Published in final edited form as:

Proc SPIE Int Soc Opt Eng. ; 7185: 71850Z-. doi:10.1117/12.809412.

Going beyond 2D: Following membrane diffusion and topography in the IgE–Fc[Epsilon]RI system using 3-dimensional tracking microscopy

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Abstract

The ability to follow and observe single molecules as they function in live cells represents a major milestone for molecular-cellular biology. Here we present a tracking microscope that is able to track quantum dots in three dimensions and simultaneously record time-resolved emission statistics from a single dot. This innovative microscopy approach is based on four spatial filters and closed loop feedback to constantly keep a single quantum dot in the focal spot. Using this microscope, we demonstrate the ability to follow quantum dot labeled IgE antibodies bound to FcεRI membrane receptors in live RBL-2H3 cells. The results are consistent with prior studies of two dimensional membrane diffusion (Andrews et al., *Nat. Cell Biol.*, **10**, 955, 2008). In addition, the microscope captures motion in the axial (Z) direction, which permits tracking of diffusing receptors relative to the “hills and valleys” of the dynamically changing membrane landscape. This approach is uniquely capable of following single molecule dynamics on live cells with three dimensional spatial resolution.

Keywords

Microscopy; Particle Tracking; Fluorescence; Quantum Dot; Membrane Signaling; Live Cell; RBL-2H3; IgE

1. INTRODUCTION

Fluorescence microscopy on live cells, even at the single molecule level, has seen large growth in a diverse set of applications over the last decade.^{1–5} For example, imaging cellular structure in three dimensions via laser scanning confocal microscopy is routinely accessible with commercial grade instruments. Custom research based tools include a variety of fluorescence correlation spectroscopy (FCS) techniques^{6–13} and single particle tracking (SPT)^{7, 14–19} aimed at measuring intracellular biomolecular dynamics. A major challenge

for applying fluorescence microscopy tools to biological systems, especially on live cells, is to be able to measure dynamics with fast time resolution (nanoseconds to milliseconds) over elapsed times scales that are biologically relevant (milliseconds to minutes). This is especially true for following transport dynamics of biomolecules in the plasma membrane. Using high speed SPT in two dimensions, Kusumi and coworkers have been able to establish a general model of hop diffusion for plasma membrane proteins and phospholipids.^{14, 20–22} However, due to technical limitations (frame storage/camera memory), a single molecule can only be observed for 10's to 100's of milliseconds. Lowering the time resolution can extend observation periods to seconds, but the loss of fast time information obscures the hop diffusion dynamics.^{14, 23}

In addition to the requirements of high time resolution and extended observation periods, live cells are inherently three dimensional, while the majority of existing microscopies are only sensitive to two dimensional (2D) space. Although diffusion on the plasma membrane is generally two dimensional, for many cells (such as a polarized cell), motion that occurs perpendicular to the XY plane may be important. Moreover, subsequent endocytosis of a membrane bound biomolecule allows for three dimensional motion of an endosome within the cell volume. Two dimensional microscopies are unable to follow such motion. A handful of researchers have recently demonstrated microscopes capable of tracking single fluorescent particles in three dimensions (3D).^{24–30} Our 3D-tracking microscope is based on confocal detection and closed loop feedback.^{28–30} Using this approach, we recently demonstrated the ability to track single quantum dots moving at rates comparable to cellular transport processes, even in environments with fluorescent backgrounds significantly higher than those found in live cells.³⁰ A unique and powerful feature of our 3D-tracking microscope includes the capability of recording detected photons as time correlated single photon counting (TCSPC) data, which allows simultaneous time resolved spectroscopy and SPT, but more importantly gives a time-tagged photon stream that potentially allows for sub- μ s positional estimation limited only by emission shot noise.³⁰ This feature of the microscope bridges the timescale gap between molecular spectroscopy and/or fast conformational fluctuations and biological signaling processes.

Here we use this 3D-tracking microscope to follow membrane dynamics of a transmembrane receptor, Fc ϵ RI, in rat mast cells (RBL-2H3). Fc ϵ RI is known to strongly bind IgE antibodies.^{31–34} When IgE–Fc ϵ RI complexes are crosslinked by multivalent antigen, a transmembrane signaling cascade is triggered that induces degranulation and changes in intracellular calcium levels.^{32, 35–38} Both the signaling cascade and the membrane dynamics have been extensively studied in this system with continued refinement of the mechanistic details.^{18, 19, 32–42} One important aspect of the signaling cascade is the ability of the cell to down regulate the process by endocytosis of stimulated receptors.^{32, 37, 38} Endocytosis leads to the formation of an intracellular compartment that undergoes three dimensional transport during maturation from an early to a late endosome. Our ultimate goals are to follow the successive series of events: receptor motion, cell stimulation via receptor crosslinking, receptor-mediated endocytosis, and intracellular endosome transport. In this report we demonstrate the ability to follow, in three dimensions, the first step in this chain of events: receptor motion within the plasma membrane. Specifically the 3D-tracking microscope is able to follow the motion of receptors located

near the side of cell, where the plasma membrane shows curvature, and the receptors are free to move in the axial direction. In addition, we find evidence of receptors located on the apical plasma membrane surface that undergo limited (~ 400 nm) axial motion indicating dynamic motion of the plasma membrane.

2. EXPERIMENTAL METHODS

2.1 Three Dimensional Tracking Microscopy

The custom confocal microscope used for three dimensional tracking microscopy has been described previously.^{28–30} The working principles and design of the microscope are shown in Figure 1. The microscope is built around an inverted microscope platform (Olympus IX-71), which allows for simultaneous laser confocal tracking and filtered white-light imaging. For the purposes of tracking quantum dot labeled membrane receptors, simultaneous wide-field imaging was performed by placing a pellicle beam splitter (8% reflective) in the output path of the microscope and imaging the reflected light onto a CCD camera (Acton/Princeton Instruments PhotonMax 512). To prevent the white-light from interfering with the tracking aspects of the microscope, the visible lamp was filtered (Semrock FF01-794/160-25) to the near infrared (800 nm), which is effectively blocked by the tracking emission bandpass filter (Chroma HQ620/60 X).

Three dimensional laser confocal tracking is based on imaging the emission from a single fluorescent particle in the focal volume onto an array of four spatial filters. The four spatial filters consist of two identical fiber bundles receiving emission from the transmitted and reflective faces of a beam splitter cube. Each fiber-bundle consists of two $50\text{ }\mu\text{m}$ optical fibers with a $55\text{ }\mu\text{m}$ center-to-center distance. The distances between the two fiber bundles to the tube lens are unequal, allowing the fiber-bundles to observe two planes in the sample separated by ~ 200 nm in the axial (Z) direction. This arrangement of spatial filters allows the microscope to collect light from four confocal volumes that are arranged at the vertices of a tetrahedron and thus provides three-dimensional spatial sensitivity. The output of each fiber is imaged onto a single-photon counting avalanche photodiode (PerkinElmer SPCM-AQR-14). The four avalanche photodiodes are read by a pulse counting board (National Instruments PCI-6602) for positional tracking feedback, and a TCSPC (Becker & Hickl SPC630) board for time-resolved spectroscopy. Sample fluorescence is excited using a 500 nm diameter beam waist formed by directing a collimated diode laser (PicoQuant LDH-P-C-470B, 470 nm, 10 MHz) to the back of a water-immersion objective ($\text{NA} = 1.2$, $M = 60$) with a dichroic beam splitter (Chroma 505DXCT). Trajectories are recorded at 200 Hz by reading the PCI-6602, estimating the current particle position by a LabVIEW Real-Time algorithm, and updating a fast closed loop 3-axis piezo stage (Physik Instrumente P-733.3DD) to recenter the particle in the focal spot. A full description of the proportional-integral-differential (PID) tracking algorithm has been given recently.³⁰ The trajectories reported here used an incident average laser power of $15\text{--}20\text{ }\mu\text{W}$.

2.2 Analysis of Trajectories from Live Cells

To highlight the fact that tracking in any spatial dimension is possible with this system, trajectories collected from live cells are classified into two types depending on the spatial

position on the cell membrane. The first type (top-down trajectories) are located on either the apical or adherent plasma membrane, which is relatively flat, and generally show little motion in the axial (Z) direction. The second type (side trajectories) are located on the side of the cell, where there is distinct curvature of the plasma membrane, and generally show extended motion in the axial (Z) direction. This type of trajectory is not accessible in 2D camera based tracking but is readily achieved with active feedback based microscopes. Due to the fact that the motion for some FcεRI complexes occurs in all three spatial dimensions, the motion characteristics are analyzed for each axis separately, in an effort to analyze in-plane from out of plane motion. The motion is quantified via the mean squared displacement (MSD),

$$MSD(\Delta t_n) = MSD(n\delta t) = \langle [S(t+n\delta t) - S(t)]^2 \rangle, \quad (1)$$

where $S(t) = (x(t), y(t), z(t))$ represents the particle's three-dimensional coordinates as a function of time (t) and t_n represents the elapsed time between positional observations. For free diffusion (Brownian motion) along any axis, MSD is linear in t_n with a slope of $2D_K$. The diffusion coefficient, D_K , can be estimated from single particle trajectories undergoing Brownian motion by linear fitting of the MSD for the first 1/4 of the trajectory duration and dividing the resulting slope by two. We refer to this estimate as the macroscopic diffusion coefficient, D_{macro} .

Molecular motion on live cell plasma membranes generally deviates from free diffusion due to the complex chemical environment in the lipid bilayer and the underlying cytoskeleton.^{14, 21, 22} For any trajectory, regardless of free or anomalous diffusion, the microscopic diffusion coefficient, D_{micro} , can be extracted by fitting the MSD between a limited time window.^{21, 22} For the X and Y axes, D_{micro} is determined between 20 and 50 ms. For the Z axis, D_{micro} is determined between 40 and 70 ms. These time windows gave good agreement between D_{micro} and D_{macro} for single particle trajectories in glycerol/water mixtures.³⁰ These time-windows were chosen to minimize the contribution of stage feedback to the real underlying molecular motion, as discussed in Section 3.1.

FcεRI receptor motion was classified using the relative deviation statistic of Kusumi et al. to differentiate free and anomalous diffusive transport modes.^{21, 22} The relative deviation along one axis, $RD(N, n)$, is given by:

$$RD(N, n) = \frac{MSD(N, n)}{2D_{\text{micro}}n\delta t}, \quad (2)$$

where N represents the number of positional observations in a trajectory and n represents the number of periods between observations where the MSD is evaluated. The RD statistics, generated from 3D random walks and averaged over the three axes, are provided in Table 1. There was no systematic difference in the RD statistics for the X, Y, or Z axes. The distributions of $RD(N, n)$ were determined from 500 random walks. RD_{min} and RD_{max} were determined from 2.5% of the trajectories with the smallest and largest $RD(N, n)$ values respectively.²¹

Trajectories that display an $RD(N, n)$ value below RD_{min} are classified as either hop diffusion or confined diffusion. Trajectories that display an $RD(N, n)$ value above RD_{max} are classified as directed diffusion.²¹ We tested the RD statistic on single particle tracking data collected from quantum dots freely diffusing in glycerol/water mixtures³⁰ and found that 91% (140 sample size) of the trajectories were classified as free diffusion. This is consistent with the RD statistic definition, where free diffusion occurs in the center 95% of the RD distribution.²¹

2.3 Materials

Both mouse monoclonal anti-DNP IgE and monovalent biotin-IgE were prepared as described previously.¹⁹ Biotin-IgE was labeled with 625 nm Streptavidin QDots (Invitrogen) in a 1:1 stoichiometry in phosphate saline buffer (+ 1% bovine serum albumin (BSA)) and stored as a 20 nM stock at 4 °C.¹⁹ For simplicity we refer to the QDot labeled IgE as QD-IgE and unlabeled anti-DNP IgE as dark-IgE. 2,4-dinitrophenyl conjugated to bovine serum albumin (DNP-BSA) was obtained from Invitrogen. Sterile 8-well chamber slides (Lab-Tek) suitable for live cell microscopy were obtained from Nalge Nunc International.

2.4 Cell Culture and Labeling for 3D Tracking

Rat basophilic leukemia (RBL-2H3) cells were cultured in MEMEagles media supplemented with 1% L-glutamine, 1% Pen-Strep, and 10% fetal bovine serum (FBS) as previously described.¹⁸ Sterile 8-well chamber slides were seeded in duplicate at three densities: 1×10^4 , 5×10^3 , and 1×10^3 cells per well, in 500 μ L total volume per well. The cells were incubated overnight at 37 °C and used, starting with the highest density, for three consecutive days in the tracking experiments. Tracking microscopy on live cells was performed at 22 °C for a period of 3–4 hours.

2.4.1 QD-IgE Labeling—Cells in 8-well chamber slides were washed with Hank's balanced salt solution (HBSS) to remove the highly fluorescent cell culture medium. The 20 nM QD-IgE stock was diluted to 50 pM in HBSS and 200 μ L aliquots were added to the 8-well chambers. The cells were then incubated at 37 °C for 15 minutes. Excess QD-IgE was removed by washing the cells four times with 200 μ L aliquots of HBSS.

2.4.2 Dark-IgE Labeling and Crosslinking—To activate the resting QD-IgE labeled RBL-2H3 cells, 200 μ L of dark-IgE (0.5 μ g/mL) was added to saturate the Fc ϵ RI membrane receptors.¹⁸ The cells were left to incubate at 37 °C for 15 minutes. Excess dark-IgE was removed by washing the cells with three 200 μ L aliquots of HBSS. RBL-2H3 cells were activated by crosslinking the IgE labeled Fc ϵ RI receptors with DNP-BSA at a concentration 0.5 μ g/mL at 22 °C. Some of the cells showed visual evidence of membrane ruffling under these conditions, though not to the extent demonstrated at 37 °C.¹⁸ The majority of the labeled Fc ϵ RI receptors under the crosslinking conditions showed immobilized behavior as previously reported.^{18, 41, 43}

3. RESULTS AND DISCUSSION

3.1 Tracking: Estimating Tracking Performance via Crosslinked FcεRI

When IgE-FcεRI complexes are crosslinked with multivalent antigen such as DNP-BSA, the receptors are known to aggregate and a large fraction appear to be immobilized.^{18, 41, 43} We use this feature of the FcεRI receptor to test the positional accuracy of the closed loop tracking algorithm and hardware. Figure 2 shows a typical trajectory of a crosslinked QD-IgE labeled FcεRI receptor located on the apical plasma membrane surface. The X, Y, and Z positions are stable at $X = 0$, $Y = 0$, $Z = 0$, with a standard deviation of 50, 53, and 125 nm respectively. We note that our 3D positional determination is made from only 5 ms of data. Higher positional accuracy could, in principle, be obtained through longer integration periods,⁴⁴ but the microscope would lose its ability to track fast, dynamic motion, such as that occurring during IgE signaling and down-regulation. The MSD data (Fig. 2(b)) can be fit with a single exponential rise,

$$L_{err}^2 \left(1 - \exp \left(\frac{-\Delta t_n}{\tau} \right) \right), \quad (3)$$

where L_{err} represents an estimate of the tracking positional error and τ gives a lower limit of the timescale of observable motion. Fitting parameters for the MSD data averaged from 7 trajectories of crosslinked FcεRI are provided in Table 2. Estimates of the tracking positional error via the stage position standard deviation and MSD fitting (Eqn. 3) are consistent with each other and are similar to error estimates based on Monte Carlo simulations of the tracking microscope.^{28–30}

The results of tracking crosslinked FcεRI receptors show that the 3D tracking microscope is capable of obtaining positional accuracy of nearly 50–80 nm in the lateral plane (XY) and 150–190 nm in the axial (Z) direction. The MSD analysis suggests that the 3D tracking microscope should be able to observe positional changes in the lateral plane on a ~ 15 ms timescale, while changes in the axial position can be observed on a slightly slower (30 ms) timescale. The difference in the response times is likely due to the smaller positional sensitivity in the Z axis³⁰ and explains why we choose slightly different time ranges used to determine D_{micro} for the lateral and axial directions (Section 2.2).

3.2 Tracking: QD-IgE Labeled FcεRI on Apical or Adherent Membrane Surfaces

Prior studies of FcεRI membrane receptor dynamics on RBL-2H3 cells, using video frame rate (33 ms/frame), reported four types of (2D) motion including free diffusion, restricted diffusion, directed diffusion, and immobile.^{18, 19} More generally, the dynamics underlying membrane diffusion in most if not all mammalian cells types has been shown to be hop diffusion with an immobile population when using faster time resolution (25 μs).¹⁴ Observing hop diffusion without sufficient time resolution to discern individual compartments (confinement) and inter-compartment hopping is known to give rise to MSD plots that look like free diffusion²³ or restricted diffusion on larger length scales.¹⁸

An example (top-down) trajectory of a mobile QD-IgE labeled FcεRI receptor is compared to a trajectory of a quantum dot freely diffusing in a glycerol/water mixture (Figure 3).

Using the RD statistic described in section 2.2, the FcεRI receptor trajectory is classified as hop-diffusion or confined diffusion, while the trajectory in glycerol/water is classified as free diffusion. Even when trajectories obtained in glycerol/water exhibit a D_{micro} value similar to a FcεRI trajectory, the RD statistic can differentiate the motional dynamics. Prior studies have shown compartment residency times in plasma membranes range from 15–100 ms depending on cell type.¹⁴ The 5 ms integration time used here is only slightly shorter than the range of residency times listed above and suggests that the microscope is at the upper limit for observing hop diffusion. If a larger integration time was used, the dynamics would likely appear to be simple Brownian motion even though the underlying mechanism is hop diffusion.^{14, 23} This is further supported by the value of D_{micro} for the FcεRI trajectory shown in Figure 3 ($0.1 \mu\text{m}^2/\text{s}$). This value is rather low compared to prior examples of intracompartiment diffusion constants, which typically are $\sim 5 \mu\text{m}^2/\text{s}$.¹⁴ When using an integration time similar to the average compartment residency time, the extracted values of D_{micro} are known to be under estimated by an order of magnitude.²³

Figure 4 shows selected trajectories of QD-IgE labeled FcεRI receptors on either the apical or adherent plasma membrane surface. All the trajectories were classified as confined or hop diffusion according to the methods laid forth in Section 2.2. The trajectories range in duration from 17 to 32 s and indicate a heterogeneity in the long term diffusion coefficients (intercompartment hopping). The lateral (XY) MSD was fit to a line between 100 ms and 0.5 s to quantify the variance in the apparent long term diffusion (Figure 5). The values of the apparent diffusion coefficients range from 0.007 to $0.08 \mu\text{m}^2/\text{s}$, consistent with prior observations of FcεRI receptors at 22°C.^{18, 41}

Although these trajectories were collected from receptors on either the apical or adherent membrane surface, and thus are largely confined to the lateral plane, there appears to be some real motion in the axial (Z) direction for time scales ~ 100 ms. The Z positions vs. time are shown for the trajectories before and after applying a 100 ms boxcar moving average (Fig. 4). Although the raw Z trajectories are very noisy, by averaging with a 100 ms sliding window, clear fluctuations that range between 200 and 400 nm in the Z position are observable for trajectories B and D. The axial MSD (Figure 5(b)) for all the trajectories indicates complex behavior after 100 ms with quasi periodic fluctuations likely caused by the errors in determining the axial position. For trajectories B and D, however, the MSD grows linearly from 0.1 to 1 s due to the sub 500 nm observable fluctuations in the Z position. At this point it is unclear what is the cause of the Z fluctuation. It is interesting that trajectory D shows clear Z fluctuations even though motion in X and Y is fairly confined. This suggests that the Z motion is partially due to dynamic membrane motion and not necessarily moving about a static membrane topography.

3.3 Tracking: QD-IgE Labeled FcεRI on the Side Membrane Surface

In section 3.2 we demonstrated the ability to track FcεRI receptors on apical and adherent membrane surfaces that are generally flat in the axial direction. The results are consistent with prior 2D tracking studies of this membrane receptor. In addition, the ability of the 3D-tracking microscope to record the 3D position in real time allowed, for the first time, the observation of limited (~ 400 nm) fluctuations in the axial position. These fluctuations likely

occur due to a dynamically fluctuating membrane surface. In this section we demonstrate the ability to track this membrane receptor on the side of the cell, where the receptor has greater motion in the axial direction. We further note this region of the cell has a much greater curvature and strain on the plasma membrane, which may affect the underlying lipid composition, phase segregation, and receptor dynamics.

Figure 6 shows a 2D representation of 3D motion for selected trajectories of QD-IgE labeled FcεRI receptors located on the side of the plasma membrane. The trajectories are colored as a function of time from start (red) to finish (violet). These trajectories generally show an extent in the axial direction near that of the lateral direction. Two dimensional projections of these trajectories (Figure 7) show that they generally lie along a line in the lateral (XY) plane, with this line corresponding to the boundary of the plasma membrane as seen from above. The projections along both the XY and XZ plane show evidence of sub micron compartmentalization, indicating that receptors on the side of the cell also undergo confined or hop diffusion as seen for receptors on the apical membrane surface. The MSD behavior for hop diffusion can be empirically described as:

$$MSD(\Delta t_n) = \frac{L_c^2}{6} \left(1 - \exp\left(\frac{-\Delta t_n}{\tau}\right) \right) + 2D_{hop}\Delta t_n. \quad (4)$$

The first term in Equation 4 is for confined motion, where L_c is the length of a square confining compartment. If the trajectory resides solely in a single compartment, then the MSD for a single axis will plateau to a value of $L_c^2/6$. The second term in Equation 4 accounts for the long term hopping from compartment to compartment. This is equivalent to the long term diffusion coefficients obtained by fitting a line between 100 ms and 0.5 s in Section 3.2.

The MSD behavior for receptors on the side of the cell are shown in Figure 8. We start by discussing the behavior of trajectory A. The short time (< 0.5 s) MSD behavior for trajectory A was classified as hop diffusion along the Y and Z axes, and free diffusion along the X axis. This is a result of the alignment of the cell membrane, where individual compartments are seen to reside in a line largely along the X axis (Fig. 7). A linear fit of the MSD for the X axis (free diffusion) and fits to Equation 4 for the Y and Z axes (hop diffusion) are shown as red lines in Figure 8. The fits indicate that the compartment length, L_c , is about 300 nm along the Y axis. We note that the L_c value along the Z axis is obscured by the larger track error along the axial direction. The hopping diffusion coefficients, D_{hop} , along Y and Z are 0.02 and 0.05 $\mu\text{m}^2/\text{s}$ respectively, while the linear fit along X gives a value of 0.05 $\mu\text{m}^2/\text{s}$. The longer time (> 0.5 s) behavior of the MSD indicates that transport along the X axis looks somewhat directed, showing micron length transport in this direction due to hopping between multiple compartments aligned along X (Fig. 7).

Trajectory B shows considerably more complex behavior than A. While trajectory B is nearly 4 times longer in duration than A, the length scale traveled for these trajectories are similar. This suggests that trajectory B undergoes time dependent dynamics with extended periods of relatively confined motion. To demonstrate this behavior, the MSD data for each axis are shown for two 10 second sections of trajectory B (Fig. 8). The first section (10–20 s,

black symbols) displays short time behavior that can be fit to the empirical hop diffusion model (Eqn. 4) for all three axes. On longer timescales (> 0.5 s) there is a dramatic change in the MSD behavior that indicates directed transport along all three axes. The MSD behavior for the second section of the trajectory (20–30s, blue symbols) shows short time behavior nearly identical to the first section, however, after 0.5 s the MSD continues to indicate hop diffusion. This observation indicates that the receptor undergoes transient confinement to a point of near immobility, followed by periods of rapid movement.

The short time hop diffusion behavior was fit to Equation 4 for both sections of the trajectory. In both sections, the length of the compartments, L_c , was limited by the tracking error (Table 2) for all three axes, which suggests that the compartment size is ~ 100 nm. The long term diffusion coefficients, D_{hop} , range between 0.002–0.01 $\mu\text{m}^2/\text{s}$ for all three axes. These values are much smaller than in trajectory A where the diffusion coefficients ranged from 0.02–0.05 $\mu\text{m}^2/\text{s}$. This result demonstrates that the same level of heterogeneity in the hopping rates exists for receptors located on the flat (apical or adherent) or curved membrane surfaces. Moreover, trajectory B in Figure 6 elegantly displays time dependent heterogeneity in the mode of transport. The notion of a mobile and immobile population of receptors, as is often inferred from bulk fluorescence recovery after photobleaching measurements, is clearly an oversimplification of the membrane dynamics. Changes in the membrane on macroscopic (10's of seconds) timescales influence receptors on microscopic (sub micron) length scales. Finally we note that the true power of the 3D tracking microscope is displayed for trajectory B (Figure 7). As this receptor moves along the side of the cell in distinct sub micron compartments, the axial position moves down nearly 1 μm , back up 1 μm , and finally down 1 μm again for a total traveled length in Z of about 3 microns. This observation could not have been made with conventional 2D video microscopy; this is a clear demonstration of the utility of directly following axial motion in fluorescence microscopy.

4. CONCLUSIONS

The motion of single Fc ϵ RI membrane receptors on live RBL-2H3 cells was recorded in full three-dimensional space. This represents the first application of a closed-loop 3D tracking microscope to single molecule live cell microscopy. The results of tracking this membrane receptor on the apical or adherent membrane surface are consistent with prior observations using conventional 2D microscopies. The trajectories showed evidence of hop diffusion behavior with long term diffusion coefficients between 0.007–0.08 $\mu\text{m}^2/\text{s}$. In addition to observing largely 2D membrane trajectories, the 3D tracking microscope could detect small fluctuations in the axial position on timescales between 0.1 and 1 s, indicating a dynamic membrane surface. The power of the 3D tracking microscope to track axial motion allowed the first observations of Fc ϵ RI receptor dynamics along the side of the plasma membrane. The trajectories along the side of the plasma membrane are essentially 2D in nature with strong confinement along a line in the lateral plane that defines the plasma membrane boundary. When projected into the axial direction, receptor trajectories on the side of the plasma membrane show axial confinement similar to lateral confinement for receptors on the apical or adherent membrane surface. Currently more observations are needed to discern if receptors located on the top and side membrane surfaces undergo statistically different

dynamics. In addition to such studies, we anticipate soon using this microscope to follow receptor-mediated endocytosis and molecular transport in polarized cells.

ACKNOWLEDGEMENTS

This work is supported by the National Institutes of Health (No. R21AI07707) and the National Nanotechnology Enterprise Development Center (NNEDC). This work was performed at the Center for Integrated Nanotechnologies, a U.S. Department of Energy, Office of Basic Energy Sciences user facility. Los Alamos National Laboratory is operated by Los Alamos National Security, LLC, for the National Nuclear Security Administration of the U.S. Department of Energy under contract DE-AC52-06NA25396. We thank Nick Andrews, Sheli Ryan, Janet Pfeiffer and Marina Martinez for preparation of materials and general training in the RBL-2H3 cell culture and handling.

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Idealized projection of the fiber optic spatial filters back into the sample space

Figure 1.

3D-tracking detection system and pinhole arrangement. Two fiber bundles described in the text are used as the four spatial pinholes for particle position detection. The array of “pinholes” project into sample space on the four vertices of a tetrahedron, allowing three-dimensional spatial resolution.

(a) Trajectory of a crosslinked FcεRI receptor. The mean stage position for all three axes is stable with small drifts (≤ 30 nm) over a 20 s observation. The count rate summed over the four detectors is shown on the bottom with an average of 284 counts/5 ms (56.8 kHz).

(b) MSD calculated from stage position data in figure 2(a). X (Open Square), Y (Open Circles), Z (Open Triangles). Solid Lines represent fits to equation 3. Fit parameters averaged over 7 trajectories are provided in Table 2.

Figure 2.

Typical tracking data from a crosslinked FcεRI receptor. Standard deviations of stage positions provide an estimate of the tracking positional errors, while MSD plots provide estimates of the timescales of observable motion limited by the closed-loop feedback.

Figure 3.

Example trajectories projected into the XY plane. Both trajectories are 18 s in duration. Left: Trajectory of a QD-IgE labeled FcεRI membrane receptor. Middle: Trajectory of a quantum dot in a mixture of glycerol/water as described in reference.³⁰ Right: Short time MSD behavior for the left (open squares) and middle (open circles) trajectories. Solid lines represent fits used to determine D_{micro} . Individual trajectories were classified as hop or free diffusion as explained in section 2.2.

Figure 4.

Top: Selected trajectories of QD-IgE labeled FcεRI receptors projected into the XY plane.

A) 17 s Duration. B) 21 s Duration C) 32 s Duration. D) 21 s Duration. Bottom: Z position vs time. Raw data (Black), 100 ms averaging (Red). Trajectories from receptors on the apical or adherent membrane surfaces.

(a) Lateral (XY) MSD for trajectories shown in Figure 4. Solid lines between 20 and 50 ms are linear fits to determine D_{micro} . Dashed lines between 0.1 and 0.5 s are linear fits to determine the long term apparent diffusion caused by hopping between compartments. The long term apparent diffusion ranges from 0.007-0.08 $\mu\text{m}^2/\text{s}$.

(b) Axial (Z) MSD for trajectories shown in Figure 4. Dashed lines between 0.1 and 1 s are linear fits. For trajectories A and C the MSD is largely stable indicating flat Z trajectories, while linear growth for B and D indicate fluctuations in the Z position on a sub 1 s timescale. Figure 4 indicate that these fluctuations are around 200-400 nm.

Figure 5.

MSD data from QD-IgE-FcεRI receptor complexes located on the apical or adherent plasma membrane surface.

Figure 6. Example 3D trajectories of QD-IgE labeled FcεRI receptors located on the side of the plasma membrane. Trajectories are colored as a function of time from start (red) to finish (violet) following a rainbow (ROYGBIV) color scheme. Trajectory durations are A) 11 s, B) 40 s.

Figure 7.

Projection of trajectories from Figure 6 in the XY and XZ planes. Left: Trajectory A. Right: Trajectory B. Trajectories are colored as a function of time from start (red) to finish (violet) following a rainbow (ROYGBIV) color scheme.

Figure 8.

MSD vs t_n for trajectories in Figure 6 for each axis. Left: Trajectory A. The MSD behavior for the three axis are shown as Black Symbols. Red lines are fits to Equation 4 for Y and Z and a linear fit for X. Right: Trajectory B. Trajectory B shows strongly time dependent behavior. The MSD behavior for two sections of trajectory shown as Black Symbols (10–20 s) (offset by 0.02 μm^2) and Blue Blue Symbols (20–30 s). Red lines are fits to Equation 4 for X, Y and Z. The time axis is broken at 0.25 s, indicating a change from linear to logarithmic scaling.

Table 1

$RD(N, n)$ statistics from Monte Carlo simulation of Brownian motion.

(N, n)	$RD_{\min}$	$RD_{\max}$
(5000,100)	0.71	1.30
(2000,100)	0.59	1.54
(1000,100)	0.44	1.81
(500,100)	0.30	2.27

Table 2

Fitting parameters for Equation 3 averaged from 7 trajectories of crosslinked FcεRI receptors. Standard deviation given in parentheses.

Axis	L_{err}/nm	τ/ms
X	68.8(0.7)	17.8(4.0)
Y	80.6(1.1)	19.0(5.5)
Z	187.5(2.3)	30.7(5.6)