COST-EFFECTIVE MULTIPLEXED IMMUNOASSAYS USING SILVER PRECIPITATION AND A DESKTOP SCANNER

G. Zhou[1,2], S. Bergeron[1,2], and D. Juncker[1,2*]

[1]Biomedical Engineering Department, McGill University, Montreal, CANADA and
[2]McGill Genome Innovation Centre, Montreal, CANADA

ABSTRACT

We introduce a multiplexed sandwich immunoassay using silver precipitation to produce a visible read-out that can be quantified using a common desktop scanner, thus eliminating the need for a fluorescent scanner and reducing the overall cost of a multiplexed test. We benchmarked our technique against a fluorescence immunosorbent microarray and found that all LOD values obtained with a desktop scanner come close to those obtained with a fluorescence scanner. We further performed parallel quantification of four proteins (EGF, IL1b, G-CSF, and TNF-RII) spiked in diluted serum and the values extracted using the two methods were found to be in good agreement (coefficient of determination R^2>0.9). This assay costs only a fraction of a fluorescence assay, thereby it may benefit the development of low-cost multiplexed immunoassays.

KEYWORDS: Microarray, Multiplexed assay, Silver precipitation, Desktop scanner

INTRODUCTION

Antibody microarrays can quantify multiple analytes simultaneously and they have been used in disease diagnosis and patient stratification. One of many challenges of translating microarray technology from research to practical patient care is the need for expensive fluorescence laser scanners. Fluorescent scanners have limited number of excitations, take several minutes to scan a single slide, and are relative expensive. In comparison, desktop scanners employers a white light source hence eliminating the need for multiple lasers and are available at a much affordable price. They have been used for quality control of manufacturing DNA microarrays[1] and quantification of cDNA expression level in animal models[2]. Gold and silver nanoparticles are widely used to label biomolecules and to produce colorimetric signals in both nucleic acid tests[3] and protein assays[4, 5], because of their strong light scattering properties[6, 7]. This work seeks to combine the silver precipitation for signal amplification and a common desktop scanner for quantification to enable a low-cost multiplexed antibody microarray format. Silver precipitation produces a visible read-out that can be read by unaided eye and quantified using a common desktop scanner, thus eliminating the need for a fluorescent scanner and reducing the overall cost of a multiplexed test. We benchmarked our platform with conventional fluorescent immunosorbent assay (FLISA) for sensitivity and quantified multiple proteins in serum in parallel.

EXPERIMENTAL

Multiplexed assay protocol involving silver precipitation

Capture antibodies and rabbit IgG-biotin (positive control) were first diluted in a printing buffer (optimized in this lab) to reach a final concentration of 10 μg/ml. The capture antibodies and IgG-biotin were spotted on a Xenobind glass slide (Xenopore Inc, Hawthorne, NJ) using an inkjet spotter (Nanoplotter 2.0, GeSiM, Grosserkmannsdorf, Germany). The slide was incubated in a 60% humidity chamber at room temperature overnight. To perform the assay, the slide was assembled with a 16-well gasket (Grace Bio-Lab, Bend, OR) and rinsed with a washing buffer (PBS buffer with 1% BSA and 0.1% Tween 20) three times, 5 min each. After rising, the slide was blocked with the same buffer for 1h, rinsed, and incubated with standards and samples for 3h. The slide was then rinsed again, incubated with biotinylated detection antibodies for 1h, rinsed, and incubated with streptavidin-HRP followed by silver precipitation or another incubation step with streptavidin-Cy3.

Signal quantification using a desktop scanner

To quantify the microarray spot intensity, a high-resolution desktop scanner (Canon LiDE 700F, Canon Canada Inc., Mississauga, ON) was used to image the slide. For FLISA, the assay results were imaged with a fluorescence scanner (Agilent G2505c, Agilent Technologies, Santa Clara, CA). The LOD was calculated as the mean of the blank test plus three times the standard deviation of this mean.

RESULTS AND DISCUSSIONS

Sandwich assay with silver precipitation

A 14-plex sandwich assay was developed for targets comprising inflammatory chemokines (*e.g.* CCL2, CCL4, CCL10), interleukins (*e.g.* IL-1β, IL-6, IL-10), growth factors (*e.g.* EGF, FGF, β-NGF), and several candidate cancer biomarkers, including FAS, G-CSF, MMP3, SPARC, and TNF-RII. Capture antibodies against the 14 proteins were spotted on a glass surface that reacts with primary and secondary amine group to create a covalent bond, resulting in 2048 100-μm spots per slide (Figure 1a-b). The slide was incubated with standards and samples, washed and incubated with biotinylated detection antibodies and HRP-labeled streptavidin. Once the assay is complete, we performed enzyme-

modulated silver precipitation. HRP produces radicals in the presence of a reducing agent and the radicals act as a charge transfer media that gives an electron to Ag(I) and reduces it to silver particles at the site of the HRP enzyme. The precipitated silver particles are measured ~200 nm in diameter (Figure 1c-d). Due to the strong scattering effects of silver nanoparticles, they can be visualized in a dark field microscope. A high sample concentration results in a microspot saturated with silver nanoparticles; whereas low concentration leads to a spot with a few nanoparticles (Figure 1e). The spot intensity is visible to the naked eye and can be quantified in a high-throughput fashion using a common desktop scanner. The scanner is 1200 dpi and 8-bit gray scale values, providing a lateral resolution of 22 µm and a moderate signal depth. Upon quantifying the spot intensity for different concentrations, a binding curve can be established, and the LOD for the protein can be calculated. This was performed for all 14 proteins spiked in buffer solution.

Technology validation

To validate this sandwich assay using silver precipitation and quantification with a low-cost desktop scanner, we performed a second set of experiments using fluorescent-labeled detection antibodies and scan the results with a $25K fluorescent scanner. We derived the binding curve and calculated LODs using both approaches. Figure 2 shows the binding curve for a few selected proteins and Table 1 summarizes the results for all proteins. We see a good agreement of detection limit and detection range between the two methods. All LOD values obtained with a desktop scanner fall in the pg/mL range, and come close to the LODs obtained with fluorescence detection with a few exceeding them. In addition, 13 of the 14 assays were correlated with an $R^2 > 0.9$. The difference is the proposed method uses an instrument that costs only ~$100 versus fluorescent scanner could cost over $25K.

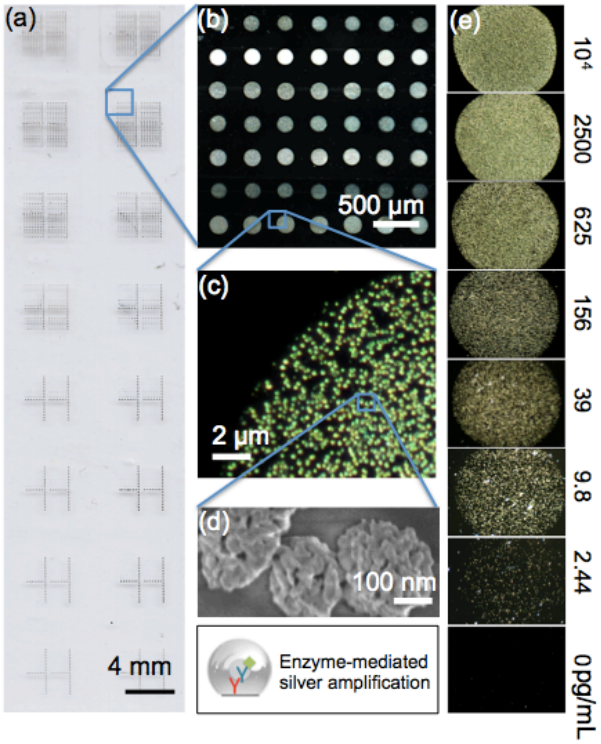

Figure 1: ***Images of a slide and of a single 14-plex assay at different magnifications.*** *(a) Slide scanned with a desktop scanner. The slide has 16 wells to incubate 16 different concentrations of proteins. (b) Array with 16 ×16 spots, comprising 14 proteins and 2 standards. Each row is one type of antibody and different columns are replicates for the same protein. (c) Each spot is approximately 150 µm in diameter, covered with silver nanoparticles. (d) Each silver particle is the result of HRP-mediated silver reduction at the antibody-protein binding site, therefore is used to quantify the protein concentration. (e) Darkfield image of microspots for a concentration between 0–10 ng/mL.*

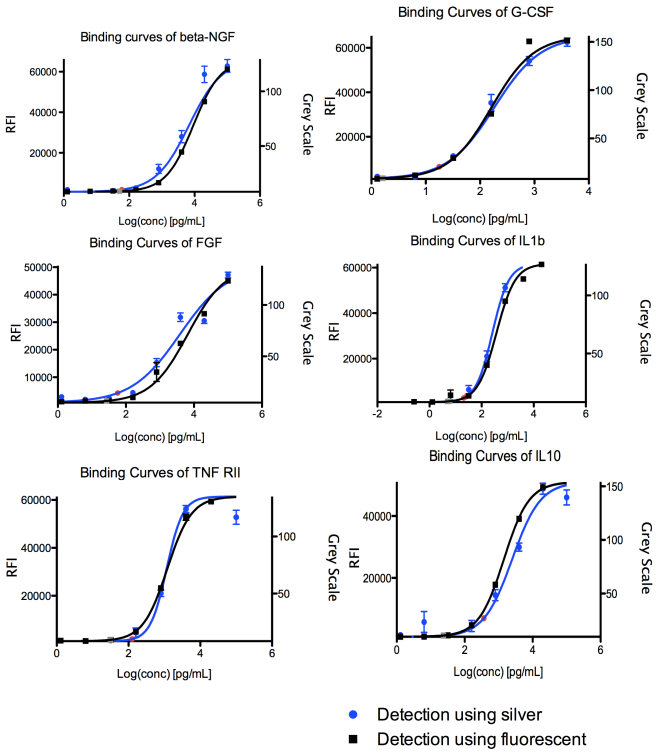

Figure 2: ***Binding curves of assays using enzyme mediated silver precipitation & fluorescence immunosorbent assays using streptavidin-Cy3.*** *The silver precipitation approach is benchmarked against the standard fluorescent detection method. The binding curves obtain using the two methods have similar ranges and slopes. All LOD values obtained using silver precipitation and a desktop scanner (~$100) are in the pg/mL range, rivaling fluorescence detection.*

Parallel measurements of multiple serum samples

To test if the silver precipitation works with a complex matrix such as serum, we spiked 1:10 diluted serum with various concentration of epidermal growth factor (EGF), interleukin 1β (IL-1β), granulocyte-colony stimulating factor (G-CSF), and tumor necrosis factor receptor-II (TNF-RII). Concentration values were measured using this assay with silver precipitation and using fluorescence immunosorbent assay. On a 16-well slide, half of the slide was used for establishing a binding curve and the other half was used to test seven diluted serum sample spiked with various concentrations of the four above-mentioned proteins. Concentration extracted from the two methods are found to be in good agreement with all $R^2 > 0.9$ (Figure 3). The axis represents the method used.

Table 1. ***Summary of LODs and correlation between silver precipitation and fluorescence measurement for 14 proteins.***
13 out of the 14 proteins show a R^2>0.9.

Protein	LOD(Ag) (pg/mL)	LOD(Fl) (pg/mL)	R^2
beta-NGF	*59.6*	*51.3*	*0.97*
CCL2/MCP-1	*97.9*	*7.8*	*0.98*
CCL4/MIP-1β	*14.8*	*12.5*	*0.95*
CCL10/IP-10	*367.4*	*23.7*	*0.94*
EGF	*44.3*	*3.9*	*0.85*
FAS	*128.2*	*186.1*	*0.95*
FGF	*57.1*	*27.0*	*0.96*
G-CSF	*17.7*	*1.6*	*0.98*
IL-1β	*25.2*	*5.0*	*0.99*
IL-6	*84.4*	*11.3*	*0.96*
IL-10	*186.6*	*362.0*	*0.97*
MMP-3	*152.8*	*202.0*	*0.93*
SPARC	*512.3*	*4930.7*	*0.93*
TNF-RII/p75	*125.2*	*31.0*	*0.95*

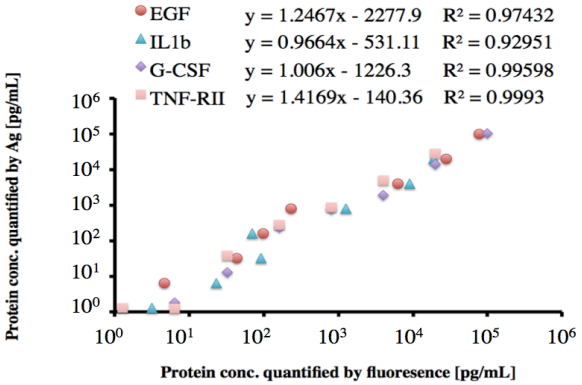

Figure 3: ***Correlation curve between the silver and fluorescence detection used to quantify seven serum samples spiked with various concentration of proteins.*** *To test if the silver precipitation can work with complex matrix such as serum, we spiked 1:10 diluted serum with various concentrations of EGF, IL-1β, G-CSF, and TNF-RII. Concentration values were measured using the two methods, and were found to be in good agreement with all R^2>0.9. The axis represents the method used.*

CONCLUSION

In summary, we have shown a low-cost multiplexed immunoassay using silver precipitation and a common desktop scanner. The cost of performing a multiplexed assay is only a fraction of a conventional FLISA. One limitation is the signal depth (8-bit) of the desktop scanner used, however it can be improved by taking duplicated measurements averaging to extrapolate finer signal levels. This method is applicable to multiplexed immunoassays and can be valuable for the development low-cost multiplexed immunoassays given a limited budget.

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CONTACT: *D. Juncker, david.juncker@mcgill.ca