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Distance Measurement by Circular Scanning of the Excitation Beam in the Two-Photon Microscope

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KEY WORDS colocalization; fluorescence microscopy; optical ruler; tracking

ABSTRACT We developed a method to measure relative distances with nanometer accuracy of fluorescent particles of different color in a two-photon scanning fluorescence microscope, with two-channel photon counting detection. The method can be used in the 10–500 nm range, for distances below the resolution limit of standard far field microscopy. The proposed technique is more efficient than the methods using raster scanning. To achieve maximum sensitivity in the radial direction, the excitation beam is moved periodically in a circular orbit with a radius of the size of the point spread function. The phase and the modulation of the periodic fluorescence signal, calculated by fast Fourier transform, gives the phase and the radial distance of the particle from the center of scanning. The coordinates of particles are recovered simultaneously in the two channels and the relative distance is calculated in real time. Particles can be tracked by moving the center of scanning to the recovered position, while measuring the distance from the second particle. Intensity data are saved and fitted later by a model accounting for light leakage between the channels. The total number of detected photons limited the accuracy of the position and distance measurement. Experiments demonstrating the advantages of the method were performed on fluorescent spheres and single dye molecules immobilized on quartz surface. *Microsc. Res. Tech.* 63: 34–49, 2004. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Fluorescence microscopy is a powerful tool for studying biological processes in vitro or in the cellular environment. Spatial distribution of fluorescent dyes attached to the cellular structures or macromolecules can be mapped inside the cell in 3D using confocal or two-photon microscopy. There are unique advantages to apply two-photon excitation, as the inherent selection of the sub-volume without the need of confocal pinholes and the reduced background detection of laser scatter compared with one photon excitation (Denk et al., 1990; König, 2000). Some other related two-photon techniques, such as fluorescence correlation spectroscopy (FCS) (Berland et al., 1995) and particle tracking (So et al., 1998), are also available to study the diffusion properties of fluorescent labeled particles in different environments of cells. In various far-field optical microscopy imaging techniques, Abbe principle establishes the achievable resolution, and additional effort has to be made to localize more precisely dye molecules or fluorescent particles. Resolving the power of the microscope is directly proportional to the wavelength of light, and inversely proportional to the numerical aperture, therefore improving these parameters can be considered to increase resolution. Recent advances in optics, based on new physical concepts, reduced the diffraction limit to around 100 nm in the z direction by increasing the aperture in the 4- π microscope (Hell, 1994) or by engineering the intensity distribution in the focal spot by stimulated emission (Klar et al., 2000). In the near-field optical microscopy, where Abbe principle does not apply lateral resolution of 50 nm can be obtained near the surface (Betzig and Trautman,

1992). Besides these physical concepts of increasing the resolution, deconvolution can also help to get some specific sub-resolution spatial information.

Point spread function (PSF) is defined as the excitation intensity distribution in the focal spot of the microscope. The shape of the PSF can be measured by a point scan method, imaging an isolated fluorescent particle, which is much smaller than the beam waist (Schneider and Webb, 1981). Although fluorescence microscopy is capable of single molecule sensitivity, the resolution of the image is not sufficient to resolve the positions of individual molecules, which are closer than the size of the PSF. For example, resolving power of the two-photon microscope for excitation wavelength $\lambda = 790$ nm and numerical aperture $NA = 1.3$ would be $0.61 \lambda/NA = 370$ nm, much larger than the size of one dye molecule (in the order of 1 nm). Only from the size or shape of the spot in the image it is impossible to distinguish between a single molecule, an aggregate, or a small fluorescent bead. When two particles are closer than the width of the PSF, their position can be determined only if their properties are well known.

There are many cases when the position of the center of mass of an isolated fluorescent particle is important,

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but resolving the shape of the particle or the distribution of fluorescent dyes inside the particle is not necessary. By fitting the measured intensity distribution by the known shape of the PSF, its center of mass can be determined with high accuracy (Bobroff, 1986). In this case, only a signal-to-noise ratio and mechanical drifts limit the position determination in the system (Ghosh and Webb, 1994). For the measurement of distances between two particles, independent measurements of particle positions are needed. For example, measurement can be performed in two different colors at the same time. Recently, a colocalization method was proposed to measure distances (larger than 10 nm) between immobilized fluorescent beads or quantum dots based on fitting the intensity distributions by a Gaussian PSF profile in a dual channel microscope (Lacoste et al., 2000). For shorter, molecular size distances, fluorescence resonance energy transfer (FRET) can be used both in bulk measurements to get average distances and between single molecules (Ha et al., 1999).

Determination of position by following the motion of the particle can be realized by different methods of tracking (Allersma et al., 1998; Berg, 1971; Ghosh and Webb, 1994; Goulian and Simon, 2000; Kubitschek et al., 2000; Saxton and Jacobson, 1997). The idea of using circular movement of the focus in the confocal system for the tracking of fluorescent molecules diffusing within membranes was recently proposed by Enderlein (2000a,b). Optimal values of the geometric parameters were determined based on simulations, the accuracy of the tracking was discussed for a given diffusion coefficient, and application for the time correlated single photon counting was described. However, circular movement of the focus was used only as a form of a quadrant detector, and the particle position was assumed to be the same as the center of scanning.

In this report, we introduce a method to measure relative distances on the scale of 10–500 nm between fluorescent particles immobilized on the surface. Instead of raster sampling of the PSF, the excitation beam is moved along a circular orbit by galvo-motor driven scanning mirrors. The idea of scanning the excitation beam on a circular orbit has several advantages. Circular scanning can be realized very fast without distortion of the path, while the tangential component of the velocity of the beam is always the same. Instead of recording the whole raster image, we can place the particle in the most sensitive (steepest) part of the PSF by choosing the radius of scanning in the order of the width of the PSF. Information about the position of the particle is recovered in every cycle during the measurement using fast Fourier transform (FFT) or later by fitting the intensity trace in every cycle by the intensity formula. The center of the particle can be localized within few nm precisions. It is limited only by shot noise, depending on the number of photons detected, and the mechanical stability of the system. Some possible sources of systematic errors of the position, such as geometric parameters, PSF shape, and leakage between the channels are discussed in this report. Our method of analysis and visualization of the stream of intensity data in the pseudo-image enables us to select easily that part of the measurement that was not distorted by photophysical effects such as blinking or photobleaching. The distance between par-

ticles of two different colors can be calculated if the fluorescence signal is collected in two channels and the position is determined in every cycle for both colors. During the 2D tracking, a routine recovered the position of the fluorescent particle to move the center of scanning on top of the particle in every cycle. This routine was used to center the circular scanning on top of the particle automatically, thereby maximizing the sensitivity of the distance measurement. After theoretical calculations, the principle of the method is illustrated on the distance measurement of immobilized fluorescent beads of 100-nm diameter. An example of the distance measurement between single dye molecules on the quartz surface is discussed. Future application possibilities and the extension in 3D distance measurement and tracking are outlined. Measurements on biological systems are in progress and will be the subject of a separate study.

PRINCIPLES OF THE METHOD

Determination of the Position by Circular Scanning in the Focal Plane

Let us consider one fluorescent particle immobilized on a surface. Fluorescence emission is detected while circular scanning of the excitation beam is performed in the plane with known radius A and frequency f . Fluorescence emission as a function of time can be calculated for this simple model system. Let us consider a typical shape for the point spread function (PSF) that is obtained in the two-photon microscope in the form of Gaussian-Lorentzian (GL) function (Berland et al., 1995). Because two-photon absorption cross-sections are generally very small and fluorescence intensity is proportional to the square of the laser intensity, fluorescence is excited only near to the focal plane in a diffraction-limited spot of GL shape. The fluorescence intensity measured when the laser spot is centered at the point $[x_s, y_s, z_s]$ and an immobilized point-like fluorescent particle located at $[x, y, z]$ are given as:

$$F(x, y, z, x_s, y_s, z_s) = \frac{I_0}{\left(1 + \frac{(z - z_s)^2 \lambda^2}{\pi^2 w_0^2}\right)^2} \times \exp\left[-\frac{4((x - x_s)^2 + (y - y_s)^2)}{w_0^2}\right] \quad (1)$$

where I_0 is the maximum fluorescence intensity (when the center of the excitation beam is at the center of the particle), w_0 is the radial beam waist, and λ is the wavelength of the excitation light. In the case of raster scanning of the image, the laser beam is moved along parallel lines, thus PSF is sampled in regular steps in an x and y direction. The center of mass can be determined by fitting Eq. 1 to the intensity distribution in the image, where x_s , y_s means the coordinates of the image pixels and z_s is the z coordinate of the plane that is in the focus. By taking the image at different planes, the PSF can be mapped in 3D, using a fluorescent particle, which is small comparable to the size of the PSF. The calibrated width of the PSF was used later to determine the position of particles.

In the distance measurement (and during the tracking) instead of raster scanning we were using scanning on a circular orbit in the xy plane (Fig. 1). For the

Fig. 1. Scheme of the scanning of the laser beam on a circular path when only one fluorescent particle is present. Immobilized particle is located at the radial coordinates $[r, \varphi]$ and the point spread function is represented by the shaded area centered at $[A, \theta]$. PSF is moving along the circular orbit of radius A represented as a dashed circle, with the center at $[0, 0]$. The actual distance d of the particle from the center of the scanning beam determines the recorded intensity.

measurements on the surface, the radial part of the PSF is sufficient to describe the fluorescence intensity, as we focus at the surface and z is constant:

$$F(d) = F_0 \exp \left[-\frac{4d^2}{w_0^2} \right] \quad (2)$$

where $d^2 = (x - x_s)^2 + (y - y_s)^2$ is the square of the distance between the center of the particle and the center of the laser beam. F_0 stands for the maximum intensity in the measured plane, including all the dependence on the axial direction. As long as the z position does not change during the measurement, the maximum intensity remains constant. The coordinates of the center of the beam scanned along a circular orbit of radius A , with frequency f as functions of time t are:

$$\begin{aligned} x_s &= A \cos(2\pi ft) = A \cos \theta \\ y_s &= A \sin(2\pi ft) = A \sin \theta \end{aligned} \quad (3)$$

Radial coordinates of the center of the beam are $[A, \theta]$. It is convenient to use radial coordinates $[r, \varphi]$ for the position of the fluorescent particle as well:

$$\begin{aligned} r^2 &= x^2 + y^2 \\ \sin \varphi &= y/r, \quad \cos \varphi = x/r \end{aligned} \quad (4)$$

Distance d of the pointlike particle from the center of the light beam in radial coordinates is given as follows:

$$d^2 = A^2 + r^2 - 2Ar \cos(\theta - \varphi) \quad (5)$$

Fluorescence intensity during circular scanning is a periodic function of time (through the angle $\theta = 2\pi ft$)

$$F(\theta) = F_0 \exp \left[-\frac{4}{w_0^2} (A^2 + r^2 - 2Ar \cos(\theta - \varphi)) \right] + B \quad (6)$$

For known radius of scanning A , beam waist w_0 and background B , the measured intensity function $F(\theta)$ can be fitted to determine the position of the particle $[r, \varphi]$ and its maximum intensity F_0 in the focal plane. The width of the peak in the intensity trace is related to radial distance r . The height of the peak is determined both by the intensity F_0 and the radial distance r , and the direction of the particle gives the center of the peak (phase φ). In this simple case, only one particle was assumed in one color detection. If there is a distribution of particles, a model for more particles can be used for the fit, where intensities from different particles add. If the particles are very close together, the fit of the fluorescence intensity profile cannot resolve well their position, unless position and properties of one of the molecules are known. To fit the position of particles at small distances (smaller than the width of the PSF), different colors and dual channel detection must be used.

Distance Measurement With Two Channels

In the previous section we have shown that by scanning the laser beam in a circular path and fitting the measured periodic intensity signal by Eq. 6, the position of the immobilized particle can be determined. For two particles of different color, the emitted intensity can be measured simultaneously in two channels, and can be fitted independently. From the independently determined position, their distance can be recovered. In real systems, however, the two emissions cannot be separated spectrally, there is usually some leakage of light between the detection channels that can be calibrated and taken into account.

Let us consider two particles at coordinates $[r_1, \varphi_1]$ and $[r_2, \varphi_2]$ of maximum intensities F_{01} and F_{02} in the measured plane. If there was not leakage between the channels, particle one would have a contribution to the intensity only in channel a and particle two only in channel b . If there was leakage between the two channels, we get for the measured intensities:

$$\begin{aligned} F_a(\theta) &= F_{01} \exp \left[-\frac{4}{w_0^2} (A^2 + r_1^2 - 2Ar_1 \cos(\theta - \varphi_1)) \right] \\ &+ \beta F_{02} \exp \left[-\frac{4}{w_0^2} (A^2 + r_2^2 - 2Ar_2 \cos(\theta - \varphi_2)) \right] + B_a \end{aligned} \quad (7)$$

$$\begin{aligned} F_b(\theta) &= \alpha F_{01} \exp \left[-\frac{4}{w_0^2} (A^2 + r_1^2 - 2Ar_1 \cos(\theta - \varphi_1)) \right] \\ &+ F_{02} \exp \left[-\frac{4}{w_0^2} (A^2 + r_2^2 - 2Ar_2 \cos(\theta - \varphi_2)) \right] + B_b \end{aligned}$$

where α and β are coefficients giving the average leakage for the particle one and two, respectively, B_a and B_b is the background. We assumed that the leakage coefficient does not change from one bead to the other and can be calibrated for the given setup and alignment before the measurement of the distances. For fitting the intensity traces in the two channels, global fit using nonlinear Levenberg-Marquart algorithm was performed by χ^2 minimization (weighted by errors) with linked parameters (Globals WE, LFD, Urbana IL). In our case, only the signal from the green spheres was leaking into the red channel. This simplifies the fitting procedure, as first the green channel data can be fitted, and then the recovered parameters together with known leakage coefficient were used in the fit of the red channel.

Limitations of the Accuracy of the Determined Center of Mass Position

Accuracy of the determined center of mass position depends on the accuracy of the calibrated parameters, the fitting process, the properties of the experimental setup, and the signal-to-noise ratio. In the following position, determination based on the circular scanning and raster scanning will be compared in terms of accuracy. The size of the PSF and the pixel size or radius of scanning are known from calibration (described in detail later in a separate paragraph). Mechanical properties of the experimental setup (reproducibility of the scanner coordinates) affect the scanning accuracy. In the circular scan, both mirrors perform oscillations driven by sinusoidal voltage; thus, at the turning points the movement of the beam is “smooth.” When the motion is fast, the amplitude may decrease, but the motion is maintained sinusoidal. In case of the raster scan, mirrors are moved by a saw-tooth voltage. Therefore, the spot has to return fast at the end of every line to start the new line. When this motion is fast, some pixels from the beginning of the line and the end have to be clipped to have in the image only the part where the motion of the beam was uniform. Based on these considerations, circular motion is at least as reproducible as the raster scan. Mechanical jitter is the property of the setup, and affects both raster scan and circular scan adding noise of particular frequencies to the data. When we measured the power spectrum of the signal, we did not find any additional frequency. Let us suppose for the calculation of the theoretical limit of the position error that calibration is perfect, and values do not change during the measurement.

Besides the experimental factors, there is a theoretical limit of the distance measurement, which is determined by the signal-to-noise ratio (SNR). Enough photons must be collected to determine the parameters of the intensity curve by the fit with a given accuracy because the fluorescence signal detected by the PMT is always limited by shot noise. Intensity F is measured as number of photons, which has a Poissonian noise $\Delta F = \sqrt{F}$. The error of the position was calculated based on chi-square statistics, following the procedure described in Bobroff (1986). This calculation determines the best fit parameters and specifies the errors of the fitted parameters by minimizing the sum of squares of the difference between the response function and the measured values at each sample point. The correspond-

ing difference $\Delta = \chi^2 - \chi_m^2$, where χ^2 denotes the sum of squares and χ_m^2 is its minimum was calculated analogously to Bobroff for response functions corresponding to the raster scan and the circular scan method. Calculations were performed for the case when signal dominates the background.

The response function (the PSF in the plane of the focus) in the raster scanned image based on the Eq. 1 will be:

$$F(F_0, x, y, x_i, y_j) = F_0 \exp \left[-\frac{4}{w_0^2} ((x - x_i)^2 + (y - y_j)^2) \right] \quad (8)$$

where fitted parameters are the maximum intensity F_0 , and the position of the particle $[x, y]$. The parameter set corresponding to the best fit (where χ^2 has minimum) is F_{0m}, x_m, y_m . The position of the scanner $[x_i, y_j]$ is known and will be changed in regular steps in the $n \times n$ matrix. The size of the PSF is represented by the beam waist w_0 , which is a parameter determined by a calibration. For the χ^2 difference we get:

$$\begin{aligned} \Delta(F_0, x, y) = & \sum_{i=1}^n \sum_{j=1}^n F_{0m}^{-1} \left\{ \left(\frac{8F_{0m}}{w_0^2} \right)^2 ((x_i - x_m)(x - x_m) \right. \\ & \left. + (y_j - y_m)(y - y_m))^2 + (S - S_m)^2 \right\} \\ & \times \exp \left[-\frac{4}{w_0^2} ((x_i - x_m)^2 + (y_j - y_m)^2) \right] \quad (9) \end{aligned}$$

The error of the coordinate x will be the largest for the given value of Δ , when $y = y_m$ and $F_0 = F_{0m}$

$$\frac{\Delta x}{w_0} = \sqrt{\frac{\Delta(F_0, x, y)}{F_{0m} N K_x}} \quad (10)$$

$$K_x = \frac{1}{N} \sum_{i=1}^n \sum_{j=1}^n 8 \frac{x_s^2}{w_0^2} \exp \left(-\frac{4(x_i^2 + y_j^2)}{w_0^2} \right)$$

The error of the coordinate is inversely proportional to the square root of the maximum intensity F_{0m} and the number of pixels $N = n^2$ in the raster scan. Coefficient K_x corresponds to the integral $tF_g(t)$ in Bobroff (1986), this time calculated numerically. We can evaluate the coefficient K_x for the different situations. To use this equation to evaluate the position error, an appropriate value of Δ must be substituted. Traditionally error limits at “1 σ ” level, corresponding to 68% confidence level, are calculated. For three parameter fit, $\Delta = 3.5$. As an example, let us calculate the error in case of a raster image of 8×8 pixels and the PSF centered in the field. The error of the parameter x was calculated numerically for different pixel sizes and was the smallest if the size of one pixel was $0.15 w_0$, that means the field is $1.17 w_0$ wide. For the coefficient K_x in the minimum of the error, we get 0.29.

A similar calculation can be performed for the circular scan, using the following intensity expression based on Eq. 6. as a response function.

$$F_i(F_0, r, \varphi, \theta_i) = F_0 \times \exp\left[-\frac{4}{w_0^2}(A^2 + r^2 - 2Ar \cos(\theta_i - \varphi))\right] \quad (11)$$

The chi-square difference will be:

$$\Delta(F_0, r, \varphi) = \sum_{i=1}^N \frac{1}{F_{0m}} \left\{ \left(\frac{8F_{0m}}{w_0^2} \right)^2 [(A \cos(\theta_i - \varphi_m) - r_m) \times (r - r_m) + A \sin(\theta_i - \varphi_m)r_m(\varphi - \varphi_m)]^2 + (S - S_m)^2 \right\} \times \exp\left[-\frac{4}{w_0^2}(A^2 + r_m^2 - 2Ar_m \cos(\theta_i - \varphi_m))\right] \quad (12)$$

The maximum excursion of r at fixed Δ occurs when $\varphi = \varphi_m$ and $F_0 = F_{0m}$ and we get it in the similar form as in case of the raster scan, with corresponding factor K_r :

$$\frac{\Delta r}{w_0} = \sqrt{\frac{\Delta(F_0, r, \varphi)}{F_{0m}NK_r}} \quad (13)$$

$$K_r = \frac{1}{N} \sum_{i=1}^N 8 \left(\frac{A \cos(\theta_i - \varphi_m) - r_m}{w_0} \right)^2 \times \exp\left[-4 \left\{ \left(\frac{A}{w_0} \right)^2 + \left(\frac{r_m}{w_0} \right)^2 - 2 \frac{A}{w_0} \frac{r_m}{w_0} \cos(\theta_i - \varphi_m) \right\}\right]$$

Similarly, the maximum excursion of φ at fixed Δ occurs when $r = r_m$ and $F_0 = F_{0m}$, with the corresponding factor K_φ :

$$\frac{r_m \Delta \varphi}{w_0} = \sqrt{\frac{\Delta(F_0, r, \varphi)}{F_{0m}NK_\varphi}} \quad (14)$$

$$K_\varphi = \frac{1}{N} \sum_{i=1}^N 8 \left(\frac{A \sin(\theta_i - \varphi_m)}{w_0} \right)^2 \cdot \exp\left[-4 \left\{ \left(\frac{A}{w_0} \right)^2 + \left(\frac{r_m}{w_0} \right)^2 - 2 \frac{A}{w_0} \frac{r_m}{w_0} \cos(\theta_i - \varphi_m) \right\}\right]$$

As an example, let us calculate the error in case of a circular scan of 64 points in one orbit (same number of measurement points as in the raster scan calculation) and the particle in the center of the scanning area. Based on numerical calculation, the error of the parameter r will be smallest if the radius of the circular path is $A = 0.5 w_0$. That means, that the error of r is the smallest if we measure intensity at the places, where $F = F_0/e$, thus where the PSF is the steepest. For the K_r and K_φ , we get 0.37; thus, the error is now smaller than for the raster scan using the same number of measurement points. When tracking is performed, during one orbit the particle moves and in the next cycle

the particle will not be in the center of the scan. In this case, a larger scanning radius was used. Enderlein (2000b) proposed a radius of $0.6 w_0$ for the scanning because there is no “hole” in the average intensity in the center, where the particle is supposed to be during the tracking. In our measurements, we generally used a radius of $0.8 w_0$ because this way a circular region that particle can reach falls into the sensitive “steepest part” of the PSF during scanning. In order to check the situation when the particle is not in the center of the orbit, numerical calculation was performed to find a radial distance corresponding to the minimum of the error. For scanning radius $A = 0.8 w_0$, the corresponding K_r factor (and K_φ) will be 0.20 for the particle in the center. For the K_φ factor, we get a minimum value of 0.13 when the radial distance is $r = 0.4 w_0$ (in this case, $K_r = 0.22$). According to this theoretical limit, to achieve an accuracy of 1 nm in the radial distance r if the radial beam waist was $w_0 = 400$ nm, the maximum F_{0m} has to be at least approximately 23,000 counts in accordance with having the coefficient K_r equal to 0.37 in Eq. 13. In this calculation, only the effect of the shot noise was considered.

The distance of the particles is calculated from the recovered coordinates as

$$d = \sqrt{r_1^2 + r_2^2 - 2r_1r_2 \cos(\varphi_1 - \varphi_2)}. \quad (15)$$

The accuracy of the distance of the particles is given by the errors of r_1 , φ_1 , r_2 , and φ_2 , that is for independent variables

$$\Delta d = \left| \frac{\partial d}{\partial r_1} \right| \Delta r_1 + \left| \frac{\partial d}{\partial \varphi_1} \right| \Delta \varphi_1 + \left| \frac{\partial d}{\partial r_2} \right| \Delta r_2 + \left| \frac{\partial d}{\partial \varphi_2} \right| \Delta \varphi_2. \quad (16)$$

The errors of the positions are determined as the errors of the fitted parameters during the fitting process. In the best geometry of the present setup, when the particles are at $0.4 w_0$ from the center of the scanning at the opposite side of the orbit, at the distance of $0.8 w_0$, the error of the distance measurement caused only by Poissonian noise in the photon counting is:

$$\frac{\Delta d_{\min}}{w_0} = \frac{0.85}{\sqrt{F_{0m}}} \quad (17)$$

According to this result, to achieve 1 nm accuracy in the distance measurement if the radial beam waist was $w_0 = 400$ nm, the maximum intensity has to be at least 115,600 counts in each channel.

Tracking Algorithm

At this point, we have a method to determine a position of immobilized particles (distance and direction) at the surface. In principle, the same method with the circular scanning could be used to determine the position of slowly moving particles in two dimensions or to find particles on the surface. It has the advantage that scanning can be fast. When enough photons are collected in a cycle to determine the position, we could follow the moving particle by moving the center of the circular scan on top of the particle at every cycle, opti-

mizing the conditions for the distance measurement. However, fitting the intensity by the intensity trace calculated from Eq. 7 is time consuming. For the analysis of the recorded data, this is not a problem, but to make calculations during the tracking measurement it is slow. We used a different approach in the tracking routine for the fast calculation of the position. From the fast Fourier transform (FFT) of the intensity trace, we get the DC as the 0th term in a Fourier series and AC as the coefficient of the 1st harmonic term. The phase of the AC term gives directly the φ coordinate of the particle, and the radial distance r can be calculated from the modulation (AC/DC) of the signal. The modulation value does not change when the scanning is started with a different phase, as the integration is made in one full period. For the purpose of this calculation, let us have $\varphi = 0$; this means that the circular scanning is started where the intensity is maximum. In this way coefficients of the sine series in the Fourier decomposition are zeros. The 0th and 1st coefficients of the cosine series are

$$DC = a_0 = \frac{1}{2\pi} \int_{-\pi}^{\pi} F_{01} \exp \left[-\frac{4}{w_0^2} (A^2 + r_1^2 - 2Ar_1 \cos \theta) \right] \times d\theta = F_{01} \exp \left[-\frac{4}{w_0^2} (A^2 + r_1^2) \right] \cdot I_0 \left(\frac{8Ar}{w_0^2} \right) \quad (18)$$

and

$$AC = b_1 = \frac{1}{\pi} \int_{-\pi}^{\pi} \cos \theta \cdot F_{01} \times \exp \left[-\frac{4}{w_0^2} (A^2 + r_1^2 - 2Ar_1 \cos \theta) \right] d\theta = 2F_{01} \exp \left[-\frac{4}{w_0^2} (A^2 + r_1^2) \right] \cdot I_1 \left(\frac{8Ar}{w_0^2} \right) \quad (19)$$

where $I_n(x)$ denotes modified Bessel function of n th order at x . The modulation of the intensity signal is defined as AC/DC:

$$MOD = 2 \cdot I_1 \left(\frac{8Ar}{w_0^2} \right) / I_0 \left(\frac{8Ar}{w_0^2} \right) \quad (20)$$

DC and modulation are plotted as a function of the distance of the particle from the center of scanning r in Figure 2, for different A/w_0 ratios. For known A and w_0 , the modulation is a monotonic function of the radial distance r only. From this dependency, we can immediately determine from the measured modulation, the r of the particle. The modulation change is steep over a distance of about $0.5 w_0$. For longer distances, it changes only very little; thus, the accuracy of the r read out from the curve is lower. In the tracking measurement, the modulation is calculated at every cycle (specified number of periods) and minimized during tracking by moving the center of the scan to the recovered position of the particle. In two-channel measurement, one of the channels (in our case green) is used for the

Fig. 2. The average fluorescence intensity (A) and modulation (B) of the signal in a circular scan as a function of the radial distance of particle calculated from Eq. 18 and Eq. 20 for different A/w_0 ratios: $A/w_0 = 1.2$ (thin), 1.0 (thick), 0.8 (dotted), 0.6 (dashed). In the measurements, the ratio was 0.8 .

feedback in tracking, but positions and distance are calculated for both channels online. In this calculation, the leakage between channels is not taken into account. Therefore, the recovered position of the red particle is not accurate. However, for tracking, the important fact is that the center of scanning is on top of one of the particles (green). Exact positions of the particles can be calculated precisely from the recorded data later by fitting the intensity with Eq. 7 for every period.

MATERIALS AND METHODS

Equipment

The experimental setup for the distance and tracking measurements is shown in Figure 3A. For excitation, we used a mode-locked (80 MHz) Tsunami 3960 C Ti:Sa laser pumped by Millennia V solid-state laser (Spectra Physics, Germany). Laser pulses were 150 fs (FWHM) long. Spontaneous beam pointing fluctuations of the laser are not synchronous with the scanning frequency and they cancel out in our analysis. Laser power was attenuated by neutral density filters resulting in 1–10 mW average intensity at the sample. Excitation wavelength was 785 nm. The light is directed into the microscope by galvanometer-driven scanning mirrors (Moving Coil Optical Scanner 6350 with series 603X dual axis driver, Cambridge Technologies, Watertown, MA) through a scan lens. Repeatability of the scanner is

Fig. 3. Schematic diagram of the scanning two-photon microscope setup (A). Calibration curve of the actual diameter of the orbit as a function of the voltage sent to the scanner mirrors at 500 Hz frequency of the circular scanning (B).

2 microradians, corresponding to 1.4 nm in the image for the given setup. The scanner mirrors are moved by voltage generated in a computer card by two shifted sine wave generators with DC offset. The average value, amplitude, and scanning frequency are controlled with computer commands from the acquisition program. Each of the axes is independent, but synchronized. For a circular scan, the two sine waves are out of phase by $\pi/2$. For the entire run, the same clock synchronizes scanner and data acquisition. Updating the position of the center of scanning is done by changing the offset values of the output wave. The radius of the orbit is set by the amplitude value. Raster scan of the image is made by the same procedure, but the computer card is programmed with two saw-tooth signals.

The apparatus is based on a Zeiss Axiovert S100TV inverted microscope (Thornwood, NY). Excitation light is reflected by the low-pass dichroic mirror (transmission between 370–630 nm, Chroma Technology Corp., Brattleboro, VT) and focused on a sample by 40 $\times$ Fluor, oil immersion objective lens of NA = 1.3 (Zeiss, Germany). The emitted light passes through the first dichroic mirror again and exits the microscope on the bottom port. Then the signal is divided into the two channels by the second dichroic mirror (Q560LP,

Chroma Technology Corp.). In the channels, before the PMTs there are additional filters (HQ525/50M, HQ610/75M, Chroma Technology Corp.) to cut out only green and red emission. Fluorescence light was focused by a lens into the photomultiplier tubes (HC120-08, Hamamatsu, Japan). Signal was amplified, discriminated (Phillips Scientific 6931 and 6930, Ramsey, NJ), and TTL pulses (corresponding to the photons) were counted by the data acquisition card (ISS, Champaign, IL). Data acquisition is synchronized with the scanner movement. We generally operate the data acquisition card at a sampling rate of 32 kHz for scanning.

Experiments are controlled by a data acquisition program (SimFCS, LFD, Urbana, IL). In a typical experiment for determining the position of particles (or molecules) on the surface, we take the image of the surface to map the particle distribution. Then the operator selects a starting location of the center of scanning from the image. We can measure with or without tracking. When the tracking option is activated, the data acquired over specified number of periods are Fourier transformed to provide DC, phase and modulation of the signal. A step of the size of the determined radial distance is made with the center of scanning in the direction of the phase. This tracking routine can be used to center on top of the particle as precisely as possible first and then tracking is turned off. Although in real-time only simple calculation is performed, we record the entire run for off-line analysis. The record contains the coordinates of the center of scanning, the counts measured by both detectors, and the calculated values of the particle position.

Spheres and Dyes

Yellow-green (F8803, 505/515) and red (F8801, 580/605) fluorospheres of 100-nm diameter were purchased from Molecular Probes (Eugene, OR). Spheres were sonicated for 10 min after diluting by deionized water to concentration of 0.001 % solids (number of spheres in 1 mL of solution is 1.36×10^{10}). For single molecule measurement, we used water solution of Alexa-488 maleimide (green) and Alexa-594 maleimide (red) (Molecular Probes, Eugene, OR) of 1 nM concentration, sonicated for 10 min. Quartz coverslips (ESCO Products, Oak Ridge, NJ) were cleaned carefully by sonication in detergent solution, ethyl acetate, and finally distilled water. Having clean coverslips is important to reduce the background counts especially in single molecule measurements. Then, 20 μ L of the sample solution was spin coated on the surface. Coverslips were fixed face down on a hanging drop microscope slide and were sealed to avoid later contamination of the surface.

Calibration

The size of the PSF is affected by small variations in the optical alignment and must be calibrated each time when the alignment is changed. The PSF was measured by scanning the image of a bright particle of a size smaller than the PSF. This spot was fitted in 2D by the theoretical intensity distribution (Eq. 1) and the particle size, and the width of the PSF was obtained in pixel units. The absolute size of the pixel of the image was calibrated using a grating with 5,000 line pairs/inch dyed with fluorescein solution. The image was scanned first with the grating positioned vertically and

intensity from all the rows was added. Then the process was repeated with the grating positioned horizontally when the columns were added. This process results in clean curves of the grating profiles, and the width of the line pairs was determined from these curves in pixel units. If we assume that the grating was manufactured precisely, the error of the calibration procedure is smaller than 1% (determined as a standard deviation of 10 independent measurements). The width and height of the image was the same within this error. The circularity of the scan was also checked this way.

The actual radius of the circular scanning must also be calibrated. In principle, the amplitude could be calculated from the known amplitude of the voltage sent to the scanner mirrors. However, for fast scanning the mirrors can move with smaller amplitude due to the mechanical properties of the system, thus the circular path shrinks. The actual radius of the scanning was determined for the frequency used in the measurements (500 Hz) by scanning the beam over the grating, and the diameter was calculated from the reconstructed intensity distribution around the orbit. This can be done only for radii of the size at least one line pair of the grating (5 μm). For the given frequency of scanning (500 Hz), the radius of the scanning was measured in the 5–30 μm range as a function of the voltage on the scanner, and linear relationship was found (Fig. 3B). The regression line goes through the zero as it is expected. The error of the fit of the slope of the curve that is of the radius of scanning is 1%. It was reasonable to assume that the relationship is linear for smaller radii and we got the actual radius by extrapolation.

The absolute position of the fluorescent spheres can be calculated only when the initial phase of the scanning is known in the laboratory coordinate system. A physical angle is introduced, meaning the starting phase of the circular scan. This can be determined also from the circular scan of the grating by comparing the orientation of the bright stripes appearing in the intensity trace with the corresponding stripes in the image of the grating. This is important for the tracking routine (to move the center of the scan to the calculated position), but in the distance measurement when the center of scanning is not moved relative measurement is done, and the physical angle does not affect the accuracy.

RESULTS

Images and Colocalization by 2D Fit of PSF

Figure 4 shows images of green and red fluorescent spheres of 100-nm diameter deposited on a quartz surface. Images of the spheres were obtained in the two-photon microscope using the usual raster scanning operation. An area the size of $8 \times 8 \mu\text{m}$ (256×256 pixels) was scanned by 20 kHz data acquisition frequency 5 times, and the intensity values were averaged. Only a region of interest the size of $1.6 \times 1.6 \mu\text{m}$ (50×50 pixels) is shown from the image colored corresponding to the intensity scale. Four examples are shown: green only (Fig. 4A), red only (Fig. 4B), and a mixture of green and red spheres (Fig. 4C and D). It can be seen clearly that red particles cannot be distinguished in the green channel. However, green particles are always

Fig. 4. Raster scanned images of the 100-nm diameter spheres immobilized on the surface: green sphere only (A), red sphere only (B), and green and red spheres (C, D). Intensity average of 5 frames is shown in the two channels. GP image is calculated as the difference of normalized images (defined in the text). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

present in the red channel, faded. Spheres of different emission were excited by two-photon process simultaneously at 785 nm. Emission spectra of the spheres overlap. Therefore, it is not possible to separate the signal from the two spheres by the dichroic mirror and filters completely; there will be always leakage from the emitted intensity of green spheres into the red channel (Fig. 4A). Based on the emission spectra, the expected leakage is $\alpha = 20\%$ (the intensity in the red channel is 20% of the value in the green). The leakage between the two channels is significant only in the case of green particles, as we expected from the spectral overlap. From the intensity ratio of the images, we estimated leakage as 30%. In the images, red spheres were always approximately 5 times brighter than green due to different excitation and detection efficiency. In our two-photon experiments, the fluorescence intensity of the spheres does not correspond to the number of dye molecules in the sphere; it is significantly lower. This reduced fluorescence can be due to quenching by the material of the sphere or self-quenching. We scanned several raster images in the beginning of the measurement to determine the PSF width. For

the radial beam waist we got (483 ± 6) nm in case C and (508 ± 6) nm in case D (Fig. 4). There was a difference between them because the measurements were done on different days and the alignment was changed. The size distribution of the spheres will affect the width of the measured PSF only by 5% (Starchev et al., 1998). We did not correct for this because the same spheres were used for the distance measurement and the determination of the PSF.

A generalized polarization (GP) image is shown in the third column of Figure 4. The GP value is defined as $GP = (I_g - gI_r)/(I_g + gI_r)$, where $g = I_{g\max}/I_{r\max}$ is a factor correcting for the different sensitivity of the channels (Parasassi et al., 1991). Images were thresholded to cut off background. The GP value gives us a simplified spectral information about the pixels of the image, and enables us to compare the image from the two channels. In this representation, spheres in the green channel have positive (blue) and red spheres negative GP values (red). In Figure 4C and D, particles of both colors were present, appearing as a non-uniform spot in the GP image. Circular scanning was performed in these areas to determine the distance of the beads. The smallest possible distance between the centers of the two spheres is their diameter, that is (100 ± 10) nm when they touch each other (assuming they are at the plane of the scan).

A straightforward method of determining the distance between the spheres would be to recover the center of mass in both colors by fitting the two-dimensional PSF in the images and calculating the distance of the centers (Lacoste et al., 2000). Leakage between the channels was not taken into account in our fit, but it affects the calculation of the center of the PSF through non-symmetric shape distortion in the red channel. However, red spheres were 5 times brighter, so the leaking intensity from the green spheres is rather small compared to that.

In order to compare the result of the raster scan with the circular scan method, a raster scan of 8×8 points was needed, where the pixel size is on the order of $0.15 w_0$. Therefore, a 64×64 pixel scan was performed the size of $8 \times 8 \mu\text{m}$ and a region of 8×8 pixels was selected, where the particle was approximately in the center. One frame was fitted to reduce the effect of possible mechanical drift between the individual frames. From the fit, the center of mass of the green and the red spheres was obtained. In this case, data were acquired during 3 seconds, and maximum counts were on the order of 3,000 in the green and 18,000 in the red channel. The distance was recovered as 85 ± 18 nm in case C and 254 ± 19 nm in case D (Fig. 4). Leakage was not taken into account.

Tracking and On-Line Calculations

The process of distance measurement by circular scanning of the laser beam involves three steps. First, an image is scanned for orientation in order to find the area where particles of both color are present at close proximity on the surface. In the second step, the tracking routine is used to center on top of the chosen particles. In the third step, circular scanning with a fixed center is performed and the intensity data are recorded. Finally, the distance between the particles is calculated from the recorded data. This protocol was

used for the measurement of fluorospheres immobilized on the surface.

The initial center position of the circular scanning was determined from an image taken with 1-kHz data acquisition frequency at a resolution of 64×64 pixels, where 1 pixel corresponds to 104 nm (around $0.25 w_0$). This image is only for orientation, and has to be taken fast because it is important that molecules do not bleach during the scanning of the image especially in the case of the single molecule measurement.

During the tracking and the distance measurement instead of raster scanning, the excitation beam performed circular scanning as described in Principles of the Method. The laser beam was scanned at 500 Hz, radius of scanning was 420 nm ($A/w_0 = 0.8$). Data acquisition frequency was 32 kHz, which means that the intensity was measured in 64 fractions around the circular path in each period. This number is chosen to be proper (power of 2) for the FFT calculation in the tracking routine. In every cycle of the measurement, 10 periods were added to get enough intensity for the determination of the position. Radial distance and the phase of the particle were calculated as described earlier in the tracking algorithm section of Principles of the Method and the center of the circle was moved to this recovered position before the next cycle. The movement of the center of scanning and the recovered particle position were displayed on the screen in real time over the image scanned in the first step of the measurement.

In this report, we focus only on the distance measurement with immobilized particles and tracking is used only to locate them precisely. One trace corresponding to tracking an immobilized particle on the surface is shown in Figure 5. The sample stage was moved upwards suddenly as indicated by an arrow to simulate a movement of the particle in one direction and the corresponding movement of the center of the scanning is shown as immediately follows the particle. Recovered positions of the particles show similar distribution as the same particles in the raster image at Figure 4C, and the calculated distance averaged for 400 cycles is (87 ± 23) nm, corresponding well with the result of the 2D fit, (85 ± 18) nm. Although it is useful to measure the distance between particles while tracking, the movement of the center of scanning introduces a source of mechanical noise in the system. For the immobilized particles, it is better to do measurement with fixed center of scanning, i.e., the tracking mechanism is turned off.

After centering the circular orbit by the tracking routine above the sphere in the first channel (it takes few seconds), the circular scan was performed with a fixed center. During the measurement, the FFT calculation of the position is made, but now the center of scanning is fixed, is not moved into the recovered position. Recovered positions of the particles are shown on the screen over the image during the measurement. The calculation shown in Figure 6 was performed for the spheres from Figure 4D, adding a different number of periods in a cycle, thus with 20- and 200-ms time bins of the position determination. It shows that recovered positions are more scattered for shorter bins due to a smaller number of detected photons (higher shot noise). In this case, the distance was (247 ± 12) nm for

Fig. 5. Real time display during the tracking of 100-nm spheres on the quartz surface by centering the circular orbit on top of the particle at the green channel. Recovered positions of particles, position of the center of scanning, and relative distance are shown. Ten periods were added in the cycle (1 cycle = 20 ms). Arrow indicates the movement of the sample stage during the tracking.

Fig. 6. Real time display of the tracking routine with fixed center of scanning of 100-nm spheres on the quartz surface. Recovered position of the spheres is shown as it was determined during the tracking routine by FFT, where 10 and 100 periods were added in a cycle (1 period = 2 ms). Recovered distance of the particles is shown.

20-ms and (246 ± 4) nm for 200-ms cycles, respectively. The distance recovered from the 2D PSF fit of the image was similar: (254 ± 19) nm. Accuracy of the calculated distance as a function of number of collected photons is analyzed in detail later.

Determination of the Position and Distance: Calculation From the Saved Data

Figure 7 shows the recorded intensity trace of the scanning in a circular orbit. For the visualization and the analysis of the saved stream of intensity data, we used a two-dimensional pseudoimage representation. A continuous stream of the intensity data was divided into sections corresponding to periods of scanning, in this case by 64 points. A 2D matrix was formed, where individual periods are in rows. Usually, the frequency of data acquisition is too high to get a sufficient amount of photons in one period. Therefore, several periods (rows) were summed. The number of summed rows to get a sufficient number of photons in the cycle can vary. In the cases shown, we added 100 rows; thus, the time length of one distance measurement is 200 ms. In columns, we get the time dependence of the intensity corresponding to the same position on the circular

path. As we expected, immobilized particles give straight band in this representation, because they stay in a fixed position around the circle. This indicates also that the system is well synchronized. Along the time axis, we can see the effect of bleaching, as the maximum intensity decreases in time. Although containing hundreds of dye molecules, 100-nm beads had a noticeable exponential photobleaching of decay times: $\tau_{\text{green}} = (10 \pm 2)$ min and $\tau_{\text{red}} = (3 \pm 1)$ min. This pseudoimage representation is very useful because we can immediately have an idea of how many particles can be distinguished in every channel, about the leakage, the bleaching, or scanning instabilities.

Figure 8A shows the results of the fit of the intensity traces of one cycle (200 ms) for the two channels by Eq. 7 using Levenberg-Marquart algorithm. Parameters used in the fit were: the width of the PSF $w_0 = 508$ nm, the radius of scanning $A = 420$ nm, the leakage coefficients $\alpha = 0.3$, $\beta = 0$, and the initial phase of the scanning 160 degrees. Background was measured in the empty area of the image $B_a = 20$ counts/cycle, $B_b = 200$ counts/cycle. First, the green channel was fitted with one component, and the result of this fit was used as fixed parameters for the fit in the red channel. Recovered value of the distance was 259 ± 9 nm, longer

Fig. 7. Pseudoimage representation of the fluorescence intensity during the tracking with fixed center of scanning of 100-nm particles. Intensity in the individual cycles of 100 periods (200 ms) as a function of time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

compared to the value calculated both by FFT (246 ± 4 nm) and by the fit of the image (254 ± 19 nm). The recovered distance by FFT can appear shortened as a result of the leakage, because the position recovered in the red channel is the center of mass of the red particle and the leaking intensity of the green. In the fit of the intensity, the leakage was included, and the position of the red particles was not distorted, as shown in Figure 8B. This fit can be made for every row in Figure 7.

Accuracy of the Position and Distance Determination

In addition to the shot noise, there are other sources of errors in the position determination. The mechanical drift of the table, the defocusing or the mechanical vibrations faster than the cycle of measurement will result in errors of the recovered position. For each method of the calculation of the particle coordinates,

Fig. 8. Circular scan was performed with fixed center of scanning on immobilized 100-nm particles. Intensity in one cycle of 100 periods (200 ms) was fitted by the model intensity function (A); Parameters of the fit were: $w_0 = 508$ nm and $A/w_0 = 0.8$. Recovered positions of the green (light gray) and red (dark gray) sphere relative to the center of scanning are compared with the calculated positions from FFT (open circles) (B).

the accuracy of the recovered values was determined as a function of the number of detected photons, and compared with the theoretical curve calculated from Eq. 13 having $K_r = 0.38$ and Eq. 17 (Fig. 9).

Error of the parameters in the fit of the intensity in one cycle can be calculated directly for every cycle from the covariance matrix, for 68% confidence level. The length of one cycle was changed to get a different total number of photons in the cycle. The accuracy of the FFT calculation of the position during the data acquisition was determined as the standard deviation of the average from the data calculated for the different cycle lengths (Fig. 9). It can be assumed that during the measurement, particles are not moving, and in every cycle we perform the measurement of the same position. Position was calculated in 10 different cycles, averaged, and the standard deviation was plotted as a function of the average number of counts in the cycle. The theoretical lower limit

Fig. 9. Accuracy of the position (A) and distance (B) measurement of the 100-nm spheres as a function of the number of detected photons. A different number of periods (1–2,000) was added in a cycle to get a different number of photons. In the case of FFT calculation, the standard error was calculated from the recovered coordinates in 10 cycles. In the case of intensity fit, the standard deviation of fitted parameters was determined in the fit of one cycle. The theoretical curve was calculated for $w_0 = 508$ nm and $N = 64$. The error of the distance was determined using Eq. 16 and 17.

is shown calculated from Eq. 13. We can see that if the cycles are longer, standard deviation grows more rapidly than the theoretical curve would suggest. This is the effect of the slow mechanical drift of the sample stage during 10 long cycles with velocity on the order of 5 nm/s. The theoretical curve is only the lower limit of the accuracy corresponding to the ideal geometry, and it is clear that in a real experiment we get only values above the curve. Error of the distance measurement was calculated from the individual error values of the coordinates using Eq. 16. Here we can also see the effect of the mechanical drift. For comparison, the error determined for the fit of the raster scanned images is shown. We can conclude, that the accuracy of the distance measurement was photon limited.

Determination of Position of More Than One Particle Per Channel

So far, we have seen cases where only one particle of each color was present. In Figure 10, a GP image

of a group of four 100-nm spheres is shown. A circular scan was performed as indicated in the image. As we expected, in the red channel two particles can be seen, as it shows up immediately in the intensity pseudoinage in Figure 10B. In the FFT calculation, only the first harmonic was taken into account. Therefore, the recovered position in the channel where two particles are present gives the center of mass of the two particles. The calculation could be improved by calculating the second and higher harmonics. However, the FFT calculation is used mainly for tracking, and the exact position can be recovered from the intensity record. In the fit of the intensity trace, we used a model for two particles. From the fit of one cycle (Fig. 11A), the positions of particles could be recovered, as shown in Figure 11B. Two red particles and one green particle were recovered, as seen in the GP image. The fourth, green particle was too far to be detected by the same measurement (using scanning).

Fig. 10. Image of four 100-nm spheres next to each other in the GP image and the circular orbit of the laser beam (A). Pseudocolor image of the intensity trace recorded from the tracking with fixed center of scanning in the two channels where one cycle is 200 ms (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Determination of the Position of Single Molecules

We have seen that the limiting factor in the position determination of the molecules is the number of photons collected. Can we detect enough number of photons before bleaching to determine the position of single dye molecules, and what will be the accuracy? As a simple test system, Alexa dye molecules were immobilized on a quartz surface by spin coating. Concentration was kept low to have only single dyes relatively far from each other. Cleaned quartz surface was uniform and free of any fluorescent spots. In the fast image of the surface with Alexa dyes, spots the size of the PSF were seen (not shown). Stepwise bleaching and blinking behavior were observed in some cases, when the

Fig. 11. Tracking with fixed center of scanning of the group of 100-nm spheres next to each other. Fit of the intensity in one cycle (A) and recovered positions of the green (light gray) and red (dark gray) spheres compared to the positions recovered from the FFT calculation (open circles) (B).

spot of the PSF size was cut by half or had black lines. During the scanning of the image, some of the emitted photons (in the order of thousand) are wasted by finding the rough position of the molecules. Circular scanning was performed with fixed center of scanning in the bright area that was still present after the scan. From the two-dimensional pseudocolor representation of the intensity trace (Fig. 12), bleaching of the single molecules can be seen as the band representing a molecule at a fixed position around the circle suddenly disappears. In this case, there was one molecule in the green channel bleached after 32 seconds. In the red channel, in the first 3 seconds there were two molecules: one was bleached after 3 seconds, the other blinked and was bleached after 9 seconds. The total number of photons detected from the dyes was on the order of 6,000 counts in both channels. As we excite the red dye more efficiently, its emission is brighter but bleaches faster. From this diagram, we can select the time interval for the distance determination. Part of the traces, where particles can be seen, was added and fitted by the intensity model (Fig. 13A). In the green

Fig. 12. Measurement of single Alexa 488 and Alexa 594 dye molecules on quartz surface. Pseudocolor representation of the intensity recorded during scanning showing stepwise bleaching and blinking of the molecules. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

channel, one component was enough to fit the peak. In the red channel, at least two components were needed, besides the leaking intensity from the green particle. This indicates the presence of two red molecules. Reconstructed positions of particles can be seen in Figure 13B. Error of the position determination was relatively large, because particles were far from the center of scanning.

DISCUSSION

We have described a novel, fast, and sensitive tool for localization of fluorescent probes immobilized on the surface. The principles of the method were first demonstrated for relatively large fluorospheres of 100 nm in diameter. Choosing this system has the advantage that it is bright as spheres contain on the order of a thousand dye molecules, but it is still significantly smaller than the size of the PSF. We have shown a measurement where the spheres were in closest proximity, touching each other presumably at a distance of 100 nm. Using the tracking algorithm, we could effectively center on top of the particles, in the region where the distance measurement would be the most accurate. The tracking algorithm was very effective. In the case of 100-nm spheres, the position of the spheres was determined in 20-ms cycles, where the average number of photons in the cycle was around 350 counts, and the precision of the distance determination was on the order of 25 nm, if averaged for 8 seconds. The distance

Fig. 13. Single Alexa dye molecules on quartz surface. Fit of the total intensity record added into one cycle (C) and reconstruction of the positions of the individual dye molecules (D).

calculation is based on the fast Fourier transform (FFT) of the modulated signal after each cycle, in real time. Although it does not take into account leakage between the channels and it is affected by the changes in the background, by minimization of the modulation the center of scanning could be moved very close on top of the particle. During the measurement, all the calculated values of the parameters can be followed, as well as the path the particle travels. The parameters of the measurement are recorded, so the data can be re-analyzed, taking into account leakage between the channels or using a model for more particles in the intensity fit. In comparison with the tracking algorithm proposed by Enderlein (2000b) where the direction is chosen by picking the quadrant of the maximum intensity and steps are of fixed size, our method calculates the position of the particle in every cycle and moves the center of scanning by required step length and direction.

Limitations of the tracking method can be seen immediately with the used parameter settings. If during

the cycle the particle moves, the recovered position would be the center of mass of the path. Considering a particle diffusing not further than the width of the PSF during one orbit (2 ms), supposing sufficient number of collected photons, the fastest trackable particle would have a diffusion coefficient $D = 15 \mu\text{m}^2/\text{second}$. This corresponds to a 28-nm diameter sphere in water; thus faster scanning is needed to track proteins in water. However, the viscosity is usually higher in the cellular environment, slowing down the diffusion. A single R-phycoerythrin molecule, for example, had a mean diffusion coefficient of $3.5 \mu\text{m}^2/\text{second}$ in the cytoplasm (Goulian and Simon, 2000), and would be trackable with settings used in our measurement. In case of linear movement, particle of velocity $v = 250 \mu\text{m}/\text{second}$ could be tracked. Molecular motors as kinesin move with a load (200 nm) with a velocity on the order of 800 nm/second (Coy et al., 1999). Thus, the tracking method can be fast enough to measure motion inside the cell, if enough photons are collected from the particles. In principle, faster circular scanning can also be used, if the particles are bright enough to be detected. This method of tracking using circular scanning of the laser beam can be extended easily to track in 3D. A piezo-scanner can be used to move the focus in the axial direction, while a circular scan is performed in the radial plane. Although it is more complicated, both the FFT calculation and the intensity trace can be calculated for this 3D orbit. We have discussed only the 2D case in this report.

If particles are immobilized, tracking can be used to center on top of them, and the distance measurement of the immobilized spheres can be realized by the circular scanning of the excitation beam with fixed center. The position of the particles can be recovered or calculated later from the saved intensity data. As the center of the scanning does not move, any number of periods can be added in a cycle for distance calculation. The first step of the analysis was the intensity pseudoinage representation of the recorded intensity data, showing important basic information about the system. Different immobilized particles appear as vertical intensity maxima at defined positions around the circular path. From the number of the vertical stripes, we can clearly see if one or more particles were present. Eventual photobleaching appears as fading of the intensity in time in the corresponding vertical stripe, its rate can be calculated by fitting the intensity change by exponential decay. Leaking intensity from the green channel into the red can be observed also and quantified. A different number of periods can be added in a cycle (time resolution changed in the vertical scale) for the analysis to achieve a sufficient number of photons for position determination with required accuracy. Large-scale motions could be seen as horizontal change if the sphere moved around the circular path (e.g., during the tracking because the center of scanning is moved, not shown). In the case of the single molecule measurement, stepwise bleaching and blinking can be seen easily from the pseudoinage representation, and measurement can be divided into different sections for analysis. From this simple representation of the recorded data, important features of the measured system can be seen by visual examination on a smaller scale than in the raster scanned image. Although we do

not get a detailed image of the surface, by circular scanning we select the most important information for the position and distance determination. In fact, the detailed image would be taken from at least $1 \mu\text{m}^2$ area where only a small number of points would sample the PSF close to the steepest and most sensitive region, and much time would be spent measuring, for example, the background in the corners. By circular scanning, a sufficient number of photons for required accuracy can be detected in a shorter time, and more effectively.

Fit of the intensities by model function was performed cycle by cycle using the Levenberg-Marquart algorithm. The accuracy was shown to be shot noise limited. On the longer time scales (20 seconds), the mechanical drift of the system affected the accuracy of the position determination and the relative distance. Distances recovered by the colocalization from the raster images or by the FFT method during tracking are in good agreement; however, both are distorted due to the leakage between the two channels. Fitting the intensity traces with the model function gives more accurate distances. In the present setup, we could achieve 4-nm accuracy in the distance determination in 200-ms cycles with the 100-nm fluorospheres. Other methods for localization or tracking of fluorescent particles have a similar accuracy of the position and distance determination. In the colocalization method of Lacoste et al. (2000), for example, 9-nm accuracy was achieved for 40-nm fluorescent beads and 13 nm for quantum dots using one photon excitation.

We discussed some important sources of the systematic errors that originate in the alignment and the calibration procedure. Attention has to be paid to the proper alignment of the microscope. All the distances are calculated basically in the units of the width of the PSF, therefore, all the changes in the size of the PSF affect the result. Calibration of the PSF width has to be accurate and carefully performed before the measurements. Everything that changes the PSF shape has an effect also on the result of the measurement. For example, if the excitation spot is elongated, an off-centered particle will appear twice around the circular period, as the tail of the PSF can reach the particle from the opposite side of the orbit also, and in the intensity fit every particle appears doubled. In principle, fitting by the intensity trace calculated using the real shape of the PSF eliminates this problem.

Single molecule sensitivity of the method was demonstrated by measuring the distances between dyes immobilized on the surface. On the order of 6,000 counts were collected from one molecule in 10–30 seconds. Of course, when measuring single fluorescent dye molecules, to achieve higher accuracy we must acquire data for a longer time, limited by the bleaching rate. The total number of photons detected would correspond to a maximum accuracy of 5 nm in distance determination. However, the recovered position of the dyes was not near the center of the scanning, as it was assumed in the position error calculation. Actually, the dyes were very far from the center, out from the orbit, which caused a larger error. In this case, the positions were determined with the errors in the order of 50 nm. The error could be reduced by using brighter particles, as quantum dots or silicon nanoparticles, and by reducing bleaching by deoxygenation or using more effective data

acquisition by avalanche photodiodes. The bleaching rate in two-photon excitation is usually higher than in one-photon measurement, but the background is reduced (Sanchez et al., 1997; Sonnleitner et al., 1999).

CONCLUSIONS

We have shown so far that the localization by tracking and the distance measurement of the fluorescent particles immobilized on the surface by the circular scanning of the excitation beam is a fast, sensitive method, and can be applied also for single molecule measurements. Our method builds on the established methods for determining the center of mass of a system of fluorescent molecules and on fitting methods, which are based on the accurate determination of the PSF. We have constructed a practical device that can be implemented on most multiphoton microscopes, widely used in biology. The idea of using the rapid circular scanning increases the SNR because of the synchronous detection and, in addition, all the data are collected in the region of the maximum sensitivity of the PSF. The method can be used both in the solution and in the cellular environment to localize fluorescent particles and measure distances between them with nanometer accuracy. Application to the biological systems is in progress and will be the topic of a separate publication.

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