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Sensitive single-molecule protein quantification and protein complex detection in a microarray format

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Abstract

Single-molecule protein analysis provides sensitive protein quantitation with a digital read-out and is promising for studying biological systems and detecting biomarkers clinically. However, current single-molecule platforms rely on the quantification of one protein at a time. Conventional antibody microarrays are scalable to detect many proteins simultaneously, but they rely on less-sensitive and less quantitative quantification by the ensemble averaging of fluorescent molecules. Here we demonstrate a single-molecule protein assay in a microarray format enabled by an ultra-low background surface and single-molecule imaging. The digital read-out provides a highly sensitive, low femtomolar limit of detection and 4 orders of magnitude of dynamic range through the use of hybrid digital-analog quantification. From crude cell lysate, we measured levels of p53 and MDM2 in parallel, proving the concept of a digital antibody microarray for use in proteomic profiling. We also applied the single-molecule microarray to detect the p53-MDM2 protein complex in cell lysate. Our study is promising for development and application of single-molecule protein methods because it represents a technological bridge between single-plex and highly multiplex studies.

Keywords

Antibody microarray; Proteomics; Sandwich immunoassay; Single molecule detection; Total internal reflection fluorescence

Single-molecule protein detection has the potential to improve systems biology and biomarker studies by providing highly sensitive quantification and a digital read-out. Single-molecule protein techniques are achieved by several methodologies, but common among them is the coupling of a single-molecule-sensitive detection modality with a method for eliminating background fluorescence. In one approach, single-molecule protein quantitation is achieved through the dilution of antibody-target complexes in low cross-section capillaries followed by detection by a sensitive photon detector[1]. In another instance, femtoliter-volume wells are used to harbor single-molecule enzyme-linked immunoassays[2]. Additionally, total internal reflection fluorescence (TIRF) imaging has provided a platform

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Conflict of interest

The authors declare no conflicts of interest.

for single-molecule quantification on planar surfaces. TIRF analysis is especially promising because of the reliability and affordability of TIRF optics and has been recently demonstrated for the digital quantification of proteins[3–6] and lipopolysaccharides[7]. Despite the quantitative advantages of all of these single-molecule methods, they are currently low throughput in that they can analyze only one target at a time.

Microarrays are advantageous for proteome and interactome profiling because they scale reliably for dozens of protein targets while relying on minimal reagent volumes [8, 9] and so have proven valuable for quantifying the abundance of many proteins simultaneously[10–13] and for detecting pairs of interacting proteins[14–18]. However, microarrays have neither the sensitivity of detection nor the precise digital read-out provided by single-molecule methods. For these reasons, a single-molecule assay for proteins that has the scalability of a microarray has been a major goal[4, 5].

Here we perform a proof-of-principle demonstration of a single molecule antibody microarray. Our procedure begins by treating a glass substrate with a nanogel coating that forms a protein resistant, hydrogel barrier of around 75 nm thickness[19]. The coating is activated with a cross-linker, making it reactive with the exposed lysines of printed antibodies. Then, monoclonal capture antibodies are printed in specified locations (fields) onto the substrates. Substrates are sealed to a flow chamber, the surface is passivated, and cell lysate is passed over the chip, binding target molecules to the antibody fields. Then, the chip is exposed to detection antibodies and finally to fluorescently-labeled streptavidin (Figure 1).

To obtain a single-molecule resolution read-out, total internal reflection fluorescence (TIRF) imaging is performed. The microscope is directed to the locations of the printed fields, and images are acquired within the center of each field. Non-overlapping viewing areas are acquired within each printed field to obtain intra-field replicates (multiple fields and multiple slides are imaged as well). Then, digital measurements are obtained by counting fluorescence objects within the printed fields and subtracting away background levels, which are measured outside of the fields.

We first sought to assess the performance difference between a digital and an analog microarray in terms of limit of detection (sensitivity) and dynamic range. To do this, we used the model assay that detects fluorescently labeled streptavidin by binding to a biotinylated protein printed on the surface (to emulate a protein target captured by a printed capture antibody). This model is a suitable estimate of performance since it incorporates (1) the printing/attachment efficiency of the capture molecule, (2) the pull-down of a protein target from solution, and (3) the non-specific binding of the protein target on the surface.

We obtained TIRF images for different concentrations of captured protein (Figure 2A). Then we analyzed the same raw data by two different methods to obtain two standard curves – one by single-molecule counting (digital) and one by conventional, ensemble intensity averaging (analog). The standard curve obtained from analog image processing provided a dynamic range from 10 pM to 100 pM (Figure 2B), and the curve obtained from digital processing of the same data provided a dynamic range from 14 fM (0.74 pg mL^{-1}) to 33 pM (Figure 2C).

So, given the same raw data, analysis by digital counting provided greater sensitivity than ensemble averaging by around 3 orders of magnitude. By combining the two standard curves (using the digital curve for the 14 fM to 33 pM range and the analog curve for the 33 pM to 100 pM range), the hybrid digital-analog curve provided around 4 orders of magnitude dynamic range. These results show that single-molecule microarrays, by providing a high resolution view of the printed antibody fields, can provide more sensitive detection than analog microarrays. Also, single-molecule microarrays can allow for extension of dynamic range by combining digital and analog standard curves. The dynamic range we observed here is well suited for the analysis of biological systems and serum biomarkers.

We applied the single-molecule antibody microarray to analyze the regulatory proteins p53 and MDM2. We generated p53/MDM2 microarrays as described above using capture antibodies for p53 and MDM2. We benchmarked the microarrays using dilutions of protein standards to validate each antibody pair individually and then tested for cross-reactivity of the antibodies in a parallelized (dual-plex) assay (Supporting Figure 1). To validate the single-molecule antibody microarray in a real world application, we quantified p53 and MDM2 protein levels in a well-characterized cell culture line, HCT116. This colorectal cancer line is known to respond to the DNA damage agent 5-fluorouracil by up-regulating p53 and its downstream targets[20, 21]. The p53/MDM2 single-molecule microarray was exposed to cell lysate, protein levels were detected in a single detection antibody step, and the slide was imaged and analyzed. Both p53 and MDM2 protein levels fell within the digital range of the assay (Figure 3A). We found p53 protein expression to increase by 1.8-fold under DNA damage conditions and MDM2 protein expression to increase by 6.6-fold under DNA damage conditions (Figure 3B). These changes are in agreement with a recent study using similar induction conditions of cancer cells (2.3-fold and 5.5-fold, respectively) [22]. The results from this indicate the single molecule microarray is well suited for cellular profiling. Promisingly, the microarray format should make the assay readily adoptable to more highly multiplexed studies.

Antibody microarrays have been widely used for the discovery of proteome-wide interactions[14–18], and we hypothesized that a single-molecule antibody microarray could be used to detect a protein complex. To achieve this, we altered the detection step of the p53/MDM2 microarray protocol by incubating only one detection antibody at a time. In this way, we could quantify molecules located in the alternate field with respect to the detection antibody. To establish the specificity of this protocol, we analyzed solutions that contained one protein (e.g. p53) but lacked the other (e.g. MDM2). In these controls, we observed low signal from two distinct immunosandwich assays: anti-p53 detection antibody binding to anti-MDM2 capture fields and anti-MDM2 detection antibody binding to anti-p53 capture fields.

After establishing the levels of cross reactivity of the assay, we analyzed cell lysate from HCT116 cells. By the two independent immunosandwiches, we observed p53-MDM2 complex levels significantly above the controls (Figure 3C). The difference in magnitude between the two lysate measurements is indicative of the difference in affinity of the pairs of antibodies in the two different immunosandwiches. The mass of signal in both of the interaction assays indicates the presence of a protein complex, which is consistent with the

known protein-protein interaction between p53 and MDM2 *in vivo* and *in vitro* [23, 24]. Thus, we present a model of how a single-molecule antibody microarray may be used in protein-protein interaction studies.

Single molecule quantification of proteins should become of greater benefit to disease diagnostics and systems biology by providing quantitation of rare proteins in biological specimens. We used standard antibody microarray reagents, a nanogel-coated surface, and TIRF imaging to provide duplex digital molecule counting in a planar format that is suitable for higher degrees of multiplexing. As with all antibody microarrays, scaling up to greater numbers of targets requires optimization to minimize cross-reactivity. In this study, we focused on the platform itself – surface architecture, antibody immobilization, and detection – which dictates assay performance to a large degree [25–27]. Using a representative set of antibodies from a popular vendor, we were able to detect with a digital read-out regulatory proteins p53 protein and MDM2 from crude cell lysate and quantify small expression changes (1.8-fold). We also modified the assay to demonstrate its utility in a protein-protein interaction study. The low femtomolar sensitivity and 4-orders of magnitude dynamic range we observed provide a proof of principle for the use of single-molecule antibody microarrays in larger-scale protein quantification and protein-protein interaction studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Scheme of the single-molecule antibody microarray method.

Figure 2.

(A) Representative TIRF images of fluoro-streptavidin captured onto the single-molecule microarray printed with biotin capture fields. (B) Standard curve generated by analyzing the data by conventional, fluorescence intensity averaging (analog processing). (C) Standard curve generated by analyzing the data by discrete molecule counting (digital processing). The single-molecule microarray enables higher sensitivity and higher dynamic range than a microarray analyzed by analog processing.

Figure 3.

(A) Single-molecule protein expression of p53 and MDM2 in cancer cells, with and without DNA damage induction. (B) Expression changes of 1.8-fold for p53 and 6.6-fold for MDM2 are detected by three biological replicates. (C) Two different digital sandwich immunoassays specifically detect the p53-MDM2 protein complex. Cell lysate and control samples were analyzed by distinct immunosandwich assays: one using printed p53 capture antibody, α p53_C, with MDM2 detection antibody, α MDM2_D, and the other using printed MDM2 capture antibody, α MDM2_C, with p53 detection antibody, α p53_D. The analysis of cell lysate

provided a mass of signal above the levels of cross-reactivity determined from controls, indicating the presence of the p53-MDM2 complex.

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