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Seeing Better through a MIST: Evaluation of Monoclonal Recombinant Antibody Fragments on Microarrays

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Automation is the key approach for genomewide and proteomewide screening of function and interaction. Especially for proteomics, antibody microarrays are a useful tool for massive parallel profiling of complex samples. To meet the requirements of antibody microarrays and to obtain a great variety of antibodies, new technologies such as phage display have partly replaced the classical hybridoma method. While the selection process for phage-displayed antibody fragments itself has been automated, the bottleneck was shifted further downstream to the identification of monoclonal binders obtained from the selections. Here, we present a new approach to reduce time, material, and waste to extend automation beyond the selection process by application of conventional microarray machinery. We were able to express recombinant antibody fragments in a single inoculation and expression step and subjected them without purification directly to an automated high-throughput screening procedure based on the multiple spotting technique (MIST). While obtaining comparable sensitivities to enzyme-linked immunosorbent assays, we minimized manual interaction steps and streamlined the technique to be accessible within the automated selection procedure.

After having sequenced the entire human genome, the current task is to understand the proteome by identification and quantification of all proteins in a given sample. So far, DNA microarrays have been employed to detect the transcription level of genes in cells.[1−3] However, it has been found that there is no stringent correlation between transcription level and protein abundance.[4,5] Furthermore, the status of a protein in terms of posttranslational modification and structure cannot be determined by DNA microarrays. To solve this problem, antibody microarrays are envisaged to take the place of DNA microarrays in proteome research.[6,7] Such arrays consist of a multitude of different antibodies that are immobilized on a solid support and allow characterization of the protein repertoire of a given sample. However, the production of such antibody microarrays and its application require the provision of highly specific and stable antibodies, possessing high affinity and showing no cross-reactivity.[8−11]

Monoclonal antibodies that recognize their cognate antigen and even the state of its modification in a specific manner are classically generated by the hybridoma technology. Nevertheless, hybridoma technology is handicapped, when it comes to generating antibodies against highly conserved antigens. Furthermore, it is not designed to deliver antibodies against the scale of an entire proteome in an economical sense. Therefore, recombinant technologies such as phage display of antibody fragments[12,13] have been developed to select antibodies in a high-throughput approach against a multiplicity of antigens, and automation of the selection procedure has already been established in the microtiter plate format.[14−17] However, one has to be aware that phage display-

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(1) Diehl, S.; Diehl, F.; El-Sayed, N. M.; Clayton, C.; Hoheisel, J. D. *Mol. Biochem. Parasitol.* **2002**, *123*, 115−23.

(2) Scheideler, M.; Schlaich, N. L.; Fellenberg, K.; Beissbarth, T.; Hauser, N. C.; Vingron, M.; Slusarenko, A. J.; Hoheisel, J. D. *J. Biol. Chem.* **2002**, *277*, 10555−61.

(3) Brown, P. O.; Botstein, D. *Nat. Genet.* **1999**, *21*, 33−7.

(4) Anderson, L.; Seilhamer, J. *Electrophoresis* **1997**, *18*, 533−7.

(5) Gygi, S. P.; Rochon, Y.; Franza, B. R.; Aebersold, R. *Mol. Cell Biol.* **1999**, *19*, 1720−30.

(6) Glökler, J.; Angenendt, P. *J Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2003**, *797*, 229−40.

(7) Kusnezow, W.; Hoheisel, J. D. *J. Mol. Recognit.* **2003**, *16*, 165−76.

(8) MacBeath, G. *Nat. Genet.* **2002**, *32*, 526−32.

(9) Templin, M. F.; Stoll, D.; Schrenk, M.; Traub, P. C.; Vohringer, C. F.; Joos, T. O. *Trends Biotechnol.* **2002**, *20*, 160−6.

(10) Angenendt, P.; Glökler, J.; Murphy, D.; Lehrach, H.; Cahill, D. J. *Anal. Biochem.* **2002**, *309*, 253−60.

(11) Haab, B. B.; Dunham, M. J.; Brown, P. O. *Genome Biol.* **2001**, *2*, RESEARCH0004.

(12) Knappik, A.; Ge, L.; Honegger, A.; Pack, P.; Fischer, M.; Wellnhofer, G.; Hoess, A.; Wolle, J.; Pluckthun, A.; Virnekas, B. *J. Mol. Biol.* **2000**, *296*, 57−86.

(13) de Haard, H. J.; van Neer, N.; Reurs, A.; Hufton, S. E.; Roovers, R. C.; Henderikx, P.; de Bruine, A. P.; Arends, J. W.; Hoogenboom, H. R. *J. Biol. Chem.* **1999**, *274*, 18218−30.

(14) Hallborn, J.; Carlsson, R. *Biotechniques* **2002**, (Suppl.), 30−7.

(15) Konthur, Z.; Walter, G. *DDT: TARGETS* **2002**, *1*, 30−6.

(16) Walter, G.; Konthur, Z.; Lehrach, H. *Comb. Chem. High Throughput Screening* **2001**, *4*, 193−205.

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derived recombinant antibodies may require some postprocessing, such as a maturation step to increase affinity, the adjustment of codon usage for efficient production in *Escherichia coli*, or the change of scaffold to the more stable Fab.[18]

In the automated approach, the selection yields a polyclonal antibody population for each antigen, which needs to be screened for monoclonal and specific binders. To ensure the successful identification of a specific antibody fragment for a single antigen, a minimum of 96 single clones out of each selection pool has to be evaluated. The use of the whole capacity of the automated system, starting with a 96-well plate harboring 96 different proteins, would therefore require the screening of 96 microtiter plates, each carrying 96 monoclonal antibody fragments. This would already result in 9216 clones to be screened by enzyme-linked immunosorbent assays (ELISA) without any controls and cross-reactivity screening.

Microarray technology is particularly designed to fulfill the requirements of high-throughput analysis in a highly parallel fashion and to reduce volume and sample consumption. Protein and antibody microarrays can be made to encompass as many as 10 000 samples on a chip within the dimensions of a microscope slide.[19] Improvements for the immobilization and detection procedures have recently been made.[10,20−24] However, in contrast to assays performed in the classical microtiter plate format, only few different samples can be analyzed on a microarray, since microarrays are lacking true multiplexing capacity. To overcome this bottleneck, we have recently introduced a novel method allowing multiplex analysis on microarrays called multiple spotting technique (MIST).[25] Here, we show its application for the high-throughput screening of monoclonal single-chain Fv (scFv) selected by phage display against multiple antigens. By immobilization of the target proteins and transfer of the recombinant antibody fragments in a second round of spotting onto the immobilized proteins, several thousands of monoclonal antibody fragments can be screened in parallel on a single chip.

EXPERIMENTAL SECTION

Materials. Cy5 monoreactive dye packs were obtained from Amersham Biosciences Europe GmbH (Freiburg, Germany), and protein L and horseradish peroxidase-conjugated protein L were from Affitech AS (Oslo, Norway). Conjugation of the Cy5-dye to the protein L was performed as recommended by the manufacturer. ABTS was obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany), TG1 cells were from Stratagene Corp. (La Jolla, CA) and epoxy-coated glass slides from Telechem International Inc. (Sunnyvale, CA).

Methods. (1) Expression of scFv's. Expression medium was prepared containing 2YT medium with 100 μg/mL ampicillin, 4.35% (v/v) glycerol, 0.1% (w/v) glucose, and 1 mM IPTG, which allows inoculation and expression in a single step without manual interaction. Inoculation of the medium was performed with overnight cultures of TG1 cells from previous selections carrying the pIT2 vector with the genetic information of the scFv. Expression was performed either in a 10-mL batch for determination of the detection limit or in 80-μL batches using 384-well plates for the screening. The plate was sealed with an airpore tape sheet (Qiagen GmbH, Hilden, Germany) and incubated overnight at 37 °C in a shaker.

(2) ELISA. A 100-μL sample of a 10 μg/mL protein solution diluted in PBS was added in each well of a 96-well immunoassay microtiter plate (Thermo Labsystems, Vantaa, Finland). After incubation for 60 min at room temperature, the plate was washed three times with 1× PBS containing 0.1% (v/v) Tween-20 (PBS-T). A 400-μL sample of blocking solution (5% (w/v) fatfree milk powder dissolved in PBS-T) was added in each well for blocking, and the plate was incubated at room temperature for 60 min. After rinsing the wells three times with 1× PBS-T, a dilution row of the TG1 cultures was prepared in 5% (w/v) fatfree milk powder dissolved in PBS-T containing 4.35% (v/v) glycerol. A 100-μL sample of the dilution row was added and incubated for 60 min at room temperature. The wells were rinsed three times with PBS-T, and 100 μL 0.02% (v/v) of horseradish peroxidase-conjugated protein L dissolved in blocking solution was transferred in each well and incubated for 60 min at room temperature. After rinsing the wells three times with PBS-T, 100 μL of 0.5 mg/mL ABTS dissolved in a reaction buffer (25 mM sodium citrate, 25 mM citric acid, 0.05% (v/v) 30% hydrogen peroxide, pH 4.0) was added to each well and scanning of the absorption at 405 nm was performed using the SpectraMax 250 (Molecular Devices Corp., Sunnyvale, CA) at different time intervals.

The ELISA for the 384-well plate was done as described for the dilution row, with the exception that 60 μL of culture was used instead of 100 μL.

(3) Screening of Binders on Microarrays. Protein solutions of 200 μg/mL were prepared using PBS and spotted onto epoxy-coated glass slides using the QArray spotting robot equipped with 16 150-μm solid tip steel pins (Genetix Ltd., Hampshire, U.K.). After spotting, the slides were incubated overnight at 4 °C. The next morning, the slides were rinsed with TBS and blocked for 15 min in 3% (w/v) fatfree milk powder dissolved in TBS containing 0.1% (v/v) Tween-20 (TBS-T). After blocking, the slides were rinsed with TBS and spun dry at 1000*g* for 1 min in a centrifuge. The slides were placed in the exactly same position as previously into the spotting robot, and the 384-well plate containing the TG1 cultures was used as a source plate for spotting. Directly after spotting, the slides were rinsed with PBS and incubated for 30 min in 1 μg/mL Cy5-labeled protein L, dissolved in blocking solution (3% (w/v) fatfree milk powder in TBS-T). The slides were rinsed with TBS and incubated twice for 15 min in TBS-T containing 0.1% (v/v) Trition X-100. After rinsing

(17) Krebs, B.; Rauchenberger, R.; Reiffert, S.; Rothe, C.; Tesar, M.; Thomassen, E.; Cao, M.; Dreier, T.; Fischer, D.; Hoss, A.; Inge, L.; Knappik, A.; Marget, M.; Pack, P.; Meng, X. Q.; Schier, R.; Sohlemann, P.; Winter, J.; Wolle, J.; Kretzschmar, T. *J. Immunol. Methods* **2001**, *254*, 67−84.

(18) Shimba, N.; Torigoe, H.; Takahashi, H.; Masuda, K.; Shimada, I.; Arata, Y.; Sarai, A. *FEBS Lett.* **1995**, *360*, 247−50.

(19) Lueking, A.; Possling, A.; Huber, O.; Beveridge, A.; Horn, M.; Eickhoff, H.; Schuchardt, J.; Lehrach, H.; Cahill, D. J. *Mol. Cell. Proteomics* **2003**, *2*, 1342−9. Epub 2003 Sep 29.

(20) Kusnezow, W.; Jacob, A.; Walijew, A.; Diehl, F.; Hoheisel, J. D. *Proteomics* **2003**, *3*, 254−64.

(21) Angenendt, P.; Glökler, J. *Methods Mol. Biol.* **2004**, *278*, 123−34.

(22) Schweitzer, B.; Roberts, S.; Grimwade, B.; Shao, W.; Wang, M.; Fu, Q.; Shu, Q.; Laroche, I.; Zhou, Z.; Tchernev, V. T.; Christiansen, J.; Velleca, M.; Kingsmore, S. F. *Nat. Biotechnol.* **2002**, *20*, 359−65.

(23) Levit-Binnun, N.; Lindner, A. B.; Zik, O.; Eshhar, Z.; Moses, E. *Anal. Chem.* **2003**, *75*, 1436−41.

(24) Angenendt, P.; Glökler, J.; Sobek, J.; Lehrach, H.; Cahill, D. J. *J. Chromatogr., A* **2003**, *1009*, 97−104.

(25) Angenendt, P.; Glökler, J.; Konthur, Z.; Lehrach, H.; Cahill, D. J. *Anal. Chem.* **2003**, *75*, 4368−72.

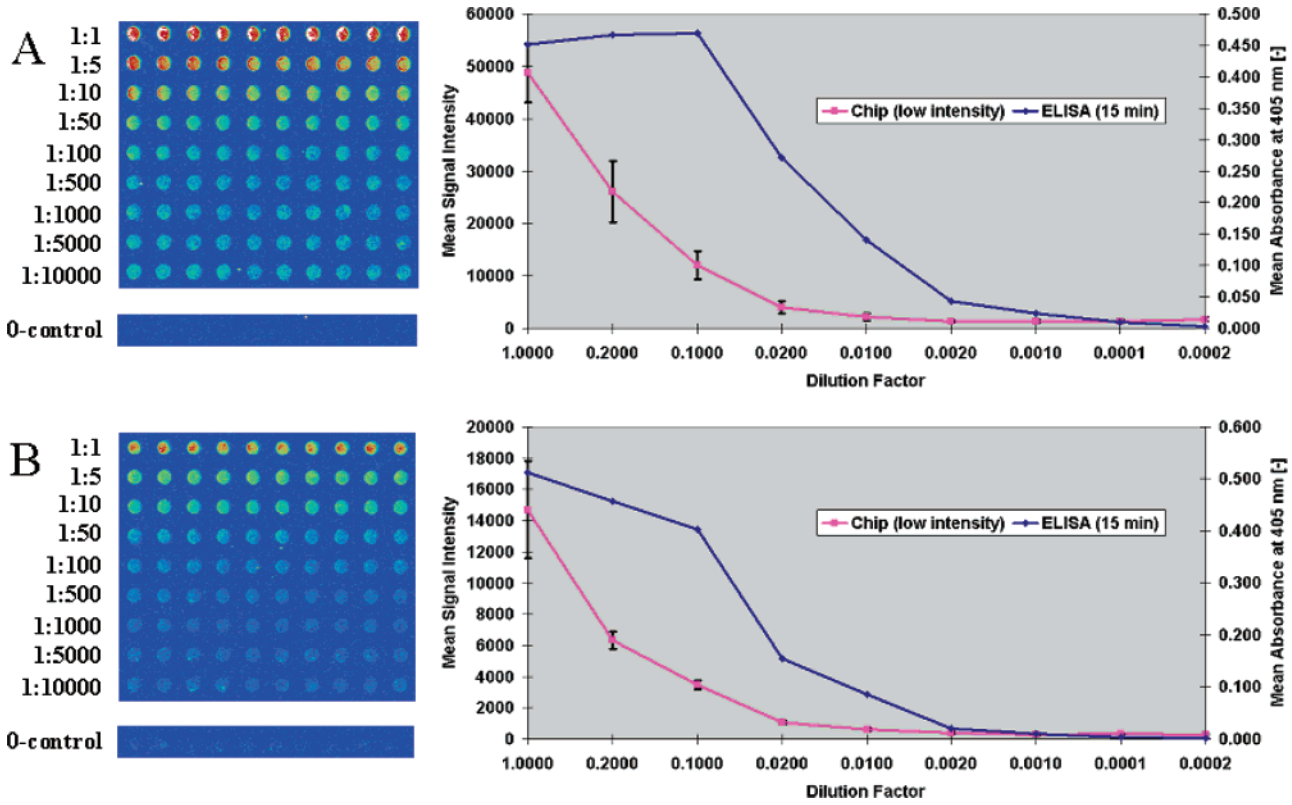

Figure 1. Dilution row of anti-polyubiquitin clone from position B1 (A) and E1 (B) using chip data from scanning with low intensities and ELISA data from scanning after 15-min incubation. Left: Scan of the dilution row as well as the negative control spotted in sets of 10 spots per concentration step. Numbers indicate the dilution. Right: Mean signal intensity and mean absorbance at 405 nm versus dilution factor with standard deviations for the chip data. Since the ELISA data were obtained from duplicates, no standard deviations were calculated for the ELISA data.

with TBS, the slides were spun dry at 1000*g* for 1 min in a centrifuge and scanned using the ScanArray 4000 (Perkin-Elmer Life Sciences, Wellesley, MA). GenePix Pro 4.1 software (Axon Instruments Inc., Union City, CA) was used to analyze the scan data.

For the dilution row microarray, dilution rows of the TG1 cultures were prepared in 5% (w/v) fatfree milk powder, dissolved in PBS-T containing 4.35% (v/v) glycerol and spotted as described onto the immobilized polyubiquitin.

RESULTS AND DISCUSSION

To ensure proper scFv production in TG1 cells and to assess the detection limit of the new technique in comparison to ELISA, an overnight culture of two monoclonal anti-polyubiquitin scFv's was prepared in expression media. During the incubation of the culture, a uniform concentration of 0.2 μg/mL polyubiquitin was spotted onto epoxy-activated slides, which were blocked after incubation at 4 °C. A dilution series of the overnight culture was prepared and spotted in sets of 10 spots per concentration onto the immobilized polyubiquitin. As a negative control, undiluted culture was also spotted onto sections of the slide, which did not contain any polyubiquitin but blocked surface. After incubation with Cy5-labeled protein L and subsequent washing, the slides were scanned at two different intensities. The scans were analyzed, and the negative control was subtracted. To allow direct comparison of the data, the same cultures were subjected to ELISA and screened in duplicates. To display the dynamic range of both techniques and allow comparison, the ELISA plates were scanned after 15 min and after overnight incubation at room temperature. After analysis and subtraction of the negative control values, two plots of signal intensity/absorption versus dilution were drawn for each clone containing both chip data and the ELISA data. One plot was generated with the data of the low-intensity scan of the chip and the data from the 15-min incubation of the ELISA plate (Figure 1), and one plot was drawn from the high-intensity scanning and the overnight incubation (Figure 2).

Both figures display the feasibility of the new technique for the screening of recombinant antibody fragments. Moreover, the figures show comparable detection limits and an equal dynamic range of the microarray experiment in comparison to ELISA. This is remarkable, since the principle of ELISA relies on the enzymatic amplification of the signal, while the microarray-based approach applies fluorescently labeled protein L. The experimental setup also indicates additional advantages of the microarray approach in comparison to the ELISA. The transfer of the assay from the ELISA plates to the microdroplets allows us to omit the incubation time, in which the scFv usually bind to their antigen. Due to the little diffusion possibilities in ~300 pL, the time required for spotting is sufficient to allow the establishment of an accelerated equilibrium within the droplet. Moreover, the microarray-based approach is advantageous, since the dynamic range of the ELISA data is dependent on the incubation time, while the dynamic range of the chip can be freely adjusted by the settings for the laser power and the photomultiplier range. This is especially important due to the diverse signal levels of the clones, which depend on

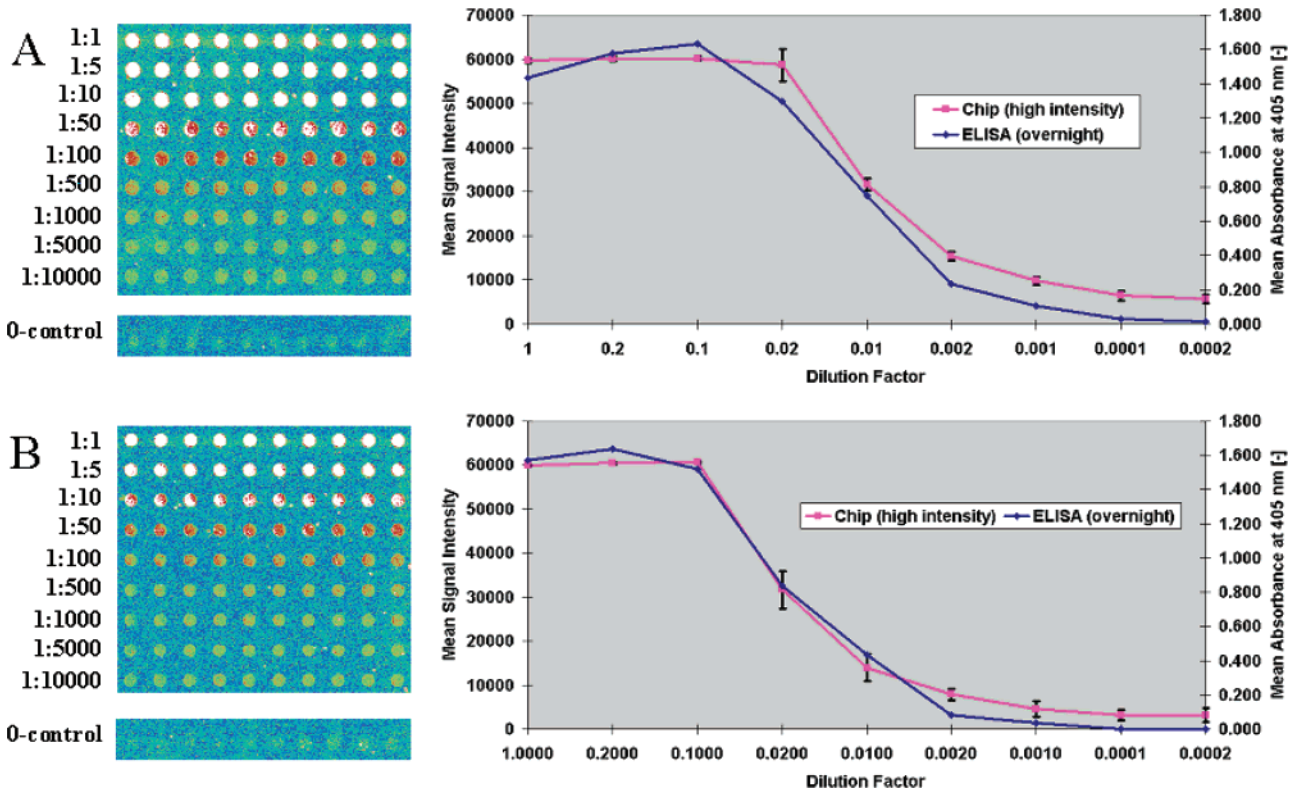

Figure 2. Dilution row of anti-polyubiquitin clone from position B1 (A) and E1 (B) using chip data from scanning with high intensities and ELISA data from scanning after overnight incubation. Left: Scan of the dilution row as well as the negative control spotted in sets of 10 spots per concentration step. Numbers indicate the dilution. Right: Mean signal intensity and mean absorbance at 405 nm versus dilution factor with standard deviations for the chip data. Since ELISA data were obtained from duplicates, no standard deviations were calculated for the ELISA data.

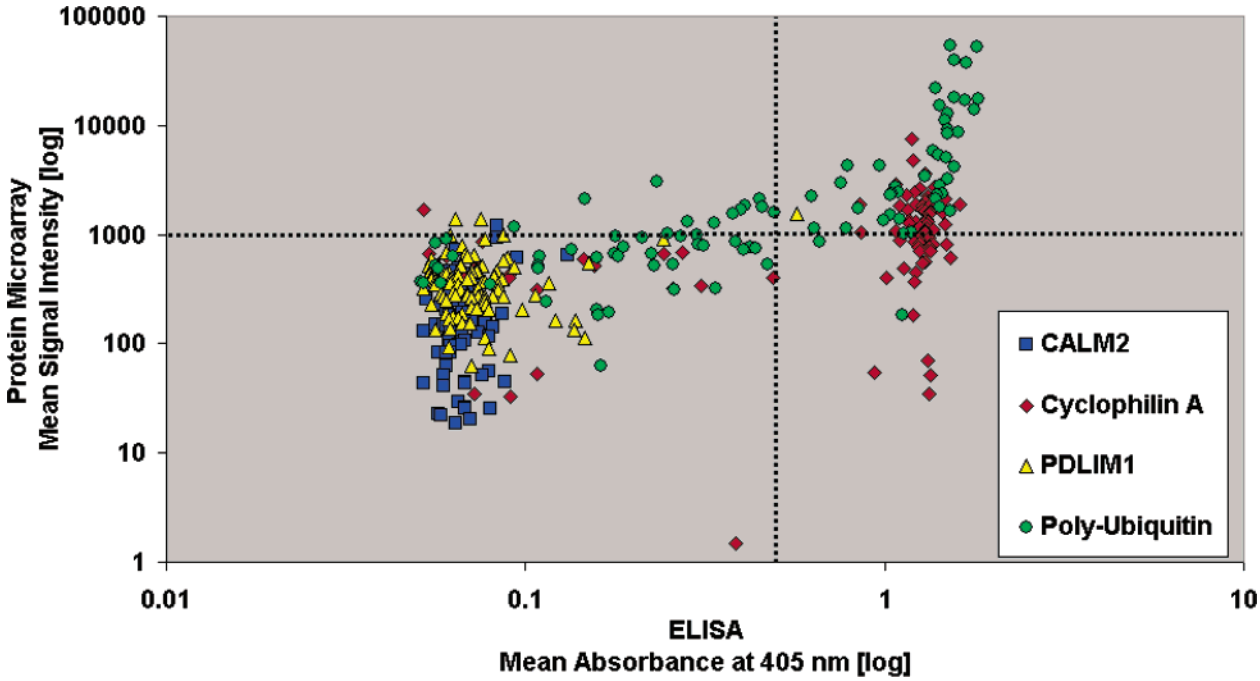

Figure 3. Scatterplot of mean signal intensity of the microarray data versus mean absorbance at 405-nm data from the ELISA. Four selections comprising 96 monoclonal scFv's were screened in parallel. Two dashed lines indicating microarray signal intensities above 1000 and ELISA absorbance values above 0.5 were introduced to allow easy localization of clear signals.

the overall success of each selection. Furthermore, the microarray approach allows the scanning at different intensities to enlarge the dynamic range and maintains the possibility to rescan the slide after analysis. Since the detection of weakly binding scFv's may require overnight incubation, the microarray approach is a much faster alternative due to the laser scanning procedure. Figures 1 and 2 also demonstrate the reliability of data from the chip assay revealing low standard deviation values. ELISA graphs are inserted

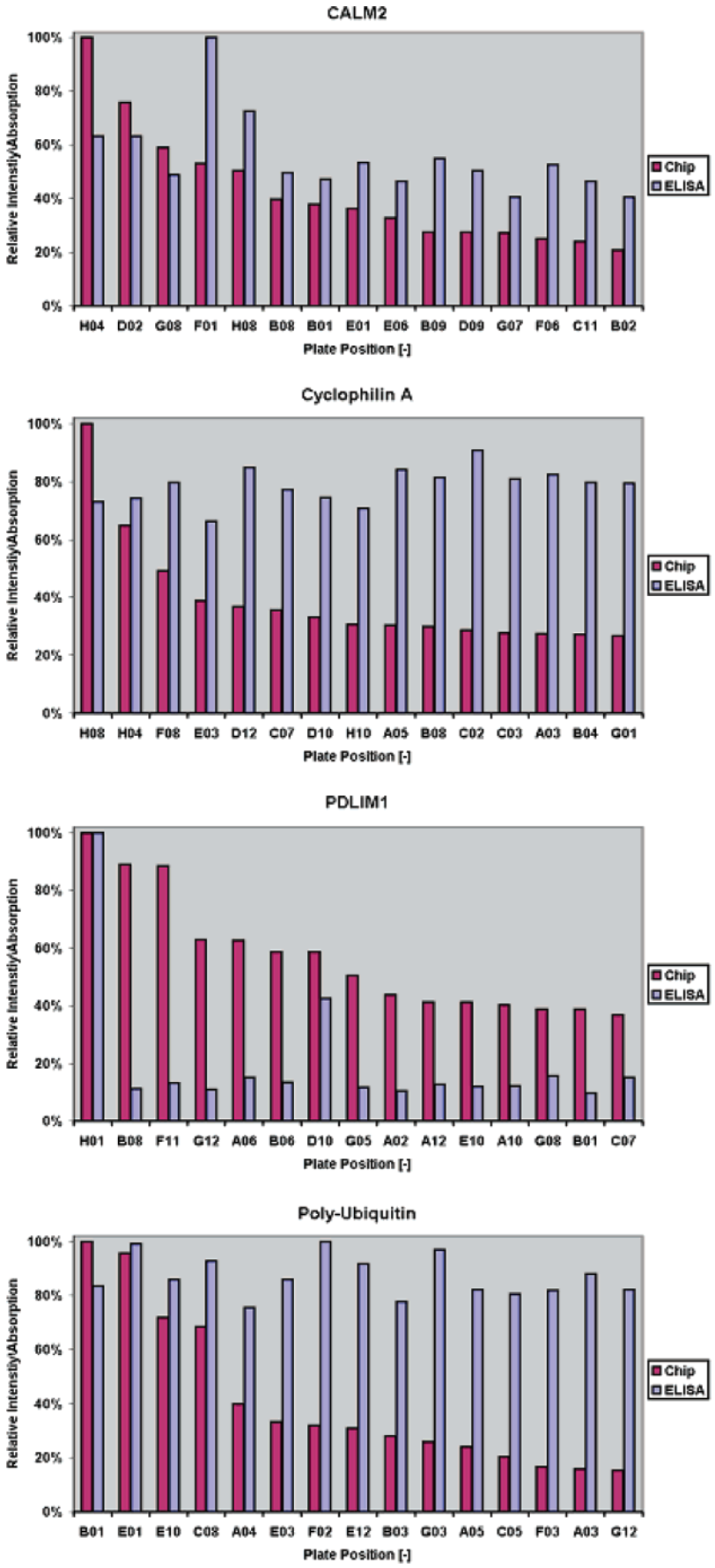

Figure 4. Ordered graphs of relative intensity/absorption of the best 15 binders for each protein. Ordering was done according to the signal intensity obtained from the microarray data.

for comparison showing mean values only.

To assess the feasibility of the technique for the selection of a multitude of recombinant antibody fragments, four sets of 96 monoclonal antibody fragments were screened, all originating from the fourth selection round of an automated phage display selection against targets of varying size. Each set was spotted in quadruplicate on its respective target and on a blocked microarray surface as a negative control. In parallel, the cultures were subjected to an ELISA to allow a comparison of the microarray-based technique to the traditional ELISA technique. Both approaches were analyzed, and the values for the negative controls were subtracted. A scatterplot of microarray data versus ELISA data was generated from all four selections (Figure 3). Additionally, a graph for the direct comparison of both experiments for the 15 best binders on the chip was drawn, to display the capability of the technique to reduce the amount of clones to be screened (Figure 4).

Figure 3 demonstrates the practicability of the new approach for the characterization of recombinant antibody fragment selections of varying qualities. While the polyubiquitin and the cyclophilin A selections provide sets with a large proportion of highly binding fragments, the PDLIM1 and CALM2 selections yield few good binders. The success of the selection depends on several factors, such as the diversity of the phage library or the format of target molecule. The latter can be observed in the selection of the polyubiquitin. While CALM2, PDLIM1, and cyclophilin A provide a monomer for the selection, the polyubiquitin consists of three ubiquitin moieties and hence increases the proportion of strong binders due to high avidity.

To allow easy categorization of the signals, two dashed lines were introduced into the scatterplot to discriminate microarray signal intensities above 1000 and ELISA absorbance values above 0.5. The majority of the clones are in the top right or bottom left quadrant, indicating the general trend of increasing signal intensities on the chip with increasing signal intensities in ELISA. However, some clones, for example, from the cyclophilin A selection, show high binding capacity in ELISA but not on the chip. Reasons for this may be the less favorable environment of the chip in contrast to ELISA. ELISA provides a comparably gentle environment, in which the proteins are predominantly kept in the liquid phase, while the microarray approach may destabilize the binders by mechanical forces, due to the second spotting step, in which the scFv's are applied by contact printing onto the immobilized protein. Moreover, the large surface-to-volume ratio of the droplets may influence the binding characteristics of some of the more sensitive scFv's. Nevertheless, such an additional selection according to stability as performed on the chip may be desirable, since the strong-binding scFv's that will be gained from such selections should be applicable to a large variety of applications with different demands with regard to stability. Additionally, it is important that none of the clones provided intense false positive signals in the chip experiment, which were not observed by ELISA. This congruence is obvious in the PDLIM1 selection, in which one single specific clone was identified, which is identical in both ELISA and on the chip. This confirms the reliable screening of large sets of clones on the chip surface. To demonstrate this consistency, diagrams displaying the results of both techniques for the 15 best-binding clones on the chip were generated for all selections (Figure 4). The diagrams confirm that the new technique can be used for the prescreening of clones from selections, after which a subset of the best binders from each selection can be rearrayed for further studies.

The reliability combined with the ease of handling and the small time requirement makes the new technique excel current solutions with regard to throughput and flexibility.[19,26] It allows the easy generation of several replicates on the chip and consumes very little reagents. Moreover, the cell culture plates that were used as source plates for the second spotting step can be used for the next step of the selection procedure, the rearraying of the strong-binding subsets. The technique does not require the transfection of the phagemid into the highly expressing HB2151 strain but works well with the TG1 strain used in the panning process. This allows the streamlining of this technique into the selection process and reduces manual interaction steps. Other advantages include the composition of the media, which includes both glucose for maintenance of the plasmid during growth and IPTG for the induction of expression, once the glucose is metabolized. This eliminates the manual addition of small proportions of IPTG to the cell cultures and promotes automation.

CONCLUSION

The secondary spotting of scFv directly from culture supernatants on the immobilized ligands facilitates the identification of positive clones using the multiple spotting technique. While most of the binding activity is preserved, the technique displays comparable sensitivity to the ELISA approach and requires only a single detection molecule like fluorescent protein L for quantification instead of the separate labeling of all antigens. This opens new applications of scFv's on microarrays, which were previously limited by the lack of binding activity of immobilized scFv's on chip surfaces. The positive clones can be identified, rearrayed, and further assayed simultaneously against a multitude of antigens for specificity by MIST. Once the specific clones are identified, the scFv's complementarity determining regions can be subcloned into another vector providing a more stable scaffold such as Fab[27] for applications such as antibody microarrays, in which stability is required. In our study, the screening has succeeded to yield most of the clones valuable for further investigations. The main advantage besides the involved robotics for high-throughput screening is the minute amount of antigens, consumables, and time necessary to screen thousands of samples in parallel. In light of already existing examples of the MIST, this study shows that many new applications for MIST may be found in the future just by introducing novel protocols.

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(26) Michaud, G. A.; Salcius, M.; Zhou, F.; Bangham, R.; Bonin, J.; Guo, H.; Snyder, M.; Predki, P. F.; Schweitzer, B. I. *Nat. Biotechnol.* **2003**, *21*, 1509−12. Epub 2003 Nov 9.

(27) Tordsson, J. M.; Ohlsson, L. G.; Abrahmsen, L. B.; Karlstrom, P. J.; Lando, P. A.; Brodin, T. N. *Cancer Immunol. Immunother.* **2000**, *48*, 691−702.