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Single Molecule Enzyme-Linked Immunosorbent Assays: Theoretical Considerations

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Abstract

We have developed a highly sensitive immunoassay—called digital ELISA—that is based on the detection of single enzyme-linked immunocomplexes on beads that are sealed in arrays of femtoliter wells. Digital ELISA was designed to be highly efficient in the capturing of target proteins, labeling of these proteins, and their detection in single molecule arrays (SiMoA); in essence, the goal of the assay is to “capture every molecule, detect every molecule”. Here we provide the theoretical basis for the design of this assay derived from simple equations based on bimolecular interactions. Using these equations and knowledge of the concentrations of reagents, the times of interactions, and the on- and off-rates of the molecular interactions for each step of the assay, it is possible to predict the number of immunocomplexes that are formed and detected by SiMoA. The unique ability of SiMoA to count single immunocomplexes and determine an average number of enzymes per bead (AEB), makes it possible to directly compare the number of molecules detected experimentally to those predicted by theory. These predictions compare favorably to experimental data generated for a digital ELISA for prostate specific antigen (PSA). The digital ELISA process is efficient across a range of antibody affinities ($K_D \sim 10^{-11}$ – 10^{-9} M), and antibodies with high on-rates ($k_{on} > 10^5 \text{ M}^{-1} \text{ s}^{-1}$) are predicted to perform best. The high efficiency of digital ELISA and sensitivity of SiMoA to enzyme label also makes it possible to reduce the concentration of labeling reagent, reduce backgrounds, and increasing the specificity of the approach. Strategies for dealing with the dissociation of antibody complexes over time that can affect the signals in an assay are also described.

Keywords

Digital ELISA; Single molecule; kinetics; PSA

1. Introduction

The sensitive detection of proteins is important in a number of scientific and clinical fields, including oncology,¹ neurology,² inflammation,³ and infectious diseases.⁴ The lower limits of detection of proteins in complex samples has, however, lagged behind those that can be achieved for measuring nucleic acids. For example, using the polymerase chain reaction (PCR), it is routinely possible to detect RNA from 50 copies of HIV per mL of blood,

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equating to an RNA concentration of about 166 zM. Using conventional immunoassays, a typical limit of detection for the p24 capsid protein of HIV is 5 pg/mL,⁵ or 0.2 pM, i.e., about 1.2 million times less sensitive than PCR in terms of target molecules and about 1200 times less sensitive in terms of virus copies. We have recently developed a method based on counting single enzyme-labeled immunocomplexes of proteins captured on paramagnetic beads in single molecule arrays (SiMoA) that is typically 1000-fold more sensitive than conventional enzyme-linked immunosorbent assay (ELISA) approaches.^{6,7} This method—which we call digital ELISA—has been used to detect proteins important in prostate cancer,^{6,7,8,9} Crohn's disease,¹⁰ and neurological disorders¹¹ at fg/mL concentrations. In this paper, we describe in detail the theoretical aspects of this digital immunoassay, including the binding kinetics of each step of the assay, and present a theoretical model for the generation of signal in the assay as a function of time, concentrations, and binding constants.

A common reaction of researchers well-versed in analog immunoassays to the data showing the LODs that can be achieved using digital ELISA—we have shown that it can detect 50 aM of PSA in 25% plasma—is incredulity. To frame the question that summarizes this viewpoint: how is it possible to detect subfemtomolar concentrations ($\sim 10^{-16}$ M) of a protein using antibodies of picomolar to nanomolar affinity ($K_D \sim 10^{-11}$ – 10^{-9} M) in a sample containing millimolar amounts (10^{-3} M) of other, non-target blood proteins? To address this skepticism, it is necessary to reconcile the experimental data with a robust theoretical model for the assay. Such a model needs to address two aspects of the assay system: analytical sensitivity and specificity. When considering theoretical aspects of analytical sensitivity, we ask the question: are sufficient molecules captured and detected given the affinity and concentrations of the chemical reagents involved, and the time of the assay? Theoretical models for sensitivity can be developed using relatively straightforward equations based on the kinetics of bimolecular interactions for each stage of the assay; an excellent description of similar, general theoretical approaches for immunoassays are provided elsewhere.¹² When addressing analytical specificity, we consider whether the signal generated by the target molecules (calculated by sensitivity) can be discerned above the signal generated by other molecular interactions that originate from non-target molecules. For example, if theoretical considerations of sensitivity indicate that 50 enzyme-labeled molecules of target will be captured and detected (well within the detection limit of SiMoA)⁶, but 1000 enzyme molecules of background signal are detected arising from non-specific molecular interactions, then it will be challenging to detect the specific signal above the noise in the background, the lower bound of which is Poisson noise ($\sqrt{1000} \approx 32$ molecules) but is more typically 7–10% (70–100 molecules) due to experimental variations. As background interactions can, in theory, involve thousands of molecular interactions, it is challenging to develop *a priori* theoretical models for specificity. Simple experiments can, however, shed light onto the major non-target interactions that give rise to background, and help optimize the immunoassays. SiMoA was designed to be highly sensitive to proteins in blood from such theoretical considerations of both sensitivity and specificity.

Other researchers have also used fundamental consideration of physico-chemical interactions to design novel immunoassays, sometimes with counter-intuitive approaches but improved performance. For example, Ekins and co-workers developed the elegant ambient analyte ligand assay¹³ that has formed the theoretical basis of “microspot” assays or planar protein arrays. This approach was based on minimizing the amount of antibody in the system (hence the use of microspots of capture antibodies deposited on a planar substrate) and measuring the fractional occupancy of the spotted antibodies. Theory indicated that this approach would make the measurement insensitive to the volume of analyte being tested and, consequently, more robust and less dependent on the precision of automated pipetting

systems. The sensitivity of this “minimal antibody” approach is, however, limited by non-specific binding of labeling reagents to the surface on which the antibodies are spotted:¹³ high concentrations of labeling reagents are needed to label low amounts of captured proteins that results in increased backgrounds and limits sensitivity. Based on our observation that backgrounds were dominated by the interactions between the labeling reagents and the immobilized capture antibodies, we have tackled the challenge of optimizing sensitivity and specificity of immunoassays from the opposite direction taken by the ambient analyte assay. Digital ELISA uses an *excess of capture antibodies immobilized on beads* to kinetically drive the system towards the bound protein state, and maximize the number of target proteins captured. As these proteins are ultimately detected using SiMoA—which we have shown is extremely sensitive to enzyme, detecting down to 220 zM⁶—only a fraction of these proteins need to be labeled using a detection antibody and enzyme conjugate. By reducing the concentration of the labeling reagents or the time of labeling, sufficient molecules can be detected *and backgrounds are greatly reduced*, making it easier to detect target molecules above non-target interactions. We believe that this approach addresses the fundamental challenges of maximizing both sensitivity and specificity of immunoassays.

In this paper, we describe mathematically the kinetics of each step of digital ELISA in terms of each bimolecular interaction. These equations indicate the efficiency of each step in terms of molecules captured, labeled, and detected. We compare the predictions generated by these equations to data acquired for a digital ELISA for prostate specific antigen (PSA). We also consider the theoretical and experimental challenge of dissociation of antibodies from immunocomplexes over time, and suggest some approaches for minimizing its effect.

2. Materials and Methods

Immunoassays for PSA based on the detection of single enzyme-labeled immunocomplexes on paramagnetic beads in single molecule arrays (SiMoA) were developed as described previously.^{6,7} These assays were performed in two steps: immunocomplex formation on paramagnetic beads, followed by detection of single enzyme-labeled proteins in arrays of femtoliter wells.

2.1. Formation of immunocomplexes on beads

Antibodies and protein standards were obtained from Biospecific. Monoclonal capture antibodies were covalently attached to 2.5- μ m diam., carboxyl-terminated paramagnetic beads (Varian) using a standard coupling method based on 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). 100 μ L samples of plasma diluted 1:4 (25 μ L plasma + 75 μ L phosphate buffered saline (PBS)) spiked with different concentrations of PSA were mixed with 500,000 capture beads in a 96-well plate, and incubated to capture the target protein. The beads were washed three times with 5 $\times$ PBS + 0.1% Tween-20. Beads were then incubated with 100 μ L of biotinylated polyclonal detection antibody and washed three times with 5 $\times$ PBS + 0.1% Tween-20. Beads were then incubated with 100 μ L of streptavidin- β -galactosidase (S β G) and washed eight times with 5 $\times$ PBS + 0.1% Tween-20. Incubation times and reagent concentrations were varied as required experimentally. Beads were then resuspended in 25 μ L of PBS.

2.2. Detection of immunocomplexes using SiMoA

Beads were loaded into arrays of 50,000 50-fL wells by centrifuging 10 μ L of bead suspension in contact with the array. The femtoliter well arrays were formed by etching optical fiber bundles, and the wells were designed to accept one bead per well as described previously.⁶ A droplet of resorufin- β -D-galactopyranoside (RGP)—a substrate to -

galactosidase—was placed on a silicone gasket material, and the femtoliter well arrays loaded with beads were sealed mechanically against the silicone gasket, creating an array of isolated femtoliter-volume reaction vessels containing RGP. White light images of the arrays were used to identify those wells that contained beads. Fluorescence images were acquired (577 nm excitation; 620 nm emission) to detect the presence (“on” bead) or absence (“off” beads) of enzymatic activity in each well containing a bead. The digital signal, i.e., the fraction of “on” beads for each array (f_{on}), was determined using image analysis. Depending on f_{on} , the average enzymes bound per bead (AEB, the unit of measurement of SiMoA) was determined. If $f_{\text{on}} < 0.7$, then AEB was determined digitally using the Poisson distribution from the fraction of active beads; if $f_{\text{on}} > 0.7$, then AEB was determined in an analog fashion using the average intensity of wells containing a bead in an array. The equations used to determine digital and analog AEB are given elsewhere.⁷

3. Results and Discussion

3.1 The Digital ELISA Process

The steps used to perform digital ELISA are shown schematically in Figure 1. In the first step (Step A), the target protein in the sample is captured on paramagnetic particles that have been functionalized with a “capture” antibody to the target protein. In the second step (Step B), the captured proteins are labeled with enzyme molecules through sequential incubations with a second “detection” antibody that is biotinylated and streptavidin- β -galactosidase. In the third and final step (Step C), the paramagnetic beads that are associated with enzyme-labeled proteins are suspended in a solution containing a fluorogenic substrate to the enzyme, and loaded into arrays of microwells with volumes around 50 femtoliters, such that each well contains no more than one bead. The arrays of microwells are sealed to trap the fluorescent product of the enzyme-substrate reaction within the volume of the microwell. The sealed arrays are imaged fluorescently on a CCD camera using standard microscope optics that can easily detect fluorescent product from a single enzyme trapped in 50 fL. Software analyzes these images to determine the fraction of beads associated with at least one enzyme (f_{on}), the average intensity of the beads in an arrays, and, by inference, the average number of enzymes associated with the beads in the array (AEB).⁷

Digital ELISA was designed with the goal of detecting most, if not all, protein molecules in a sample by counting single molecules. Bead-based capture has the potential to result in very efficient capture and labeling of protein molecules from a kinetic perspective. The single molecule arrays make it possible, given sufficiently large arrays, to singulate and count *all* of the labeled protein molecules. By combining these two very efficient processes, digital ELISA is a highly efficient process, and the ability to detect most of the protein molecules in a sample is what lies at the heart of its high sensitivity. In the following section, we will describe the kinetics of each of the three steps in digital ELISA, determine their theoretical efficiency, and compare these predictions to experimental data.

3.2. Sensitivity of Digital ELISA

3.2.1. Capture of Proteins on Beads (Step A)

3.2.1.1. Equilibrium Aspects: The maximum efficiency of the capture of protein molecules on antibody-coated paramagnetic particles (Step A in Fig. 1) can be predicted by considering the equilibrium between free protein in solution (L) and the antibody on beads (Ab) resulting in bound protein (AbL) on beads (eq. 1), and the conservation of the total concentrations of antibody (Ab_{total}) and protein (L_{total}) in the system (eqs. 2 and 3):

$$[Ab_{total}] = [Ab] + [AbL] \quad (2)$$

$$[L_{total}] = [L] + [AbL] \quad (3)$$

From the definition of the dissociation constant ($K_D = k_{off}/k_{on} = [Ab][L]/[AbL]$) it follows from eqs. 1-3 that:

$$[AbL]^2 - (K_D + [L_{total}] + [Ab_{total}]) [AbL] + [L_{total}] [Ab_{total}] = 0 \quad (4)$$

The concentration (and, therefore, the number) of bound or captured protein molecules, $[AbL]$, can be determined by solving quadratic eq. 4 using values of K_D , the overall concentration of protein in solution ($[L_{total}]$), and the concentration of antibody on the beads $[Ab_{total}]$. The “thermodynamic” efficiency of protein capture at equilibrium is defined as $[AbL]/[L_{total}]$.

The variation of $[AbL]$ as a function of ($[L_{total}]$ (from, say, pM down to aM) can be modeled from experimentally-derived estimates of $[Ab_{total}]$ at different values of K_D . Based on measuring depletion of the capture antibody from solution during functionalization of the beads, we estimate that each 2.5- μ m-diam. bead is modified with approximately 274,000 antibodies. This loading density corresponds to each antibody occupying an area equivalent to a square of side 15 nm. As antibodies are known to be approximately 15-20 nm long and 6-15 nm wide, we are confident in this estimate. Typical assays use 500,000 beads in 100 μ L of sample, corresponding to $[Ab_{total}] = 2.3$ nM. Antibodies that are used in immunoassays such as ELISA typically have K_D in the range 10 pM to 10 nM.^{14,15} Figure 2A shows plots of the number of AbL complexes formed on 500,000 beads as a function of $[L_{total}]$ for K_D ranging from 10 pM to 10 nM based on eq. 4. Figure 2B shows the average number of protein molecules captured per bead as a function of bead number assuming $K_D = 1$ nM. These plots focus on the range of protein concentrations that are most relevant to digital ELISA, i.e., from 100 aM to 10 pM. The inset tables show the capture efficiencies; capture efficiency is effectively independent of concentrations until significant fraction of the antibodies are bound to a protein molecule, i.e., at nanomolar concentrations, well above the protein concentrations of interest here.

Figure 2A shows that almost all proteins are captured for $K_D \leq 10^{-10}$ M. At nanomolar dissociation constants, the capture efficiency is still high (~70%): a 1000-fold reduction in antibody affinity only resulted in a 30% drop in capture efficiency. This observation suggests that, at equilibrium, digital ELISA will work effectively over a broad range of antibody affinities. In terms of the absolute number of molecules that need to be detected over this range, we have previously shown that single molecule arrays can detect down to 10 enzyme labels in 100 μ L, equivalent to approximately 0.00002 enzyme labels per bead. Provided protein molecules are efficiently labeled with enzyme (see Section 3.2.2), this detection limit is well below the average number of captured molecules predicted by eq. 4 (right-hand y-axis of Fig. 2A). For example, incubating 100 aM of protein with 500,000 beads modified with a capture antibody with $K_D = 10^{-9}$ M would give rise to 0.008 captured molecules per bead on average. From a kinetic perspective, assuming typical affinities of antibodies there are plenty of proteins captured at equilibrium on beads from solutions containing attomolar concentrations and lower to be detected using SiMoA.

Fig. 2B shows that digital ELISA is also effective over a wide range of bead concentrations. While capture efficiency decreases with the number of beads (inset table), the relevant parameter for SiMoA detection (molecules per bead) actually increases as the bead concentration drops. This competing effect (fewer beads means lower capture efficiency but greater molecules per bead for a given capture number) starts to be less important at <100,000 beads. It is, in theory, possible to use very low bead concentrations in digital ELISA because of this effect. Two considerations, however, mean that particularly beneficial results are obtained when the bead amount is between 200,000 and 500,000 beads. First, delivering at least 100,000 beads using the current methods for loading helps to provide sufficient beads in wells to detect background AEB values on the order of 0.008 without significant Poisson noise. As described previously, the number of beads loaded drops quickly below 100,000 beads delivered that may lead to insufficient numbers of beads detected. Second, fewer beads can result in longer diffusional distances between beads during capture, slowing the kinetics of capture as described in Section 3.2.1.2.

3.2.1.2. Kinetic Aspects: While the previous section indicates that, at equilibrium, digital ELISA is efficient over a wide range of experimental conditions, it does not consider the time dependence of kinetics that will determine how long it takes to reach equilibrium. Next, we will consider two possible kinetically limiting processes of bead-based capture of proteins that affect the generation of the SiMoA signal as a function of time: adsorption and diffusion.

3.2.1.2.1. Adsorption Kinetics: The rate of formation of antibody-protein complexes is determined by the balance between the on- and off-rates of the protein in these complexes. From eq. 1 to 3, the overall rate of formation of antibody-protein complexes is given by:

$$\frac{\partial [AbL]}{\partial t} = k_{on} ([Ab_{total}] - [AbL]) ([L_{total}] - [AbL]) - k_{off} [AbL] \quad (5)$$

There is no analytical solution to eq. 5, but theoretical values of [AbL] as a function of t can be generated numerically, from known values for k_{on} , k_{off} , $[Ab_{total}]$, and $[L_{total}]$. Figure 3 shows plots of [AbL] as a function of time determined numerically using eq. 5, assuming $[Ab_{total}] = 2.3 \text{ nM}$ (500,000 beads in 100 μL as described in Section 3.2.1.1) and $[L_{total}] = 1 \text{ fM}$. These plots were generated using k_{on} and k_{off} values ranging from 10^4 – $10^6 \text{ M}^{-1} \text{ s}^{-1}$ and 10^{-3} – 10^{-6} s^{-1} , respectively, corresponding to dissociation constants from 1 nM to 10 pM. These kinetic parameters are typical for the antibodies that are selected to develop ELISAs.^{14,15} To verify that these kinetic parameters were representative of commercial reagents and to experimentally test eq. 4 and 5 for one particular digital ELISA, we determined on- and off-rates and dissociation constants for five antibodies using a biosensor (FortéBio, Menlo Park, CA). K_D was determined in the classical fashion by measuring the on- and off-rates of the antibodies to a surface presenting the target protein (e.g., PSA). We have measured using a biosensor on and off rates for five antibodies that we have used in digital ELISAs for PSA, tau, and TNF- α ; k_{on} ranged from 9×10^4 to $1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, and k_{off} ranged from 1.3×10^{-3} to $3 \times 10^{-6} \text{ s}^{-1}$, with K_D from 1 nM to 12 pM. For example, the PSA capture antibody used in the experiments presented here had kinetic parameters $k_{on} = 2.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 3 \times 10^{-6} \text{ s}^{-1}$, and $K_D = 12 \text{ pM}$ (in agreement with the manufacturers specification).

It is clear from Fig. 3 that the rate of protein capture is determined largely by the on rate of the binding reaction for ELISA quality antibodies. After a 1000 s (17 minutes) incubation, for $k_{on} = 10^6 \text{ M}^{-1} \text{ s}^{-1}$ the capture efficiency was high, ranging from 67% to 89% for dissociation constants from 1 nM down to 10 pM, respectively. For $k_{on} = 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and

$10^4 \text{ M}^{-1} \text{ s}^{-1}$, the capture efficiencies are 20% and 2.3%, respectively, independent of K_D . The optimal capture antibody, therefore, is one with a high on-rate. That said, as SiMoA is so sensitive to the enzyme used to label captured proteins (see below), that for highly efficient protein capture it has been experimentally necessary to label only a fraction of those captured. Therefore, even with antibodies with lower on rates ($k_{\text{on}} \sim 10^4 \text{ M}^{-1} \text{ s}^{-1}$) it is possible to detect captured proteins using SiMoA. For example, in 1000 s capture efficiency equal to 2.3% results in an AbL concentration of 0.023 fM from a 1 fM solution of protein. Assuming each of these complexes were labeled with an enzyme (see below), then the concentration of enzyme would be 23 aM, much higher than the analytical sensitivity of the method (220 zM). Furthermore, this capture efficiency would give rise to 0.3% active beads, around the typical background targeted to minimize Poisson noise in these assays. Femtomolar detection of proteins is, therefore, feasible using antibodies with these lower on rates. More experimental studies on on-rate against SiMoA signal are needed to test these theoretical calculations.

3.2.1.2.2. Diffusion-limited Kinetics: Our first generation single molecule immunoassays focused on capturing proteins directly within the microwell arrays that had been modified with antibodies. While this approach had the same high capture efficiencies at equilibrium as for the bead-based approach shown in Fig. 1, in practice the detection efficiency was reduced as compared to bead-based assays because of kinetic limitations imposed by diffusion. Calculations indicated that protein capture efficiency in this case was diffusion limited, and it could take more than 3 months for a femtomolar concentration to reach equilibrium via diffusion with an array presenting antibodies. The use of beads helps to overcome the diffusion limitation of using a fixed, planar and unstirred substrate to capture proteins, by distributing the capture component throughout the sample.

If we consider 500,000 beads evenly distributed throughout 100 μL of solution, then each bead can be viewed as occupying a cube of side 58 μm . The time (t) it takes for a protein to diffuse an average distance $\langle x \rangle$ is given by $t = \pi \langle x \rangle^2 / 4D$, where D is the diffusion coefficient of the protein.¹⁷ A protein such as PSA that has a diffusion coefficient $D = 8.5 \times 10^{-7} \text{ cm}^2/\text{s}$,¹⁸ takes approximately 31 s to diffuse 58 μm . In other words, all of the protein molecules in the system are within about 30 s of diffusion of a bead. This fact means that the bead based approach is not limited by diffusion as transport to a fixed planar capture substrate would be.

At very low concentrations, where there are fewer protein molecules than beads, we also have to consider collision frequency as a potential kinetic limitation of digital ELISA: each protein molecule has to collide with a bead for it to have a chance of being captured. At protein concentrations of 1 fM, there are 60,000 protein molecules in 100 μL for 500,000 beads. It is useful, therefore, to view the sample volume being broken up into cubic cells of side $l = \langle x \rangle = 58 \mu\text{m}$ that each contains a single bead, and about 1 in 8 cells containing a single protein molecule. Consider a single PSA molecule diffusing by a random walk through such a cell: how many collisions with the bead will occur in 31 s or over a 1000 s incubation period? To provide an analytical solution to this problem, we consider the beads as fixed and randomly distributed throughout the cells and transform the random walk of the protein to a straight cylinder of diameter $2a$ (where a is the hydrodynamic radius of PSA) and length Λ , and then determine the probability that the projected areas of the bead and PSA molecule are coincident. Based on this approach, the number of collisions that will occur in time T is given by the product of the number of beads (or cells) traversed (Λ/l) and the fractional area overlap of the bead and PSA molecule as the molecule passes through each cell ($(\pi r^2 + \pi a^2)/l^2$), where r is the radius of the bead. The characteristic step length (λ) and jump time (τ) of a molecule undergoing random walk can be related to its diffusion coefficient via the Einstein-Smoluchowski equation, such that, $D = \lambda^2/2\tau$.¹⁷ For an

incubation time T , the total distance travelled by the PSA molecule, Λ , therefore, is given by $\Lambda = \lambda T / \tau = 2DT / \lambda = DT / a$. The total number of collisions (n) in time T is therefore given by eq. 6:

$$n = \frac{\pi DT}{a l^3} (r^2 + a^2) \quad (6)$$

In the case of PSA mixed with 2.5- μ m-diameter beads, $D = 8.5 \times 10^{-7} \text{ cm}^2/\text{s}$, λ is assumed to be twice the hydrodynamic radius of the PSA molecule ($a = 3 \text{ nm}$),¹⁹ and $r = 1.25 \text{ }\mu\text{m}$. In one diffusional timeframe ($T = 31 \text{ s}$), eq. 6 indicates that the PSA molecule will collide with the bead 22 times on average. During a 1000 s incubation, each PSA molecule will collide with a bead 712 times unless it is captured. Based on these considerations, theory indicates that the capture of protein molecules on beads will not be limited by diffusion or low collision frequencies.

To test the analytical solution given by eq. 6, we set up a rudimentary two-dimensional random walk simulation for a single PSA molecule in a square of side 58 μm containing a randomly placed bead with diameter of 2.5- μm . The step size was set to 6 nm and the jump time to 212 ns; 500 simulations were performed and each simulation was stopped when the PSA molecule first coincided with the bead. In each of these 500 simulations over 31 s, the PSA molecule hit the bead 68% of the time. The mean time to collision was 13.3 s. The mean number of collisions in the diffusion time ($T = 31 \text{ s}$) was therefore about 1.6.

Using these two methods, we estimated the number of collisions per 31 s to be 2–20. As these models do not take into account convective effects or the motion of the bead itself, we assume they underestimate the collision frequency. As the bead and protein molecule are likely to collide several times during a typical 1000 s incubation, we do not believe that the dynamics of capture by beads will limit the sensitivity of this method.

3.2.2. Labeling of Captured Proteins with Enzymes (Step B)

3.2.2.1. Equilibrium Aspects: In digital ELISA, the proteins captured by antibodies immobilized to beads are labeled with a biotinylated detection antibody that is then labeled with S β G (Fig. 1). We will consider the kinetic equilibrium of each of these two steps separately.

To calculate the efficiency of labeling the captured protein by a detection antibody, eq. 4 can be used with the detection antibody as the “free ligand” (equivalent to L_{total} in eq. 4) and the capture antibody-protein complex as the immobilized capture agent (equivalent to Ab_{total} in eq. 4). Figure 4 shows plots of the labeling efficiency of different concentrations of AbL complex by detection antibody as a function of the concentration of detection antibody used (at fixed K_D) and as a function of K_D (at a fixed detection antibody concentration). The plot in Fig. 4A assumed $K_D = 1 \text{ nM}$ for the detection antibody and a high efficiency capture antibody (i.e., $[AbL] \approx [L_{\text{total}}]$). These assumptions are similar to those for the capture and detection antibody pair for the PSA digital ELISA presented here.

As can be seen from Fig. 4, the labeling efficiency is insensitive to the concentration of captured proteins across the analytical region of interest. In our assays, we typically use detection antibody concentrations of 0.1 $\mu\text{g/mL}$, i.e., around 1 nM, to ensure sufficient labeling and also minimize the primary source of non-specific binding (see below). Fig. 4A shows that this concentration should result in a capture efficiency of ~50% for $K_D = 1 \text{ nM}$. Similar trends can be seen as a function of the affinity (K_D) of the detection antibody (Fig. 4B). Lower affinity detection antibodies would need greater concentrations to achieve

higher labeling efficiencies, but this approach would also lead to higher non-specific binding.

The efficiency of labeling by S β G of the complexes formed between the captured protein and detection antibody can also be determined from eq. 4 with S β G as the “free ligand” (equivalent to L_{total} in eq. 4) and the capture antibody-protein-biotinylated detection antibody complex (AbL-detAb) as the immobilized capture agent (equivalent to Ab_{total} in eq. 4). Figure 5 shows plots of the labeling efficiency of different concentrations of AbL-detAb complexes by enzyme conjugate as a function of the concentration of S β G used typically in digital ELISA (1–150 pM). Fig. 5 assumes a $K_D = 10^{-15}$ M for the interaction between S β G and the biotinylated detection antibody,²⁰ and an efficiency in the formation of AbL-detAb complexes calculated using parameters typical for the PSA digital ELISA, i.e., those shown in Fig. 2A and Fig. 4A, for an overall efficiency of about 50%. Fig. 5 shows that all of the complexes will be labeled by enzyme across the concentration range of interest and the range of label concentrations used typically. At concentrations of S β G of 15 pM (the concentration typically used in the digital ELISA for PSA), the complex will be 100% labeled at equilibrium.

The calculations plotted in Fig. 5 assumed that each detection antibody had a single biotin group that was competent for binding S β G. The detection antibodies have, however, on average 8 biotin groups appended.⁷ The number of fluorescent product molecules at low ratios of analytes to beads, however, indicate that only single enzymes bind to the detection antibody, presumably because of steric reasons. Furthermore, the variation in the fraction of active beads follows a Poisson distribution as consistent with a unimolecular interaction between detection antibody and S β G. That said, repeating the calculations assuming 8 biotin groups per detection antibody yields essentially the same efficiency for labeling the detection antibodies with at least one enzyme as in Fig. 5, i.e., 100%.

3.2.2.2. Kinetic Aspects: The kinetic variation of labeling the captured proteins with detection antibody and enzyme conjugate can also be modeled by consideration of the adsorption and diffusional kinetics in turn.

The adsorption kinetics of labeling AbL complexes with detection antibodies (to form AbL-DetAb) can be modeled numerically using an equation similar to eq. 5. Figure 6 shows plots of [AbL-DetAb] as a function of time determined numerically, assuming $[Ab_{\text{total}}] = 2.3$ nM (500,000 beads in 100 μ L), $[L_{\text{total}}] = 1$ fM, and $[DetAb] = 1$ nM as is typical for digital ELISA. As for protein capture, these plots were generated using k_{on} and k_{off} values ranging from 10^4 – 10^6 $\text{M}^{-1} \text{s}^{-1}$ and 10^{-3} – 10^{-6} s^{-1} , respectively, corresponding to dissociation constants from 1 nM to 10 pM. This chart also contains a plot of the predicted kinetic profile of the the PSA detection antibody used in the experiments described here ($k_{\text{on}} = 3.9 \times 10^5$ $\text{M}^{-1} \text{s}^{-1}$, $k_{\text{off}} = 8.6 \times 10^{-4}$ s^{-1} , and $K_D = 2$ nM).

As for capture of proteins, it is clear from Fig. 6 that the rate of labeling of captured protein by detection antibodies is determined largely by the on-rate of the binding reaction. The optimal detection antibody, therefore, is one with a high on-rate. That said, for antibodies with slower on-rates, signals can be increased by increasing the labeling efficiency of the enzyme step (see below). As an example of typical labeling efficiencies as a function of time, 1 nM of detection antibody used in the PSA digital ELISA is predicted to label approximately 22% of the captured proteins within 1000 s, and over the incubation period in the assays previously published (60 min),⁷ the calculations indicate that 31% of captured proteins are labeled.

The effect of diffusion of the detection antibody on the kinetics of protein labeling can be modeled by determining the diffusional timescales and lengths as described for protein capture in Section 3.2.1.2.2. The IgG molecule has a diffusion coefficient $D = 3.89 \times 10^{-7} \text{ cm}^2/\text{s}$,²¹ so it takes approximately 69 s to diffuse the average inter-bead distance of 58 μm . Based on eq. 6 and assuming a hydrodynamic radius $a = 5.5 \text{ nm}$,²¹ each detection antibody will collide with a bead 12 times within 69 s or 625 times during a typical incubation time of 60 mins. As the concentration of detection antibody is typically 1 nM, there are about 120,000 antibody molecules in each “cube” occupied by a bead. This number of molecules would, therefore, translate to about 75 million collisions in 60 min. These calculations indicate that the labeling of protein with capture antibodies will not be limited by diffusion.

The adsorption kinetics of labeling of AbL-DetAb complexes with S β G (to form AbL-DetAb-S β G) can be modeled numerically the same way as the detection antibody labeling step. Figure 7 shows plots of [AbL-DetAb-S β G] as a function of time determined numerically using eq. 6, assuming $[\text{Ab}_{\text{total}}] = 2.3 \text{ nM}$, $[\text{L}_{\text{total}}] = 1 \text{ fM}$, and $[\text{DetAb}] = 1 \text{ nM}$, at several different concentrations of S β G. These plots were generated assuming $k_{\text{on}} = 5.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{\text{off}} = 5.1 \times 10^{-9} \text{ s}^{-1}$ for the interaction between streptavidin and biotin.²⁰ The calculations also assume that each detection antibody has one biotin group that is able to bind one enzyme conjugate.

Fig. 7 demonstrates that, at the concentrations of S β G typically used in digital ELISA (15–50 pM), only a fraction of the AbL-DetAb complex are being labeled with an enzyme. For example, after a 30 min incubation with $[\text{S}\beta\text{G}] = 15 \text{ pM}$ (as used for the PSA digital ELISA and others) only 13% of the complexes are labeled with an enzyme. This under-labeling is deliberate and arises from the ability to use the high sensitivity of SiMoA to minimize the concentration of enzyme conjugate used in the assay that gives rise to background signals that limit assay sensitivity. Complete labeling of these complexes is readily achieved kinetically, e.g., incubation of 150 pM for 1 h would result in 94% of complexes being labeled, but this process would increase non-specific binding, and increase the average enzymes per bead such that the full dynamic range and sensitivity of SiMoA would not be exploited. These considerations are discussed more fully in Section 3.2.4.

The effect of diffusion of S β G on the kinetics of labeling the complexes with an enzyme can be modeled by determining the diffusional timescales and lengths as described for protein capture in Section 3.2.1.2.2. Assuming that S β G has a diffusion coefficient close to that of β -galactosidase ($D = 2.7 \times 10^{-7} \text{ cm}^2/\text{s}$),²² it takes approximately 99 s for the enzyme conjugate to diffuse the average inter-bead distance of 58 μm . Based on eq. 6 and assuming a hydrodynamic radius $a = 8 \text{ nm}$,²³ each S β G molecule will collide with a bead 8 times within 99 s or 149 times during a typical incubation time of 30 mins. As the concentration of detection antibody is typically 15 pM, there are about 1,800 S β G molecules in each “cube” occupied by a bead. This number of molecules would, therefore, translate to about 269,000 collisions in 30 min. These calculations indicate that the labeling of AbL-DetAb complex with enzyme by S β G will not be limited by diffusion.

Another important kinetic issue, other than the association of complexes, is the dissociation of complexes, once the bulk reagent is removed. As with other assays based on immunocomplex sandwiches, dissociation can lead to loss of signal and variation in signal depending on the time between reagent removal and detection. Dissociation can occur at several molecular interaction points in the immunocomplex: dissociation of the protein from the capture antibody; dissociation of the detection antibody from the captured protein; and dissociation of S β G from the biotin on the detection antibody. The off-rate of the interaction of streptavidin with biotin is of the order of 10^{-9} s^{-1} , so we do not expect dissociation of S β G from the detection antibody to cause a significant loss of signal over the time course of

the digital ELISA experiment. Given similar off-rates between capture antibody and protein, and protein and detection antibody in solution, we believe that the *effective* off-rate of the protein from the capture surface will be much lower than the off-rate of the detection antibody from the captured protein for the following reason. The captured protein is surrounded by a large number of unoccupied capture antibodies on the bead. When the protein dissociates from a capture antibody, there is a high probability of rebinding to the surface, and also there is the chance of multivalent interactions between the protein and capture antibodies. Reduced effective off-rates of molecules bound to a surface have been observed by others¹² and can, in some instances, effectively lead to a molecule being irreversibly bound. Incidentally, we believe that these surface-enhanced “avidity” effects allow us to rigorously wash the beads after protein capture ensuring the captured proteins remain on the surface while weakly bound molecules are removed (See Section 3.3). Detection antibody, however, is bound to a single protein molecule on a bead; rebinding or multivalent interactions cannot occur in this instance. The enhanced affinity effects do not exist for this interaction and the off-rate is likely to be close to that in solution. We, therefore, believe that dissociation effects will be dominated by the dissociation of the detection antibody from the captured protein.

We have demonstrated the effect of dissociation of detection antibody by varying the time between removal of the solution of detection antibody and array imaging. Figure 8A shows plots of AEB against the time between removal of the solution of detection antibody and imaging of the arrays. Fitting of the decrease in AEB with this time difference yielded an off-rate $k_{\text{off}} 10^{-4} \text{ s}^{-1}$ the same order of magnitude as determined using the biosensor. This phenomenon can lead to sample-to-sample imprecision of AEB values if the time between when dissociation begins and imaging varies. There are several options for minimizing the impact of dissociation on the precision and accuracy of digital ELISA. The first, and least invasive, method is to ensure that every sample experiences the same timing of incubation, washing, and detection during an experiment. In this way, the time for dissociation is fixed and, while there might still be loss of signal from dissociation, it will be constant for all samples and not affect the precision of the assay. In practice, this approach means that each sample has to be processed individually. The automated instrumentation that we are developing for digital ELISA, in fact, is based on sequential handling of individual samples removed from a 96-well plate to negate the impact of dissociation. The second approach is to apply a mathematical correction to the data to correct for differences in time allowed for dissociation before detection occurs. While this approach is feasible, it is dependent on having a stable dissociation rate and the experimental variation in this rate that we have observed from day-to-day and between batches of reagents limits its usefulness. A third approach is to “freeze” the labeled immunocomplex on the beads to stop dissociation occurring and allow for difference in time between incubations and imaging. We have demonstrated this approach by adding sucrose to the solution in which the beads are loading into the arrays of microwells. After loading the beads, the arrays are dried without loss of enzymatic activity. In this manner, the immunocomplexes are “frozen” and dissociation is stopped or greatly slowed. Arrays can then be imaged at increasing times without reduction of signal. Fig. 8B shows a series of experiments where beads were loaded into arrays in solutions containing 10% sucrose and dried, and the arrays were imaged at times ranging from 5 min to 2 hours after bead loading. The coefficients of variation (CV) of AEB for these three runs were 4.4%, 4.5%, and 7.5% indicating that the effect of dissociation had been eliminated.

3.2.3. Detection of enzymes associated with beads using SiMoA (Step C)—The final step of the digital ELISA process is to detect the enzymes that are associated with immunocomplexes on beads (Step C, Fig. 1). As SiMoA is capable of detecting single enzymes, the efficiency of this process is very high. Careful measurements of enzyme

kinetics demonstrated a wide distribution in the activity of single enzymes.^{7,24} Typical conditions for generating signal from single enzymes indicate that almost all of the enzymes will generate sufficient signals to be detected. Using a model system based on capturing SβG using beads presenting high concentrations of biotin, we showed that the efficiency of capturing and detecting enzyme was >70%.⁶ The reason for not achieving 100% detection of enzyme in that assay was not clear, but it could have been due to incomplete binding of SβG, non-specific binding of SβG to plastic, or to incomplete detection of single enzymes.

Given the high efficiency of detecting single enzymes, the limit to sensitivity and achieving the goal of “detecting every molecule in the system” is bead loading efficiency. While digital ELISA is a ratiometric method, the sensitivity at extremely low AEB will be limited by the number of beads detected. Using the 50,000-well glass arrays and loading beads via centrifugation described above, we are able to detect approximately 25,000–30,000 beads from 200,000 beads used in a digital ELISA, an overall efficiency of <15%. To improve this efficiency and detect the remaining 85% of enzymes, three improvements in the SiMoA process are needed. First, arrays with more wells are required. We have developed arrays based on microreplication in plastics with 216,000 wells.²⁵ These manufacturing processes, based on the same methods used to create DVDs, make it possible to generate arrays with millions of wells. Second, CCD cameras are needed with sufficient resolution and chip size to image large arrays. Using current scientific cameras with resolutions >8 megapixels, we are able to image approximately 200,000 wells. The availability of low cost cameras currently limit our ability to image more beads. Finally, the efficiency of delivery of magnetic beads to the wells needs to be improved. Dead volume and the limited area that the wells occupy on the array surface mean that bead delivery is an inefficient process. Typically hundreds of thousands of beads are required to deliver tens of thousands of beads to wells. While this limitation is generally unimportant for a ratiometric method, the low end sensitivity and consequently the overall dynamic range of the method could be improved by getting more beads into wells. We are working on magnet designs and fluidic structures to minimize dead volume and improve flux of beads to the wells.

Imprecision is another important consideration when counting enzymes in digital ELISA. We have shown that SiMoA is Poisson noise limited, i.e., the imprecision is equal to $\sqrt{N}/N$, where N is the number of enzyme-associated beads detected.⁶ In general, to keep the imprecision due to Poisson noise less than 10% in digital ELISA, we optimize the labeling steps to ensure that at least 100 beads are detected in the absence of analyte.

3.2.4. Overall efficiency of digital ELISA—Based on the models presented in Sections 3.2.1 to 3.2.3 it is possible to predict the overall efficiency of a digital ELISA assay based on the number of beads, the number of antibodies per bead, the on- and off-rates of the capture and detection antibodies, the concentrations of detection antibody and SβG, the times of the variation incubations, and the volume of incubation. Table 1 shows a summary of the efficiencies of the various capture and labeling steps for three concentrations of PSA (0.1 pg/mL = 3.33 fM, 1 pg/mL = 33.3 fM, and 10 pg/mL = 333 fM) both at equilibrium and for the incubation times tested experimentally. These efficiencies were converted to a total number of molecules captured and labeled, so, from knowing the number of beads used, AEB values can be predicted. These values have been compared to the experimental AEB values subtracted from the background AEB values to generate the PSA specific signal (Table 1 and Figure 9). We have published extensively on the PSA digital ELISA^{6,7} and have performed in depth preclinical analytical testing of the reagents,⁹ so it provides a convenient first test of the mathematical models presented here.

Figure 9 shows plots that compare the predicted AEB values to those determined experimentally both in this study and also in previous publications.⁷ The predictions and

experimental data are in good agreement (Table 1), and most lie within 2-fold of each other. Given the multiple steps and the number of parameters in these models, this level of agreement is remarkable. While these data only represent one target protein, they do suggest that the simplicity of the digital ELISA and the ability to determine numbers of molecules detected directly will allow extensive testing of theoretical models for immunoassays. These data also indicate that assays could be designed (concentrations, incubation times, etc.) before any experiments are done based on knowledge of antibody kinetic parameters.

Table 1 shows that, at equilibrium, digital ELISA is efficient overall and that the design of digital ELISA does, indeed, make it possible to detect most of the molecules in the system. A comparison of equilibrium conditions and those used in a typical assay, indicate how the signals are kinetically controlled. Typically, we observe background signals that are equivalent to adding 1 fM of analyte to the sample as described previously (AEB < 0.005). We note that if the system was allowed to reach equilibrium, such a 1 fM background signal would correspond to the AEB 0.03. As our noise floor of about 100 active beads in a total number of beads detected of 25,000, corresponding to AEB = 0.004, we must kinetically control the signals in digital ELISA to make use of the full dynamic range and sensitivity of the method. In the “120-60-30 min” assay that we have published previously, Table 1 shows that by using a low concentration of SβG, we reduced the labeling efficiency from close to 100% to about 13% bringing the background down to the noise floor, so while the overall efficiency is relatively low (2.9%) the full dynamic range of the arrays are utilized. Another approach to kinetically controlling the signals is to use shorter incubation times with higher concentrations. Table 1 and Figure 9 shows several examples of “10-10-10 min” assays where higher concentrations of both detection antibody and enzyme conjugate can be used to achieve the same AEB values in shorter times. In general, this process of optimizing the concentrations of detection antibody and SβG for fixed incubation times to bring backgrounds down to the Poisson noise floor is carried out every time a new assay is developed.

One point of note is that the largest differences between theory and experiment are for conditions where incubation times are short and enzyme concentrations are high. The reason for this deviation is not clear. One possibility is that the very short incubation times result in greater variation in assay timing for this manual process and increases the chance of deviation from theoretical model. Another possibility is that at high labeling efficiencies (the deviation seems to increase with enzyme label efficiency) the binding isotherms are not described by simple bimolecular interactions. Another possibility is lot-to-lot variation in antibody loading: comparison of identical conditions for two lots of capture beads (rows 3 and 4 of Table 1) suggest that coupling efficiency may cause the majority of the deviation from theory.

3.3. Specificity of Digital ELISA

Section 3.2 demonstrated that in terms of sensitivity, SiMoA is efficient and does indeed make it possible to detect femtomolar concentrations of proteins using antibodies with nanomolar affinity. The second aspect to being able to detect a protein using digital ELISA is the specificity of the process. Given that femtomolar concentrations can be captured, labeled and detected, how is it possible to detect that in a matrix that contains thousands of other proteins, many of which are present at concentrations that are 10^{12} -fold higher? It is much more challenging to model specificity quantitatively as was possible for sensitivity. Qualitative arguments about the design of digital ELISA do, however, shed light on how very high specificity can be achieved. Consider a capture antibody with a nanomolar K_D . Many manufacturers provide information of the specificity of ELISA antibodies for closely related proteins. Assuming that the K_D of the antibody with the protein of highest cross-reactivity is 1 μ M (1000-fold specificity); any higher affinity would indicate that the

antibody is not useful at all as a specific reagent. The specificity of the antibody for the protein in question is therefore about 1000-fold in concentration, not useful when trying to discriminate 10^{12} -fold differences in concentration. The use of a second labeling antibody greatly improves the specificity of the assay, and is one of the strengths of ELISA in general. Assuming that the second antibody also has nanomolar affinity for the target protein, but has lower cross-reactivity than the detection antibody, say, $K_D = 1$ mM, as presumably it would be a badly designed antibody pair to bind with equal affinity to a cross reactive protein. In this case the selectivity would be $(10^{-9} \times 10^{-9}) / (10^{-3} \times 10^{-6})$, i.e., a selectivity of about 10^9 -fold. This qualitative specificity starts to approach the discrimination we have demonstrated for PSA (detecting femtomolar concentrations against millimolar concentrations of other serum and plasma proteins). We believe that the greater specificity observed has roots in the surface effects that we have described above. Some preliminary experiments where we have immobilized capture antibodies on biosensors and observed the off-rates of the target protein indicate that the off-rates may be much lower than when a protein and antibody interact in solution. The lower off-rates are likely due to rebinding effects on surfaces and possible multivalent interactions.¹² These experiments indicated that the proteins were bound almost irreversibly, but that was difficult to verify as the off-rates were approaching the limits of the biosensor capabilities. The very low off-rates of proteins captured on beads meant that we are able to wash the beads vigorously after sample incubation without any noticeable loss of signal. This vigorous washing presumably washes away many proteins of much higher concentration but much lower affinity. These surface effects could contribute another 10^2 – 10^6 -fold discrimination (estimated from the effective dissociation constant of $<10^{-11}$ M for the protein-capture complex) and make the overall specificity of the assay $>10^{11}$ -fold. While some of these arguments are speculative, similar effects have been discussed in the literature previously.¹² Detailed studies on off-rates measured using biosensors on a variety of protein targets are needed to fully understand how the specificity of ELISA is increased by effects related to surface avidity.

Despite the lack of quantitative models for specificity, the high specificity achieved by digital ELISA (detection of 5×10^{-17} M of a target protein in 10^{-4} M of non-target proteins in plasma), suggests that the qualitative arguments are useful. We have not observed significant background signals from matrix proteins;⁶ all of the background signals seem to arise from the interaction of the labeling reagents with the capture antibodies, a phenomenon that the high sensitivity of SiMoA is ideally suited to reducing.

Conclusions

As for other immunoassays, the performance of digital ELISA is dependent on the analytical sensitivity of the technique and the specificity of the reagents used. Using a relatively simple set of equations based on bimolecular interactions for each step of the assay, it is possible to predict the analytical sensitivity to target proteins from knowledge of concentrations, time, and the affinity of the key interactions. Digital ELISA is a method that literally counts the number of molecules during detection, so provides an excellent way to compare the number of molecules predicted by theory and those detected in experiments. We have shown a remarkable concordance between these simple equations and experimental data for one protein, indicating that, as well as being of great potential in medical diagnosis, digital ELISA could be a useful tool for testing theoretical aspects of immunoassays.

Mathematical modeling of specificity is more challenging, given the large number of potential interactions that can give rise to background signals that are not related to the concentration of the target protein. In our experience with digital ELISA, however, those background signals are dominated by the interaction of the labeling reagents with the capture antibody immobilized on beads. By designing digital ELISA to have a

thermodynamic sink of capture antibodies that capture a large fraction of the target proteins, and SiMoA having extremely high sensitivity to enzyme label, it is possible to under-label those proteins by reducing the concentration of enzyme conjugate in particular, thereby reducing backgrounds and increasing specificity. This general approach of maximizing the capture of proteins and single molecule sensitivity seems an attractive theoretical approach to designing immunoassays.

By exploring different dissociation constants, on-, and off-rates of antibodies using these equations, it appears that digital ELISA is, in principle, effective over a broad affinity that are encountered with high quality ELISA reagents ($K_D \sim 10^{-11}$ – 10^{-9} M). It does appear, however, that the generation of signal is mostly limited by on-rate of the antibodies, so that antibodies of nanomolar affinity with high on-rates would appear to be good choices for this technology. The greatest challenge in implementing this technology from a kinetics perspective is dissociation of detection antibody that can lead to reduction in signals as the time from removal of detection antibody solution to single molecule imaging increases. We have implemented two methods to deal with this dissociation issues: use of automation to ensure that the timing of incubation and imaging are the same for every sample in an experiment; and, chemically “freezing” the immunocomplex on the beads so that they do not dissociate.

As with other immunoassay technologies, the kinetic parameters of the interactions of the antibodies with the target protein play a prominent role in the performance of the assay. Given the widespread availability of biosensor technologies that can make these kind of measurements, it is surprising how little use of these kinetic data are made when evaluating antibody reagents for use in immunoassays. In the future, we hope to perform more in depth studies of the effect of on- and off-rates of antibodies on digital ELISA to extend the testing of the equations presented here beyond PSA.

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Figure 1. Digital ELISA based on the detection of single immunocomplexes in arrays of femtoliter wells. **Step A:** Capturing of single protein molecules on paramagnetic beads coated in capture antibodies; **Step B:** Labeling of capture proteins with a biotinylated detection antibody and then with an enzyme conjugate; and **Step C:** Loading of beads suspended in enzyme substrate into arrays of femtoliter-sized wells for isolation and detection of single molecules.

Figure 2.

(A) Plots of the number of protein molecules captured on antibody-presenting beads (No. of AbL) against concentration of protein in solution calculated from eq. 4, assuming 500,000 beads each presenting 274,000 antibodies in 100 μ L of sample, and K_D values of 10^{-11} M (crosses), 10^{-10} M (open circles), 10^{-9} M (filled triangles), 10^{-8} M (filled squares). The right axis shows the average number of protein molecules captured per bead. The inset table indicates the calculated capture efficiency ($= [\text{AbL}]/[\text{L}_{\text{total}}]$) in the range of $[\text{L}_{\text{total}}]$ shown as a function of K_D . (B) Plots of the average number of protein molecules captured per bead against concentration of protein in solution calculated from eq. 4, assuming $K_D = 10^{-9}$ M and 1,000,000 beads (closed circles), 500,000 beads (filled squares), 100,000 beads (filled triangles), 50,000 beads (crosses), and 10,000 beads (open diamonds) in 100 μ L of sample each presenting 274,000 antibodies. The inset table indicates the calculated capture efficiency in the range of $[\text{L}_{\text{total}}]$ shown as a function of the number of beads.

Figure 3. Plots of the concentration of captured protein ($[AbL]$) as a function of time determined from eq. 5 assuming an antibody concentration of 2.3 nM and a protein concentration of 1 fM. The top horizontal of the chart ($[AbL_{total}] = 1 \times 10^{-15} \text{ M}$) equates to 100% capture of protein.

Figure 4.

Plots of the fraction of captured protein complexes labeled by a detection antibodies against the concentration of captured protein ([AbL]) determined from eq. 4 as: (A) the concentration of detection antibody is varied from 1 pM to 100 nM, with the K_D of the interaction of the detection antibody and captured protein fixed at 1 nM; and (B) the K_D of the interaction of the detection antibody and captured protein was varied from 10 nM to 1 pM, with the concentration of detection antibody fixed at 1 nM.

Figure 5.

Plot of the fraction of captured protein-detection antibody complexes (AbL-detAb) labeled by the enzyme conjugate (SβG) against the concentration of captured protein-detection antibody complexes ([AbL-detAb]) determined from eq. 4, at concentrations of SβG ranging from 1 pM to 150 pM. The calculations assume $K_D = 10^{-15}$ M for the interaction of the biotinylated detection antibody and SβG, and that each detection antibody presents one biotin group that is able to bind SβG.

Figure 6.

Plots of the concentration of captured protein labeled with a detection antibody ([AbL-DetAb]) as a function of time determined from an equation analogous to eq. 5 assuming a capture antibody concentration of 2.3 nM, a protein concentration of 1 fM, and detection antibody concentration of 1 nM. $[AbL-DetAb] = 0.996 \times 10^{-15}$ M equates to the concentration of labeled protein if the efficiency was 100%.

Figure 7.

Plots of the concentration of captured protein labeled with a detection antibody ($[AbL-DetAb-S\beta G]$) as a function of time determined from an equation analogous to eq. 5 assuming an antibody concentration of 2.3 nM, a protein concentration of 1 fM, detection antibody concentration of 1 nM, and $S\beta G$ at four concentrations. The red dotted line indicated the concentrations of $AbL-DetAb-S\beta G$ at which each $AbL-DetAb$ conjugate is labeled with an enzyme ($[AbL-DetAb-S\beta G] = 0.498 \times 10^{-15}$ M under these conditions).

Figure 8.

Plots of AEB against time for digital ELISA for solutions containing 1 pg/mL PSA: (A) where the arrays were imaged at different times after removal of detection antibody solution. The experimental data (closed circles) were fitted to an exponential dissociation curve (black line). The fit indicated a dissociation rate of $\sim 10^{-4} \text{ s}^{-1}$; and (B) As (A), but where the beads were loaded into the arrays in solutions that also contained 10% sucrose and were dried. This process eliminated the characteristic decay caused by dissociation.

Figure 9.

Plots of AEB against concentration of PSA from experimental data (closed symbols) and theoretical calculations based on eq. 5 (open symbols) for: A) An assay with capture, detection and enzyme incubation times of 120, 60, and 30 min, respectively, and standard labeling reagent concentrations; these data were previously published in ref. 7; B) Assay with short incubation times and standard labeling reagent concentrations; and C) Assay with short incubation times and labeling reagent concentrations five-fold higher than standard. The theoretical values are taken from Table 1 that explains the details of the calculations.

Table 1

Calculated efficiencies of capture and labeling steps in digital ELISA, and comparison of corresponding predicted AEB values to experimental data. The parameters entered into eqs. 4 (equilibrium conditions) and 5 (all others) are those used in PSA digital ELISA: volume = 100 μ L; number of beads = 500,000; number of antibodies per bead = 274,000; $k_{on} = 2.67 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 3.13 \times 10^{-6} \text{ s}^{-1}$, and $K_D = 12 \text{ pM}$ for capture antibody; $k_{on} = 3.92 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 8.57 \times 10^{-4} \text{ s}^{-1}$, and $K_D = 2.2 \text{ nM}$ for detection antibody. The concentration of detection antibody and enzyme conjugate are indicated for each condition. The incubation times are indicated by X-Y-Z min, where X is the capture time, Y is the detection label time, and Z is the enzyme label time, respectively.

Condition	[PSA] (pg/mL)	[PSA] (fM)	Capture Efficiency	Detection label efficiency	Enzyme label efficiency	Overall Efficiency	Number of molecules labeled	Predicted AEB	Experimental AEB-AEB _{[PSA] = 0}
Equilibrium	0.1	3.33	99.5%	23.4%	100%	23.24%	46,644	0.0933	na
[DetAb] = 0.67 nM	1	3.33	99.5%	23.4%	100%	23.24%	466,440	0.9329	na
[SbG] = 15 pM	10	333	99.5%	23.4%	100%	23.24%	4,664,402	9.329	na
120-60-30 min	0.1	3.33	98.3%	22.9%	12.9%	2.91%	5,849	0.0117	0.0146 ^a
[DetAb] = 0.67 nM	1	33.3	98.3%	22.9%	12.9%	2.91%	58,489	0.1169	0.1444 ^a
[SbG] = 15 pM	10	333	98.3%	22.9%	12.9%	2.91%	584,669	1.169	1.557 ^a
120-60-30 min	0.1	3.33	98.3%	31.0%	30.9%	9.42%	18,900	0.0378	0.0338 ^b
[DetAb] = 1 nM	1	33.3	98.3%	31.0%	30.9%	9.42%	188,994	0.3780	0.2719 ^b
[SbG] = 40 pM	10	333	98.3%	31.0%	30.9%	9.42%	1,889,745	3.779	2.432 ^b
120-60-30 min	0.1	3.33	98.3%	31.0%	30.9%	9.42%	18,900	0.0378	0.0473 ^b
[DetAb] = 1 nM	1	33.3	98.3%	31.0%	30.9%	9.42%	188,994	0.3780	0.2949 ^b
[SbG] = 40 pM	10	333	98.3%	31.0%	30.9%	9.42%	1,889,745	3.779	3.340 ^b
10-10-10 min	0.1	3.33	30.5%	16.6%	4.5%	0.23%	457	0.0009	0.0036
[DetAb] = 1 nM	1	33.3	30.5%	16.6%	4.5%	0.23%	4,562	0.0091	0.0186
[SbG] = 15 pM	10	333	30.5%	16.6%	4.5%	0.23%	45,720	0.0914	0.1858
10-10-10 min	0.1	3.33	30.5%	56.8%	20.6%	3.57%	7,163	0.0143	0.0042
[DetAb] = 5 nM	1	33.3	30.5%	56.8%	20.6%	3.57%	71,634	0.1433	0.1021
[SbG] = 75 pM	10	333	30.5%	56.8%	20.6%	3.57%	716,266	1.433	1.172

Condition	[PSA] (pg/mL)	[PSA] (fM)	Capture Efficiency	Detection label efficiency	Enzyme label efficiency	Overall Efficiency	Number of molecules labeled	Predicted AEB	Experimental AEB-AEB _{[PSA] = 0}
10-10-10 min	0.1	33.3	30.5%	77.4%	37.0%	8.73%	17,526	0.0351	0.0111
[DetAb] = 10 nM	1	33.3	30.5%	77.4%	37.0%	8.73%	175,262	0.3505	0.1571
[SbG] = 150 pM	10	333	30.5%	77.4%	37.0%	8.73%	1,752,433	3.505	1.691
10-10-10 min	0.1	3.33	30.5%	16.6%	11.6%	0.59%	1,174	0.0023	0.0015
[DetAb] = 1 nM	1	33.3	30.5%	16.6%	11.6%	0.59%	11,740	0.0235	0.0406
[SbG] = 40 pM	10	333	30.5%	16.6%	11.6%	0.59%	117,390	0.2348	0.4400
10-10-10 min	0.1	3.33	30.5%	56.8%	46.0%	7.96%	15,977	0.0320	0.0192
[DetAb] = 5 nM	1	33.3	30.5%	56.8%	46.0%	7.96%	159,772	0.3195	0.1994
[SbG] = 200 pM	10	333	30.5%	56.8%	46.0%	7.96%	1,597,583	3.195	2.225
10-10-10 min	0.1	3.33	30.5%	56.8%	70.8%	12.3%	24,614	0.0492	0.0132
[DetAb] = 5 nM	1	33.3	30.5%	56.8%	70.8%	12.3%	246,137	0.4923	0.2108
[SbG] = 400 pM	10	333	30.5%	56.8%	70.8%	12.3%	2,461,206	4.922	2.348

^aData taken from ref. 7.

^bData generated for two preparations of capture beads