REGULAR ARTICLE

Systematic comparison of surface coatings for protein microarrays

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To process large numbers of samples in parallel is one potential of protein microarrays for research and diagnostics. However, the application of protein arrays is currently hampered by the lack of comprehensive technological knowledge about the suitability of 2-D and 3-D slide surface coatings. We have performed a systematic study to analyze how both surface types perform in combination with different fluorescent dyes to generate significant and reproducible data. In total, we analyzed more than 100 slides containing 1152 spots each. Slides were probed against different monoclonal antibodies (mAbs) and recombinant fusion proteins. We found two surface coatings to be most suitable for protein and antibody (Ab) immobilization. These were further subjected to quantitative analyses by evaluating intraslide and slide-to-slide reproducibilities, and the linear range of target detection. In summary, we demonstrate that only suitable combinations of surface and fluorescent dyes allow the generation of highly reproducible data.

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1 Introduction

Inspired by the success of DNA microarrays, the application of protein microarrays is also becoming popular in research and development toward new diagnostic systems. A broad spectrum of research applications is described in the literature to date, and has been used to diagnose type I allergy [1, 2] and to identify autoantigens in human autoimmune diseases [3] for cancer cells and cancer serum profiling [4, 5] and for the analysis of signal transduction pathways [6]. Protein microarrays allow researchers to process many samples in one experiment and to work with low sample volumes, making this approach time saving and cost efficient [7]. However, protein microarray technology still needs to be improved, especially for routine applications.

Parameters like surface coating and detection method are key to the success of microarray experiments. Suitable slide surfaces should bind capture proteins in a high amount while the binding of all other proteins should be minimized. Nonspecific binding of proteins would otherwise cause high background levels and impede the readout of relevant signals. Considerable effort has been made to improve the specific immobilization of proteins on modified glass surfaces, using a number of different strategies [8–11]. These mainly involve covalent attachment of proteins *via* different functional groups that are coupled to the surface. The most commonly used functional groups are epoxy, aldehyde, and *N*-hydroxysuccinimide (NHS)-ester. In contrast, aminosilane coatings immobilize proteins *via* electrostatic forces. To-

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Abbreviations: Ab, antibody; **gam**, goat antimouse; **IgG**, immunoglobulin G

gether, these are commonly classified as 2-D surfaces. The use of a 3-D polymer matrix that embeds the sample in a structural environment is an alternative way to immobilize proteins. This type of surface does not involve the cross-linking of capture molecules with the surface and therefore does not disturb potential functional sites or regulatory domains of these proteins. Several commercial products are available both for 2-D and for 3-D surfaces.

While various slide surfaces have thus far been compared [12–14], the influence of fluorescent dyes on protein microarray experiments has not been systematically analyzed for different slide surfaces. Here, we studied eight 2-D and 3-D slide surface coatings differing in their chemistry and in their mode of protein immobilization, and compared their performance with two different Alexa Fluor dyes. Parameters like spot diameter, LOD, and S/N were analyzed separately for each surface type. In a quantitative analysis, we demonstrate that the reliability of protein microarrays strongly depends on the proper combination of slide surface and fluorescent dye.

2 Materials and methods

2.1 Antibodies (Abs) and proteins

In this manuscript, immobilized Abs and proteins are specified as "probes" and those used for incubation as "targets" [15]. We spotted four different control probes, 15 different mAbs, and four different recombinant proteins on each microarray. The array layout is shown in Fig. 1. The spotted control probes were: Streptavidin, Alexa Fluor 532-conjugated; goat antimouse (gam) immunoglobulin G (IgG), Alexa Fluor 532-conjugated; streptavidin, Alexa Fluor 647-conjugated; and gam IgG, Alexa Fluor 647-conjugated (molecular probes). Each control probe was spotted in eight different concentrations (100, 75, 50, 37.5, 25, 18.75, 12.5, and 6.3 μg/mL), diluted in 80% PBS/20% glycerol. The 15 different affinity purified mAbs that were spotted recognize different epitopes of the following antigens: HLA-A,B,C, β2-microglobulin, HLA-DR α-chain, CD3 ε-chain, CD5, CD19, CD22, EpCAM (a detailed description of Abs can be found in the supplementary material), delivered in PBS in two concentrations (200 and 100 μg/mL). For the production of mAbs, hybridoma cells were grown in a MiniPERM bioreactor (Vivascience, Goettingen, Germany). Purification of immunoglobulins was carried out by affinity chromatography over protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Freiburg, Germany). The purity of the eluted material was assessed by SDS-PAGE under reducing conditions and was usually higher than 95%. Two recombinant GST fusion proteins (DKFZp564O123/GST, 49.9 kDa, spotted concentrations 0.3 and 0.15 mg/mL; GFP/GST, 53 kDa, spotted concentrations 2 and 1 mg/mL) and two recombinant His6-NusA fusion proteins (DKFZp564H1322 His6-NusA, 113.5 kDa, spotted concentrations 4 and 2 mg/mL; DKFZp564D152 His6-NusA, 105.1 kDa, spotted concentrations 2 and 1 mg/mL) were used [16, 17]. The proteins were affinity purified and verified by CBB-stained SDS-PAGE. Protein concentrations were determined by Micro BCA

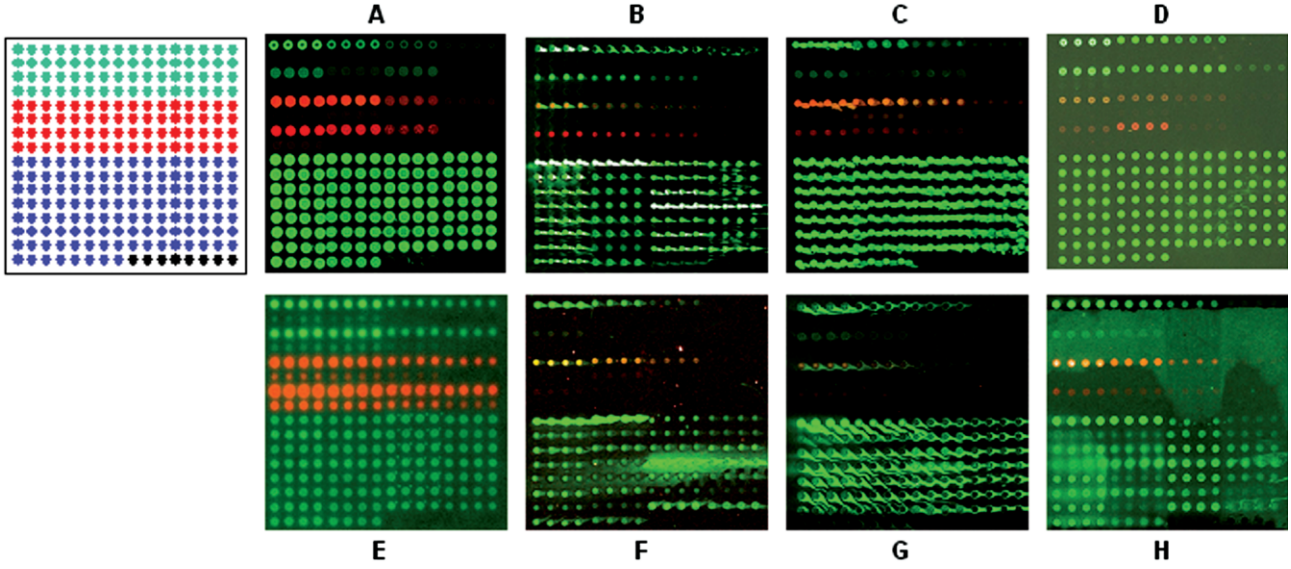

Figure 1. Visualization of surface and spot homogeneity. Representative images of one array of different surface types are shown after incubation with target gam IgG, Alexa Fluor 532-conjugated. All surfaces except for the FAST surface were incubated with 1.6 μg of target (FAST slide: 0.32 μg). Four replicate spots were generated for each probe. Array scheme on the left: green spots–Alexa Fluor 532-conjugated control probes; red spots–Alexa Fluor 647-conjugated control probes; blue spots–15 mAbs spotted in two different concentrations (from left to right: 200, 100 μg/mL); black spots–recombinant fusion proteins. Slide surfaces used: (A) HydroGel, (B) Nexterion slide H, (C) PATH, (D) FAST, (E) CAST, (F) QMT epoxy, (G) QMT protein slide, and (H) silylated slide.

Assay (Pierce, Rockford, IL, USA) as *per* the supplied protocol. Gam IgG, Alexa Fluor 532-conjugated, and gam IgG, Alexa Fluor 647-conjugated (Molecular Probes), were used as targets for the spotted mAbs.

2.2 Surfaces and spotting of arrays

Ab and protein solutions were prepared in 384-well plates (ABgene, Surrey, UK) containing 25 μL of probe *per* well. A noncontact printer with a piezoelectric dispensing technique (BioChipArrayer, Perkin Elmer) with four glass tips spotted 1 nL of the protein solutions in 80% PBS/20% glycerol in an ordered array on different slide surface coatings (see Table 1). Each slide contained the same array format. One array consisted of 16 columns and 18 rows; each protein was spotted four times within one array. Each array was replicated four times on each slide. The horizontal and vertical spacing between the spots was 400 μm. Glass tips were washed in 5% Tween/PBS containing 40 mM NaOH during the spotting process. After spotting, the microarrays were stored dry in a light-protected slide box at 4°C for up to 1 month.

2.3 Preparation of dye-conjugated Abs

AntiGST and antiHis Abs (Amersham Biosciences, Freiburg, Germany) were diluted in PBS Dulbecco's (GIBCO) to a final concentration of 200 μg/mL. Fifty microliter of 0.1 M Na_2CO_3 buffer (pH = 8.4) was added. Alexa Fluor 647 (1 mg) (Molecular Probes) was dissolved in 100 μL DMSO, water free (Fluka, Munich, Germany), and 15 μL of the reactive dye solution was added to the Ab solution. The mixture was incubated for 1 h at room temperature in the dark with continuous stirring. To separate the conjugate from unreacted labeled reagent, the Ab-dye solution was filtered over a spin column (Bio-Spin disposable chromatography columns, BioRad) filled with 0.2 mL Sephadex G-25 Superfine (Amersham Biosciences) slurried in PBS/2 mM NaN_3. The major protein fraction was collected and further analyzed. The final Ab concentration and the molar ratio of dye to Ab were calculated by means of absorption measurement at 280 and 650 nm. AntiGST: 0.036 mg/mL, molar ratio dye/Ab: 7.3; antiHis: 0.078 mg/mL, molar ratio dye/Ab: 8.9.

2.4 General protocol for assay on microarray

Slides were blocked in blocking buffer containing 4% PEG 6000 (Merck, Darmstadt) in PBS at room temperature for 1 h with gentle agitation. The edges of the slide were carefully wiped until dry, and then 53 mm × 22 mm hybridization chambers (Schleicher & Schüll) were filled with 800 μL Ab solution. Target proteins were diluted in incubation buffer (90% blocking buffer/10% DMSO (AppliChem GmbH, Darmstadt)). The following amounts of target protein were applied (referring to 800 μL incubation volume): antiGST, Alexa Fluor 647-conjugated: 0.29 and 0.06 μg; antiHis, Alexa Fluor 647-conjugated: 0 031 and 0.013 μg; gam IgG, Alexa Fluor 532-conjugated, and gam IgG, Alexa Fluor 647-conjugated: 1.6, 0.8, 0.55, 0.4, 0.32 μg. Incubation was carried out in the dark for 30 min at room temperature. Arrays were washed three times in PBS/0.2% IGEPAL (CA-630, Sigma, nonionic detergent), once in PBS and once in distilled water, and centrifuged until dry in a custom-made slide adapter. Finally slides were scanned using a ScanArray ExpressHT scanner (Perkin Elmer, Wellesley, USA) with a resolution of 10 μm *per* pixel. Laser power and PMT were adjusted for each surface type separately.

Table 1. Description of surfaces and results for spot diameter, S/N, and lower LOD. Spot diameter (μm), SD is given in parentheses; S/N, 95% confidence interval is given in parentheses; lower LOD (μg/mL). A, B, C, D, E surfaces are 3-D and F, G, H are 2-D surface coatings

Slide surface	Name (provider)	Surface coating	Spot diameter	S/N	Lower LOD green color channel	Lower LOD red color channel	Slide replicates
A	HydroGel™ (Perkin Elmer)	Polyacrylamide	242 (54)	31 [19, 42]	18.75	18.75	36
B	Nexterion™ slide H (SCHOTT Nexterion AG)	Hydrogel, based on an organic polymer	199 (44)	12 [8, 16]	12.5	37.5	12
C	PATH™(Clinical MicroArrays)	Modified NC	184 (40)	3 [3, 4]	18.75	12.5	8
D	FAST (Schleicher & Schuell)	NC	190 (15)	4 [-1, 9]	37.5	50 strong variability	8
E	CAST (Schleicher & Schuell)	NC	209 (32)	1 [1, 1]	12.5	12.5	18
F	QMT epoxy/Nexterion™ slide E (SCHOTT Nexterion AG)	Epoxy functionalized	196 (42)	10 [9, 11]	50	50	10
G	QMT Protein Slide (SCHOTT Nexterion AG)	Aldehyde functionalized	221 (58)	6 [1, 11]	50	75 strong variability	4
H	Silylated slides (Greiner)	Aldehyde functionalized	191 (34)	4 [0.4, 8]	18.75	25	5

2.5 Data analysis and statistics

Image analysis was performed with Gene Pix Pro 3.0 (Axon Instruments, California) to determine spot diameter. The mean pixel values of feature and background were used as the signal and background intensities, respectively. Statistical analysis was performed with the statistical platform R [18].

3 Results

3.1 Homogeneity of background and spots

Visual inspection of slide surface and spot homogeneities revealed that surfaces A–C, F, and G produced a more consistent overall background than D, E, and H (Fig. 1). A and D showed clear Ab spots, whereas for C, F, and G the spots were smeared. Spots generated with higher protein and Ab concentrations were also smeared on slide B. Most of the spots were obscured by background fluorescence for surface H.

3.2 Spot diameter, S/N, and LOD

The impact of the slide surfaces on the spot diameter was determined with the help of image analysis software. The spots of all replicate slides for each surface type were regarded (Table 1). The spot diameters ranged from 184 μm (SD: 40 μm, surface B) to 242 μm (SD: 54 μm, surface A).

To compare the S/N (signal intensity divided by background intensity) for different surface types we averaged over all spots of one microarray. Only spots of probes supposed to be recognized by the target were considered. The overall S/N of slide replicates for each surface type was calculated by linear regression. In Table 1 the ratios and the 95% confidence intervals are listed. The highest S/N was found for surface A with a value of 31 and a corresponding 95% confidence interval ranging from 19 to 42. Similar S/Ns showed surfaces B and F ((B: 12 (8, 16) and F: 10 (9, 11)). The lowest S/N was found for surface E 1 (1, 1).

To measure the binding capacity for each surface type, the LOD was evaluated for the labeled control probes. No saturation effect or upper LOD was found for any surface type across all spotted concentrations ranging from 6.25 to 100 μg/mL. We did not evaluate higher concentrations because typical sample concentrations of Abs are about 250 μg/mL. The goal is rather to spot as little of the expensive Abs and proteins as possible. Signal intensities were plotted against the concentration for each probe and replicate spot (Fig. 2). We used three criteria to determine the lower LOD for each color channel: (i) the average signal intensity of all replicate spots of the control probes must be twofold higher than those of the buffer spot replicates; (ii) the statistical significance calculated using a one-sided t-test must have a $p < 0.05$; (iii) criteria 1 and 2 had to be fulfilled by 75% of

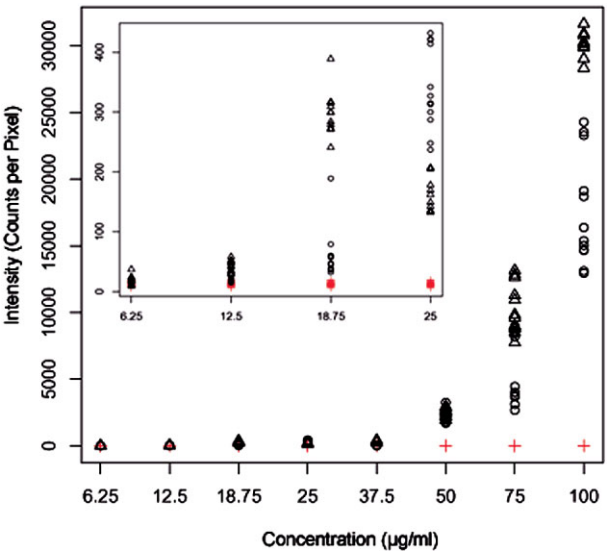

Figure 2. Determination of the LOD. Plotted were 16 replicate spots for streptavidin, Alexa Fluor 532-conjugated (circle), and 16 replicate spots for gam IgG, Alexa Fluor 532-conjugated (triangle), against different probe concentrations for surface type A (HydroGel). Red crosses represent the replicate buffer spots. Inset shows the magnification for the concentration range of 6.25–25 μg/mL.

slide replicates of each surface type. The results are shown in Table 1. Surface type E revealed the lowest LOD at a concentration of 12.5 μg/mL in both color channels. In contrast, surface type G detected the probes only when spotted at concentrations higher than 50 μg/mL. Also slide type D showed in the green color channel a LOD at 50 μg/mL and a high variability. Surface type B revealed a lower LOD in the green color channel (12.5 μg/mL), while C detected spotted probes above the same concentration.

3.3 Specificity

We next examined the specificity of the targets gam IgG, antiGST, and antiHis Abs on different surface types. Because mAbs and recombinant His- and GST-fusion proteins were spotted on the same microarray we always had negative controls on the array. Only spots of probes supposed to be recognized by the target were regarded as positive, all other as negative (control probes were not counted). The mean positive and negative signal intensities and the ratios were initially calculated for each target/probe pair individually, and then combined for each surface and are presented in Table 2. High positive and low negative signal intensities were measured using surfaces A–C and G. Surface E yielded almost equal intensity levels for positive and negative spots. The microarrays were then subjected to a detailed visual inspection to analyze whether the targets specifically recognized their appropriate probes. We found that the target protein gam IgG recognized all spotted Abs on surfaces A–D

Table 2. Specificity of targets gam IgG, antiHis, and antiGST Abs on different slide surfaces. Only spots of probes supposed to be recognized by the target were regarded as positive and all others as negative. Mean positive and negative signal intensities are presented. Ratio is the positive signal divided by the negative signal. Slide surfaces: (A) HydroGel, (B) Nexterion slide H, (C) PATH, (D) FAST, (E) CAST, (F) QMT epoxy, (G) QMT protein slide, and (H) silylated slide

	A	B	C	D	E	F	G	H
Positive signal	12 194	13 163	5 156	13 062	1453	6102	12 516	4126
Negative signal	156	478	74	1682	1373	206	191	235
Ratio	78	28	70	8	1	30	66	18

and F–H. No signals above background were measured in this experiment on surface E with gam IgG, Alexa Fluor 647-conjugated (Table 3). AntiGST recognized both spotted recombinant GST proteins on surfaces A, B, D, and F–H (Fig. 3). On surface C, only the GFP-GST protein and on surface E no signals for both recombinant GST proteins were detected. The spotted His-fusion proteins were identified with antiHis on surfaces A, B, and D. All other surfaces did not allow for the detection of recombinant His-proteins.

3.4 Quantitative analysis

3.4.1 Reproducibility of control probes

Based on the results described above, slide types C–H were excluded from further analysis. Surfaces C and E–H showed only confined detection of recombinant proteins and Abs. Surface D revealed a low S/N and a strong variability for the LOD in the red color channel. For the following analysis ten slides for surface types A (HydroGel) and B (Nexterion slide H), respectively, were examined.

Intraslide and slide-to-slide reproducibilities for the spotted control probes, streptavidin and gam IgG, both Alexa Fluor 532-conjugated or Alexa Fluor 647-conjugated, respectively, were evaluated. To test the intraslide reproducibility we compared the intensity of 16 replicate spots within a slide. In contrast, for testing the slide-to-slide reproducibility equal spots on different slides were evaluated. The CV was calculated as the slope obtained by linear regression of SD *versus* signal intensity. The results for both surface types are shown in Table 4. For surface type A, intraslide and slide-to-slide reproducibilities (CV = 20%, CV = 61%) were found to be higher for Alexa Fluor 647-conjugated control probes. For surface type B, however, the Alexa Fluor 532-conjugated probes revealed the best intraslide (CV = 19%) and slide-to-slide (CV = 55%) reproducibilities.

3.4.2 Linear range of target detection

To quantitatively assess the linear range of target detection, five slides were incubated with target gam IgG, Alexa 532-conjugated, and another five slides were incubated with gam IgG, Alexa 647-conjugated, for surfaces A and B, respectively. Different amounts of target were applied (1.6, 0.8, 0.55, 0.4, 0.32 µg) on each slide. To determine if any of the spotted Ab concentrations, Ab probes, or conjugated dyes would influence linear range, the data set was analyzed for each of these parameters separately. The intensity on each Ab spot having been replicated 16 times (over four subarrays) was analyzed as a function of the amount of target (Fig. 4A and B). The coefficient of determination (R^2, range: $0 < R^2 \leq 1$) was evaluated by a linear regression fit. The calculated R^2 values for each Ab sample were averaged and are listed in Table 5. Detailed results for the different Abs are given in supplementary material. For surface type A and the Alexa Fluor 532-conjugated target, the R^2 was 0.77 with a spotted Ab concentration of 100 µg/mL and 0.85 for a spotted Ab concentration of 200 µg/mL. The same surface revealed lower R^2 values with Alexa Fluor 647-conjugated target: R^2 was 0.44

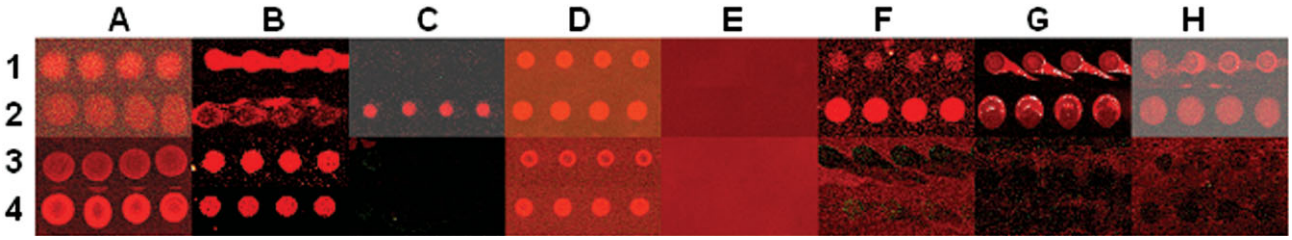

Figure 3. Detection of different probes by target Abs antiGST and antiHis, both Alexa Fluor 647-conjugated. Represented are four replicate spots for each of the following probes: 1, DKFZp5640123-GST; 2, GFP-GST; 3, DKFZp564H1322-His6-NusA; 4, DKFZp564D152-His6-NusA. Surfaces used: (A) HydroGel, (B) Nexterion slide H, (C) PATH, (D) FAST, (E) CAST, (F) QMT epoxy, (G) QMT protein slide, and (H) silylated slide.

Table 3. Recognition of different spotted protein and Ab probes by their respective target gam IgG, antiHis, and antiGST Abs on different surface types. ++, Strong signal detection; +, weak signal detection; –, no signal detection. Slide surfaces: (A) HydroGel, (B) Nexterion slide H, (C) PATH, (D) FAST, (E) CAST, (F) QMT epoxy, (G) QMT protein slide, and (H) silylated slide

	Slide surface							
Probe	A	B	C	D	E	F	G	H
mAbs	++	++	++	++	+[a)]	++	+	+
DKFZp5640123-GST	++	++	–	+	–	++	++	++
GFP-GST	+	+	++	++	–	++	++	++
DKFZp564H1322-His6-NusA	+	++	–	++	–	–	–	–
DKFZp564D152-His6-NusA	++	+	–	++	–	–	–	–

a) No signals were detected with gam IgG, Alexa Fluor 647-conjugated.

Table 4. Intraslide and slide-to-slide CV and corresponding 95% confidence interval for spotted control probes streptavidin and gam IgG, each conjugated with either Alexa Flour 532 or Alexa Fluor 647 dye

Slide surface	Alexa Fluor 532		Alexa Fluor 647	
	Intraslide % CV	Slide-to-slide % CV	Intraslide % CV	Slide-to-slide % CV
HydroGel (A)	36 [35, 37]	101 [96, 105]	20 [18, 21]	61 [53, 68]
Nexterion slide H (B)	19 [18, 21]	55 [51, 59]	33 [30, 36]	81 [74, 88]

for spotted Ab concentration of 100 μg/mL while R^2 was 0.64 for 200 μg/mL. For slide type B, higher R^2 values were detected in the red color channel: $R^2 = 0.73$ (100 μg/mL) and $R^2 = 0.64$ (200 μg/mL) as compared to the green color channel $R^2 = 0.32$ (100 μg/mL) and $R^2 = 0.55$ (200 μg/mL). No systematic influence of spotted Ab probe was found.

3.4.3 Intraslide reproducibility of target detection

To assess the intraslide reproducibility of target detection, the slides described in Section 3.4.2 were evaluated. The signal intensity of each Ab spot was averaged over 16 replicates. The CV was calculated as the slope obtained by linear regression of SD *versus* signal intensity. CVs were evaluated separately for each color channel, each spotted Ab concentration, and each surface type (Table 5). The CV ranged from 19 to 37% for surface A. The reproducibility was found about two-fold higher with Alexa Fluor 532-conjugated target than with the other dye. For slide type B the CV ranged from 13 to 19% and showed no significant dependence on spotted Ab concentration or conjugated target dye.

4 Discussion

In this study, we compared eight different protein microarray surfaces and the influence of fluorescent dyes on their applicability in protein microarray experiments. Parameters were defined and evaluated for each surface individually. First, spot diameter, S/N, and the LOD were analyzed. Generally, low spot diameters are preferred because they allow for a higher analyte density on the chip. In consequence, higher signal intensities and optimal S/Ns can be achieved [19, 20]. The spot diameter ranged from 184 (PATH) to 242 μm (HydroGel), and these differences were not statistically significant. However, high S/N require low background intensities, which we found to be strongly influenced by the respective surface. For surface type A (HydroGel), we found the highest S/N which was much better than that of any other surface type tested. Surface type B (Nexterion slide H) and surface type F (QMT epoxy) showed comparable S/N and followed HydroGel in the ranking. Surfaces coated with NC revealed very low S/N as well as the 2-D surfaces G (QMT protein) and H (silylated slide). The LOD of spotted control probes, which were already conjugated to dye, reflects the capacity of surfaces to immobilize proteins. We found no upper LOD in the spotted concentration range of 6.25–100 μg/mL. Higher concentrations were not tested as it is desirable for Ab and recombinant protein microarrays to work with low sample concentrations. On E (CAST), the LOD was best compared to all other surface types, whereas for D (FAST) and G, we found strong variations in the red color channel. The thicker surface layer of CAST slides compared to FAST slides might explain this effect. A greater binding capacity of the CAST slides could result in higher signal intensities already for low concentrations. Strong variations in the detection of low protein concentrations might be caused by a nonhomogeneous surface layer. In case of QMT protein slide, which belongs to the group of 2-D surfaces, variable densities of functional groups might cause

Table 5. R^2-values and intraslide reproducibility of target protein detection. Listed are mean R^2 values and coefficients of variation with the corresponding 95% confidence interval for two different probe concentrations (upper value: 100 μg/mL; lower value: 200 μg/mL)

Slide surface	gam IgG, Alexa Fluor 532-conjugated		gam IgG, Alexa Fluor 647-conjugated	
	R^2	Intraslide % CV	R^2	Intraslide % CV
HydroGel (A)	0.77	20 [18, 21]	0.44	37 [35, 39]
	0.85	19 [18, 20]	0.64	36 [35, 38]
Nexterion slide H (B)	0.32	15 [13, 17]	0.73	13 [12, 13]
	0.55	12 [10, 13]	0.64	16 [15, 18]

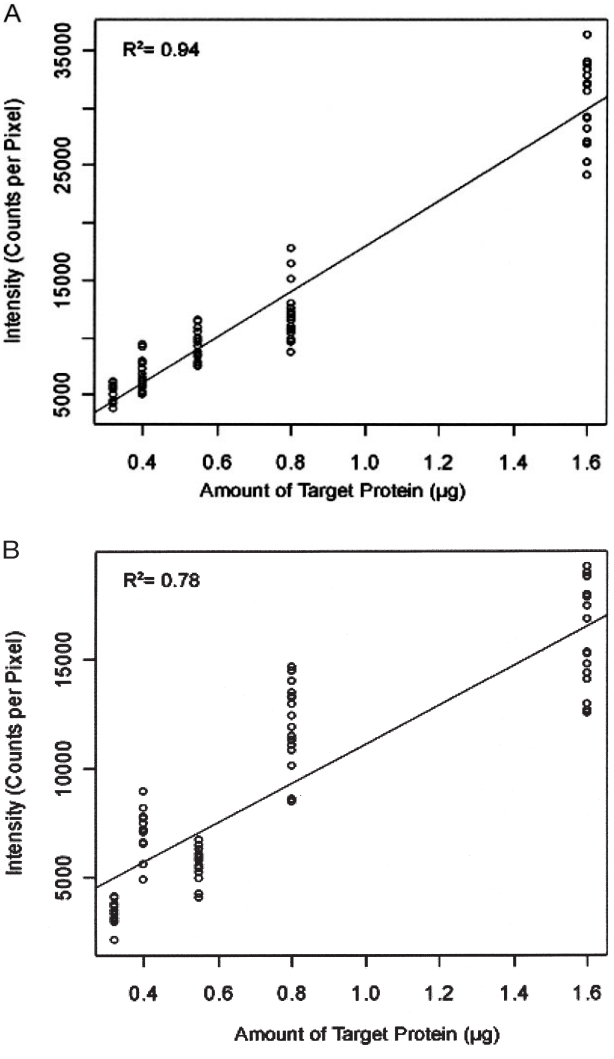

Figure 4. Linear range of target detection. Graphs show signal intensities plotted *versus* the amount of target for one mAb W6/32. Each point represents the signal intensity of one of 16 replicate spots on the same microarray, which was incubated with the indicated amount of target. (A) HydroGel–Incubation with target gam IgG, Alexa Fluor 532-conjugated, probe concentration: 200 μg/mL. (B) Nexterion slide H–Incubation with target gam IgG, Alexa Fluor 647-conjugated, probe concentration: 100 μg/mL.

differences in protein binding capacity. In summary, 3-D surfaces revealed lower limits of detection than 2-D surfaces. This could be explained with an increased binding capacity of polymer matrices compared to coatings containing functional groups.

Second, the specificity of targets to detect their matching probes on different surface types was analyzed by probing with gam IgG, antiHis, and antiGST Abs. Specificity was found highest for A (HydroGel), C (PATH), and G (QMT protein). Then we analyzed in more detail which of the targets recognized their respective probe. This revealed that the target gam IgG indeed detected all of the spotted mAbs except those spotted on surface types E (CAST), G (QMT protein), and H (silylated slide). Weak signals were detected for G and H and no signals on E (CAST) for the target gam IgG, conjugated with Alexa Fluor 647. The differences in detection of recombinant protein were even stronger. Especially, the high molecular mass His6-NusA fusion proteins (DKFZp564H1322, 113.5 kDa; DKFZp564D152, 105.1 kDa) could not be detected on C (PATH), E (CAST), F (QMT epoxy), G (QMT protein), and H (silylated slide) surfaces. However, this effect cannot be attributed to the mass of these proteins alone, as Abs (150 kDa) were successfully immobilized on the same surfaces. Rather, the His epitope might not have been accessible to the Ab after protein binding to the surface. It must be noted that the surface E (CAST) revealed signals for spotted dye conjugated control probes whereas the target Abs gam IgG, antiHis, and antiGST did not detect their respective antigens on that surface.

Third, the reproducibility of spotted control probes as well as the linear range and intraslide reproducibility were quantitatively assessed. We concentrated on surfaces A (HydroGel) and B (Nexterion slide H) as these had been identified to have the highest S/Ns and to perform best in Ab and protein detection. We compared intraslide and slide-to-slide reproducibility of the measured intensities of the spotted control probes, using the two conjugated Alexa Fluor dyes. Our measurements revealed a strong relationship between the surface and the applied dye. Hydrogel surfaces showed higher reproducibility for Alexa Fluor 647-conjugated probes while the performance of Nexterion slide H was best with Alexa Fluor 532-conjugated probes.

The linear range of the detected signals depending upon the amount of target is a fundamental criterion for the successful application of protein microarrays, for example, in clinical diagnosis [6]. Microarrays were incubated with five different amounts of target ranging from 0.32 to 1.6 μg *per* slide. These amounts were selected to guarantee that the probe was always in excess of the target. Thus we excluded saturation effects and ensured that measured intensities represented physiological intensity levels in profiling experiments [19]. As expected, the gam IgG Ab did not reveal any dependence on the spotted Ab type, despite these Abs are directed against different antigens.

Finally, we determined the useful combinations of slide surfaces and fluorescent dyes. A linear regression fit revealed a higher reproducibility and data quality when HydroGel was incubated with Alexa Fluor 532-, as compared to Alexa Fluor 647-conjugated target proteins. In contrast, the results obtained with the Nexterion slide H surface showed superior results when incubated with Alexa Fluor 647- as compared to the Alexa Fluor 532-conjugated proteins. The enhanced data quality and reproducibility achieved with the proper surface-dye combinations was also reflected by decreased intraslide coefficients of variation. It must be noted that it makes a significant difference whether the intensity of spots of already conjugated probes is measured or of the target itself. The latter case is of greater importance for most applications, *e.g.*, in diagnosis.

In summary, we have demonstrated that determining the optimal combination of surface and dye is a critical and thus far unnoticed parameter that significantly influences the reproducibility and reliability of protein microarray experiments. Our results suggest to conduct protein profiling experiments using only one dye, which is in contrast to common "color flip experiments," where the same proteins are conjugated to different dyes.

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