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Protein Expression Profiling of Formalin-Fixed Paraffin-Embedded Tissue Using Recombinant Antibody Microarrays

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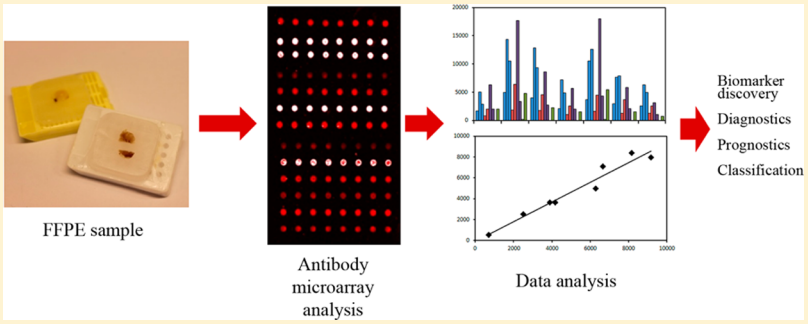
S *Supporting Information*



ABSTRACT: Proteomics, the large-scale analysis of proteins, is a rapidly evolving field with an increasing number of key clinical applications, such as diagnosis, prognosis, and classification. In order to generate complete protein expression profiles, or protein atlases, any crude sample format must be addressable in a rapid, multiplex, and sensitive manner. A common and clinically central sample format, formalin-fixed, paraffin-embedded (FFPE) tissue material, holds great potential as a source for disease-associated biomarker signatures. However, despite major efforts, extraction and subsequent profiling of proteins from FFPE tissue has proven to be challenging. In this proof-of-concept study, we have demonstrated for the first time that proteins could be extracted, labeled, and subsequently profiled in a multiplex, sensitive, and reproducible manner using recombinant scFv antibody microarrays. Thus, we have added FFPE samples to the list of sample formats available for high-throughput analysis by affinity proteomics, paving the way for the next generation of biomarker-driven discovery projects.

KEYWORDS: *antibody microarrays, FFPE, protein expression profiling*

INTRODUCTION

Proteomics, the large-scale analysis of proteins, has raised great hope in deciphering multiplex disease-associated biomarker panels for improved diagnosis, prognosis, classification, evidence-based therapy selection, etc., taking the next step(s) toward personalized medicine.[1−4] In the quest for delineating biomarkers, the access to large well-characterized sample cohorts of high quality is critical, and a wide variety of clinical sample formats have been addressed, such as serum, plasma, urine, and tissue, including formalin-fixed, paraffin-embedded (FFPE) tissue sections. In this context, FFPE material is of particular interest, since the majority of human biopsy specimens are currently stored long-term as FFPE blocks and thus could provide an essential source for multiplexed proteomics-based retrospective biomarker discovery efforts.[5,6] However, this sample format has traditionally been analyzed for diagnostic tumor pathology using immunohistochemistry (IHC), targeting only one or a few markers at a time,[6,7] making the multiplexity and throughput key technical issues.

For this purpose, major efforts have been made to make FFPE tissue compatible with classical proteomic technologies.[8−12] However, the extensive formalin-induced cross-linking of proteins when stored as FFPE tissue sections, essential for allowing preservation of tissue integrity over time and maintaining histomorphological information important for diagnosis using IHC, also leads to key difficulties in efficiently mining proteins for proteomic analysis. Robust methods for extracting soluble peptides as well as full-length intact proteins from FFPE tissue sections compatible with various mass-spectrometry-based setups and reverse-phase protein arrays (RPPA) have only recently been developed.[8−12] Notably, a high concordance has been observed with respect to both

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Table 1. Summary of the Different Antibodies Used for the Antibody Microarray Analysis

antigen group	specificity (target)	antibody clone	antibody format	affinity (K_d, M)	validation[a] of specificity[21−23,25,27,29−33]
	C1q	Ab1	scFv	6×10^9	A, B, C, E, F
	C3	Ab2	scFv	1×10^8	A, B, C, E, F
	C4	Ab3	scFv	1×10^8	A, B, E
	C5	Ab4	scFv	9×10^7	A, B, E
	IL-4	Ab5	scFv		A, E
	IL-8	Ab6	scFv		A, E, F
	IL-12	Ab7	scFv		A, E, F
B-cell lymphoma associated proteins	OSPBL-3[b]	Ab8−Ab11	scFv		A, F
	SOX-11	Ab12, Ab13	scFv, IgG (Ab13)		A, F
	KIAA0882	Ab14−Ab20	scFv, IgG (Ab15,17,19)		A
cancer associated protein	Mucin-1	Ab21−Ab28	scFv, IgG (Ab28)		A
peptide motifs	M-34 (SEAHLR)	Ab29	scFv		A, D
	M-35 (TNDESK)	Ab30	scFv		A, D
	M-14 (DFAEDK)	Ab31	scFv		A, D
	M-33 (LSADHR)	Ab32	scFv		A, D
	M-17 (SSAYSR)	Ab33	scFv		A, D
	M-1 (EDFR)	Ab34	scFv		A, D
	M-32 (QEASFK)	Ab35	scFv		A, D

[a]A = validation of antibody specificity using stringent selection protocols and evaluating reactivity against pure analyte and mixtures of pure analytes; B = validation of antibody specificity by analyzing a crude human serum sample with known levels of several serum analytes (used as a standardized proteome sample); C = validation of antibody specificity by affinity pull-down assays and subsequent MS analysis targeting the crude standardized proteome sample in B; D = validation of antibody specificity by affinity pull-down assays and subsequent MS analysis targeting various crude tissue extracts (e.g., liver homogenate); E = validation of antibody specificity targeting crude serum, plasma, urine, and/or tissue extracts using orthogonal methods such as ELISA, MSD, protein arrays; F = validation of antibody specificity targeting crude human proteomes (e.g., serum, plasma) using blocking/spiking approaches. [b]Oxysterol binding protein-like 3.

protein yield and abundance between fresh frozen and formalin-fixed tissue when targeting digested proteins[13,14] as well as intact proteins.[8] Although the freshly collected samples did globally deliver a higher amount of data, this could potentially be counterbalanced by a wider availability of FFPE samples and of retrospective information about the patients' clinical status.[13,14] Despite the success, the applicability of RPPA and mass spectrometry for protein expression profiling of FPPE tissue sections is still hampered because of issues related to sensitivity, reproducibility, sample complexity, and/or throughput, demonstrating the need for additional proteomic methodologies.

In this context, antibody-based microarrays has been established as a promising affinity proteomic technology for rapid and sensitive protein expression profiling of complex samples in a highly multiplex manner.[1,15−17] Recently, we and others have successfully used this methodology for deciphering candidate biomarker signatures for diagnosis, prognosis, classification, and evidence-based therapy selection for various cancers and inflammatory conditions; for review see refs 1 and 16−18. To date, we have developed and designed high-performing recombinant antibody microarrays capable of targeting crude, directly labeled proteomes, including plasma, serum, urine, tissue extracts, cell lysates, and intact cells,[16,19−23] as well as digested proteomes.[24]

In this conceptual study, we have designed the first recombinant antibody microarray technology platform for multiplexed protein expression profiling of proteins recovered from FPPE tissue sections. The results showed that crude, directly biotinylated FFPE extracts could be targeted and that a range of proteins displaying different properties could be profiled in a sensitive and reproducible manner in a single experiment.

MATERIAL AND METHODS

Antibodies

In total, 35 different antibodies (denoted Ab1−Ab35) were used in this study, targeting four different groups of antigens: 7 immunoregulatory proteins (Ab1−Ab7), 3 B-cell lymphoma-associated proteins (Ab8−Ab20), 1 cancer-associated protein (Ab21−Ab28), and 7 peptide motifs (Ab29−Ab35[25]) (Table 1). Notably, while the first and third groups of antibodies had been selected using intact, native antigen for selection,[26] the antibodies targeting B-cell lymphoma associated proteins had been generated using protein epitope signature tags (PrESTs), 100−150 amino acids long.[27,28] The last group of antibodies, targeting peptide motifs, displayed reactivity toward the motif whether the protein was intact or digested and native or denatured, as long as the motif was accessible.[24] Thirty human recombinant single-chain Fragment variable (scFv) antibodies were selected from the n-CoDeR library[26] in-house[25] or by BioInvent International AB (Lund, Sweden). Noteworthy, these scFv are all based on the same constant scaffold, VH-3-23/VL1-47, differing only in their complementarity determining regions, making their overall biophysical behavior (e.g., stability, adsorption abilities, and nonspecific scaffold binding) as similar as possible. The specificity, affinity (normally in the 1−10 nM range),[21,26] and on-chip functionality of the phage display derived scFv antibodies were ensured by using (i) stringent phage-display selection protocols and (ii) a molecular design, adapted for microarray applications.[16,18] The specificity of these antibodies has previously been validated using pure analytes, mixtures of pure analytes, well-characterized standardized crude serum samples (no such FFPE sample(s) currently at hand), and orthogonal methods, such as mass spectrometry (serum/tissue extract pull-down assays), ELISA, MesoScaleDiscovery (MSD) assay, immunohistochemistry,

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and/or cytometric bead assay, as well as using spiking and blocking experiments in crude sample formats (e.g., serum, urine, and tissue extracts) (Table 1).[21−23,25,27,29−33] Five full IgG human antibodies were constructed from scFv fragments and produced as previously described.[34]

Production of scFv Antibodies

All scFv antibodies were produced in *E. coli* in 100-mL cultures and purified from culture expression supernatants using affinity chromatography on Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany). ScFvs were eluted using 250 mM imidazole, extensively dialyzed against PBS (pH 7.4), and stored at 4 °C until use. The protein concentration was determined by measuring the absorbance at 280 nm (average 400 μg/mL, range 40−1300 μg/mL).

FFPE Tissue Samples

Anonymized formalin-fixed, paraffin-embedded 10−15-μm thick tissue sections mounted on microscope slides from a total number of 10 patients (10 slides/patient) were collected, including tissue sections from two breast cancer (BC) patients and two healthy (N) individuals (Dept. of Pathology, Skåne University Hospital, Malmö), along with four mantle cell lymphoma (MCL) patients and two follicular lymphoma (FL) patients (Dept. of Pathology, Skåne University Hospital, Lund, Sweden). All specimens were fixed in 10% neutral-buffered formalin for about 24 h in accordance with clinical protocols.

Deparaffinization and Extraction

Deparaffinization and protein extraction of all FFPE samples were performed on about 2 cm^2 tissue sections, thus normalizing the amount of tissue used from each sample. Except for three MCL patients and one FL patient (one extraction/patient), two slides per patient were randomly selected, subsequently extracted, and handled as individual samples (n = 16) (Table 2). Deparaffinization and extraction was performed using Qiagen's Qproteome FFPE Tissue Kit (Qiagen, Hilden Germany).[20] Only FFPE samples fixed according to the recommended settings (in 4−10% formalin for 14−24 h) were used for protein extraction. Briefly, the paraffin was removed by soaking the slides in xylene (Sigma-Aldrich, St. Louis, MO, USA), and the tissue was rehydrated with a graded ethanol series (100%, 96%, and 70%) followed by double-distilled water. Extraction buffer (proprietary information) (Qiagen) was added to the tissue material prior to incubation at 100 °C for 20 min in order to reverse the formalin cross-linking, followed by an incubation at 80 °C for 2 h to ensure maximal protein solubilization. Finally, the material was centrifuged for 15 min at 14000 × g, and protein extracts were stored at 4 °C until further use. Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. No information about the percentage of tissue protein that can in general be extracted using this extraction kit or whether this might change depending on fixation/extraction conditions was available from the supplier, and this was not further investigated in this study.

Table 2. Total Protein Concentration after Extracting Each Sample Type[a]

sample	extraction	protein yield [μg/mL]
MCL	1	860
	2	1800
	3	3400
	4	1200
	5	1100
FL	1	390
	2	550
	3	850
BC	1	640
	2	570
	3	500
	4	470
N	1	330
	2	330
	3	2100
	4	810

[a]Ten FPPE slides were collected per patient (number of patients = 10, including 4 MCL, 2 FL, 2 BC, and 2 N), and 1 or 2 slides per patient were then randomly selected, subsequently extracted, and handled as individual samples (n = 16).

Labeling of Protein Extracts

Protein extracts were desalted using Zeba Desalt Spin Columns, 0.5 mL, MWCO 7 kDa (Pierce, Rockford IL, USA). The extracted proteins were then labeled using the Single-color Bio-ULS Cell Lysate Protein Labeling and Fluorescent Detection Kit (Kreatech Diagnostics, Amsterdam, The Netherlands), according to the manufacturer's instructions. Briefly, 25 μg of protein was added to a mixture of purified water, BIO-ULS, and buffer and was then incubated at 50 °C for 1 h. Next, nonreacted BIO-ULS was removed, and the buffer was simultaneously exchanged to a sample buffer included in the kit using ULS-Trap columns.

Production of Antibody Microarrays

The antibody microarrays were produced, using two non-contact dispensers (Biochip Arrayer1, Perkin-Elmer Life & Analytical Sciences, Wellesley, MA, USA, and sciFLEXARRAYER S11, Scienion AG, Berlin, Germany), which deposit about 300 pL/drop, using piezo technology. The antibodies (40−1300 μg/mL) were arrayed onto black polymer MaxiSorp Slides (NUNC A/S, Roskilde, Denmark) by spotting two drops at each position, and the first drop was allowed to dry out before the second drop was dispensed. The spots, about 150 μm in diameter, were deposited at a pitch-to-pitch distance of 300 μm. The arrayed antibodies were immobilized through adsorption, an interaction that has previously been found to be sufficiently strong to withstand the subsequent array washing/handling steps.[21,23] Eight replicates of each scFv antibody were arrayed to ensure adequate statistics. Unless otherwise stated, 6 subarrays with 28 × 8 antibodies (no. of antibodies × no. of replicates) (clones Ab1−Ab28) were spotted onto each slide. In one experiment, 35 × 8 antibody-based subarrays (clones Ab1−Ab35) were used.

Antibody Microarray Analysis

The analysis of the FFPE tissue extracts was carried out using reagents from the Single-color Bio-ULS Cell Lysate Protein Labeling and Fluorescent Detection Kit (Kreatech Biotechnology, Amsterdam, The Netherlands). The subarrays were blocked with 60 μL blocking buffer for 15 min at RT and washed with 2 × 50 μL washing buffer. Labeled protein samples (500 μg/mL) were then diluted 25 times in sample buffer, and 50 μL was added to each subarray. The arrays were incubated for 1.5 h at RT in a humidity chamber, after which they were washed with 2 × 50 μL washing buffer. For visualization of the

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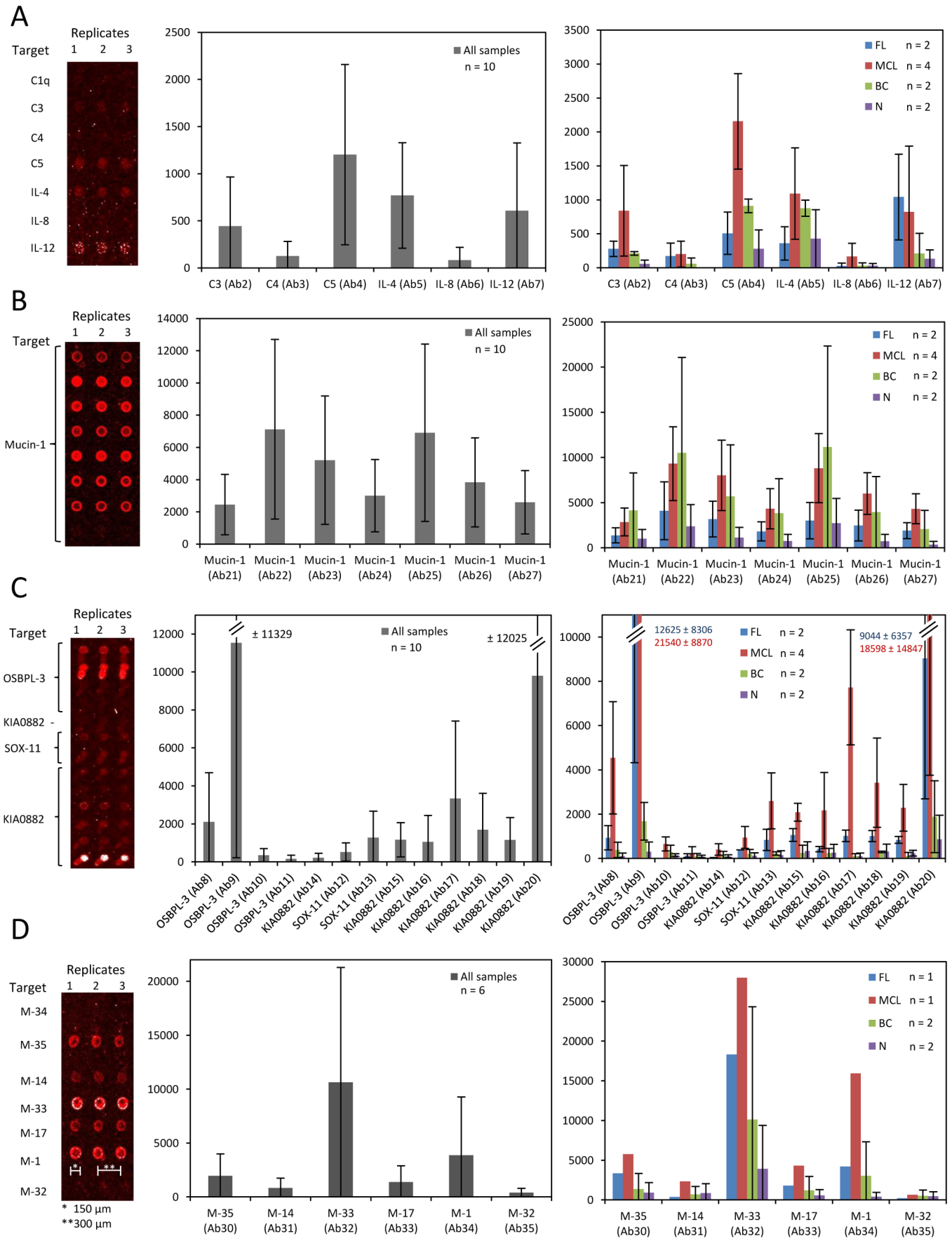

Figure 1. Protein expression profiling of FFPE tissue extracts. Microarray image for a representative sample and the corresponding mean signal intensities for each antigen when the samples were grouped either all together (gray bars) or according to sample type (colored bars) when targeting

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Figure 1. continued

(A) immunoregulatory proteins, (B) a cancer associated protein, (C) B-cell lymphoma associated proteins, and (D) peptide motifs. The microarray image in panel D also includes a dimension scale bar, representative for all arrays, indicating the pitch-to-pitch spot distance (300 μm) and spot diameter (150 μm). No detectable signal intensities were observed for the M-34 antibody (Ab29), the C1q antibody (Ab1), and one of the Mucin-1 specific antibodies (Ab28). The latter two were therefore set to represent the baseline (i.e., cutoff value). The plotted values have all been corrected for the baseline (cutoff) value.

arrays, 50 μL 1 μg/mL Alexa-647 conjugated streptavidin in sample buffer was added to each subarray and incubated for 45 min at RT in a humidity chamber protected from light. Next, the arrays were washed and soaked in PBS for 2 min, followed by purified water for 1 min, and finally dried under a stream of nitrogen gas. The slides were then immediately scanned using a confocal microarray scanner (ScanArray Express, Perkin-Elmer Life & Analytical Sciences) with 5 μm resolution using two different scanner settings, 90/90 and 100/100 (PMT Gain/laser power (%)). The intensity of each spot was quantified using ScanArray Express software V4.0 (Perkin-Elmer Life & Analytical Sciences) using the fixed circle method. To automatically remove any potential spot irregularities, the two highest and two lowest values were automatically excluded, and the result was based on the mean intensity calculated from the four remaining spots.

Blocking Experiment

In order to evaluate the antibody specificity, microarray blocking experiments were performed on all eight scFv antibodies (clones Ab21− Ab28) directed against Mucin-1. To this end, the standard microarray procedure, described above, was modified with an extra step prior to adding the sample. In this extra step, 50 μL 0.07 mg/mL unlabeled recombinant Mucin-1 (MUC1(32TR)-Tn-Fc), was added, incubated for 1 h at room temperature in a humidity chamber, and then washed with washing buffer, before the standard procedure was resumed. The recombinant protein MUC1-(32TR)-Tn-Fc, consisting of 32 tandem repeats of MUC1, carrying Tn glycosylation, fused to mouse IgG2a Fc,[25] was kindly provided by Dr. G. C. Hansson (Department of Medical Biochemistry and Cell Biology, University of Gothenburg, Gothenburg, Sweden).

Mass Spectrometry

To extend the range of specificity analysis (see the section Antibodies), affinity pull-down assays were performed for two of the scFv antibodies (Ab1 (a-C1q) and Ab2 (a-C3)). To this end, a standardized human serum sample, representing the most complex, crude proteome sample at hand with known levels of several analytes (e.g., C1q and C3), thus representing a standardized proteome sample, was targeted. Purified scFvs were immobilized onto magnetic beads (Dynabeads, Invitrogen Dynal, Oslo, Norway) as previously described.[32] The scFv-coated magnetic beads were then incubated with human serum (diluted in PBS) for 90 min. Bound proteins were eluted, digested, and analyzed using mass spectrometry (a Micromass ESI-QTOF Ultima API (Waters)). Only spectra from ions with charge state +2 and +3 were acquired for MS/MS analysis. The evaluation of obtained data was carried out using Mascot Distiller (version 2.4.1) for database searches. The searches were performed against the SwissProt (2013_08.fasta) with the *Homo sapiens* taxonomy filter (20267 sequences after taxonomy fileter) and decoy approach. The following search parameters were used: enzyme: trypsin; missed cleavages: 1; fixed modification: carbamidomethyl (C); variable modification: methionine oxidation. A peptide mass tolerance of 100 ppm and fragment mass tolerance of 0.1 Da was used.

RESULTS

In this study, we have for the first time applied recombinant scFv antibody microarrays for multiplex protein expression profiling of FFPE tissue. To demonstrate proof-of-concept, we extracted and directly labeled individual FFPE tissue sections from cancer patients (n = 8) (breast cancer (BC), follicular lymphoma (FL), and mantle cell lymphoma (MCL)) and healthy controls (N) (n = 2) (Table 2) and profiled 18 biomarker targets comprising 4 different groups of proteins using 35 well-characterized (e.g., specificity and on-chip functionality) antibodies (Table 1). Key assay parameters were assessed, including extraction yield, sample compatibility, reproducibility, limit of detection, and specificity.

Protein Extraction Yield

First, the yield of the protein extraction step was evaluated by measuring the total protein concentration of each extract. To this end, 16 individual extractions were performed on 2 cm^2 FFPE sections from four different sample types, including MCL, FL, BC, and N (Table 2). The results showed that 100 μL with a mean total protein concentration of 987 μg/mL (range of 330−3440 μg/mL) was obtained. The results agreed well with the anticipated yield (500−1600 μg/mL) set by the manufacturer for BC tissue, thus demonstrating the applicability of the approach.

Sample Compatibility

Second, the compatibility of crude, directly biotinylated FFPE tissue extracted proteins with recombinant antibody microarray-based profiling was assessed (Figure 1). Tissue extracts from MCL, FL, BC, and N were profiled on 35 × 8 antibody arrays targeting four groups of antigens (n = 18) (Table 1), including immunoregulatory proteins (n = 7) (Figure 1A), cancer-associated protein (n = 1) (Figure 1B), B-cell lymphoma-associated proteins (n = 3) (Figure 1C), and peptide motifs (n = 7) (Figure 1D). The set of antigens was chosen to reflect antibodies that had been selected against mainly intact, native antigens (Figure 1A,B), small protein fragments, PrESTs, (Figure 1C), or short peptide motifs (Figure 1D). Of note, the latter set of antibodies was known to display reactivity whether the antigen(s) was intact, digested, native, and/or denatured, as long as the linear epitope was accessible. The results showed that adequate spot morphologies and low nonspecific (background) binding were obtained regardless of antigen or sample type (Figure 1). The latter conclusion was supported by the observations that (i) low/no signal intensities were observed for areas outside the antibody-coated spots (blocked by milk proteins), (ii) high signal-to-noise ratios (in average 200−500, range 30−1800) were obtained for all antibody-coated spots that were detectable, and (iii) no signal intensities were observed for three arrayed scFv antibodies (of which two were subsequently used as negative controls). Further, the data showed that 16 of 18 targeted

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A

Aim		Samples extracted in the same tube	Samples labeled in the same tube	Samples analyzed on the same slide	All steps performed same day	Coefficient of determination (R^2)
Overall reproducibility	i	Yes	Yes	Yes	Yes	0.93-0.98
Effect of workflow parameters	ii	Yes	Yes	No	Yes	0.91-0.97
	iii	Yes	No	No	Yes	0.96-0.99
	iv	No	No	Yes	Yes	0.80-0.96
	v	No	No	No	No*	0.80-0.97

*All steps performed on different days.

B

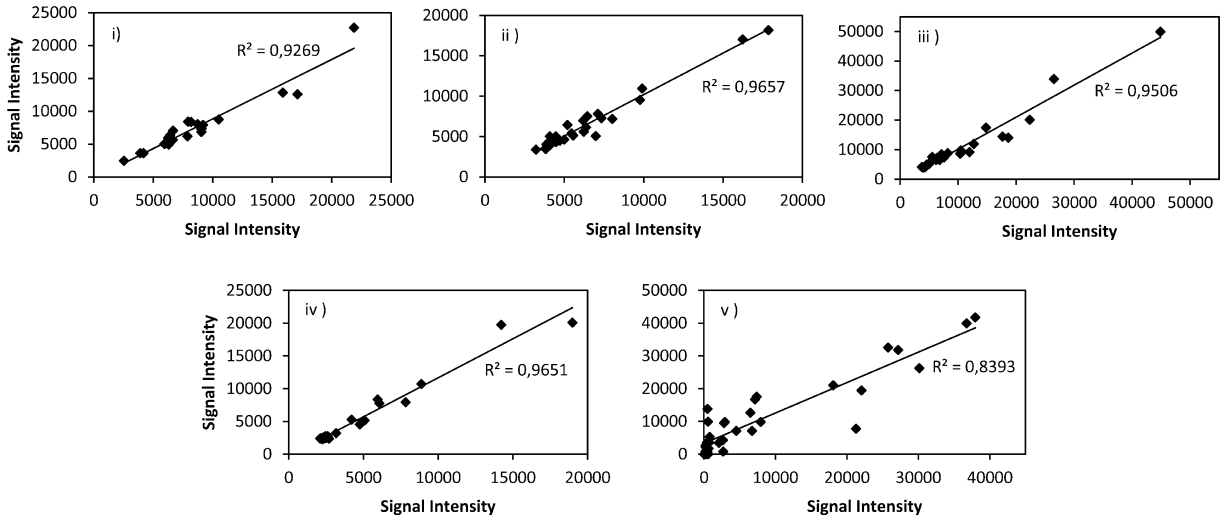

Figure 2. Reproducibility of the assay. (A) Summary of the various assay step combinations (denoted comparisons i–v) for which the reproducibility was assessed, represented by the coefficient of determination. By "samples extracted in the same tube" the label "YES" means that FFPE sections from the same tumor were extracted in the same tube, and the label "NO" means that FFPE sections from the same tumor were extracted in different tubes on the same or a different day. By "samples labeled in the same tube" the label "YES" means that FFPE sections from the same tumor were labeled in the same tube, and the label "NO" means that FFPE sections from the same tumor were labeled in different tubes on the same or a different day. (B) Representative linear regressions for each assay step combination are shown (comparison i/sample MCL4; comparison ii/sample MCL1; comparison iii/sample MCL2; comparison iv/sample BC1; comparison v/sample MCL1). Each data point represents signal intensities from the same sample in two analyses (handled according to A)) and plotted against each other. Each signal intensity is based on the mean value of 4 replicate antibody spots. The 28 antibody array layout was used for comparisons i–iv, while the 35 antibody array layout was used in comparison v. Antibodies Ab1, Ab28, and Ab29 were not included in the analysis since no detectable signals were observed.

antigens could be successfully detected in at least one or more of the samples, while only the complement protein C1q and the peptide motif M-34 were not detectable in any sample (Figure 1). In addition, when viewing all samples (n = 10) as one broad group (i.e., including different patients suffering from different indications), dynamic signal intensities were, as might be expected, observed for each individual antigen, ranging from small (e.g., M-32) to large (e.g., Mucin-1), reflecting sample-related differences in antigen expression levels (i.e., biological differences) (Figure 1, gray bars). These differences were further refined and demonstrated by viewing the four sample groups individually (i.e., including different patients, but suffering from the same indication) (Figure 1 colored bars). Hence, crude FFPE tissue extract was found to be a sample format compatible with multiplexed expression profiling using recombinant antibody microarrays.

Technical Reproducibility

The mean spot-to-spot reproducibility, expressed as coefficient of variation (CV) was found to be 9.6% (range 3.7–14.6%). Next, the reproducibility of the entire assay was evaluated and expressed in terms of coefficient of determination, R^2, using all samples and antibodies that gave detectable signals (Figure 2 and Supplementary Figure 1). As outlined in Figure 2A, all of the individual steps of the assay were assessed in different combinations, including the extraction, labeling, and array assay steps, along with whether the individual steps had been performed on the same day or not. Representative linear regressions for the different assay step combinations are shown in Figure 2B. In general, the results showed high assay reproducibility for the entire setup, with R^2 values ranging from 0.80 to 0.99. In more detail, the overall reproducibility, i.e., when the same batch of extracted and labeled sample was analyzed on duplicate subarrays on the same slide, was found to be high (R^2 of 0.93–0.98) (Figure 2 and Supplementary Figure

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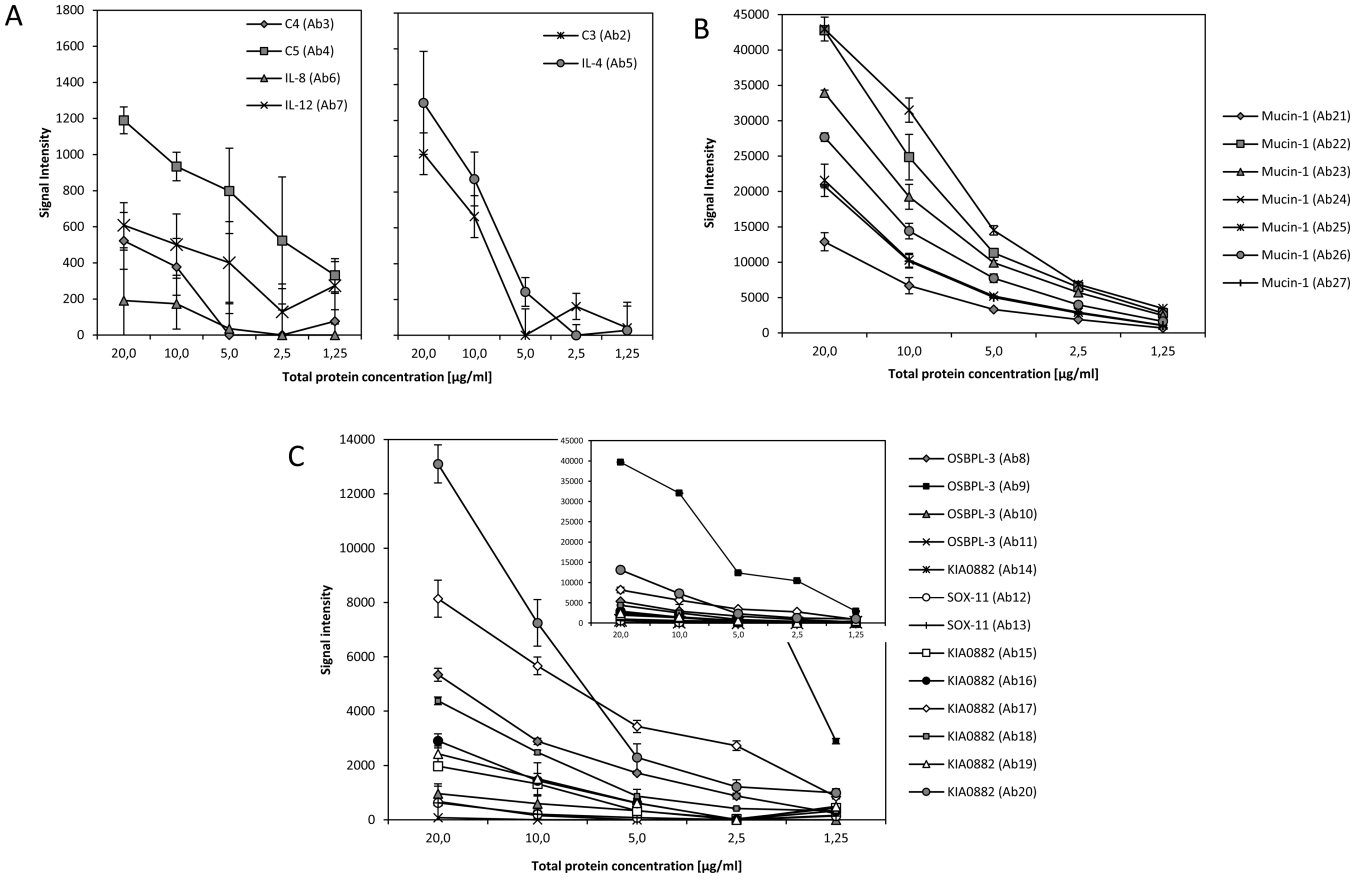

Figure 3. Limit of detection, presented in terms of total protein concentration. Protein expression profiling of serial dilutions of a representative MCL sample (MCL3), targeting (A) immunoregulatory proteins, (B) a cancer associated protein, and (C) B-cell lymphoma associated proteins, is shown. No detectable signal intensities were observed for the C1q antibody (Ab1) and one of the Mucin-1 specific antibodies (Ab28), and these were therefore set to represent the baseline (i.e., cutoff value). The plotted values have all been corrected for the baseline (cutoff) value.

1). In fact, the R^2 values were high (0.91−0.99) regardless of whether the extracted sample was labeled in the same tube (batch) and/or analyzed on the same slide (comparisons i−iii). In contrast, the R^2 values dropped (0.80−0.97) when the tissue sample was split and extracted in different tubes (batches) (comparisons iv and v), indicating that this step contributed most to the technical assay variability.

Limit of Detection

Next, the limit of detection (LOD), in terms of total protein concentration, was determined, and representative results obtained for one MCL sample are shown in Figure 3. Serial dilutions (20 to 1.25 μg/mL total protein) of FFPE tissue extracts were analyzed on a 28 × 8 antibody array, targeting immunoregulatory proteins (Figure 3A), a cancer-associated protein (Figure 3B), and B-cell lymphoma-associated proteins (Figure 3C). While 2 antigens could not be detected (C1q and M-34) (data not shown), the results showed that the LOD was 10 μg/mL total protein, corresponding to 500 ng protein, for 5 antibodies (IL-8/Ab6, OSBPL-3/Ab11, SOX-11/Ab12, SOX-11/Ab13, and KIA0882/Ab14) (Figure 3A,C). For the remaining 21 antibodies targeting antigens belonging to all three antigen groups, the LOD was found to be in the range of 1.25−5 μg/mL, corresponding to 62.5 and 250 ng of total protein (Figure 3). The observation that different antibody clones targeting the same antigen resulted in different LODs might be explained by differences in (i) antibody concentration), (ii) antibody affinity, and/or (iii) antibody reactivity toward FFPE extracted proteins (how well the epitopes were preserved) and highlighted the advantage of using multiple clones per antigen. Taken together, the setup was found to display adequate sensitivity for multiplex protein expression profiling of crude FFPE extracts.

Specific Detection

To extend the range of specificity analysis (Table 1), affinity pull-down assays were performed for two of the scFv antibodies (Ab1 (a-C1q) and Ab2 (a-C3)). To this end, a standardized human serum sample, representing the most complex, crude proteome sample at hand with known levels of several analytes (e.g., C1q and C3), thus representing a standardized proteome sample, was targeted. The results showed that the two antibodies specifically captured C1q and C3, respectively (Supplementary Figure 2 and Supplementary Table 1).

Finally, in order to evaluate that the antibody specificity was retained despite the harsh conditions the FFPE sample proteins had been subjected to during both initial fixation and subsequent extraction, blocking experiments were performed. Results, illustrated for the seven scFv antibodies targeting the cancer-associated protein Mucin-1, which gave detectable signal intensities in five different FFPE extracts, are shown in Figure 4. Ab28 is not included as is no detectable signal intensity was observed in these experiments (instead used as negative control). In the blocking experiments, the arrays were incubated with pure, unlabeled Mucin-1, prior to adding the labeled FFPE extracts. The results showed that the four cancer

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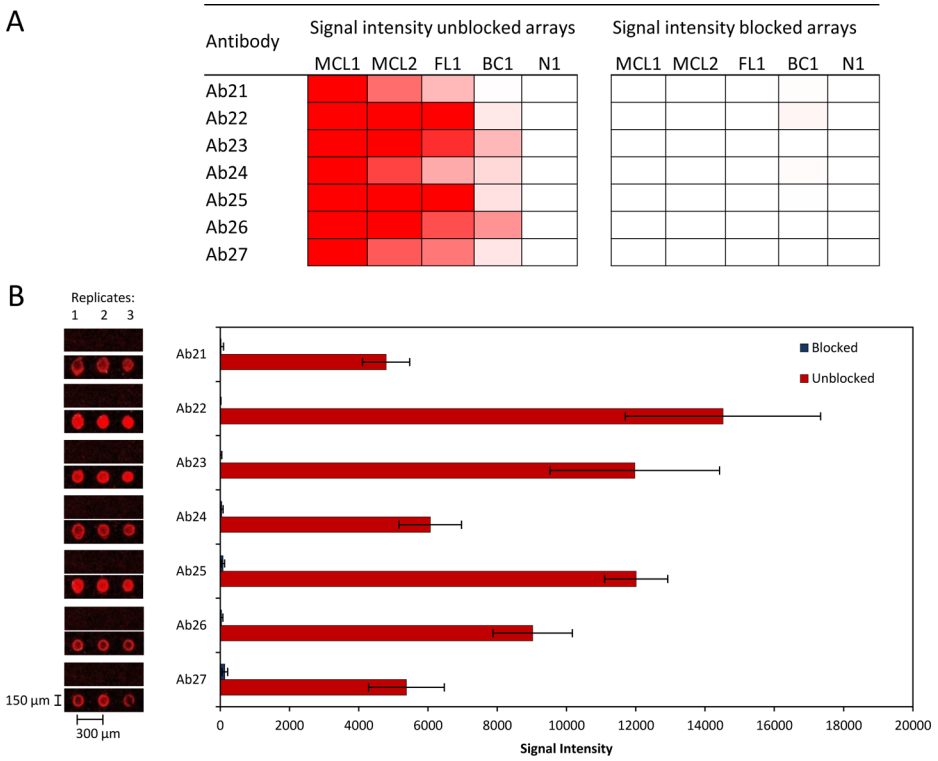

Figure 4. Antibody specificity evaluated by blocking experiments. (A) Signal intensities, as represented by a heat map (red-highest signal, white-lowest signal), of the 7 different detectable scFvs targeting a cancer associated protein (Mucin-1) when analyzing 5 different FFPE sample extracts, with and without addition of unlabeled Mucin-1 antigen to the array prior to adding the sample. The eighth a-mucin antibody (Ab28) is not shown as no detectable signal intensity was observed (instead used as negative control). (B) Microarray image and corresponding signal intensities for all 7 scFvs when analyzing a representative sample (MCL2). A dimension scale bar, representative for all arrays, indicating the pitch-to-pitch spot distance (300 μm) and spot diameter (150 μm), is shown.

FFPE extracts that displayed medium to strong signals for Mucin-1 could all be specifically blocked with antigen in all cases (Figure 4A,B). The FFPE extract from one healthy control did not display any reactivity toward Mucin-1, regardless of whether the array had been blocked with antigen (Figure 4A). Thus, the data implied that the specificity of the antibodies was retained, further supporting the compatibility of FFPE extracts with antibody microarray-based analysis.

DISCUSSION

Accessing the vast archives of retrospective clinically well-defined FFPE sample collections for disease proteomics driven projects will be essential, but our ability to explore the proteomes of FFPE tissues is still in an early stage and will require additional technology development to make it practicable.[11] In light of this, we have here presented the first proof-of-concept study for multiplexed, high-throughput protein expression profiling of FFPE material using recombinant scFv antibody microarrays in a specific and sensitive manner.

A key concern in any proteomics-based assay of FFPE extracts is whether the protein can be extracted in an assay-compatible format. Previously, FFPE extracts have been successfully analyzed using mass spectrometry, targeting predominantly digested samples, i.e., tryptic proteomes, and RPPA, addressing denatured and/or native proteins, providing strong support for retrospective proteomic analysis of FFPE tissue sample for biomarker discovery.[8−14] In fact, encouraging work has recently been published showing successful extraction of native proteins from FFPE material, and analyses of such extracts have been comparable to that of fresh frozen tissue with regard to protein identification, abundance, and immunoreactivity.[8,12] Of note, immunoreactive, nondegraded full length proteins could be extracted, as demonstrated by a high correlation between Western blots and RPPAs for several proteins, such as β-actin, HER2, PAI-1, EFGR, and E-cadherin.[5,8,35] To the best of our knowledge, we showed here for the first time that proteins could be extracted from FFPE samples in a format also compatible with recombinant scFv antibody microarrays. In addition, we also showed that the (epitope) structure of a majority of the targeted antigens appeared to be preserved, as reflected by a retained specific immunoreactivity. This was evaluated by using antibodies that had been stringently selected against a comprehensive range of proteins targets, including (i) short peptide motifs (4−6 amino acids long), (ii) short unique protein fragments, PrESTs (100−150 amino acids long),[28] as well as (iii) intact, full length native proteins. In fact, only 3 of 35 antibodies, targeting two soluble proteins (C1q and Mucin-1) and one peptide motif (M3-34) failed to display any detectable signals. Of note, Mucin-1 was readily detected by seven other Mucin-1 antibodies, highlighting the benefit of using several antibodies targeting the same antigen.[16] The lack of antibody reactivity could be explained by reasoning that (i) the antigen was not present in the sample, (ii) the antigen was present, but below LOD, (iii) the epitope was destroyed during the extraction procedure and/or sterically blocked during the labeling procedure (e.g., for the Mucin-1 specific antibody), and/or (iv) a free C-terminal is required for binding (only for the M-34 specific antibody).[24] Furthermore, the fact that low nonspecific background binding

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was observed in general further supported the notion that proteins with retained structural properties rather than denatured structures with "sticky" properties were extracted. In this context, it should be noted that we used well-characterized antibodies displaying high specificity.[21−23,25,27,29−33] Here, we also extended this range of specificity analysis by performing affinity pull-down assays linked to mass spectrometry (Ab1 and Ab2), as well as blocking experiments (Ab21−Ab28), further supporting the observed antibody specificities.

The assay sensitivity is the next key factor to consider when targeting crude proteomes, such as FFPE tissue extracts. The sensitivity of competing MS-based experiments is in general limited to high to medium abundant analytes in conventional discovery projects.[36] In the case of RPPA, it is also inherently challenging to target truly low-abundant analytes in arrayed complex samples containing high-abundant proteins.[37−41] Still, Berg et al. have reported that they were able to detect HER2 in FFPE tissue extract spotted at a concentration of 0.5 μg/mL.[5] Notably, by adopting various signal amplification systems, the RPPA sensitivity could be improved, generating LODs in the range of femtograms of pure protein per spot.[40,41] In comparison, we found the assay sensitivity, expressed in terms of total protein concentration, to be as low as 1.25−5 μg/mL, corresponding to 62.5−250 ng total protein added per array, without using any signal amplification, for 21 of the antibodies (and in the range of 500 ng total protein for another set of 5 antibodies). As the concentration of the specifically targeted proteins could be estimated to be significantly lower than the total protein concentration, the true LOD could be expected to be well below the 62.5 ng total protein per sample here observed. Hence, the data implied that the observed sensitivity of our setup compared well to competing setups and should be acceptable in clinical settings. In addition, the promising route of signal amplification remains to be explored to further enhance our assay sensitivity if so required. In this context, it might be of interest to note that the non-amplified assay sensitivity of our conventional recombinant antibody microarrays has been shown to be in the sub-picomolar range when targeting crude serum samples.[21]

In previous RPPA work, the assay reproducibility (CV values) was reported to be 0−38% for interarray experiments, while the reproducibility between triplicate extractions from the same sample was 6−36%.[5] We evaluated all steps of the assay, and the reproducibility, expressed in terms of coefficient of determination (R^2), was found to be in the range of 0.80−0.99. Also in our case, the extraction step was found to have a significant impact on the reproducibility. It should, however, be noted that we used different tissue sections from the same tumor when we compared the outcome of different extractions. The issue of (tumor) tissue heterogeneity is a well-known fact,[42−44] which affects all (proteomic) technologies attempting to analyze tissue sections, and a potential limitation one should be aware of in any FPPE tissue-based study. While this could influence the resulting protein expression map, it would not per se affect the ability of the recombinant antibody array technology to profile FFPE samples. In other words, it is the extraction step (and tissue selection step) itself that will be critical in order to minimize variations. This could, if so required, be further addressed in future work by adopting laser capture microdissection (LCM), which allows more specific and homogeneous cell types to be isolated from the FFPE tissue before extraction and subsequent antibody array analysis, thus avoiding (minimizing) the heterogeneity altogether.[45] Precision LCM sessions are able to acquire several thousand cells (10,000 cells would give about 100 ng total protein), which would then be within the observed range of LOD for the current recombinant antibody array setup.

In conclusion, in this proof-of-concept study, we have designed the first technology platform for protein extraction, labeling, and expression analysis of FFPE tissue samples using recombinant scFv antibody microarrays. By this, we have extended the range of crude sample formats compatible with multiplexed, sensitive, and specific antibody array-based profiling in a high-throughput format. Hence, we have paved the way for a new attractive way of addressing the extensive repertoire of retrospective clinically well-defined FFPE sample collections, setting the stage for the next generation of biomarker-driven discovery projects.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BC, breast cancer; CV, coefficient of variation; FFPE, formalin-fixed paraffin-embedded; FL, follicular lymphoma; IHC, immunohistochemistry; LCM, laser capture microdissection; LOD, limit of detection; MCL, mantle cell lymphoma; N, healthy controls; PrEST, protein epitope signature tag; RPPA, reverse-phase protein array; scFv, single chain fragment variable

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