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Three dimensional time-gated tracking of non-blinking quantum dots in live cells

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Abstract

Single particle tracking has provided a wealth of information about biophysical processes such as motor protein transport and diffusion in cell membranes. However, motion out of the plane of the microscope or blinking of the fluorescent probe used as a label generally limits observation times to several seconds. Here, we overcome these limitations by using novel non-blinking quantum dots as probes and employing a custom 3D tracking microscope to actively follow motion in three dimensions (3D) in live cells. Signal-to-noise is improved in the cellular milieu through the use of pulsed excitation and time-gated detection.

Keywords

Single particle tracking; time-gating; quantum dot; microscopy

1. INTRODUCTION

Single particle tracking (SPT) is a time-tested and powerful tool in science. From a historical perspective, the observation of the motion of single pollen particles in an optical microscope by Robert Brown in 1828 clearly contributed to the atomistic view of matter, with the theoretical underpinnings of macromolecular diffusion put forth by Albert Einstein in 1905. [1, 2] Since this early pioneering work, technical advances in light sources (e.g. lasers and light emitting diodes), the development of charged coupled device (CCD) cameras, new modes of image contrast and better imaging probes, and higher-grade optical components have opened the door to the types of problems that can be addressed by single particle tracking. Biophysical examples of the power of single molecule and particle tracking approaches include studies of how bacteria respond to nutrient gradients (chemotaxis)[3],

direct observation of heterogeneous motion of lipids and receptors in the plasma membrane of live cells[4], and a precise (sub-diffraction limit) measure of the step size of motor proteins such as kinesin[5] or myosin[6].

In conventional single particle tracking, a fluorescent or scattering probe is attached to the target molecule of interest (e.g. protein, lipid, or nucleic acid) and its motion is recorded in a series of two dimensional (2D) images on a CCD camera placed in the focal plane of a microscope. While 2D tracking techniques are well matched to 2D problems (such as motion in a flat cellular membrane)[7], intracellular (and membrane bound) motion is inherently three dimensional (3D).[8-10] To this end, a number of research groups have developed 3D tracking methods to follow the motion of single molecules and particles. Methods for 3D SPT in a microscope include camera-based approaches[11-15] and methods based upon keeping the molecule in the center of a confocal microscope by active feedback[8-10, 16-22]. Camera-based approaches have the advantage that a large number of molecules can be tracked simultaneously, but are generally limited by the depth of focus of the microscope objective employed, which is $\sim 1\ \mu\text{m}$ for the high numerical aperture microscope objectives needed for single molecule fluorescence imaging. While methods for 3D molecule/particle tracking via confocal feedback have a greater Z tracking range, only a single molecule is monitored at a time. However, in addition to the superior Z tracking range, the confocal feedback approach has a number of other advantages, including an insensitivity to large backgrounds (due to the spatial filtering inherent in confocal microscopy)[21], orders of magnitude less photo-damage than wide-field methods, and the ability to perform time-resolved spectroscopy on the molecule being followed[9, 10]. Confocal feedback methods have been used to follow individual bacteria[3], individual quantum dots[17, 18] [8-10, 20, 21], single organic dyes[22], and single fluorescent proteins[22] moving at biologically relevant transport rates.

The choice of the label to be used for SPT (e.g. organic dye, fluorescent protein, quantum dot, or larger scattering particle) is often dictated by the type (protein, lipid, or nucleic acid) and location (*ex vivo*, on a membrane, or in the cytosol) of the biomolecular target to be studied. For intracellular proteins in live cells, fluorescent proteins clearly have substantial advantages as labels for SPT, due to their ability to be genetically expressed in tandem with a target protein of interest. However, fluorescent proteins and organic dyes (in the absence of an oxygen scavenging system) often photobleach quite rapidly ($< 1\ \text{s}$ at excitation intensities required for single molecule imaging).[22] Longer trajectories can be obtained with quantum dots or large scattering particles such as colloidal gold, with the caveat that these probes need to be produced and introduced exogenously to the cell for molecular labeling. For outer membrane receptors or lipids, quantum dots or larger scattering particles (such as colloidal gold) become more viable labels for SPT. Quantum dots, in particular, have distinct advantages for following the motion (and internalization) of cellular receptors. These advantages include: 1. Relatively small size (tens of nm in diameter) when compared to colloidal gold (typically hundreds of nm in diameter) 2. Orders of magnitude more photostable than organic dyes or fluorescent proteins. 3. Narrow emission bands coupled with broad excitation spectra that permit a large number of different emission colors with a single excitation wavelength.[23]

One drawback of QDs as labels for SPT is the fact they blink on and off, with these time-scales being power law distributed.[24, 25] While methods have been developed to account for QD blinking when reconstructing molecular trajectories in 2D image sequences[26-28], molecular motion clearly is not being recorded while the QD is in a dark state. Moreover, for 3D tracking using active feedback, QDs that enter a dark state for a period greater than the time it takes the QD to move out of the optical probe volume terminates the measured trajectory.[21] To overcome the limitations of QD blinking in SPT experiments, we and others have been exploring the use of a new functional class of non-blinking or blinking suppressed QDs for SPT.[10, 29] In addition to suppressed blinking, these new QDs, which have been extensively studied for a variety of applications[30-46], also have enhanced photo- and chemical stability when compared to conventional QDs. These superior properties were created through a CdSe/CdS core/shell QD comprised of a CdSe core (2–6 nm) and an unusually thick shell comprised of 8–20 monolayers of CdS. These thick-shell or so-called ‘giant’ QDs (gQDs) were recently further optimized to afford essentially uninterrupted emission under extended continuous excitation.[42]

While the steady photon emission, resistance to photobleaching, relatively small size, and large Stokes shift all make gQDs useful probes for SPT[23], here we exploit another useful feature of QDs and gQDs, namely, their long fluorescence lifetime, to improve signal-to-noise when tracking in large background environments.[47] In particular, the long lifetime of quantum dots enables time-gating to be employed to improve signal-to-noise. In time-gating, pulsed excitation and time resolved detection are used to measure the arrival time of a detected photon with respect to an excitation laser pulse. For time gating, detected photons that are coincident (or arrive within a given time window) of the excitation pulse are discarded in the analysis, which can dramatically reduce background (if the measured background has a shorter lifetime than the QD). Time-gating has been previously employed to improve signal-to-noise in single molecule detection in flowing sample streams by discriminating against Raman light scattered by the solvent (water).[48-51] Time-gating has also been used to improve the signal-to-noise in confocal microscopy and in photon pair correlation (fluorescence photon anti-bunching) measurements.[10, 46] Here, we demonstrate that time-gating can be used to improve the signal-to-noise for 3D molecular tracking applications of QDs in real-time. The combination of a non-blinking probe, 3D tracking methods, and superior signal-to-noise of time-gated tracking enables following the motion of individual allergy receptors (IgE-FcεRI) for several minutes on and inside live rat mast cells.

2. EXPERIMENTAL METHODS

2.1 Giant QD functionalization, labeling, and characterization

Giant quantum dots (gQDs) were synthesized by successive ion layer absorption (SILAR) as described previously. [30, 52] Ligand exchange was used to coat the dots in a custom ligand consisting of a dihydrolipoic acid (DHLLA) group for anchoring to the CdS surface, a hydrophilic polyethylene glycol (PEG) linker with ~12 subunits, and a terminal carboxylate group employed to make the gQDs water soluble and useful for subsequent carbodiimide-mediated bioconjugation to streptavidin. Bioconjugation of the streptavidin gQDs (gQD-SA)

to either dinitrophenol (DNP) or IgE (via streptavidin-biotin chemistry) was performed as described previously.[52] Figure 1 shows a schematic of the gQD conjugation.

After ligand exchange, the hydrodynamic diameter was determined to be ~20 nm via 2D single-particle tracking (LM-12 Nanosight Ltd.) and atomic force microscopy (AFM, Veeco Instruments Bioscope SZ). The average brightness per particle (BPP) and approximate concentration of the gQDs were determined using fluorescence correlation spectroscopy (FCS).

2.2 Creating stable transfected GFP-clathrin mast cells

RBL-2H3 cells were transfected using an Amaxa device (Lonza) following the manufacturer's protocol. Stable transfectants were obtained using the appropriate selective agent as described in Cleyrat et al.[53]

2.3 Cell culture and labeling

RBL-2H3 cells were cultured in MEM media (Cellgro) supplemented with 10% fetal calf serum, 1% Pen-Strep, and 1% L-Glutamine (Life Technologies), and incubated at 37 °C with 5% CO₂ and 100% humidity. Cells were seeded to chamber slides (Nunc) at $\sim 1 \times 10^4$ cells per well in 0.5 mL complete MEM without Phenol Red.

For the time-gated raster scanning imaging experiments, the cells were rinsed in their imaging well three times with 200 μ L of Hanks buffered saline solution (HBSS) buffer to remove the MEM media and incubated for 15 min at 37 °C with 500 pM gQD-IgE in HBSS. After rinsing three times with 200 μ L of HBSS, the cells were incubated for 30 min at 37 °C with 1 μ g/mL unlabeled IgE to fully activate all the Fc ϵ RI receptors. After again rinsing three times with 200 μ L of HBSS, the cells were ready for 3D tracking where they were held at ~35 °C using an objective heater (TC-1-100, Bioscience Tools). To stimulate the RBL cells, 1–2 μ g/mL DNP-BSA in HBSS was added. For tracking of gQD-DNP, the RBL cells were incubated with unlabeled IgE as just described, followed by ~25 pM gQD-DNP in HBSS for ~2 min before rinsing with HBSS.

For 3D tracking experiments, cells in 500 μ L of MEM media were spiked with 2 μ L of 1.36 mg/mL unlabeled IgE in PBS and incubated. The cells were rinsed three times with 200 μ L of MEM to remove excess unlabeled IgE. Immediately prior to tracking, the cells were mixed gQD-DNP in MEM to a final gQD-DNP concentration of ~7 pM in the sample chamber.

2.4 Tracking microscope

Our 3D tracking microscope is shown schematically in Figure 2 and is described in detail elsewhere.[8-10, 19-22, 51] In brief, the 3D tracking microscope is a stage-scanning confocal microscope that uses a unique spatial filter geometry and active feedback of the stage position to keep the fluorescent probe molecule centered in the field of view of the microscope. The system is built upon a commercial microscope platform (Olympus IX-71) and uses a 60 $\times$ 1.2 NA water immersion microscope objective for fluorescence excitation and to collect the emitted light. Fluorescence excitation of QDs is provided by 1-2 μ W

average power from a 405 nm pulsed diode laser (PicoQuant LDH-P-C-405) operating at a pulse repetition rate of 10 MHz. This excitation light is directed into the sample via a dichroic beam splitter (Semrock 405 DiO2-R405) in the microscope and under-fills the back aperture of the microscope objective to create a beam waist (w_0) of ~500 nm at the focal plane in the sample. The fluorescence light emitted by the sample is collected by the objective, transmitted through the dichroic beam splitter, and spectrally filtered by an emission bandpass filter (Semrock FF01-641/75) prior to being focused with an externally mounted tube lens (25 cm focal length achromat, Thorlabs), with the manufacturer's tube lens having been removed from the microscope. This emitted light is split into two paths by a 50/50 beam splitting cube (Thorlabs). The light in each beam path is focused midway between the two adjacent optical fibers. These custom made fiber-pairs (Polymicro Technologies) consist of two 50 μm diameter optical fibers with a center-to-center fiber separation of ~55 μm . Each fiber serves as a spatial filter for the single element detector associated with this fiber. An image of these fibers (as seen with a microscope) is shown in Figure 3A. The outputs of the four optical fibers are coupled to four independent inputs of a quad single photon-counting avalanche photodiode module (PerkinElmer SPCM-AQ4C). Of particular importance is that one pair of fibers is oriented in the plane parallel to the optical table whereas the other is oriented vertically with respect to the optical table, with each fiber pair located at a slightly different distance to the tube lens than the other pair. With this arrangement, the projection of the fiber faces back into the object space forms a 3D tetrahedron, as depicted by the colored spheres in Fig. 3B. A QD in the dead center of the tetrahedral detection volume would produce equal counts on all four detectors (labeled D0, D1, D2, and D3), with any motion away from the center leading to an asymmetric count distribution. This asymmetry in count distribution forms the basis for an error signal for a Proportional Integral Differential (PID) feedback loop that drives a fast XYZ piezo stage (Physik Instrumente P-733.3DD) that repositions the sample once every 5 ms in an effort to re-center the QD in the optical probe volume. Photon counting and PID feedback is performed using National Instruments hardware (a PCI-6602 Board for counting detected photon TTL pulses and a PCI-6731 board for analog control of the Piezo stage) that is controlled by custom software written in LabVIEW RealTime.

2.4 Time gating

The four independent outputs of the SPCM-AQ4D detectors are each split into two identical output channels using four PicoQuant PSM 50 modules. Currently, one set of these channels (four detector outputs) are directly fed into a PicoQuant HydraHarp 400 module to enable time-correlated single photon counting while tracking. In the past, the other four of these channels were directly fed into the National Instruments 6602 Board for photon counting and subsequent stage motion. Here, to enable hardware based time-gating for 3D tracking in real-time, the output of the four detectors are conditioned (using a constant fraction discriminator module) and fed into four time-to-amplitude converter modules (three Tennelec TC863s and one Canberra 2145) that have a single channel analyzer (SCA) output. The TAC measures the photon arrival times relative to the laser sync pulse in reverse start-stop mode. The SCA outputs a TTL pulse when the time between the laser sync and photon arrivals is longer than a selected time threshold. A threshold of ~4 ns chosen for this work.

2.5 Simultaneous Spinning Disk Microscopy

Previously, we have recorded a white light image of the media (cell or fluid) around the QD being tracked in the microscope.[9, 10] This enabled a gross measure of QD location with respect to a cell (e.g. is the QD on the outer membrane or inside a cell). To improve the contextual information obtained during our 3D tracking experiments (e.g. is this receptor near a particular protein complex, in an early or late endosomal compartment, etc), we have recently added the ability to perform Nipkow spinning disk microscopy during 3D tracking experiments. This system uses an LED for fluorescence excitation and a commercial Crest-X Light spinning disk unit. Incorporation of the system into the microscope is shown schematically in Figure 2. A more detailed description of the hardware and software integration of this system with our tracking microscope will be provided in a forthcoming manuscript.

3. RESULTS AND DISCUSSION

As discussed above in the **Introduction**, time-gating is an established method to reduce the background in fluorescence measurements in instances where the signal has a longer fluorescence lifetime than the background. [48-51] However, even for molecules or quantum dots with long fluorescence lifetimes, any gate applied to suppress the background also results in a loss of signal. Additionally, while dedicated TAC/SCA modules can gate and measure the time elapsed between a laser pulse and the detected fluorescence photon in real-time, there is a substantial dead-time associated with these modules (on the order of a microsecond), which lowers the number of counts observed at high count rates. As such, prior to time-gated tracking, we measured and modeled the signal lost by the TAC/SCA modules as a function of count rate (Figure 4) and performed calculations to find the optimum time-gate for tracking QDs (fluorescence lifetime of 16 ns) in a background dominated by fluorescent proteins that have a fluorescence lifetime of 3.5 ns (Figs. 5 and 6).

The analytic fit in Figure 4 is derived from the following considerations. First, we assume without a gate that the total counts measured (c) comes from fluorescence (fc) and a constant background (dc) due to detector dark counts, e.g., $c = fc + dc$. Assuming the fluorescence decay has a lifetime of τ , the fraction of the fluorescence counts (fc) that makes it through the gate is given by:

$$fc_{tg} = \int_{t_{tg}}^{\frac{1}{L_{rep}}} a \cdot e^{-\frac{t}{\tau}} dt = a\tau \left(e^{-\frac{t_{tg}}{\tau}} - e^{-\frac{1}{\tau L_{rep}}} \right) \quad (1)$$

Where t_{tg} is the length of the time-gate, L_{rep} is the laser repetition rate, and a is the amplitude of the fluorescence decay that depends upon laser excitation power, overall

detection efficiency, and fluorophore concentration. Since $\frac{1}{L_{rep}} \gg \tau$ is usually fulfilled, Eqn. 1 simplifies to:

$$fc_{tg} = a \cdot \tau \cdot e^{-\frac{t_{tg}}{\tau}} \quad (2)$$

Expressing the time gated fluorescence, fc_{tg} , in terms of the fluorescence in absence of the gate, fc , which is given by:

$$fc = \int_0^\infty a \cdot e^{-\frac{t}{\tau}} dt = a\tau \quad (3)$$

yields

$$fc_{tg} = fc \cdot e^{-\frac{t_{tg}}{\tau}} \quad (4)$$

In addition to the reduction in counts due to the time-gate, there is also a loss of counts due to the overall system dead time, which here is dominated by the dead time of the TAC/SCA module, which we denote as t_{dead} . We note that the dead time of the SPAD units

(manufacturer specified as 50 ns) and the laser pulse period, $\left(\frac{1}{L_{rep}}\right)$, or 100 ns are other potential limits to the observed count rates in time-correlated single photon counting measurements, but these are substantially shorter than the manufacturer specified dead time of the TAC/SCA modules (0.65 μ s). At low count rates, the observed counts in the presence of dead-time effects (C_{obs}) should be nearly identical to the count rate in the absence of any dead time effects (c). At high count rates, the maximum detected count rate is limited to $1/t_{dead}$.

Here, we approximate this behavior as $C_{obs} = \frac{1 - e^{-c \cdot t_{dead}}}{t_{dead}}$. Combining this dead-time loss with the losses from the time gate yields the following functional form for the time-gated counts as a function of the un-gated counts, (c):

$$c_{tg} = (dc_{tg} + fc_{tg}) \frac{1 - e^{-c \cdot t_{dead}}}{c \cdot t_{dead}} = \left[dc(1 - t_{tg} \cdot L_{rep}) + fc e^{-\frac{t_{tg}}{\tau}} \right] \frac{1 - e^{-c \cdot t_{dead}}}{c \cdot t_{dead}} \quad (5)$$

As can be seen, this model (Fig. 4, solid red line) fits our measured data (Fig. 4, blue squares) well. For the data, we plot the fractional loss in the time gated count rate (from a solution of QDs having a 16 ns fluorescence lifetime) as a function of the un-gated count rate, with the loss in counts being calculated from the un-gated signal recorded by the HydraHarp module. In the fit, the dark count rate dc (1700 counts per second) the laser repetition rate L_{rep} (10 MHz) and the fluorescence lifetime τ (16 ns) are fixed, with the length of the time-gate t_{tg} and the dead time t_{dead} of the TAC/SCA as freely floating fit parameters. The fit yields a 4.4 ns time-gate and a TAC/SCA dead time of approximately 4 μ s for this TAC/SCA-detector pair, with similar results obtained for the other 3 TAC/SCA-detector pairs. We note the TAC/SCA dead time from these measurements ($\sim 4 \mu$ s) is substantially longer than the manufacturer specified dead time (0.65 μ s). The reason for this discrepancy is currently unclear. As mentioned above, other system dead-times (detectors, 50 ns) or the reciprocal of the laser repetition rate (100 ns) are too short to be a substantial factor in this discrepancy.

After establishing that we had acceptable and calculable signal losses for a ~ 4.4 ns time gate at count rates comparable to single QD tracking applications (15-25 kHz per detector or 60-100 kHz summed on all four detectors), we extended our calculations to consider the optimal time-gate for tracking QDs (with a fluorescence lifetime of 16 ns) in a background

of fluorescent protein constructs having a 3.5 ns fluorescence lifetime. For these estimates, we define the time-gated signal-to-noise ratio, SNR_{tg} , as:

$$SNR_{tg} = \frac{f c_{tg}}{d c_{tg} + b g_{tg}} \quad (6)$$

or

$$SNR_{tg} = \frac{f c e^{-\frac{t_{tg}}{\tau}}}{d c (1 - t_{tg} \cdot L_{rep}) + b g e^{-\frac{t_{tg}}{\tau_{bg}}}} \quad (7)$$

where bg is the background fluorescence count rate, bg_{tg} is the background fluorescence count rate with the applied time gate and τ_{bg} is the fluorescence lifetime of the background.

To find the time gate that yields the maximum signal-to-noise ratio, one can take the derivative of SNR_{tg} with respect to t_{tg} and set this equal to zero. This yields:

$$\frac{d}{dt_{tg}} SNR_{tg} = \frac{-\frac{f c e^{-\frac{t_{tg}}{\tau}}}{\tau} \left(d c (1 - t_{tg} \cdot L_{rep}) + b g e^{-\frac{t_{tg}}{\tau_{bg}}} \right) - f c e^{-\frac{t_{tg}}{\tau}} \left(-\frac{b g}{\tau_{bg}} e^{-\frac{t_{tg}}{\tau_{bg}}} - d c \cdot L_{rep} \right)}{\left(d c (1 - t_{tg} \cdot L_{rep}) + b g e^{-\frac{t_{tg}}{\tau_{bg}}} \right)^2} = 0 \quad (8)$$

which simplifies to:

$$\left(\frac{b g}{\tau_{bg}} - \frac{b g}{\tau} \right) e^{-\frac{t_{tg}}{\tau_{bg}}} = \frac{d c}{\tau} - \frac{d c \cdot t_{tg} \cdot L_{rep}}{\tau} - d c \cdot L_{rep} \quad (9)$$

While Eqn. 9 cannot be analytically solved for t_{tg} , this expression does show that the proper choice of time-gate does not depend upon the overall fluorescence intensity, but does depend upon the lifetimes of the signal and background as well as the background and dark count rates. Solving Eqn. 9 numerically for two different values of the dark count rate (1000 Hz and 3500 Hz, which correspond roughly to the highest and lowest dark counts of the 4 detector SPCM-AQ4C unit employed in the tracking experiments) yields the curves shown below in Figure 5.

As discussed above, the optimal time-gate depends upon the fluorescence background count rate that varies from sample to sample. For a fixed dark-count rate of 3.5 kHz, signal level of 20 kHz, and a fluorescence background rate of 5 kHz, we show in Figure 6 how the time-gated signal-to-noise (light blue), total count rate (black), fluorescence signal count rate (red), fluorescence background count rate (green), and dark count rate (dark blue) are affected as a function of the size of the time gate. We note for these parameters (which are comparable to those encountered in live cell tracking experiments), the optimal time-gate is approximately 7 ns, with the expected signal-to-noise ratio being relatively insensitive to the precise choice of gate width.

Prior to time-gated 3D tracking experiments, we show the advantages of time-gating to reduce the fluorescence background stemming from fluorescent proteins used to label other cellular compartments/structures in Figure 7. This fluorescence image is of QD-labeled IgE-Fc ϵ RI receptors on the outside of rat mast cells that are stably transfected to produce yellow fluorescent protein (YFP)-tubulin. While the tubulin structure is clearly seen in the raw (non-time gated) image on the left due to detection of the red tail of the YFP emission in the QD detection channel (Fig. 7A), the time-gated image (Fig. 7B) clearly shows individual QDs as puncta that would be easy to identify and track in the absence of such structure. From a tracking perspective, while we have previously demonstrated an insensitivity of our 3D tracking methods in high background environments, the background in those measurements (fetal bovine serum) was spatially homogeneous.[21] For a spatially inhomogeneous (e.g. structured) background as shown in the raw (un-gated) image shown in Figure 7A, the tracking algorithm we employ is expected to work less effectively than for a homogeneous background. In particular, the error signal we employ for feedback is based on differences between appropriate combinations of the four detectors. [54] While a homogeneous background is essentially subtracted out in calculating the error signal, a spatially heterogeneous (patterned) signal is not, leading in a best case scenario to a tracking offset and a worse case scenario of the tracking algorithm transitioning from following the molecule of interest to following the spatially patterned cellular background. As can be seen in the gated image (Fig. 7B), time-gating reduces the short-lived protein fluorescence substantially, essentially removing the spatially patterned background, providing a solid foundation for 3D tracking.

As discussed above, we have added Nipkow spinning disk microscopy (see Fig. 2) to our 3D tracking system primarily to improve the contextual information obtained during 3D tracking experiments. However, one potential drawback to using fluorescent protein labeled cell lines is that direct excitation of the fluorescent proteins by the tracking laser beam may contribute to a large background (as shown in Fig. 7A), even for band pass filters chosen for QD tracking in the red. As we show above in Fig. 7B, this background can be substantially suppressed by using pulsed excitation and time-gated detection. Below, we further show how time-gated tracking (to remove the contribution of YFP signal erroneously used in the tracking algorithm) combined with spinning disk microscopy of YFP labeled cell lines can be used to obtain long single particle trajectories in high background environments with enhanced contextual information.

In Figure 8 we show a 3D hardware time-gated trajectory of a gQD-DNP-IgE-Fc ϵ RI receptor complex obtained on a stimulated rat mast cell. From the white light image obtained during tracking, (Fig. 8B), it would be difficult to even determine if this QD complex is associated with a cell, as highly stimulated mast cells such as this one are very difficult to visualize by transmitted light microscopy. The gQD position, however, is still clearly visible as the bright spot in the image. However, the spinning disk image (shown in Figure 8C) clearly shows this gQD-DNP-IgE-Fc ϵ RI receptor complex is associated with a cell. In fact, the spinning disk image also shows that the cell with which the gQD-Fc ϵ RI complex is associated has a neighboring cell which is also not visible in the white light image.

A photon pair correlation was calculated from the TCSPC data collected by the HydraHarp module during the track in order to verify the trajectory shown in Figure 8 is of a single gQD-DNP-IgE-FcεRI receptor complex and not an aggregate of multiple gQD-DNP-IgE-FcεRI receptor complexes. As was done previously[10], photons arriving 4 to 40 ns after the laser pulse were used in the calculation, to minimize the contribution of bi-exciton emission to the photon pair correlation (Figure 8E and F). The ratio of the area of the central ($t=0$) peak area to the average area of the outer peaks can be used to count (or at least set an upper limit on) the number of independent quantum emitters.[21] For the photon pair correlation histogram shown Fig. 8E, the ratio of the center to the outer peaks is 0.37, well below the 0.5 limit that corresponds to 2 quantum emitters, signifying this trajectory is unequivocally of a single quantum dot. It is worth reiterating that the TCSPC data collect by the HydraHarp is independent of the hardware time-gating modules used for tracking.

A major advantage of fluorescence imaging while tracking is the structural information of the cell obtained from the fluorescence image. Here, the gQD-FcεRI's location relative to clathrin coated pits on the stimulated cell membrane and the nuclear envelope can be visualized from the spinning disk image. This, in conjunction with the ability to identify and image stimulated cells that are difficult to see with white light imaging, are primary advantages of the simultaneous confocal tracking/fluorescence imaging system over previous implementations that only had a white light image. This opens up the possibility for population studies to correlate receptor motion with cellular morphology or to examine protein interactions important for IgE-FcεRI signaling and downregulation.

4. CONCLUSIONS

While it has long been recognized that quantum dots are superior probes for tracking cellular membrane dynamics due to their relatively small size, large Stokes shift, and excellent photostability, here we exploit another useful feature of quantum dots, namely, their long fluorescence lifetime, to maximize the signal-to-noise in 3D molecular tracking applications. In particular, we show how cellular backgrounds (due to Raman scattered laser light, cellular auto-fluorescence, or fluorescently labeled protein constructs) can be substantially diminished through the use of pulsed excitation and time-gated detection. Perhaps most importantly, the background suppression afforded by time-gating makes it easier to perform 3D SPT experiments on cell lines that are stably transfected with fluorescent proteins. The ability to track single QD labeled proteins in cell lines expressing a large concentration of fluorescent proteins, combined with spinning disk microscopy, greatly increases the contextual information obtained in the measured 3D trajectories. Future work along these lines include fluorescent labeling of other kinases and proteins important for IgE-FcεRI signaling and downregulation by endocytosis.

Here, we have used QDs as a long-fluorescence lifetime probe, but similar time-gating methods could be used for other probes that have even longer fluorescence lifetimes compared to cellular auto-fluorescence, such as lanthanide doped microspheres.[55] Time-gating where the long-lived (rather than short lived) fluorescence is gated out could also be applied to short-lived fluorescence or prompt scattering signals (such as Raman or surface enhanced Raman probes[56]) in backgrounds dominated by longer-lived fluorescence.

We emphasize that the time-gated detection methods demonstrated here are well matched to the single element detector/confocal feedback methods employed by ourselves and others for 3D molecular tracking.[8-10, 16-22] This provides another distinct advantage of confocal feedback 3D tracking methods over camera-based alternatives, as nearly all CCDs can not be gated (or turned off or on) with nanosecond timing accuracy.

While we have demonstrated that improved signal-to-noise can be obtained by time-gated detection using QDs as probes, our method of time-gating in real time (using TAC/SCA modules) is not without signal loss. While signal loss due to the time gate is inevitable, it would be beneficial to minimize the loss in signal due to the deadtime of the TAC/SCA modules. In particular, for time-gating, there is no need to actually record/measure the time between a laser pulse and a detected photon (as is typically done in time-correlated single photon counting), one only needs to make sure photons coincident within a given time-window of the laser pulse are discarded. In future work, fast digital logic gates may prove to be a superior technology to TAC/SCA modules for nearly deadtime-free time-gated 3D tracking.

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Figure 1.

A) Schematic of the gQD conjugation scheme. B) Separation of streptavidin-conjugated gQDs (gQD-SA) from streptavidin (SA) via a size-exclusion column. C) Gel electrophoresis of gQD and gQD-SA shows shift in mass upon gQD-SA binding. Adapted from Reference 10.

Figure 2.
Schematic of the tracking system. See text for details.

Figure 3.

A) Image of fiber pairs viewed through an optical microscope. A lamp was used to illuminate the other end of the fibers to provide contrast in this image. B) Idealized projection of the fiber optic faces back into object space. The four detection probe volumes are labeled D0, D1, D2, and D3 and form a 3D tetrahedron. Feedback in X is provided by the difference of D0 and D1, feedback in Y is provided by the difference of D2 and D3, whereas feedback in Z relies on the difference of D0+D1 and D2+D3.

Figure 4.

Measurements (blue squares) and model (red line) of loss of signal due to TAC modules and time-gate. These are shown for an approximately 4.4 ns gate as a function of the gated signal for a suspension of QDs in solution for one of the four TAC/SCA module-detector combinations. The length of the time gate and the TAC dead time are freely floating fit parameters.

Figure 5.

Optimal time-gate as shown as a function of the fluorescent background count rate for two different values of detector dark counts (black curve is 1000 Hz, red curve is 3500 Hz) that correspond to the minimum and maximum dark count rates for the four detectors of the SPCM-AQ4C unit.

Figure 6.

The anticipated time-gated signal-to-noise (light blue), total count rate (black), fluorescence signal count rate (red), fluorescence background count rate (green), and dark count rate (dark blue) as a function of the size of the time gate for parameters similar to those for single QD tracking in live cells (20 kHz signal, 5 kHz fluorescence background, detector dark counts of 3500 Hz).

Figure 7. Images of QD-FcεRI on rat mast cells stably transfected with YFP-tubulin taken without (A) and with (B) a ~4 ns time gate. Note the un-gated (raw) image on the left clearly shows intracellular structure, whereas the image on the right shows mainly QD-FcεRI located on the cell membrane. A linear blue-scale between minimum and maximum pixel values has been applied to each image.

Figure 8.

A hardware time-gated track of gQD-DNP-IgE-FcεRI on the membrane of an RBL cell. The Rainbow color scheme denotes the passage of time. A) A 3D representation of the gQD-DNP-IgE-FcεRI trajectory. B) White light image of the cell at 20 s in the trajectory. C) Spinning disk image of the GFP-clathrin taken at 20 s. D) The count-rate of the particle after time-gating which was used by the tracking algorithm. E) The lifetime histogram of the gQD and background fluorescence calculated from the non-hardware gated TCSPC module. The software time gate used for calculating a time-gated photon pair correlation (from the HydraHarp TCSPC data) is shown by the black vertical lines. F) Photon anti-bunching curve calculated from the TCSPC data after applying a 4-40ns time-gate in post processing.