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Bioanalysis

Miniaturization of multiplexed planar recombinant antibody arrays for serum protein profiling

Background: Antibody-based microarrays are a developing tool for high-throughput proteomics in health and disease. However, in order to enable global proteome profiling, novel miniaturized high-density antibody array formats must be developed. **Results:** In this proof-of-concept study, we have designed a miniaturized planar recombinant (single-chain Fragment variable). antibody array technology platform for multiplexed profiling of non-fractionated, directly labelled serum samples. The size of the individual spot features was reduced 225-times (78.5 μm^2/spot) and the array density was increased 19-times (38,000 spots/cm^2). These miniaturized, multiplexed arrays were produced, using a desktop nanofabrication system based on dip-pen nanolithography technology, and interfaced with a high-resolution fluorescent-based scanner. The reproducibility, sensitivity, specificity, and applicability of the set-up were demonstrated by profiling a set of well-characterized serum samples. **Conclusion:** The designed antibody array platform opens up new possibilities for large-scale, multiplex profiling of crude proteomes in a miniaturized fashion.

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Background

Antibody-based microarray is an established technology within high-throughput proteomics with proven capability of delivering protein expression profiles, or protein maps, of complex proteomes in both health and disease [1–4]. Classical antibody microarrays, composed of up to several hundred different antibodies, displaying 18 × 10^3 μm^2 (Ø ~150 µm) sized spot features and an array density of ≤ 2,000 spots/cm^2, have so far been produced [2,3]. Considering the size and complexity of the human proteome [5], the classical microarray designs will, however, not be able to harbor the number of probes required (>20,000 probes/array) to perform global proteomics, demonstrating the need for novel, miniaturized high-density array designs [2,6].

To this end, the first generations of conceptual antibody (protein) nanoarrays, based predominantly on planar-, well-, nanowire-, and cantilever-based layouts, have been generated [7–17]. In these efforts, the possibilities to produce miniaturized nanoarrays, with <0.8 μm^2 (Ø < 1 µm) sized spot features, at even high density (>100,000 spots/cm^2) were outlined. But despite the success, these nanoarray set-ups are associated with several key technical constraints. First, according to the ambient analyte theory [18], miniaturizing the spot diameter below 1 µm is likely to offer little or no assay benefit, and reducing the spot size beyond a certain limit will in fact impair assay sensitivity [19]. Second, the set of technologies at hand for producing high-density antibody nanoarrays have not been designed for multiplexing, that is, functionalizing the individual features with different antibodies [6–17,20–22]. Consequently, the current antibody nanoarray layouts have been produced by functionalizing all features with the same antibody, resulting in mainly 1-plex arrays. Third, methodologies for sensitive sensing of nano-sized arrays have so far only been established for low-density array layouts [9]. Fourth, the availability of a renewable antibody source,

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Key terms:

Proteome
All proteins present in a given sample at a given time-point.

Antibody microarray
- Miniaturizued assay in which a set of antibodies are deposited one-by-one onto a solid support in an array pattern, and used to detect the corresponding antigens.

Dip-pen nanolithography
- Technology in which the tip of an atomic force microscope cantilever is used as a 'pen' to write (print) ink, for example, protein onto a surface.

Multiplexed assay
- One assay used to detect several (>1) analytes at the same time.

scFv antibody
- Fusion protein of the variable regions of the heavy (VH) and light chains of antibodies capable of specifically binding antigen.

with proven high on-chip performances, for large-scale profiling represents a major issue [2]. Hence, additional efforts will be required to establish a viable miniaturized high-density antibody array technology platform.

Recently, a desktop tool designed for multiplexed deposition, simultaneously printing of up to 12 different proteins, with individual features in the 0.78 μm^2/spot (Ø 1 µm) to 78.5 μm^2/spot (Ø 10 µm) size range (size-dependent on tip-surface contact-time), based on dip-pen nanolithography (DPN) technology was presented [23]. It is noteworthy that sub-micron sized spot features (Ø 10 µm) would still be compatible with both high-density array layouts (< 510,000 spots/cm^2) and fluorescence-based sensing of high-density arrays using conventional, high-resolution scanners. In fact, fluorescence is by far the most commonly used read-out system for conventional antibody microarrays [24] (for review see [2]). Interestingly, the viability of DPN-generated arrays visualized using fluorescence-based sensing was recently indicated for a 1-plex monoclonal antibody array [25].

In this discovery study, we have designed the first miniaturized planar recombinant antibody array technology platform, with significantly reduced spot features (78.5 μm^2/spot) and increased array density (38,000 spots/cm^2), for multiplexed assay of crude, directly labelled serum samples. To this end, we uniquely combined a probe source based on a renewable, large recombinant single-chain Fv (scFv) antibody library, microarray adapted by molecular design [2], with the next generation of a DPN®-based desktop tool, NanoArrayer 3000, interfaced with a high-resolution fluorescent-based scanner.

Experimental section

Antibodies

Twenty-eight human recombinant scFv antibody fragments, directed against eight human complement proteins (CPs), eight human cytokines, and one non-human serum protein (Supplementary Table 1S), were stringently selected from a large phage display library, as previously described [26].

Production & purification of scFv antibodies

All scFvs were produced in *Escherichia coli* and purified from expression supernatants using affinity chromatography on Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany) [27]. The degree of purity and integrity of the scFvs were evaluated using 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA). The protein concentrations were determined by measuring the absorbance at 280 nm (average concentration 0.72 mg/ml, range of 0.18–1.72 mg/ml). The specificity, affinity (1–10 nM range), and on-chip functionality of the phage-display derived scFvs were ensured by using: stringent selection protocols [26]; multiple clones (one to four) per target molecule; and/or a scFv library microarray adapted by molecular design [2,28]. The specificity of several scFv antibodies have previously been validated using well-characterized, standardized serum samples, and orthogonal methods, such as mass spectrometry, ELISA, Meso Scale Discovery assays, cytokine bead arrays and/or microarray spiking and blocking experiments (Supplementary Table 1S) [4,27–31] [Unpublished Data; Wingren *et al.*].

Serum samples & antigen

In total, six well-characterized (de-identified) human serum samples, including a large pool of normal human sera (NS), and five complement-deficient serum samples, that is, samples with known significantly altered levels of one or several CPs were obtained from the Institute of Laboratory Medicine, Section of Microbiology, Immunology and Glycobiology, Lund University (Sweden; Supplementary Table 2S). It is noteworthy that NS is used as a validated reference in the daily routine analysis of CPs in clinical samples. The levels of CPs in the complement-deficient sera were determined using electro immunoassay (rocket immunoelectrophoresis) and/or turbidometry (C3 and C4). The analytical range of these methods ranged from 1–6% up to 400% of the normal serum concentration of the protein (electro-immunoassay), and 0.05–20 g/l (C3) or 0.01–4 g/l (C4) (turbidometry). Purified C1q were purchased from ElectraBox Diagnostica (Tyresö, Sweden). Cholera toxin subunit B (CT), originating from *Vibrio cholerae*, was purchased from Sigma (St. Louis, MO, USA).

Labeling of samples

The serum samples (~90 mg/ml) were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) according to previously optimized labeling protocol for serum proteome [27,31]. CT was spiked into the serum samples to a final concentration of 280 nM. The serum samples were labeled at a molar ratio of biotin: protein (15:1) assuming an average molecular weight of 50 kDa for the serum proteins [32]. Unreacted biotin was removed by extensive dialysis against PBS (pH 7.4) for 72 h using a Slide-A-Lyser (MWCO 3.5 kDa) (Pierce). The samples were aliquoted and stored at -20°C until further use.

C1q was labeled with Alexa-Fluor-647 at a molar ratio of 10:1 dye: protein, according to the recommendations of the manufactures (Invitrogen). Briefly, $NaHCO_3$ (pH 8.5) was mixed with 10 µl C1q (1 mg/ml) to a final concentration of 0.1 M. Alexa-Fluor-647 (Invitrogen) was added to the protein mixture to a final concentration of 0.02 mM. The sample was first incubated on ice for 5h, with careful mixing every 20 min, and then dialyzed against 40% (v/v) glycerol in PBS (pH 7.4) for 72h using a Slide-A-Lyser (MWCO 3.5 kDa) (Pierce). The samples were aliquoted and stored at -80°C until use.

Production & handling of scFv arrays

The scFv arrays were produced using a desktop nanofabrication system, NanoArrayer 3000, based on DPN technology (NanoInk Inc, Skokie, IL, USA). Prior to dispensing, the tips, DPN Probes Type M-ED Side M2 with 12 modified "A frame" cantilevers (DPN pen) (NanoInk Inc.), were plasma cleaned for 1 min at low RF-value, using a gas mixture of Oxygen/Argon (20%/80%) at 1.4 bar using a Plasma Cleaner (PDV-002) (Harrick Plasma, Ithaca, NY, USA). The antibodies (0.1 to 0.5 mg/ml) where diluted in printing buffer (NanoInk Inc.), and Alexa-Fluor-555 cadaverine disodium salt (Invitrogen) was added to a final concentration of 56 µg/ml (position marker). Then, 400 nL of the antibody printing buffer solutions were loaded into inkwell cartridge (NanoInk Inc.) and the DPN probes were subsequently dipped into these antibody solutions by the NanoArrayer 3000. The scFvs were printed on black polymer Maxisorb (NUNC A/S), Roskilde, Denmark) or Nexterion Slide E (Schott AG, Mainz, Germany) and the dispensing was performed at ambient temperature and humidity. Eight replicates of each scFv were arrayed to ensure adequate statistics. Each slide could hold 18 sub-arrays arranged in a 3 × 6 format (to fit the slide module assembly used, see below), where each 12-plexed sub-array was functionalized with nine specific scFv antibodies, one control scFv antibody, one positive control (Streptavidin-alexa647), and one negative control (PBS). In total, three 12-plexed array layouts denoted A to C were generated (Supporting Information Table 3S). The produced scFv antibody array slides were then placed in a slide module assembly (NanoInk Inc) and dried over night at room temperature. Next, the arrays were blocked with 5% (w/v) fat-free milk powder (Semper AB, Sundbyberg, Sweden) in PBS for 1 h. All incubations were performed at room temperature. The arrays were washed three times with 0.05% (v/v) Tween-20 in PBS (PBS-T) and then incubated with 60 µl biotinylated serum sample or directly labeled C1q (0.046 ng/ml to 460 ng/ml) for 1 h. All serum samples were diluted 1:10 (resulting in a total serum dilution of 1:450, corresponding to approximately 0.2 mg/ml) in 1% (w/v) fa- free milk powder and 1% (v/v) Tween-20 in PBS (PBS-MT). Then the arrays were washed three times with PBS-T. Any subarrays incubated with biotinylated serum samples were incubated with 1 µg/ml Alexa-647 conjugated streptavidin (Invitrogen) diluted in PBS-MT for 1 h. Finally, the arrays were washed three times with PBS-T and one time with PBS and directly dried under a stream of nitrogen gas and immediately scanned. To evaluate the printing efficiency, 0.024 mg/ml alexa647-labelled BSA (Saveen-Werner, BDH Chemicals (Poole, Great Britain) were printed in eight replicates per sub-array, including 81 sub-arrays on seven slides.

Analysis of scFv arrays

The arrays were scanned using a high-resolution fluorescent scanner, InnoScan®900 (Innopsys, Carbonne, France) at three different scanning setting (Detection gain at 635nm: 15, 50 and 100%). The slides were loaded and scanned one by one. All arrays were scanned with 1 µm/pixel resolution (the resolution, a factor of two above the maximum resolution, was selected to best match the diameter of the spots) and the Mapix software (V.4.6.2) (Innopsys) was used to quantify the mean intensity of each spot using the fixed circle method, a standard approach for quantifying array spots. The method applies user defined parameters of the spot diameter (10 µm) and background diameter (spot diameter plus 1 pixel), in that. background is measured locally outside each spot. The local background was then subtracted from the spot intensity and to compensate for possible local defects, the highest and the lowest signal intensities were automatically excluded [2,4], and each data point represents the mean value of the remaining six replicates. For protein analytes displaying saturated signal, values from lower scanning settings were chosen. The LOD was defined as the analyte concentration corresponding to a signal 2× SD above the negative control. The

dynamic range was defined as the signal intensity that lies between 2× SD above the negative control and below the maximum (saturated) signal.

Results & discussion

In this proof-of-concept study, we have designed and evaluated miniaturized planar recombinant antibody arrays for multiplexed profiling of crude serum samples. To this end, 27 human recombinant scFv antibodies, targeting 17 high- (μM to nM range) and low-abundant (pg/ml) serum analytes, were used as probes (Supporting Information Table 1S). Six well-characterized serum samples, with known expression levels ofeight complement proteins, were used as model system (Supporting Information Table 2S). It should be noted that the probes were selected from a phage display library, microarray adapted by molecular design, thus representing a large, renewable probe source with proven on-chip performances [2,28]. Early studies have outlined the risk of using off-the-shelf readily available antibodies, not designed and/or optimized for the application, showing that only a fraction of the commercially available polyclonal and monoclonal antibodies displayed adequate on-chip performances as probes [33,34].

First, 18 miniaturized sub-arrays, organized into a 6 × 3 layout to match the footprint of the 18-well reaction gasket, were printed per slide using the DPN®-based desktop tool, NanoArrayer 3000 (Figure 1A). In each sub-array, 12 different proteins (reagents) were simultaneously deposited eight-times, resulting in 12 × 8 (no. of reagents × no. of replicates) patterns. Notably, the multiplexity of the sub-arrays could be increased in incremental steps of 12 by loading the printer with a new 12-DPN pen and a 12-inkwell prepared with the next set of reagents. In addition, we have ongoing work indicating the capability of using 48-DPN pens/48-inkwells (exploratory designs) for simultaneous deposition of 48 different proteins (reagents), thus generating 48-plex arrays and multiples thereof [Unpublished Data, Petersson *et al.*,]. In comparison, standard ink-jet printers used to produce conventional antibody microarrays (18×10^3 μm^2 (Ø ~150 μm) sized spots) deposit the proteins, either one-by-one or up to eight simultaneously, depending on the printing mode capability and number of nozzles, and the multiplexity is increased by washing the nozzles and reloading them with new reagents.

The 12 × 8 arrays were generated with 78.5 μm^2 (Ø 10 μm) sized individual spots, orientated at a generous pitch-to-pitch distance of 40 × 66 μm (Figure 1A) (with 14 × 14 μm as smallest cut-off limit). Hence, an array density of ~38,000 spots/cm^2 was obtained, with ~510,000 spots/cm^2 representing the upper practical limit. This set-up should represent a sufficiently dense array layout even for large-scale discovery efforts. In comparison, we have previously on a regular basis generated and applied conventional recombinant scFv antibody microarrays, displaying 18 × 10^3 μm^2 (Ø ~150 μm) sized spot features and an array density of <2,000 spots/cm^2 [4]. Hence, we have generated a miniaturized recombinant antibody microarray set-up for which the size (area) of the individual spot features was reduced 225 times and the array density was increased at least 19 times. In this context, it might be of interest to note that we have previously generated recombinant antibody nanoarrays with even smaller spot features (≥0.03 μm^2/spot, and ≥ Ø 0.2 μm) [14,15], but these 1-plex arrays all suffered from the key technical constraints outlined above (see Introduction).

The spot morphology (shape and homogeneity) was found to be adequate as judged by visual inspection, as illustrated in Figure 1A. The spot diameter was evaluated for ten scFv antibodies printed in eight replicates per sub-arrays, including 108 sub-arrays on six slides, after the capture of biotinylated targets, thus incorporating printing efficiency of functional scFv antibodies into the measurements. The results showed that the spot diameter ranged between 8.3 and 10.2 μm, to some extent reflecting antibody concentration (data not shown), giving a CV of 10.9%. Furthermore, we also printed directly labelled BSA to study the printing efficiency *per se*. To this end, labelled BSA was printed in eight replicates per sub-array, including 81 sub-arrays on seven slides. The spot morphology (shape, homogeneity) was found to be adequate (visual inspection) with consistent spot signal intensities (CV of 12%). Hence, the performance of the printing process was found to be adequate.

In the printing process, two planar slides, known to perform well in conventional antibody microarrays [31,35], including black polymer Maxisorb slides and Nexterion slide E, were evaluated as support. The advantage of using a simple planar surface is that the need for more elaborate surface architectures, e.g. nanowires [13], that in turn also would determine (limit) the array layout *per se*, could be bypassed. While the Maxisorb slide was found to be too soft to be printed on, creating indentations and heterogeneous spots (Supplementary Figure 1S), adequate spot morphologies, low non-specific background binding, and dynamic signal intensities were observed for sub-microarrays on Nexterion slides (Figure 1A). Hence, Nexterion slide E was used as support throughout the remaining part of the study.

Next, the technical reproducibility of the entire set-up was evaluated by repeatedly analyzing replicates of crude, biotinylated serum samples, and determining

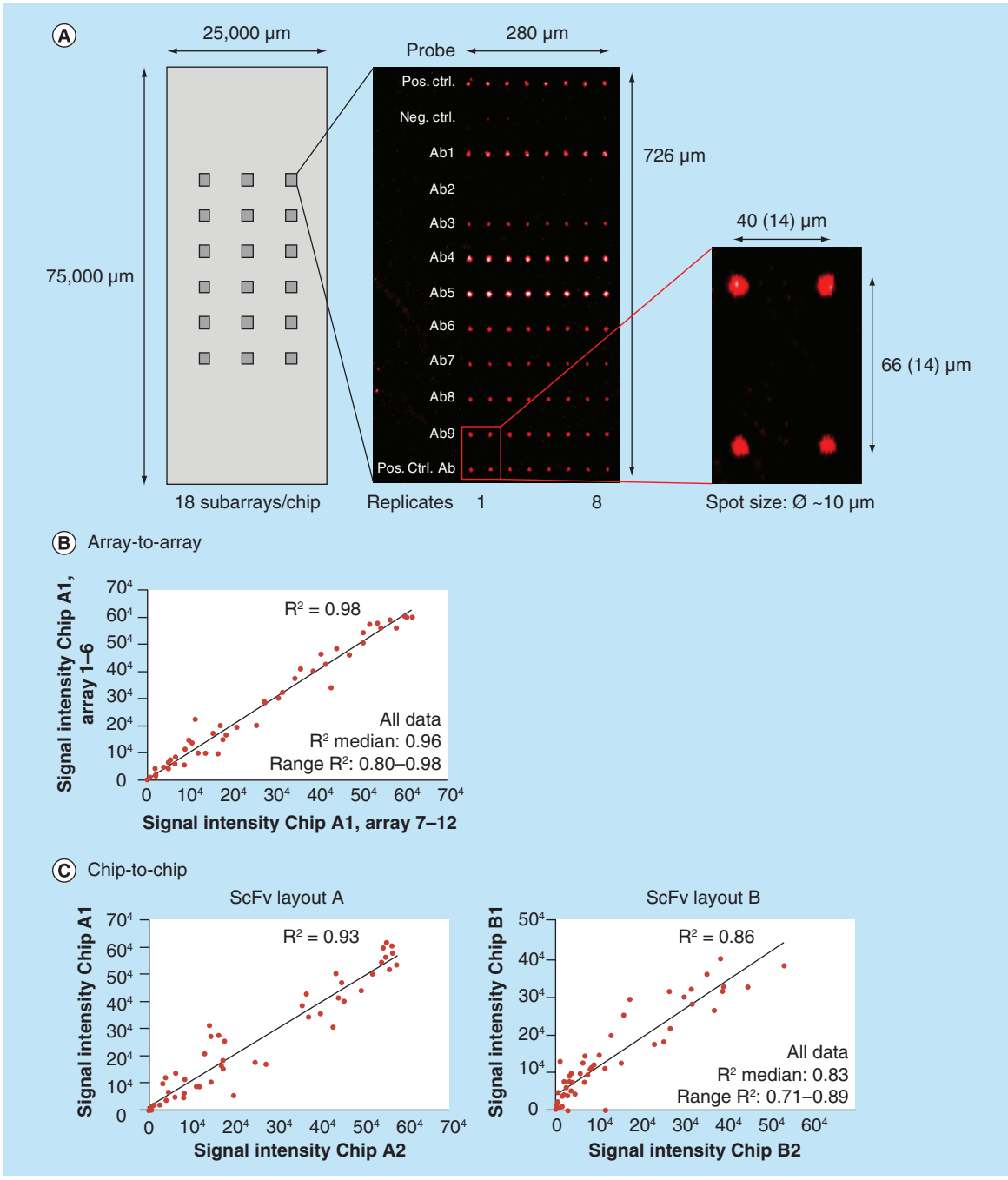


Figure 1. The miniaturized recombinant antibody array set-up. (A) The miniaturized set-up. **(B)** Array-to-array reproducibility: a representative correlation plot, illustrated for the data associated with array layout A. **(C)** Chip-to-chip variability: two representative correlation plots for data associated with array layouts A and B are shown. ScFv: Single-chain Fv.

the: spot-to-spot variation; array-to-array variability; and chip-to-chip variability. The data was reported in terms of CV value or coefficient of determination (R^2). First, the spot-to-spot reproducibility was assessed for all 27 scFv antibodies, deposited in eight replicates/array, by analyzing six serum samples on 83 representative arrays (layout A - 24 arrays on 2 slides, layout B - 48 arrays on 4 slides, and layout C - 11 arrays on 1 slide) (Supporting Information Table 3S). The results showed that a mean CV value of 12% (range 5 to 27%) (by omitting one antibody clone the range was reduced to 5 to 21%) was obtained. In comparison, the established and (more) optimized conventional antibody microarrays regularly display a spot-to-spot variability of <10% (recombinant antibody microarrays) [36] and <20% (polyclonal and monoclonal antibody micro-

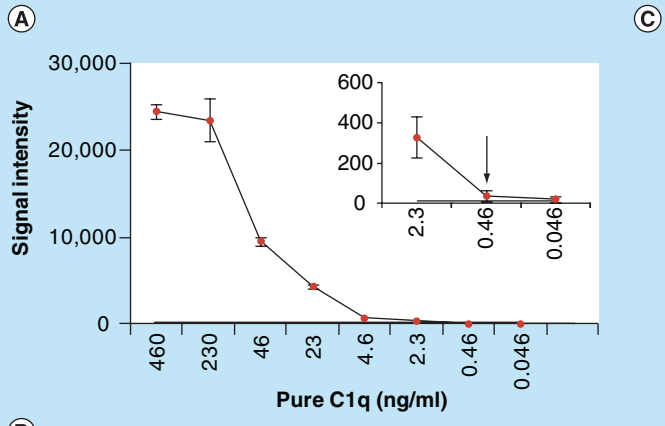
arrays [37,38]. In the case of antibody nanoarrays, the reproducibility is a key critical topic (often < 30% [15]) that still remains to be fully demonstrated [7–17,25].

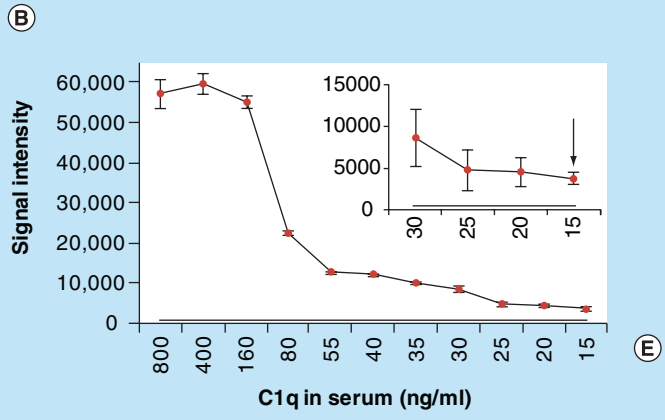
Second, the array-to-array variability, i.e. samples analysed on different sub-arrays, but on the same slide, was investigated for 18 scFv antibodies, deposited in eight replicates/array, by analyzing six serum samples on 72 representative sub-arrays (layout A - 24 arrays on 2 slides, and layout B – 48 arrays on 4 slides). The results showed that a median R^2-value of 0.96 (range 0.80–0.98) was obtained (Figure 1B). A representative correlation plot, illustrated for the data associated with array layout A, is also shown in Figure 1B. In comparison, conventional antibody microarrays regularly display R^2-values of > 0.9 (recombinant antibody microarrays) [4,36] and > 0.7 (polyclonal and monoclonal antibody microarrays) [38].

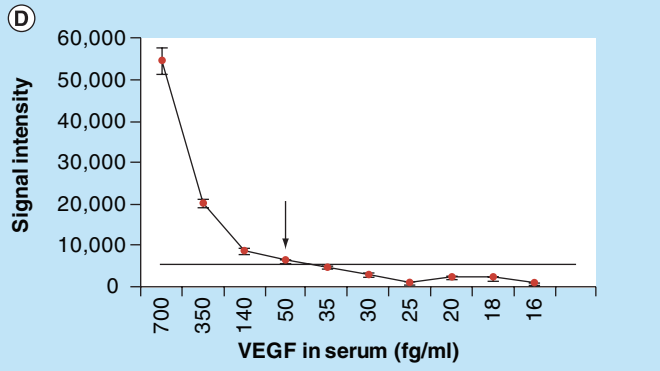
Third, the chip-to-chip variability, that is,. a sample analyzed on different sub-arrays and different slides (and different days), was assessed for 18 scFv antibodies, deposited in eight replicates/array, by analyzing six serum samples on in total 72 representative sub-arrays (layout A – 24 arrays on 2 slides, and layout B – 48 arrays on four slides). Two representative correlation plots for data associated with array layouts A and B are shown in Figure 1C. The data showed that median R^2-values of 0.93 (array layout A) and 0.83 (range 0.71–0.89) (array layout B) were obtained. As might be expected (Figure 1C &B), the R^2-values dropped somewhat when introducing additional assay variables, that is, when running the assay on different slides and days. In comparison, conventional antibody microarrays regularly display R^2-values > 0.8 [4,36,38]. Taken together, the miniaturized recombinant antibody array set-up was found to display an adequate reproducibility, and in the same range as that observed for conventional antibody microarrays [4,36–38], for review see [2].

The LOD was evaluated by analyzing serial dilutions of directly labeled sample in the format of pure antigen (C1q) and/or crude serum samples. In the latter case, a well-characterized, standardized normal serum sample (NS) was applied (Supplementary Tables 1S & 2S). LOD was defined as the analyte concentration corresponding to a signal that is 2× SD above the background signal (negative control). In the case of the complement protein (CP) C1q, LOD was found to be about 0.46 ng/ml targeting pure analyte (Figure 2A), while the LOD increased about 35 times (LOD ≤ 15 ng/ml) when addressing crude serum, NS, with known concentration of C1q (Figure 2B). Furthermore, targeting a set of additional seven CPs of á priori known concentrations in NS, the LOD was found to be 10–1200 ng/ml (Figure 2C). The fact that different antibody clones targeting the same antigen, but different epitopes, displayed different LODs is often observed [27,31], and might be explained by differences in: affinity; antibody concentration; and/or epitope masking upon biotinylation. Next, the LOD was determined for a set of nine low-abundant cytokines of estimated concentration in NS (Figure 2D & E, & Supplementary Table 1S). The results indicated that the LOD was in the range of 3–180 fg/ml. Hence, the results showed that both high- and low-abundant serum analytes could be detected in crude, directly labeled proteomes, indicating on adequate assay sensitivity well in line with what is required for affinity-proteomics-based efforts (for review see [1–3]).

The ambient analyte theory [18,19] explains the theoretical background to why the assay sensitivity should be more sensitive for miniaturized assays, such as antibody microarrays versus conventional antibody immunoassays. It also postulates that miniaturizing the spot diameter below 1 µm is less likely to provide any additional assay benefit (set-up dependent), and the assay sensitivity will in fact be impaired rather than improved if the spot size goes below a certain limit (set-up dependent) [19]. To investigate the impact of our miniaturizing on the LOD, we first compared the LOD for our miniaturized recombinant antibody arrays (spot size - 78.5 μm^2 and Ø 10 µm) with that of our previously developed conventional recombinant antibody microarrays (spot size – 18 × 10^3 μm^2 and Ø ~150 µm) [27,31] (Figure 2C). These set-ups are as identical as possible (e.g., using the same recombinant antibody clones), essentially differing only in spot size and choice of solid support. The results showed that the LOD was two- to 500-times worse (reduced) by miniaturizing the spot area 225-times (78.5 vs. 18 × 10^3 μm^2) and spot diameter 15-times (Ø 10 vs. 150 µm). The LOD for other high-end conventional antibody microarray set-ups, for example, [24,39] (not as directly comparable due to more substantial differences in assay set-up, such as different antibodies (both format and clones), different sample labelling protocols etc) are also high (pM range over fg/ml range), enabling truly low-abundant analytes, such as cytokines, to be targeted. Hence, also compared with those platforms, the same trend of reduced LOD was indicated. The observation that the miniaturization did not lead to an improved assay sensitivity in general might be explained by that: different fluorescence-based scanners and accompanying softwares were used (InnoScan®900 (Innopsys, Carbonne, France vs. Scan Array Express, PerkinElmer Life & Analytical Sciences, Wellesley, MA, USA); different solid supports were used; we compared the 1th generation of miniaturized arrays with a highly optimized conventional microarray set-up [27,31,36]; and/or we have reduced the spot size beyond the opti-







C

ScFv	LOD (ng/ml)	LOD conventional recombinant Ab microarrays (ng/ml)†
C1q	≤15	0.2
C1s	50	6.1
C3 (1)	1200	2.4
C3 (2)	240	n.d.
C3 (3)	170	n.d.
C3 (4)	170	n.d.
C4 (1)	400	42
C4 (2)	100	n.d.
C4 (3)	80	n.d.
C4 (4)	140	n.d.
C5 (1)	35	1.6
C5 (2)	35	n.d.
C1-INH (1)	80	1.0
C1-INH (2)	170	n.d.
C1-INH (3)	170	n.d.
Factor B (1)	≤70	1.0
Factor B (2)	≤70	n.d.
Properdin	10	2.2

E

ScFv	LOD (fg/ml)	LOD conventional recombinant Ab microarrays (fg/ml)†
VEGF	50	35
IL-6	3	1
IL-8	3	2/25
IL-12	30	7/45
IL-4	3	1
IL-16	40	n.d.
IL-18	180	n.d.
IL-1α	50	12.6
TNF-β	30	7

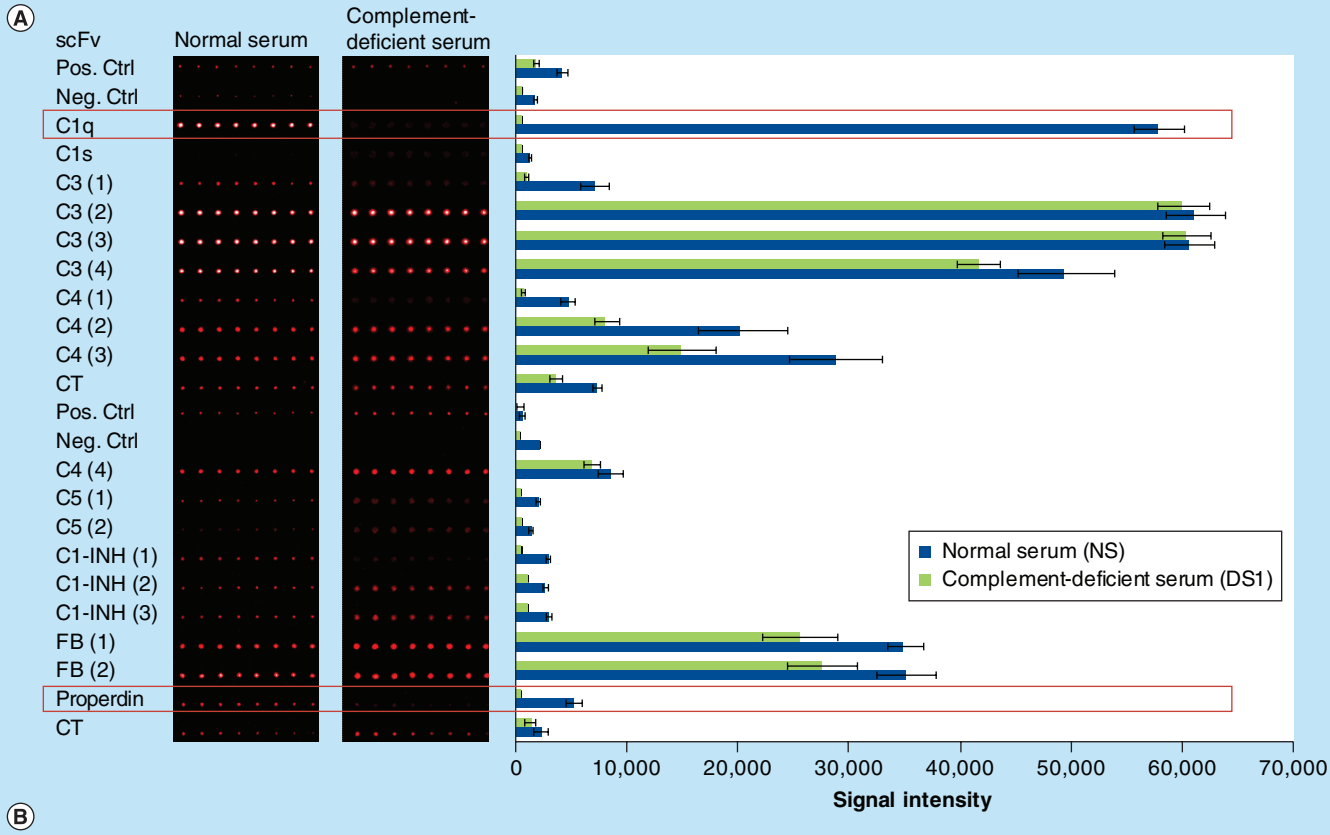
Figure 2. LOD for the miniaturized recombinant antibody array platform. (A) Titration of pure, directly labeled C1q. The LOD cut-off is shown as a solid line, and LOD is indicated by an arrow. **(B)** Titration of crude, directly labeled normal serum (NS), screened by the anti-C1q antibody. The LOD cut-off is shown as a solid line, and LOD is indicated by an arrow. **(C)** LOD for eight complement proteins in crude, directly labeled, and compared with the corresponding LOD for our conventional recombinant antibody microarray set-up. **(D)** Titration of crude, directly labeled NS, screened by the anti-VEGF antibody. The LOD cut-off is shown as a solid line, and LOD is indicated by an arrow. **(E)** LOD for nine cytokines in crude, directly labeled NS, and compared with the corresponding LOD for our conventional recombinant antibody microarray set-up.
†Data from [26,30] and [Unpublished data, Wingren *et al.*]
ScFv: Single-chain Fv.

mal settings according to the ambient analyte theory (reflecting both spot size and antibody affinity) [19].

Furthermore, the sample titration experiments enabled us to estimate the dynamic range, and representative titration curves are shown in Figure 2A, B & D. The results indicated that the dynamic range of the assay, defined as the range of analyte concentrations that lies between 2× SD above the background (nega-

tive control) and below the maximal signal, was in the range of $\leq 10^3$. Hence, this feature of the miniaturized recombinant antibody array set-up was similar to, or only slight worse, than that observed for recombinant antibody nanoarrays [14], conventional recombinant antibody microarrays [4], and conventional polyclonal and monoclonal antibody microarrays [24,39–40] (for review see [2,41]).

Finally, to demonstrate proof-of-concept for the applicability of the set-up, we performed multiplexed protein expression profiling of eight CPs in six well-characterized serum samples, including NS and five CP deficient serum samples (denoted DS1 to DS5) (Figure 3 and Supplementary Table 2S). It is noteworthy that only approximately 0.13 µL of serum was consumed per array, illustrating the wealth of information that could be generated using miniaturized antibody, while consuming minute amounts of a clinical sample. In addition, the array assay took less than 3.5 h to run, further illustrating the applicability of the set-up.



B

	C1q		C1-INH (2)		C3 (2)		C4 (2)		C5 (1)(2)		FB (2)		Properdin	
	Clinical lab. data (%)	scFv array data (%)	Clinical lab. data (%)	scFv array data (%)	Clinical lab. data (%)	scFv array data (%)	Clinical lab. data (%)	scFv array data (%)	Clinical lab. data (%)	scFv array data (%)	Clinical lab. data (%)	scFv array data (%)	Clinical lab. data (%)	scFv array data (%)
NS	100	100	100	100	100	100	100	100	100	100	100	100	100	100
DS1	2	0	n.d	40	97	101	82	43	88	42	56	79	5	6
DS2	n.d.	32	n.d	6	11	3	152	117	118	105	n.d	74	130	100
DS3	93	90	n.d	117	6	14	85	122	22	22	n.d	93	121	129
DS4	68	81	155	104	107	102	0	14	117	128	n.d	103	130	339
DS5	n.d.	92	n.d	198	142	104	121	293	0	0	n.d	99	n.d	279

Figure 3. Serum protein expression profiling of eight complement proteins in six well-characterized human serum samples, using miniaturized recombinant antibody arrays. (A) Representative array image of normal serum (NS) and a complement deficient serum (DS1), deficient complement proteins indicated with a red box. **(B)** Comparison of the known clinical protein expression levels of the complement proteins, determined using orthogonal methods, with the antibody array data.
ScFv: Single-chain Fv.

Representative array images of NS and DS1 (C1q and properdin deficient) are shown in Figure 3A. The results showed that significantly lower signal intensities were, in particular, observed for both C1q and properdin. Next, we compared the array signal intensities with the known expression levels of the CPs determined using orthogonal methods. Representative results, illustrated by those obtained for C1q, C1-INH, C3, C4, C5, FB, and properdin, are displayed in Figure 3B. The results showed that the array data correlated well with the expected expression profile of the CPs. In more detail, the significantly reduced expression levels (<70%) of e.g., C1q (DS1), C3 (DS2 and DS3), C4 (DS4), C5 (DS3), and properdin (DS1) could readily be detected. Furthermore, medium to small (<50%) changes in the expression levels, including both down-regulated (e.g., C1q in DS4, and FB in DS1) and up-regulated (e.g., properdin in DS3 and C5 in DS2) levels, could also be monitored. Taken together, we have generated proof-of-concept for the first generation of a miniaturized recombinant scFv antibody array platform. The set-up was demonstrated to provide a rapid, sensitive, and reproducible profiling of non-fractionated, directly labeled proteomes in a highly specific manner, thus paving the way for large-scale, multiplex profiling of crude proteomes in a miniaturized fashion.

Future perspective

We believe that miniaturized (recombinant) antibody microarrays will be an essential tool for large-scale, multiplexed profiling of crude proteomes, such as serum, in both health and disease. It opens up new possibilities for biomarker discovery, and in the second wave, for miniaturized assay set-ups suitable for clinical settings, requiring minimal amounts of both reagents and samples, and potentially also for point-of-care applications.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/BIO.13.342

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Executive Summary

Experimentals

- We have designed a miniaturized planar recombinant single-chain Fv antibody array technology platform for multiplexed profiling of non-fractionated, directly labelled serum samples.
- The size of the individual spot features was reduced 225 times (78.5 μm^2/spot) and the array density was increased 19 times (38,000 spots/cm^2).

Results & discussion

- The set-up was demonstrated to provide a rapid, sensitive, and reproducible profiling of non-fractionated, directly labeled serum proteins in a highly specific manner.
- The designed antibody array platform opens up new possibilities for large-scale, multiplex profiling of crude proteomes in a miniaturized fashion.

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