

SCANNING NEAR-FIELD OPTICAL MICROSCOPY OF LIVE CELLS IN LIQUID

R. Januškevičius^a, V. Vaičiškauskas^a, D.J. Arndt-Jovin^b, and T.M. Jovin^b

^a *Department of Nonlinear Optics and Spectroscopy, Institute of Physics, Savanorių 231, LT-02300 Vilnius, Lithuania*

^b *Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany*

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A scanning near-field optical microscope (SNOM) is applied to fluorescence imaging of biological samples in liquid, including live cells. The SNOM is mounted on a Zeiss Axiovert 135 TV fluorescence microscope. For feedback we use tuning fork shear force method. The scanning tip is produced from a 125 μm optical fibre (8.3 μm core diameter) in a commercial Sutter P-2000 pipette puller and is coated with aluminium. Other commercial tips have also been used. Coarse z -axis adjustment is hydraulic, and fine positioning is accomplished with piezoelectric tube units. We have constructed the original liquid chamber, which allows long term stability of scanning and highest values of the Q factor (300 or more). The depth of liquid layer was less than 40 μm . Near-field images – the topography and distribution of membrane fluorescence of live human epithelial A431 cells, stably transfected with an EGFP fusion protein of the epidermal growth factor transmembrane receptor protein (EGFR, erbB1), were obtained in liquid.

Keywords: scanning near-field optical microscopy, fluorescence microscopy, sub-diffraction limit, live cells

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1. Introduction

Fluorescence microscopy is a powerful experimental tool for visualisation of biological specimens and the determination of the distribution of fluorescently labelled objects within the sample. Scanning near-field optical microscopy (SNOM) can simultaneously map topographic and optical properties (fluorescence, absorption) on and of the surfaces. The spatial resolution of traditional optical microscopy with conventional far-field optics is limited by diffraction to approximately $\lambda/2$. This limit does not apply to near-field microscopy, in which a miniature optical probe is scanned over a sample surface at nanometre distances. In this case the resolution is defined mainly by the physical size of the aperture.

SNOM is a highly useful tool for investigating the long-range lateral distribution of labelled objects in the 100–1000 nm range and has been used to probe various biological molecules and systems, such as green fluorescent protein (GFP) in bacteria [1], cells labelled with fluorescent anti-erbB2 monoclonal antibodies [2], and fluorescently labelled plasma membranes of fixed human skin fibroblasts [3]. Data collected from dry biological samples may exhibit artefacts caused by drying

of the samples [3]. Furthermore, imaging by SNOM has been performed primarily on fixed cells [4, 5].

Several feedback mechanisms have been proposed for imaging samples under water [6, 7]. The best resolution reported to date for SNOM operated in liquid on hard samples is 60 nm [8]. A shear-force non optical “tuning fork” type distance control method is known to have a very large quality factor Q [9] and is very effective in the SNOM systems operating in air. Unfortunately, the quality factor sharply decreases upon immersion of the fibre tip into water. The liquid depth must be minimized (50–100 μm) in order to reduce the damping of tip oscillation and the consequent loss in the Q factor of resonance caused by the drag force of the liquid [5, 10]. The vibrational mode of tuning fork is conserved upon full immersion in water [11]. Hydrophobic tips were proposed by Sommer and Franke [12].

For application of this microscopy technique to the investigation of soft samples, such as living cells in liquid, entirely new requirements emerge. This concerns the adjustment of distance between the tip and the sample. In water, a very precise control of the gap between the tip and the object is essential to prevent catastrophic tip-sample collision. Living cells have a significant

thickness (typically $\sim 10 \mu\text{m}$) and need to be imaged in an aqueous environment. For these reasons there is very scarce experimental research with live cells [14] with high spatial resolution. Only 200–300 nm resolution was achieved using the ion conductance as a distance control mechanism [13]. Using a diving bell concept an 80 nm resolution was achieved with the Q factor 200 [14, 15]. However, the increase of the Q factor and as a consequence the scanning speed remain the main problems in the live cell microscopy.

There is a second factor necessitating a modification of the tip construction for experiments in water: due to the similarity of refractive indexes of water and the quartz core, the total internal reflection is frustrated and the light does not reach the tip. Very high refraction index fibre tips should be used, or the tip of the waveguide should be coated with metal.

Here we present the key features of the high Q factor microscope as well as the original results of topographic and fluorescence views of live cells. Our results are promising with respect to the maintenance of a stable and very shallow cell (only 20–40 μm layer of water) for further development of SNOM.

2. Materials and methods

The SNOM is mounted on a Zeiss Axiovert 135 TV fluorescence microscope. To ensure the recognition of the object of interest with a high optical resolution (Fig. 1(a, b)), we operate in the illumination mode using shear force for feedback control.

We use a commercially available controller and software (RHK SPM 100 with control module V-SCAN 100). The scanning tip is produced from a 125 μm optical fibre (Siecor, 8.3 μm core diameter) by a heating and pulling sequence in a commercial Sutter P-2000 pipette puller and is coated sequentially with 30 nm Cr and 120 nm Al films. A coated fibre tip prevents light from exiting into the liquid before reaching the end of the probe. The tip cone angle is kept relatively small ($\sim 20^\circ$). Commercially available tips (Nanonics, Shearforce NSOM Fiber Probe 488, ϕ 50 and 100 nm) have also been used.

The fibre tip is glued to the tuning fork with cyanacrylate. In order to simplify this process, the fibre tip and the tuning fork are first fixed on the scanning head and then glued together; a piezoelectric plate is also attached (1 mm thickness, $5 \times 5 \text{ mm}^2$ area, material PIC151, $d_{33} = 450 \text{ pm V}^{-1}$, Physik Instrumente, Germany) (Fig. 1(c)). The scanning head has manual coarse xy -position adjustment and a coarse hydraulic

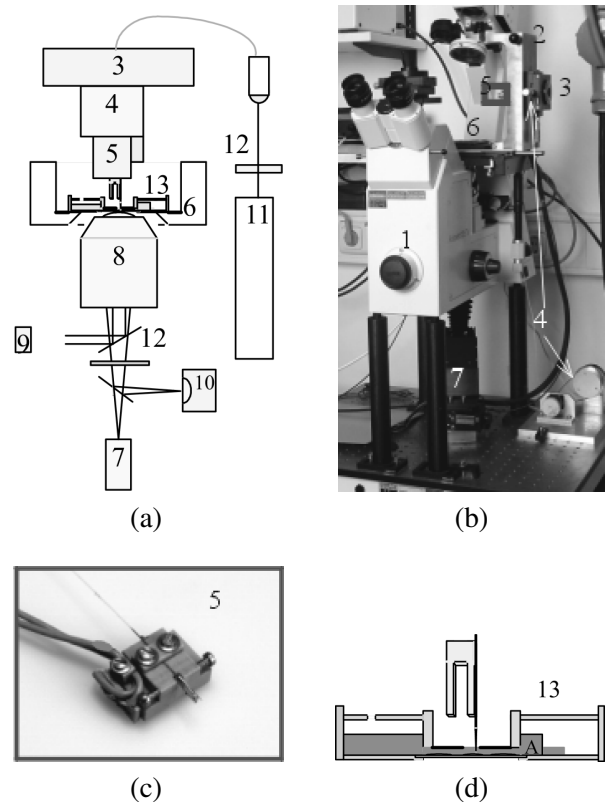


Fig. 1. SNOM designed for imaging of live cells. (a) Schematic diagram and (b) photo view of microscope. 1 is Zeiss Axiovert 135 TV fluorescence microscope, 2 is uncovered holder, 3 is coarse xy position adjustment with microstage, 4 is hydraulic approach unit, 5 is holder of fibre and of tuning fork, 6 is nanostage used for XYZ scanning, 7 is photodiode, 8 is objective, 9 is a Hg lamp, 10 is eye piece, 11 is laser, 12 is filter, 13 is liquid chamber. (c) Schematic diagram: holder of fibre and of tuning fork. (d) Schematic diagram of liquid chamber.

approach unit in the z direction. Due to the use of a hydraulic approach, the microscope is relatively impervious to mechanical and acoustic noise.

The piezo plate is driven by a signal from a generator incorporated into a lock-in amplifier (Stanford Research model 830). The signal from the tuning fork (Bürklin, quartz 32768 Hz) is pre-amplified (Princeton Applied Research model 5113) and detected with the lock-in amplifier, the output of which is used as the feedback signal to the RHK controller. An offset applied to this signal defines the set-point of the feedback loop and thereby the working distance between tip and sample. The PI nanostage (x - y scanning) and piezoelectric tube (z scanning) is driven from the RHK controller through a PI driver (Physik Instrumente, module E-863.10). Scanning data (fluorescence) are collected with the same RHK controller.

For imaging, the 488 nm line of an $\text{Ar}^+ - \text{Kr}^+$ mixed gas laser (Performa, Spectra Physics, Mountain View, CA) was stabilized by a laser intensity stabilizer (Cam-

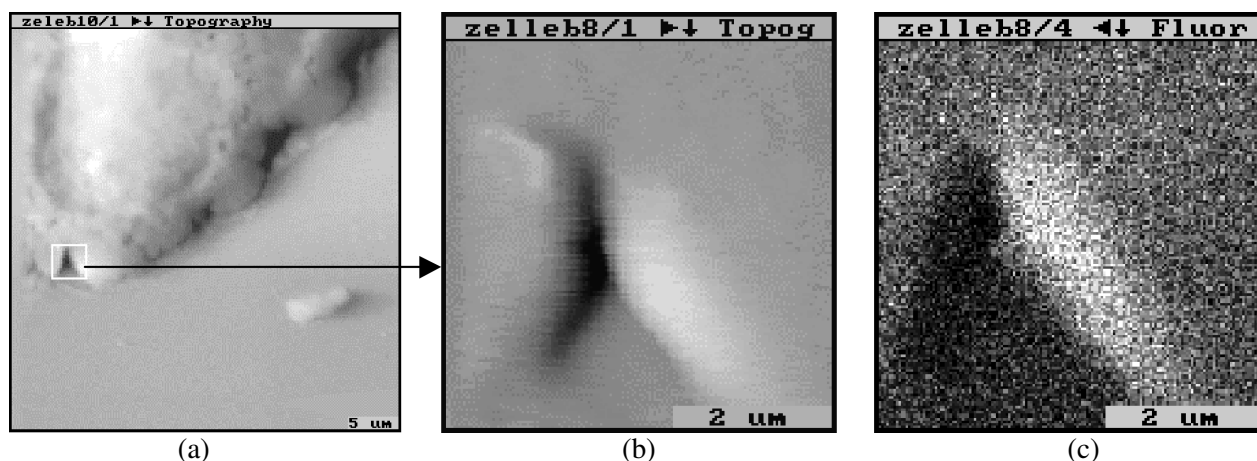


Fig. 2. Live human A431 cells (A4 erbB2 Y13). (a, b) Topographic image, (c) fluorescence image.

bridge Research & Instrumentation, Woburn, MA) and coupled into the fibre. In the experiments reported here, the fluorescence was selected by a bandpass filter (505 nm) and detected with a single-photon counting avalanche photodiode (SOCM-AQR-13, EG&G Optoelectronics, Vaudreuil, Quebec, Canada). Measurements were performed at room temperature under ambient conditions.

2.1. Liquid chamber

To operate the SNOM in liquid, we designed a chamber for a 20–40 μm layer of liquid (Fig. 1(d)). The chamber consists of two separate parts, the bottom part of which is a cap with a central hole to accommodate 18 mm diameter glass cover slips. The top part (cover) has a central 2 mm diameter hole. The evaporation liquid is compensated by replenishment through a capillary tube from chamber A. The distance between the glass cover slip and the top hole in the cover determines the depth of liquid. This liquid chamber con-

struction permits scanning in an aqueous environment for ~ 3 hours without refilling of liquid.

3. Results and discussion

The simultaneously scanned topographic and fluorescence images of live human A431 cells (A4 erbB2 Y13) are shown in Fig. 2. The scan area is $40 \times 40 \mu\text{m}^2$ (Fig. 2(a)) and $5 \times 5 \mu\text{m}^2$ (Fig. 2(b, c)). The height of the cell in the scanned area is $4.8 \mu\text{m}$. Live human A431 cells transfected with EGFP-EGF fusion protein (EG33) in liquid are shown in Fig. 3. An area of 128×128 pixels was scanned with an integration time of 3 ms/pixel for fluorescence light. The image of the cell was scanned in an aqueous salt solution. Bright regions in the fluorescence image correspond to areas with high fluorescence and a high concentration of the EGFP-EGF fusion protein. Previous SNOM studies demonstrated that a related receptor (erbB2) is clustered into small domains on cell surfaces [2].

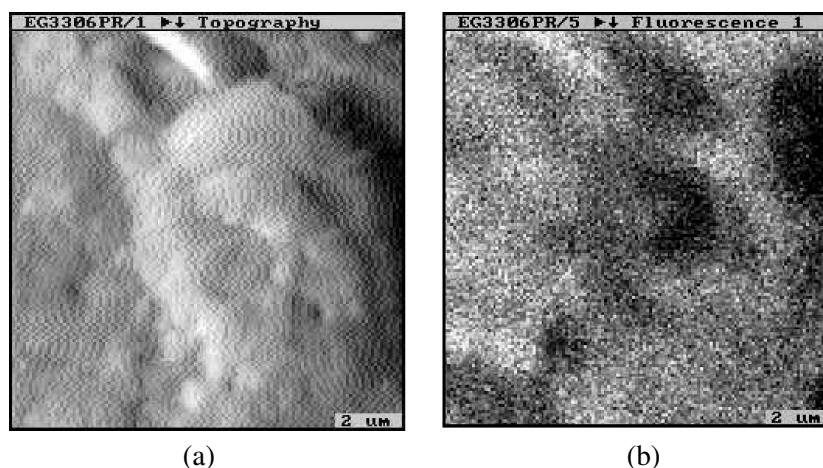


Fig. 3. Live human A431 cells transfected with EGFP-EGF fusion protein (EG33) in liquid. (a) Topographic image, (b) fluorescence image.

Scanning was accomplished with a small cone angle tip to minimize the water drag force. The tip was immersed $\sim 40 \mu\text{m}$ into the liquid in our setup. In this case the quality factor Q decreased only by about 5–7% (compared to air) which is much better when compared to that obtained in [15]. This kind of tip exhibits a low interaction force with the cell, thus reducing damage to the latter. Using this concept we routinely obtain Q factors of 300 or higher.

Obtaining an intense fluorescence signal in illumination mode requires the operation at a lower light intensity in comparison to a SNOM in which the tip performs both the illumination and the detection functions.

There are, however, potential hazards for cell damage during operation, e. g., fast scan speed and/or in the event of unsuccessful approach of the tip to the sample. We used a scan speed of $10 \mu\text{m/s}$ and a fast feedback control (low integration time). For cells exhibiting substantial motion, this scanning speed did not suffice for the acquisition of full images. However, several scans of the same part of the cell were often possible before the quality of the topographic view declined. To minimize damage to the cell, we performed the approach of the tip in two steps: first to the sample outside the cell, and then to the cell itself.

Because the cells have a significant thickness, the excitation light propagating to the far-field would illuminate distant fluorophores, causing a relatively high background fluorescence signal upon which the near-field signal is superimposed [16]. To compensate for this effect, certain further design modifications are required.

4. Conclusions

In this work we have demonstrated the sub-diffraction resolution on the membrane of live cells using SNOM. The original construction of the liquid chamber allows for the long term stability of scanning and the highest values of the Q factor (300 or more). In the scan area the liquid layer is shallow ($20\text{--}40 \mu\text{m}$). Using such a cell the images of soft human epithelial A431 cells in liquid with the 120 nm spatial resolution have been obtained. The obtained high values of the Q factor enable multiple scans of the same cell and prevent the cell damage. Our results are promising in further investigation of the distribution of fluorescently labelled objects on the surface of live cells.

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GYVŲ LĄSTELIŲ SKENUOJANTI ARTIMO LAUKO MIKROSKOPIJA SKYSTYJE

R. Januškevičius^a, V. Vaičiškuskas^a, D.J. Arndt-Jovin^b, T.M. Jovin^b

^a *Fizikos institutas, Vilnius, Lietuva*

^b *Makso Planko Biofizinės chemijos institutas, Göttingenas, Vokietija*

Santrauka

Sukurtas ir sukonstruotas optinis artimo lauko mikroskopas pritaikytas gyvų ląstelių ir kitų biologinių objektų, esančių tirpaluose, fluorescenciniam signalui fiksuoti. Mikroskopas sumontuotas ant fluorescencinio Zeiss Axiovert 135 mikroskopo. Skenavimui naudotos adatos, pagaminamos kaitinant ir tempiant šviesolaidį, kurios vėliau metalizuojamos. Sukonstruota speciali skystinė kiu-

vetė, pritaikyta biologiniams objektams skenuoti tirpale. Mažas tirpalo gylis kiuvetėje minimaliai įtakoja adatos jautrį skenuojant bandinio paviršių ir nekritiškai sumažina adatos rezonanso Q faktorių. Tai leidžia skenuoti lengvai pažeidžiamą gyvų ląstelių paviršių. Gauti eksperimentiniai gyvų žmogaus epitelio A431 ląstelių, žymėtų EGFP baltymu, artimo lauko fluorescencijos ir topografijos vaizdai.