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Molecule by Molecule Direct and Quantitative Counting of Antibody−Protein Complexes in Solution

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We have used two-color fluorescence coincidence detection to directly count individual protein−antibody complexes of protein G or herpes simplex virus labeled with one or more red- and blue-excited antibodies. This allowed quantitative measurement of the concentration of the protein−antibody complexes over 3 orders of magnitude down to the femtomolar level. Single molecule measurements in diluted serum are also possible. The sample preparation is simple, takes place in solution, and requires no separation. Both the antibody affinity and complex dissociation rate are important in determining the sensitivity of the method. At present, the sensitivity limit of 50 fM is determined by the encounter rate of the labeled analyte with the probe volume. This method can be used to detect and quantitate proteins and to measure the stoichiometry, equilibrium constant, and dissociation rate of protein−protein complexes at low concentrations.

The development of a general method to sensitively detect and quantify protein analytes and to measure protein−protein interactions is important in clinical diagnosis and the field of proteomics, since an equivalent of the polymerase chain reaction, widely used for ultrasensitive DNA detection, does not exist for proteins. Current methods are largely based on protein capture onto a surface, using immobilized antibodies and then detection with a second antibody coupled to an enzyme, which generates an amplified signal by catalysis of a chemical reaction that produces a detectable color. Other more recent methods have used antibodies[1,2] or antibody-coated beads[3] that are tagged with DNA, which can be amplified by PCR to increase sensitivity. In this paper, we have taken the principle of simultaneous detection of the fluorescence of two independently excited fluorophores, recently applied by us and others for the fluorescence detection of nucleic acids,[4−9] and used this for ultrasensitive detection of proteins and virus particles. Our approach is general and is based on the direct counting of individual protein analytes tagged with labeled antibodies in solution, molecule by molecule, without the need for any separation or amplification steps. Since the number of protein analytes is counted directly, the measurement is quantitative with a large dynamic range. The principle of the method is to label the protein target with red-excited and blue-excited antibodies by simply adding the labeled antibodies to the solution to be analyzed, as shown in Figure 1A. The antibodies can bind to the same or different antigens on the target. The only requirements are that two or more antibodies bind to one target molecule and that the dissociation rate of the antibodies is slow compared to the duration of the experiment. Coincidence bursts of fluorescence are detected only in cases in which a target molecule labeled with both a red- and blue-excited antibody diffuses into the probe volume. Target molecule labeled with all red- or all blue-excited antibodies will not give coincident events, but only single-color events (red or blue). At the low concentrations used, below 50 pM, there is a low probability of a red- and a blue-excited antibody entering the probe volume at the same time. This constitutes a statistical background of coincident events below which it is not possible to detect the target. The background from impurities is also significantly reduced by 2 orders of magnitude relative to a single-color experiment, since it is very unlikely that an impurity will fluoresce in both channels when red-excited and blue-excited simultaneously due to the wide spectral separation.[4] This, as we shall demonstrate, enables the measurement to be performed in the presence of serum.

EXPERIMENTAL SECTION

Chemicals and Reagents. Sodium hydrogen carbonate ($NaHCO_3$), hydroxylamine, protein G, bovine serum albumin (BSA), rabbit immunoglobulin G (IgG, 95%), and human serum (minus IgA/IgM/IgG) were all purchased from Sigma-Aldrich Company (Dorset, U.K.). Alexa Fluor 488 *N*-hydroxylsuccinimide ester and Alexa Fluor 647 *N*-hydroxylsuccinimide ester were

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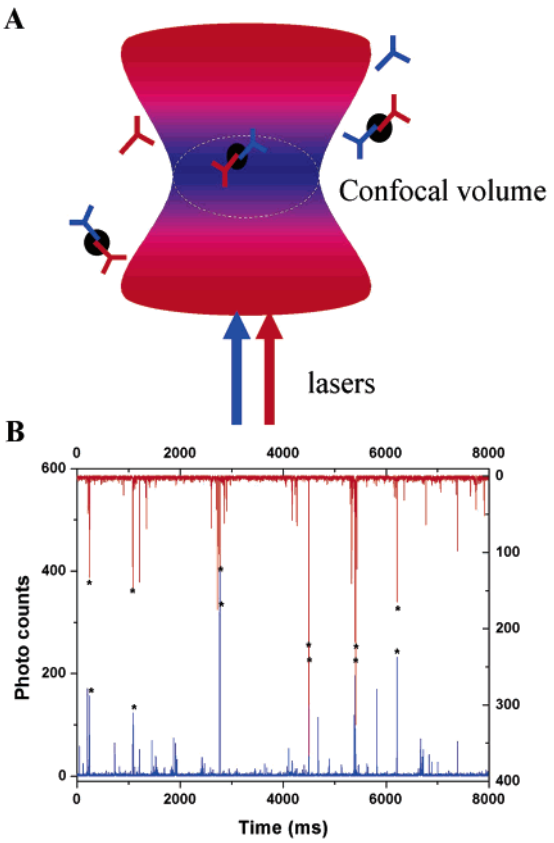

Figure 1. Two-color single-molecule coincidence detection. The principle of the method. Coincidence events are detected when a protein molecule labeled with a red-excited and a blue-excited antibody enters the probe volume. There are also coincident events when unbound red-excited and blue-excited antibodies enter the probe volume at the same time. This constitutes a statistical background below which it is not possible to detect dual-labeled proteins. (B) Fluorescence bursts on both the blue- and red-excited channels for protein G/IgG complexes in PBS buffer formed in solution at initial concentrations of 50 pM protein G and 50 pM IgG labeled with Alexa 647 and 50 pM IgG labeled with Alexa 488. Coincident bursts on both channels are marked with an asterisk. The integration time was 5 ms, and the excitation laser power was 300 μW at 488 nm and 100 μW at 633 nm.

purchased from Molecular Probes Europe BV (Leiden, The Netherlands). PBS (10 mM phosphate, 150 mM NaCl, 2 mM NaN_3, pH 7.2), $NaHCO_3$ (0.1 M, pH ~ 8.3), and hydroxylamine−HCl (1.5 M, pH 8.5) buffers were all prepared using ultrapure MilliQ water (resistivity > 18 MΩ cm).

Preparation of Alexa 488- and Alexa 647-Labeled Rabbit IgG. The protocol provided by Molecular Probes, in Amine-Reactive Probes, was used to label the rabbit IgG. Briefly, rabbit IgG was dissolved in the 0.1 M $NaHCO_3$ buffer (pH 8.3) to a concentration of 5 mg/mL, then 1 mL of the freshly prepared IgG solution was added to a vial of 1 mg of Alexa Fluor 488 *N*-hydroxylsuccinimide ester. After the dye was thoroughly dissolved and mixed with the protein, the solution was stirred for 1 h at room temperature. The labeling reaction was terminated by addition of 100 μL of the 1.5 M hydroxylamine−HCl (pH 8.5). The resulting mixture was then loaded onto a Bio-Rad BioGel P-30 column with PBS buffer as the eluting buffer. The first yellowish fluorescent fraction, labeled rabbit IgG, was collected. The degree of labeling, determined by measuring the UV absorbance at 280 and 494 nm and using the equations provided by Molecular Probes, was seven fluorophores per IgG molecule. Alexa 647-labeled rabbit IgG was prepared using the same procedure, except the IgG solution was added to a vial of Alexa Fluor 647 *N*-hydroxylsuccinimide ester. The average labeling, detected by measuring the UV absorbance at 280 and 650 nm, was eight fluorophores per IgG molecule.

Production of Purified HSV Virions and LP2 IgG. A gH-negative mutant of herpes simplex virus type 1 (HSV-1) which lacks the gene encoding glycoprotein H, named HFEMdelUL22Z,[10] was propagated in a helper cell line, CR1, which supplies gH in trans.[11] Tissue culture medium from infected cells was clarified by centrifugation at 2000×*g* for 10 min, and virus particles were then pelleted from the supernatant by centrifugation at 18 000 rpm for 2 h in a Beckman type 19 rotor at 4 °C. The pellets were resuspended in a small volume of PBS and sonicated before being layered on 30 mL of 15−30% Ficoll gradients in PBS. The gradients were centrifuged at 12 500 rpm for 90 min in a Beckman SW28 rotor at 4 °C, and the visible band at the center of the gradient was harvested, diluted with PBS and pelleted by centrifugation at 21 000 rpm in an SW28 rotor. The final pellet was resuspended in PBS, and aliquots were stored at −70 °C. Virus particle numbers were estimated by comparison with latex particles of known concentration using negatively stained preparations as described by Watson et al.[12] LP2 is a mouse monoclonal antibody that recognizes the HSV envelope glycoprotein gD.[13] LP2 was purified from the tissue culture supernatant from hybridoma cells producing this antibody by immunoaffinity chromatography on a protein A sepharose column and eluting the LP2 with 0.1 M glycine, pH 3. LP2 concentrations were determined by measuring the optical density at 280 nm. This antibody was labeled with Alexa 647 or Alexa 488 by the same labeling procedure as above. The average labeling numbers are 14 and 12 for Alexa 647 and 488 dyes, respectively.

Single-Molecule Experimental. The apparatus used to achieve two-color single-molecule fluorescence coincidence detection has been described in a recent publication.[5] Briefly, two overlapping laser beams (488 nm, Argon ion, model 35LAP321-230, Melles Griot, and 633-nm model 25LHP151 He−Ne laser, Melles Griot) were directed through a dichroic mirror and oil immersion objective (Apochromat 60×, NA 1.40, Nikon) to be focused 5 μm into a 1-mL sample solution supported in a Lab-TeK chambered coverglass (Scientific Laboratory Suppliers Ltd, U.K.).

Fluorescence was collected by the same objective and imaged onto a 50-μm pinhole (Melles Griot) to reject out of focus fluorescence and other background. Green and red fluorescence were then separated using a second dichroic mirror (585DRLP, Omega Optical Filters). Green fluorescence was filtered by long-pass and band-pass filters (510ALP and 535AF45, Omega Optical Filters) before being focused onto an avalanche photodiode, APD (SPCM AQ-161, EG&G, Canada). Red fluorescence was also filtered by long-pass and band-pass filters (565ALP and 695AF55, Omega Optical Filters) before being focused onto a second APD

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(SPCM AQR-141, EG&G, Canada). Dark count rates for the two APDs were found to be below 100 counts/s. Outputs from the APDs were coupled to two PC implemented multichannel scalar cards (MCS-Plus, EG&G, Canada), and the synchronous start output of one MCS card was used to trigger the second to eliminate any time delay between the two cards. This was confirmed by use of a fast oscilloscope.

For single-molecule coincidence experiments, a 10 nM stock solution of the protein G−IgG complex in PBS was made by adding 30 nM protein G to 30 nM Alexa 488-labeled IgG and 30 nM Alexa 647-labeled IgG with the same volumes. The stock solution of complex was diluted to 50 pM in PBS and left for 30 min before experiments. Coverslips modified with BSA were used in all experiments to minimize surface absorption, and all experiments were carried out at room temperature. The excitation laser powers were adjusted to 100 μW for the red laser and 300 μW for the blue laser to give comparable counts on both channels. We collected data for 180 min for the fluorescence coincidence experiment with a 5-ms bin time on both MCS cards. The average brightness of a fluorescence burst was 470 counts with an average background of 10 counts. A threshold of 50 counts was used on each channel to count coincident events. For experiments in which the protein G concentration was varied, we took a stock solution of 10 nM IgG and mixed it with stock solution of protein G at the desired ratio of protein G to IgG. This was then left for 15 min and then diluted to the appropriate final concentration in PBS. For experiments in serum, we made a stock solution of the protein G−IgG complex at a concentration of 10 nM in human serum (from Sigma), which was diluted to the appropriate concentration in PBS for measurements. The background, due to two differently labeled antibodies in the probe volume at the same time, was determined experimentally by following the same protocol as described above but using only PBS instead of protein G.

For the single-virus coincidence experiments, HSV-1 particles were diluted to 5 pM in PBS, then 25 pM Alexa 488-labeled LP2 and 25 pM Alexa 647-labeled LP2 were added. This sample was further diluted for the low concentration coincidence counting experiment. All experiments were carried out at room temperature. The excitation laser powers were the same as in the protein G experiment. We collected data for 180 min for the fluorescence coincidence experiment with a 10 ms bin time on both MCS cards. The average brightness of a fluorescent burst was 850 counts, and the average background was 18 counts in these experiments. A threshold of 100 on each channel was used to count coincident events.

RESULTS AND DISCUSSION

We studied the well-characterized interaction between protein G (MW 20 kDa) and IgG. The IgG was either labeled with Alexa 488 (blue-excited) or Alexa 647 (red-excited). Protein G has up to three available binding sites for IgG with a subnanomolar dissociation constant.[14] Typical data obtained with both labeled antibodies and protein G at 50 pM concentration are shown in Figure 1B, where coincident events are marked with asterisks. There are, on average, eight flurophores per antibody. The heterogeneity in the number of fluorophores per antibody contributes to the variation in fluorescence intensity for individual antibodies, with both red and blue excitation. This also results in a broad ratio distribution for the protein G−IgG complex, peaking at a ratio of 0.5, as expected for a complex with one red and blue antibody, as shown in Figure 2.

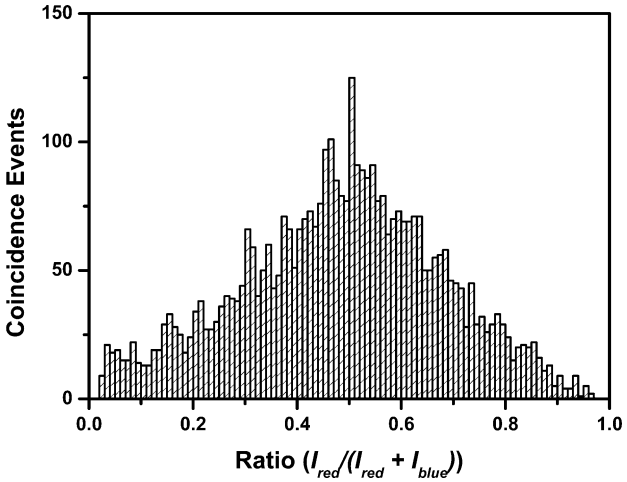

Figure 2. Ratio histogram for the protein G−IgG complex.

However, for coincident counting, we are only interested in fluorescence events above a certain threshold, so this variation does not affect the measurement. Not all of the events are coincident for two reasons. First, single labeled protein G and free antibodies are also present. Second, the overlap between the red- and blue-excited probe volumes is imperfect. The overlap was measured previously to be 30%, so some molecules diffuse through a region that is excited just by the red or blue laser only.[4] Note the good signal-to-noise ratio, typically 50:1, on both channels for individual protein−antibody complexes shown in Figure 1B.

We made measurements to determine the mean stoichiometry of the IgG−protein G complex and its dissociation rate. A comparison of the average fluorescence counts of the complex, under both red and blue excitation, with the labeled antibodies only gave a ratio of 1.1 ± 0.2 for the red- and blue-excited antibody per complex. Thus, under our experimental conditions, there are two IgG antibodies bound to a single protein G. The third site may not be accessible or may have a lower affinity. The stability of the complex once diluted for single-molecule measurements is also important. We therefore evaluated the dissociation rate of the protein G−IgG complex by measuring how the number of coincident events changed during the experiment (Figure 3A). We found that the dissociation rate for one antibody from the complex was 3120 ± 800 s^{-1}. Thus, during a typical 3-h measurement, the complex has dissociated to approach its equilibrium value. By using the equilibrium number of coincident events and also the number of red only and blue only events, we could estimate the equilibrium constant for IgG_2/protein G complex $\rightarrow$ 2IgG + protein G, since we know the red, blue, and overlapped excited volumes and the coincidence detection efficiency (20%),[4] and hence, we can calculate the concentration of all species. This gave an equilibrium dissociation constant value of 2300 pM[2]. We assumed that both antibody binding events are independent with the same dissociation constant (K_d), as has been observed in radiochemical labeling experiments.[14] We then obtained a K_d value of $\sim$50 pM, for a bound antibody dissociating from protein G, which is in good agreement with the literature.[14] This shows that

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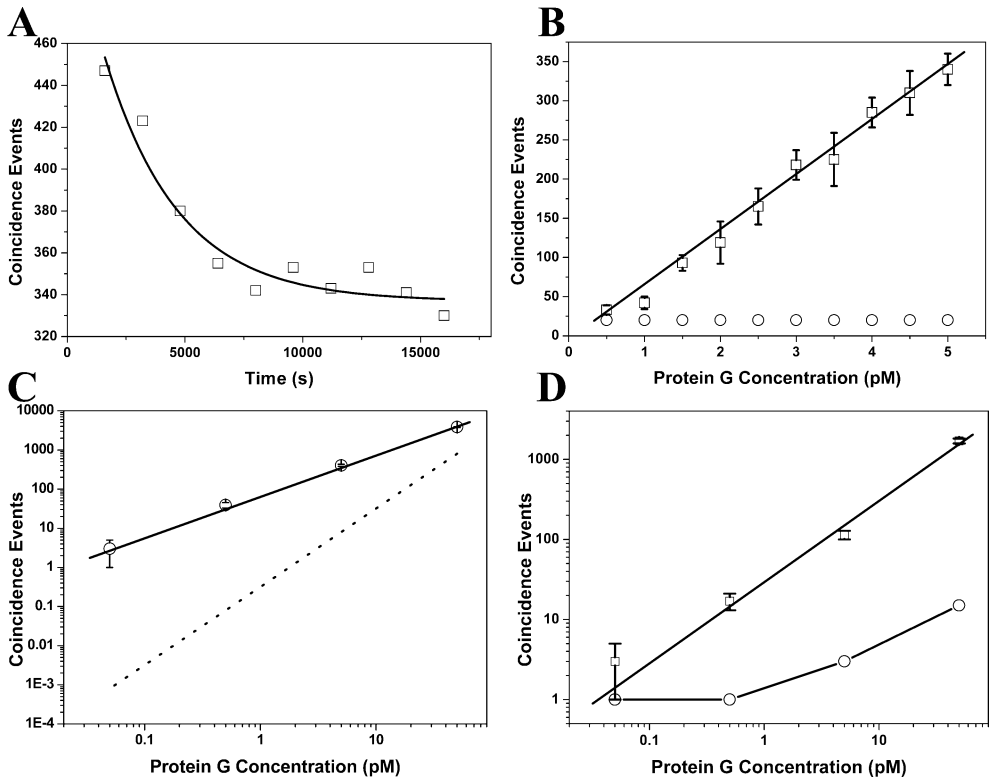

Figure 3. Detection of protein G. (A) Off-rate measurement on the protein G−IgG complex performed at an initial protein G−IgG complex concentration of ~50 pM. The off rate was estimated to be 3120 ± 800 s^{-1} by fitting the data to a single-exponential decay. (B) Dependence of the number of coincident events with the solution concentration of protein G (0.5−5 pM) in a 5 pM solution of IgG labeled with Alexa 647 and 5 pM IgG labeled with Alexa 488. The data were fitted by a linear function. The experimental background with the antibodies only is also shown (open circles). (C) Dependence of the number of coincident events on the solution concentration of protein G. The total IgG concentration, with equal amounts of Alexa 488- and Alexa 647-labeled IgG, was twice the protein G concentration. The data were fitted by a linear function. The background with antibody only is shown as a dotted line. This is based on the experimental background measurement at 50 pM and then extrapolation to lower concentrations (see text for details). (D) Dependence of the number of coincident events on the solution concentration of protein G from samples measured in human serum (diluted 100-fold with PBS buffer). The total IgG concentration, with equal amounts of Alexa 488- and Alexa 647-labeled IgG, was the same as the protein G. The data were fitted by a linear function. The experimental background with the antibodies only is also shown (open circles).

the fluorophore labeling of the antibodies does not interfere with the antibody binding.

There are two limiting cases for the number of coincident events expected as a function of protein G concentration. We mix the stock solutions well above the binding constant for the complex and always have at least one red- and blue-excited antibody per protein G in the solution. In this case, all the protein G will have two antibodies bound. We will not detect red−red or blue−blue complexes, but only half of the complexes labeled red−blue or blue−red. If the complex dissociates slowly when diluted for measurement, then the number of coincident events detected is proportional to the number of protein G molecules in solution. The other limit is when equilibrium is reached rapidly, and then the number of coincident events will depend on the equilibrium constant for complex formation and also the protein G and IgG concentrations. We measured the number of coincident events as a function of protein G concentration in the presence of 10 pM total IgG. There is a clear linear dependence of number of coincident events with protein G concentration (Figure 3B). At 1 pM or lower protein G concentration, it is not possible to detect coincident events above the statistical background due to red- and blue-excited IgG's being randomly present in the probe volume at the same time.

To obtain higher sensitivity, it is necessary to decrease the IgG concentration to reduce the statistical background. The results of doing this are shown in Figure 3C. In this case, the total concentration of IgG used was twice the protein G concentration. The number of coincident events is directly proportional to protein G concentration over 3 orders of magnitude. This indicates that for these low concentration experiments, the number of protein G molecules detected is dominated by the number of complexes initially formed in the stock solution. The equilibrium number of complexes would be much lower. The background was measured experimentally at 50 pM. The value obtained was higher than expected if the background was statistical and due to the Poisson probability of red- and blue-labeled antibodies' being present in the probe volume at the same time. This suggests some weak interaction between antibodies to form a dimer. We assumed that the amount of dimer present is proportional to the square of the antibody concentration, as expected for equilibrium between monomers and dimers. We then extrapolated from the measured background level at 50 pM to lower concentrations, since it is not possible to make the measurement directly. The number of coincident events counted is significantly higher than the background at all concentrations so that the sensitivity is only limited by the encounter rate of the protein G−IgG complex with the

probe volume. In this case, relying only on diffusion, the limit is ~50 fM. Thus, while we have the sensitivity to detect a single protein G−IgG complex, the lowest concentration that can be measured is limited by the rate individual molecules diffuse into the probe volume.

We then repeated the experiment using samples made in human serum (Figure 3D). This is a more complex solution in which there is the possibility of other proteins interfering with the antibody binding. The samples were diluted 100-fold into PBS buffer, and the number of coincident events was counted. Again, the number of coincident events was linearly proportional to the protein G concentration over 3 orders of magnitude, indicating no interference from other proteins in the human serum. However, in this case, the background coincident events were significantly higher because of fluorescent impurities in the serum, so we used a threshold of 100 for data analysis. The limit of detection sensitivity was ~50 fM.

To show the generality of the method and demonstrate the potential for virus detection, we then performed experiments on herpes simplex virus type 1. The virus particle is ~120 nm in diameter, comprising an icosahedral nucleocapsid surrounded by a lipid bilayer embedded with multiple virus-specific membrane glycoproteins, including glycoprotein D (gD).[15] We used fluorophore-labeled antibodies to gD for these experiments. We found in this case that the binding was irreversible over the 3-h measurement time. Experiments with the labeled antibodies only gave no detected coincidence events and, hence, a zero statistical background. This is because multiple antibodies bind to the virus, so a higher fluorescence threshold was used for coincidence detection, rejecting two differently labeled antibodies in the probe volume at the same time. On the basis of the mean fluorescence intensity for a labeled virus compared to an antibody alone, there were 10 antibodies in total per virus under the conditions of the experiments. This is in agreement with the ratio of antibody to virus used in these experiments (10:1). The observation of a very slow antibody off-rate ensures that virus-bound antibodies do not dissociate during the experiment. The number of coincident events was linearly proportional to the number of viruses over 3 orders of magnitude, down to a concentration of 50 fM, where the encounter rate with the probe volume was again limiting (Figure 4).

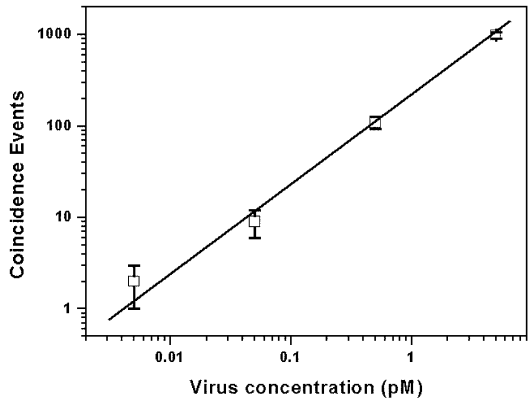

Figure 4. Detection of herpes simplex virus. Dependence of the number of coincident events on the concentration of HSV-1. The total concentration of the LP2 antibody, with equal amounts of Alexa 488- and Alexa 647-labeled antibody, was 10 times the HSV-1 concentration. The data were fitted by a linear function.

These experiments have shown that direct counting of individual target protein molecules or viruses gives a linear dependence of signal with the analyte concentration over 3 orders of magnitude, allowing quantitative measurements. The detection limit for both protein G and HSV in PBS buffer is not due to signal-to-noise or background coincidence, but rather, the rate at which the analyte molecules encounter the probe volume (and thus, the acquisition time for an experiment). The fact that we can quantitatively detect both viruses of 120 nm in diameter and protein G molecules of diameter 3.5 nm using the same apparatus with a similar detection limit supports that the method is general for the detection of most biological particles and complexes. The only requirement is for antibodies labeled with two different fluorophores; therefore, it should be straightforward to modify existing methods. Specificity could also be improved by the use of two different antibodies that bind to different antigens on the target protein, as has been employed in other methods.[16] However, the experiments with protein G show that both the affinity of the antibody for the target and the complex dissociation rate will be important parameters in real applications of this method. For high sensitivity, it is important that the antibody labeling of the analyte is done above the dissociation constant. The antibody concentration determines the sensitivity limit due to background events, so the antibody affinity needs to be as high as possible to reduce this background. It is also important that the complex does not dissociate or dissociates slowly during the time scale of the experiment. Thus, for both these reasons, high-affinity antibodies will be required, although temperature or measurement conditions could also be changed to minimize dissociation. In addition, for an actual quantification assay, it would be necessary to make measurements at different fixed antibody concentrations to reduce the background events to a level at which the concentration of the sample could be determined. Potentially of greater value may be the use of this method to directly determine the stoichiometry of complexes, equilibrium constants, and dissociation rates without the use of radioactive labeling. We have demonstrated previously that single-molecule coincidence detection can be used to quantitatively measure the interactions between RNA molecules.[5] The adaptation of this approach to proteins will allow their detection and identification and also the measurement of protein−protein, protein−DNA, and protein−RNA interactions. The method should be particularly useful for studying the proteome, the interactions of low-abundance proteins and proteins that are expressed at low levels.

There are a number of possible strategies that could be employed to further improve this detection limit, if required. Flow or use of a laser scanner[6] could be employed to increase the encounter rate of the analyte with the probe volume. Since the residence time of the analyte in the probe volume will be reduced, so will the fluorescence signal. This can be partially compensated by increasing the laser power, although this is limited as a result of saturation effects. Alternatively, secondary antibodies could be used to amplify the signal. Another approach would be to

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concentrate the analyte prior to analysis by affinity purification using, for example, magnetic beads. Recently, single-molecule measurements have been demonstrated in microfluidic devices.[17] It therefore may be possible to use a microfluidic device for sample preparation and flow in combination with single-molecule coincidence detection. Importantly, we have demonstrated that single-molecule detection is possible from samples in diluted serum, opening up the possibility of direct detection in clinical samples. For experiments in diluted serum, at 50 fM, the level of coincident background is limiting the sensitivity. This could be lowered, if required, by the use of a larger spectral separation between the two fluorophores, excitation using red sources, or increasing the signal for individual analytes.

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In summary, we have demonstrated that direct counting of coincident fluorescence events allows quantitative measurement of protein analytes at the femtomolar level. This appears to be a general method to detect and quantify proteins and to characterize macromolecular complexes.

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