High-resolution axial and lateral position sensing using two-photon excitation of fluorophores by a continuous-wave Nd:YAG laser

Ernst-Ludwig Florin, J. K. Heinrich Hörber, and Ernst H. K. Stelzer[a)]
Cell Biophysics Programme, European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, Postfach 10.2209 D-69117 Heidelberg, Germany

(Received 2 January 1996; accepted for publication 14 May 1996)

The change in position of fluorescent beads captured inside the focal volume of optical tweezers is monitored using fluorescence emission induced by two-photon absorption of a continuous-wave Nd:YAG laser ($\lambda = 1064$ nm). The displacement of a bead due to interactions with its environment leads to a fluorescence intensity variation that is used to design a novel spatial sensor. We determine changes in the axial position of a CY3-labeled latex bead with a diameter of 1.03 μm to a precision better than 10 nm. At an intensity of 600 mW/ μm^2 the two-photon bleaching rate is lower than 50% per 2000 s. © *1996 American Institute of Physics.* [S0003-6951(96)00630-4]

Optical tweezers are based on the interaction of a laser beam with dielectric objects.[1] The tweezers generate a force field with components pointing towards the optical axis and in some locations towards the direction of the incoming beam of light.[2] Particles can, therefore, be trapped laterally and axially. Recently, it has been shown that the continuous-wave (cw) laser power in an optical trap is sufficient to induce a two-photon absorption process.[3]

In this letter we show that the position of a fluorescently labeled latex bead (the probe) relative to the focus of a beam (as defined by geometrical optics) can be monitored by the fluorescence emission intensity. The emitted intensity is highest when the object is close to the focus and decreases as the probe's distance from the focus increases. The laser beam of the optical tweezers then serves two purposes: (a) it determines the position of the probe with respect to the geometrical focus as the origin of an inertial reference frame, and (b) it induces the emission of a signal that is proportional to the probe's position in the coordinates of the inertial reference frame. When moving the optical trap the bead will follow. However, mechanical interaction of the beads with objects in its microscopic neighborhood can prevent the bead from following the movement of the laser beam. The change in its position (relative to the moving focus) results in a corresponding intensity change and is recorded as a function of the position of the focus. Our setup is, therefore, meant to be used as a sensitive surface probing device. The experiments presented in this letter serve two purposes: first they demonstrate that a two-photon absorption phenomenum occurs; second they show how the fluorescence emission could be applied to measure the elastic properties of soft biological material such as neuronal cells.

The instrument is built around an inverted microscope (Axiovert 35, Carl Zeiss, Oberkochen, Germany) equipped with a Plan-Neofluar 100×1.3 (Carl Zeiss) microscope objective lens (Fig. 1). A computer controls the power of a Nd:YAG laser (DPY421 QII, Adlas, Lübeck, Germany) and the angular positions of two orthogonally oriented scan mirrors (040E, LSK, Stallikon, Switzerland) that move the focus laterally in the object plane. The movement along the optical axis is achieved by a piezo-ceramic device (P-841.40, PI, Waldbronn, Germany) with an internal feedback system that moves the sample stage only. A photo diode mounted behind a deflecting mirror is used to monitor the laser power. The movement of the bead can be observed using standard microscope video equipment. The fluorescence intensities have been measured in photon-counting mode with a photomultiplier (R2949, Hamamatsu, Japan), a photon-counting unit (C3866, Hamamatsu) and a gated counter (EMBL). Fluorescence emission spectra were collected by replacing the photomultiplier with the fiber bundle entrance of a CCD-based spectrophotometer (Intraspec IV, Oriel, U.S.). CY3-labeled latex beads (1.03 μm, L5282, Molecular Probes) were diluted in 1% sodium azide in water and always freshly prepared. The aqueous medium with the beads was mounted between two cover slips held in a tight aluminum frame. The neurons were cultured as described elsewhere.[4]

To characterize the process responsible for the signal from the probe, a single fluorescent latex bead was captured by optical tweezers and kept for the duration of the experiment. The fluorescence emission spectrum (data not shown) was recorded during a period of 5 s. The laser power was approximately 480 mW in the focal plane. The spectral shape is identical to a typical spectrum found by excitation of CY3 (absorption and emission maxima at 530 and 565 nm, respectively). In Fig. 2 the emission intensity is displayed as a function of the excitation intensity in a log–log plot. It summarizes the data of two experiments in which the excitation intensity was first increased and then decreased. The line indicates a fit assuming a power dependence resulting in the exponent 2.01. The crosses indicate the experimental data. The error is less than 1% in the upper right-hand corner and 2%–3% in the lower left-hand corner. To evaluate dye consumption (i.e., bleaching rate) the fluorescence emission of a single latex bead was monitored for more than 20 min. The fluorescence intensity dropped at a rate of 50%/2000 s. The laser power was again 480 mW in the focal plane.

Figure 3 documents an experiment in which a single fluorescent latex bead was captured and first pressed against a cover slip and afterwards against a neuron cultivated close by on the same cover slip. The fluorescence emission intensity was recorded as a function of the focus displacement

[a)]Electronic mail: stelzer@embl-heidelberg.de

 0003-6951/96/69(4)/446/3/$10.00

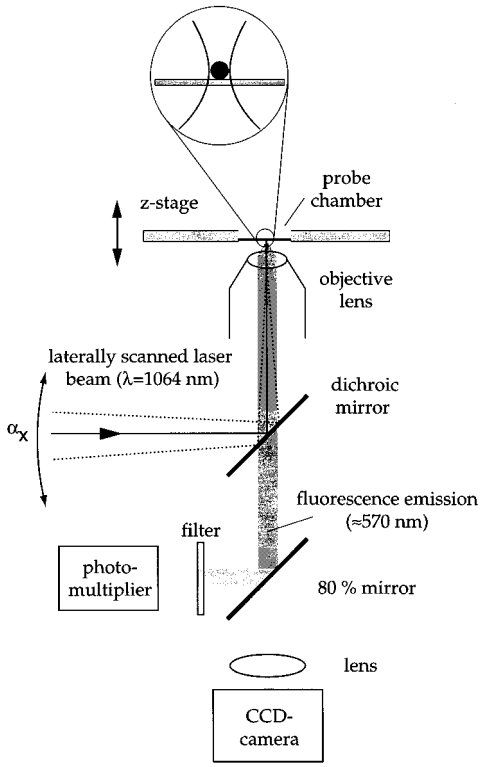

FIG. 1. A dichroic mirror directs the laser beam into a microscope objective lens that focuses the light onto a water-immersed fluorophore-labeled latex bead. The light is used to move the bead and to excite the fluorophore in the latex bead. A CCD camera provides a view of the probe as it is moved inside the chamber, and a photomultiplier detects the fluorescence emission. The filter discriminates against the excitation light and is removed when the spectrometer is used instead of the photomultiplier. Not shown is the scan unit that moves the laser beam of the optical tweezers laterally.

along the optical axis. Initially (left-hand side in Fig. 3), the bead had not touched the surface and the intensity increases as the spherical aberration decreased towards the water glass interface.[5] As the bead came in contact with the cover glass surface (+++), it was pushed away from the focal volume and the intensity decreased. The sharp initial edge indicates that the response of the bead to the hard cover glass surface was immediate. The response to the biological material of the neuron (○○○) was delayed, but after squeezing the cell for 150 nm both curves became linear. The slope of the intensity response caused by pressing the bead against the glass surface was 98%/μm while the slope of the response to the neuron was 85%/μm. Similar shapes are known from atomic force microscopy (AFM) experiments on platelets.[6] Both experiments were performed repeatedly at a speed of about 10 s per scan. Each point corresponded to an average of 500 count rates each recorded for a period of 1 ms and achieving values as high as 3000 photons. The error bars for the intensity are smaller than the marker size: 2%–3% in the approach to the surface and less than 2% in the linear part.

The spectrum is identical to the fluorescence emission spectrum of CY3 and its intensity is proportional to the square of the excitation intensity. The data acquired in the experiments, therefore, proves that the system is sensitive to the fluorescence emission induced by a two-photon absorption process. This process, thus, confines the volume as in a

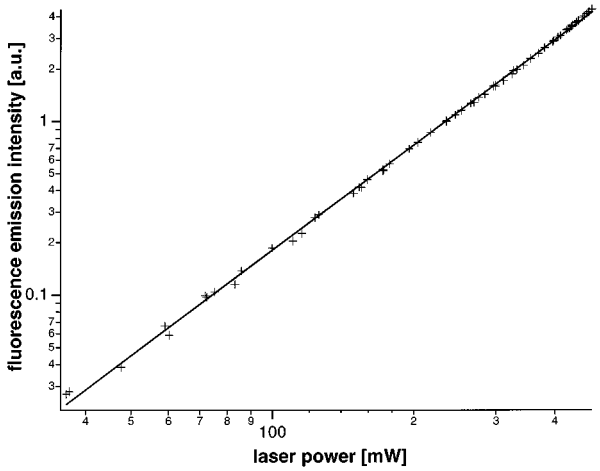

FIG. 2. Emission intensity recorded as a function of the excitation light intensity. The experiment was performed after a fluorescently labeled latex bead with a diameter of 1.03 μm had been captured by the optical tweezers. The excitation power in the focal plane was estimated to be 480 mW.

confocal fluorescence microscope based on a two-photon absorption process.[7,8]

Resolution is correctly defined as the distance between two point objects or two infinitely thin lines and a certain contrast. In our instrument we only have a single point and we quantify how well we determine the position of a bead, i.e., where the bead has moved in the inertial reference frame defined by the laser beam. Unlike resolution, the position can (at least in principle) be determined with an infinitely high precision. In practice, this value is only limited by the signal-to-noise-ratio. In this arrangement it is closely related to the photon counting rate and finally limited by the rate at which fluorophores are consumed. However, even at a very high-laser power the bleaching rate is so low that it will not affect a precise position determination.

From the data shown in Fig. 2 we know that the intensity can be easily measured with an accuracy of better than 1%. Since the axial position sensitivity as shown in Fig. 3 is 98%/μm we conclude that the position along the optical axis

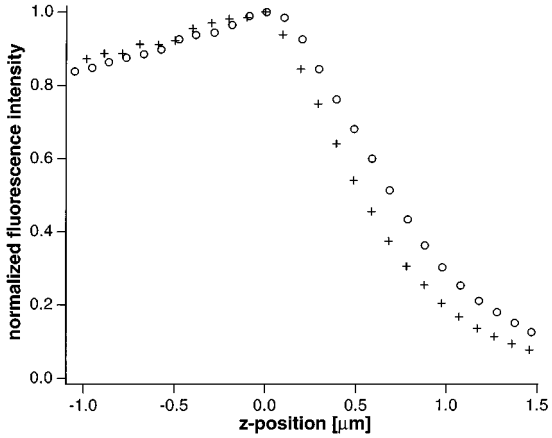

FIG. 3. The fluorescence emission of a bead captured by optical tweezers and monitored as a function of the relative axial position to the cover glass surface. Negative values indicate a position above the specimen (i.e., in solution). (+++) The probe approaches the cover slip. (○○○) The probe approaches the soft biological material in neurons on the same cover slip.

can be determined with an accuracy of better than 10 nm. In another set of experiments (data not shown) we determined an up to three times higher lateral position sensitivity that agrees with other experiments and theoretical evaluations.[8]

The fact that the two curves in Fig. 3 have different slopes can be interpreted as the elastic response of the plasma membrane (including bilayer, cytoskeleton, etc.) of the neuronal cell. Assuming that both the optical tweezers and the plasma membrane act as hook springs we conclude that (under these conditions) the spring constant of the plasma membrane in neurons is 6–10 times the spring constant of the optical tweezers.

If we wish to produce an image that reflects the object height above a reference position, the intensity has to be recorded at each point in the object. This is simplified by the fact that the intensity is linear with the position. A careful evaluation shows that an image of size 500×500 will be recorded within 5 min at an axial resolution of 10 nm and within 1/2 min at an axial resolution of 30 nm.

Traditional scanning probe microscopes (e.g., AFM) require a direct mechanical connection between the tip and the actuator.[9] This makes them most useful for the observation of surfaces. The technique outlined in this manuscript requires no direct connection and can, therefore, be used in all three dimensions inside transparent objects. In the approach of the probe to the neuron, the actuator passed the cell's cytoplasm. During the entire experiment the neuron was kept alive in the same sterile, temperature-controlled environment used during the observation in a video microscope and for various biochemical experiments,[4] i.e., under favorable physiological conditions. Another distinct advantage is that the regime of forces is at least one, maybe even two orders of magnitudes lower than in AFMs and, therefore, probably better suited for a completely different range of force measurements required for complex biological systems.

We conclude that the novel technique described in this letter will become a major tool for the analysis of forces in the pN-regime as well as a three-dimensional microscope for applications in which an excellent axial and lateral resolution are required.

The authors thank S. Albrecht, M. Hartung, T. Wohland, and S. Lindek for helping us with the spectrometer and the optical tweezers and for their critical comments on the manuscript. The neuron was kindly provided by A. Pralle, L. Meyn, and C. Dotti.

[1] A. Ashkin, J. M. Dziedzic, J. E. Bjorkholm, and S. Chu, Opt. Lett. **11**, 288 (1986).
[2] A. Ashkin, Biophys. J. **61**, 569 (1992).
[3] J. Liu, G. J. Sonek, M. W. Berns, K. Konig, and B. J. Tromberg, Opt. Lett. **20**, 2246 (1995).
[4] K. Goslin and G. Banker, *Culturing Nerve Cells*, edited by G. Banker and K. Goslin (MIT, Cambridge, MA, 1991), pp. 251–282.
[5] S. Hell, G. Reiner, C. Cremer, and E. H. K. Stelzer, J. Microsc. **169**, 391 (1993).
[6] M. Radmacher, M. Fritz, C. M. Kacher, J. P. Cleveland, and P. K. Hansma, Biophys. J. **70**, 556 (1996).
[7] W. Denk, J. H. Strickler, and W. W. Webb, Science **248**, 73 (1990).
[8] E. H. K. Stelzer, S. Hell, S. Lindek, R. Stricker, R. Pick, C. Storz, G. Ritter, and N. Salmon, Opt. Commun. **104**, 223 (1994).
[9] G. Binnig, C. F. Quate, and Ch. Gerber, Phys. Rev. Lett. **12**, 930 (1986).