Attomolar detection of proteins in serum using single molecule enzyme-linked immunosorbent assays.

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Introduction

The clinical use of protein biomarkers for the differentiation of healthy and disease states, and for monitoring disease progression, requires the measurement of low concentrations of proteins in complex samples. Current immunoassays measure proteins at concentrations above 10^{-12} M, whereas the concentration of the majority of proteins important in cancer, neurological disorders, and the early stages of infection are thought to circulate in the range from 10^{-16} to 10^{-12} M. The isolation and detection of single protein molecules provides a promising approach for measuring extremely low concentrations of proteins. Here, we report an approach for detecting thousands of single protein molecules simultaneously using the same reagents as the gold standard for detecting proteins, namely, the enzyme-linked immunosorbent assay (ELISA). This method—which we call digital ELISA—has been used to detect proteins in serum at sub-femtomolar concentrations and to measure prostate specific antigen (PSA) in 30 individuals after radical prostatectomy surgery, down to 400 aM (14 fg/mL).

Methods

Our approach makes use of arrays of femtoliter-sized reaction chambers—which we term Single Molecule Arrays (SiMoA)—to isolate and detect single enzyme-labeled protein molecules (1). This "digital ELISA" is shown schematically in **Figure 1**. A sandwich antibody complex is first formed on microscopic beads and the bound complexes are labeled with an enzyme reporter molecule. When assaying samples containing extremely low concentrations of protein, the ratio of protein molecules (and the resulting enzyme label complex) to beads is small (typically less than 1:1) and, as such, the percentage of beads that contain a labeled immunocomplex follows a Poisson distribution, leading to single immunocomplexes on individual beads. It is not possible to detect these low numbers of enzyme labels using standard detection technology (e.g., a plate reader), because the fluorophores generated by each enzyme diffuse into a large assay volume (typically 0.1–1 mL), and it takes hundreds of thousands of enzyme labels to generate a fluorescence signal above background. SiMoA enables the detection of very low concentrations of enzyme labels by confining the fluorophores generated by individual enzymes to extremely small volumes (~50 fL), leading to a high local concentration of fluorescent product molecules. To achieve this localization in an immunoassay, beads are loaded into an array of femtoliter-sized wells. The loaded array is then sealed against a rubber gasket in the presence of a droplet of fluorogenic enzyme substrate, isolating each bead in a femtoliter reaction chamber. Beads possessing a single enzyme-labeled immunocomplex generate a locally high concentration of fluorescent product in the 50-fL reaction chambers. By acquiring time-lapsed fluorescence images of the array using standard microscope optics, it is possible to distinguish beads associated with a single enzyme molecule ("on" well) from those not associated with an enzyme ("off" well). Imaging the arrays also allows tens to tens of thousands of single immunocomplexes to be detected simultaneously. The protein concentration in the test sample is determined by simply counting the number of wells containing both a bead and fluorescent product relative to the total number of wells containing beads. The hardware and software used to generate SiMoA data is shown in **Figure 2**.

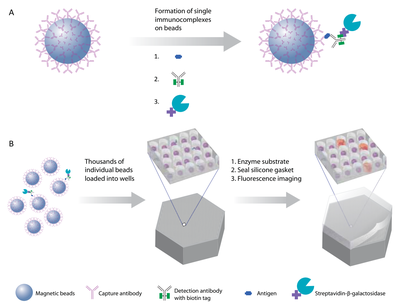


Figure 1. Digital ELISA based on arrays of femtoliter wells. (A) Capturing and labeling single protein molecules on beads using standard ELISA reagents. (B) Loading of beads into femtoliter well arrays for isolation and detection of single molecules. (C) SEM image of a small section of a femtoliter well array after bead loading. 2.7-µm-diam. beads were loaded into an array of wells with diameters of 4.5 µm and depths of 3.25 µm. (D) Fluorescence image of a small section of the femtoliter well array after signals from single enzymes are generated.

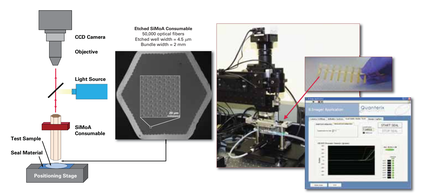
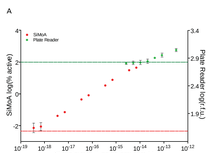


Figure 2. Prototype SiMoA Instrumentation. A prototype SiMoA instrument has been developed for assay validation and to support pharmaceutical and diagnostics collaborations. The SiMoA consumable is manufactured by etching tens of thousands of reaction vessels into the end of an optical fiber bundle. Strips of 8 fiber bundles are sized to sample one column of a microtiter plate, enabling convenient processing of up 96-samples. The current bench-top instrument automatically seals the reaction vessels and concurrently reads the array of over 50,000 single molecule fluorescent assays. Sample preparation is done on a separate automated fluid handling workstation capable of processing hundreds of samples per shift.

Results

A) Enzyme label Sensitivity

Initially, we assessed the potential gains in sensitivity to enzyme label that can be achieved by singulating enzyme-labeled molecules compared to a traditional, ensemble measurement. We created populations of beads with well-characterized enzyme-to-bead ratios by mixing 400,000 beads presenting biotin with a range of concentrations of an enzyme conjugate, streptavidin-β-galactosidase (SβG), commonly used as a label in ELISA. The ensemble of beads was read out in 100 µL on a fluorescence plate reader after 1 h incubation with 100 µM resorufin-β-D-galactopyranoside (RGP), a fluorogenic substrate for β-galactosidase. The detection limit of the capture assay on the microtiter plate reader was 15 fM of SβG (**Figure 3**). For SiMoA detection, the ratio of active wells to the total number of beads was determined: **Figure 3** shows a log-log plot of the percentage of beads that contained an enzyme as a function of bulk SβG concentration. The calculated limit of detection (LOD) was 220 zeptomolar. The sensitivity of SiMoA to intrinsic label was therefore ~10–20 enzymes in 100 µL (or about 20 to 30 yoctomoles), corresponding to an increase in sensitivity over ensemble measurements of a factor of about 68,000. Chemiluminescence detection of alkaline phosphatase has an LOD of about 30 aM, i.e., about 100 times higher than SiMoA. The linear dynamic range of digital detection of enzyme labels by SiMoA was from 3.5 fM down to 350 zM, i.e., about four logs.



[SbG] (aM)	Average # single complexes	Average % active	Measurement %CV	Poisson %CV
0	1	0.0016%	87%	122%
0.35	3	0.0086%	75%	55%
0.7	5	0.0099%	63%	46%
3.5	22	0.0413%	10%	21%
7	38	0.0713%	15%	16%
35	237	0.4461%	1%	7%
70	385	0.8183%	5%	5%
350	1787	3.3802%	2%	2%
700	4036	7.5865%	5%	2%
3500	15634	30.6479%	3%	1%
7000	24836	44.5296%	1%	1%

Figure 3. Digitization of enzyme-linked complexes greatly increases sensitivity compared to bulk, ensemble measurements. (A) Log-log plot of signal output (% active beads for SiMoA, or r.f.u. for plate reader) as a function of the concentration of an enzyme conjugate (streptavidin-β-galactosidase or SβG) captured on biotinylated beads. The limit of detection for the ensemble, analog technology (plate reader) was 15×10^{-15} M (15 fM; green line). The limit of detection for the digital technology (SiMoA) was 220×10^{-21} M (220 zM; red line). Error bars are based on the standard deviation over three replicates for both technologies. (B) The imprecision of SiMoA is determined by the Poisson noise of counting single events.

B) Sub-femtomolar detection of proteins and DNA

We developed digital ELISAs for PSA and TNF-α, and a digital assay for DNA based on detection of single enzyme labels using SiMoA. **Figure 4** shows data from digital assays for PSA, TNF-α, and DNA. The human forms of the proteins were spiked into 25% bovine serum to be representative of clinical test samples. DNA was detected in buffer to be representative of purified nucleic acid preparation techniques. Using digital ELISA to detect PSA in 25% serum, an LOD of ~50 aM (1.5 fg/mL) was determined from this experiment, equating to an LOD in whole serum of ~200 aM. For comparison, a leading commercial PSA assay (ADVIA Centaur, Siemens) reports an LOD of 3 pM (0.1 ng/mL) in human serum, and the most sensitive previously reported assay for PSA had an LOD of 10 fM (2). The single molecule assay reported here is, therefore, more sensitive than the commercial assay by a factor of 15,000, and more sensitive than other ultra-sensitive methods by a factor of at least 50. The detection limit determined from the TNF-α digital ELISA was ~150 aM (2.5 fg/mL), corresponding to ~600 aM in whole serum; the highest sensitivity commercially-available ELISA for TNF-α has an LOD of 21 fM (0.34 pg/mL) in serum (R&D Systems, Inc.). SiMoA, therefore, imparts an improvement over the most sensitive TNF-α assay of a factor of 35. The LOD of the digital DNA sandwich assay was 135 aM, corresponding to about 8000 copies.

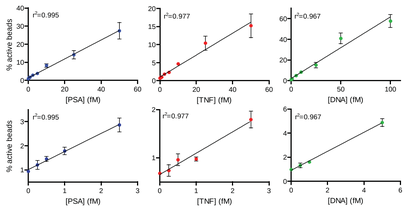


Figure 4. Sub-femtomolar detection of proteins in serum and DNA in buffer using digital ELISA. Plots of % active beads against analyte concentration for: (A) Human PSA spiked into 25% serum, (B) Human TNF-α spiked into 25% serum, and (C) DNA in buffer.

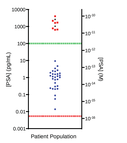
C) Detection of PSA in patients after radical prostatectomy

To demonstrate the possible diagnostic value of detecting very low concentrations of proteins in human clinical samples using digital ELISA, PSA was measured in serum samples from patients who had undergone radical prostatectomy (RP) surgery. After RP, the vast majority of PSA is eliminated, and levels fall below the detection limit of standard commercial assays (3 pM or 0.1 ng/mL). Regular monitoring of these patients for return of PSA can detect recurrence of prostate cancer, but several years may pass post-surgery for biochemical recurrence to be detected by current immunoanalyzers. The ability to accurately quantify PSA levels in post-prostatectomy patients at very low concentrations (<3 fM or 100 fg/mL) should provide early indication of recurrence if PSA levels increase. First, we validated the PSA digital ELISA for specificity to PSA using control standards (Bio-Rad) and serum from healthy individuals (ProMedDx) that had been assayed using the ADVIA Centaur PSA assay (**Figure 5**).

	Centaur ng/mL	SiMoA ng/mL
Bio-Rad Control 1	0.838	1.06 ± 0.21
Bio-Rad Control 2	2.47	2.66 ± 0.36
Normals		
ProMedDx S376	2.1	1.60
ProMedDx S378	2.3	1.70
ProMedDx S381	2.9	2.14
ProMedDx S388	4.1	3.95
ProMedDx S395	0.93	0.63
ProMedDx S396	0.9	0.77
ProMedDx S397	1.2	0.66

Figure 5. Comparison of PSA digital ELISA and commercial immunoanalyzer (ADVIA Centaur, Siemens). PSA samples from Bio-Rad (controls) and ProMedDx (serum from healthy individuals) that had previously been tested on the Centaur were tested using digital ELISA. The PSA concentrations of the healthy serum samples determined using SiMoA were (24±12)% lower than those originally determined on the ADVIA Centaur. The systematic bias between the two technologies can be explained by a difference in the PSA used to generate calibration curves or the cycle of freeze-thaw that the samples experienced before being tested with digital ELISA.

We then used digital ELISA to measure PSA in the sera of patients who had undergone radical prostatectomy. **Figure 6** shows PSA levels measured using digital ELISA in the serum of 30 RP patients (age 60–89) whose blood was collected at least six weeks post-surgery. The PSA levels in the sera of all 30 patients were below the detection limit of commercial assays. PSA was successfully detected in all 30 patients using digital ELISA, with concentrations ranging from 14 fg/mL to 9.4 pg/mL, with an average of 1.5 pg/mL. Further clinical studies are required to establish the diagnostic benefit of measuring PSA at fg/mL levels in RP patients.



Patient ID	[PSA] (pg/mL)	Dose %CV	Patient ID	[PSA] (pg/mL)	Dose %CV	Patient ID	[PSA] (pg/mL)	Dose %CV
5600	9.39	6%	5590	0.22	22%	5580	0.30	4%
5599	0.75	10%	5589	0.85	17%	5579	1.22	15%
5598	2.71	12%	5588	2.33	3%	5578	0.090	91%
5597	1.79	12%	5587	1.06	13%	5577	1.92	6%
5596	2.46	17%	5586	1.29	22%	5576	0.014	286%
5595	0.32	21%	5585	0.49	84%	5575	0.79	63%
5594	1.63	15%	5584	0.056	136%	5574	1.62	20%
5593	1.15	12%	5583	1.33	26%	5573	0.22	32%
5592	3.46	9%	5582	4.76	9%	5572	1.04	20%
5591	0.21	25%	5581	1.57	31%	5571	0.24	21%

Figure 6. Digital detection of PSA in serum samples of patients who had undergone radical prostatectomy. The concentrations of PSA were determined using digital ELISA in serum samples from RP patients (●), healthy control samples (■), and Bio-Rad PSA control samples (▲). RP patient samples were obtained from SeraCare Life Sciences (Milford, MA), and all had undetectable PSA levels as measured by a leading clinical diagnostic assay (ADVIA Centaur); the green line represents the detection limit of the ADVIA Centaur PSA assay (100 pg/mL or 3 pM). All 30 patient samples were above the detection limit of the PSA digital ELISA, shown by the red line (0.006 pg/mL or ~200 aM), with the lowest patient PSA concentrations measured at 0.014 pg/mL (~400 aM) using digital ELISA.

Conclusions

- An approach—called digital ELISA—for detecting single proteins from blood using tradition enzyme label reagents and single molecule arrays has been developed.
- The sensitivity to enzyme label of SiMoA is in the zeptomolar range, and is 100x more sensitive than the detection of alkaline phosphatase by chemiluminescence
- Digital ELISA has enabled the measurement of clinically important proteins in serum at femtogram per milliliter concentrations using robust procedures, e.g, PSA has been detected down to <10 fg/mL in serum.
- Digital ELISA enabled the detection of PSA in the serum of all samples from patients who had undergone radical prostatectomy. The average concentration in this cohort was 1.5 pg/mL, with the lowest concentration detected being 0.014 pg/mL.
- We believe that the digital ELISA approach described here has the potential to revolutionize the detection of proteins in blood and other bodily fluids, and to facilitate the early diagnosis and treatment of disease in a number of diseases.

References

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