



Single Molecule Probe Scanning Optical Force Imaging Microscope for Viewing Live Cells

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Abstract. We have developed an imaging system that combines the soft compliance of an optical trap with the sensitivity of single particle tracking to image forces on/in live cells using a single molecule probe. The probe used is a single (or few) molecule of interest that is conjugated with a single 40 nm colloidal gold probe. The colloidal gold/membrane protein complex, freely diffusing on a live cell, is held in a laser trap while the cell is scanned underneath. Computer control allows for synchronization of the cell scan and capture of the probe position. Resistance to the dragging of the probe images a fine structure of barriers in the membrane of live cells.

1. Introduction

Since the advent of scanning force microscopy, the desire for precise and ultrasensitive methods of force imaging has increased. Initial success of such techniques as atomic force microscopy [1] in imaging hard samples drove research toward softer, biological samples (see for example [2, 3]). The complication of using AFM with its stiff (on a biological scale) cantilever is that the probe quickly overwhelms a live cell and thus the soft details are lost (or the sample must be pre-fixed and is thus dead at the time of imaging). The force required to extend a soft polymer such as the red blood cell structural protein, spectrin, to its full length can be less than 20 pN with a nominal spring constant of $k_B T / (b * L) = 0.004$ pN/nm (for spectrin of length, $L = 200$ nm and persistence length, $b = 5$ nm, $k_B T$ is the thermal energy), and forces less than 1 pN can displace a lipid bilayer under low tension. The stiffness of current commercial cantilevers is on the order of 1–10 pN/nm and thus cannot easily sense the soft biological samples without strongly disturbing them. As such, a technique with a softer touch is desired for live cell imaging studies.

The technique of optically trapping colloidal objects in a highly focused laser beam has the required soft spring like characteristics. Optical trapping of micron-sized dielectric spheres was first demonstrated by Ashkin [4]. Small metallic particles, of a diameter less than the wavelength of light can also be trapped at a reduced trapping potential. The trapping potential has, generally, a spring constant of approximately 0.001–0.005 pN/nm, for 40 nm diameter gold particles (for the

laser powers and setup we use). This allows for a compliance matching between the probe and the sample.

It has also been shown that small gold particles could be conjugated to single proteins in a live cell outer membrane and viewed through contrast enhanced bright-field video microscopy [5, 6]. This technique, known as single particle tracking (SPT) allowed direct observation of the movement of single (or small numbers of) molecules in the membrane of live cells. Tracking of the colloidal particle/membrane molecule complex is accomplished through a cross-correlation between an initial image of the particle (the kernel) and the subsequent captured frames from the video of the trajectory, following the method of Gelles et al. [7]. As such, the complexity of the membrane, which classically was expected to be a simple 2-dimensional fluid with freely floating imbedded proteins (the *fluid mosaic model* of Singer and Nicolson [8]), begun to be probed. The membrane was shown to contain a complex compartmentalized structure due to both the actin scaffolding (spectrin in erythrocytes) and lipid-based domains, see Figure 1a.

A combination of the techniques of optical trapping and single particle tracking has been used to look in depth into the forces applied to a single protein in the membrane of a live cell [9, 10]. The stiffness of the actin cytoskeleton has been estimated at 0.001–0.01 pN/nm by this method, making actin network behave as a weak spring. Also, previous work in optical force imaging has been presented by the groups of Hörber [11] and Webb [12].

Here we have extended this combination of optical trapping and single particle tracking to develop a single molecule probe scanning force imaging microscope that has the position accuracy of single particle tracking, the soft compliance of optical tweezers and single molecule resolution due to the use of a 40 nm colloidal gold/membrane protein probe. The technique involves (see Figure 1b) trapping a *freely* diffusing membrane protein (that is, not one that is frozen due to direct binding to the cytoskeleton) conjugated to a single colloidal gold probe in an optical trap. The cell is then scanned below the trap and the resistance to dragging is measure through a lag of the probe behind the trap potential center. Scans are performed as a function of trap stiffness (in the range of 0.001–0.005 pN/nm depending on the laser power employed) and scan rate (0.5–2 $\mu\text{m/s}$).

2. Experimental Details

The setup is based around an Olympus IX-70 inverted microscope, as shown in Figure 2, where we have had the nosepiece raised to allow entrance of a both the epi-port beam for fluorescence observation and the trapping laser beam from the side. The trapping laser used is a ND:YVO₄ 4W Laser at 1064 nm wavelength (T20-B10-106Q, Spectra Physics, Palo Alto, CA). The infrared laser is used to minimize the visible light toxicity to the live cells. The trapping beam is optically expanded 5 \times (lens from Sigma Koki) before being steered into the back of the objective lens to overfill the entrance of the objective lens. Steering is accomplished

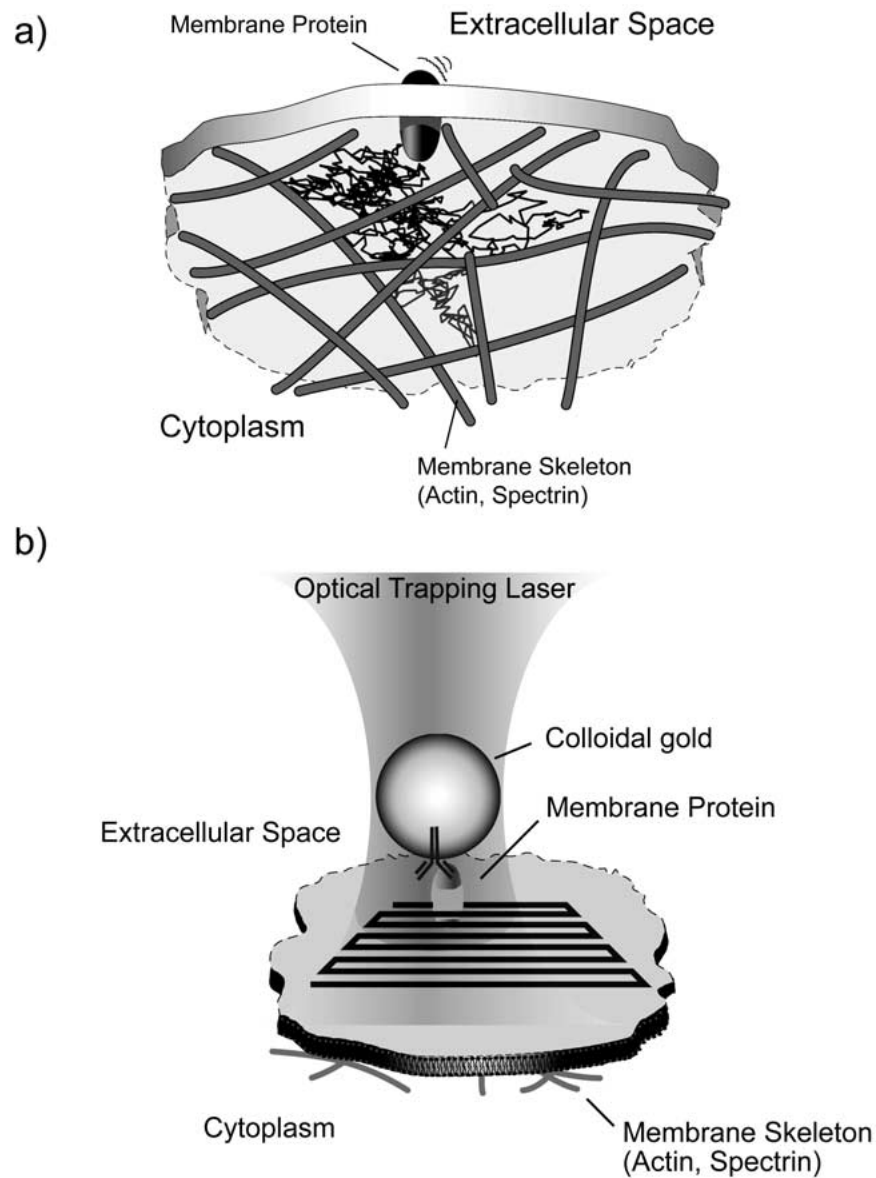


Figure 1. a) The cellular plasma membrane consists of a fluid lipid bilayer, which may be inhomogeneous, that contains embedded proteins. Some of these proteins span the bilayer, extending into both the intracellular and extracellular spaces. A fraction of these proteins are immobilized through attachment to the membrane cytoskeleton, the cytoplasmic structural scaffolding of the cell consisting mainly of actin and actin binding proteins, like spectrin. The close proximity of the skeleton network to the membrane presents steric barriers to free protein diffusion. Diffusion across the barrier occurs when the fence fluctuates out of the way (actin or membrane fluctuation due to thermal noise) or the fence opens (actin failure). b) To directly image the barriers, a membrane on a living cell protein tagged through a monoclonal antibody with a 40 nm colloidal gold particle is trapped in a laser optical tweezers while the cell is scanned underneath. Deviation in the probe from the center of the trap against the direction of scanning times the spring constant of the trap determines the force on the probe and is used to force image the barriers to free diffusion.

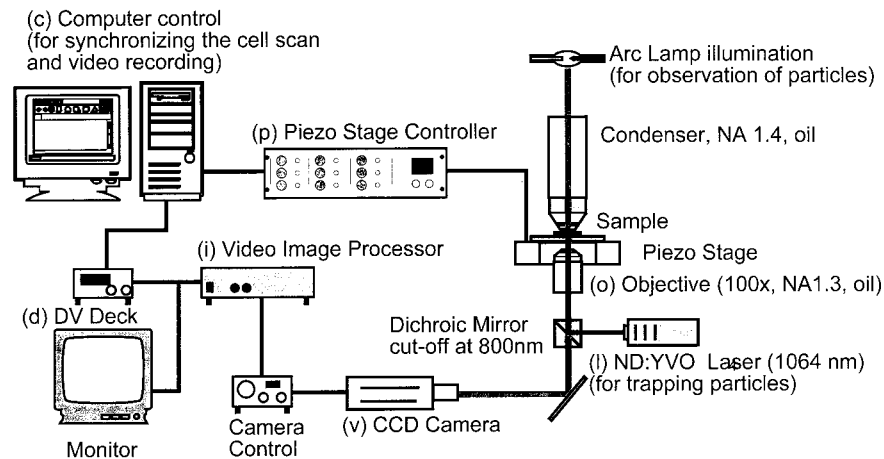


Figure 2. The Scanning Optical Force Imaging microscope consists of an Olympus IX-70 microscope modified to allow entrance of the trapping laser from the side. The laser beam (1064 nm) from a (1) Spectra Physics ND:YVO₄ laser was expanded 5 times and steered into the (o) objective lens (Zeiss 100× 1.3NA Plan Apochromat). Scanning of the (p) piezo stage (Physik Instrumente) and (v) video synchronization is (c) computer controlled. The image of the trapped probe is recorded through a (v) Hamamatsu CCD camera, contrast enhanced through an (i) Olympus XL-10 Image Processor and taped on (d) Digital Video (Sony) for later analysis.

by a pair of beam steering mirrors and a dichroic mirror (cutoff at 800 nm) in the microscope's optical path. The beam is focused into the object plane through adjustment of a micrometer actuator on the second expander lens.

Scanning of the cell beneath the focused beam is accomplished through computer control of a scanning piezo stage (Physik Instrumente, Germany). The stage has sub-nanometer resolution with full travel of 50 μm in closed loop operation.

The probe used is a colloidal gold particle of diameter 40 nm. A sparse number of ligand or monoclonal antibody (or its Fab fragment) is conjugated to the colloidal gold (British Biocell, Cardiff, UK) through physical adsorption above the pI of the protein. The small diameter helps in limiting the number of membrane molecules bound to the probe (as opposed to 200 nm or larger latex particles as are used by other groups [13, 14]). The colloid with the sparsely bound protein is stabilized by further addition of 20 kD polyethyleneglycol (PEG) polymer (Sigma). This also helps reduce the chance of nonspecific interactions between the gold and the cell surface.

Probe position was calculated from contrast enhanced bright field images taped on digital video for post experiment analysis (DSR-20, Sony). Synchronization of the piezo stage and video imaging is done through stripping the sync pulse from the video image with a LM1881 IC. The sync pulse is then used as the trigger for both the start of the computer controlled scan and the computer initiation of the video recorder.

Trap stiffness was calculated through the fluctuation amplitude. Such calculation requires observation of the gold particle in a time window shorter than a millisecond (assuming a trap width of ~ 500 nm and a diffusion rate for the gold particle of 10^{-7} cm²/s). As such we used a Photron Ultima 40 CMOS based camera to track a trapped but otherwise free floating single gold probe in the sample medium at framing rates of 4500 fps. Trap stiffness ranges from 0.001–0.004 pN/ μ m for the range of laser powers employed here.

Specifically, our test probe consisted of the membrane protein CD44 conjugated with a 40 nm colloidal gold particle. The gold particle was prepared by incubating the 40 nm colloidal gold with anti-CD44 monoclonal antibody (clone OX-50) at room temperature with shaking for 1 hour at a molar ratio of 0.044 (antibody/gold particle) to ensure monovalency. 20 kD PEG polymer was added to a final concentration of 0.5% (w/v) and incubated 15 minutes at room temperature with shaking to stabilize the colloidal and reduce nonspecific interactions. The gold/antibody solution was washed three times and finally resuspended in Hank's Balanced Salt Solution (HBSS) containing 0.5% (w/v) 20 kD PEG.

The cells used were Normal Rat Kidney (NRK) Fibroblasts grown in Ham's/F12 supplemented with 10% fetal bovine serum. Cells were used 24–48 hours after plating onto clean coverglass. Cells were washed in HBSS then 50 μ L of the antibody conjugated gold solution was added. The majority of gold solution was removed and excess HBSS with 0.5% 20 kD PEG. A sealed chamber was then made using 0.15 mm spacers and sealing with paraffin wax. Experiments were completed at room temperature within 30 minutes of sealing the chamber.

3. Results and Discussion

The membrane protein CD44 was employed as a probe to a live NRK cell. CD44 has a single transmembrane spanning helix with a small, 72 amino acid, cytoplasmic domain (for a review of the structure and function of CD44 see Lesley et al. [15]). This small intracellular domain is a probe that senses a thin slice under the membrane in a live, dynamic cell.

The free diffusion of a membrane protein can also report the fine structure of the membrane in live cells. The diffusion of our CD44/gold probe conjugate (prior to trapping by the laser) is no different. Shown in Figure 3 is the trajectory of a single probe over 1 sec shown at a time resolution of 500 Hz, in a live normal rat kidney (NRK) cell. The trajectory is grayed as a function of time to show the progression of the molecule (black to light gray). There is a reduction in D_{MACRO} , the long time diffusion coefficient (calculated from asymptote of the mean squared displacement (MSD) at long times) compared to D_{micro} , the short time diffusion coefficient (calculated from the mean squared displacement over 4–8 ms) that implies anomalous or *hop* diffusion. Specifically, it has been proposed that the membrane cytoskeleton *fence* structure corrals the protein during its random walk, thus allowing fast diffu-



Figure 3. Shown is the trajectory of a colloidal gold-tagged CD44 molecule in the plasma membrane of a live NRK cell. The trajectory was recorded at a frame rate of 500 frames/second (2 ms time resolution). The progression from black to light gray shows the time course. The changes in tone are chosen to show plausible locations of barriers to free diffusion.

sion on short time scales yet restricting long time travel through slow hops between corrals. The division in grayscale in Figure 3 was chosen to show plausible corrals. A full statistical analysis of the MSD– Δt plots [16] of the trajectories obtained imply that the corrals are approximately 660 nm in diameter on average and the CD44 protein escapes every 1.8 s on average.

To further explore the domain-like structure of the membrane, the CD44/gold conjugate was used as the probe for single molecule scanning probe imaging on live NRK cells. The small cytoplasmic domain is a probe that senses a thin slice under the membrane in a live cell. Figure 4 shows the result of a scan (forward scans only) across the cell over the same line repeatedly. Light areas imply a shear resistance of about 0.2 pN. The scanning rate was 1 $\mu\text{m/s}$ with a trap stiffness of 1.8 pN/ μm . The y -axis of Figure 4b shows the cycle number of repeated scanning and is equivalent to time. Figure 4a shows a single scan (the third of Figure 4b) where an array of high resistance barriers is seen with a separation on the order of 0.5 μm as expected from the free diffusion studies mentioned above.

The images formed through direct monitoring of the resistance to dragging show a periodic array of barriers. These barriers may correspond to direct imaging of the cytoskeletal network of actin fibers found at the internal lipid face. Also, note that the position of the barriers shift over time revealing the dynamics of the

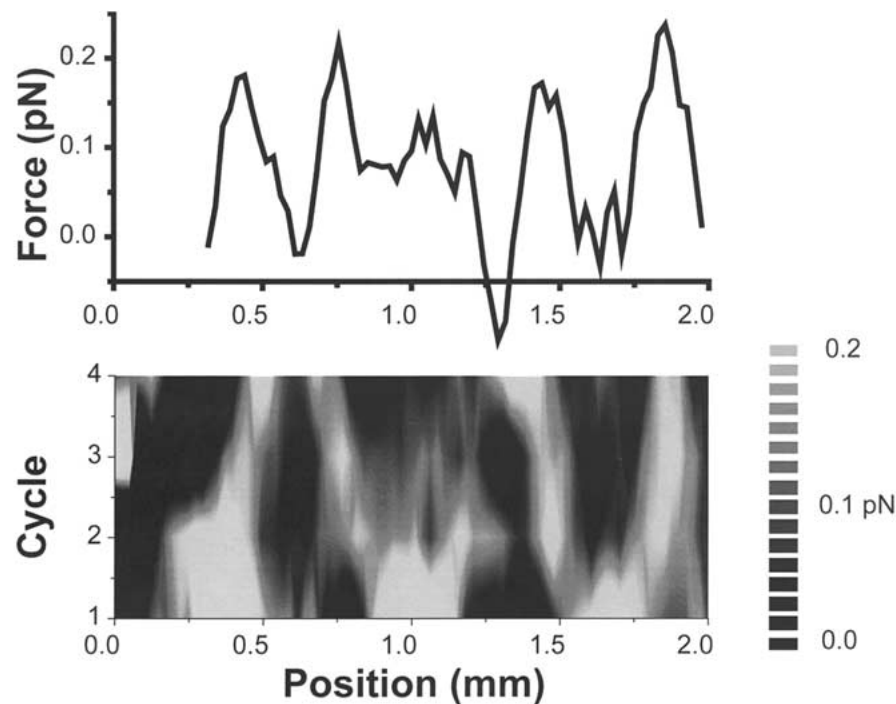


Figure 4. Repeated scanning of the same line. Shown are a) the force recorded along a single scan line as a function of time and b) four repeated scans presented as an image with the y-axis denoting the cycle. Note the array of high force regions (light regions, maximum force of 0.2 pN) implying barriers to free diffusion due to the cytoskeletal actin meshwork. Dynamics of the membrane skeleton is imaged over time. The upper plot shows the forces found along the third cycle. Scan length $2 \mu\text{m}$, scan speed 1.8 pN/s, 4 cycles, forward scans only.

membrane skeleton. We are currently following this line of research in both one- and two-dimensional scanning (to be published).

We have described an instrument with a compliance matched to the soft compliance of the cell, which can shear force image the membrane of living samples using a single molecule probe. Application of this to the fine structure and dynamics of the subsurface scaffolding of the plasma membrane is underway. As studies of live samples become more detailed we begin to understand the mechanisms of control being used in cell life. As such, we begin to get a handle on such important and vital processes as cell-cell adhesion and cell signaling, which are required for life.

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