Ultrastable combined atomic force and total internal fluorescence microscope

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Combining atomic force microscope (AFM) with other microscopy techniques has expanded the range of potential applications for single molecule investigations dramatically. Particularly hybrid instruments with total internal reflection fluorescence (TIRF) excitation have opened new routes in life sciences. Here we present a novel design for such a hybrid microscope, which overcomes the limitations of conventional combinations caused by their limited mechanical stability. A thorough analysis of the noise spectra and a comparison of the different designs and the different operation modes are given. With this instrument we demonstrate single molecule manipulation by AFM and simultaneous TIRF imaging. © *2009 American Institute of Physics.* [DOI: 10.1063/1.3148224]

I. INTRODUCTION

The atomic force microscope (AFM) has revolutionized nanoscience in many ways. It is now widely used in biophysics as a tool to image nanoscale structures, as well as to measure and apply forces in a well-defined manner.[1–3] While AFM techniques are very precise in terms of spatial and force resolution, even the fastest AFMs currently are orders of magnitude slower in terms of imaging compared to optical methods.[4] On the other hand traditional optical microscopy provides fast readout but is limited by diffraction. Therefore it is obvious that AFM and single molecule fluorescence microscopy are complementing each other in a favorable manner.[5–7] As the AFM is a tool to operate on surfaces, the choice of total internal reflection fluorescence (TIRF) as illumination source for single molecule microscopy is convenient. However positional noise of the sample on the nanometer length scale that could have been neglected in sole single molecule TIRF microscope setups become an issue. Such fluctuations hamper high-precision AFM measurements.

In previous studies we have investigated in great detail the effect of mechanical vibrations of the AFM on the measured unbinding forces of molecular complexes.[8] It is obvious that a fluctuating cantilever, but in the same way a fluctuating sample position, causes force fluctuations in the molecular complex under investigation, which add to the externally applied force. Since bond rupture is a nonequilibrium process, these fluctuations do not average out to zero but reduce the force, which has to be applied externally to break the bond. Particularly when stiff and short handles are used as linkers, fluctuation in the relative distance between tip and sample surface may lead to drastic artifacts.

In optical microscopy, such fluctuations, e.g., caused by vibrations of the microscope, stay often unnoticed since they are below the optical resolution limit. It is particularly the direct coupling of mechanical noise via the oil immersion, which is essential for TIRF, which causes severe problems. Also in high-resolution fluorescence techniques such as 4π-stimulated emission depletion (STED), photo-activated localization microscopy (PALM), or stochastic optical reconstruction microscopy (STORM) such fluctuations remain unnoticed, since their characteristic time typically lies above the integration time of the detectors and thus are averaged out. Not so in mechanical experiments, where such position fluctuations result in force fluctuations which may result in bond ruptures. To cope with this issue we analyzed potential sources of such fluctuations and as a consequence of our analysis developed a very compact microscope setup featuring a solid connection between an immersion objective lens, the AFM, and the sample. By mechanically decoupling the focusing mechanism from the sample and the AFM head, vibrations limiting the force resolution could be eliminated virtually completely.

II. SAMPLE POSITION FLUCTUATIONS IN TIRF MICROSCOPY

Several different methods are commonly used for fluorescence excitation in the evanescent field (TIRF).[9,10] Whereas prism-based systems allow for use of variety of glass substrates and incident angles, and are not limited by the numerical aperture (NA) of the objective lens, combining them with an AFM is difficult. An objective-type TIRF setup installed on an inverted microscope as it was introduced by Tokunaga *et al.*[11] offers the advantage of using an objective lens with high numerical aperture for excitation as well as for collection of emission, therefore leaving the upper half space above the sample free. A scanning probe microscope (SPM) such as the AFM may then be placed above the sample for simultaneous imaging, force measurements, and manipulation of the sample. Several implementations of this concept are already reported in the literature.[5–7,12–15]

Since high NA-objective lenses have very short working distances, objective-type TIRF can only be performed on

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thin cover slips, with a thickness of up to 0.15 mm. These thin glass substrates are prone to deformation and vibrations, coupling through the system acoustically an even more important mechanically, and through the air, which hampers high-resolution AFM measurements. While sound coupling into the system can be damped effectively by placing the whole setup in a soundproof box, mechanical vibrations are still hard to handle. Oscillations below 100 Hz, as arising from the building, can be effectively damped by active tables, but higher frequencies and especially noise sources inside the microscope itself still have to be eliminated.

A high NA-objective lens needs immersion oil to be operated to match the refractive index of the glass substrate. When using the objective lens for adjusting the focus, as it is done with virtually all commercially available objective-type TIRF setups so far, additional vibrations from, e.g., a focusing piezo are coupled into the glass cover slip. Moreover, moving the objective lens for controlling the focus during the experiment introduces drift mediated by the immersion oil's viscosity. However, for TIRF illumination the focus needs to be adjusted very precisely, because the evanescent field is only a few hundred nanometers in depth. During long measurements temperature changes as well as buffer evaporation may cause the surface to drift out of focus. Hence when operating commercially available focus piezo stages of the objective lens in a closed loop in order to correct for this focus drift, strong vibrations couple into the sample through the immersion oil, thereby making high-resolution AFM experiments virtually impossible.

III. HYBRID TIRF/AFM

In the evolution of SPMs, compact designs, as rigid as possible, have proven to minimize both vibrations and thermal drift. This design principle should provide the best solution for the problems with the mechanical stability discussed above.[16]

We kept our AFM head as short and compact as possible (see Fig. 1). Cantilever deflection is measured with an infrared superluminescent laser diode SLD-37-HP (Superlum Diodes Ltd, Cork, Ireland), which minimizes interferences of the reflected beam and keeps the entire optical spectrum open for fluorescent measurements. Servo motors controlling the z-position of the head and the position of the deflection sensing photodiode allow for automatic compensation of lever and probe drift during long term measurements.

The major difference of the presented setup to yet existing ones is the optimization of the mechanical stability of the sample-cantilever entity. Whereas in conventional microscopes the objective lens is mounted on a revolver and/or focusing piezo, our setup contains a high NA-objective lens, which is rigidly mounted in a massive aluminum block directly connected to the AFM head, as Fig. 1 shows. This way the objective is integrated directly in the AFM head, maximizing the mechanical stability. The sample is located on the cover slip, glued over a hole drilled into the bottom of a Petri dish. The latter is clamped tightly to the xy-piezo scanner by strong magnets in order to be able to move the sample. It is adjusted with micrometer screws for coarse alignment. Modern objective lenses with high NA, such as the Nikon 100× lens (CFI Apo TIRF 100×, NA 1.49, Nikon Inc., Tokyo, Japan) we used here, allow variation in the penetration depth of the evanescent field, depending on the distance of the beam from the rim of the objective lens. This is achieved by shifting the laser orthogonally to the optical axis of the objective lens. This way the penetration depth can be varied between approximately 200–500 nm.

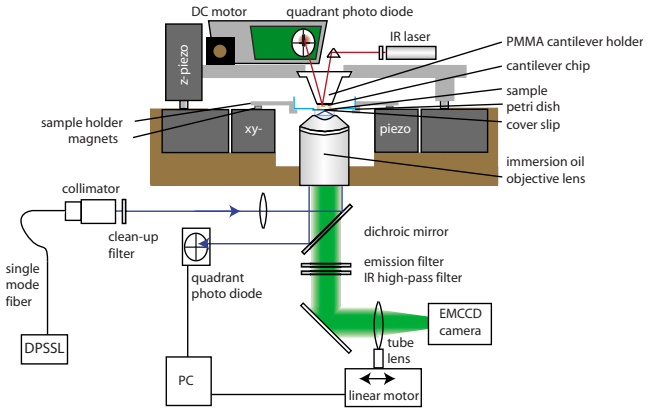

FIG. 1. (Color online) Schematic drawing of the combined AFM/TIRF microscope. TIRF excitation is performed by a diode pulsed solid-state laser coupled to a single-mode fiber. Using a collimator lens and a biconcave lens for focusing the beam on the back focal plane of the objective lens, wide-field TIRF illumination is achieved. Fluorescence emission passes through an appropriate filter set and is collected by the EMCCD camera. The tube lens is used for focusing and therefore mounted on a linear translation stage controlled by a PC that reads the position of the back-reflected beam from a quadrant photo diode. With this feedback a constantly sharp image can be ensured.

Focusing is facilitated by moving the tube lens via a motorized high-accuracy linear translation stage M-605, (Physik Instrumente, Karlsruhe, Germany) which focuses the fluorescence light from the sample onto the chip of the electron multiplying charge coupled device (EMCCD) camera (Andor iXon DU-897, Andor Technology, Belfast, Ireland). This motor is completely mechanically decoupled from the rest of the instrument and does not need to stand on the vibration insulation table.

In order to build a most compact instrument, for the excitation path, the light of a solid-state diode pulsed laser (532 nm DPSS Laser, CrystaLaser, Reno, NV, USA) is coupled into a single-mode optical fiber and used for fluorescence excitation via a compact slider unit consisting of a collimator lens, a clean-up filter, a lens for focusing the beam into the back focal plane of the objective lens, and an adjustable dichroic mirror that directs the light to the sample. This unit can be replaced easily with another one containing an alternative filter set, when working with a different laser. In total internal reflection the back-reflected beam hits a quadrant photo diode, which measures its position. This way a change in the lateral position of the reflected beam and hence a change in distance between the objective lens and the cover slip can be determined. Finally this information can be used for the adjustment of the focus via the tube lens.

By moving the tube lens via the linear translation stage, the relative distance from the charge coupled device chip and therefore the magnification is altered. This effect can be

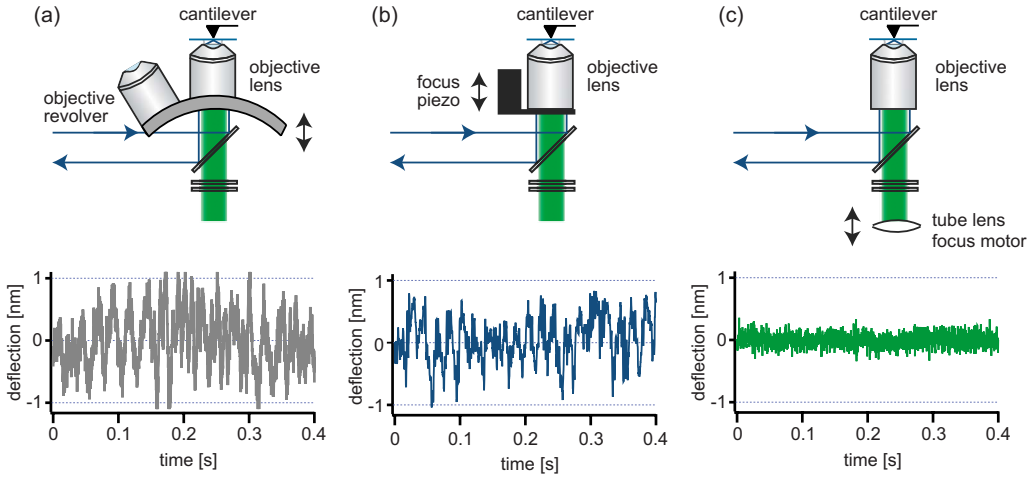

FIG. 2. (Color online) Calculated change in magnification as a function of the tube lens position, respectively, the corresponding sample position for the presented home-built microscope.

quantified by elementary optics, reducing the microscope to a one-lens system. The resulting focal length f and the position of the principal planes H_1 and H_2 are determined by the focal lengths of the objective f_1, the tube lens f_2, and their relative distance d as follows.

$$f = \frac{f_1 f_2}{f_1 + f_2 - d}, \quad H_2 = \frac{d f_2}{f_1 + f_2 - d}.$$

With the given image distance $b = f_2 + x - H_2$ the object distance $g = fb/(b-f)$ and the magnification $M = b/g$ can be calculated for each tube lens position x. Figure 2 shows the results for the described setup. Typical sample drift of 1 μm can be corrected by moving the tube lens by about 10 mm. In this range the magnification changes stay below 0.5%. Even when moving the tube lens by the maximum range of the linear motor (± 25 mm) the change in magnification is below 2.0%. This effect has thus of minor influence on the imaging qualities of the microscope, also in terms of aberration correction.

IV. EXPERIMENTAL RESULTS

In order to compare the setup described above with other combined AFM-TIRF hybrids we measured the height fluctuations of the cover slip. For quantification amplitude spectra were taken with an AFM cantilever contacting the surface. All measurements were performed using a Veeco MLCT-C Cantilever at a contact force of approximately 1 nN. As first reference we used a home-built compact AFM-TIRF microscope with piezo adjustable objective lens. As a second reference a commercially available setup consisting of standard inverted microscope Zeiss Axiovert 200F, (Carl Zeiss AG, Jena, Germany) with a MFP-3D AFM, (Asylum Research, Santa Barbara, CA) and alternatively a Nanowizard II AFM (JPK Instruments, Berlin, Germany) was used. The results are plotted in Figs. 3 and 4 for direct comparison.

The combined conventional setup [Fig. 4(a)] shows the highest noise of all. Since the spectra recorded with the different AFM heads were virtually indistinguishable we conclude that the mechanics of the inverted microscope dominates the resonances. The bridge of the inverted microscope acts as a tuning fork. Vibrations are transduced via the objective revolver through the immersion oil, leading to fluctuations of the sample of approximately 1 nm and more. For high-resolution single molecule force spectroscopy such fluctuations are not tolerable!

Using a piezo driven single objective lens directly attached to the AFM such as in the home-built setup used as reference is an improvement, but as soon as the focusing piezo is operated in closed loop, simultaneous AFM measurement become very noisy [Fig. 4(b)]. Vibrations at the electrical carrier frequency of 50 Hz (and multiples) as well as other resonance frequencies of the instrument become clearly visible. As the spectra in Fig. 3 show, not only in the active focus regulation mode, but even in a passive mode with the feedback switched off, the vibrations in the microscope are coupled through the objective into the sample.

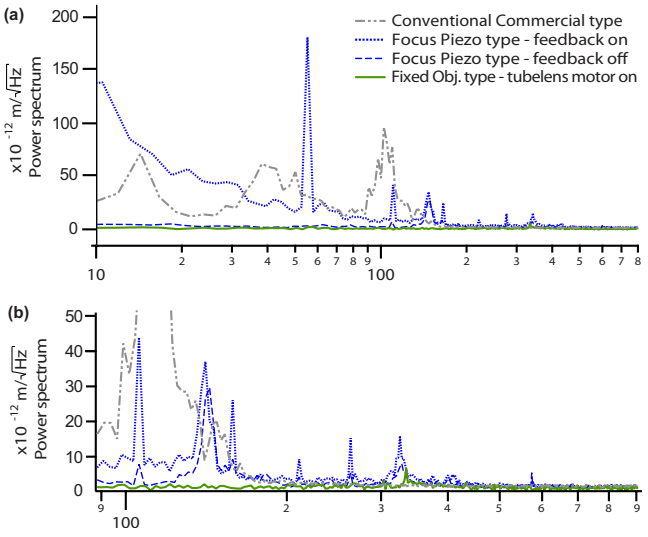

FIG. 3. (Color online) (a) Noise spectra of sample fluctuation recorded on the surface. While the conventional setup shows a broad distribution of frequencies, the home-built microscope with focus piezo exhibits mainly sharp resonance peaks, even when the closed-loop feedback of the focus piezo is switched off. The noise level is drastically reduced in the setup with fixed objective lens even with the tube lens focus motor switched on. A magnified view is given in (b).

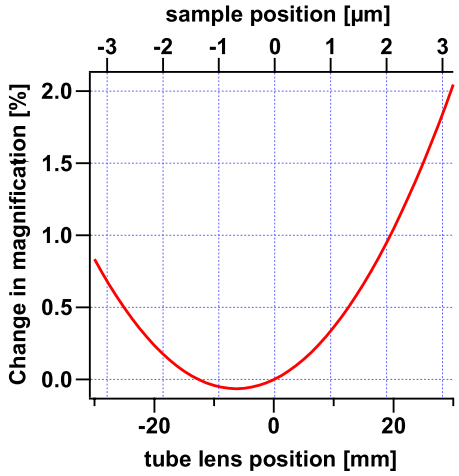

FIG. 4. (Color online) Comparison of the different setups and corresponding height fluctuations of the cover slip surface taken by contacting the surface of the cover slip with the AFM cantilever tip. (a) represents the combination of a standard inverted microscope and a commercial AFM head, (b) shows a setup with objective lens focus piezo, and (c) the new approach with moveable tube lens for focusing.

Moreover, adjusting the focus by moving the objective lens introduces drift that gets especially disturbing when performing experiments over hours.

In contrast, when operating the new setup with fixed objective but moveable tube lens cover slip vibrations are reduced to a minimum, while a sharply focused optical image is assured at all times [Fig. 4(c)]. Spectra that were taken while readjusting the focus position are virtually indistinguishable from those with fixed focus since the movable tube lens is mechanically decoupled from the AFM/sample part.

The combined AFM-TIRF instrument presented here was successfully used for simultaneous single molecule experiments such as single molecule cut-and-paste procedures as described in Ref. 17. Using molecular handles with hierarchical forces fluorescently labeled biomolecules were picked up from a depot, transported, and deposited with about 10 nm precision to a target area (Fig. 5). Figure 5(b) shows the rupture force curves measured on the depot area (left) and the target area (right), which exhibit very small noise comparable to a stand-alone AFM. The focusing mechanism described above made it possible to obtain sharp images of single fluorophores [Figs. 5(c) and 5(d)] at all times while allowing for high-resolution force spectroscopy in parallel for many hours.

V. CONCLUDING REMARKS

During day-to-day use, this combined TIRF-AFM setup proved to be a versatile instrument for many kinds of single molecule experiments.[17–19] The compact design with decoupled focusing succeeded in reaching a maximum of force resolution on the AFM side, while at the same time assuring a constantly sharp image for state-of-the-art single molecule microscopy.

Although we only show here only the combination of TIRF microscopy and AFM force spectroscopy, the principle of the setup described above can also be applied to any combinations of optical and SPMs where high sensitivity and

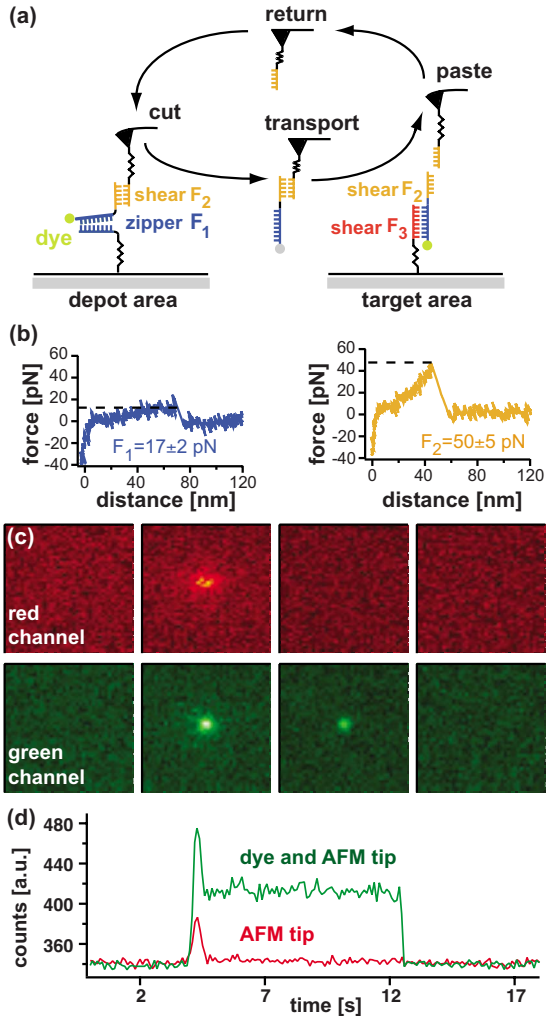

FIG. 5. (Color online) Single molecule cut-and-paste experiment performed using the combined TIRF-AFM microscope. (a) Fluorescently labeled DNA strands are transported by the AFM using a specific handle sequence on the tip and an anchor sequence on the cover slip surface. (b) High-resolution force spectra (d) and single molecule fluorescence time traces could be recorded simultaneously for many hours.

nanometer resolution is required. If no laser is used for TIRF excitation of the sample, or the laser's wavelength would interfere with the measurements, an IR laser could be used instead for determination of the cover slip distance by measuring the position of the totally reflected beam. Looking ahead, future AFM heads could already include an objective lens built in. This would not only allow for placing them directly on any (commercial) inverted microscope, but also would lower the AFM's center of mass, hence making it even more inert to interfering vibrations.

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