Detection and identification of proteins are typically achieved by analyzing protein size, charge, mobility and binding to antibodies, which are critical for biomedical research and disease diagnosis and treatment. Despite the importance, measuring these quantities with one technology and at the single-molecule level has not been possible. Here we tether a protein to a surface with a flexible polymer, drive it into oscillation with an electric field, and image the oscillation with a near field optical imaging method, from which we determine the size, charge, and mobility of the protein. We also measure antibody binding and conformation changes in the protein. The work demonstrates a capability for comprehensive protein analysis and precision protein biomarker detection at the single molecule level.
Proteins play a central role in biochemical processes in living systems1–3. They also serve as drugs, drug targets, and disease biomarkers4,5. Detecting and identifying proteins are thus the most elementary tasks in biomedical research, and in disease diagnosis and therapeutics6–8. Various technologies have been developed for protein analysis, and the most important ones include electrophoresis, mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA) and western blot (WB)9–13. These technologies are often used in combination to separate and identify proteins based on charge, size (mass), and specific binding to antibodies. Although ubiquitous in both research labs and industry, they are destructive, involving protein fragmentation and denaturation10,13. They also lack single-molecule analysis capability required for studying heterogenous processes and for improving sensitivity in precision diagnosis14,15.

Several technologies have been demonstrated to detect single molecules16–20, including two label-free optical imaging methods. One is an indirect method, which takes advantage of the spectral feature of the molecules22–24. The other one images interference from scattered light (iSCAT), from which the protein size is quantified20,21. Single-molecule electrophoresis20 and anti-Brownian electorokinetic (ABEL) trap30 have been developed to measure the charge or mobility of single molecules trapped by an electric field. However, simultaneous imaging of size, charge, mobility, and binding of proteins on a single platform has not been possible. Different proteins may have similar sizes, simultaneous detection of multiple intrinsic properties of a protein is thus a key requirement for protein identification and function analysis25,31,32. This is an important reason that electrophoresis, MS and WB are the workhorses in biomedical research.

Here we report a method to image single proteins, measure the size and charge of each protein simultaneously, determine the mobility and analyze antibody binding of the protein in real time. Proteins in this work are resolved individually on a sensor surface, thus requiring no separation. The method is analogous to electrophoresis, MS and WB in terms of analyzing proteins based on mass (size), charge, mobility, and to ELISA in terms of antibody binding, but it is achieved with one detection platform and at the single-molecule level. We further show that the method allows label-free detection of binding-induced conformation changes in single proteins.

Results

Detection principles. To achieve single-protein imaging capability, we tether proteins to an indium tin oxide (ITO) coated glass slide via polyethylene glycol (PEG), a flexible polymer, and drive the proteins into vertical oscillation by applying an alternating electric field to the ITO surface (Fig. 1a, b). To determine the oscillation, the ITO slide is placed on the objective of a CMOS imager. The light intensity detected by the camera is given by (see “Methods”),

\[ I = 2|u_e + u_r||u_e\cos(\phi), \]

where \(u_e\) and \(u_r\) are the evanescent wave, scattered wave, and reflected wave from ITO, respectively, and \(\phi\) is the phase shift of the scattered wave relative to the combined evanescent and reflected wave. Because the evanescent field is localized near the ITO surface within ~100 nm, the scattered light is extremely sensitive to the protein-surface distance. As the protein oscillates, so does the scattered wave, which is recorded as an image sequence (Fig. 1c). We perform fast Fourier Transform (FFT) on each pixel of the image sequence with 1 s integration time and apply a band pass filter at the frequency of the applied field to extract the oscillation amplitude while rejecting random noise (Supplementary Fig. 2). The FFT image resolves a protein as a circle, centered at the ITO surface and surrounded by a ring-like signal, which is the scattered wave from the protein and planar evanescent wave on the sensor surface. Spatial Fourier Transform (k-domain) of the image further reveals the characteristic two-ring feature (Fig. 1e, and “Methods” for imaging principle)33. The FFT image provides size, charge, and mobility of the protein as we show below.

The protein oscillation amplitude (\(\Delta z_0\)) is determined by the entropic force of the PEG tether and electrical driving force. The entropic force is described by the freely jointed chain (FJC) model, which predicts an entropy force proportional to the PEG displacement for small oscillation amplitude34. Thus, the protein oscillation amplitude is given by (“Methods”)

\[ \Delta z_0 = \frac{E_0(\Delta z_0)}{k_{\text{PEG}}} q, \]  

where \(E_0(\Delta z_0)\) is the amplitude of the field, \(q\) is the charge of the protein, and \(k_{\text{PEG}}\) is the entropic spring constant of the PEG tether. Equation (2) shows that the oscillation amplitude is proportional to the applied electric field, but this is valid only at low fields, where the oscillation amplitude is smaller than the PEG length. When the field is sufficiently large, the oscillation amplitude reaches a plateau as the PEG tether is stretched to its most accessible length (e.g., 80%)31,35. This behavior has been observed for all the proteins studied here, and Fig. 1g shows the result for bovine serum albumin (BSA) as an example.

To quantify \(\Delta z_0\) we consider that the evanescent field decays exponentially from the ITO surface into the solution with a decay length of \(d\). Consequently, the FFT image contrast, \(\Delta C(\Delta z_0, D_H)\), is given by (Methods),

\[ \Delta C(\Delta z_0, D_H) = \beta(D_H) \left[1 - \exp\left(-\frac{\Delta z_0}{d}\right)\right], \]

where \(D_H\) is the protein diameter and \(\beta\) is the strength of the evanescent wave scattering by a protein, which depends on the protein size. In the high-field plateau regime, the PEG tether is almost fully stretched, such that \(\Delta z_0\) is close to the linear length of PEG (\(L_{\text{PEG}}\)), and the corresponding FFT image contrast, \(\Delta C(\Delta z_0 = L_{\text{PEG}}, D_H)\), is maximum. Knowing \(\Delta C(\Delta z_0 = L_{\text{PEG}}, D_H)\), Eq. (3) allows determination of \(\beta\), from which \(D_H\) is extracted with a calibration curve (see below).

Once \(\beta\) is known, \(\Delta z_0\) at different applied electric fields can be determined from the measured \(\Delta C(\Delta z_0, D_H)\) with Eq. (3). This allows us to extract the charge of the protein (\(q\)) with Eq. (2) together with \(E_0\) and \(k_{\text{PEG}}\) determined with the procedures described in Methods and Supplementary Fig. 1. Finally, we obtain the protein mobility (\(\mu\)) from charge (\(q\)) and size (\(D_H\)) according to \(\mu = q/(3\pi n D_H)\), where \(n\) is the solution viscosity.

We applied the method to proteins with different sizes and charges.

Measuring size, charge, and binding of IgG. The first example is goat immunoglobulin G (IgG), which has a molecular weight of 150 kDa and is negatively charged (pH = 7.4). Figure 2a shows the FFT image of several IgG molecules captured when the ITO surface potential is modulated with amplitude, \(U_0 = 8\) V. The FFT image contrast and the extracted oscillation amplitude (\(\Delta z_0\)) increase with the potential amplitude and reach plateaus around 8 V (Fig. 2b). From the \(\Delta z_0\) vs. \(U_0\) plots we determined the size and charge of each IgG (Fig. 2c). The oscillation is in phase with the applied potential, indicating that the protein moves towards...
the surface (thus more scattering) when the potential increases. This is expected for negatively charged IgG, which is attracted toward the surface when it is positively charged (Supplementary Fig. 2).

We analyzed 186 IgG molecules and obtained diameter, charge and mobility histograms (Fig. 2d). The histograms reveal pronounced peaks at 10.4 nm, $-5.0 \, \text{e}$ (e, the elementary charge, is $1.6 \times 10^{-19} \, \text{C}$) and $-0.86 \times 10^{-8} \, \text{m}^2\text{V}^{-1}\text{s}^{-1}$, respectively. These mean values agree with the values measured by the dynamic light scattering experiment performed in this work (see below) and by small-angle X-ray scattering, nuclear magnetic resonance, and capillary electrophoresis reported in literature (Supplementary Tables 1, 2). The mean charge is also close to the estimated value at the buffer pH (Supplementary Table 3). The diameter histogram displays a small secondary peak located at a larger diameter, which is attributed to the formation of aggregations (Fig. 2d). Small secondary peaks also appear in the diameter and charge histograms of other proteins (e.g., Fig. 3c, Supplementary Fig. 3d, h).

To ensure imaging of single IgG molecules, we studied anti-IgG binding to the IgG tethered on the surface (Fig. 2e). This was performed by first flowing blank PBS over the surface while driving the IgG to oscillate in the plateau regime. After reaching a stable baseline, 130 nM anti-IgG in PBS was introduced to allow binding of anti-IgG to the oscillating IgG on the surface. The FFT image contrast and the apparent diameter of IgG increase (Fig. 2f, Supplementary Fig. 4b, and Supplementary Video 1), indicating binding of anti-IgG to IgG. After measuring anti-IgG binding to IgG, blank PBS was introduced to allow study of unbinding of anti-IgG from IgG. We observed decrease in the FFT image contrast and diameter associated with the unbinding process. To ensure specific binding of anti-IgG to IgG, we performed a control experiment by introducing an antibody (anti-human IgG) that should not bind to the IgG (goat IgG). Indeed, we did not detect any change in the FFT image of the goat IgG (Supplementary Fig. 4c, d).

The standard deviations of the measured IgG diameter and charge determined from the histograms shown in Fig. 2d are 3.4 nm and 1.2 e, respectively. They reflect heterogeneity of the 186 different IgG molecules tethered on the surface, rather than measurement precision (see “Methods” and Supplementary Fig. 13 for measurement error analysis). To examine the precision in size measurements, we repeatedly measured and plotted the diameter of one IgG molecule before and after binding to an anti-IgG from IgG. We observed decrease in the FFT image contrast and the apparent diameter of IgG increase (Fig. 2f, Supplementary Fig. 4b, and Supplementary Video 1), indicating binding of anti-IgG to IgG. After measuring anti-IgG binding to IgG, blank PBS was introduced to allow study of unbinding of anti-IgG from IgG. We observed decrease in the FFT image contrast and diameter associated with the unbinding process. To ensure specific binding of anti-IgG to IgG, we performed a control experiment by introducing an antibody (anti-human IgG) that should not bind to the IgG (goat IgG). Indeed, we did not detect any change in the FFT image of the goat IgG (Supplementary Fig. 4c, d).
IgG, showing well separated bound and unbound states (Fig. 2g). The standard deviations of the IgG and anti-IgG/IgG molecules are 0.21 and 0.33 nm, respectively, which reflect the measurement precision. We attribute the molecular heterogeneity to the different orientations of the tethered proteins, because the NHS group in the PEG can potentially react with any amine group (lysine residue) exposed on the surface of the protein.

To further confirm the imaging of anti-IgG binding to IgG, we performed an end-point assay by incubating IgG tethered on the surface with 33 nM anti-IgG. The diameter histogram obtained after incubation shows two peaks located at 10.3 and 13.2 nm, respectively (Fig. 2h). The former is IgG, and the later corresponds to anti-IgG/IgG complex, which has an estimated mass of ~2× of IgG from the diameter. This is expected for anti-IgG/IgG complex because anti-IgG and IgG have similar masses. The charge histogram also reveals two peaks, located at ~4.8 e and ~7.2 e, which are associated with IgG and IgG/anti-IgG complex, respectively (Fig. 2i). As an additional control, we measured and confirmed the binding and unbinding of anti-IgG with IgG using a surface plasmon resonance setup, a well-established independent detection technology (Supplementary Fig. 5).

Measuring size and charge of BSA and lysozyme. We applied the method to lysozyme (MW = 14 kDa) and observed lower FFT image contrast than IgG, which is expected because of its smaller size than IgG (Supplementary Fig. 3a). The oscillation has a ~180° phase shift relative to the applied potential (Supplementary Fig. 2). This is opposite of IgG but expected because lysozyme is positively charged at pH of 7.4. Similar to IgG, the lysozyme oscillation amplitude increases with the potential modulation amplitude and then approaches a plateau at large potential amplitude (>9 V) (Supplementary Fig. 3b). We determined $D_0$, $q$, and $\mu$ of the individual lysozyme molecules and constructed histograms for these quantities (Supplementary Fig. 3c, d). The mean values of $D_0$, $q$, and $\mu$ are 4.1 nm, 4.3 e and $1.8 \times 10^{-5}$ m$^2$ V$^{-1}$ s$^{-1}$, respectively. The measured $D_0$ and $\mu$ are consistent with the dynamic light scattering data (see below) and the charge agrees with the expected value (Supplementary Table 3).

Another example is BSA (MW = 66 kDa), which is smaller than IgG but larger than lysozyme. As shown in Supplementary Fig. 3e, the BSA image contrast is lower than IgG but greater than lysozyme, which is consistent with the size of the molecule. We plotted BSA oscillation amplitude vs. potential modulation (Fig. 2f).
amplitude and observed similar dependence as IgG and lysosome, namely, a low-field linear regime followed by a high-field plateau regime (Supplementary Fig. 3f). The measured $D_H$, $q$, and $\mu$ are $8.3\,\text{nm}$, $-5.3\,\text{e}$ and $-1.2\times10^{-8}\,\text{m}^2\text{V}^{-1}\text{s}^{-1}$, respectively (Supplementary Fig. 3h). These results agree with the values from the dynamic light scattering (Fig. 4c and Supplementary Fig. 15) and calculated charge (Supplementary Table 3). We summarize the results for IgG, lysozyme and BSA, as well as other proteins and complexes in Supplementary Table 4.

Conformation change of calmodulin. In addition to the size, charge and mobility of single proteins, the present method provides valuable information on understanding the conformation changes in proteins. For most proteins, the conformation change is expected to be accompanied by size and/or charge changes and can thus be determined by the present method. To demonstrate this capability, we studied Ca$^{2+}$ binding to calmodulin (CaM), a protein that mediates various Ca$^{2+}$ signaling processes, such as muscle contraction, inflammation, and fertilization. CaM has two globular domains, each containing two EF-hand motifs, so it can bind up to four Ca$^{2+}$ and causes conformation and charge changes in CaM (Fig. 3a). We tethered CaM to an ITO surface, incubated it in buffers with and without Ca$^{2+}$, and measured the oscillation vs. potential modulation amplitude in each buffer (Fig. 3b). CaM with Ca$^{2+}$ reaches a plateau at a larger potential amplitude than that without Ca$^{2+}$. This is because Ca$^{2+}$ binding reduces the net charge of CaM. We determined $D_H$, $q$, and $\mu$ for CaM and Ca$^{2+}$/CaM complex from the oscillation vs. potential modulation amplitude and observed similar dependence as IgG and lysosome, namely, a low-field linear regime followed by a high-field plateau regime (Supplementary Fig. 3f). The measured $D_H$, $q$, and $\mu$ are $8.3\,\text{nm}$, $-5.3\,\text{e}$ and $-1.2\times10^{-8}\,\text{m}^2\text{V}^{-1}\text{s}^{-1}$, respectively (Supplementary Fig. 3h). These results agree with the values from the dynamic light scattering (Fig. 4c and Supplementary Fig. 15) and calculated charge (Supplementary Table 3). We summarize the results for IgG, lysozyme and BSA, as well as other proteins and complexes in Supplementary Table 4.

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amplitude plot and obtained histograms from hundreds of CaM and Ca\textsuperscript{2+}/CaM complex images (Fig. 3c). The results show that \( D_H \) of CaM increases from 5.3 to 6.0 nm upon binding to Ca\textsuperscript{2+}, which is consistent with literature values\textsuperscript{37} and attributed to Ca\textsuperscript{2+} binding-induced conformation change in CaM. \( q \) and \( \mu \) for CaM are \(-6.5 e \) and \(-2.0 \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} \), respectively, which change to \(-5.1 e \) and \(-1.4 \times 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1} \) after binding to Ca\textsuperscript{2+}. We further verified the size and charge changes by performing dynamic light scattering measurement (Fig. 4c).

To demonstrate the capability of real-time conformation and charge detection, we also monitored Ca\textsuperscript{2+} binding to CaM by first driving CaM into oscillation, and then alternatively flowing 1 mM Ca\textsuperscript{2+} and 1 mM ethylene glycol tetracetic acid (EGTA) solutions over the surface. EGTA is known to cause unbinding of Ca\textsuperscript{2+} from CaM via chelation with Ca\textsuperscript{2+}, so the experiment allowed us to repeatedly monitor the binding and unbinding of Ca\textsuperscript{2+} to a CaM molecule and the associated size and charge changes of CaM (Fig. 3d–g, Supplementary Fig. 6, and Supplementary Video 2). The real-time data are consistent with the end-point measurements carried out by incubating CaM in Ca\textsuperscript{2+} and Ca\textsuperscript{2+} free solutions. We determined the measurement precision by measuring the size and charge of the same single molecule repeatedly. The statistics (Fig. 3e, g) shows that the standard deviations for the size and charge are 0.3 nm and 0.5 e, respectively. The fundamental limit of this technology is shot noise (Methods). As such, a better a signal-to-noise ratio (SNR) is expected by increasing the incident light intensity. This will allow us to detect smaller molecules and improve the precision by minimizing the effect of ITO charging (Methods).

Size calibration. The protein size (\( D_H \)) in this work is determined with calibration by imaging polystyrene (PS) nanoparticles of different diameters (\( D_H = 40–140 \) nm). These nanoparticles are larger than the proteins and can be directly imaged with the setup by subtracting the background from each image, allowing us to obtain the image contrasts vs. size (Fig. 4a). We confirmed that the image contrasts were due to single particles rather than aggregates with scanning electron microscopy (SEM) (Supplementary Fig. 7). The power relation between the image contrast and \( D_H \) is \( 2.1 \pm 0.2 \), smaller than 3 expected from a simple scattering model. This discrepancy is attributed to the roughness of the ITO surface as shown by the AFM experiment and simulation (Supplementary Fig. 8). Electric double layer-enhanced light scattering has been reported, which may also contribute to the observed optical signals here\textsuperscript{27}. The calibration curve was used to determine the size of protein–PEG complex (\( D_{H,\text{app}} \)) (Fig. 4b).

### 2D identification of single proteins

Two-dimensional (2D) gel electrophoresis is a powerful technology that identifies proteins...
based on size and isoelectric point (or mobility at different pH). The present single-molecule imaging method can perform protein analysis in an analogous manner at the single-molecule level. This capability is shown in Fig. 4d, which plots different proteins and protein–ligand complexes according to mobility and size. The proteins and complexes in the 2D-plot are well separated, allowing identification of proteins like 2D electrophoresis. Binding of IgG to anti-IgG shifts the IgG region to a new position in the 2D-plot, which is analogous to the WB technology, providing further identification of the protein. Figure 4d also shows that 1D histograms based on either size or mobility alone are not capable to differentiate different proteins and complexes, which underscores the importance of simultaneous size, charge and mobility measurements.

Covalement tethering the proteins to the surface allows us to continuously track the same molecules for sufficiently long time and switch solutions in the measurements without losing the molecules, which is a challenging task for most single-molecule trapping methods. Compared to single-molecule methods that do not require surface attachment such as ABEL trap and iSCAT, the tethering proteins are more likely to have fixed orientations, thus the broad distribution in measured size and charge reflect the orientational heterogeneity of the proteins that otherwise hard to obtain. For example, lysozyme has a more asymmetrical shape than calmodulin, and it shows a smaller standard deviation in size. Since the PEG tethers the protein via nonselective NHS-methoxylsilane, lysozyme, calmodulin, and BSA were purchased from Sigma-Aldrich. Goat IgG (anti-digoxigenin) was purchased from Abcam. Goat antimouse IgG and rabbit anti-goat IgG were purchased from Invitrogen. Secretory IgA (from human colostrum, MW = 385 kDa) and IgM (from human plasma, MW = 950 kDa) were purchased from Athens Research and Technology. Polymerized nanoparticles were purchased from Bangs Labs and the hydrodynamic diameters were determined with dynamic light scattering. Biotin particles were purchased from Nanocs. Deionized (DI) water with resistivity of 18.2 MΩ cm was purchased from VWR. (3-Glycidyloxypropyl)trimethoxysilane, lysozyme, calmodulin, and BSA were purchased from Sigma-Aldrich Supplies. Streptavidin was purchased from VWR. (3-Glycidyloxypropyl)trimethoxysilane, lysozyme, calmodulin, and BSA were purchased from Sigma-Aldrich. Goat IgG (anti-digoxigenin) was purchased from Abcam. Goat antimouse IgG and rabbit anti-goat IgG were purchased from Invitrogen. Secretory IgA (from human colostrum, MW = 385 kDa) and IgM (from human plasma, MW = 950 kDa) were purchased from Athens Research and Technology. Polymerized nanoparticles were purchased from Bangs Labs and the hydrodynamic diameters were determined with dynamic light scattering. Biotin–PEG–NH₃ (~15% length variation) was used to tether the protein to the streptavidin-functionalized ITO surface. The protein (IgG, lysozyme, BSA, CaM, IgA, or IgM) was first incubated with the biotin–PEG–NH₃ tether at 10:1 ratio to form a PEG–protein complex in 1× PBS overnight at 4 °C. The solution containing protein–PEG complex was then added to streptavidin coated ITO slides and incubated for 2 h to allow biotin–streptavidin binding. Finally, the ITO slide was rinsed with 100× diluted PBS to remove free protein molecules in the solution.

**Calibration curve.** 100× diluted PBS was placed on top of the ITO surface, and PS nanoparticle solution was added to allow binding of the nanoparticles to the surface. An image sequence was recorded at 800 frames/s for 5 s. The hydrodynamic diameter of each PS nanoparticle sample was measured with dynamic light scattering.

**Signal processing.** An 80 Hz FFT filter (bandwidth = 1 Hz) was applied to the recorded image sequence in time domain to remove random noises (Supplementary Fig. 2) using MATLAB. The temporal FFT image was converted to k-space using a Fast Fourier transform algorithm, and a spatial filter was applied to remove spatial noises (Supplementary Fig. 10i). A region of interest (ROI) with 10 × 10 pixels was selected for each protein, and the mean intensity within the ROI ($I_p$) was used to determine the contrast of the protein. An adjacent region of the same size was selected as a reference region, and the mean intensity of the reference region ($I_f$) was also determined. The contrast of the protein was determined with $ΔC$($ΔI_m$, $ΔI_d$) = ($I_p$ − $I_f$)/$I_f$, where $I_f$ is the mean intensity within the ROI without FFT filter. The size and charge of each protein were determined based on the FFT image contrast.

**Equation of motion of tethered protein molecules.** The motion of a protein tethered to a surface by PEG is determined by

$$m \frac{d^2 z}{dt^2} + \frac{dz}{dt} + k_{PEG} \cdot z = qE + F_r,$$  

where $m$, $z$, $c$, and $q$ are the mass, displacement, damping coefficient, and charge of the protein, $k_{PEG}$ is the spring constant of the PEG tether, arising from the entropic force, $E$ is the applied electric field, $F_r$ is the stochastic force on the PEG and the protein due to thermal fluctuation, which has an average of zero. For a protein with molecular weight of 100 kDa ($m = 1.7 \times 10^{-19}$ g) under an electric field oscillating at 80 Hz, the first and the second terms are $10^{-3}$ and $10^{-6}$ pN, respectively, which are much smaller than the entropic force estimated below.

To estimate the entropic force associated with the conformation change of the PEG tether, we use the FJC model[14,39], which leads to an entropic force of

$$f_{entropy} = k_{PEG} \cdot z = 3k_BT \frac{qE}{nB^2},$$

where $k_B$ is the Boltzmann constant, $T$ is temperature, $b$ is the Kuhn length of PEG, and $n$ is the number of segments with length of $b$. For PEG10k, $b = 0.55$ nm, $n = 113$, and $k_{PEG} = 3.62 \times 10^{-19}$ N/m. The entropic force is 22.8 pN when the PEG is stretched to 63 nm, which is many orders of magnitude greater than the first and second terms of Eq. (4). This simplifies Eq. (4) to $k_{PEG} \cdot z = qE$, which is Eq. (2). For a sinusoidal electrical field, $E = E_0 \cos(\omega t)$, where $E_0$ is the field amplitude and $\omega = 2\pi f$ is the angular frequency. The sinusoidal oscillation of the displacement with amplitude $Δz$ takes the form of $z = Δz \sin(\omega t + θ)$, where $θ$ is the phase shift of the oscillation with respect to the field. Both the oscillation amplitude and phase shift can be determined by performing FFT on the image sequence.

**Measuring the applied electric field and protein charge.** Because the ionic concentration varies with the distance from the surface, the applied electric field varies with the molecule-surface distance, which is denoted as $E = E_0(Δz, U_0)e^{-qU}$. To measure the electric field, we tethered 40 nm streptavidin coated gold nanoparticles (AuNPs) to a surface with PEG10k linkers31, swept the potential negatively from 0 to −2 V, and recorded particle-surface distance ($Δz$) changes vs. potential ($U$) (Supplementary Fig. 1a, b). Because AuNPs are negatively charged at pH = 7.4, the negative potential sweep pushes them away from the surface, leading to decrease in the image intensity (Supplementary Fig. 1c). The intensity decreases exponentially with the potential and reaches a minimum when the PEG is fully stretched (Supplementary Fig. 1d). By converting the image intensity into particle-surface distance ($Δz$), we obtained $Δz_p$ vs. $U_p$ for the AuNP (Supplementary Fig. 1e). The plot shows a linear regime followed by a plateau regime, which is observed for all the proteins studied here (Figs. 2b, 3b, 4d, 6b).
Supplementary Fig. 3b, f). At the transition from the linear to plateau regimes, the polymer is almost fully stretched (Supplementary Fig. 3b, f). At the transition from the linear to plateau regimes, the ARTICLE NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-18547-w | www.nature.com/naturecommunications

that each pattern in the contrast images was due to single particle rather than aggregates. The contrast images show good correlation with the SEM images as what we observed in Fig.4a. We measured the sizes, and plotted the size vs. contrast in logarithmic scale (Supplementary Fig. 4c, d).

When a protein is present on the surface, it scatters the evanescent wave, generating a scattered wave \( u_s \), given by

\[
u_s(r, r') = \frac{k_a}{\lambda} u_e(r) e^{-i \cdot q \cdot (r-r')}
\]

where \( r' \) is the location of the protein and \( r \) is the location on the image, \( \beta \) is a scattering coefficient related to the polarizability of the molecule, \( d \) is the decaying constant of the evanescent wave, and \( k \) is the wave number of evanescent wave. The superposition of the two waves, together with light reflected from the ITO surface \( u_i \), is

\[
u(r, r') = u_i(r) + u_s(r) + u_e(r, r')
\]

The overall reflected light detected by the camera \( I \) is given by

\[
I = |u_i(r) + u_s(r) + u_e(r, r')|^2
\]

The image contrast of the particle is described by

\[
I(r, r') = |u_i(r) + u_s(r) + u_e(r, r')|^2 - |u_i(r) + u_e(r, r')|^2
\]

which is proportional to the cubic power of the diameter \( \Delta_{t,\beta}^5 \). The observed size dependence of the image contrast is slower than cubic power (between 2 and 3), which is attributed to surface roughness, as discussed below.

Effect of PEG tether. The size obtained here includes contribution from the PEG tether. To extract the diameter of the protein \( D_H \), the following equation is used, where \( D_H \) is the apparent diameter of the protein

\[
 D_H = D_H^{\text{app}} - D_{\text{PEG}}
\]

where \( D_{\text{PEG}} \) is the diameter of PEG coil measured with dynamic light scattering.

Length of PEG tether. The PEG tether used in this work has a molecular weight of 10 kDa, which consists of 225 ethylene glycol units, each has a length of 0.278 nm. The linear length of the PEG is thus 0.278 nm × 225 = 63 nm.

Extracting oscillation amplitude with FFT. The image sequence records the oscillation of protein molecules over time. Plotting the local image contrast vs. time reveals periodic oscillation of a protein (e.g., IgG and lysozyme) (red dashed line, Supplementary Fig. 2a, c). The FFT amplitude spectrum shows a sharp peak located at the frequency of the applied electric field (red line, Supplementary Fig. 2b, d). We performed this FFT analysis on each pixel of the image sequence (Fig. 1c), extracted the oscillation amplitude averaged over 1 s, and constructed an FFT image (oscillation amplitude image) shown in Fig. 1d.

Probability of anti-IgG binding to IgG. We estimated probability \( P(t) \) of anti-IgG binding to IgG by measuring the kinetic constants with surface plasmon resonance (Supplementary Fig. 5). In terms of the kinetic constants, \( P(t) \) takes the form of,

\[
P(t) = \frac{P_0}{1 + k_1 \cdot P_0 [\text{anti-IgG}] + k_2}
\]

where \([\text{anti-IgG}]\) is the concentration of anti-IgG in the solution, and \( k_1 \) and \( k_2 \) are given in Eq. 9, which is applicable to the dissociation rate constants, respectively. In the anti-IgG binding experiment shown in Fig. 2c, [anti-IgG] = 130 nM and \( t = 50 \text{s} \), which leads to \( P(t) = 0.36 \) according Eq. 9, indicating 36% chance of an IgG binding with an anti-IgG.

Additional data of anti-IgG binding to IgG. Using the measured size and charge (Fig. 2h–i), we determined the mobility for IgG and IgG/anti-IgG complex and the mobility histogram (Supplementary Fig. 4a). Unlike the size and charge histograms, the mobility shows only one peak. This is because mobility is an intensive quantity, which shows that the image contrast is originated from the interference between \( u_i \) and \( u_e \) (where \( q \) is the phase shift). Using Eq. (14), we computed an image, which closely resembles the experimental image (Supplementary Fig. 8b). Equation (14) shows that the image intensity scales with \( \beta \), which is proportional to the cubic power of the diameter \( \Delta_{t,\beta}^5 \). The observed size dependence of the image contrast is slower than cubic power (between 2 and 3), which is attributed to surface roughness, as discussed below.

Effect of surface roughness. The above analysis assumes a perfect surface. In practice, ITO surface has finite roughness and atomic force microscopy (AFM) images reveal grain-like features on the surface (Supplementary Fig. 8a). The surface roughness is particularly important for small objects, such as protein molecules, which are comparable with or smaller than the grains. We simulate the surface roughness effect by including an additional term, \( \eta_{\text{rough}} \), in Eq. (13),

\[
I(r, r') = |u_i(r) + u_s(r) + u_{\text{rough}}(r, r')|^2 + |u_i(r) + u_{\text{rough}}(r, r')|^2
\]

which leads to increased background and also slower dependence of the image contrast on the protein size. Using the grain size of the ITO measured from the AFM images, we performed numerical simulation of the size dependence of the image contrast. The simulation shows that 1000 small polystyrene particles randomly distributed on the surface around a polystyrene particle of interest with diameter varying from 20 to 150 nm. The size distribution of the small particles used for simulation is based on the AFM measurement, which varied from 0 to 25 nm, with an average diameter of 3 nm. The logarithmic plot of the image contrast vs. diameter shows a slope of ~2.2 (Supplementary Fig. 8c).

Measuring the size of IgA and IgM. To further confirm that the image contrasts are due to single molecules, we measured the image contrasts of immunoglobulin A (IgA) and immunoglobulin M (IgM) (Supplementary Fig. 9), which are dimer and pentamer of IgG, and determined sizes using the image contrast vs. size plot (Fig. 4b). The size of IgA and IgM are determined to be 13.9 ± 4.5 and 17.6 ± 6.0 nm, respectively, close to the values measured with dynamic light scattering (14.3 ± 4.5 and 21.4 ± 6.0 nm).

Surface charging effect and background noise. A bare ITO surface also responds to the applied electric field and gives rise to a background. This response arises from the charge-dependent refractive index of ITO. To evaluate this effect, we modulated a bare ITO slide with potential \( U_i = 10 \text{V} \) and \( f = 80 \text{ Hz} \) and observed the FFT images (Supplementary Fig. 10b). The features shown in the images are due to the grains of the ITO surface, which affect limits of detection for the diameter \( \Delta_{t,\beta}^5 \) and charge \( q \). We converted the features in the ITO background image to the equivalent \( D_{\text{II}} \) and \( q \) images using the calibration curve in Fig. 4b and the Einstein equation (assume the mobility is \( 1 \times 10^{-7} \text{m}^2 \text{V}^{-1} \text{s}^{-1} \), the typical value for proteins). The results show the distributions of \( D_{\text{II}} \) and \( q \) associated with surface roughness (Supplementary Fig. 10b, c).

In single-molecule detection, the charge-induced features overlap with the protein images and affect detection accuracy (Supplementary Fig. 10g). To reduce this effect, we performed k-space FFT to the temporal FFT image and converted the image to k-space, which shows two rings originated from the interference of scattered field and evanescent field (Supplementary Fig. 10b). We apply a filter to block part of the low frequency region (Supplementary Fig. 10i), where the background features are located, and the result shows most of the features are removed (Supplementary Fig. 10i). We applied the same filter to the images in Supplementary Fig. 10a–c to remove the background features and obtained the distributions of \( D_{\text{II}} \) and \( q \) from which the limits of detection were estimated to be ~0.5 nm for diameter, and 0.3 e for charge (Supplementary Fig. 10d–f).
SNR estimation. The evanescent wave (E_e) propagates along the ITO surface and is scattered by the sample particle or protein on the surface. The scattered wave (E_s) is given by 50,51

\[ E_s = \frac{k^3}{4\pi} a^3 e^{-\theta_r} e^{-\sqrt{3}k r_{sy}}. \]

where \( k = 2\pi/\lambda \) is the wave number, \( a \) is the polarizability of the particle, \( r_{sy} \) is the distance from the particle to the point of the field on the surface, \( \theta_r \) is the decay constant of evanescent field, and \( \delta \) is phase shift between the plane wave and the circular wave (scattered wave). The polarizability \( a \) is given by

\[ a = 4\pi a n_i^3 \left( n_i^2 - n_{i,o}^2 \right) / \left( n_i^2 + 2n_{i,o}^2 \right), \]

where \( n_i \) is the radius of the particle, \( n_i \) and \( n_{i,o} \) are the refractive index of particle and water, respectively. For a polystyrene (PS) particle with radius of 6 nm, \( a \) is about 4 \( \times \) 10^{-25} m^6.

The overall light collected by the camera \( I_{\text{total}}(\theta, \phi) \) is the superposition of \( E_s \) and light reflected by the ITO surface \( E_r \),

\[ I_{\text{total}}(\theta, \phi) = |E_r + E_s|^2 = |E_r|^2 + 2Re\left\{E_r^* E_s + E_s^* E_r \right\} + |E_s|^2. \]

The first term \( |E_r + E_s|^2 \) in Eq. (19) is the background without the particle, and the third term is negligible for small particles. We note all the particles in this work are sufficiently small. Equation (19) can thus be simplified as

\[ I(\theta, \phi) \propto 2Re\left\{E_r^* E_s \right\}. \]

By combining with Eqs. (16) and (17), Eq. (20) can be written as

\[ I(\theta, \phi) \propto \begin{cases} 2 \frac{k^3}{4\pi} a^3 e^{\theta_r} e^{-\sqrt{3}k r_{sy}} \cos(\theta_r + r_{sy}) + \delta), \end{cases} \]

The evanescent light intensity has ~5 times enhancement over the incident light field, which provides sufficient enhancement given that the surface condition is more complex than that in the standard frequency. The variation in determined diameter is ~3.3%. The PEG length is also related to the spring constant, which can affect the interpretation of charge (see below). (b) PEG spring constant. Each PEG tether may have different spring constant due to the variation in length and different local environment. The variation in spring constant is ~7% measured by AFM force spectroscopy93. (c) Determining the plateau of oscillation. The plateau and the transition point in the oscillation amplitude vs. applied potential plot (Fig. 1g) are used to determine the size and charge. The error in determining the maximum extension is ~10%, which leads to ~3.3% variation in size and 10% variation in the potential (or charge). (d) Measuring the applied electric field. The field is determined based on the charge of gold nanoparticles, which has a variation of 5%. (e) Surface charging effect introduces 1 nm error in the slipping layer thickness for the protein is determined to be 1 nm. Using these parameters, we plotted \( \sigma_{\text{eff}}/\sigma_{\text{total}} \) ratio vs. ionic strength (Supplementary Fig. 11), showing that the effective charge is ~20% of total charge for 1× PBS, and ~90% in 100× diluted PBS.

Measurement error. The derivation of size and charge involves several parameters of the PEG and the applied field, thus the variation of these parameters can affect the accuracy in size and charge. We estimate the variations here. (a) PEG length. The PEG10k has a length variation of ~15%, which leads to ~10% variation in the measured intensity according to Eq. (3). Because intensity is proportional to the square of diameter, the variation in determined diameter is ~3.3%. The PEG length is also related to the spring constant, which can affect the interpretation of charge (see below). (b) PEG spring constant. Each PEG tether may have different spring constant due to the variation in length and different local environment. The variation in spring constant is ~7% measured by AFM force spectroscopy93. (c) Determining the plateau of oscillation. The plateau and the transition point in the oscillation amplitude vs. applied potential plot (Fig. 1g) are used to determine the size and charge. The error in determining the maximum extension is ~10%, which leads to ~3.3% variation in size and 10% variation in the potential (or charge). (d) Measuring the applied electric field. The field is determined based on the charge of gold nanoparticles, which has a variation of 5%. (e) Surface charging effect introduces 1 nm error in the slipping layer thickness for the protein is determined to be 1.4 nm. Using these parameters, we plotted \( \sigma_{\text{eff}}/\sigma_{\text{total}} \) ratio vs. ionic strength (Supplementary Fig. 11), showing that the effective charge is ~20% of total charge for 1× PBS, and ~90% in 100× diluted PBS.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability. MATLAB code for fast Fourier Transform is provided in Supplementary Note 2.

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