

Particle Tracking in a 2-Photon Excitation Microscope

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Introduction

Within the past few years, single-particle tracking techniques have been increasingly used for studying biological systems. In contrast to large ensemble measurements, in which only average quantities are obtained, single-particle methods provide information about the distribution of molecular properties in inhomogeneous systems. Analysis of single molecule trajectories is useful to understand several aspects of the motion of molecules and their interactions with the environment.

Most of the methods used so far for particle tracking in fluorescence microscopy rely on ultrafast video cameras that can detect single particles with accuracy of 10-100 nm in the range of ms [1]. The main limitation of these methods is that they can only detect particles moving within the focal plane, and thus they are generally limited to applications such as studying molecular processes in membranes.

To localize and track fluorescent particles in three dimensions, most techniques are based on imaging the sample with a camera at different z-positions and then analyzing the resulting image z-stacks as a function of time [2, 3].

These methods have a time resolution in the range of seconds and can only be applied to slow moving particles. In addition, as they require repetitive illumination of large sample volumes, their use in biological applications is limited because of photodamage and bleaching.

Set Up

Microscope Set Up

The tracking experiments were carried out with the Olympus IX70 microscope. The two-photon excitation source was a mode-locked titanium-sapphire laser (Mira 900, Coherent, Palo Alto, CA) pumped by an argon ion laser (Innova 300, Coherent) tuned to 920 nm. The laser power at the sample was in the range 1-10-mW, except when indicated. The light is directed into the microscope by two galvomotor-driven scanning mirrors (Cambridge Technologies, Watertown, MA) through a scanning lens. During the tracking procedure, the two scanning mirrors are moved independently by two synchronized voltage sine waves shifted 90° relative to each other and generated in a computer card (three-axis card, ISS, Champaign, IL). As a consequence, the laser moves in a circular path. The frequency of the circular orbit was 250 Hz. The position of the scanning center is determined by the offset values of the sine waves.

The laser light is reflected with a low-pass dichroic mirror (transmission between 370 and 630 nm, Chroma Technology, Brattleboro, VT) and focused on the sample with a 20x (dry) 0.75-NA objective. Fluorescence emission collected by the objective passes through the dichroic and a short-pass filter, exiting the microscope through the side port. A Hamamatsu H7422P-40 photomultiplier tube was used as a detector with its output amplified, passed through

a photon counting discriminator (PX01 Photon Counting Electronics, ISS), and counted with a data acquisition card (ISS). The experiments are controlled by a data acquisition program (SimFCS, Laboratory for Fluorescence Dynamics, Champaign, IL).

Tracking Routine

The tracking routine starts with a fast raster scan of a large area of the sample in which the particle of interest can be observed. Then, we select the particle to be tracked by clicking on top of its image. This directs the laser beam to the chosen particle by changing the DC offset values of the output of the 3-axis card for the x, y, and z coordinates. This point is considered as the initial coordinates for the tracking.

During each cycle of the tracking routine, the excitation beam traces n circular orbits in a plane above the particle and n orbits in a plane below (Fig. 1). The diameter of the orbit is equal to the x,y-width of the point spread function (PSF), and the scanned z planes are separated in a distance equal to the z-width of the PSF.

In a typical experiment, the frequency of the z-square wave is 8 or 16 times slower than that of the x and y sine waves. Thus, the x,y-scanning mirrors trace n = 4 or 8 circular orbits in each focal plane (2n orbits per cycle). The fluorescence intensity is averaged over the n orbits in each plane to improve the signal/noise ratio (S/N).

Fluorescence data is collected at high frequency ($f_{\text{data}} = 16$ or 32 kHz) as the laser moves around the particle.

As demonstrated by Kis-Petikova and Gratton [4] and Berland et al. [5], the fluorescence intensity (F) during the scanning is a periodic function of time (t):

$$F(t) = \frac{2F_0 / \pi}{1 + \frac{\lambda^2 (z_p - z_s(t))^2}{w_0^4 \pi^2}} \exp \left[-\frac{2 \left[(x_p - x_s(t))^2 + (y_p - y_s(t))^2 \right]}{w_0^2 + \frac{\lambda^2 (z_p - z_s(t))^2}{w_0^2 \pi^2}} \right] + B \quad [1]$$

where w_0 is the beam waist, λ is the wavelength, B is the background intensity, and F_0 is a constant. The subscripts p and s refer to the particle and the scanner coordinates, respectively.

For the scanning routine described above, the coordinates of the scanner vary as a function of time as follows:

$$\begin{aligned} x_s(t) &= r_{xy} \cos(2\pi f_{\text{orbit}} t) \\ y_s(t) &= r_{xy} \sin(2\pi f_{\text{orbit}} t) \\ z_s(t) &= \begin{cases} r_z & 0 < \frac{f_{\text{orbit}} t}{n} < 1, \quad 2 < \frac{f_{\text{orbit}} t}{n} < 3, \dots \\ -r_z & 1 < \frac{f_{\text{orbit}} t}{n} < 2, \quad 3 < \frac{f_{\text{orbit}} t}{n} < 4, \dots \end{cases} \quad [2] \end{aligned}$$

where r_{xy} is the xy-circular orbit radius, r_z is half the amplitude of the z-square wave and f_{orbit} is the frequency of the circular orbit. According to these equations, the absolute position of the particle (x_p, y_p, z_p) can be determined by measuring the fluorescence intensity while moving the laser beam as a function of time. Fig. 1 represents intensity profiles simulated for particles situated at different positions relative to the center of the scanner. It is clear that the intensity signal is very sensitive to the position of the particle.

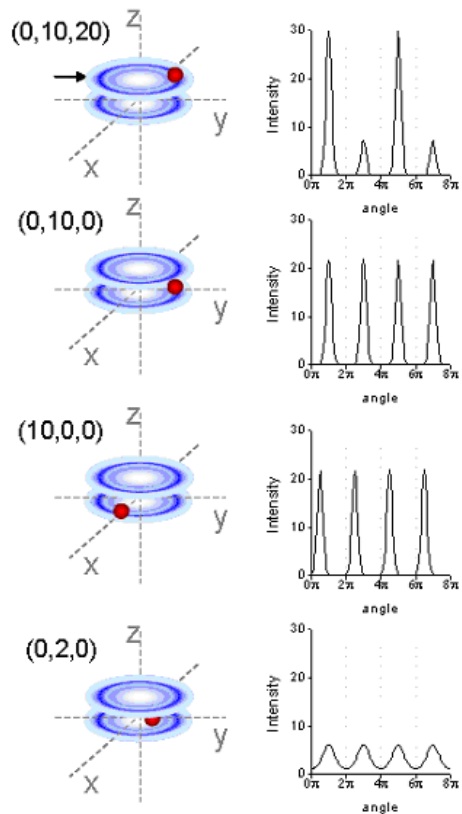


Figure 1. Intensity profile determined during two cycles of the tracking routine represented as function of the angle of rotation of the laser for different relative positions of the particle with respect to the center of scanning (right panels). Each cycle of the tracking routine consisted in two orbits, each one at a different z-plane. The left panels show diagrams of the relative position of the particle (red circle) respect to the center of scanning. The laser orbits are represented with blue ovals. The arrow shows the starting point of the tracking cycle.

After each cycle of the tracking routine, the coordinates of the particle are determined on the fly from the phase and modulation of the fluorescence signal calculated by Fast Fourier Transform. Then, the center of scanning is moved to this new position and a new cycle of the tracking routine starts. In other words, during the tracking routine, the scanner follows the particle by changing its position to that calculated for the particle in the previous cycle. In an ideal tracking experiment, the scanner is always on top of the particle and the positions of the scanner and the calculated for the particle are identical.

Results

The performance of the method was studied in different calibration experiments. First, we determined the error of the tracking method by following 500-nm fluorescent beads immobilized onto a coverslip. The calculated error in the particle position was 20 nm along each axis, using $f_{\text{data}} = 32 \text{ kHz}$, $f_{\text{orbit}} = 250 \text{ Hz}$, $n = 8$, $r_{x,y} = 0.7 \text{ }\mu\text{m}$ and $r_z = 2.5 \text{ }\mu\text{m}$. This value was calculated by averaging the standard deviations obtained for the x, y and z coordinates after 600 tracking cycles.

Then, we moved the coverslip in known trajectories and determined the trajectories of the fluorescent beads. Fig. 2 shows an experiment in which we tested the tracking method for distances in the nm range. We used a nanometric stage (Mad City Labs: Nano-Bio 2) with 0.7 nm position accuracy in the closed loop scanning mode. The beads were moved in defined steps ranging from 20 to 100 nm. Fig. 2A shows the trajectory recovered for a particle moving in steps of 60 nm in the x direction.

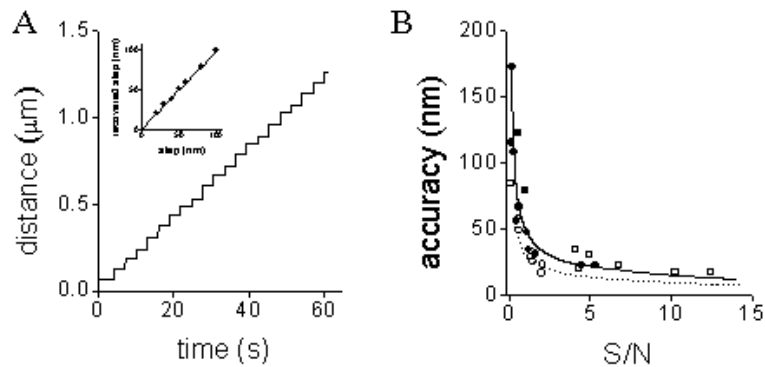


Figure 2. Tracking accuracy. (A) Trajectory recovered for beads moving in 60 nm steps. The inset to the figure show the average step size recovered plotted as a function of the input step size. (B) Dependence of the tracking accuracy on the signal/noise ratio. The position of fixed beads with diameters (nm): 552 (□), 100 (○), 24 (■) and 14 (●) where determined at laser powers in the range 0.1-15 mW, using the following parameters for the tracking routine: $f_{\text{data}} = 32$ kHz, $f_{\text{orbit}} = 250$ Hz, $n = 16$, $r_{x,y} = 0.7$ μm and $r_z = 2.5$ μm . The continuous line represents the fitting of the following function: $\text{accuracy} = \alpha(\text{S/N})^\beta$ with $\alpha = 49 \pm 5$ nm, $\beta = 0.54 \pm 0.08$. The dashed line represents the theoretical accuracy.

Figure 2B shows the accuracy on the determination of the position measured for beads of different sizes (10-500 nm diameter) as a function of the signal to noise ratio, which was modified by varying the excitation laser power. The figure shows that the accuracy of the tracking is approximately constant for S/N higher than 2 and increases with square root dependence for lower values of S/N, as predicted by theory. Also, the accuracy is independent of the size of the fluorescent beads. In addition, Figure 2B shows the theoretical accuracy of the particle position as a function of S/N. These values were obtained by using the tracking program to simulate and track a particle with diffusion coefficient equal to zero, variable brightness and considering a PSF of dimensions identical to that determined experimentally. The accuracy was determined by tracking the simulated particle using the same set of parameters that were used in the experiment shown in Fig 2B. It can be observed that the theoretical limit (~1 nm) cannot be reached experimentally due to instrumental factors such as thermal and mechanical jitter that contribute to the noise of the measurements.

Conclusion

In conclusion, we determined that the tracking method can follow the motion of fluorescent particles in 3-dimensions with a precision of 20 nm using an integration time of 32 ms.

Using two-photon microscopy makes this method well suited for biological applications since it provides significantly lower out-of-focus photodamage and photobleaching than other fluorescence microscopies [6]. Also, during the tracking the laser is focused in a small volume surrounding the particle, resulting in reduced photodamage of the rest of the sample.

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