3D single-molecule tracking using one- and two-photon excitation microscopy
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ABSTRACT

Three dimensional single-molecule tracking (3D-SMT) has revolutionized the way we study fundamental cellular processes. By analyzing the spatial trajectories of individual molecules (e.g. a receptor or a signaling molecule) in 3D space, one can discern the internalization or transport dynamics of these molecules, study the heterogeneity of subcellular structures, and elucidate the complex spatiotemporal regulation mechanisms. Sub-diffraction localization precision, sub-millisecond temporal resolution and tens-of-seconds observation period are the benchmarks of current 3D-SMT techniques. We have recently built two molecular tracking systems in our labs. The first system is a previously reported confocal tracking system, which we denote as the 1P-1E-4D (one-photon excitation, one excitation beam, and four fiber-coupled detectors) system. The second system is a whole new design that is based on two-photon excitation, which we denote as the 2P-4E-1D (two-photon excitation, four excitation beams, and only one detector) system. Here we compare these two systems based on Monte Carlo simulation of tracking a diffusing fluorescent molecule. Through our simulation, we have characterized the limitation of individual systems and optimized the system parameters such as magnification, z-plane separation, and feedback gains.

Keywords: single-molecule tracking, confocal microscopy, two-photon excitation, Monte Carlo simulation

1. INTRODUCTION

Detection of single molecules by laser-induced fluorescence is a powerful tool to characterize biological and chemical processes. However, fast diffusion of small molecules in solution makes it difficult to observe a target molecule longer than 1 millisecond, the time typically required for a molecule to cross the field of view of a microscope objective. Therefore, there has been a driving force to develop single-molecule tracking (SMT) techniques that enable observation of nanoscale objects in free solution or inside live cells for extended period of time. Up to now many two dimensional SMT schemes have been introduced and provided information about nuclear transport, membrane diffusion, and molecular motor movement. While the results of 2D tracking are enlightening, cells are intrinsically three dimensional and anisotropic. To achieve 3D SMT, several methods have been proposed, including defocused imaging, multi-plane imaging, astigmatism, and double-helix point spread function. A common limitation of these camera-based approaches is the Z tracking range (approximately ±0.5 μm), due to the shallow depth of focus of a high-numerical-aperture objective that is necessary for single-molecule imaging. In addition, camera-based tracking usually suffers from a large fluorescence background. This is because of its wide-field, epi-illumination scheme. Moreover, these approaches are bounded in temporal resolution by the camera frame rate (~1ms for fast cameras).

In our lab, two 3D-SMT microscopes have been built that offer several distinguished advantages. The first system is a confocal-feedback tracking microscope that was originally developed in Werner’s lab. Here we denote this confocal system as the 1P-1E-4D system, since it is built upon one-photon excitation, one excitation beam, and four fiber-coupled detectors. The second system is a new design developed by us that uses two-photon excitation and four spatiotemporally demultiplexed excitation beams to encode the position of a fluorescently tagged molecule. As only one single-photon detector is needed, we denote this system as the 2P-4E-1D (two-photon excitation scheme, four excitation beams, and one detector) system. Since both of our tracking microscopes utilize single-photon counting modules (SPCMs) that have instrument response time much shorter than 1ms, they can follow fast motion on timescale orders of magnitude faster than tracking from image. We point out that only a few groups built their tracking systems based on SPCMs. The key advantage of SPCM-based tracking systems lies in time-correlated single photon counting (TCSPC), which enables lifetime measurement while tracking a molecule. In terms of the signal-to-noise ratio, background fluorescence

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is strongly suppressed in our tracking systems by two different mechanisms: 1P-1E-4D utilizes pinholes for spatial filtering, while 2P-4E-1D exploits intensity-squared dependence of two-photon absorption. Two-photon excitation also provides the benefits of minimal photobleaching and large penetration depth, making 2P-4E-1D especially suited for tracking in thick tissues. For our systems, the Z tracking range is not limited by the depth of focus of the objective, but by the travel range of piezoelectric motion stage (30 μm for 1P-1E-4D and 100 μm for 2P-4E-1D). The increase in Z tracking range allows for molecular tracking throughout the entire mammalian cell (typically 10 μm).

In order to optimize our tracking microscopes and understand the differences between the two systems, here we have produced detailed Monte Carlo simulations of our tracking microscopes. Fluorescence excitation, detection, and feedback control algorithm were modeled by a set of parameters that can be manipulated and readily applied in experiments. We have also taken into account the real-world factors, such as photon detection efficiency, background counts on the detector (e.g. Poisson noise and Raman scattering from water), and response time of the piezoelectric stage and galvanometer mirrors.

2. METHOD

The 1P-1E-4D system has been previously reported. The sample suspended on a coverslip is mounted upon a three-axis, piezoelectric stage. We focus 470 nm laser at 15 μW through a water-immersion objective (60x, NA=1.2). The fluorescence is collected by the same objective and split evenly onto two optical fiber bundles. Each fiber bundle consists of two φ50 μm multimode fibers with a center-to-center spacing 55 μm. The fibers are connected to a 4-channel avalanche photon diode (APD) array. The fiber bundles are orthogonally installed and slightly offset along the optical axis, resulting in a tetrahedral arrangement of the four confocal volumes in sample space. The XYZ position of a fluorescent molecule is estimated from the differences in photon counts of four channels. Feedback control of the piezoelectric stage is responsible for bringing the fluorescent molecule back to the center of focus whenever it diffuses away.

In the 2P-4E-1D system, 860 nm 20mW Ti:sapphire laser is used for two-photon excitation. Our approach requires splitting individual laser pulse into four separate pulses, each of which is offset spatially and temporally (Fig. 2 right panel, intensity contour surface of the four spatiotemporally demultiplexed beams). The lateral offset is achieved by imparting small angular deviations into each beam via steering mirrors, and the axial separation of beam pair is controlled by altering the divergence of one of the beam pairs with two telescope lenses. The temporal offsets are introduced simply by varying the propagation distance of the 4 excitation beams in free space. Using simple time-gated analysis, all detected photons can be assigned to a specific excitation volume, while only one detector (photomultiplier) is needed in this scheme. We are currently refining the design and working on experiments. Detailed setup schematic will be reported in the near future.

The error signals of the 1P-1E-4D and 2P-4E-1D schemes are very similar, and can be formulated collectively in Eq. 1. \( D_i (i = 1 \sim 4) \) represents the number of photons collected during an update cycle by each APD (for 1P-1E-4D), or in each time gate (for 2P-4E-1D). The voltages applied to drive the piezoelectric stage motion and galvanometer mirror deflection are proportional to the error signals \( E_x, E_y, E_z \), with a constant gain factor for each axis \( (K_x, K_y, K_z) \). The gain factors are important parameters that need to be optimized.

\[
\begin{align*}
E_x &= \frac{D_1 - D_3}{D_1 + D_3} \\
E_y &= \frac{D_2 - D_4}{D_2 + D_4} \\
E_z &= \frac{(D_1 + D_3) - (D_3 + D_4)}{(D_1 + D_3) + (D_3 + D_4)}
\end{align*}
\]

3. MODELING

Of primary concern in our tracking schemes is the power to resolve the position of a target molecule in 3D space. Tracking error comes from background fluorescence, detector shot noise, and fluorescence fluctuation dynamics.
To better understand the tracking accuracy, we have established a MATLAB program to create random walk of a diffusing molecule, monitor the fluorescence detection of this molecule, and simulate the feedback tracking of the molecule. The realization of this Monte Carlo simulation lies in successful modeling of the molecular detection function (MDF) and a number of instrument-related parameters such as shot noise in the photon detection process, detectors quantum efficiency, background counts, and response time of the piezo-electric stages. MDF describes the probability of detecting fluorescence from a target at various positions in sample space. It is the product of excitation intensity \( I(\vec{r}, z) \) and photon collection efficiency function \( \text{CEF}(\vec{r}, z) \) for one-photon fluorescence excitation. For two-photon excitation, MDF is the product of \( I^2(\vec{r}, z) \) and \( \text{CEF}(\vec{r}, z) \).

CEF is a map of the relative photon counts collected from a dipole emitter for a given location in sample space. It's dependent on objective’s numerical aperture and effective active area of the detector (limited by diameter of pinholes or optical fibers used in confocal imaging). CEF is assumed to be unity for our 2P-4E-1D system as the PMT in use has a large photosensitive area. For our 1P-1E-4D, the objective magnification \( M \) plays a significant role in determining the CEF, considering that the effective size of CEF is roughly the physical size of the pinhole divided by \( M^2 \). Here we have employed a semi-geometric approximation of \( \text{CEF}(\vec{r}, z) \) in simulation, which has been proved to be reasonably accurate for typical confocal imaging.

Previously the excitation laser beam is assumed to have a Gaussian-Lorentzian geometry in the focal region. Indeed most single-molecule fluorescence detection employs a Gaussian laser beam as the excitation source. The assumption of Gaussian-Lorentzian geometry is sufficiently correct as long as the paraxial approximation holds. However, when focusing a Gaussian beam through a high-numerical-aperture objective, this approximation is no longer valid. Richard and Wolf started from the assumption that the objective is perfectly aplanatic, and gave a set of compact expressions of electrical field \( \vec{e}(P) \) near the focal point of the objective (Eq. 2-4). The assumption of aplanatic imaging properties can be satisfied for state-of-the-art high-numerical-aperture objectives. The excitation intensity \( I(\vec{r}, z) \) is proportional to \( |\vec{e}(P)|^2 \).

\[
\begin{align*}
e_x(P) &= -i\alpha(I_0 + I_z \cos 2\phi_p) \\
e_y(P) &= -i\alpha I_z \sin 2\phi_p \\
e_z(P) &= -2\alpha I_1 \cos 2\phi_p \\
\end{align*}
\]

where
\[
\begin{align*}
I_0 &= I_0(kr_p, \theta_p, \alpha) = \int_0^\alpha \cos \theta \sin \theta (1 + \cos \theta) J_0(kr_p \sin \theta \sin \theta_p) e^{ikr_p \cos \theta \sin \theta_p} d\theta \\
I_1 &= I_1(kr_p, \theta_p, \alpha) = \int_0^\alpha \cos \theta \sin^2 \theta J_1(kr_p \sin \theta \sin \theta_p) e^{ikr_p \cos \theta \sin \theta_p} d\theta \\
I_2 &= I_2(kr_p, \theta_p, \alpha) = \int_0^\alpha \cos \theta \sin \theta (1 - \cos \theta) J_2(kr_p \sin \theta \sin \theta_p) e^{ikr_p \cos \theta \sin \theta_p} d\theta \\
n \sin \alpha &= NA
\end{align*}
\]

Fig. 2 shows the excitation intensity contour surface and a stack of MDF slices at different depths. Each slice has the size of 700x700 nm. In 1P-1E-4D system, the projections of fiber bundles’ input faces in sample space are initially set to be located at \( z = \pm 0.125 \) μm, symmetrical about the excitation focal plane at \( z = 0 \). In 2P-4E-1D system, the excitation foci are also set to be located at \( z = \pm 0.125 \) μm for comparison. Note that at the \( z = \pm 0.125 \) μm planes, two MDFs start to merge, indicating that the spatial resolving power fades away. Not limited by the flattening of MDF at large \( Z \) depth, 2P-4E-1D exhibits a longer \( Z \) resolvable range as compared to that in 1P-1E-4D. On the other hand, 2P-4E-1D has MDFs with much larger apparent sizes, due to longer excitation wavelength and the lack of spatial filtering. A broadly distributed MDF implies that 2P-4E-1D could be more susceptible to background noise, which could deteriorate molecular tracking in complex biological systems.
Figure 1. Schematic of the focusing geometry through an objective. A point source is assumed to be at infinity and give rise to a linearly polarized (along the X direction) monochromatic plane wave at the entrance pupil of the objective. The objective here is perfectly aplanatic, which transforms an incident planar wavefront into a segment of a spherical wavefront. The origin \((\vec{r} = 0, z = 0)\) of the sample space coordinate system is at the focus of the laser beam. \(\theta_p\) and \(\phi_p\) are the polar angle and the azimuthal angle, respectively.

A. 1P-1E-4D Design

Molecular Detection Function (MDF) = Excitation Volume \(\times\) Collection Efficiency Function (CEF)

Figure 2. Comparison of molecular detection functions (MDFs). (A) In the 1P-1E-4D design, the calculation indicated that the spatial resolving capability of MDFs fades away at a distance of 0.25 \(\mu\)m from the center of the MDFs (located at \(\pm 0.125 \mu\)m), mainly due to the flattening of collection efficiency function (CEF) at this Z depth and a single excitation volume. As the spatial resolving capability disappears, target molecule can be lost. (B) In the 2P-4E-1D design, the MDFs are dominated by the spatiotemporally demultiplexed two-photon excitation volumes, as the PMT has a large active area (i.e. CEF-1).

4. SIMULATION AND RESULTS

The position of target molecule is encoded in error signals. An off-line characterization of error signals is therefore crucial for predicting the real-time tracking performance of our systems. Error signals are independent on incident laser power (assuming no optical saturation), but dependent on relative excitation intensity distribution \(\bar{l}(\vec{r}, z)\). For each tracking system, we have calculated \(\bar{l}(\vec{r}, z)\) with two different methods. One relies on the commonly used Gaussian-Lorentzian beam expression;\(^{27}\) the other uses Wolf equations (Eq. 2-4).\(^{29}\) The resulting error signals are shown in Fig. 3.
The linear portion of error signals is the resolvable, “lock-in” region, in which our “direct proportional” tracking control loop works excellently. In this linear region, we notice the Gaussian expression always produces smaller error signals as compared to those produced by the Wolf equations (Fig. 3A and 3B). For 1P-1E-4D, the difference between the two types of error signals is negligible simply because of the dominant influence of CEF(\(\vec{r}, z\)) in MDF calculation. However, for 2P-4E-1D, the difference is evident due to the square of \(I(\vec{r}, z)\) and a constant CEF(\(\vec{r}, z\)). It’s noteworthy that the slope of \(Z\) error signal given by the Gaussian expression is less than 50% of that given by the Wolf equations. Since the slope of error signals is directly related to the position sensitivity and hence the tracking accuracy, a Monte Carlo simulation based on Gaussian beam assumption will dramatically underestimate the tracking accuracy of 2P-4E-1D along the \(Z\) direction.

Based on the Wolf excitation field expressions, we have compared the error signals of 1P-1E-4D and 2P-4E-1D. The two systems exhibit almost identical \(E_x\), which suggests they will perform equally well in terms of 2D tracking in the XY plane. The \(Z\) error signal (\(E_z\) in Fig. 3B) agrees well with our prediction from Fig. 2 – while 1P-1E-4D has a smaller \(Z\) “lock-in” range (0.25 μm versus 0.6 μm), its steeper \(E_z\) could offer a more sensitive tracking response in the \(Z\) direction. In other words, while 2P-4E-1D may be able to track faster moving molecules, this could come with a price of lower tracking accuracy in the \(Z\) direction.

**Figure 3.** Error signals comparison of 1P-1E-4D (blue curve) and 2P-4E-1D (red curve). Solid lines: Error signals derived from the Wolf excitation field expressions. Dashed lines: Error signals derived from the Gaussian-Lorentzian excitation expression. \(E_x\) has the same dependence on \(y\) as \(E_x\) on \(x\). The \(z\)-plane separation of the fiber input faces (projected into sample space) in 1P-1E-4D and \(z\)-plane separation of the excitation volumes in 2P-4E-1D are both set to be at 0.25 μm for comparison. The linear region of error signal is where the target molecule can be effectively tracked. \(E_x\) is steeper than \(E_z\), meaning our tracking systems are more sensitive to XY displacement than \(Z\) displacement. As a result, the XY tracking accuracy is always better than the tracking accuracy in \(Z\).

There are many parameters that govern the system’s performance, and some of them are coupled together. We have used Monte Carlo simulation to optimize the optical configuration. The target molecule is modeled as diffusing in free space with diffusion coefficient \(D = 1.5 \mu m^2/s\). The photon count in each detector follows the Poisson distribution, with the average being molecule position dependent fluorescence signal plus a constant background noise that approximates Raman scattering from water. Once the photon counts fall below the specified threshold, the tracking is terminated. Fig. 4A and 4B shows the dependence of tracking duration of 1P-1E-4D on objective magnification and fiber bundle axial separation in sample space (top row); along with variation of tracking duration of 2P-4E-1D as a function of excitation volumes lateral and axial separation (bottom row). Careful arrangement of the detection/excitation volumes has resulted in a median tracking duration more than 1.7 seconds of a target molecule diffusing at \(D = 1.5 \mu m^2/s\).
We have also optimized the feedback gain factors with tracking error as the performance metric. The results are shown in Fig 5. As expected, both 1P-1E-4D and 2P-4E-1D have much better tracking accuracy in lateral direction than in axial direction. Due to the symmetry of optics, $K_x$ and $K_y$ are always set to be equal. Tracking accuracy in XY is only loosely dependent on $K_z$; similarly, tracking accuracy in Z is insensitive to $K_x$ and $K_y$. For both our systems, while the Z tracking error is large (>200 nm), the overall accuracy is still below the diffraction limit. XY tracking errors are ~100 nm. To verify these results experimentally, we are currently tracking immobilized fluorescent particles moved by an independent motion stage in prescribed patterns.
Fig 5. Tracking error over a range of feedback gain parameters ($K_{xy}, K_z$) for tracking the Brownian motion of a fluorescent particle with diffusion coefficient $D = 0.5 \, \mu m^2/s$. Panel A-D: 1P-1E-4D. Panel E-H: 2P-4E-1D. Each pixel represents the mean squared distance ($\sigma$) between molecule position and stage position for a given set of ($K_{xy}, K_z$). Panel D and Panel H are the total mean squared distance $\sigma_{total} = (\sigma_x^2 + \sigma_y^2 + \sigma_z^2)^{1/2}$. For each ($K_{xy}, K_z$), $\sigma$ is averaged from twenty 1-sec runs. Tracking errors are only shown for ($K_{xy}, K_z$) that enables at least ten 1-sec tracking out of the twenty runs. For both our systems, while the Z tracking error is large (>200 nm) and dominates in $\sigma_{total}$, the overall accuracy is still below the diffraction limit. XY tracking errors are ~100 nm.
5. CONCLUSION

We have evaluated a number of instrumentation parameters and estimated the tracking accuracy of the two 3D-SMT systems (1P-1E-4D and 2P-4E-1D) using Monte Carlo simulation, as the first step towards developing new 3D tracking microscopes. In our model, we have found that the traditional Gaussian-Lorentzian excitation profile significantly underestimates (more than 2-fold) the Z error signal in the 2P-4E-1D scheme. The Wolf equations, on the other hand, provide a more precise description of excitation intensity distribution, which is crucial for designing a system to perform single-molecule tracking through a high-numerical-aperture objective. Error signal comparison between the two systems indicates their equal performance in 2D molecular tracking. In the Z direction, while 2P-4E-1D offers a larger “lock-in” region, it comes with the cost of lower tracking accuracy (i.e. smaller slope of $E_Z$). For both tracking systems, our simulation shows that the tracking accuracy in XY is at least twice better than that in Z.

We have optimized the optical configuration of our tracking systems, as well as the gain factors. The quality metrics that we have used are tracking duration and tracking accuracy. Under optimized condition, tracking accuracy better than the diffraction limit of light can be achieved in all three dimensions.

In our simulation, MDF and error signals are determined computationally. Their comparison with experimental data will be the first step to verify our simulation results. In the future we can perform more realistic simulation by combining experimental MDF with simulated random walk and feedback control.

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