supporting Information Figure 1). The coupled targets were based on use of chicken lysozyme as an scFv selection target and were chosen to identify positive binding events (chicken lysozyme), scFvs discriminating between closely related antigens (quail, turkey, and duck lysozymes; Figure 1a; Supporting Information Figure 2), scFvs showing nonspecific binding (yeast alcohol dehydrogenase (ADH), ubiquitin, and neutravidin alone), and scFv expression levels (anti-SV5 antibody recognizing the peptide tag at the scFv C-terminus). The four lysozyme antigens were purified to single band purity (Figure 1b) from the eggs of chicken, quail, turkey, and duck, tested for specific lysozyme activity using EnzChek

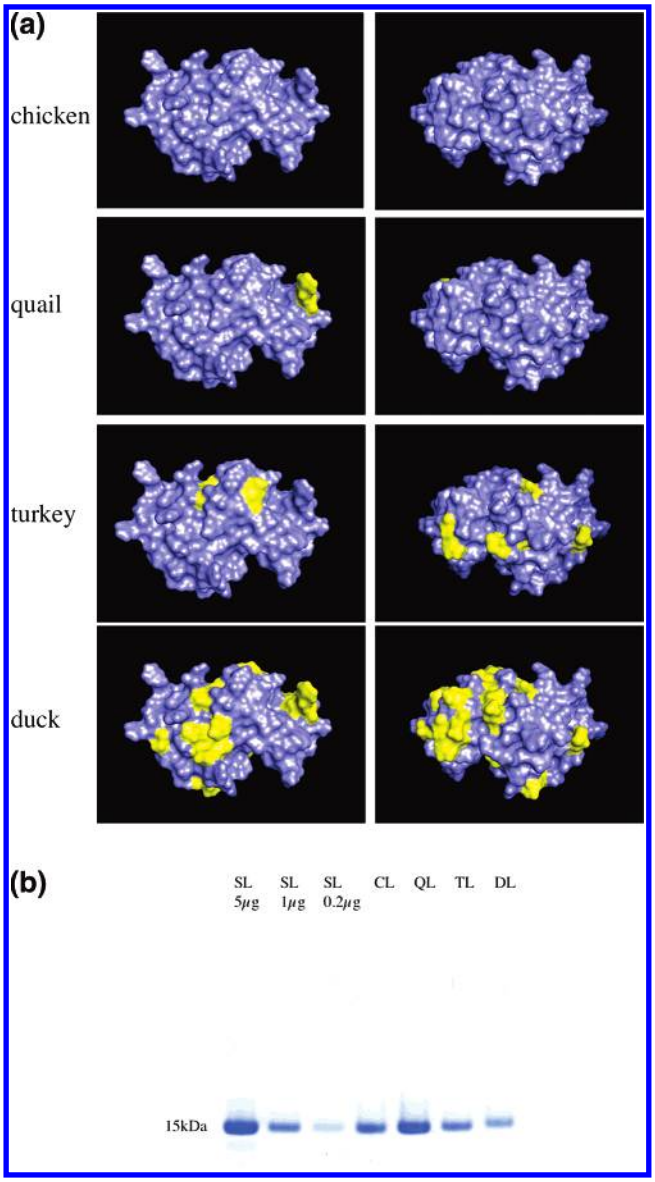


**Figure 1.** (a) Solution NMR structure of chicken lysozyme (1E8L) and PyMOL software was used to indicate mutations between lysozymes from different species. All mutations are colored in yellow, and each structure is shown in 2 views, the one on the right being rotated by 180°. Quail lysozyme differs by 4 amino acid mutations, of which only 1 is surface-exposed. Turkey lysozyme differs by 7 mutations, of which 5 are surface-exposed. Peking duck lysozyme differs by 23 mutations, of which 19 are surface-exposed. (b) SDS-PAGE analysis of lysozymes purified by ionic exchange chromatography. Sigma chicken lysozyme standards (SL), chicken lysozyme (CL), quail lysozyme (QL), turkey lysozyme (TL), and peking duck lysozyme (DL).

Lysozyme Assay Kit (Molecular Probes, Ayriss et al. in preparation), and conjugated with 1−2 biotins per molecule. Following antigen coupling, the 8 individual microsphere sets were washed and pooled in equal numbers to create the multiplex (Figure 2a).

To use scFvs for multiplexed flow cytometry, they must be fluorescently labeled with a dye that is distinguishable from the dye used to label the microspheres. Furthermore, to obtain quantitative comparisons of scFv binding, it is important that scFvs are uniformly labeled. For these reasons, we avoided the

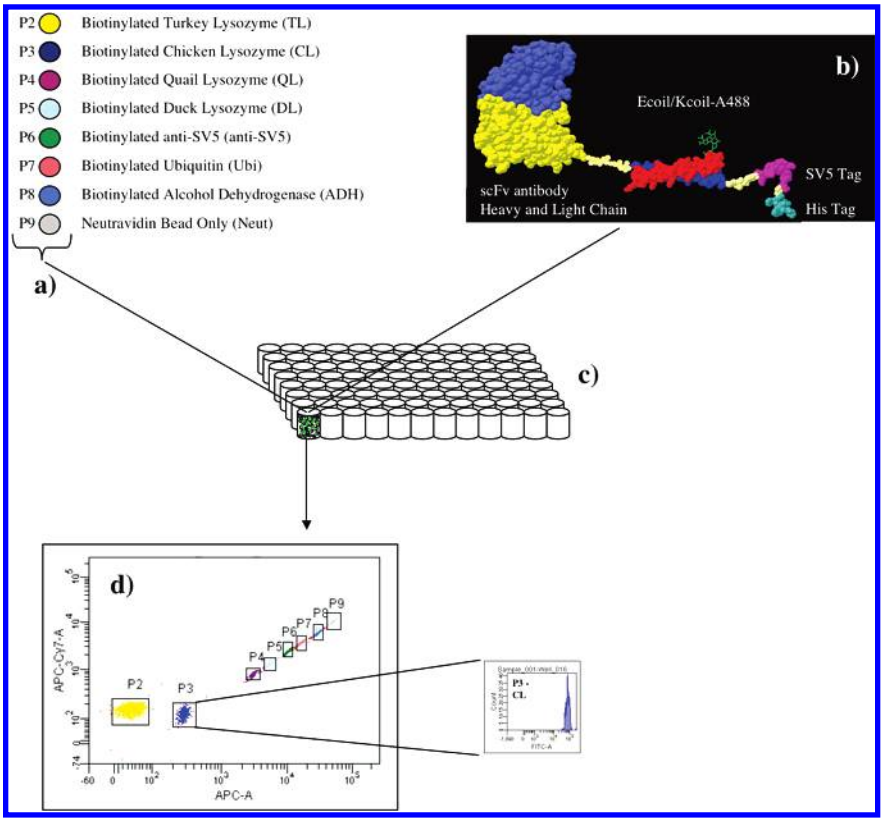


**Figure 2.** Preparation of samples for flow cytometry analysis. (a) The 8 microsphere multiplex: biotinylated antigens were coupled with different colored microsphere sets, facilitated by the biotin/neutravidin interaction. Incubation of the test scFv binder with the multiplex allows analysis against numerous targets within the same test sample. (b) Diagram of scFv coiled-coil complex used for screening. This diagram is assembled from existing D1.3 modeled structures. scFvs were fluorescently labeled using synthetic Ecoil and Kcoil peptides that form heterodimers. Kcoil labeled with Alexa488 binds to scFv expressed as Ecoil fusions. (c) Sample preparation and analysis was carried out in 96 well format. (d) The Becton Dickinson LRSII Flow Cytometer was used for data analysis. The bead multiplex was separated into gates by excitation using a 633 nm laser through APC-Cy7 (780/60BP) and APC (660/20BP) detectors. The mean fluorescent value of each gate, representing bound labeled scFv, was recorded following excitation using a 488 nm laser through a FITC (530/80BP) detector.

use of fluorescently labeled secondary antibodies and chose to use the-coiled coil system intead.[51] This is based on a pair of synthetic coiled coils, E and K, which bind to one another with an affinity of 64 pM.[52] The K-coil, fluorescently labeled with Alexa488 (K-coil-A488), was prepared synthetically, while the E-coil was appended to the C-terminus of different scFvs in the vector pEP-E-coil (Pavlik et al., in preparation). In addition to the E-coil, pEP-E-coil also contained the SV5 tag[53] as well as the His6 tag (in the order scFv−E-coil−SV5−His tag, Figure 2b). In preliminary experiments (data not shown), we were able to show that E-coil scFv fusions were released directly into the media supernatant, that they could be efficiently labeled with the K-coil-A488, and that results obtained with purified scFvs were similar to those obtained with nonpurified scFvs released directly into the culture supernatant.

Individual transformants were picked into minimal media and transferred into autoinduction media[54] for overnight growth. The scFv's in the culture supernatant were fluorescently labeled in a one-step process by the addition of synthetic K-coil-A488. This was followed by the addition of the microsphere multiplex to the fluorescently labeled scFv's in a 96 well format (Figure 2c), with each well containing a different scFv. All wash steps were eliminated from the screening system without detriment, maximizing throughput capability and reducing required hands-on time. The samples were directly analyzed using the LSRII flow cytometer (Becton Dickinson), and binding events were determined by comparing the mean fluorescence of each individual microsphere set (Figures 2d and 3).

**Optimizing Screening Parameters with Control scFv Binding Ligands.** Screening parameters were optimized using two well-characterized positive control scFv's recognizing lysozyme, and a negative control scFv recognizing ubiquitin. The lysozyme scFv positive controls were derived from the monoclonal antibodies, D1.3[55] and HyHel10,[56] while the anti-ubiquitin scFv (anti-ubiquitin, Pavlik, P., unpublished results) was selected from a large phage antibody library.[9] The scFv genes were subcloned into the pEP-E-coil vector and the proteins prepared as positive controls without purification. These were analyzed against the 8 microsphere multiplex. The results in Figure 3 demonstrate that positive, negative, cross-reactive binding events, and corresponding expression levels (anti-SV5) are clearly defined: D1.3 binds chicken and quail lysozyme strongly, turkey and duck lysozyme very weakly, and the negative control antigens (ADH, ubiquitin, and neutravidin) not at all. In contrast, the anti-ubiquitin scFv binds ubiquitin, but none of the lysozymes or negative control antigens. All three scFv's are well-expressed, with the SV5 microspheres capturing significant amounts of each scFv. The analytical data for D1.3 (Figure 3) shows that the number of gated events (approximately 600) is consistent for each individual bead set, indicating that each antigen-coated microsphere is represented equally within the

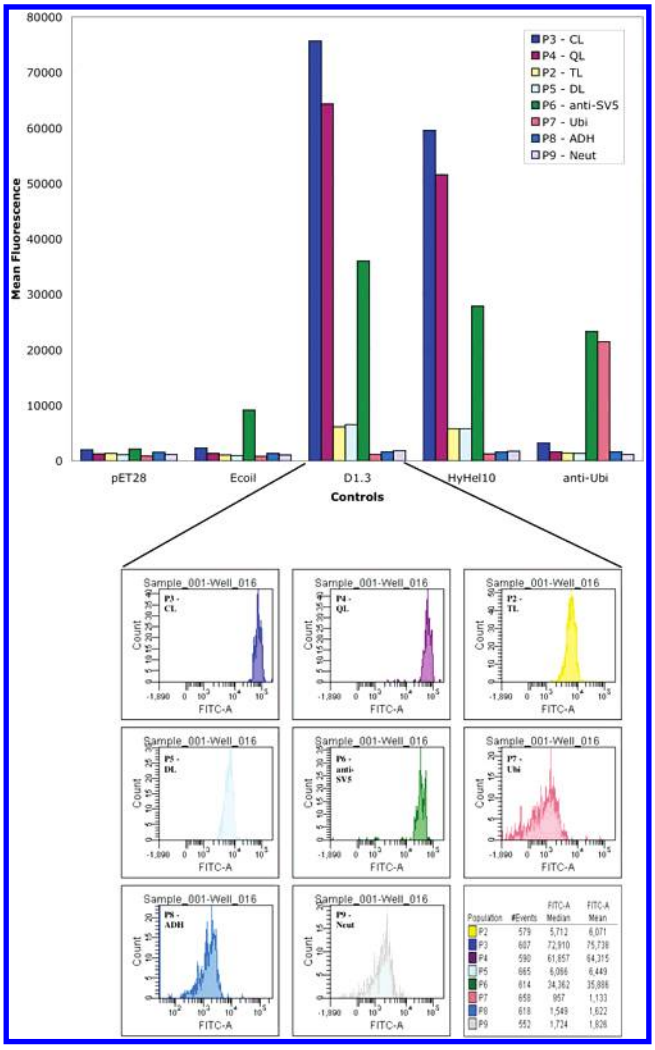


**Figure 3.** Optimizing screening parameters with negative and positive controls. The positive control scFvs D1.3, HyHel10, and anti-ubiquitin (anti-Ubi) show specific binding to their respective antigens, chicken lysozyme and ubiquitin. The SV5 value indicates the level of protein expression. Analysis of BL21 transfomants containing pET28 and pEP-Ecoil (Ecoil) vectors demonstrates the insignificant background binding of free Kcoil-A488 to labeled microspheres. Inset: Raw flow cytometry data output for D1.3 binding to the bead multiplex.

bead multiplex. These results clearly demonstrate the sensitivity of the analysis and the ability to identify positive binding events.

As the free K-coil-A488 was added in excess over the scFv-E-coil, experiments were also carried out to determine the level of background binding of the free K-coil-A488 to the microspheres within the culture supernatant. In this case, two controls were used. The first consisted of the culture supernatant produced from pET28 BL21 transformants (pET28) expressing no recombinant protein, and the second, pEP-E-coil transformants (E-coil), expressing the E-coil fused to the SV5 and His6 tags, as a small peptide, with no additional coding sequence. In the absence of scFv, SV5 Tag, or E-coil, no signal was obtained on any of the microspheres, while with the E-coil-SV5-His6 fusion, signal was obtained only with the SV5 microspheres, demonstrating the specificity with which K-coil-A488 bound E-coil fusions, even in the absence of scFv. These experiments show that background levels of fluorescence produced by free K-coil-A488 were insignificant and did not obscure true binding events.

**Screening a Selection Output by High-Throughput Multiplexed Flow Cytometry.** To apply this screening technology to the output of a real selection, DNA encoding scFv's from the third round output of a selection, carried out against chicken lysozyme, was subcloned into the pEP-E-coil vector. In total, 261 clones were picked, and the proteins were expressed as E-coil fusions, fluorescently labeled with synthetic K-coil-A488, and screened in multiplex, against a panel of eight different antigens. Analysis of the results revealed that the mean fluorescence of each SV5 value was at least 3 times above background, demonstrating that all of the 261 scFv proteins were expressed (data not shown). The binding profile of the total scFv population is summarized in the pie chart shown in Figure 4a, and representative clones are shown in detail in Figure 4b. As can be seen, 32% of the output did not recognize any of the lysozymes (e.g., Q2E12 and Q2G6 in Figure 4b), consistent with standard selection outputs, while 18% recognized all the lysozymes (Q2C10 and Q2E8 in Figure 4b). The rest of the clones were distributed among seven different lysozyme combinations, with 96% of the positives recognizing the selection antigen (chicken lysozyme), and almost 60% recognizing at least chicken and quail lysozyme, which differ by only a single surface amino acid. Interestingly, the 4% of clones which did not recognize chicken lysozyme, recognized turkey lysozyme. The classification of these scFv's as negative was based on the fact that positive binders were required to show signals 3-fold greater than background, and under this definition, weak binders are identified as negative. As the purpose of the experiment was to demonstrate the power of this multiplex technology to characterize positive binders, scFv's were not sequenced. and individual clones may be represented more than once in the population analyzed. To account for variations in scFv expression levels (Supporting Information Figure 4), the binding data for each scFv is normalized to the corresponding SV5 signal and plotted as a percentage of the mean fluorescence standardized to the SV5 value. We assessed the reproducibility of the screening platform by repeating the whole procedure using scFv-E-coils, independently prepared from the same glycerol stocks, labeling them with K-coil-A488, and repeating the analysis. Although the binding results showed some variation in absolute values, the patterns of reactivity were essentially identical in the two experiments (Figure 4c). We also tested the stability of the prepared bead samples by storing them for 14 days at 4 °C after initial analysis (Figure 4d). Following storage, the samples were reanalyzed, and the results show that even though the mean fluorescent signal had decreased slightly, probably due to proteolysis, the scFv binding profiles were reproducible (Figure 4e), demonstrating excellent stability over a 14 day period.

**Multiplex Screening Results Are Comparable to ELISA.** As the Enzyme-Linked Immunosorbent Assay (ELISA) is the 'gold standard' for screening potential affinity reagents, we compared results obtained with multiplexed flow cytometry with those obtained by ELISA. To provide a valid comparison, the ELISA was carried out in a format comparable to that used for multiplexed flow cytometry, with biotinylated antigens immobilized onto a neutravidin-coated plastic surface. scFv-E-coils released into the bacterial culture supernatant were directly added to each antigen coated plate, washed, and analyzed using our standard reporter combination of anti-SV5[53] and Alkaline Phosphatase labeled anti-Mouse IgG (Dakocyto-

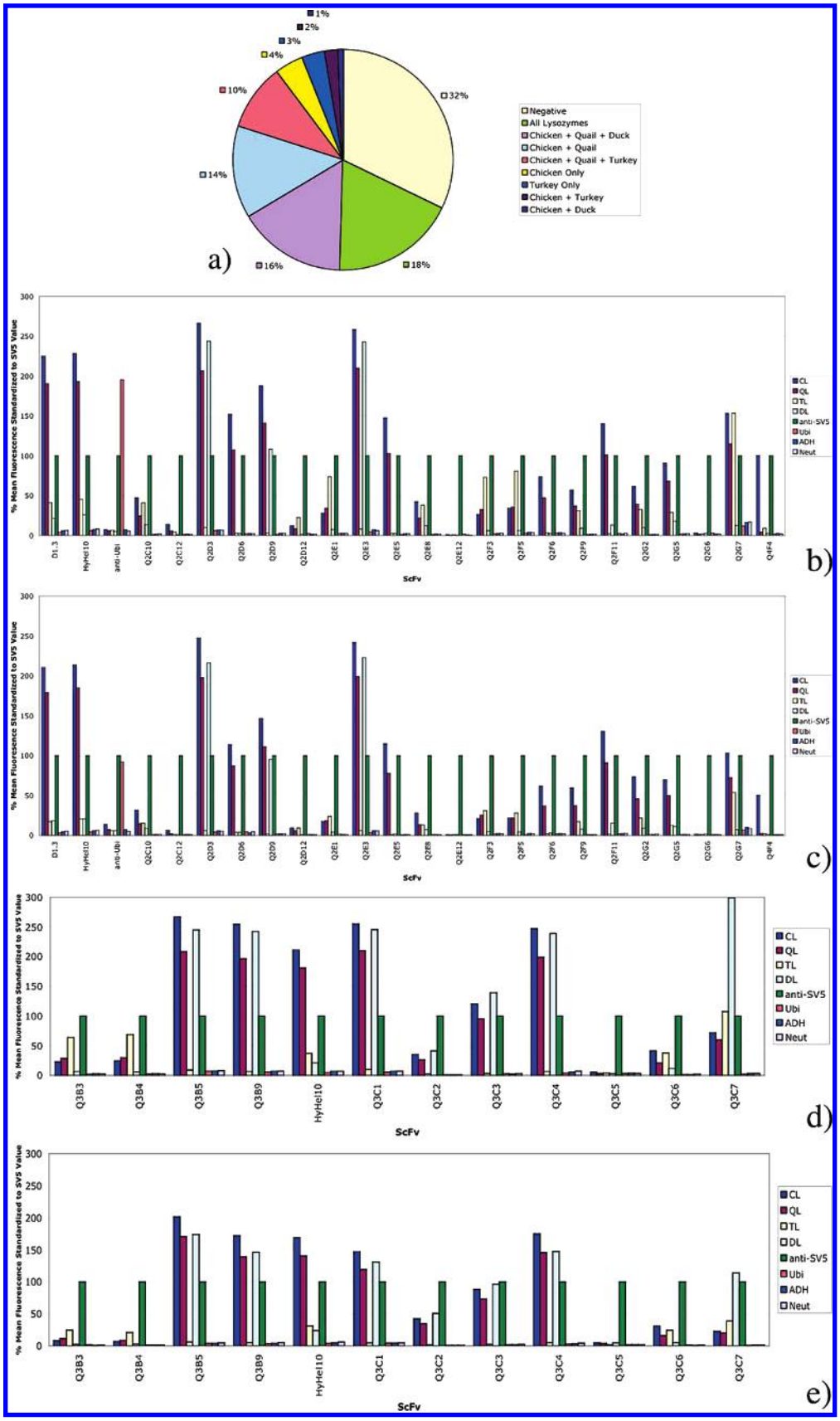


**Figure 4.** Multiplex screening using flow cytometry. (a) The binding profile summary of the total scFv population screened. (b) The binding profile of representative scFvs. Each value has been standardized to the corresponding SV5 mean fluorescent signal. (c) Independent reanalysis of representative scFv's analyzed in panel b. scFvs were independently expressed, labeled, and analyzed. Results show that the flow cytometry results are reproducible. (d) Subset of scFvs samples analyzed at time 0. and (e) after 14 days at 4 °C. The binding profiles of the scFv's are consistent between data sets showing that prepared samples can be stored at 4 °C for up to 14 days prior to analysis.

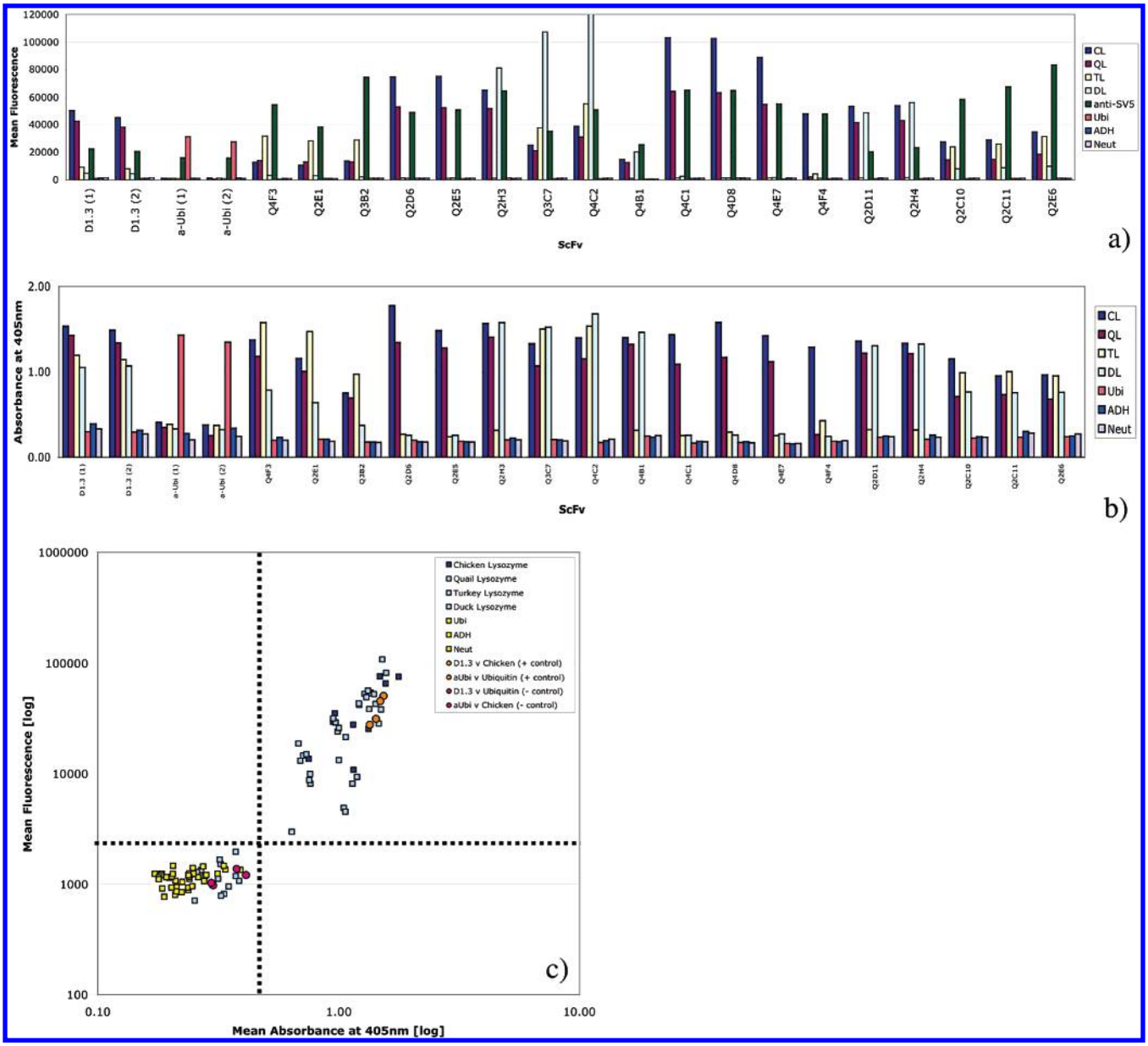


**Figure 5.** Comparison of ScFv binding results produced in 'multiplex' by (a) flow cytometry and (b) ELISA. (c) A scatterplot in which each point represents a single scFv/antigen pair. Known positive controls (D1.3 vs chicken lysozyme and anti-ubiquitin vs ubiquitin) as well as known negative controls (D1.3 vs ubiquitin; anti-ubiquitin vs chicken lysozyme; selected scFvs vs ADH, ubiquitin, and neutravidin) are separately indicated as described in the key.

mation). The number of scFv's analyzed compared was restricted by the inability of the ELISA to multiplex, requiring one well per assay. The ELISA experiment was designed within a 384 well plate to accommodate two replicates for each of the 8 antigens and for 22 different scFv samples. The results show that the scFv binding profiles analyzed by multiplexed flow cytometry (Figure 5a) and ELISA (Figure 5b) are comparable. In addition, flow cytometric data include expression levels of scFv using the anti-SV5 antibody beads. Similar analysis in the ELISA format would require capture and detection antibodies and was not performed. Analysis and comparison of the results in the two different data sets shows that the background is lower and sensitivity is higher in flow cytometry compared to ELISA. This is confirmed by scatter plot analysis, in which the ELISA signals are compared to those obtained by multiplexed flow cytometry (Figure 5c). The scattering of data points using both techniques is clearly defined into two separate populations indicating the positive and negative scFv binders. However, flow cytometry provides a wider dynamic range in the discrimination between different positives, and a narrower range between the different negatives. This is in contrast to the ELISA results, in which the opposite was true. This demonstrates that the identification of positive clones is easier with flow cytometry than it is with ELISA.

**Multiplex Identification of Ligands with Specific and Selective Binding Profiles.** Two scFv antibodies, Q4F4 and Q2E5, were chosen for further analysis due to their interesting binding profiles. Flow cytometry results indicated that Q4F4 specifically bound chicken lysozyme and showed no cross-reactivity with any other of the other lysozymes, including quail (Figure 6a), which differs by only 1 very conservative surface amino acid change (R68 to K68; Figure 1). The second scFv antibody Q2E5 was chosen because, based on flow cytometry data, it was shown to bind to only chicken and quail lysozymes (Figure 6b).

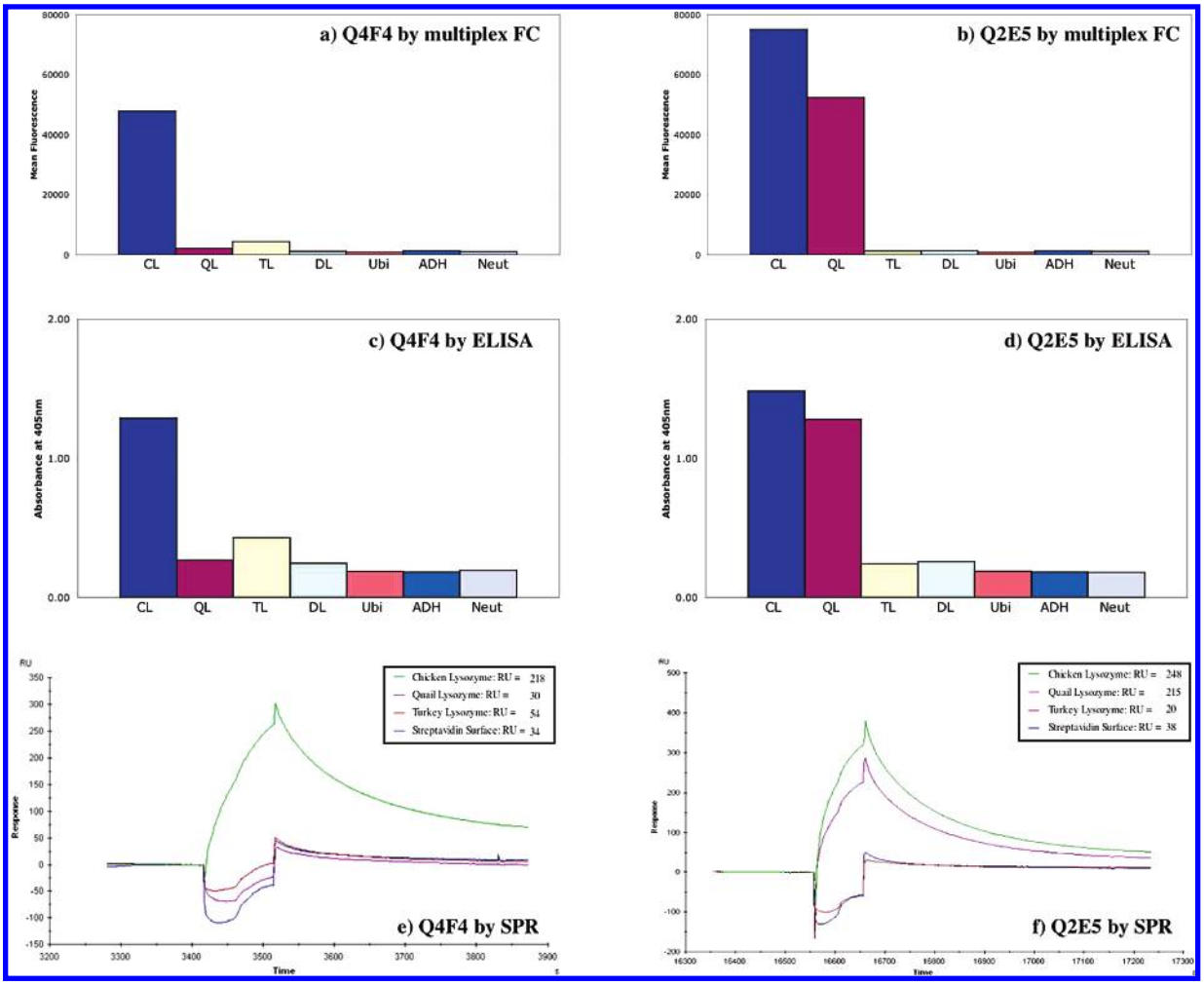


**Figure 6.** Results obtained by multiplex screening are reproducible by ELISA and Surface Plasmon Resonance (SPR) analysis. Multiplex flow cytometry results of (a) unpurified Q4F4 and (b) unpurified Q2E5. ELISA results of (c) unpurified Q4F4 and (d) unpurified Q2E5. SPR analysis of (e) purified Q4F4 (binds CL only with an average (3) response of 218 units) and (f) purified Q2E5 (binds CL and QL only with an average (3) response of 248 and 215 units, respectively). Both ScFv's were tested in replicates of 3 on the same chip, and each flow cell was saturated with antigen in an order to prevent cross-contamination. The chip surface remained stable following regeneration with 10 $\mu$L of 10 mM glycine, pH 2.

The binding profiles of the two chosen scFv's were reproduced and confirmed by ELISA (Figure 6c,d) and surface plasmon resonance (Biacore) analysis (Figure 6e,f). These results demonstrate that not only are multiplexed flow cytometry results reproducible in two additional analytical systems, but they are sensitive enough to be exploited for the identification of binding ligands that discriminate between very closely related antigens in a primary screen.

## Discussion

The use of combinatorial libraries for the generation of antibodies that bind defined targets continues to increase, especially with the proliferation of projects to develop antibodies against thousands of different proteins.[58,59] As a result, there is a growing need for the development of high-throughput screening systems able to rapidly provide a complete functional profile of specific binders, to ensure lead candidates are accurately characterized and quickly advance to the next stage of assessment. A significant bottleneck in the development of HTS technologies is the inability to comprehensively analyze individual affinity reagents against numerous targets simultaneously in a single analytical test. As a result, screening usually involves a primary screen in which potential positives are identified, followed by a secondary screen, in which the properties of such putative positives are confirmed and expanded. We have overcome this problem by exploiting the multiplex capabilities of flow cytometry and have developed a method that is high-throughput, efficient, and highly reproducible, providing data in the primary screen more characteristic of that usually obtained in secondary or even tertiary screens.

All technical aspects of the method were engineered toward efficiency, by minimizing the number of steps required for sample preparation while retaining the ability to analyze large numbers of samples, in multiplex, in a minimum period of time. Important factors included scFv expression by autoinduction, analysis of unpurified protein, one-step fluorescent labeling of scFvs using the Ecoil−Kcoil heterodimer duplex, the use of neutravidin-coated microspheres with biotinylated antigens, the elimination of microsphere wash steps immediately prior to analysis, and the preparation and analysis of samples in a 96 well format.

We used D1.3, HyHel10, and anti-ubiquitin positive controls to define the experimental method, determine the analytical parameters of the flow cytometer, and demonstrate that

**Table 1.** Comparison of Parameters Required by ELISA and BD LSRII Flow Cytometer for Ligand Binding Analysis

| parameters of screening system | LSRII Flow Cytometry | ELISA |
|---|---|---|
| Volume of scFv culture supernatant per sample | 50 $\mu$L | 50 $\mu$L |
| Number of replicates for each antigen per scFv sample | 500 | 1 |
| Minimum amount of each antigen (15 kDa) needed to screen 1 scFv sample | 0.015 $\mu$g | 1 $\mu$g |
| Minimum amount of each antigen (150 kDa) needed to screen 1 scFv sample | 0.15 $\mu$g | 1 $\mu$g |
| Minimum amount of each antigen (15 kDa) needed to screen 96 scFv samples | 1.5 $\mu$g | 96 $\mu$g |
| Minimum amount of each antigen (150 kDa) needed to screen 96 scFv samples | 15 $\mu$g | 96 $\mu$g |
| Can the screening system accommodate multiplexing? | Yes | No |
| Can prepared samples be stored? If so, for how long? | Yes; Upto 14 days at 4 °C | No |
| Time to screen 96 samples | 30 min | 3 h |

binding events could be identified. In addition, negative controls were included to ensure that free fluorescent K-coil did not obscure true binding events. Initial results showed that positive, negative, and cross-reactive binding events were clearly defined and that the experimental parameters were optimized. The results also showed that the sensitivity of the instrument was capable of identifying binding ligands that could discriminate between closely related antigens. This was first addressed with the D1.3 positive control, where the binding profile showed binding to chicken lysozyme at a level more than 50 times above background (average of mean fluorescence from anti-ubiquitin, ADH, and neutravidin microspheres) and slightly lower levels of reactivity with quail lysozyme, which differs by one conserved surface amino acid mutation (R68 to K68). The D1.3 scFv also cross-reacted with turkey and duck lysozyme, but at significantly lower levels due to the presence of more surface amino acid mutations. The discrimination of D1.3 binding between such closely related antigens clearly demonstrates the sensitivity of the instrumental analysis.

Screening results incorporating data from 261 scFv antibodies showed that multiplex analysis clearly identified positive, negative, and cross-reactive binding events from a population selected against chicken lysozyme. As expected, we identified a large proportion of ligands (65%) that primarily bound chicken lysozyme, but in addition, also recognized additional lysozyme antigens in a multitude of different, specific, binding profiles. In addition, comparison of the flow cytometry with the corresponding ELISA data shows that the results produced, using the two different analytical methods, are equivalent, a necessary step for flow cytometry to become an accepted and trusted alternative screening method. This was confirmed in our further analysis of two scFv's (Q2E5 and Q4F4) with interesting binding properties. scFv Q4F4 bound chicken lysozyme and showed no cross-reactivity with any other antigen including quail lysozyme, even though they differ by only a single conserved surface amino acid; whereas Q2E5 bound only chicken and quail lysozymes. We demonstrated that the binding profile of each scFv was consistent when analyzed by three different methods: multiplexed flow cytometry, ELISA, and surface plasmon resonance.

Although microarrays are usually used to screen single antibodies against many antigens, or vice versa, a microarray method has been published,[34,35] in which antigens are arrayed first and antibodies subsequently arrayed in perfect register. Although this is not a true multiplex assay, in the sense that different binding events are analyzed pairwise, rather than simultaneously in a single test under identical conditions, it is capable of generating similar data. However, the correlation between microarray and ELISA signals reported varied over a 1000-fold range, unlike the 10-fold range we have seen when comparing flow cytometry to ELISA (Figure 5c). Two factors are likely to be important in the improved quality of data obtained with flow cytometry compared to microarrays. The first is the large numbers of microspheres analyzed, which allows the application of far more stringent statistical methods with improved confidence measures, and the second, that their size allows microspheres to remain in suspension for several hours, providing near-fluid-phase reaction kinetics,[60] quite unlike the poorly understood and complicated reaction kinetics occurring in microarrays.[61,62] In addition, as no protein spotting is needed, it is easier to keep proteins in a functional, nondenatured state with multiplexed flow cytometry.

As we developed the flow cytometry method and generated results, it became clear that, in addition to the multiplex capability, flow cytometry offered many other additional advantages compared to ELISA. As shown in Table 1, the analytical time parameters of ELISA are not efficient in a high-throughput screening research and development environment. The time taken to screen 96 samples against a single antigen by ELISA takes approximately 3 h, counting incubations with the scFv, anti-SV5, and anti-mouse-AP antibodies and pipetting time, but can be significantly longer when antibodies are analyzed simultaneously against multiple antigens in multiple plates. In addition, prepared samples have to be analyzed as soon as they are prepared. In comparison, we have demonstrated that flow cytometry samples are stable at 4 °C for up to 2 weeks once prepared, and once plates are prepared, it takes only 30 min to screen 96 clones. This element of flexibility is an important factor in a research environment where thousands of samples are being prepared and screened simultaneously.

Another significant difference and major advantage of using flow cytometry screening is the efficiency of antigen consumption. For each clone analyzed, flow cytometry measures approximately 500 replicates (microspheres) for each antigen, compared to only a few replicates for each antigen with ELISA. In addition, the amount of antigen required for flow cytometric analysis is considerably less than the amount needed for ELISA. The flow cytometry method as described here uses 100 pmol of each antigen (equivalent to 1.5 $\mu$g of a 15 kDa protein) for the analysis of 100 different scFv's. In comparison, ELISA requires 6667 pmol of each antigen (equivalent to 100 $\mu$g of a 15 kDa protein) for analysis of the same number of scFv's, 67 times more for the antigen used here.

However, we believe that there are opportunities for additional significant reductions in antigen requirements. We were able to show that the microsphere capacity becomes saturated at an antigen concentration of 0.4 nM (Supporting Information Figure 3), corresponding to 0.04 pmol per sample of 2 $\mu$L microspheres ($2 \times 10^4$ microspheres) under the assay conditions used. In a full screening experiment 200 $\mu$L microspheres are used. The conditions used here assumed that saturation was fully scalable (i.e., 200 $\mu$L microspheres becomes saturated with 4 pmol antigen). However, it may be possible

to saturate microspheres at the same antigen concentration, but in lower volumes, leading to further reductions in antigen consumption. Furthermore, the present configuration of the flow cytometer (LSRII) used requires a large sample volume (300 $\mu$L), of which less than 10% is actually analyzed. This presents additional opportunities for reduction of the amount of antigen required, should this become a limiting factor.

We have developed a screening method that has the potential to streamline high-throughput analysis. Multiplexed flow cytometry provides a flexible screening alternative to current high-throughput methods and produces a complex analytical profile of results that are consistent and reliable. The increase in comprehensive data output, combined with the reduction in analysis time and antigen consumption, provides significant advantages over standard ELISA methods. By combining high-throughput screening capabilities with multiplexed technology, we have re-defined the parameters for initial identification of novel binding ligands recovered from combinatorial libraries.

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**Supporting Information Available:** Figures showing the uniformity of neutravidin-coated microspheres; the protein sequence alignment of chicken, quail, turkey, and duck lysozymes; the binding capacity of the neutravidin-coated microspheres; the relationship between mean bead fluorescence signal from anti-SV5 beads and scFv-Ecoil concentration; and additional information on the anti-ubiquitin antibody and the pEP-E-coil vector used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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