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Scanning FCS, a novel method for three-dimensional particle tracking

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Abstract

We describe a novel method to track fluorescent particles in three dimensions with nanometre precision and millisecond time resolution. In this method, we use our two-photon excitation microscope. The galvomotor-driven *x*–*y* scanning mirrors allow the laser beam to move repetitively in a circular path with a radius of half the width of the point spread function of the laser. When the fluorescent particle is located within the scanning radius of the laser, the precise position of the particle in the *x*–*x* plane can be determined by its fluorescence intensity distribution along the circular scanning path. A *z*-nanopositioner on the objective was used to change the laser focus at two planes (half width of the point spread function apart). The difference of the fluorescence intensity in the two planes is used to calculate the *z*-position of the fluorescent particle. The laser beam is allowed to scan multiple circular orbits before it is moved to the other plane, thus improving the signal to noise ratio. With a fast feedback mechanism, the position of the laser beam is directed to the centre of the fluorescent particle, thus allowing us to track a particle in three dimensions. In this contribution we describe some calibration experiments performed to test the three-dimensional tracking capability of our system over a large range.

Introduction

Studying molecular dynamics in cellular systems is important for the understanding of cellular processes in living cells. Several optical microscopy techniques have been employed for this purpose. Fluorescence recovery after photobleaching is frequently used to study the motion of molecules in artificial or natural membranes; however, it is limited to a spatial thickness of 0.5–1 μm. Fluorescence correlation spectroscopy can measure the diffusion constant and molecule concentration at a fixed sample position, in solution, on the membrane or inside the cell. Fluorescence cross-correlation spectroscopy can study dynamic molecular interactions. Scanning fluorescence correlation spectroscopy can explore multiple sample positions, yielding diffusion constants, molecule concentrations and spatial correlation. These techniques measure the properties of a small number of molecules each time, but sample many molecules over an extended period. Thus molecules with distinct properties can be differentiated by statistical analysis of each individual molecule, unlike cuvette measurement, which averages the properties of all molecules. To further study the property of individual molecules, particle tracking can be used. With ultra-fast video cameras, particles can be tracked with high precision and good time resolution. However, these methods are limited to particles moving within the focal plane of the lens objective [1,2]. On the contrary, we are developing a new method to track single or multiple particles in three dimensions. Our method is not limited by the sample depth, or large axial or radial movement of the particle and the tracking can last for hundreds of seconds as long as the particle remains fluorescent. As an example, the tracking method has been applied to the study of chromosome dynamics in living cells.

Key words: fluctuation spectroscopy, fluorescence, particle tracking.

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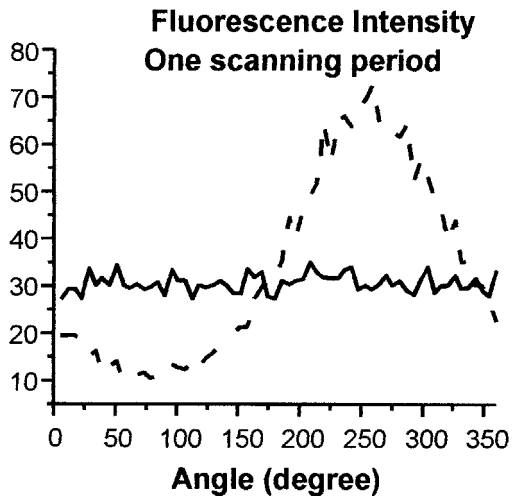
Methods and instrumentation

The particle-tracking method described here was developed on a home-build two-photon-excitation scanning microscope, which was described in a previous paper [3]. The inverted microscope was an Olympus IX70. The laser beam was directed into the microscope by two galvomotor-driven scanning mirrors (Cambridge Technologies, Watertown, MA, U.S.A.) through a scanning lens. The two scanning mirrors are moved by voltages generated independently in a computer card (3-axis card). When they are synchronized to perform two sine waves shifted 90° by voltage generators, the laser beam moves in a circular path at the desired frequency and radius depending on the amplitude and frequency of the sine wave. A *z*-nanopositioner is placed underneath the objective (60× Olympus/water) to enable changes of the focal plane of the laser beam. The *z*-nanopositioner is also driven by voltage generated in the 3-axis card (square wave) with a DC offset. The centre of the circular path and the focal plane can be changed by changing the DC offset values of the output waves independently.

Experiments are controlled by a data-acquisition program (SimFCS; Laboratory for Fluorescence Dynamics, Urbana, IL, U.S.A.). The measurements are carried out in the

Figure 1 | Fluorescence intensity profiles obtained from one scanning period

The continuous line was obtained when the particle coincides with the centre of the scanning circle. The dashed line was obtained when the particle was shifted with respect to the centre of the scanning orbit.



following manner: with a fast raster scan, the fluorescence image of a large area of the sample is obtained. The particle of interest can be chosen by simply clicking on the image. This will change the DC offset value of the 3-axis card output voltage. This point is used as the initial position for tracking. Then the laser beam scans in a circular orbit with radius equal to half of the width of the point spread function at a frequency of 250 Hz. Fluorescence intensity is collected at high frequency (32 000 Hz) as the laser moves in circles around the particle. If the centre of the particle coincides with the centre of the scanner orbit, the fluorescence intensity collected along the orbit is constant. If the particle is shifted with respect to the centre of the scanner orbit, the fluorescence intensity would depend on the laser position in the orbit.

Figure 1 shows an example of the fluorescence intensity collected in both cases. The intensity profiles of multiple scanning orbits (four or eight, depending on the signal level) were averaged to improve the signal-to-noise ratio. The fast Fourier transform of the average intensity profile provides the DC as the 0th term in a Fourier series and AC as the coefficient of the first harmonic term. The phase of the AC term gives directly the φ coordinate of the particle, and the radial distance between the centre of the scanner and the centre of the particle can be calculated from the modulation (AC/DC) of the signal. For known scanning radius and width of the point spread function, the modulation is a monotonic function of the radial distance d only. From this dependence we can immediately determine the position of the particle in the radial plane.

To determine the particle position along the z-axis, the laser beam is alternately focused at two different planes located at half the width of the axial point spread function from each other. This is achieved by sending a square wave (voltage) from the 3-axis card to the z-nanopositioner underneath the objective. In each plane, the laser completed a certain number of circular scans (four or eight) before changing to the second plane. Because the point spread function is symmetrical, when the particle is in the focal plane, the total fluorescence intensity measured above and below the focal plane will be the same. If the particle is in a different plane, there will be a difference in the intensities measured at the two scanning planes. Based on this difference, the position of the particle on the z-axis can be easily determined. Thus the position of the particle in (x, y, z) can be determined after one full cycle. The recovered coordinates are used to calculate the new DC offset value, resulting in the laser beam moving directly to the centre of the particle. The calculation and updating of the (x, y, z) position of the particle can be done on the fly, enabling us to track particles in real time with no range limitation of the particle movement other than the entire field of view.

A threshold in fluorescence intensity was set to differentiate target molecules from background. When the measured fluorescence intensity falls below the threshold, it is assumed that the scanner was no longer tracking the target molecule. The computer will instruct the 3-axis card to increase its voltage, thus increasing the scanning radius of the laser beam. By increasing the searching area, the position of the lost molecule can be determined rapidly, and the centre of the laser beam orbit jumps to that position resuming tracking. The target molecule will be lost only when its fluorescence intensity falls below the threshold level.

Results

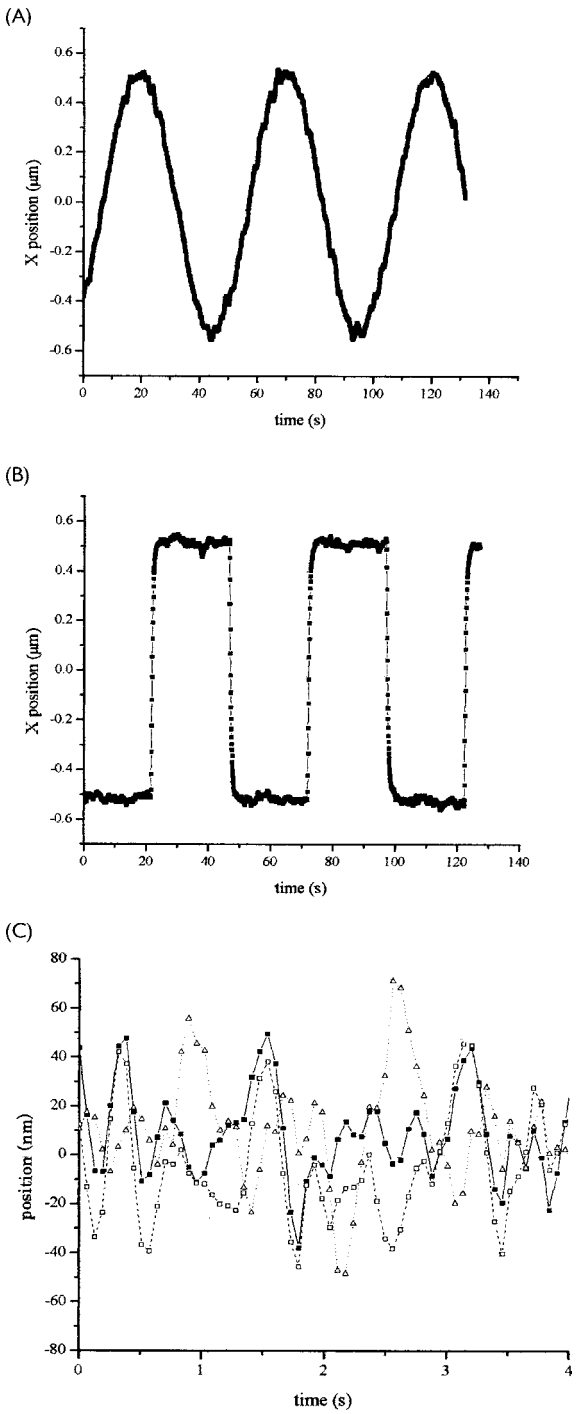
In the previous section, we described the method for tracking of particles in three dimensions. To test the accuracy and performance of this method, we mounted an LVDT (linear voltage differential transformer) feedback-controlled xy piezo stage on the microscope which allows us to move the sample in defined trajectories in the xy plane by inputting a given voltage to the piezo stage.

We dispersed fluorescent beads on to a coverslip and applied to the x- or y-direction of the piezo stage a sine or square wave with various amplitudes corresponding to distances in the range 0.1–10 μm at a frequency of 0.02 Hz. The recovered amplitudes of the sine wave were linearly related to the input amplitudes with slopes of 1.026 ± 0.003 (x-axis), 1.078 ± 0.005 (y-axis) and ordinates equal to zero. Figures 2(A) and 2(B) show examples of the recovered trajectories of beads with a motion amplitude of 1 μm. In both cases, the shape of the trajectories agreed with that of the input waves.

To test the tracking in the z-direction, we mounted the piezo stage perpendicular to the microscope stage. Under this condition, the sample could be moved along the optical axis of the microscope. By following a procedure similar to that described above, we verified that the trajectories recovered for particles moving in the z-direction agreed with those of the input waves. The recovered amplitude of the sine wave

Figure 2 | Trajectory of fluorescent beads

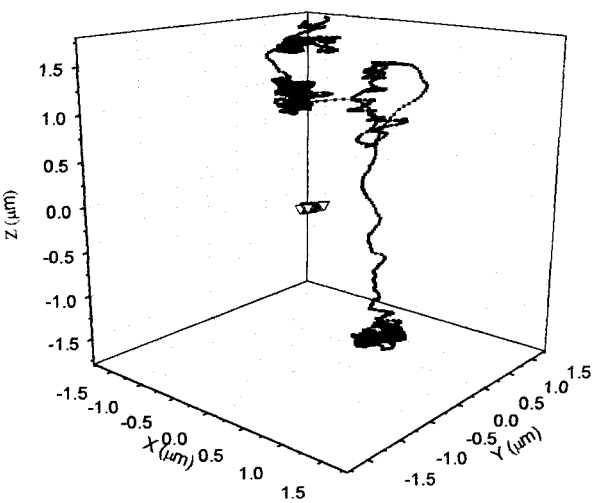
Fluorescent beads (500 nm) on a coverslip were moved 1 μm in sine (**A**) or square (**B**) waves by applying 0.1 V at 0.02 Hz to the *xy*-piezo stage. The recovered trajectories are plotted as a function of time. (**C**) Error in the recovered positions corresponding to the sine-wave trajectories of (**A**); ■, *x*-axis; □, *y*-axis; Δ, *z*-axis.



was linearly related to the inputted amplitudes with a slope of 0.97 ± 0.02 and ordinate equal to zero. Taken together, these results show that the method allows an accurate tracking of particles in three dimensions.

Figure 3 | Tracking beads in agarose

Fluorescent beads (500 nm) were suspended at 80°C in a solution composed of 20 mM phosphate buffer (pH 7.4 at 25°C) and 0.1% (■) or 0.3% (▽) agarose. The samples were cooled down up to room temperature and the motion of the beads in the gel was studied using the tracking procedure described in this contribution. The trajectories shown in the figure correspond to beads tracked for 100 s and 40 s, respectively.



To evaluate the error of the tracking procedure in the determination of a particle position, we measured trajectories of 500-nm fluorescent beads placed on a coverslip (Figure 2C). The standard deviation for the positions of these fixed particles was 20 nm in the *x*, *y* or *z* axis.

As an example of application of the tracking method, we studied the diffusion of fluorescent beads in agarose gel. In this case, the particles are free to move in three dimensions and the motion is only restricted by the gel network.

Figure 3 shows a trajectory of a bead diffusing in a 0.1% agarose gel. The motion of the particle can be described as a transient trapping with jumps among different cavities of the gel. The figure also shows a trajectory obtained for a bead in 0.3% agarose gel. As can be observed, the bead was confined to a cavity and hardly moved. This description agrees with that observed by Ragan [4] for 100-nm beads in agarose gels.

Discussion

In this work, we have described a method for tracking particles in three dimensions in which a laser beam orbits around a particle, allowing its fast and precise localization and tracking. To our knowledge, most of previous methods described for tracking of particles make use of fast CCD cameras with a maximum speed of 30 frames/s [5] or quadrant detectors. Using cameras, *z*-sectioning is required for tracking in three dimensions, which slows down the process.

In contrast, our tracking procedure allows the determination of a particle position in 64 ms (in three dimensions) or 16 ms (in two dimensions). In the calibration experiments shown in this work, we were able to recover the trajectories

of particles with nanometre accuracy. Although we are not describing in any detail the results obtained in biological samples, we have applied the tracking system to follow the motions of chromosomes in living cells.

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