ACSNANO

www.acsnano.org

ACCESS

Three-Dimensional Two-Color Dual-Particle Tracking Microscope for Monitoring DNA Conformational Changes and Nanoparticle Landings on Live Cells

Yen-Liang Liu,* Evan P. Perillo, Phyllis Ang, Mirae Kim, Duc Trung Nguyen, Katherine Blocher, Yu-An Chen, Cong Liu, Ahmed M. Hassan, Huong T. Vu, Yuan-I Chen, Andrew K. Dunn, and Hsin-Chih Yeh*

Cite This: https://dx.doi.org/10.1021/acsnano.9b08045

III Metrics & More



ABSTRACT: Here, we present a three-dimensional two-color dual-particle tracking (3D-2C-DPT) technique that can simultaneously localize two spectrally distinct targets in three dimensions with a time resolution down to 5 ms. The dual-targets can be tracked with separation distances from 33 to 250 nm with tracking precisions of ~15 nm (for static targets) and ~35 nm (for freely diffusing targets). Since each target is individually localized, a wealth of data can be extracted, such as the relative 3D position, the 2D rotation, and the separation distance between the two targets. Using this technique, we turn a double-stranded DNA (dsDNA)-linked dumbbell-like dimer into a nanoscopic optical ruler to quantify the bending dynamics of nicked or gapped dsDNA molecules in free solution by manipulating the design of dsDNA linkers (1-nick, 3-nt, 6-nt, or 9-nt single-strand gap), and the results show the increase of k_{on} (linear to bent) from 3.2 to 10.7 s⁻¹. The 3D-2C-DPT is then applied to observe translational and rotational motions



of the landing of an antibody-conjugated nanoparticle on the plasma membrane of living cells, revealing the reduction of rotations possibly due to interactions with membrane receptors. This study demonstrates that this 3D-2C-DPT technique is a new tool to shed light on the conformational changes of biomolecules and the intermolecular interactions on plasma membrane.

KEYWORDS: 3D particle tracking, rotational movement, nanoscopic optical ruler, DNA conformation change, antibody-receptor interactions

Single-particle tracking (SPT) has enabled direct observations of particle dynamics with sub-millisecond temporal resolution and submicron spatial resolution inside complex biological systems.^{1,2} With decades of efforts, SPT has provided insights into actions of motor proteins,^{3,4} dynamics of cell membranes,^{5–7} transport of mRNA,^{8,9} internalizations of viral particles,^{10,11} searching mechanisms of DNA-binding proteins on the genome,^{12,13} ligand-induced endocytosis of membrane receptors, and intermolecular interaction of viruslike nanoparticles to transmembrane receptors.^{14,15} While SPT is applied to probe cellular properties such as viscoelasticity, cytoskeleton framework, stress fluctuations, and cell states,^{16–19} most of the SPT techniques today focus on monitoring the translational dynamics of particles. The rotational dynamics of particles, on the other hand, can provide complementary information to the chemical and biological processes, such as how viral particles interact with cellular structures when they enter the cells,²⁰ and how T-cell receptors interact with antibodies on lipid bilayers.²¹ Single gold nanorods can even serve as rotational microrheology probes to monitor the polymer gelation process.²²

Received: October 11, 2019 Accepted: June 29, 2020





Figure 1. 3D-2C-DPT TSUNAMI system setup. (A) Spatiotemporally multiplexed two-photon excitation and temporally demultiplexed detection scheme of the TSUNAMI system. (B) The system splits the pulse laser into four beams, forming a tetrahedral profile. (C) Using an additional PMT and a TCSPC routing method, the data collected from the tetrahedral profile can be converted into photon-count rates found in each time-gated domain for multiple particle localization. Please note the plots are schematics to explain the multiplexed detection process. (D) The error signals obtained from the time-gated domains can then be analyzed, giving the x, y, z coordinates and the relative location of the two particles in polar coordinates (azimuthal and elevation angles). (E) Preparation of a dual-color dumbbell-shaped DNA tracer: a 150 bp dsDNA linker with two different colored 40 nm beads on each end. The theoretical distance between the two NPs is 100 nm. Modified DNA linker that causes it to flex or bend in free diffusion. The inset image shows a single dumbbell-shaped DNA tracer under 2P-LSM. (F) Key parameters acquired from 3D-2C-DPT: 3D position, 2D rotation, separation distance.

Six methods have been proposed to measure the rotation of a particle (see Yu's recent review²³ for details), including (i) using Janus particles^{24–26} or colloidal clusters, 27,28 (ii) tracking with digital holographic microscopy,^{29,30} (iii) tracking with polarized fluorescence correlation spectroscopy (FCS) on ellipsoids or rods,³¹ (iv) imaging with defocused dipole emission, 32 (v) imaging with a dual-particle system, 21,33,34 and (vi) tracking gold nanorods with differential interference contrast (DIC) microscopy.³⁵ Both the polarization detection and the conventional dual-particle system, which depend on 2D imaging methods, have one critical drawback: the elevation angles θ and $-\theta$ are indistinguishable. Janus particles have the same issue, which makes the quantitative analysis of the particle rotation complicated due to the uncertainty of the particle orientation. While digital holographic microscopy generates a 2D holographic image that encodes the 3D details of the object in 6 modes (3 translational modes and 3

rotational modes),³⁰ the complexity of processing and fitting the holographic data makes it a computationally expensive process.

Active feedback 3D-SPT was pioneered by Berg using a system with six photomultiplier tubes (PMTs)³⁶ and flourished in the past two decades: orbital 3D tracking,³⁷ confocal 3D tracking,³⁸ tetrahedral detection 3D tracking,^{39,40} tetrahedral excitation 3D tracking,⁴¹ and dynamic photon localization tracking.⁴² However, there is no simple SPT method that can probe both the particle's translational and rotational dynamics in 3D space. Inspired by these 3D-SPT techniques, we propose a three-dimensional, two-color, dual-particle tracking microscope (3D-2C-DPT) that can simultaneously localize two spectrally distinct targets in three dimensions with a time resolution down to 5 ms using only two PMTs. The targets can be tracked with separation distances ranging from 33 to 250



Figure 2. Correction of fluorescence crosstalk. The crosstalk of green fluorescence emission creates a phantom in the detection channel where the PMT is mounted with a bandpass filter (605 nm/64 nm). (A) Reconstructed trajectories from signals of the tracking channel (green) and those of the detection channel (red). The crosstalk creates a phantom for 3D-2C-DPT. The center-to-center distance between the 200 nm green fluorescent nanoparticle and its phantom is 5 nm. (B) Photon count rates of both channels. (C) This plot shows the separation distance (d), azimuth (θ), and elevation angles (φ) along the time trace. The histogram represents the distribution of the separation distances. These two polar histograms show random distributions of azimuth and elevation angles. (D) These four types of immobilized fluorescent NPs were tracked to evaluate the fluorescence crosstalks from green emission into the red channel or red emission into the green channel. The primary and secondary detectors were mounted with the corresponding bandpass filters. For instance, the primary detector was mounted with a green emission filter to track green NPs, and the second detector was mounted with a red emission filter. The average crosstalk of green emission into the red channel is about 24% of its photon-count rate in the green channel.

nm with static tracking precision of ~ 15 nm and dynamic tracking precision of ~ 35 nm (Notes S1 and S2).

The 3D-2C-DPT microscope is the multicolor extension of our 3D-SPT technique known as TSUNAMI (Tracking Single particles Using Nonlinear And Multiplexed Illumination⁴¹) which was inspired by the four-detector confocal tracking setup developed by the Werner group.40 Built on spatiotemporally multiplexed two-photon excitation, TSUNAMI requires only one detector for three-dimensional particle tracking. Being a two-photon microscope by nature, TSUNAMI enables multicolor excitation with a single wavelength, facilitating the development of the 3D-2C-DPT technique that tracks two spectrally distinct targets simultaneously with two detectors. Our 3D-2C-DPT microscope provides unambiguous orientation determination of a dual-particle system. Since each particle can be individually localized in a 3D space by the two PMTs, a wealth of data can be extracted, including the 3D position and the fluorescence lifetime of each target, and the relative 2D rotation and the separation distance between the two targets. The dual-particle system employed in this study is different from the one used in the traditional 2D rotational measurement-our system is composed of two fluorescent nanoparticles (NPs) (green and red) connected by a single double-stranded DNA (dsDNA) linker (termed a tracer or a dumbbell-like dimer). The coordinates of the green and red NPs define the orientation of the tracer. Therefore, the analysis of DPT trajectories can reveal the translational and rotational movement of the dumbbell-like dimer and offer the information on coordinates (x, y, and z), rotation angles (θ and φ), separation distance (d), and photon-count rates (B1 and B2). Using this technique, we turn the dsDNA-linked

dumbbell-like dimer into a nanoscopic optical ruler to quantify the bendability of nicked or gapped dsDNA molecules by manipulating the design of dsDNA linkers (single-strand nick, 3-nt, 6-nt, or 9-nt single-strand gaps). We can observe the conformational changes of the DNA complex using our 3D-2C-DPT system. In addition, we take advantage of the capability of rotational tracking to monitor the landing of an antibody-conjugated NP on the plasma membrane of living cells.

RESULTS AND DISCUSSION

Multiple Particle Localization. The 3D-2C-DPT works by coregistering the location of the secondary particle relative to the primary target being tracked. Dual-particle localization is realized through two significant modifications to the hardware and the algorithm of the standard TSUNAMI microscope. First, the microscope requires an additional PMT (PMT2, Figure 1A) and a time-correlated single photon counting (TCSPC) routing module. The PMT2 collects the light emitted from the secondary fluorophore in a separate spectral channel. The routing module routes the second channel to the same TCSPC board (SPC-150, Becker and Hickl) (Figure S1). Second, an algorithm to process the raw photon-count data from the second channel within the same 3D space and to coregister the secondary target relative to the primary target is needed to perform dual-particle localization. For a two-color fluorescent dimer residing somewhere inside the excitation tetrahedron (Figure 1B), its 2P emission is collected by the two PMTs. By TCSPC detection, each detected photon is assigned to a specific time gate (G1a-G4a for PMT1 and G1b-G4b for PMT2) in the fluorescence decay histogram



Figure 3. Validation of two-color localization. (A) Reconstructed dual-particle trajectory of a pair 200 nm green and red beads fixed on a coverslip. (B) Its corresponding separation distance (d) and azimuth (θ) and elevation angles (φ) along the time trace and the corresponding histograms. (C) 2P fluorescence image of red and green 40 nm fluorescent beads fixed to a coverslip. Zoom: a magnified view of a single red and green bead pair separated by a distance of 54.8 nm, scale bar is 200 nm. The Gaussian fit centroid (+) of each image channel is overlaid with the trajectory points projected onto 2D images (green and red dots). The reconstructed 3D trajectory of both beads measured for 2 s and with the centroid-to-centroid distance of 51 nm. (D) A plot shows the root-mean-square error (RMSE) of the two-color colocalization technique relative to the Gaussian-based image analysis using paired beads in the sample of 40 nm green and red NPs, along with other mixtures (G40-R40 nm linked with 90 bp or 150 bp dsDNA linker, a pair of green and red 200 nm NPs).

(Figure 1C) and therefore attributed to an individual excitation volume. An animation, Movie S1, was created to explain the demultiplexed detection process. Please note that Figure 1B,C are schematics to demonstrate the detection process. A representative raw signal summed up in each channel for a 2 s trajectory is shown in Figure S2.

The photon-count data of the primary target were processed in real-time during the tracking to lock the multiplexed laser beams on the primary target, and the photon-count data of the secondary target were saved for reconstructing the secondary trajectory later. The localization for the second channel can be performed using the same arithmetic method outlined in the Materials and Method section. The critical difference is that this localization takes place in postprocessing and does not affect the position of the xy galvo mirror actuators. The postprocessed error signals were calculated from eqs 1–6. The resulting localization is a position relative to where the point spread function (PSF) was at the current time of localization (*i.e.*, the current position of the primary target, Figure 1D). To move the relative localizations into the global frame of reference, all that is required is to shift the position by the amount of the current location of the primary target. Using this technique, two particles can be tracked simultaneously with no loss in temporal resolution.

In this study, we created a dumbbell-like NP dimer which was composed of a green NP, a red NP, and a dsDNA linker. Figure 1E shows the steps for creating the NP dimer. The vector from the centroid of the green NP to the centroid of the red NP defines the orientation of the optically anisotropic NP dimer (Figure 1D). Therefore, the 3D-2C-DPT TSUNAMI is able to reveal the translational and rotational movement of the dumbbell-like NP dimer and to offer a wealth of information: coordinates (x, y, and z), rotation angles (θ and φ), separation distance (d), and photon-count rates (B1 and B2) (Figure 1F). Movie S2 shows a representative trajectory and its trajectoryderived parameters. It should be noted that this system can only track a second particle up to 250 nm away from the

Article



Figure 4. Translational and rotational tracking of a freely diffusing dual-particle. (A) Reconstructed dual-particle trajectory of a dual-color dumbbell-shaped DNA tracer: a 150 bp dsDNA linker with two different colored 40 nm beads on each end. The theoretical distance between the two beads is 100 nm. (B) Dynamic parameters extracted from the trajectory analysis. (C, D) MSD and MSAD curves derived from a series of trajectories of dumbbell-shaped tracers diffusing in dextran solutions with different concentrations. The solid lines represent the averaged MSD or MSAD curves, and the ribbons represent the standard deviations. (E) The translational (D_t) and rotational (D_r) diffusion coefficients were extracted by MSD and MSAD curve fittings, respectively. The error bars represent standard errors, and the numbers of trajectories of 75% dextran-40, 50% dextran-40, 30% dextran-450, 20% dextran-450, and 10% dextran-450 are 50, 108, 68, 68, and 55, respectively.

primary particle, which limits the method in general to coupled particles and not to two independently moving particles.

Calibration and Localization Precision Calibration of the 3D-2C-DPT Technique. To validate the 3D-2C-DPT system, we conducted a series of calibration experiments to obtain the best data-processing parameters and to verify the accuracy of these parameters. We first tracked fluorescent NPs fixed on coverslips to correct spectral crosstalk in dual-color fluorescence (Figure 2A,B). The fluorescence crosstalk from the green NPs into the red channel needed to be taken into account, and the bleed-through of the green emission into the red channel was around 24% of fluorescence intensity detected in the green channel (Figure 2D). Therefore, the bleedthrough signal had to be corrected before we could reconstruct secondary trajectories (Figure S3). Besides, the bleed-through signal can be taken as the fluorescence emission from a pseudo-secondary-target which we called a phantom. As the phantom was perfectly colocalized with the primary target, we were able to precisely quantify the static and dynamic localization precisions by measuring the separation distance between the primary target and its phantom on immobile (Figures S4 and S5) or freely diffusing green fluorescent NPs (Figure S6). The localization graphs in xyz for trajectories of fixed 40 and 200 nm green NPs are shown in Figures S4 and S5. The xyz positions fluctuated around the centroids of particles with the standard deviations (static tracking precisions) of 15, 14, and 34 nm in xyz for a 200 nm green NP and 25, 21, and 39 nm in xyz for its phantom; and 19, 21,

and 42 nm in *xyz* for a 40 nm green NP and 23, 21, and 46 nm in *xyz* for its phantom.

To demonstrate the capability of two-color colocalization, we fixed and then tracked a pair of carboxylate-modified 200 nm green and amine-modified 200 nm red NPs on a coverslip (Figure 3A). The experimental centroid-to-centroid distance agreed well with the theoretical distance (200 nm) with the correction of fluorescence crosstalk. The narrow distributions of separation distance (214 \pm 17 nm, mean \pm standard deviation (SD)), azimuth angle $(-94 \pm 6^{\circ})$, and elevation angle $(20 \pm 5^{\circ})$ reflected the excellent tracking precision of our 3D-2C-DPT system (Figure 3B). To further validate the accuracy of DPT-derived separation distances, we compared the distances derived from the trajectories with Gaussian fit with the distances derived from the two-photon laser scanning microscope (2P-LSM) images (Figure 3C). Since the signal-tonoise in each 2P-LSM image was very high due to a long exposure time (0.83 s per frame) and averaging over several frames (5 frames), the 2P-LSM-derived distances were regarded as the ground truth in the comparison (Figure 3C). The typical standard deviation for the centroid fit in the 2P images was around 5 nm, whereas the SPT trajectories had the errors in the range of 20-50 nm. By plotting the DPT-derived separation distances against the ground truth, a clear correlation was observed, with the root-mean-square error (RMSE) less than 19 nm (Figure 3D) by repeatedly imaging and tracking many NP pairs in the sample of 40 nm green and red NPs, along with other mixtures (40-40 nm NP pairs

www.acsnano.org

Article



Figure 5. Quantification of DNA bendability using 3D-2C-DPT. (A) Series of representative time traces of separation distance measured from a variety of dumbbell-shaped DNA tracers with different designs of DNA linkers. The tracking experiment was conducted in 50% dextran-40. The separation distances of fixed and freely diffusing 200 nm green NPs are also shown as the control groups. Using EB analysis, the transitions of stacked and unstacked states were identified, and the solid black lines indicate the transitions along the time trace. (B) Histograms built from all the separation distance traces of each group. These histograms were fitted with a two-component Gaussian mixture model. The fitted mean, fitted standard deviation of separation distance, and the number of trajectories are labeled in each histogram. (C) Apparent stacking (k_{on}) and unstacking (k_{off}) rates extracted using the EB analysis. The error bars represent standard errors. (D) Schematic representation of the stacked-to-unstacked conformational transition of the dumbbell-shaped DNA tracer.

linked with 90 bp or 150 bp dsDNA, pairs of 200 nm green and red NPs).

The translational and rotational Brownian motion of dimers was characterized by tracking freely diffusing dimers at various diffusion rates (Figure 4). A representative trajectory and its dynamic information are shown in Figure 4A,B, and the video of this trajectory is presented in Movie 2. Trajectories were processed into mean-squared displacement (MSD) and meansquared angular displacement (MSAD) curves (Figure 4C,D), and the corresponding translational (D_t) and rotational (D_r) diffusion coefficients were estimated by fitting MSD and MSAD curves (Figure 4E). Both MSD and MSAD curves show the characteristics of freely diffusing traces, both translationally and rotationally. The tracers exhibit higher diffusion coefficients within the dextran solutions with lower viscosities. However, the rotational movement is less sensitive to the viscosity of the dextran solution (Figure 4E), which is also reflected by the indistinguishable rotational tracking accuracy (the values extrapolated from the MSAD curves at time lag equal to zero). The geometry and mechanical property of the dsDNA-linked dumbbell-like NP dimers might hinder us from correctly measuring the rotational diffusion coefficients of dimers. The static tracking precisions of the translational and rotational movements were estimated using the MSD and MSAD curves of pairs of 200 nm green and red NPs fixed on coverslips (Figure S7).

Conformational Changes of Nicked and Gapped DNA Structures. We applied this 3D-2C-DPT technique to study conformational changes of nicked and single-strand gapped 150 bp dsDNA in free diffusion with the format of dumbbelllike NP dimer (Figure 5). These 150bp DNA duplexes contain a single nick or a single-strand gap of 3-nt, 6-nt, or 9-nt as depicted in Table S1. With subdiffraction-limit spatial resolution in particle tracking and 5 ms temporal resolution, we monitored the change of the geometric distance between the primary and secondary targets (Figure 5A). Figure 5A shows the separation distances of representative trajectories collected from six experimental conditions: immobile green NPs, freely diffusing green NPs, and NP dimers with nicked or single-strand gapped dsDNA linkers. In the case of tracking green NPs, we see a static separation distance (between a green NP and its phantom) of ~30 nm, which represents the localization precision. The right-skewed distribution for green 200 nm NPs (Figure 5A) could be attributed to (i) higher background noise caused by other freely diffusing particles, (ii) the particle motion during the finite exposure time, (iii) the anisotropic tracking precision of in xy and z, and (iv) photobleaching effect. As described in Note S2, the measured separation distance increased while the red NPs became dimmer. These factors of dynamic error can be taken as systematic errors while evaluating the bendability of DNA. For the dimers with nicked and gapped DNA linkers, we observe a switching behavior between two states, 100 nm (the fully extended length) and 60-80 nm (with kinked DNA linkers) (Figure 5A). This result indicates that the DNA is flexing or bending during free diffusion, which allows for the NPs to occupy a range of separation distances during the trajectory. The histogram of separation distances built from all the collected traces can be well fitted by a two-component Gaussian mixture model centered at ~100 and 60-80 nm (Figure 5B). Our assumption of two-state conformational changes is supported by the Boulard group's study⁴³ in which they found that the gapped DNA exhibited two conformational states: one is close to B-DNA, and the other is significantly kinked at the gap which reduces the size of the cavity. For the nicked structure, its histogram of separation distance is very

Anti-EGFR IgG

100 nm

Α

y _4 μm

С

Star y 0.5 μm Monolayer LNCaP | Nuclei / Plasma membrane Azimuth, θ (°) 6 0 6 81 08 Ε Track duration = 58 s -180 ш



Figure 6. Landing of an anti-EGFR IgG-conjugated anisotropic dimer on the plasma membrane. (A) 3D isocontour model of the monolayer of LNCaP cells with staining for the nuclei (blue) and plasma membrane (pink). The dual-particle trajectory of the antibody-conjugated dimer was coregistered with the cell isocontour model. (B) Zoomed-in view of the dual-particle trajectory; the dimer was tracked for a duration of 58 s. (C) The highlighted subtrajectories are the subtrajectories within the time windows 0-5 s (I), 20-25 s (II), and 50-55 s (III), respectively. (D) These rose histograms show the distributions of the azimuth and elevation angles within their corresponding time windows. (E) Time traces of angles and translational diffusivities.

close to the intact dsDNA, but it displays enhanced local flexibility. This result agrees with other researchers' findings using molecular dynamics simulations⁴³ and electrophoresis.⁴ For the gap structure, we observed that the separation distance decreased as the gap became longer (Figure 5B), which means that the single-strand gaps enhance the bendability of DNA duplexes.

To characterize the kinetics of the conformational changes of these DNA duplexes, we revised and applied an empirical Bayes (EB) method^{45,46} to the analysis of the time traces of separation distances. The black lines indicate the binary-state sequences identified by the EB method (Figure 5A). In EB estimation, the variation in parameter values predicted by the prior distribution is matched to the variation in inferred parameter values over the population of trajectories, enabling a consensus kinetic model to be learned from the simultaneous analysis of a large population of single trajectories (see the Materials and Methods section for a more detailed description). Therefore, we were able to unbiasedly specify the rates of transition between conformational states (stacked and unstacked) of the nicked and gapped DNA. Through the EB analysis, our results demonstrate that the single nick and gaps do modulate the stacked-to-unstacked conformational transition of the DNA duplexes, and the rates were proportional to the length of single-strand gaps (Figure 5C). The removal of two GC pairs in 6-nt- and 9-nt-gapped dsDNA (see schematic in Table S1) might contribute to the abrupt

increase of k_{on} rates from 5 s⁻¹ (3-nt-gap) to around 12 s⁻¹ (6nt-gap and 9-nt-gap). The Frank-Kamenetskii group has demonstrated that two types of interactions contribute to the stability of nicked or gapped DNA double helix: stacking between adjacent base pairs and pairing between complementary bases.44 The melting free energy of the GC dinucleotide stack (-2.70 kcal/mol) is lower than those of AT-containing dinucleotide stacks ranging from -0.12 to -2.04 kcal/mol. Therefore, the removal of GC base pairs could increase the flexibility of the gapped DNA double helix. In a nutshell, this study demonstrated that the single nick and gaps enhanced the flexibility and facilitated the conformational changes of the DNA duplexes.

Landing of Dumbbell-Shaped Anti-EFGR IgG NP on the Plasma Membrane. To demonstrate the capability of translation and rotation tracking in live cells, we explored the application in which an antibody-conjugated dumbbell-like NP landed on the plasma membrane of a live cell. We monitored the landing process and analyzed the changes in the translational and rotational movements before and after the touchdown. The touchdown events were determined by visual inspection when the DPT trajectories were coregistered with the 3D stacked cell images. The changes in NP dynamics could also serve as indicators of the landing. The anti-epidermal growth factor receptor (EGFR) IgG NP is the revised version of the previous dumbbell-shaped dimer with a single nicked DNA linker: the green NP is conjugated with anti-EGFR

G

antibodies instead of biotinylated polyethylene glycol (PEG). Due to the design of our tracer, the rotational freedom of the DNA-linked dual-particle system is a convolution of the DNA bending and the rotational freedom of NPs. Figure 6A,B shows a representative 3D trajectory of a tracer landing on a fluorescently labeled cell (see Movie S3). Three subtrajectories (I, II, III) are highlighted in Figure 6C, and the corresponding rotation angles of these subtrajectories are plotted in the rose histograms (Figure 6D). While the translational diffusivity remains at around 0.01 μ m²/s, the variances of rotational angles decrease along time (Figure 6D,E). The confinement of rotational movement could be attributed to the interaction between antibodies and the EGFRs on the plasma membrane. More trajectories are in Figures S8-S10 and their corresponding videos in Movies S4-S6. Using our approach, we were able to interrogate the critical transition processes in which the NP interacts with the ligands on the plasma membrane.

Fluorescence colocalization can be used to determine the separation distance of binding targets, binding kinetics, conformational changes, or even the orientations of a target construct. Förster resonance energy transfer (FRET) based nanoscopic optical rulers have served as a key tool for measuring conformational changes and intramolecular distances of single biomolecules.⁴⁷⁻⁵² However, the length scale of a FRET ruler is limited to a maximum of 10 nm because the FRET process originates from dipole-dipole interactions. To break the FRET barrier, scientists developed long-range optical rulers based on plasmonic NP-based surface energy transfer (or plasmon coupling) to further increase the accessible distance range up to 80 nm (depending on the size and coating of the NPs).^{53–55} However, the instrumentation of FRET and plasmonic resonance energy transfer relies on a slow camerabased approach that is not amenable to probing targets freely diffusing in the 3D space and providing any rotational information. Our 3D-2C-DPT system not only extends the accessible distance up to 250 nm with tracking precision within 40 nm but also allows for the direct observation of DNA flexing dynamics in free solution.

The TSUNAMI system is capable of having an arbitrary number of detectors limited only by the spectral overlap of conventional fluorophores (typically a maximum of 4). Built upon two-photon microscopy, both the 3D orbital tracking system developed by the Gratton group⁵⁶ and the TSUNAMI system developed by us use a non-descanned detection configuration that bypasses the need of a pinhole for spatial filtering, which not only enables the collection of scattered photon (using a PMT) but also facilitates the colocalized detection with additional detectors for multicolor experiments. Both TSUNAMI and the 3D orbital tracking systems have added a second detector (this work) or a second spectroscope⁵⁶ for dual-particle tracking or particle emission spectrum measurement. In contrast, for confocal-feedback SPT using avalanche photodiodes, 14,40,57 the addition of a second color channel requires 3-5 extra avalanche photodiodes for spatial filtering,⁵⁸ thus increasing the difficulty in optics alignment. For camera-based tracking techniques, the multicolor detection has been made possible by various methods, including imaging with multiple cameras,⁵⁹ segmented dedicated fields of view,⁷ diffractive optics,⁶⁰ and point-spread-function engineering.⁶¹ However, the use of a camera limits the temporal resolution to 50 ms⁶¹ and the working depth in scattering samples.⁶²

Over the past three decades, there have been remarkable developments in single-molecule detection techniques that have revealed the mechanical properties of DNA. These approaches can be generally divided into two categories: active (with an external force on DNA) and passive (no applied force) methods (see Maher's review article⁶³). The singlemolecule force spectroscopies have revealed highly dynamic and mechanical processes of DNA conformational changes and DNA-protein interactions at the molecular level (see Nagy's review⁶⁴). However, single-molecule force spectroscopies cannot be used to study the mechanics of DNA molecules shorter than its persistence length (150 bp).⁶⁵ Passive methods would be the more appropriate approaches to quantify the inherent flexibility and bendability of DNA at short length scales (within 150 bp). Recently, Ha's group developed a single-molecule FRET cyclization assay for monitoring the cyclization of single DNA molecules in real-time and quantified the looping and unlooping rates at the single-molecule level.⁶⁶ Whereas this study identified the extreme bendability of dsDNA shorter than 100 bp, the DNA samples were tethered to the surface of coverslips, which means that the DNA molecules were constrained in a hemispherical space for conformational changes. In contrast, our 3D-2C-DPT technique is able to measure the end-to-end distance of freely diffusing DNA molecules at the temporal resolution of 5 ms with a tracking precision of \sim 35 nm. Nevertheless, the use of fluorescent nanoparticles could have an impact on the dynamics of DNA molecules.

Despite the extensive efforts that have been made in recent years to study the translational dynamics of biomolecules on plasma membrane,² only a few research groups have reported the rotational movement. The Sandoghdar group demonstrated that the SV40 viral particle exhibited sliding, tumbling, and rocking motions on lipid bilayers.²⁰ The Yu group monitored the rotational dynamics of receptor-mediated particle uptake in live cells⁶⁷ and interrogated the interaction of ligand–receptor binding.²¹ The Fang group quantified the rotational dynamics of gold nanorods during endocytosis.³⁵ These few studies have successfully demonstrated the rotational motion during particle-receptor interaction on the biomembrane. However, the dynamics of a particle landing on the plasma membrane is still unclear. Whereas the Yang¹⁴ and Welsher¹⁵ groups revealed the translational movement of the landing of viral-like NPS on a 3D cell surface using 3D SPT, to the best of our knowledge, the transition from rotational Brownian diffusion to binding to the plasma membrane has never been captured using a high-spatiotemporal resolution particle tracking technique. In this study, using our 3D-2C-DPT technique, we have successfully observed the transition from rotational Brownian diffusion to binding to the plasma membrane during the landing of an antibody-conjugated NP.

The parallax-quantified differential interference contrast (DIC) microscope developed by the Fang group is capable of visualizing the rotation movement of transferrin-coated gold nanorods in 3D at millisecond temporal resolution.³⁵ While their method surpasses the issue of photobleaching and provides infinite observation window in time, our technique is advantageous in several aspects: (i) better temporal resolution (5 ms vs 33 ms) with a comparable spatial resolution (static tracking precision: 15 nm vs 12 nm in *xy*, 34 nm vs 17 nm in *z*), (ii) the capability of measuring the separation distance between paired particles, and (iii) fully differentiable rotational angles (azimuthal: 2π vs $\pi/2$;

elevation: π vs $\pi/2$). Regarding rotational angles, we calculate the angles based on the coordinates of particles, but Fang's method determined the orientation of a gold nanorod by solving two even functions related to intensities of DIC images. Therefore, Fang's method can only determine the rotational angles within the ranges from 0 to $\pi/2$.

CONCLUSIONS

In summary, we developed and validated a 3D-2C-DPT technique for the visualization of translation and rotational motions of dumbbell-shaped tracers, which enabled us to investigate the DNA bending kinetics in a free solution as an optical ruler. Furthermore, we demonstrated the capability of monitoring the landing of the anti-EGFR IgG-conjugated dumbbell-shaped tracers on the plasma membrane and revealed that the interactions between antibody and EGFRs confined the translational and rotational motions of tracers. These results demonstrate that our 3D-2C-DPT technique is a powerful tool to study the conformational changes of biomolecules and the intermolecular interactions on plasma membrane.

MATERIALS AND METHODS

Sample Preparation of the Dual-Colored DNA Linker. Biotinylated single-stranded DNA (ssDNA) was conjugated onto 40 nm NeutrAvidin-labeled red fluorescent NPs (F8770, Thermo Fisher Scientific) and 40 nm NeutrAvidin-labeled green fluorescent NPs (F8771, Thermo Fisher Scientific) separately at a 1:2.3 ratio (10 μ L of 0.1 μ M NPs mixed with 10 μ L of 0.23 μ M ssDNA in 1× TE buffer). The two solutions were left to conjugate overnight in the vortex mixer before saturating the biotin-binding site with 10 μ L of 1 mM mPEG-Biotin (Methoxy PEG Biotin, Molecular Weight 1000, PG1-BN-1k, NANOCS). The samples were then returned to the vortex mixer for a few hours before combining the green and red nanoparticles and the target 150 nt ssDNA stoichiometrically (1:1:1). The sample is once again returned to the vortex mixer for another night, making sure to prevent light from reaching the samples. In order to obtain the best density for imaging, the two-color dsDNAlinked dumbbell-like dimer was diluted down 2000 times and mixed into 1× TE buffer with various concentrations of dextran (dextran-40, Cat. 1179708; dextran-450, Cat. 31392, Sigma-Aldrich) for the freely diffusing experiment before being transferred onto optical imaging 8well chambered coverglass (155409, Thermo Fisher Scientific). For antibody-conjugated two-color dsDNA-linked dumbbell-dimer, the mPEG on the green fluorescent NPs was replaced by biotinylated anti-EGFR monoclonal antibodies (Clone Ab-3, MS-311-B, Thermo Fisher Scientific). The green fluorescent NP was incubated with 1 μ M anti-EGFR antibody instead of mPEG-biotin.

Setup of TSUNAMI Microscope. TSUNAMI (Tracking of Single particles Using Nonlinear And Multiplexed Illumination) is a feedback-control tracking system which employs a spatiotemporally multiplexed two-photon excitation and temporally demultiplexed detection scheme (Figure 1A). Sub-millisecond temporal resolution (under high signal-to-noise conditions) and subdiffraction tracking precision in all three dimensions have been previously demonstrated.^{41,68} Tracking can be performed in a live cell to monitor the movements of fluorescent nanoparticle-tagged EGFRs^{41,69,70} or ballistically injected fluorescent NPs.¹⁹

The TSUNAMI microscope has been described in detail in our previous study.⁴¹ In brief, excitation of 835 nm from a $\text{Ti:Al}_2\text{O}_3$ laser (Mira 900, Coherent) was used for tracking experiments. The optical multiplexer split the primary laser beam into 4 beams which were then delayed by 3.3, 6.6, and 9.9 ns (relative to Beam 1), respectively. These 4 beams then passed through a galvo scanning system (6125H, Cambridge Technology) and were focused through a 60× N.A. 1.3 silicone oil objective (UPLSAPO 60×, Olympus) to create a tetrahedral excitation profile (Figure 1B). The photon-count rates

in the four time-gated domains fluctuate according to the position of the particle in the excitation profile (Figure 1C). The laser power was adjusted on the basis of the sizes of fluorescent beads to control photon count rates within the range 200–900 kHz. For 40 nm (F8771, Thermo Fisher Scientific) and 200 nm (F8811, Thermo Fisher Scientific) green fluorescent beads, we use an average laser power of \sim 2 and \sim 0.2 mW per beam at the objective back aperture, respectively. The background fluorescence signal is on the order of 5 kHz.

Three-Dimensional Two-Color Dual-Particle Tracking Microscope (3D-2C-DPT). To perform dual-particle localization with the TSUNAMI microscope, we implemented an additional PMT (PMT2, Figure 1A) and a TCSPC routing module (a schematic shown in Figure S1) in the TSUNAMI microscope. The second PMT collects the light emitted from the second fluorophore in a separate spectral channel (G1b-G4b, Figure 1C). The routing module (HRT-41, Becker and Hickl) is required for routing the second detection channel to the same TCSPC board (SPC-150, Becker and Hickl). Additionally, an algorithm is needed to register the raw photon-count rate from the second channel within the detection profile that locks on the primary tracking target. The algorithm works to coregister the location of the secondary particle relative to the primary particle (Figure 1D), which means that the system would not be able to track the secondary particle if it moves out of the excitation profile which locks on the primary target.⁷¹ The coregistration of the secondary target is achieved by taking the ratio of the summed photon counts in each of the four time gates in the second PMT. Then, the location of the secondary target is coregistered relative to the primary target following the formulas below.

$$\widehat{x_a} = K_x E_{xa} = K_x \frac{(G_{2a} - G_{1a})}{(G_{2a} + G_{1a})}$$
(1)

$$\widehat{y_a} = K_y E_{ya} = K_y \frac{(G_{4a} - G_{3a})}{(G_{4a} + G_{3a})}$$
(2)

$$\widehat{z_a} = K_z E_{za} = K_z \frac{(G_{4a} + G_{3a}) - (G_{2a} + G_{1a})}{(G_{1a} + G_{2a} + G_{3a} + G_{4a})}$$
(3)

$$\widehat{x_{b}} = \widehat{x_{a}} + K_{x}E_{xb} = \widehat{x_{a}} + K_{x}\frac{(G_{2b} - G_{1b})}{(G_{2b} + G_{1b})}$$
(4)

$$\widehat{y_b} = \widehat{y_a} + K_y E_{yb} = \widehat{y_a} + K_y \frac{(G_{4b} - G_{3b})}{(G_{4b} + G_{3b})}$$
(5)

$$\widehat{z_b} = \widehat{z_a} + K_z E_{zb} = \widehat{z_a} + K_z \frac{(G_{4b} + G_{3b}) - (G_{2b} + G_{1b})}{(G_{1b} + G_{2b} + G_{3b} + G_{4b})}$$
(6)

Here, $(G_{1a}, G_{2a}, G_{3a}, G_{4a})$ and $(G_{1b}, G_{2b}, G_{3b}, G_{4b})$ were the total photon counts in each time gate from PMT1 and PMT2. Error signals (E_{xa}, E_{ya}, E_{za}) and (E_{xb}, E_{yb}, E_{zb}) were multiplied by proportional constants (K_x, K_y, K_z) to estimate the location. This dual particle tracking setup and technique allow us to obtain key information about the two-color dsDNA-linked dumbbell-like dimer, such as the distance between the ends of the DNA linker and the orientation of the DNA linker in 3D space in a freely diffusing solution (Figure 1F).

Calibration for Two-Color Localization. At the start of each tracking experiment, we calibrated the tetrahedral profile to obtain consistent and optimal tracking precision. A coarse realignment with the 200 nm green beads was conducted before tracking fixed 40 and 200 nm green beads to acquire the data needed for calibration (Figure 2). Using the calibration data, we determined the fluorescence intensity scaling factor, the crosstalk correction factor (Figure S3), and the proportional constant (Note S3). Finally, the coregistration of two spectrally distinct NPs was validated using an image-based colocalization obtained by overlaying the trajectory with 2-photon scanning results (Figure 3).

www.acsnano.org

Two-Photon Laser Scanning Microscope (2P-LSM). The excitation light for 2P-LSM shared the same path as the nondelayed beam in the multiplexer, and the other three beams were blocked with shutters during laser scanning. The emission light was collected through the same objective, split into three color bands by two dichroic mirrors, and then condensed onto each PMT (H7422PA-40, Hamamatsu). The first dichroic mirror picks of the blue-green spectrum with a reflection edge at 550 nm (FF552-Di02-25×36, Semrock), and the second dichroic splits the remaining light into orange and red components with an edge at 640 nm (FF640-FDi01-25×36, Semrock). Each PMT has a bandpass filter specific to the fluorescent targets used for the experiment: 520/44 nm single-band bandpass filter (FF01-520/44-25, Semrock) for nuclear staining (Hochest 33258, H3569, Thermo Fisher Scientific) and green fluorescent NP (F8780, Thermo Fisher Scientific), 605/64 nm single-band bandpass filter (FF01-605/64, Semrock) for red fluorescent NP (F8770, Thermo Fisher Scientific), and 675/67 nm single-band bandpass filter (FF02-675/67, Semrock) for membrane stain (Cellmask Deep Red, C10046, Thermo Fisher Scientific). The mirror scanning and data acquisition were handled by a single DAQ card (PCIe-6353, National Instruments) with a custom code interface in LabVIEW. Pixel dwell times were 2.6 μ s which correspond to a frame rate of approximately 1.4 Hz. Image sizes were typically 34×34 μm^2 (512 × 512 pixels).

We use detector 1 for both 2P-LSM and 2P-3D-DPT. Because we only use one of the four beams (the nondelayed beam) for 2P-LSM, the shift between the nondelayed beam and the centroid of the tetrahedral profile is 250 nm in the *xy* plane and 500 nm in *z*. The Abbe diffraction limits for our 2P-LSM are ~260 nm in *xy* and ~500 nm in *z* (objective lens of 1.3 NA and a peak emission of 675 nm from Cellmask deep). Therefore, the shift was corrected with the coregistration of trajectories and 3D-stacked images.

The 2P-LSM raw images were read into MATLAB from binary files and denoised with a median filter before a 1D interpolation along the z dimension. To segment the cellular compartments, we used a simple intensity threshold technique that converts the image to a binary. Thresholds were selected at each z plane to account for variation in noise and brightness through the z-stack. The binary images were used as a mask to plot the cell isocontour. The trajectories were acquired with the same analog output device as the 2P-LSM images, and they were directly overlaid with the cell compartment isocontour with no conversion or scaling required.

Extracting Dynamic Parameters from MSD and MASD. The typical approach to analyze the translational movement of a single-particle trajectory started with the calculation of mean-squared displacement (MSD),^{72,73} which describes the average squared distance $(d^2, r\Delta \vec{\varphi}(t))$ is the position vector) that the particle has explored in space at a given time lag (Δt):

$$\langle \Delta \vec{r}^2(t) \rangle = \langle (\vec{r}(t + \Delta t) - \vec{r}(t))^2 \rangle \tag{7}$$

The translational diffusivity (D_t) is extracted from 3D trajectories by fitting the MSD curves. The D_t was defined as the linear MSD fitting result of the first 5 MSD points:⁶⁹

$$\lim_{t \to \infty} \langle \Delta \vec{r}^2(t) \rangle = \langle (r(t + \Delta t) - r(t))^2 \rangle = 6D_t \Delta t$$
(8)

To reveal the rotational movement, we applied the mean-squared angular displacement (MSAD) method to analyzing the rotation of the dumbbell-shaped dimer.²⁸ The tracer's orientation vector, \hat{u} , was defined as the vector that extends from the centroid of a green bead to the centroid of a red bead (Figure 1D). The cross-product $\hat{u} \times \hat{u}(t + \Delta t)$ gives the direction of the instantaneous axis of rotation for a vector rotational displacement $\Delta \vec{\varphi}(t)$, whose magnitude is given by $y |\Delta \vec{\varphi}(t)| = \cos^{-1}(\hat{u} \cdot \hat{u}(t + \Delta t))$. The total angular displacement is calculated with the following integral:⁷⁴

$$\vec{\rho}(t) = \int_0^t \Delta \vec{\varphi}(t') \, \mathrm{d}t' \tag{9}$$

The unbounded MSAD is then given by

$$\langle \Delta \vec{\varphi}^2(t) \rangle = \langle [\vec{\varphi}(t + \Delta t) - \vec{\varphi}(t)]^2 \rangle \tag{10}$$

$$\lim_{t \to \infty} \langle \Delta \vec{\varphi}^2(t) \rangle = 4D_t \Delta t \tag{11}$$

Thus, we were able to measure the translational and rotational diffusivities through MSD and MSAD, respectively.

Measurement of DNA Bending Kinetics. To characterize the DNA bending dynamics, we developed an algorithm that was adapted from an empirical Bayesian hidden Markov model (HMM). This Bayesian model was first introduced to analyze single-molecule FRET time traces.⁴⁵ We have recently used this HMM approach to characterize the hybridization kinetics of single diffusing DNA strands in solution⁴⁶ and in live cells.⁷⁵ Instead of measuring changes in FRET efficiency, our method quantified the transitions between stacked and unstacked dsDNA by measuring the end-to-end distance of dual particle-tagged dsDNA (Note S4). Since the HMM only reports the estimated transition matrix, instead of the stacking-unstacking rates of our interest, a conversion is needed. The conversion from the transition matrix to transition rates is described in Note S5. Therefore, the rates of transition between conformational states of DNA bending, $k_{\rm on}$ (stacked to unstacked) and $k_{\rm off}$ (unstacked to stacked), were extracted from the HMM analyses. The histograms of the end-to-end distance of DNA probes were built from the traces of two-color dualparticle trajectories. The histograms were then fitted by a Gaussian mixture model with two components.

3D-2C-DPT on Live Cells. A prostate cancer cell line, LNCaP, was a gift from Dr. Chun-Liang Chen at the University of Texas Health Science Center at San Antonio. LNCaP cells were grown in RPMI1640 (22400089, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (16140-071, Thermo Fisher Scientific) and 1% penicillin-streptomycin (15140122, Thermo Fisher Scientific). For tracking experiments, cells were seeded onto optical imaging 8well chambered coverglass (155411, Thermo Fisher Scientific). The maximum diffusivity measurable is approximately 4.3 μ m²/s as determined previously.⁴¹ To slow down the fast-moving NP (diffusivity of ~2-5 μ m²/s in water), we conducted the tracking experiment in the cell culture medium supplemented with the 30% dextran-40 while maintaining the same level of physiological osmolarity (300 mOsm). Staining of the plasma membrane (Cellmask Deep Red, C10046, Thermo Fisher Scientific) and nucleus (Hochest 33258, H3569, Thermo Fisher Scientific) allows the coregistration of the trajectory with cellular landmarks. 2P-LSM images were taken of the surrounding cellular environment before tracking to reconstruct the 3D cell images. Then, the trajectory and 3D stacked cell images were coregistered by postprocessing.

Hoechst 33258 and Cellmask Deep Red were commonly used for staining live cells. Dextran-40 was approved by the FDA for the treatment of shock or impending shock, venous thrombosis, and pulmonary embolism through intravenous administration. The osmolarity of the cell medium with 30% dextran-40 was maintained at the same level as the physiological osmolarity (300 mOsm). The live cells were stained right before the tracking experiment, and both staining and tracking were completed within 30 min. More than 90% of cells survived after exposure to Hoechst, Cellmask Deep Red, and dextran sulfate for 90 min, agreeing well with the previous reports. $^{76-78}$

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.9b08045.

Minimum and maximum separation distance, accuracy, and precision of the DPT tracking system; calibration of two-color colocalization; Bayesian method to measure DNA bending kinetics; conversion of transition matrix to stacking–unstacking rates; TSUNAMI feedback control schematic for two-color dual-particle-tracking; crosstalk of green fluorescence emission shortens the estimated distance between green and red fluorescent particles; and DNA sequence $(\ensuremath{\text{PDF}})$

Movie S1: multiplexed excitation and demultiplexed detection of 3D-2C-DPT (MP4)

Movie S2: 3D-2C-DPT of a dumbbell-like dimer in 30% Dextran-450 (MP4)

Movie S3: landing of an anti-EGFR-conjugated dimer on living cells 1 (MP4)

Movie S4: landing of an anti-EGFR-conjugated dimer on living cells 2 (MP4)

Movie S5: landing of an anti-EGFR-conjugated dimer on living cells 3 (MP4)

Movie S6: landing of an anti-EGFR-conjugated dimer on living cells 4 (MP4)

AUTHOR INFORMATION

Corresponding Authors

Yen-Liang Liu – Graduate Institute of Biomedical Sciences and Center for Molecular Medicine, China Medical University, Taichung 40402, Taiwan; Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States; ◎ orcid.org/0000-0002-1635-4335; Phone: +886-912378780; Email: allen.liu@mail.cmu.edu.tw

Hsin-Chih Yeh – Department of Biomedical Engineering and Texas Materials Institute, The University of Texas at Austin, Austin, Texas 78712, United States; orcid.org/0000-0001-6654-5626; Phone: +1-512-471-7931; Email: tim.yeh@ austin.utexas.edu; Fax: +1-512-471-0616

Authors

Evan P. Perillo – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States; Nanostring Technologies, Seattle, Washington 98109, United States

Phyllis Ang – Department of Electrical and Computer Engineering, The University of Texas at Austin, Austin, Texas 78712, United States; Department of Computer Science, Duke University, Durham, North Carolina 27705, United States

Mirae Kim – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Duc Trung Nguyen – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Katherine Blocher – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Yu-An Chen – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Cong Liu – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Ahmed M. Hassan – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Huong T. Vu – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Yuan-I Chen – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States Andrew K. Dunn – Department of Biomedical Engineering, The University of Texas at Austin, Austin, Texas 78712, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acsnano.9b08045

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Robert A. Welch Foundation to H.-C.Y. (F-1833), the Texas 4000 to H.-C.Y., National Institutes of Health to H.-C.Y. (GM129617) and A.K.D. (NS108484 and NS082518), and National Science Foundation to H.-C.Y. (1611451). Y.-L.L. is a recipient of the YingTsai Young Scholar Award of China Medical University (CMU108-YTY-01) and also a recipient of the Young Scholar Fellowship Program from the Ministry of Science and Technology (MOST) in Taiwan (MOST 108-2636-E-039-001).

REFERENCES

(1) Kusumi, A.; Tsunoyama, T. A.; Hirosawa, K. M.; Kasai, R. S.; Fujiwara, T. K. Tracking Single Molecules at Work in Living Cells. *Nat. Chem. Biol.* **2014**, *10*, 524–532.

(2) Manzo, C.; Garcia-Parajo, M. F. A Review of Progress in Single Particle Tracking: From Methods to Biophysical Insights. *Rep. Prog. Phys.* **2015**, *78*, 124601.

(3) Gelles, J.; Schnapp, B. J.; Sheetz, M. P. Tracking Kinesin-Driven Movements with Nanometre-Scale Precision. *Nature* **1988**, *331*, 450– 453.

(4) Yildiz, A.; Forkey, J. N.; McKinney, S. A.; Ha, T.; Goldman, Y. E.; Selvin, P. R. Myosin V Walks Hand-Over-Hand: Single Fluorophore Imaging with 1.5-nm Localization. *Science* **2003**, *300*, 2061–2065.

(5) Fujiwara, T.; Ritchie, K.; Murakoshi, H.; Jacobson, K.; Kusumi, A. Phospholipids Undergo Hop Diffusion in Compartmentalized Cell Membrane. J. Cell Biol. **2002**, *157*, 1071–1082.

(6) Dahan, M.; Levi, S.; Luccardini, C.; Rostaing, P.; Riveau, B.; Triller, A. Diffusion Dynamics of Glycine Receptors Revealed by Single-Quantum Dot Tracking. *Science* **2003**, *302*, 442–445.

(7) Andrews, N. L.; Lidke, K. A.; Pfeiffer, J. R.; Burns, A. R.; Wilson, B. S.; Oliver, J. M.; Lidke, D. S. Actin Restricts FcepsilonRI Diffusion and Facilitates Antigen-Induced Receptor Immobilization. *Nat. Cell Biol.* **2008**, *10*, 955–963.

(8) Shav-Tal, Y.; Darzacq, X.; Shenoy, S. M.; Fusco, D.; Janicki, S. M.; Spector, D. L.; Singer, R. H. Dynamics of Single mRNPs in Nuclei of Living Cells. *Science* **2004**, *304*, 1797–1800.

(9) Park, H. Y.; Lim, H.; Yoon, Y. J.; Follenzi, A.; Nwokafor, C.; Lopez-Jones, M.; Meng, X.; Singer, R. H. Visualization of Dynamics of Single Endogenous mRNA Labeled in Live Mouse. *Science* **2014**, *343*, 422–424.

(10) Lakadamyali, M.; Rust, M. J.; Babcock, H. P.; Zhuang, X. Visualizing Infection of Individual Influenza Viruses. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 9280–9285.

(11) Arhel, N.; Genovesio, A.; Kim, K.-A.; Miko, S.; Perret, E.; Olivo-Marin, J.-C.; Shorte, S.; Charneau, P. Quantitative Four-Dimensional Tracking of Cytoplasmic and Nuclear HIV-1 Complexes. *Nat. Methods* **2006**, *3*, 817.

(12) Knight, S. C.; Xie, L.; Deng, W.; Guglielmi, B.; Witkowsky, L. B.; Bosanac, L.; Zhang, E. T.; El Beheiry, M.; Masson, J.-B.; Dahan, M. Dynamics of CRISPR-Cas9 Genome Interrogation in Living Cells. *Science* **2015**, *350*, 823–826.

(13) Zhou, Y.; Wang, P.; Tian, F.; Gao, G.; Huang, L.; Wei, W.; Xie, X. S. Painting a Specific Chromosome with CRISPR/Cas9 for Live-Cell Imaging. *Cell Res.* **2017**, *27*, 298.

(14) Welsher, K.; Yang, H. Multi-Resolution 3D Visualization of the Early Stages of Cellular Uptake of Peptide-Coated Nanoparticles. *Nat. Nanotechnol.* **2014**, *9*, 198–203.

(15) Hou, S.; Welsher, K. An Adaptive Real-Time 3D Single Particle Tracking Method for Monitoring Viral First Contacts. *Small* **2019**, *15*, 1903039.

(16) Guo, M.; Ehrlicher, A. J.; Jensen, M. H.; Renz, M.; Moore, J. R.; Goldman, R. D.; Lippincott-Schwartz, J.; Mackintosh, F. C.; Weitz, D. A. Probing the Stochastic, Motor-Driven Properties of the Cytoplasm Using Force Spectrum Microscopy. *Cell* **2014**, *158*, 822–832.

(17) Panorchan, P.; Lee, J. S.; Kole, T. P.; Tseng, Y.; Wirtz, D. Microrheology and ROCK Signaling of Human Endothelial Cells Embedded in a 3D Matrix. *Biophys. J.* **2006**, *91*, 3499–507.

(18) Fakhri, N.; Wessel, A. D.; Willms, C.; Pasquali, M.; Klopfenstein, D. R.; MacKintosh, F. C.; Schmidt, C. F. High-Resolution Mapping of Intracellular Fluctuations Using Carbon Nanotubes. *Science* **2014**, *344*, 1031–1035.

(19) Le, V.; Lee, J.; Chaterji, S.; Spencer, A.; Liu, Y.-L.; Kim, P.; Yeh, H.-C.; Kim, D.-H.; Baker, A. B. Syndecan-1 in Mechanosensing of Nanotopological Cues in Engineered Materials. *Biomaterials* **2018**, *155*, 13–24.

(20) Kukura, P.; Ewers, H.; Müller, C.; Renn, A.; Helenius, A.; Sandoghdar, V. High-Speed Nanoscopic Tracking of the Position and Orientation of a Single Virus. *Nat. Methods* **2009**, *6*, 923.

(21) Yu, Y.; Gao, Y.; Yu, Y. "Waltz" of Cell Membrane-Coated Nanoparticles on Lipid Bilayers: Tracking Single Particle Rotation in Ligand–Receptor Binding. *ACS Nano* **2018**, *12*, 11871–11880.

(22) Sun, H.; Wang, Z.; He, Y. Direct Observation of Spatiotemporal Heterogeneous Gelation by Rotational Tracking of a Single Anisotropic Nanoprobe. *ACS Nano* **2019**, *13*, 11334–11342.

(23) Gao, Y.; Yu, Y.; Sanchez, L.; Yu, Y. Seeing the Unseen: Imaging Rotation in Cells with Designer Anisotropic Particles. *Micron* 2017, 101, 123–131.

(24) Anker, J. N.; Kopelman, R. Magnetically Modulated Optical Nanoprobes. *Appl. Phys. Lett.* **2003**, *82*, 1102–1104.

(25) Anthony, S. M.; Hong, L.; Kim, M.; Granick, S. Single-Particle Colloid Tracking in Four Dimensions. *Langmuir* **2006**, *22*, 9812–9815.

(26) Sanchez, L.; Yi, Y.; Yu, Y. Effect of Partial PEGylation on Particle Uptake by Macrophages. *Nanoscale* **201**7, *9*, 288–297.

(27) Hunter, G. L.; Edmond, K. V.; Elsesser, M. T.; Weeks, E. R. Tracking Rotational Diffusion of Colloidal Clusters. *Opt. Express* **2011**, *19*, 17189–17202.

(28) Edmond, K. V.; Elsesser, M. T.; Hunter, G. L.; Pine, D. J.; Weeks, E. R. Decoupling of Rotational and Translational Diffusion in Supercooled Colloidal Fluids. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 17891–17896.

(29) Fung, J.; Martin, K. E.; Perry, R. W.; Kaz, D. M.; McGorty, R.; Manoharan, V. N. Measuring Translational, Rotational, and Vibrational Dynamics in Colloids with Digital Holographic Microscopy. *Opt. Express* **2011**, *19*, 8051–8065.

(30) Yu, X.; Hong, J.; Liu, C.; Cross, M.; Haynie, D. T.; Kim, M. K. Four-Dimensional Motility Tracking of Biological Cells by Digital Holographic Microscopy. *J. Biomed. Opt.* **2014**, *19*, 045001.

(31) Yamamoto, J.; Oura, M.; Yamashita, T.; Miki, S.; Jin, T.; Haraguchi, T.; Hiraoka, Y.; Terai, H.; Kinjo, M. Rotational Diffusion Measurements Using Polarization-Dependent Fluorescence Correlation Spectroscopy Based on Superconducting Nanowire Single-Photon Detector. *Opt. Express* **2015**, *23*, 32633–32642.

(32) Karedla, N.; Stein, S. C.; Hähnel, D.; Gregor, I.; Chizhik, A.; Enderlein, J. Simultaneous Measurement of the Three-Dimensional Orientation of Excitation and Emission Dipoles. *Phys. Rev. Lett.* **2015**, *115*, 173002.

(33) Uspal, W. E.; Eral, H. B.; Doyle, P. S. Engineering Particle Trajectories in Microfluidic Flows Using Particle Shape. *Nat. Commun.* **2013**, *4*, 2666.

(34) Liu, B.; Böker, A. Measuring Rotational Diffusion of Colloidal Spheres with Confocal Microscopy. *Soft Matter* **2016**, *12*, 6033–6037.

(35) Chen, K.; Gu, Y.; Sun, W.; Wang, G.; Fan, X.; Xia, T.; Fang, N. Characteristic Rotational Behaviors of Rod-shaped Cargo Revealed by Automated Five-dimensional Single Particle Tracking. *Nat. Commun.* **2017**, *8*, 887.

(36) Berg, H. C. How to Track Bacteria. *Rev. Sci. Instrum.* 1971, 42, 868-871.

(37) Levi, V.; Ruan, Q.; Kis-Petikova, K.; Gratton, E. Scanning FCS, a Novel Method for Three-Dimensional Particle Tracking. *Biochem. Soc. Trans.* **2003**, *31*, 997–1000.

(38) Cang, H.; Wong, C. M.; Xu, C. S.; Rizvi, A. H.; Yang, H. Confocal Three Dimensional Tracking of a Single Nanoparticle with Concurrent Spectroscopic Readouts. *Appl. Phys. Lett.* **2006**, *88*, 223901.

(39) Lessard, G. A.; Goodwin, P. M.; Werner, J. H. Three-Dimensional Tracking of Individual Quantum Dots. *Appl. Phys. Lett.* **2007**, *91*, 224106.

(40) Wells, N. P.; Lessard, G. A.; Goodwin, P. M.; Phipps, M. E.; Cutler, P. J.; Lidke, D. S.; Wilson, B. S.; Werner, J. H. Time-Resolved Three-Dimensional Molecular Tracking in Live Cells. *Nano Lett.* **2010**, *10*, 4732–4737.

(41) Perillo, E. P.; Liu, Y.-L.; Huynh, K.; Liu, C.; Chou, C.-K.; Hung, M.-C.; Yeh, H.-C.; Dunn, A. K. Deep and High-resolution Three-Dimensional Tracking of Single Particles Using Nonlinear and Multiplexed Illumination. *Nat. Commun.* **2015**, *6*, 7874.

(42) Hou, S.; Lang, X.; Welsher, K. Robust Real-Time 3D Single-Particle Tracking Using a Dynamically Moving Laser Spot. *Opt. Lett.* **2017**, *42*, 2390–2393.

(43) Roll, C.; Ketterlé, C.; Faibis, V.; Fazakerley, G. V.; Boulard, Y. Conformations of Nicked and Gapped DNA Structures by NMR and Molecular Dynamic Simulations in Water. *Biochemistry* **1998**, *37*, 4059–4070.

(44) Protozanova, E.; Yakovchuk, P.; Frank-Kamenetskii, M. D. Stacked–Unstacked Equilibrium at the Nick Site of DNA. *J. Mol. Biol.* **2004**, *342*, 775–785.

(45) van de Meent, J.-W.; Bronson, J. E.; Wiggins, C. H.; Gonzalez, R. L., Jr Empirical Bayes Methods Enable Advanced Population-Level Analyses of Single-Molecule FRET Experiments. *Biophys. J.* 2014, 106, 1327–1337.

(46) Liu, C.; Obliosca, J. M.; Liu, Y.-L.; Chen, Y.-A.; Jiang, N.; Yeh, H.-C. 3D Single-Molecule Tracking Enables Direct Hybridization Kinetics Measurement in Solution. *Nanoscale* **2017**, *9*, 5664–5670.

(47) Förster, T. Experimentelle und Theoretische Untersuchung des Zwischenmolekularen Übergangs von Elektronenanregungsenergie. *Z. Naturforsch. A* 1949, *4* (5), 321–327.

(48) Stryer, L.; Haugland, R. P. Energy Transfer: A Spectroscopic Ruler. *Proc. Natl. Acad. Sci. U. S. A.* **1967**, *58*, 719–726.

(49) Stryer, L. Fluorescence Energy Transfer as a Spectroscopic Ruler. Annu. Rev. Biochem. 1978, 47, 819-846.

(50) Ha, T.; Enderle, T.; Ogletree, D.; Chemla, D. S.; Selvin, P. R.; Weiss, S. Probing the Interaction Between Two Single Molecules: Fluorescence Resonance Energy Transfer between a Single Donor and a Single Acceptor. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, 93, 6264–6268.

(51) Weiss, S. Fluorescence Spectroscopy of Single Biomolecules. Science 1999, 283 (5408), 1676–1683.

(52) Zhuang, X.; Bartley, L. E.; Babcock, H. P.; Russell, R.; Ha, T.; Herschlag, D.; Chu, S. A Single-Molecule Study of RNA Catalysis and Folding. *Science* **2000**, *288*, 2048–2051.

(53) Sönnichsen, C.; Reinhard, B. M.; Liphardt, J.; Alivisatos, A. P. A Molecular Ruler Based on Plasmon Coupling of Single Gold and Silver Nanoparticles. *Nat. Biotechnol.* **2005**, *23*, 741–745.

(54) Reinhard, B. M.; Siu, M.; Agarwal, H.; Alivisatos, A. P.; Liphardt, J. Calibration of Dynamic Molecular Rulers Based on Plasmon Coupling Between Gold Nanoparticles. *Nano Lett.* **2005**, *5*, 2246–2252.

(55) Yun, C.; Javier, A.; Jennings, T.; Fisher, M.; Hira, S.; Peterson, S.; Hopkins, B.; Reich, N.; Strouse, G. Nanometal Surface Energy Transfer in Optical Rulers, Breaking the FRET Barrier. *J. Am. Chem. Soc.* **2005**, *127*, 3115–3119.

(56) Hellriegel, C.; Gratton, E. Real-Time Multi-Parameter Spectroscopy and Localization in Three-Dimensional Single-Particle Tracking. J. R. Soc., Interface **2009**, *6*, S3–S14.

(57) Sahl, S. J.; Leutenegger, M.; Hilbert, M.; Hell, S. W.; Eggeling, C. Fast Molecular Tracking Maps Nanoscale Dynamics of Plasma Membrane Lipids. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 6829–6834.

(58) Keller, A. M.; DeVore, M. S.; Stich, D. G.; Vu, D. M.; Causgrove, T.; Werner, J. H. Multicolor 3-Dimensional Tracking for Single-Molecule Fluorescence Resonance Energy Transfer Measurements. *Anal. Chem.* **2018**, *90*, 6109–6115.

(59) Suzuki, K. G.; Fujiwara, T. K.; Sanematsu, F.; Iino, R.; Edidin, M.; Kusumi, A. GPI-Anchored Receptor Clusters Transiently Recruit Lyn and $G\alpha$ for Temporary Cluster Immobilization and Lyn Activation: Single-Molecule Tracking Study 1. *J. Cell Biol.* **2007**, 177, 717–730.

(60) Broeken, J.; Rieger, B.; Stallinga, S. Simultaneous Measurement of Position and Color of Single Fluorescent Emitters Using Diffractive Optics. *Opt. Lett.* **2014**, *39*, 3352–3355.

(61) Shechtman, Y.; Weiss, L. E.; Backer, A. S.; Lee, M. Y.; Moerner, W. Multicolour Localization Microscopy by Point-Spread-Function Engineering. *Nat. Photonics* **2016**, *10*, 590.

(62) Kim, K. H.; Buehler, C.; Bahlmann, K.; Ragan, T.; Lee, W.-C. A.; Nedivi, E.; Heffer, E. L.; Fantini, S.; So, P. T. Multifocal Multiphoton Microscopy Based on Multianode Photomultiplier Tubes. *Opt. Express* **2007**, *15*, 11658–11678.

(63) Peters, J. P.; Maher, L. J. DNA Curvature and Flexibility In Vitro and In Vivo. Q. Rev. Biophys. 2010, 43, 23-63.

(64) Neuman, K. C.; Nagy, A. Single-Molecule Force Spectroscopy: Optical Tweezers, Magnetic Tweezers and Atomic Force Microscopy. *Nat. Methods* **2008**, *5*, 491.

(65) Seol, Y.; Li, J.; Nelson, P. C.; Perkins, T. T.; Betterton, M. Elasticity of Short DNA Molecules: Theory and Experiment for Contour Lengths Of. $6-7 \mu m$. *Biophys. J.* **2007**, 93, 4360–4373.

(66) Vafabakhsh, R.; Ha, T. Extreme Bendability of DNA Less than 100 Base Pairs Long Revealed by Single-Molecule Cyclization. *Science* **2012**, 337, 1097–1101.

(67) Gao, Y.; Yu, Y. How Half-Coated Janus Particles Enter Cells. J. Am. Chem. Soc. 2013, 135, 19091–19094.

(68) Liu, C.; Liu, Y.-L.; Perillo, E. P.; Jiang, N.; Dunn, A.; Yeh, H.-C. Improving Z-Tracking Accuracy in the Two-Photon Single-Particle Tracking Microscope. *Appl. Phys. Lett.* **2015**, *107*, 153701.

(69) Liu, Y.-L.; Perillo, E. P.; Liu, C.; Yu, P.; Chou, C.-K.; Hung, M.-C.; Dunn, A. K.; Yeh, H.-C. Segmentation of 3D Trajectories Acquired by TSUNAMI Microscope: An Application to EGFR Trafficking. *Biophys. J.* **2016**, *111*, 2214–2227.

(70) Perillo, E. P.; Liu, Y.-L.; Liu, C.; Yeh, H.-C.; Dunn, A. K. In Single Particle Tracking Through Highly Scattering Media with Multiplexed Two-Photon Excitation; SPIE BiOS, International Society for Optics and Photonics, 2015; pp 933107–933107-8.

(71) Perillo, E. P. Nonlinear Microscopy Methods for Imaging and Particle Tracking in Thick Biological Specimen. Ph.D. Dissertation, The University of Texas at Austin, Austin, Texas, May 2017.

(72) Martin, D. S.; Forstner, M. B.; Käs, J. A. Apparent Subdiffusion Inherent to Single Particle Tracking. *Biophys. J.* **2002**, *83*, 2109–2117.

(73) Saxton, M. J.; Jacobson, K. Single-Particle Tracking: Applications to Membrane Dynamics. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 373–399.

(74) Mazza, M. G.; Giovambattista, N.; Stanley, H. E.; Starr, F. W. Connection of Translational and Rotational Dynamical Heterogeneities with the Breakdown of the Stokes-Einstein and Stokes-Einstein-Debye Relations in Water. *Phys. Rev. E* 2007, *76*, 031203.

(75) Chen, Y.-I.; Chang, Y.-J.; Nguyen, T. D.; Liu, C.; Phillion, S.; Kuo, Y.-A.; Vu, H. T.; Liu, A.; Liu, Y.-L.; Hong, S. Measuring DNA Hybridization Kinetics in Live Cells Using a Time-Resolved 3D Single-Molecule Tracking Method. *J. Am. Chem. Soc.* **2019**, *141*, 15747–15750.

(76) Fried, J.; Doblin, J.; Takamoto, S.; Perez, A.; Hansen, H.; Clarkson, B. Effects of Hoechst 33342 on Survival and Growth of Two Tumor Cell Lines and on Hematopoietically Normal Bone Marrow Cells. *Cytometry* **1982**, *3*, 42–47.

(77) Choksakulnimitr, S.; Masuda, S.; Tokuda, H.; Takakura, Y.; Hashida, M. *In Vitro* Cytotoxicity of Macromolecules in Different Cell Culture Systems. *J. Controlled Release* **1995**, *34*, 233–241.

(78) Fisher Scientific, Invitrogen CellMask Deep Red Plasma Membrane Stain. https://www.fishersci.ca/shop/products/molecular-probescellmask-deep-red-plasma-membrane-stain/c10046 (accessed on June 28, 2020).

М