Three-dimensional thermal noise imaging

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We present a scanning probe microscope based on optical tweezers for three-dimensional imaging of the topology of transparent material in the nanometer range. A spherical nanoparticle serves as a probe. An optical trap moves it through the sample (e.g., a polymer network), while the position of the particle center is recorded by three-dimensional interferometry. Accessible volumes are reconstructed from the histogram of thermal position fluctuations of the particle. The resolution in determining the position of surfaces in three dimensions is about 20 nm. © *2001 American Institute of Physics.* [DOI: 10.1063/1.1423404]

Conventional scanning probe microscopes (SPM), such as the scanning tunneling microscope (STM),[1] the scanning force microscope (SFM),[2] or the scanning near-field optical microscope (SNOM)[3,4] achieve their extraordinary lateral resolution by a precise positioning of the probe (STM tip, AFM tip, or optical fiber) at the sample surface. An image is acquired by scanning the probe across the surface in a predefined pattern. The precise lateral positioning is achieved by a stiff physical connection of both the probe and the sample with the microscope frame. However, it is exactly this connection that prevents them from probing three-dimensional objects such as polymer network or the interior of a live cell. In transparent material, the physical connection can be replaced by optical tweezers that stably trap a dielectric particle, which serves as a probe.

The first attempts to build such a scanning probe microscope based on optical tweezers were reported by Malmquist *et al.*[5] A silica nanosphere in the vicinity of a pair of carbon fibers was used as a light source and the light scattered from the object was recorded. Ghislain *et al.* used an optically trapped prolate glass stylus to record height profiles on a photoresist surface.[6] The first two-dimensional images from neuronal processes were recorded by Florin *et al.*[7] using a 200 nm fluorescently labeled latex bead and the two-photon excitation process.

Thermal fluctuations of the probe were discussed from the beginning as the limiting factor for the resolution of optical tweezers based scanning probe microscopes.[5] The reason is that typical force constants acting on the probe (k_{OT}) are three orders of magnitude smaller than those of soft SFM cantilevers (k_{SFM}) and allow for large position fluctuations. The magnitude of thermal position fluctuations for a harmonically bound particle as a function of the spring constant k can be estimated from the equipartition theorem $\langle \Delta x \rangle = \sqrt{k_B T/k}$, where $k_B T$ denotes the thermal energy. Whereas thermal excitations at room temperature lead to Å size position fluctuations of the SFM cantilever ($k_{\mathrm{AFM}}=60$ mN/m) perpendicular to the surface, the fluctuations of the probe in an optical trap reach tens to hundreds of nanometers along all three dimensions. Thus the particle probes an entire contact area instead of a single point for each position of the optical trap. Probing three-dimensional objects with a conventional raster scanning strategy therefore leads to a mediocre spatial resolution.

In this letter we describe a scanning probe microscope based on optical tweezers that uses the thermal position fluctuations of a nanosphere in the optical trap to scan objects in three dimensions. The function of the optical tweezers is to restrict the Brownian movement of the probe to the volume element under investigation, and to manipulate its coarse position in the sample. The probe fluctuations are recorded with nanometer spatial and microsecond temporal resolution, and the scanned objects are reconstructed from three-dimensional position histograms of the position fluctuations.

To demonstrate the principle of the microscope, we imaged an agar gel (Bacto-Agar, Difco). These gels are widely used in cell biology and molecular biology as substrates for cell culture, bacterial cultures, and chromatography. Agar is a semisolid with large pores, it is transparent in the visible and the near-infrared range and can be prepared under various buffer conditions.

Our samples were prepared at a concentration of 0.25 wt/vol by dissolving the agar powder in bidistilled water and heating it at 90–100 °C for half an hour. The gel was then stored in a refrigerator at 4 °C for later use. Prior to an experiment, the agar gel was boiled in a microwave oven for 30 s. This ensured that the agar was completely in the fluid phase. Latex beads (Fluospheres, L-5242, Molecular Probes) with a nominal diameter of 216 ± 8 nm were added to the fluid at a concentration of about one bead per ($20\times20\times20$ $\mu\mathrm{m}^3$). This low concentration was used to avoid optical trapping of multiple beads. The fluid was immediately filled into the sample chamber. The agar gel solidifies within minutes at room temperature (23 °C). The experiments were performed with the photonic force microscope (PFM),[7] a scanning force microscope based on optical tweezers with a three-dimensional position detector for the trapped particle.[8] A 1064 nm laser beam (IRCL-500-1064S, CrystaLaser, USA) is coupled into a custom-built inverted microscope frame (EMBL, Germany) (Fig. 1). The laser power in the focal spot was estimated to be between 30 and 50 mW, which is sufficient to trap 200 nm latex beads at a distance of about 2 μm

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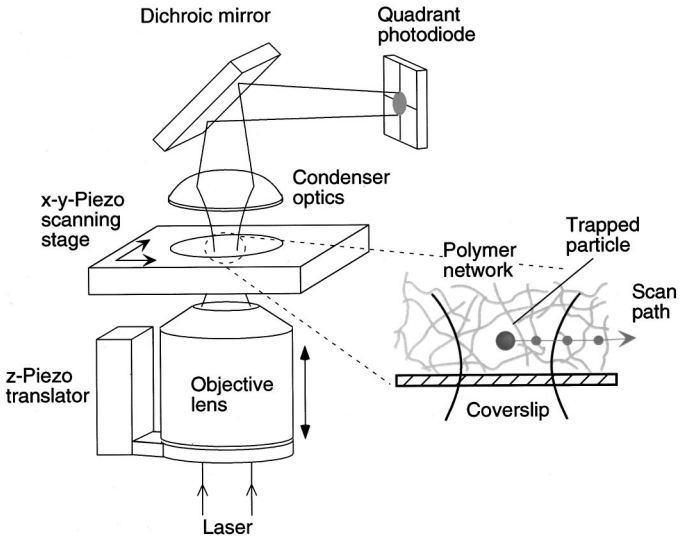

FIG. 1. Experimental setup and procedure. To image a part of an agar network, a 216 nm latex bead is trapped by optical tweezers at low laser power and then scanned stepwise through the network. The dots on the scan path indicate the different energy minimum position of the optical trap around which the bead fluctuates and probes its local environment.

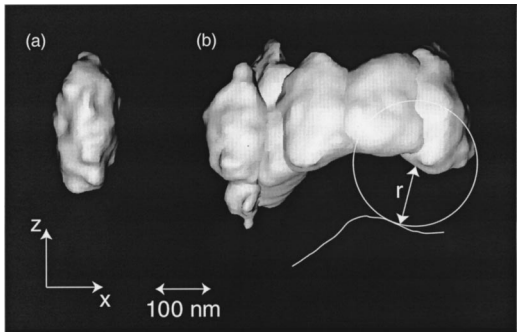

FIG. 2. Frequency isosurfaces of 5 counts per voxel of a 216 nm bead trapped by optical tweezers in solution (a) and in an agar network (b). The position of the trap was laterally scanned in the network with a stepwidth of 80 nm. The object's surface (white line) was calculated taking the radius of the bead into account (white circle). The data acquisition time at each position was 0.8 s.

above the cover slip. The trap is positioned along the optical axis by a piezotranslator (PiFoc, Physik Instrumente, Germany) and in the x, y plane by a piezoscanning stage (NPS-xy-100A and NPS 3220, Queensgate Instruments, UK). Both have a precision better than 1 nm. To measure the three-dimensional position of the particle inside the trap, the forward scattered laser light from the particle and the unscattered laser light are collected by a condenser lens and projected onto a quadrant photodiode (QPD) (S5981, Hamamatsu, Herrsching, Germany) where they generate an interference pattern. For small displacements, the differential signals from the QPD are proportional to the lateral displacement of the particle inside the trap and the sum signal is proportional to the axial displacement.[8] This detection scheme allows us to measure the position of the bead center with nanometer spatial and microsecond temporal resolution.

A particle trapped by optical tweezers explores the trapping potential by thermal fluctuations. The probability of finding the particle at a given volume element in an optical trap is described by the Boltzmann distribution.[9] The distribution can be measured by recording the position of the particle over a time interval and calculating a position histogram. The maximal energy level explored depends on the total recording time and the position autocorrelation time.[10] Typical levels are on the order of several k_BT. Figure 2(a) shows a frequency isosurface of a position histogram at a level of five counts per voxel for a 216 nm bead. The center position of the bead was recorded for 0.8 s with a data acquisition rate of 100 kHz and the histogram was calculated with a binwidth of 8 nm.

The bead explored a volume of about ± 60 nm in the lateral and about ± 150 nm in the axial (z) direction. The volume is elongated along the z axis because single beam gradient traps have smaller force constants along the optical axis than within the object plane. Its roughness reflects the counting statistics in the histogram at low frequencies.

This experiment demonstrates an important point: The bead explores a volume of about $300\times120\times120$ nm^3 at one trap position. Thus thermal position fluctuations can be regarded as three-dimensional ''natural scanners'' for the bead position, which replace the point-by-point scanning of piezotranslators in conventional scanning probe microscopes and provide the natural extension into three dimensions. Objects brought into the probe volume will be randomly probed from all directions. Large objects can be explored by moving the central trap position around the object. The presence of an object restricts the volume accessible to the bead, which is shown in Fig. 2(b). In this experiment, the bead was captured in the sample, an agar network, and the optical trap was then moved in 80 nm steps along the x axis. The thermal position fluctuations were recorded for 0.8 s at each trap position. The corresponding frequency isosurfaces show a clear constraint at their lower end, which results from the interaction of the bead with an agar filament. This surface is the result of a convolution of the bead with the object. The actual position of the object's surface has to be calculated taking the radius of the bead into account [white line and circle in Fig. 2(b)].

To probe larger volumes, the optical trap was scanned stepwise with a stepwidth of 80 nm along the x and y axes, and a stepwidth of 160 nm along the z axis. $8\times8\times2$ steps were scanned corresponding to a probed volume of $600\times600\times300$ nm^3. Three-dimensional histograms with a binwidth of 8 nm were calculated from the fluctuation recordings at each trap position and then combined to a single histogram by correcting for their relative displacement.

The chosen stepwidth in combination with the data acquisition rate and time ensured that the bead would have visited each voxel at least 15 times if there was no object. According to Poisson statistics, the probability of finding the particle only 5 times in a given voxel by statistical fluctuations is below 1% under the given experimental conditions. Therefore, a frequency isosurface at 5 counts per voxel is a sensitive indicator for the presence of an object.

Figure 3 shows the frequency isosurface of the position histogram recorded in the agar network. The volume within the isosurface represents the volume accessible to the bead during the scan. The volume elements not accessible to the bead (channels), indicated that two agar filaments (af1 and af2) crossed the probed volume and a third filament (af3) touched it at the border.

The minimal diameter of the channels [Fig. 3(a)] or the

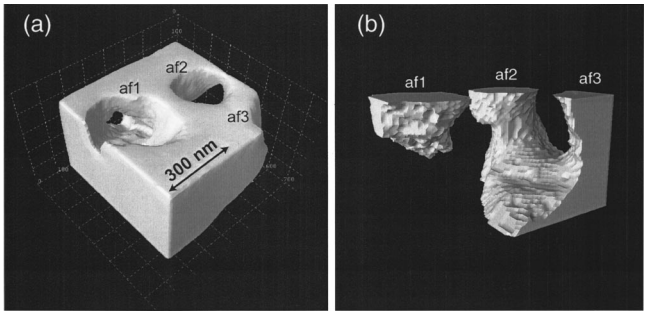

FIG. 3. 3D thermal noise image of an agar network. The volume accessible to the bead fluctuations is within the frequency isosurface (a). Volumes elements which are not accessible because of the presence of agar filaments appear either as channels (af1 and af2) or as additional constraint of the overall volume (af3). The inverse image (b) represents the volume not accessible to the bead.

objects [Fig. 3(b)] is about the diameter of the bead (216 nm) and demonstrates a mechanical amplification effect that results from the probe-sample convolution (Fig. 4). When a bead probes a mechanically stiff filamentous object with a diameter d_{object}, then the radius of the cylinder in the three-dimensional scanning probe image is at least $r_{\text{bead}}+r_{\text{object}}$. Consequently, even a molecular thin filament will appear at least with diameter of the bead. The actual diameter of the agar filaments has to be on the molecular scale, as expected from electron microscope studies.[11] Thus, one would expect considerable thermal position fluctuations of the filaments as well. These object fluctuations lead to a softening of the bead–object interface and thus allow channel diameters smaller than the diameter of the bead. This effect was indeed found in all images recorded (data not shown).

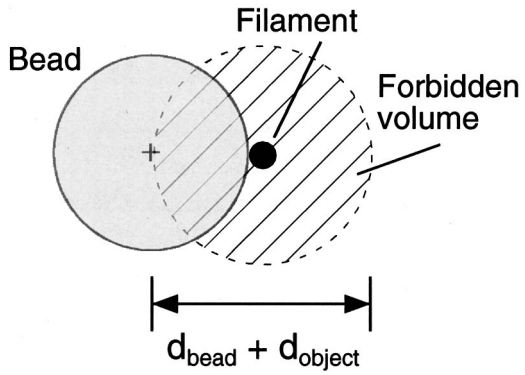

FIG. 4. Mechanical amplification effect. An mechanical stiff filamentous object with a diameter d_{object} will appear in a thermal noise scan as a cylinder with a minimal diameter of $d_{\text{min}}=d_{\text{bead}}+d_{\text{object}}$.

Several factors determine the resolution in three-dimensional scanning probe microscopy. The most important one is the diameter of the bead. Only cavities with openings larger than the bead diameter can be explored. A second factor that determines the resolution is the number of position measurements for a certain volume, because the number of measurements determines the meaningful binning of the position histogram and thus the resolution of the image, i.e., how well the position of object surfaces can be determined. We calculated this resolution from the roughness of the frequency isosurfaces in Fig. 3 to be about 20 nm. The precision in measuring the position of the probe plays only a minor role, because here we achieve a nanometer or better, which is superior in precision to the binwidth of the position histograms. Finally, as in conventional scanning force microscopy, the contact area between the probe and the sample, which depends on the radius of the probe and the properties of the sample such as its mechanical stiffness, contributes to the resolution.

The speed of thermal noise imaging depends on the viscous drag on the probe particle, which is proportional to the bead diameter. Therefore, smaller probe particles will increase both, the imaging speed and the resolution in thermal noise imaging. The smallest beads we could stably trap were $\approx$30 nm gold beads.

In summary, we described a three-dimensional scanning force microscope based on optical tweezers. We demonstrated that thermal noise imaging solves the key problem of Brownian motion of the probe in the optical trap. Moreover, we demonstrated that Brownian motion of the probe is an efficient ''natural'' 3D scanner on the nanometer scale.

In addition to applications in life sciences, we expect 3D scanning probe microscopy to also become a general method in various areas of physics such as soft matter physics.

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