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Phase contrast and DIC illumination for AFM hybrids

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Abstract

High-resolution optical microscopy is an essential pre-requisite for life science force microscopy, particularly for applications in cell biology and medicine. Identification and validation of cells is typically established with techniques like phase contrast microscopy or differential interference contrast microscopy. The option to select or monitor individual cells online with such light microscopy techniques while performing atomic force microscopy (AFM) measurements is therefore extremely beneficial. Here, we report two conceptually different strategies to implement these light microscopy techniques in a fully functional AFM head at the ultimate resolution of the Abbe diffraction limit.

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Keywords: Phase contrast; Differential interference contrast (DIC); Atomic force microscope (AFM); Single-molecule force spectroscopy

1. Introduction

Most approaches to implement high-resolution optical microscopy in a hybrid instrument were hampered by the requirement of atomic force microscopy (AFM) [1–4] to utilize one half space of the experimental volume. Therefore, high-resolution optics was limited to epi or TIRF illumination [5,6]. In this paper, we integrated in the first approach a LED-based illumination ring into the cantilever holder, replacing the ring diaphragm in the condenser of the conventional phase contrast setup. In the second approach we used the reflecting surface of the AFM cantilever as a mirror to illuminate the sample in a quasi-trans differential interference contrast (DIC) mode from the epi side. The results of both strategies are compared to each other and to the corresponding standard geometries of illumination. Stability and versatility of the setup was demonstrated by

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simultaneous single-molecule force spectroscopy. A thorough discussion of the advantages and of potential future expansions is given.

2. Materials and methods

2.1. Experimental setup

The experimental setup used for the studies described here consists of a home-built AFM stand-alone type placed on top of an inverted optical microscope (Zeiss Axiomat, Carl Zeiss Inc., Oberkochen, Germany). This well-proven type of optical microscope was chosen because of its unparalleled mechanical stability. This hybrid instrument was hung from the ceiling of the lab suspended by rubber bungee cords for vibration insulation. The resulting cutoff frequency of 0.4 Hz for the mechanical crosstalk was found to be sufficient to dampen out building vibrations. The stage of the Axiomat was replaced by a massive aluminum plate stiffening the setup against higher frequency pick-up from sound. The system was optimized towards single-molecule force spectroscopy applications and proved to be sufficiently sensitive and stable to resolve single receptor ligand unbinding events between life cell surfaces [7–9].

2.2. Phase contrast illumination by LED ring

The cantilever holder of the AFM was designed such that a ring of 12 surface mount LEDs (Fairchild Semiconductor Inc., South Portland, ME, USA, smLED, QTLP650C-Y, ultra-miniature chip type 1206, AlInGaP technology, continuous forward current $I = 20$ mA, peak wavelength $\lambda = 590$ nm) could be integrated without interfering with the laser beams of the beam bounce cantilever deflection sensor (see Fig. 1a). Therefore, the ring could not be completely closed (see Fig. 1b), resulting in a slight and tolerable anisotropy of the illumination. The direction and the position of the LEDs was chosen such, that they illuminated the sample under the aperture angle window defined by the phase contrast ring Ph#3 of the 100× Planapo Oil (N.A. = 1.30) objective of the Axiomat. The LEDs were soldered together as a ring, open at one side, and installed in the Plexiglass holder, in which a corresponding chamfer was millcut before. Fig. 1b shows the result: all LEDs (bright squares) are focused onto the phase contrast ring (dark circular ring) without spill. Normally, this phase contrast ring in the rear focal plane of the objective is illuminated by Köhler illumination through a ring diaphragm. The invention of the phase contrast microscope by Frits Zernike made it possible to observe thin transparent objects of higher refractive index

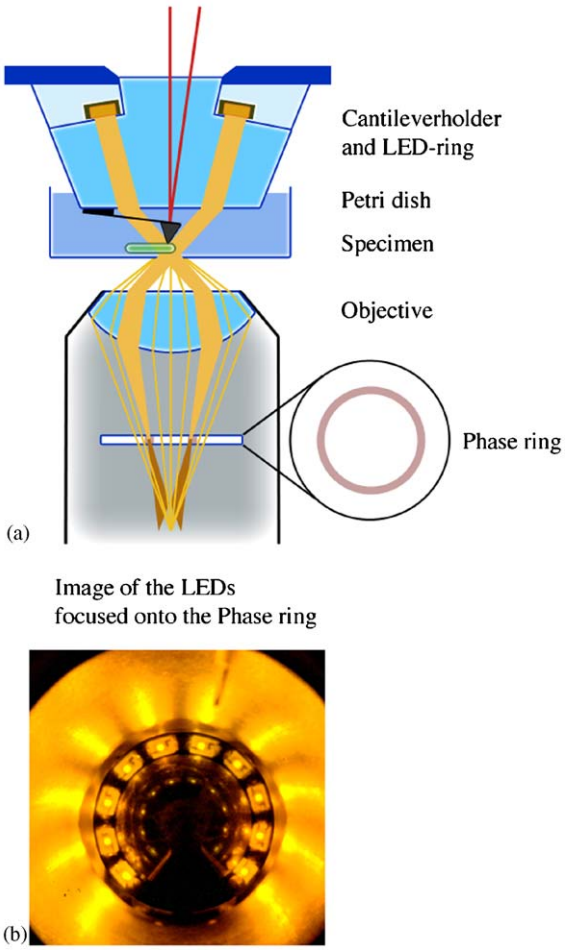

Fig. 1. (a) Ray trace schematics of the LED ring illumination for phase contrast microscopy. (b) Image taken from the rear focal plane of the objective through the Bertrand lens showing the LEDs superimposed with the phase ring.

(so-called phase objects), invisible by normal bright field illumination, in vivo without changing their characteristics. For a detailed explanation of phase contrast microscopy see Refs. [10–12]. As can be seen in Fig. 1b, the local illumination by LEDs close to the sample fulfils the same purpose, leaving the full upper half space for the AFM.

2.3. Setup for quasi-trans DIC

The setup shown in Fig. 2 is mainly that of an epi DIC alignment used for imaging opaque specimens like semiconductor chips. In contradiction to normal epi DIC we do not examine a structured reflective surface itself but only use a plain reflector to image a specimen in a focal plain right below. The needed constituents in the microscope (polarizer, Nomarski prism, analyzer) for our quasi-trans DIC technique are the same as needed for standard epi DIC. Although the surfaces of cantilever and cantilever chip facing the microscope objective are not coated with a special metallic layer (the upper side is gold coated for the purpose of laser beam deflection) they are suitable for quasi-trans DIC illumination due to their evenly plain surface qualities (chip: glass; cantilever: silicon nitride). By using the reflective chip of the cantilever as a mirror for the DIC illumination the sample could be illuminated in the full field of view (see Fig. 3a). The end of the cantilever with its tip was then used as a mirror to exactly zoom into the structure to be investigated on the cell by AFM (see Fig. 3b). Thus, the well-known techniques of trans DIC and reflected light DIC microscopy (see Refs. [13–15]) are combined to examine biological specimens in a way that pseudo three-dimensional images (corresponding to the gradient of the refractive index) of transparent samples are obtained.

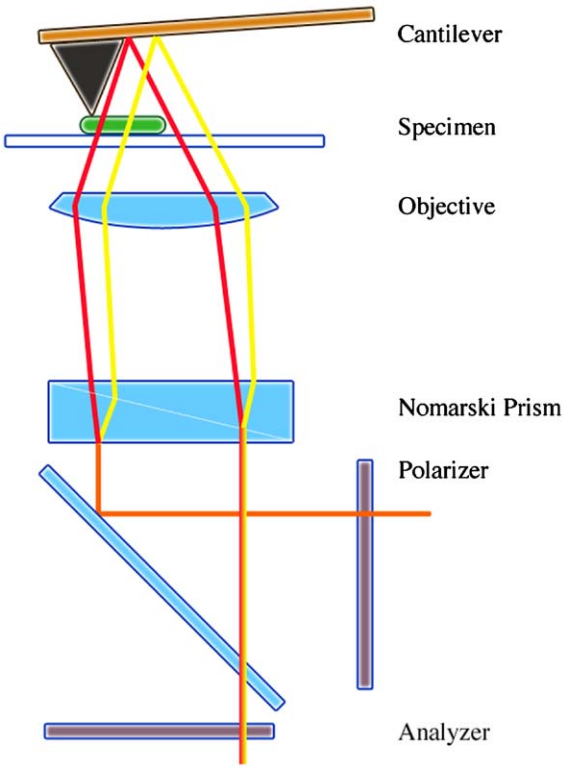

Fig. 2. Ray trace schematics of the quasi-trans differential interference contrast illumination (DIC), using the reflecting AFM cantilever as mirror.

2.4. Cantilever preparation

Commercially available cantilevers (Veeco Inc., Santa Barbara, CA, USA, #MLCT-AUHW, silicon nitride, back side coating: 15 nm Cr bottom layer, 60 nm Au top layer; front side coating: none) were used. Functionalization for the adhesion measurements was carried out as follows: the untreated cantilever was fully covered with a 50 μl droplet of poly-l-lysine solution (Sigma-Aldrich Chemie GmbH, Munich, Germany, P4707, 0.01%, sterile) which was allowed to incubate for 2 h at room temperature. Afterwards the cantilever was rinsed in water and directly installed in the AFM.

2.5. Sample preparation

To explore the full range of optical resolution, we glued a 0.17 mm thick coverglass over a hole punched into the plastic Petri dish used for this study. Human epithelial cells were taken from the inner side of the cheek and directly placed onto the coverglass in the Petri dish. A volume of 2 ml of phosphate-buffered saline (PBS, Sigma-Aldrich Chemie GmbH, Munich, Germany) was added. For about 5 min the cells were allowed to settle

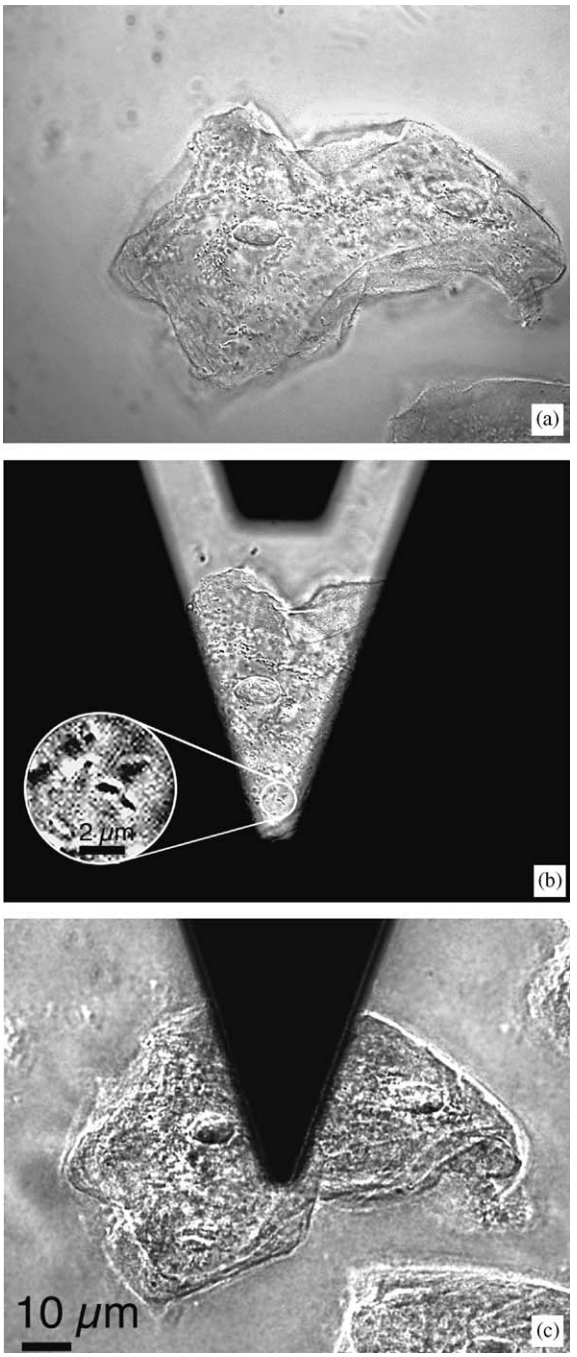

Fig. 3. Micrographs taken in parallel to AFM experiments. (a) Epithelial cell imaged with quasi-trans DIC reflected from the chip. (b) Epithelial cell imaged with quasi-trans DIC reflected from the cantilever. Inset: magnified view of organelles of the epithelial cell. (c) Epithelial cell imaged with LED ring phase contrast.

down and bind to the glass surface. Then we changed the PBS medium again to remove eventually remaining saliva. The so-prepared sample remained intact for at least 2 h of measurement.

3. Experimental results

3.1. Quasi-trans (epi) DIC

Fig. 3a shows two epithelial cells illuminated in quasi-trans DIC mode from the reflecting surface of the cantilever chip. Details of the cellular organization are visible such as the two nuclei and inner organelles like mitochondria and endoplasmic reticulae. Due to the 6° slope at which the cantilever chip is mounted onto the Plexiglass holder, the illumination is slightly anisotropic. However, this only alters the brightness, not the contrast. The quasi-trans DIC illuminated micrograph of the same cells is shown in Fig. 3b with the cantilever as a reflector. Here the cell under the cantilever tip is monitored with optical resolution at the Abbe limit of the objective while an AFM-based experiment, e.g. force spectroscopy, is performed. The identity of the target can be corroborated in this way. In addition, changes, modifications, etc. may be recorded online as they occur. The inset in Fig. 3a displays the magnified section showing micrometer-sized structures, either organelles or bacteria, in or on the epithelial cell. Sub-structures in the dimension of tenths of micrometers are clearly observable and demonstrate the limit of high-resolution optical microscopes. In additional experiments we used fully gold-coated cantilevers and cantilever chips as reflectors for quasi-trans DIC microscopy. The recorded images do not show any significant differences compared to those obtained through reflection at the silicon nitride side. Furthermore, reflection by the silicon nitride surface does not show any phase shifts or interference with beams potentially reflected by other metal layers. The results show that all kinds of available cantilevers are able to produce good DIC images as long as they provide an evenly mirroring surface.

3.2. LED phase contrast

The LED ring phase contrast image (Fig. 3c) shows the two cells together with the AFM cantilever. This image complements the DIC image since it shows the entire field of view except the one shadowed by the cantilever. This option to monitor the whole cell and especially its edge is important to infer its vitality. Compared to the DIC images, the phase contrast images have a higher contrast but seem to be somewhat lower in resolution.

The main reason for this is the relatively wide radiation angle of the used LEDs. A second reason for a certain lack of resolution is the missing segment in the LED ring.

The improvement of the illumination geometry will be the topic of further studies. The use of new types of LEDs with a better defined emission geometry and an additional annular diaphragm below the LED ring will be first approaches. The cells shown in Fig. 3a–c are all kept in a buffer solution in a home-made glass-bottom Petri dish.

3.3. Combined AFM experiments and imaging

The motivation for this development was the need to correlate a certain kind of molecular interaction measured by AFM with the morphology of the cell [16,17]. For this purpose, both the force spectroscopy experiment and the light microscopy had to be carried out simultaneously at a resolution high enough to resolve such characteristic features of the cell like mitochondria and lysosoms. The corresponding single-molecule force spectroscopy experiments will be reported in detail in a future publication. Here, only one example of a typical force curve is given in Fig. 4. The epithelial cell was contacted with an AFM tip which was functionalized with poly-l-lysine to mediate the adhesion to negatively charged membrane markers. Upon retraction, the cytoskeleton-anchored proteins, which bound to the tip, provide enough stability so that several membrane tethers are pulled out of the cell surface. These tethers rupture in quantized steps of roughly 80 pN in force upon further retraction of the tip.

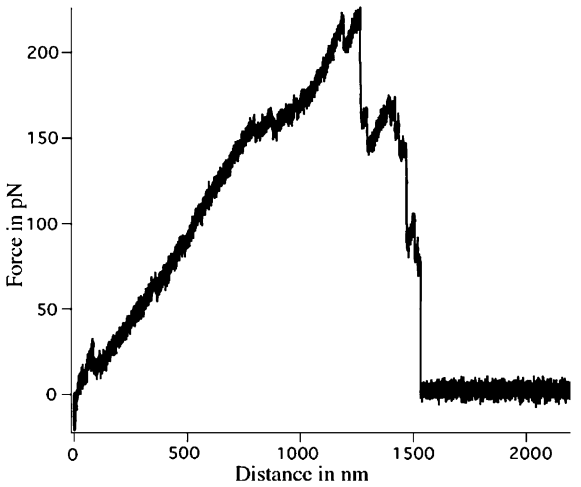

Fig. 4. Protocol of a force spectroscopy scan on an epithelial cell. Once brought into contact, the cell membrane attaches to the adherent AFM-tip via cytoskeleton anchored membrane proteins. Upon retraction several membrane tethers are pulled which rupture one by one, lowering the force acting on the cantilever by approximately 80 pN each.

4. Concluding remarks

During day-to-day use, this combination of high-resolution optical microscopy with scanning probe techniques turned out to be an extremely versatile extension of the experimental options. Localization and identification of cells and their organelles became a reliable and easy procedure prior to and during force spectroscopy. Moreover, experiments with small objects like bacteria became possible and are routine now. We built the LED ring into the cantilever holder of our home-made AFMs but it may easily be implemented in commercial instruments.

LED ring phase contrast and quasi-trans DIC illuminaton complement each other in a very beneficial way: while the quasi-trans DIC technique allows the observation of a specimen directly under the cantilever and its tip, the phase contrast illumination provides an image of the area surrounding the cantilever. If needed, the full field of view can be seen by DIC illumination from the cantilever chip.

Furthermore, phase contrast and differential interference contrast microscopy can be combined

with other optical techniques like fluorescence imaging to obtain supplementary information [18,19]. Expansion and improvements of our hybrid system will therefore be the matter of future work.

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