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ABSTRACT
A microscopy platform that leverages the arrival time of individual photons to enable 3D single-particle tracking of fast-moving (translational diffusion coefficient of $\approx 3.3 \mu m^2/s$) particles in high-background environments is reported here. It combines a hardware-based time-gating module, which enables the rate of photon processing to be as high as 100 MHz, with a two-photon-excited 3D single-particle tracking confocal microscope to enable high sample penetration depth. Proof-of-principle experiments where single quantum dots are tracked in solutions containing dye-stained cellulose, are shown with tracking performance markedly improved using the hardware-based time-gating module. Such a microscope design is anticipated to be of use to a variety of communities who wish to track single particles in cellular environments, which commonly have high fluorescence and scattering background.

I. INTRODUCTION
There is increasing recognition that molecular actions are difficult to divorce from the surrounding context, as exemplified by those of biological macromolecules. This, in a sense, is not surprising; a significant and ongoing effort of physical chemistry/chemical physics is to fully comprehend the effect of the solvent on a variety of different unimolecular and bimolecular reactions. Elucidating the local-medium dependence in a complex environment presents a much greater challenge than understanding a small-molecule reaction in a homogeneous solvent, however. In particular, biological environments are diverse, as well as being both spatially and temporally heterogeneous, with many experiments showing that a variety of weak chemical interactions can have significant effects on protein stability and, thus, function. It is, therefore, essential that the capable microscopic and spectroscopic techniques are implemented to bear on such problems in the native biological context.

A promising new approach is to follow, in real-time, the 3D movements of a single nanoscale object with nanometer-localization precision in cellular milieu while simultaneously acquiring the object’s contextual environment or correlating the object’s trajectory with a separately acquired image. This concept has been shown to provide a great deal of information on the microsecond local dynamics of biosystems, information not obtainable using other imaging methods. The core technology that enables these works is real-time 3D single-particle tracking (RT-3DSPT). Utilizing RT-3DSPT in tissue samples with a high time resolution,
on the order of tens of μs, proves a further challenge. If an experiment is performed to determine the localization of a particle in a dye-labeled specimen, then a high luminescence background will contaminate the tracking signals. Even if the sample is not incubated with any labeling dye, there is still the fact that cells autofluoresce and scatter. It is due to this that, even with extremely emissive probes, their signal will be overwhelmed by background noise in biological environments. Using the bright Atto633 dye, Chen et al. were, in a recent experiment, only able to reach a signal-to-background ratio (SBR) of 2.5 when measuring the annealing of DNA strands in vivo. Thus, an experimental method that enables isolating mostly photons from the particular probe is desired.

Discriminating probe signals from the background can, in principle, be enhanced by analyzing and incorporating spectral information in real-time while tracking the probe. Spectroscopic signatures from single nanoscale probes that have been measured using the RT-3DSPT technique include polarization, wavelength, and excited-state lifetime. Among them, the excited-state lifetime, which is realized as photon arrival time in single-photon-counting experiments, appears to be conceptually most straightforward for use in discriminating signal from background. Indeed, using photon arrival times to discriminate the signal origin has been in use for a long time in a variety of biological and non-biological imaging contexts. For imaging experiments, it provides an additional contrast mechanism to preferentially use photons from specific labels. Dahan et al. reported that the signal-to-background ratio of the quantum dot (QD) stained mouse 3T3 cells was increased by a factor of 15 by time gating the photons collected. In the context of 3D single-particle tracking, an experiment in which the tracking probe has a long luminescence lifetime and the surrounding cellular context is labeled with dyes of shorter lifetimes may be considered; the shorter lifetime photons may be time-gated away, hence affording to track only the longer-lifetime probe while excluding the background by only using photons that arrive at longer times after the excitation pulse. A cartoon illustrating this idea is shown in Fig. 1.

The ideal probe for RT-3DSPT should be bright, as it is via the photons that we are able to counter particle motion, and it should be photostable so that the tracking experiment can be as long as possible. If we wish to time-gate the photons we collect, the probe should have a long luminescence lifetime to be most distinguishable from the background. Giant quantum dots (gQDs) with reduced blinking behavior are, thus, very promising candidates to meet these requirements due to being bright, photostable, and having long luminescence lifetimes. DeVore et al. first used gQDs and time gating simultaneously in real-time 3D single-particle tracking to improve the signal-to-background ratio (SBR). With this improvement in SBR, they were able to track gQDs with a translational diffusion coefficient of ~0.1 μm²/s in dye-labeled rat mast cells. In that initial demonstration, however, there was significant dead-time due to the programming-based implementation, and thus, the tracking feedback rate was 200 Hz, insufficient to follow faster dynamics. Specifically, the time-to-amplitude converter module added 4 μs of dead-time, limiting the possible feedback rate. Since biological phenomena occur on diverse time scales, it is essential that instrumentation be able to capture fast dynamics. As examples for biological time scales of interest, the diffusion coefficient of a 100-nm diameter particle in water is 4.3 μm²/s. In real biological surroundings, a 35.8 kDa protein showed a diffusion coefficient of ~3.8 μm²/s in an E. coli cell, and the motor protein, Kinesin-1, exhibited a diffusion coefficient of ~1.4 μm²/s in lipid bilayers. It would not be possible to observe these phenomena using the earlier software-based design for time gating due to the low tracking feedback rate. Consequently, an implementation of time-gated real-time 3D single-particle tracking (TG-RT3DSPT) with improved tracking capability is necessary.

In this work, we describe a hardware-based implementation that enables the tracking of particles with considerably higher translational diffusion coefficients in high-background environments. Specifically, we demonstrate tracking particles with translational diffusion coefficients of up to (3.26 ± 0.58) μm²/s in a noisy artificial biological environment where the SBR was improved from 2.4 ± 1.3 to 5.9 ± 1.5. The best SBR improvement observed was from 2.4 ± 1.3 to 204 ± 61, a two-order-of-magnitude enhancement over prior work in which the SBR is improved from ~2.0 to ~3.2, representing a factor of 1.6 improvement. In our implementation, we use two-photon excitation because it improves the penetration depth into cellular and tissue or plant-cell samples, as well as further increasing the SBR by reducing both laser and Raman scatterings and excluding out-of-focus light.

II. EXPERIMENTAL METHODS

Figure 2 displays the experimental scheme of the time-gated real-time 3D single-particle tracking instrument, composed of an excitation module, a tracking module, a time-gating module, and a time-correlated single-photon counting (TCSPC) module.

A. Excitation module

The excitation light source of the two-photon confocal microscope was an 800 nm 80-MHz ultrafast laser (Ti: Sapphire oscillator, Tsunami, Spectra Physics). To facilitate gating of the long-emission lifetime of gQDs, the repetition rate of the oscillator was reduced using a Pockels cell (Conoptics, Model 25D and Model 320) to 40 MHz, providing a 25 ns delay between pulses.
B. Tracking module

The implementation of the real-time 3D single-particle tracking used in this study has been described before. For completeness, it is sketched below. The technique relies on an active feedback system that uses a 3D piezoelectric stage to compensate for the motion of the particle being tracked, locking the particle in the focus of the stationary microscope objective. This was achieved as follows: The emission from a two-photon-excited probe was split in a 1:9 ratio, with 10% of the emission going to an Electron Multiplying Charge-Coupled Device (EMCCD) camera (Roper Scientific) and 90% going to the tracking optics. A dichroic with a cutoff at 750 nm (FF749-SDi01-25 x 36 x 3.0, Semrock) was used to reflect the emission to the tracking optics and the monitoring camera after going through a 710-nm short-pass filter (SPF) (FF01-715/SP-25, Semrock). 50% of the light sent to the tracking optics was projected using an optical cantilever of 2 m in length onto two orthogonal prism mirrors. Each prism mirror, one for the X direction particle motion and the other for the Y particle direction, splits the signal onto two single-photon-counting avalanche photodiodes (APDs, Perkin Elmer, SPCM-AQRH-13). When a particle is centered in X or Y, the reading on the two corresponding APDs is equal. However, when −single nm deviations in the particle position occur, the detectors will have an imbalance in their signal that is used for feedback control, with the stage counteracting this motion to return the detectors to balance by moving the particle back to the center of the microscope focus. In this way, the X and Y motions are compensated for, ”locking” the particle at the center of the focus in X and Y. To compensate for any motion in the Z (optical axis) direction, 50% of the light sent to the tracking optics was projected through a slightly axially offset pinhole (10 μm diameter here), which, when imaged onto an APD, provided a quasi-linear index into the deviation in Z when such an APD intensity was normalized against the four X–Y APDs. The signals from the APDs were sent into the gating module (see Sec. II C) before being used by a field programmable gate array (FPGA, National Instruments, 7833R) to generate the feedback signals necessary, at a rate of 100 kHz, for the piezoelectric stage (Physik Instrumente, P-561.3DD) to keep the particle at the center of the focal volume. This, thus, gives the technique a time resolution of 10 μs. A ~10 nm localization precision is generally achievable for all three XYZ axes using this tracking technique. The piezoelectric stage used here has a translation range of 70 x 70 x 20 μm³.

C. Gating module

The outputs of the APDs were time-gated at the hardware level, using a constant fraction discriminator (CFD) and a logic gate in a manner illustrated in Fig. 3. The essence of the technique, similar to time-correlated single-photon counting (TCSPC), is this: the CFD takes the signal of a photon arrival from an APD and produces a pulse of temporal gating width (W_G) specified by the user. The logic gate then detects if this pulse overlaps with the laser reference pulse train, specifically the 40 MHz trigger output of the Pockels cell hardware, and if it does overlap with the reference pulse train, the pulse is accepted and 1 photon count is sent to the tracking optics. Thus, as shown in Fig. 3, by increasing the W_G, we may accept photons that arrive earlier after the excitation pulse, more likely to be associated with shorter-lifetime species such as fluorescent dyes. Conversely,
The timing diagram of the hardware-based time-gated technique. The dashed signals indicate either the laser pulse suppressed by the electro-optical modulator or the photon rejected by the logic gate. By decreasing it, we may only accept photons that arrive later after the excitation pulse, more likely to be associated with longer-lifetime species such as gQDs.

To facilitate the use of such a concept, the signals from the APDs and the laser reference pulse train were synchronized using a nanosecond delay unit (Phillips Scientific, Dual NanoSecond Delay Module, Model 7192), and the outputs of these APDs and laser reference pulse were synchronized using the excitation laser pulse with low intensity. After this synchronization had been achieved, a level translator (Phillips Scientific, 16 Channel Logic Level Translator, Model 726) was implemented to convert the laser reference pulse, more likely to be associated with longer-lifetime species such as gQDs.

To avoid the pile-up effect, the excitation power in the TCSPC experiment was adjusted such that the photon counts detected were lower than 1% of the excitation rate. Translational diffusion coefficients ($D_y$ values) from the single-particle tracking data were calculated using the previously described maximum likelihood estimation (MLE) method. All analysis codes were written in MATLAB 2019a.

E. Sample preparation

Two variants of SiO$_2$ coated gQDs (gQDs@SiO$_2$), with reduced blinking behavior, were used as trackers to evaluate the performance of the time-gated tracking technique. These samples have different lifetimes and emission spectra, and, hereafter, are referred to as A-gQDs and B-gQDs (or A-gQDs@SiO$_2$ and B-gQDs@SiO$_2$). A-gQDs were synthesized using the methods described before. The method of synthesizing the B-gQDs will be the subject of an upcoming report.

F. Scanning two-photon microscopy

Scanning two-photon microscopy was used to obtain raster-scan images of solid phase samples to evaluate the time-gated performance, which will be discussed in Sec. III B. Two-photon microscopy images were taken by scanning the piezostage across a 2D area (100 × 100 pixel$^2$, 20 × 20 μm$^2$, 200 nm/pixel) and reading out the emission at each pixel (5 ns dwell time/pixel) using an APD (Perkin Elmer, SPCM-AQRH-13). The signal from the APD was also sent into time-gated module described in Sec. II C to generate $W_G$ dependent two-photon images.

G. Steady-state measurements

Transmission spectra of optics were acquired using a Cary 8454 UV–vis spectrometer (Agilent Technologies). Absorption and emission spectra of the samples were recorded using a SpectraMax M5 (Molecular Devices) and a Fluorolog-3 (Horiba-Jobin Yvon), respectively. Emission spectra were corrected for the wavelength sensitivity of the spectrometer using a correction function supplied by the manufacturer.

H. Data analysis

Multiexponential functions were fit to the lifetime decays using a convolute-and-compare algorithm. To avoid the pile-up effect, the excitation power in the TCSPC experiment was adjusted such that the photon counts detected were lower than 1% of the excitation rate. Translational diffusion coefficients ($D_y$ values) from the single-particle tracking data were calculated using the previously described maximum likelihood estimation (MLE) method. All analysis codes were written in MATLAB 2019a.
used to provide a high-fluorescence environment. To show that the background emission was, indeed, suppressed by the gating module and not by the use of optical filters, the transmissions of the dichroic mirror and short-pass filter are also shown here and represented by dashed lines. As depicted in Fig. 4, photons from both dyes and gQDs were able to be detected by the tracking optics, from 450 to 680 nm in wavelength. The emission of the dye-stained cellulose solution shows two prominent peaks, at 530 and 680 nm, from DTAF and SYTO 62, respectively. The A-gQDs, as expected, show a narrow emission band centered at 620 nm, whereas the B-gQDs show an emission band centered at 650 nm.

The luminescence decay behavior of gQDs and dye-stained celluloses as a function of time was characterized using the TCSPC module. To match the excitation condition in the tracking experiment, the excitation wavelength was also set at 800 nm for lifetime measurements. The data and fits are shown in Fig. 5. The lifetimes of both sets of gQDs (~13.4 and ~24.0 ns for A-gQDs and B-gQDs, respectively) were considerably longer than the lifetime of the dye-stained cellulose mixture of ~2.84 ns. A triexponential model was found to yield the best fit for both sets of gQDs. The fit parameters are shown in Table I. The relatively large values were observed from the fits due to the unextinguished 80 MHz from the Pockels cell that caused the oscillations shown in the residuals of Fig. 5. The key fact from these experiments is that clearly, both sets of gQDs exhibit considerably longer lifetimes than the dye-stained cellulose mixture and most other fluorescent dyes commonly used in staining cells and/or single-molecule spectroscopy. Thus, these gQD probes should be ideal for TG-RT3DSTPT experiments.

**B. Time-gated scanning two-photon microscopy**

The effectiveness of the gating module was first examined using a series of two-photon-excited raster-scan images of a surface containing a mixture of A-gQDs and dye-stained cellulose flakes acquired using different WGs. These are shown in Fig. 6. Photon counts of the gQD response were obtained from the amplitude of a 2D Gaussian fit, and the photon counts from cellulose flakes were acquired by averaging photon counts in a 2 × 2 μm² cellulose flake area. Varying the W GS resulted in changing the number of photons detected from each area and, thus, the image contrast.

As is shown in Fig. 6, when the W G was set at 20 ns, minimal gating occurred and the intensities from the cellulose flakes and the gQDs were both clearly visible. When the minimum W G, 5 ns, was applied, mostly photons from the gQD could be observed. The signal-to-background ratio and photon counts from the mixture of gQD–cellulose flakes in relation to the W G are plotted in Fig. 7. The signal-to-background ratio is calculated using

\[
\text{SBR} = \frac{I^0 + B}{B},
\]

where \(I^0\) is the signal intensity in the absence of background B. As can be seen from Fig. 7, when the W G was decreased from 20 to 5 ns, the SBR increased from 2.9 ± 1.6 to 45 ± 43. The photon counts from the gQD and cellulose flake decreased from (42 ± 11) to (11.7 ± 4.4) kcps and from (14.4 ± 3.5) to (0.26 ± 0.02) kcps, respectively.
Quantitatively, the apparent photon counts from the sample after time gating can be described by the theoretical model,

\[ I = a \sum_{j=0}^{\infty} \int_{t_{\text{L}}-t_0-W_G}^{t_{\text{L}}-t_0} f(t + jT_L) \, dt, \]

where \( I \) is the photon counts of the species under consideration, \( f(t) \) is the lifetime behavior of the species in question, \( T_L \) is the delay between two excitation pulses, \( W_G \) is the gating width, and \( t_0 \) is the time zero, i.e., the excitation arrival time. Here, the species being considered includes emission from both the probe and the background. The sum over \( j \) is to account for the possibility of photons arriving from an excitation pulse that occurred before the previous one. In practice, when the lifetime is shorter than 200 ns, the sum can be truncated at \( j = 50 \) to reduce the computation cost (see supplementary material, Sec. S5). The \( a \) parameter is an empirical scaling factor for matching the model with observed photon counts and is the only fit parameter here. These parameters are visualized in Fig. 8.

To compare Eq. (2) with the experiment (cf. Fig. 6), instead of using the lifetime profile measured in solution for \( f(t) \), an additional measurement was conducted to determine the emission lifetime of gQDs at the solution–coverslip interface (see supplementary material, Sec. S4) to take into account potential changes in photophysics in different local environments. Using the interfacial lifetime profile for \( f(t) \), the fits are done by adjusting the \( a \) factor to obtain the best agreement with the data, which yields reasonable agreement with the trend of A-gQD photon counts vs \( W_G \) and between the dye-stained cellulose flake photon counts vs \( W_G \). The fit results are represented by the solid lines in Fig. 7. These data clearly validate the hardware-based time-gating approach, finding a substantial improvement in the signal-to-background ratio with decreasing \( W_G \).

### C. Lifetime-gated real-time 3D single-particle tracking

After the effectiveness of the gating module was verified, the lifetime gated tracking experiments were carried out. In practice, in the real-time 3D single-particle tracking concept used here, a trade-off between SBR and photon counts must be considered. If an infinitely high SBR is achieved but only one photon is detected every millisecond, this is not going to enable tracking except for a particle diffusing with an exceptionally low translational diffusion coefficient. The information is required, via photon counts on each detector, as to where the particle is in real-time. Thus, this need for photons with the ability to exclude light from short-lifetime species must be balanced.

The optimal time delay between two consecutive excitation pulses to measure a lifetime with the most accuracy is \( \geq 10 \times T_L \) the lifetime in question.\(^{22}\) For A-gQDs, this would correspond to a \( T_L \) of 200 ns between pulses, i.e., a 5 MHz repetition rate. To ensure

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \tau_1 )</th>
<th>( a_1 )</th>
<th>( \tau_2 )</th>
<th>( a_2 )</th>
<th>( \tau_3 )</th>
<th>( a_3 )</th>
<th>( \langle \tau \rangle ) (ns) \textsuperscript{1)}</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS cellulose</td>
<td>2.842 ± 0.066</td>
<td>1</td>
<td>9.5 ± 1.2</td>
<td>0.370 ± 0.021</td>
<td>48.0 ± 5.4</td>
<td>0.196 ± 0.026</td>
<td>13.4 ± 1.8</td>
<td>10.3</td>
</tr>
<tr>
<td>A-gQDs</td>
<td>1.07 ± 0.18</td>
<td>0.443 ± 0.057</td>
<td>3.32 ± 0.14</td>
<td>0.568 ± 0.043</td>
<td>27.8 ± 2.1</td>
<td>0.346 ± 0.045</td>
<td>145 ± 13</td>
<td>11.1</td>
</tr>
<tr>
<td>B-gQDs</td>
<td>3.32 ± 0.14</td>
<td>0.568 ± 0.043</td>
<td>27.8 ± 2.1</td>
<td>0.346 ± 0.045</td>
<td>145 ± 13</td>
<td>0.086 ± 0.010</td>
<td>24.0 ± 4.4</td>
<td>11.1</td>
</tr>
</tbody>
</table>

\textsuperscript{1)} \( \langle \tau \rangle = \sum a_i \tau_i \).

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**FIG. 6.** Two-photon scanning microscope images as a function of \( W_G \). The \( W_G \) is 20, 15, 10, and 5 ns for (a)–(d), respectively. The sample was a spin-coated and air-dried mixed dye solution stained Avicel PH101 cellulose and A-gQDs. The excitation power was 0.9 mW. The scale bar in each subplot represents 3 \( \mu \m\).**

**FIG. 7.** Measured A-gQD and dye-stained cellulose flake counts (red and green dots, error bars are ±standard deviation) and corresponding fits using Eq. (2) (red and green lines). The SBR at each \( W_G \) is shown as a black dot, and error bars show ±standard deviation. The statistical data are obtained from 26 gQDs and 27 cellulose flakes.
that the lifetime measurement is not distorted by the pile-up effect, however, this would correspond to a maximum amount of detected photons of 250 kcps, a large number of which would then be rejected by the gating module. This maximum assumes that each of the five detectors observes a photon at max 1% rate of excitation. Shorter delays between excitation pulses would enable considerably higher photon flux at the detectors at the expense of perhaps a less severe improvement in the SBR. Given the system of photon flux at the detectors at the expense of perhaps a less severe improvement in SBR by tracking events at different interplay of the maximum number of photon counts vs the improvement in SBR by tracking events at different $W_G$ at various $T_L$ values. The results are shown in Fig. 9.

The simulations appear to suggest that the trade-off between photon numbers and SBR is slightly more weighted to the photon numbers here. At 20, 40, and 80 MHz, there is a greater range where a lower tracking error is observed than at 2.5 MHz. In this case, the range where the tracking error is low is extremely small and has a higher error than at higher repetition rates. Thus, given the desire to have stable tracking and to have enough steps of $W_G$ to improve the SBR, a 40 MHz repetition rate has been used in the tracking experiments. Note that this choice is dependent on a number of parameters, described more in detail in Sec. S6. To be brief, if the two-photon cross sections, the lifetime, the emission spectra of the probe or background, the probe or background’s quantum yield, the downstream optics, the quantum efficiency, and the instrument response of the detector as a function of wavelength change, the tracking error vs $W_G$ is also anticipated to change. This will, in turn, require tuning of the repetition rate and the $W_G$ in order to lead to the least tracking error. To find the “optimal” $W_G$ for a particular experiment is a complex multi-parameter problem, as all experimental parameters in some way contribute to the tracking stability.

Thus, in principle, a change in any of these factors, with particular emphasis on probe and/or background, does imply that parameters for the time-gated tracking experiment must be re-optimized on a per scientific problem basis.

Based on simulations, experiments to examine at what $W_G$ the TG-RT3DSPT gave the most stable results were performed at a 40 MHz repetition rate. A sample of the dye-stained cellulose and A-gQDs was suspended in a nominally 2/1 v/v glycerol/water mixture (26.53 mPa s, or cP, viscosity). At $W_G = 5$ ns, the photon counts from A-gQDs in time-gated 3D tracking experiment were less than 50 kcps, which was not able to provide stable real-time 3D tracking. Thus, we tracked A-gQDs at 10, 15, and 20 ns $W_G$ settings and, as a reference of comparison, without using the time-gating module (a $W_G$ of 25 ns). The fluorescence background, determined by averaging over ten different cover slip regions of a sample solution prepared without A-gQDs, was (250 ± 40) kcps. A variety of different metrics that report on the time-gated 3D tracking performance are shown in Table II, and a representative tracking trajectory is shown in Fig. 10.

As is seen in Table II, when the $W_G$ was decreased from 25 to 10 ns, the total photon counts from the gating module decreased from ~750 to ~70 kcps, and the signal-to-background ratio increased from ~2.4 to ~12.6. More relevant metrics to assess the time-gated tracking performance are the mean track length and the highest translational diffusion coefficient we may measure; longer track lengths naturally imply that more data from the track can be extracted, and higher particle translational diffusion coefficients imply that the tracking system may measure faster particles and, thus, performs better. This, coupled with the fact that the mean track

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**Figures:**

- **Fig. 8.** This diagram depicts the parameters that may vary in the gating experiment. $T_G$ is the delay between two excitation pulses, $W_G$ is the gating width, $t_0$ is the time zero, and $T_G$ is the delay time between $t_0$ and the overlap between the $W_G$ and the next reference pulse. The maximum value of the lifetime curves are normalized to 1 for clear visual comparison.

- **Fig. 9.** Simulated tracking error, i.e., distance the stage deviates from the particle position, vs $W_G$ for a variety of different laser repetition rates. $W_G$ is normalized to 1 at maximum $W_G$ so that different repetition rates can be compared. The dots represent 5 ns steps in $W_G$ for the different repetition rates, as 5 ns is the smallest step size by which we may change the $W_G$ with the hardware used here. Values are mean values from 1000 particle trajectories, and data ± standard deviation is presented in Fig. S8.
lengths are the longest at this \( W_G \), shows that at \( W_G = 20 \) ns, the time-gated 3D tracking system performs best for this probe. This clearly demonstrates that both in simulation and in experiment, the best tracking is a trade-off between the SBR and photon counts; too many background photons degrade tracking performance: the mean track length decreases as the \( W_G \) decreases from 20 to 10 ns. Too few photons and tracking becomes difficult to achieve: as the \( W_G \) decreases from 20 to 10 ns, the mean track length decreases as well.

As further evidence of the improvement in tracking stability in high-background environments engendered by the gating module, the volume % of glycerol in the A-gQD/stained cellulose solution was decreased to increase the mobility of the gQDs. At \( W_G = 10 \) ns, the highest diffusion coefficient where tracking remained possible was \( \sim 1.19 \) \( \mu m^2/s \), which rises to \( \sim 3.10 \) \( \mu m^2/s \) and then \( \sim 3.26 \) \( \mu m^2/s \) for \( W_G = 15 \) ns and 20 ns, respectively. These data clearly demonstrate that our system enables fast-moving objects to be tracked in high-background environments. Indeed, the previous software-level gating module only was able to track a probe of diffusion coefficient \( \sim 0.1 \) \( \mu m^2/s \); thus, the implementation, described in this work, represents a 33-fold improvement in tracking performance in high-background environments.

An example of a successful tracking experiment is shown in Fig. 10, of a track that appears to cross a cellulose flake (video included in the supplementary material), with the fluorescence from the flake visible on the EMCCD recording but sufficiently reduced by the gating as to not interrupt the tracking of the A-gQD. The extracted diffusion coefficients of each axis are shown in Figs. 10(c) and 10(d). These coefficients were derived from the underlying 1D diffusion coefficients, calculated using literature MLE equations. The Kalman filter corrected apparent diffusion coefficient \( D_c \) for each axis is represented by black dots. The MLE for a 1D diffusion coefficient was given by

\[
\hat{D} = D_c - \frac{\sigma_p^2}{\delta^2},
\]

where \( \delta \) is the time lag duration and \( \sigma_p \) is the localization precision. The error in \( \hat{D} \) was expressed by

\[
\sigma_D = \sqrt{\frac{2(\sigma_p^2 + \sigma_D^2)}{N \delta^2}},
\]

where \( N \) refers to the number of statistically independent time lags of duration \( \delta \) available in the trajectory. These formulas were used to calculate 1D diffusion coefficients and their errors by inputting the \( \delta_{\min} \) that minimized \( \sigma_D \). The translational diffusion coefficient was given by their average, \( \hat{D}_T = (\hat{D}_x + \hat{D}_y + \hat{D}_z)/3 \), and its error by \( \sigma_{\hat{D}_T} = \sqrt{\frac{2(\sigma_x^2 + \sigma_y^2 + \sigma_z^2)}{3N \delta^2}} \) for the sample trajectory in Fig. 10, the translational diffusion coefficient is \( (1.14 \pm 0.24) \mu m^2/s \).

To further demonstrate the use of the gating module, tracking using B-gQDs in the presence of 3-\muM DTAF was examined. As the B-gQDs have a longer average lifetime than the A-gQDs (see Table 1), it was anticipated that lower \( W_G \) values than the case of the A-gQDs would lead to higher tracking performance. In addition, the two-photon cross sections of B-gQDs appear to be considerably higher than the A-gQDs, meaning more photons are created at a lower excitation flux. This also suggests that shorter \( W_G \)s may be used as more photons may be discarded without a major impact on tracking performance. Simulations also suggest this to be the case.

### Table II. Statistical results of the time-gated 3D tracking experiment using A-gQD under different \( W_G \)s.

<table>
<thead>
<tr>
<th>( W_G ) (ns)</th>
<th>Success rate* (%)</th>
<th>Mean track length (s)</th>
<th>Photon counts (kcps)</th>
<th>SBR*</th>
<th>N*</th>
<th>Fastest tracked particle (( \mu m^2/s ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>6.9</td>
<td>3.71 ± 0.37</td>
<td>750 ± 210</td>
<td>2.4</td>
<td>129</td>
<td>1.14 ± 0.17</td>
</tr>
<tr>
<td>20</td>
<td>32</td>
<td>14.3 ± 5.8</td>
<td>420 ± 65</td>
<td>5.9</td>
<td>82</td>
<td>3.26 ± 0.58</td>
</tr>
<tr>
<td>15</td>
<td>17</td>
<td>8.6 ± 4.6</td>
<td>210 ± 43</td>
<td>7.8</td>
<td>93</td>
<td>3.10 ± 0.46</td>
</tr>
<tr>
<td>10</td>
<td>3.4</td>
<td>4.1 ± 1.7</td>
<td>70 ± 15</td>
<td>12.6</td>
<td>67</td>
<td>1.19 ± 0.20</td>
</tr>
</tbody>
</table>

*These results were obtained using a sample with a diffusion coefficient of \( \sim 1.2 \mu m^2/s \). The trajectories longer than 3 s were considered as valid for analysis. Full details as to the count level of A-gQD and dye-stained cellulose are provided in Table S1.

**Number of successful tracks used in analysis.

The faster diffusing particles were prepared by adjusting the water/glycerol ratio of the solution. The diffusion coefficient was extracted using a single trajectory.
TABLE III. The statistical result of the time-gated 3D tracking experiment using B-gQD under different W_Gs.

<table>
<thead>
<tr>
<th>W_G (ns)</th>
<th>Success rate (%)</th>
<th>Mean track length (s)</th>
<th>Photon counts (kcps)</th>
<th>SBR</th>
<th>N^2</th>
<th>Fastest tracked particle (μm^2/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>8.7</td>
<td>4.3 ± 1.8</td>
<td>2800 ± 280</td>
<td>2.5 ± 2.1</td>
<td>84</td>
<td>1.22 ± 0.23</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>8.1 ± 1.1</td>
<td>800 ± 110</td>
<td>204 ± 61</td>
<td>66</td>
<td>2.42 ± 0.39</td>
</tr>
</tbody>
</table>

1These results were obtained using a sample with a diffusion coefficient of ~1.2 μm^2/s. Trajectories longer than 3 s were considered as valid for analysis. Full details as to count levels of B-gQD and DTAF at each W_G are provided in Table S1.

2Number of successful tracks used in analysis.

3Samples of different mobilities were prepared by adjusting the water/glycerol ratio of the solution. The diffusion coefficient was extracted using a single trajectory.

(see supplementary material, Sec. S8), and this is, indeed, supported by experiment. For example, at a W_G of 10 ns, whereas the performance of tracking A-gQDs was equivalent to that of an un gated A-gQD-tracking experiment (W_G = 25 ns), the mean trajectory length for 10-ns time-gated tracking of B-gQDs showed 1.9 times better performance over an un gated B-gQD-tracking experiment (~4.3 vs ~8.1 s) as well as much greater improved signal-to-background ratio (from ~2.5 to ~204). The results are summarized in Table III, and once again, it is highlighted that tracking stability and optimization are a trade-off between photon numbers and background exclusion and that different probes will enable more or less background to be excluded and maintain good tracking behavior. It also clearly points out that the gating module enables a variety of probes to be used to track single particles in high-background environments. An example track is included in Fig. S10, showing that the B-gQD can be tracked over a long period (12.1 s in this example track) with no loss of tracking caused by the background photons.

IV. CONCLUSIONS

Demonstrated here is a hardware-based implementation of an emission lifetime-gated real-time 3D single-particle tracking instrument. It allows signals associated with short-lifetime background emission and scattering to be excluded, enabling significantly improved tracking stability of longer-lifetime probes. Specifically, we have shown a 33-fold improvement in terms of the mobility of particles that may be tracked comparing to the prior work\(^5\) and that it is possible to achieve, while maintaining stable tracking, an ~100-fold improvement in the signal-to-background ratio with the use of longer-lifetime QDs as probes. We have also implemented a simulator for such experiments, which may be used to explore the trade-off between photon counts and background exclusion as a function of the characteristics of the probe, the background, the repetition rate, and the gating width.

This implementation of a time-gated real-time 3D single particle tracking system, which should, in the future, be able to be coupled to other imaging\(^6,7\) and control\(^8,9\) modalities, can be expected to broaden the scope of experimentally accessible systems, e.g., to enable new studies of nanoscale dynamics in biological and other complex systems that otherwise exhibit prohibitively high backgrounds.

SUPPLEMENTARY MATERIAL

See the supplementary material for the procedure of silica over-coating of QDs, list of chemicals purchased and storage procedure, cellulose sample preparation, further two-photon scanning microscope images, movie of tracking an A-gQD in a stained cellulose solution, demonstration of the convergence of Eq. (2), full description of the TG-3DSPT simulator, and TG-3DSPT simulations for B-gQDs.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

DATA AVAILABILITY

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

REFERENCES


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