

High-Performance Low-Cost Antibody Microarrays Using Enzyme-Mediated Silver Amplification

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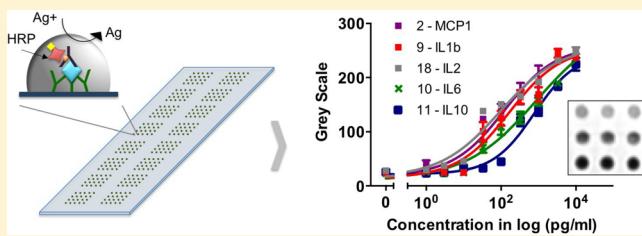
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Supporting Information

ABSTRACT: Antibody microarrays can detect multiple proteins simultaneously, but the need for bulky and expensive fluorescence scanners limits their adaptation in clinical settings. Here we introduce a 15-plex enzyme-mediated silver enhanced sandwich immunoassay (SENSIA) on a microarray as an economic alternative to conventional fluorescence microarray assays. We compared several gold and silver amplification schemes, optimized HRP-mediated silver amplification, and evaluated the use of flatbed scanners for microarray quantification. Using the optimized assay condition, we established binding curves for 15 proteins using both SENSIA and conventional fluorescence microarray assays and compared their limits of detection (LODs) and dynamic ranges (DRs). We found that the LODs for all proteins are in the pg/mL range, with LODs for 12 proteins below 10 pg/mL. All but two proteins (ENDO and IL4) have similar LODs (less than 10-fold difference) and all but two proteins (IL1b and MCP1) are similar in DR (less than 1.5-log difference). Furthermore, we spiked six proteins in diluted serum and measured them by both silver enhancement and fluorescence detection and found a good agreement ($R^2 > 0.9$) between the two methods, suggesting that a complex matrix such as serum has a minimal effect on the measurement. By combining enzyme-mediated silver enhancement and consumer electronics for optical detection, SENSIA presents a new opportunity for low-cost high-sensitivity multiplex immunoassays for clinical applications.

KEYWORDS: antibody microarray, high-sensitivity assay, multiplex assay, signal amplification, enzyme-mediated silver precipitation, and flatbed scanner



INTRODUCTION

Parallel quantification of multiple proteins may reveal crucial information about a complex disease that is otherwise unavailable with a single protein target. Antibody microarrays can quantify multiple proteins simultaneously and are common in disease diagnosis and patient stratification,¹ thereby benefiting early diagnosis of diseases such as breast cancer.² Most high-sensitivity multiplex microarray tests rely on fluorescence detection and need a laser scanner for image acquisition. Depending on the number of lasers available and the type of detectors used, a scanner costs between \$40K and \$250K. They are also sensitive to shock and dust and require regular maintenance. The high cost of acquisition and upkeep of a fluorescence scanner limits the adaptation of microarray technology in clinics. Furthermore, organic fluorophores are prone to photobleaching and cause assay signals to fade. Therefore, a stable nonfluorescent reporter molecule and a low-cost imager may accelerate the application of antibody microarrays for disease diagnosis.

Alternative imaging methods have been proposed to quantify nonfluorescent signals, for example, microscopy,³ colorimetric

scanners,⁴ interferometry-based imagers,⁵ CD players,⁶ and smartphones.⁷ Microscopes are widely available in many laboratories but do not always have an automated stage and thus have limited scanning capability. Colorimetric scanners are cheaper than fluorescence scanners but still cost about \$5K (e.g., ArrayIt Spotware Colorimetric microarray scanner). Interferometry-based imagers measure minute changes in optical path length due to a local change of refractive index at the sensor surface, which is a very sensitive method, but require a special substrate (Si wafer coated with 100 nm SiO₂) with tailored optical properties and specialized instrumentation. CD players are an affordable method as well but require customization, while smartphones often necessitate an adapter for scientific imaging. In contrast, flatbed scanners have homogeneous illumination, generate distortion-free images, and have recently been used for biomedical imaging,⁸ such as for an immunoassay in conjunction with silver amplification showing a simplex assay in buffer solution with ng/mL

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sensitivity.⁹ Although promising, it was not clear from the study whether silver amplification is suitable for high-sensitivity multiplex assays and whether the resolution of a flatbed scanner is sufficiently high for imaging microarray spots as the assay works with a 5 mm spot size defined by a PDMS sheet with a hole cut out.

Gold nanoparticles (AuNPs) are one of the most widely used reporter molecules, notably in lateral flow assays, to generate a nonfluorescent signal.¹⁰ Silver amplification is often used in applications such as electrophoresis,¹¹ Western blotting,¹² and tissue staining to further enhance the signal.¹³ Several studies have used silver amplification (AgNO_3 + hydroquinone) for protein assays against a variety of targets reaching an LOD between 1 and 10 ng/mL,^{6,9,14,15} with one landmark study showing its application in a yes–no HIV and syphilis screening test targeting low-resource countries.¹⁶ However, the sensitivities of these assays are orders of magnitude worse than the gold standard enzyme-linked immunosorbent assay (ELISA), which typically falls in the pg/mL range. Mirkin and colleagues improved the sensitivity of silver amplified assays using DNA-coded AuNPs.^{17,18} In their work, detection antibodies (dAbs) were conjugated to DNA-coded AuNPs and then were captured on magnetic beads coated with capture antibodies (cAbs) in the presence of an analyte. The DNAs were released from the AuNPs and concentrated on a microarray, followed by silver amplification. The concentrating step improves the assay sensitivity to attomolar (aM), but the assay requires customized AuNPs and several additional assay steps. In another work, the same group used the same DNA-conjugated AuNPs in a two-step AuNP-catalyzed Au amplification, and although no binding curve or LOD was determined, they found that they could detect 300 aM of PSA in buffer.¹⁹ Whereas multiplexing was demonstrated with a 3-plex assay, only protein spiked-in buffer was tested and again only qualitative yes–no assays were shown.

The use of silver amplification is largely focused on chemical amplification using Au nanoclusters (subnanometer to 10 nm) or AuNPs (10 to 100 nm) as catalytic centers. Interestingly, enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (ALP) can also catalyze and induce the growth of silver particles in the absence of AuNPs. Although not fully understood, a widely accepted explanation is that silver ions are precipitated to particles by electrons that are generated during the redox reaction of H_2O_2 . Enzyme-mediated silver precipitation has been used in applications such as (1) silver-enhanced *in situ* hybridization (SISH) to assess HER2 genes in breast cancer tissues as an alternative to fluorescent *in situ* hybridization (FISH);^{20,21} (2) electric-based detection of DNAs by measuring the conductivity of percolating silver particles between two Au electrodes;²² and (3) electrochemical quantification of proteins by measuring the stripping current during the deposition or nicking of silver particles.^{23–25} These approaches demonstrate high specificity of enzyme-mediated silver amplification, but multiplexing remains a challenge and may require complex addressable electronics.

In this work, we present a multiplex silver enhanced sandwich immunoassay (SENSIA) on a microarray using a flatbed scanner for assay read-out. After first comparing chemical and enzymatic gold and silver amplification methods, we selected HRP-mediated silver amplification, optimized its reaction conditions on a microarray, and configured a scanner for optimal microarray imaging. After optimizing the SENSIA protocol, we developed a 15-plex assay and used it to quantify

candidate cancer biomarkers including cytokines, growth factors, and cancer-related proteins. The performance of SENSIA was compared with fluorophore-linked immunosorbent assay (FLISA) by quantifying the LODs and DRs in both buffer and serum. In addition, we tested the specificity of the antibodies used in SENSIA and the possibility of storing SENSIA slides for delayed analysis.

MATERIAL AND METHODS

Reagents

Antibodies and proteins were purchased from R&D Systems (Minneapolis, MN) as summarized in Table S1 in the Supporting Information (SI), reconstituted, and stored according to the supplier's instructions. All dAbs were purchased in biotinylated form. Streptavidin (SA)-conjugated HRP and Cy3 were obtained from Life Technology (Burlington, ON), SA-Au from Diagnostic Consulting Network (Carlsbad, CA), SA-nanogold from Nanoprobe (Yaphank, NY), silver precipitation kits from Sigma-Aldrich (St. Louis, MO) and Nanoprobe, pooled human serum from Jackson Immuno-research Laboratory (West Grove, PA), and all other chemicals from Sigma-Aldrich.

Microarray Fabrication

cAbs against 15 target proteins and biotinylated goat anti-rabbit IgG as calibrants were diluted in a printing buffer (31% 2,3-butanediol and 2.5 M betaine in PBS) to a final concentration of 100 $\mu\text{g}/\text{mL}$. The cAbs (100 $\mu\text{g}/\text{mL}$, 2 nL per spot) were printed using an inkjet spotter (Nanoplotter 2.0, GeSiM, Grosserkmannsdorf, Germany) and a customized contact spotter (Nanoplotter 2.1, GeSiM), the latter of which was used in the long-term storage experiment. The printed slide (Xenopore, Hawthorne, NJ) was incubated overnight at RT in a 65% humidity chamber.

SENSIA Protocol

The printed slide was assembled with a 16-well gasket (Grace Bio-Lab, Bend, OR) and rinsed with washing solution (PBS buffer with 0.1% Tween20) three times, 5 min each. It was blocked with 100 μL of the blocking solution (PBS buffer with 3% BSA and 0.1% Tween20) for 1 h, incubated with 100 μL of sample (a three-fold dilution series with a starting concentration of 10 ng/mL) for 3 h, 100 ng/mL biotinylated dAb for 1 h, and 4 $\mu\text{g}/\text{mL}$ SA-HRP for 30 min. The 16-well gasket was then replaced with a single-well gasket. The slide was rinsed again with washing solution and DI water and then incubated with 1 mL of 50% silver amplification solution diluted in DI water. The silver precipitation kit consists of three reagents, and the diluted reagents were added in sequence 5 min apart. All incubation steps were performed on a rotary shaker.

Comparison of Different Signal Amplification Methods

A similar protocol to SENSIA was followed. Antirabbit IgGs (100 $\mu\text{g}/\text{mL}$, 4 nL) were immobilized on the slide and incubated with biotinylated rabbit IgG (10 pg/mL to 40 ng/mL, 100 μL). The slide was incubated with SA-Au or SA-nanogold (500 ng/mL, 100 μL) or SA-HRP or SA-AP (4 $\mu\text{g}/\text{mL}$, 100 μL) for 30 min while shaking constantly, followed by Au or Ag amplification protocols, as recommended by the supplier or as described in the literature.^{16,19}

Comparison of SENSIA and FLISA

The protocols for FLISA and SENSIA were mostly identical, except the last step where in SENSIA the biotinylated dAbs

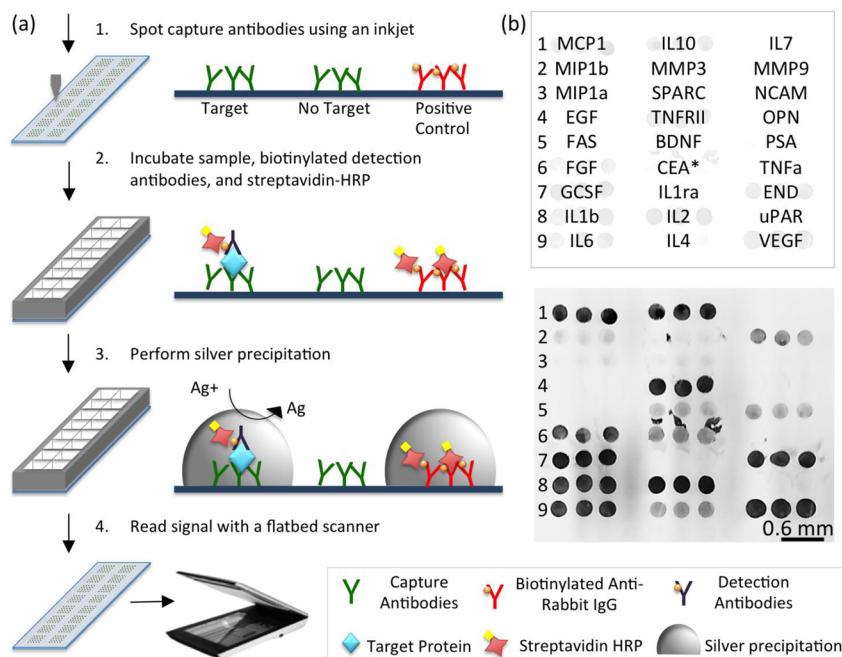


Figure 1. Schematic of multiplex SENSIA. (a) Typical multiplex SENSIA assay protocol: (1) spotting cAbs on a microarray slide using an inkjet spotter; (2) clamping a 16-well gasket onto the slide to allow sequential incubation of a blocking solution (3% BSA and 0.1% Tween20 in PBS), samples containing various concentrations of proteins, biotinylated dAbs, and finally SA-HRP; (3) applying the silver amplification solution; and (4) acquiring the image using a flatbed scanner. (b) Spotting pattern of cAbs against 27 proteins, repeated in each well, and an example assay result.

were bound to SA-HRP followed by silver amplification and in FLISA, the biotinylated dAbs were directly bound to SA-Cy3.

Analysis of Cross Reactivity in Singleplex and Multiplexed Assays

A singleplex assay was performed for all 15 proteins individually and compared with a multiplex assay to verify the assay specificity. The singleplex assay was carried out on the same microarray slide, following a similar protocol as the multiplex assay, except each well was incubated with one of the 15 proteins and its dAb; whereas for multiplex assay, the wells were incubated with a mixture of all proteins and a mixture of all dAbs.

Parallel Measurement of Multiple Serum Samples

Six proteins, MCP, GCSF, IL1b, IL6, TNFRII, and IL2, were spiked at a concentration of 1 ng/mL in 10% serum in PBS, and each protein was subsequently diluted two-fold in the 10% serum to constitute five samples containing 1, 0.5, 0.25, 0.125, and 0 ng/mL of each protein. Each sample was divided in two aliquots and quantified by both silver and fluorescence detection. The extracted concentrations were compared and correlated using least-squares linear regression, and the Pearson coefficient of determination, R^2 , was calculated.

Image Acquisition and Data Analysis

The SENSIA assay was imaged four times using a flatbed scanner (LiDE700F, Canon, Mississauga, ON) at 1200 dpi and the FLISA slide using a fluorescence scanner (G2505c, Agilent Technologies, Santa Clara, CA). Microarray Profile, a plug-in in Fiji (an open-source software), was used to extract the median value of each spot as well as the local background of each spot to get the relative spot intensity. Median values, instead of mean values, were used to dampen the effect of high-intensity speckles in the microspots, possibly due to dusts or scanning artifacts. The extracted data were imported into Prism 6 (GraphPad Software, Lo Jolla, CA) for further calculation of

binding curves, LODs and DRs. A nonlinear four-parameter logistic (4PL) regression fit was used to derive the binding curves. LODs were calculated as the mean of zero concentration plus three times the standard deviation of the zero concentration. DRs were defined as the concentration range at which the signal intensity is 5% above and below the minimal and maximal asymptote of the binding curves. A detailed derivation of DR is provided in the SI eqs 1–4.

RESULTS AND DISCUSSION

Our study uses the same assay protocol as in a classical antibody microarray with fluorescence detection in a sandwich assay format but replaces fluorescent labels with silver precipitation to amplify the signal and quantify the binding using a flatbed scanner. Figure 1 illustrates the main concept of SENSIA and the spotting pattern of 27 cAbs, each with three replicates. With an average spot size of 250 μm in diameter and a center-to-center pitch of 400 μm , a density of 400 spots per cm^2 was achieved. Given that the smallest spot that can be dispensed by the inkjet spotter is 80 μm in diameter and a minimal pitch of 250 μm , a high-density array is possible on SENSIA slides. In Figure 1b, we observed a “comet-tail” pattern on row 6 middle column (CEA), which was due to a high concentration of cAb being used during spotting.²⁶ Although 100 $\mu\text{g}/\text{mL}$ was found to be the optimal spotting concentration for most proteins for maximal binding capacity without saturating the spot, cAb against CEA may have a high affinity to the slide and needs to be diluted further in future experiments for optimal spot morphology. (The smear pattern did not affect the signal intensity of the neighboring spots in the current experiment.)

Optimization of SENSIA Protocol

First, we compared five signal amplification methods involving nanoparticles (Figure 2a and Figure S1 in the SI): (i) large

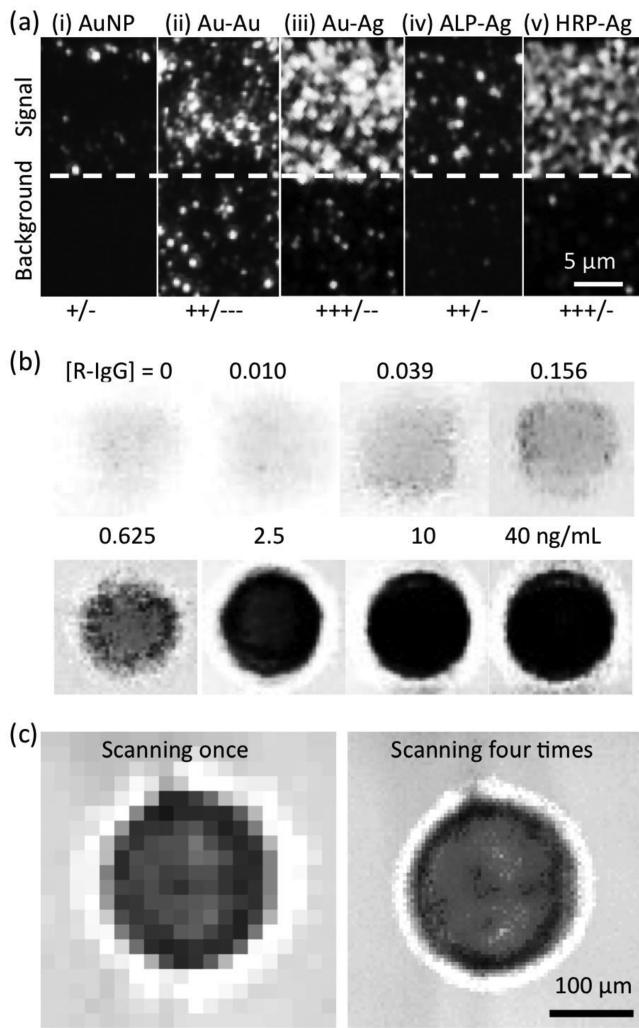


Figure 2. SENSIA assay optimization. (a) Comparison of different signal amplification protocols: (i) AuNPs, (ii) small AuNP-catalyzed Au precipitation, (iii) small AuNP-catalyzed Ag precipitation, and (iv) ALP- and (v) HRP-catalyzed Ag precipitation. Rabbit IgGs-biotin (1 ng/mL) were captured on a glass surface and incubated with various SA conjugations, followed by various signal amplification procedures, and the results were imaged under a light microscope in dark-field mode. The “+” and “−” represent qualitative signal intensity at the signal or the background regions, respectively. (b) Characterization of the selected HRP-catalyzed silver precipitation for rabbit IgG concentration ranging from 10 pg/mL to 40 ng/mL. (c) Scanned image of a microarray spot obtained following single or multiple scans.

AuNPs conjugated directly to dAbs, (ii) small AuNP-catalyzed Au precipitation, (iii) small AuNP-catalyzed Ag precipitation, and (iv) ALP- and (v) HRP-catalyzed Ag precipitation. Direct labeling of antibodies with large AuNPs (i) resulted in nearly no background but also a weak signal, while small AuNP-catalyzed Au or Ag precipitation (ii–iii) yielded strong signals but also some background due to spontaneous precipitation. Enzyme-catalyzed silver precipitation (iv–v) produced low background, and HRP, in particular, yielded a strong signal and reacted fast (15 min vs 2 h when catalyzed by ALP) and therefore was selected as the amplification method and for further optimization.

Next, we evaluated the HRP-catalyzed silver amplification in a one-step assay using an antirabbit IgG as the cAb and 1 ng/mL biotinylated rabbit IgGs as the analyte. We found that the

assay signals are susceptible to small changes in the incubation time. To mitigate the time-dependent variation, we slowed down the reaction by either conducting the assay at a low temperature (4 °C vs RT) or using diluted silver solution (1:1, 1:2, and 1:3 dilution in DI vs no dilution). The two conditions resulting in the best signal-to-background ratio were: (1) no dilution at 4 °C for 15 min or (2) using 1:1 dilution with DI at RT for 15 min. Any further dilution of the silver solution requires a longer incubation time up to 30 min, giving enough time for the background to develop but only improving the signal marginally. The second condition was used for subsequent assay development. Figure 2b shows a series of microarray spots of rabbit IgG ranging from 10 pg/mL to 40 ng/mL, with the spot intensity proportional to the analyte concentration. Figure S2 in the SI shows improved signal uniformity across the slide and reduced variability in spot intensity under the optimized condition, and Figure S3 in the SI shows CV of silver spot intensities within each well and between wells, demonstrating high reproducibility of silver amplification.

Finally, we configured the flatbed scanner for maximal resolution for quantifying a microarray assay. The scanner used in this work has a signal depth of 8-bit and a lateral resolution of 1200 dpi. To maximize its performance, we operated the scanner in manual mode and set it to scan the slides four times. Figure 2c shows that the multiscan reduces pixilation and is expected to reduce noise owing to oversampling and averaging.

Binding Curves of Multiplexed SENSIA

We qualitatively evaluated reagent-driven cross-reactivity^{2,27} among 27 antibody pairs that have a general relevance to cancer and selected the 15 pairs that showed the least cross-reactivity for the antibody microarray. The selection process and criteria are described in Table S1 and Figure S4 in the SI. The selected 15 proteins include interleukins IL1b, IL2, IL4, IL6, and IL10, chemokines MCP1, proteases MMP9, growth factors BDNF, ENDO, FGF, and VEGF, and a few cancer-related antigens CEA, GCSF, TNFRII, and PSA. We established binding curves for each protein (Figure 3) and calculated their LODs and DRs (Table 1). Most binding curves exhibited a characteristic sigmoid shape with plateaus at both high and low concentrations, as expected. Two proteins (IL4, MMP9)

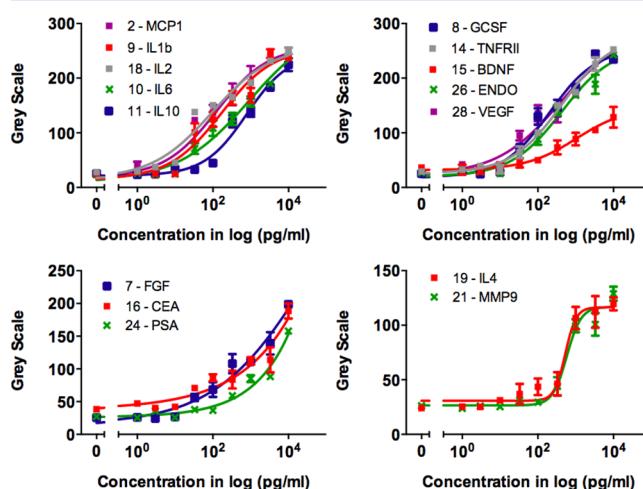


Figure 3. Binding curves of 15 proteins measured using SENSIA. The concentration ranges from 1 pg/mL to 10 ng/mL. The error bars are standard deviation of three replicated spots from a single experiment.

Table 1. LODs and DRs for 15-Plex Assays Measured by SENSIA and by FLISA

	LOD (pg/mL)		DR (in log)	
	SENSIA	FLISA	SENSIA	FLISA
BDNF	114	783	3.96	2.84
CEA	6.87	9.43	4.12	4.38
ENDO	2.09	97.9	3.98	3.41
FGF	1.38	1.22	4.41	5.08
GCSF	6.32	5.56	3.69	4.11
IL10	9.05	6.02	3.31	4.49
IL1b	1.29	1.36	4.10	5.65
IL2	3.27	3.25	4.32	5.18
IL4	182	2220	0.85	0.67
IL6	1.15	1.99	5.52	5.79
MCP1	0.69	0.34	4.07	5.68
MMP9	184	442	1.12	2.13
PSA	49.3	396	2.05	2.40
TNFRII	6.46	32.7	3.96	3.70
VEGF	7.55	3.55	4.91	4.97

showed a relatively narrow dynamic range, and three proteins (CEA, FGF, and PSA) did not reach a plateau at high concentrations. As shown in Table 1, the LODs for all proteins were below 10 pg/mL except BDNF, IL4, MMP9, and PSA, which were between 50 and 200 pg/mL. Therefore, silver amplification works well in a multiplex microarray assay.

Comparison of SENSIA and FLISA

Next, we compared the assay performance between silver amplification (SENSIA) and fluorescence (FLISA). The protocols for FLISA and SENSIA were mostly identical except the last step, where in SENSIA the detection antibodies were bound to SA-HRP followed by silver amplification and in FLISA they were bound directly to SA-Cy3. Figure 4 shows a representative binding curve of the 15 proteins quantified using SENSIA and FLISA. The *y* axis was adjusted so that the fitted curves are aligned for easy visual comparison. We found that most binding curves exhibit very similar sigmoid characteristics. Interestingly, assays for BDNF and ENDO that do not perform ideally in SENSIA are worse in FLISA. Furthermore, the narrow dynamic range of IL4 and MMP9 showed a similar trend in FLISA as in SENSIA, and MCP1 has a relatively high background in FLISA but not in SENSIA. These discrepancies may be attributed to the difference in the amplification methods, as SENSIA requires a washing step with DI water before undergoing silver amplification at a pH of 3.0. The washing step and low pH condition may have washed off some bound reagents, thus introducing a bias to the assay signal. Referring to Table 1, we see that the LOD of SENSIA is better than that of FLISA for nine proteins (BDNF, CEA, ENDO, IL1b, IL4, IL6, MMP9, PSA, and TNFRII) and for the remaining six proteins, FLISA performs only marginally better than SENSIA. Nevertheless, the LODs for both methods fall within a 10-fold difference for 13 proteins (all except ENDO and IL4). DR of FLISA is better than that of SENSIA for 11 proteins (all but BDNF, ENDO, IL4, and TNFRII), yet 13 proteins (all but except IL1b and MCP1) have a less than 1.5-log difference. Overall, the LOD and DR between SENSIA and FLISA are in good agreement, while some differences exist for a small number of proteins that may be due to the differences in protocols. These results indicate that SENSIA could serve as a highly sensitive alternative to FLISA.

Analysis of Cross Reactivity in Singleplex and Multiplex Assays

Multiplexed sandwich assays, where reagents such as dAbs are applied as a mixture, are susceptible to reagent-driven cross-reactivity due to the “sticking” of reagents to one another and to nontargeted proteins.²⁷ To verify that reagent-driven cross-reactivity did not affect this 15-plex assay, we spiked 1 ng/mL of each protein in PBS buffer and quantified each protein in both singleplex and multiplex formats (Figure S5 in the SI). We applied a two-side *t* test with 95% confidence interval and found that despite apparent differences between the singleplex and multiplex signals for several proteins (BDNF, CEA, GCSF, IL2, and MCP1), only two (CEA and MCP1) are statistically significant, indicating that for most proteins the measurements are statistically indistinguishable. SENSIA introduces a different signal amplification method to fluorescence, which is not expected to address the cross-reactivity issue. However, the experiments shown here suggest that SENSIA may be combined with an antibody colocalization microarray format² or a snap-chip,²⁸ which allows scaled-up multiplexing while avoiding reagent-driven cross-reactivity.

Parallel Measurements of Multiple Serum Samples

Complex samples such as serum can interfere with the detection and quantification of proteins owing to matrix effects.^{29,30} In order to demonstrate that SENSIA works well with complex samples, we selected 6 of the 15 proteins, IL1b, IL2, IL6, GCSF, MCP1, and TNFRII, as they have a relatively wide dynamic range and a low LOD. We quantified them using both SENSIA and FLISA and correlated the extracted concentrations using a least-squares linear regression curve. Figure 5 shows that five proteins (all except MCP1) have an *R*² greater than 0.9, and two proteins (IL1b and IL6) exhibit heteroscedasticity (large variation at high concentrations). The matrix effect of serum and the presence of endogenous proteins could account for both observations. It is known that serum can cause nonspecific background and have an inhibitory effect on some proteins³¹ and thus introduce protein-dependent bias or concentration-dependent variation. Likewise, the presence of endogenous proteins in the serum and an usual elevation of such proteins in pooled serum, for example, serological level of IL1b in the clinics ranges from 0.03 to 0.2 ng/mL,³² GCSF 0.02 to 0.6 ng/mL,³³ MCP1 ca. 0.4 ng/mL,³⁴ and IL2 0.14 to 0.32 ng/mL,³⁵ can cause the assay signal to extend beyond the linear range of the binding curves. In such a scenario, small variations in signal could lead to large changes in the concentration determined based on the binding curves that were established in buffer. Furthermore, several proteins (IL2, IL6, GCSF, and MCP1) show a higher variance in the SENSIA measurement (large error bars along the *x* axis), which could be due to a lower dynamic range (10-bit vs 16-bit) and a lower resolution (22 μ m vs 5 μ m) of the flatbed scanner compared with a fluorescent scanner. Nevertheless, $>0.9R^2$ values for five of the six proteins suggest a good agreement between the two methods and that SENSIA can be used to quantify proteins in serums and yield similar results as FLISA.

Long-Term Storage of SENSIA Slides

For many clinical applications it is useful to preserve slides for future examination. Unlike organic fluorescent molecules, silver precipitates are stable and their signal intensity does not fade. To test this, a SENSIA slide was scanned immediately after the assay was completed and one month later (Figure S6 in the SI). Using a *t* test, the gray-scale intensities of seven of the eight

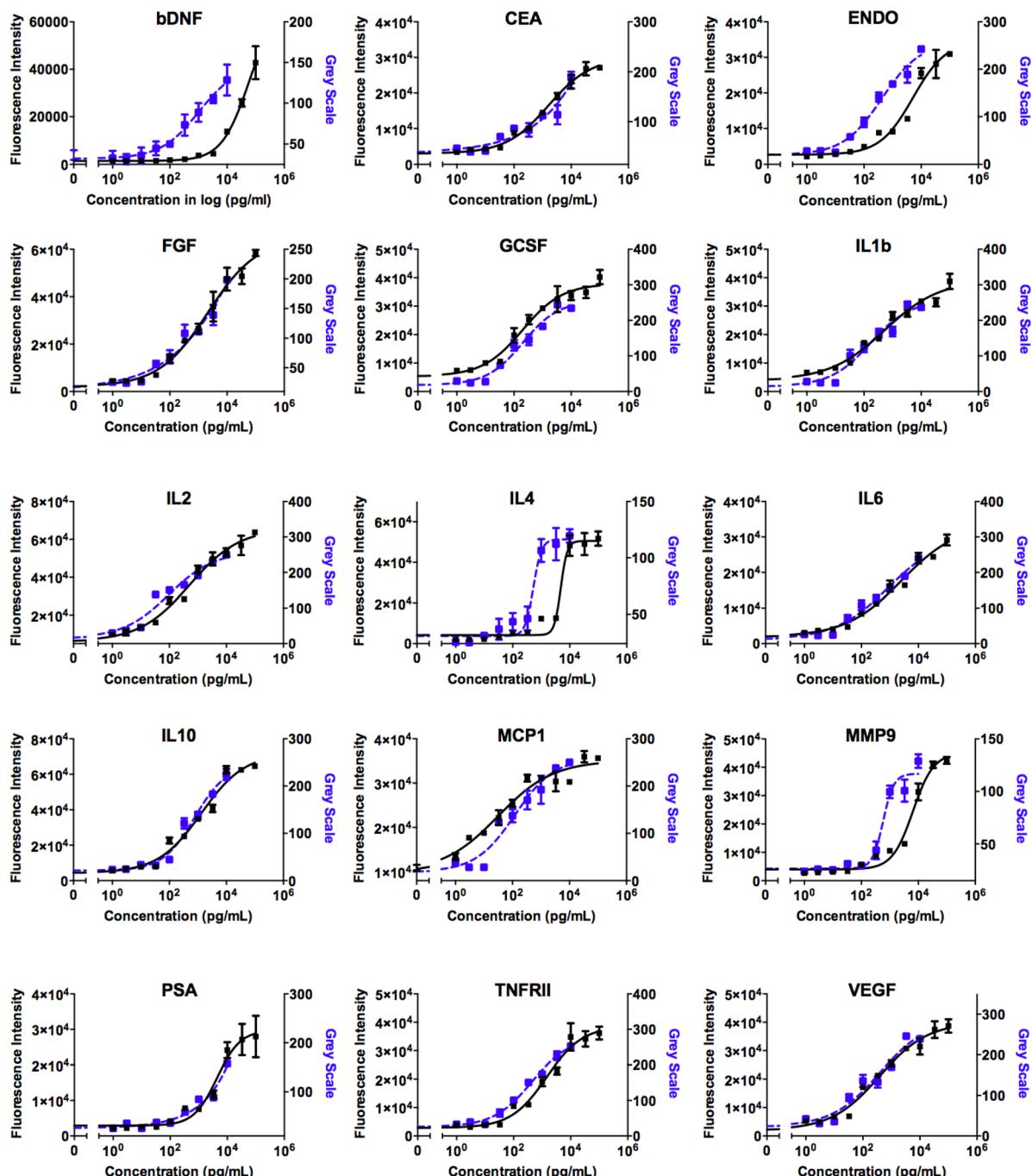


Figure 4. Binding curves of 15 proteins obtained by SENSIA (blue) and by FLISA (black). The maximal and minimal asymptotes of the binding curves are overlaid for visual comparison of detection range and overall trend of the binding curves. The error bars represent standard deviation of three replicated spots.

MCP1 samples were found to not be statistically different between the two scans, while one sample was statistically different (P value = 0.047). Taken as a whole, one month storage at room temperature had little effect on the signal level, and long-term storage of SENSIA slides appears possible.

CONCLUSIONS

In this work, we applied silver precipitation to a sandwich assay on antibody microarrays at a density of 400 spots per cm² and analyzed the results using a flatbed scanner. We validated SENSIA by comparing it with fluorescence microarray assays, achieving comparable LODs and DRs, minimal cross reactivity

between reagents, and a good agreement between results obtained by the two methods, suggesting that SENSIA may serve as an inexpensive and sensitive alternative to fluorescence microarray assays.

The key contributions of this work include: (1) optimizing enzymatic mediated silver amplification and configuring a flatbed scanner for optimal microarray analysis, (2) establishing 15-plex sandwich immunoassays with standard curves and low pg/mL LODs and validating that there is minimal cross-reactivity between the proteins and antibodies on the panel, and (3) evaluating and benchmarking the performance of antibody microarrays analyzed by SENSIA using a \$100 flatbed

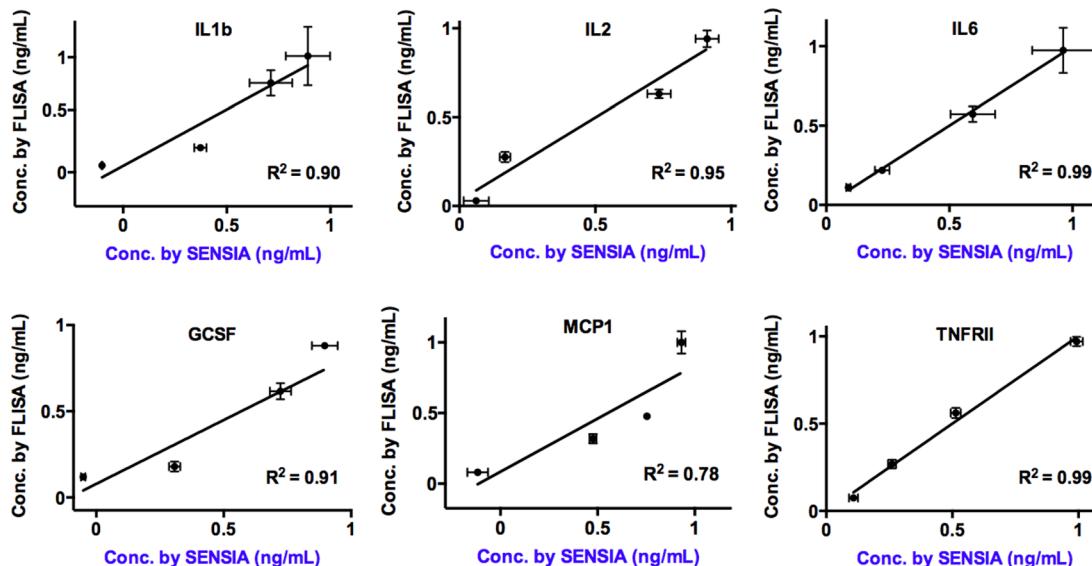


Figure 5. Comparison of protein concentrations measured in serum obtained by SENSIA and by FLISA. The error bars represent standard deviations of three measurements of the same sample measured by SENSIA or FLISA. R^2 is calculated from Pearson correlation coefficient.

scanner and by FLISA using a \$50K fluorescence scanner and demonstrating comparable results in both buffer solution and serum samples.

Although the reagent consumption of SENSIA is similar to FLISA, the main advantage is the significant cost reduction in scanning device, which makes multiplex assays more accessible to researchers and clinicians who do not have a fluorescence scanner. Furthermore, flatbed scanners typically operate in a reflective mode, thus compatible with both transparent and opaque (nitrocellulose) slides as well as membranes or even paper, giving the users more flexibility in the choice of substrates. Finally, the stability of the precipitated silver permits delayed analysis, which may be another attractive feature in research and clinics where archive and reanalysis of samples is important.

■ ASSOCIATED CONTENT

S Supporting Information

Supporting Information includes additional material and methods, derivation of assay dynamic range, optimization of SENSIA protocol, selection of antibodies for multiplex assays, analysis of cross reactivity in singleplex and multiplex assays, and long-term storage of SENSIA slides. 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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Sensor Systems. D.J. acknowledges support from Canada Research Chair (CRC).

■ ABBREVIATIONS

SENSIA, silver enhanced sandwich immunoassay; FLISA, fluorescence-linked immunosorbent assay; ELISA, enzyme-linked immunosorbent assay; LOD, limit of detection; DR, dynamic range; cAb, capture antibody; dAb, detection antibody; AuNP, gold nanoparticle; HRP, horseradish peroxidase; ALP, alkaline phosphatase; SA, streptavidin; SISH, silver in situ hybridization; FISH, fluorescence in situ hybridization; BDNF, brain-derived neurotropic factor; CEA, carcinoembryonic antigen; EGF, epidermal growth factor; ENDO, endoglin; FAS, FAS/tumor necrosis factor receptor superfamily 6; FGF, fibroblast growth factor; GCSF, granulocyte colony stimulating factor; IL1b, interleukin 1 β ; IL1ra, interleukin 1 receptor antagonist; IL2, interleukin 2; IL4, interleukin 4; IL6, interleukin 6; IL7, interleukin 7; IL10, interleukin 10; MCP1, monocyte chemotactic protein-1; MIP1a, macrophage inflammatory protein 1 alpha; MIP1b, macrophage inflammatory protein 1 beta; MMP3, matrix metallopeptidase 3; MMP9, matrix metallopeptidase 9; NCAM1, neural cell adhesion molecule 1; OPN, osteopontin; PSA, prostate specific antigen; SPARC, secreted protein acidic and rich in cysteine; TNFa, tumor necrosis factor receptor alpha; TNFRII, tumor necrosis factor receptor II; uPAR, urokinase plasminogen activator surface receptor; VEGF, vascular endothelial growth factor

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