Simultaneous Topography and Fluorescence Imaging of Cells Using Agilent's 6000 ILM AFM

Application Note

Christian Rankl, Gerald Kada
Agilent Technologies

Josef Madl, Julian Weghuber
Institute for Biophysics, University of Linz, Austria

Introduction

Since its invention in the late eighties of the last century the atomic force microscope (AFM) has become an invaluable tool for studying the micro- and nanoworld of living organisms. Especially its ability to measure at room temperature and in aqueous environments with sub-nanometer resolution make it a perfect tool for visualizing proteins and complex biological structures in their native state. Despite that great ability it has some limitations, like a limited field of view (<100 µm) and a quite slow scan speed (taking an image lasts minutes). This makes it difficult to find the region of interest on an inhomogeneous sample, like cells grown on a glass substrate. In addition to that, AFM imaging is restricted to contrast mechanisms based on mechanical forces between tip and sample surface, which does not allow discriminating different proteins.

In contrast to that, optical microscopy has a field of view in the mm range and images can be done within several milliseconds, allowing scanning over several mm² within seconds. Optical techniques like brightfield illumination, phase contrast and differential interference contrast (DIC) reveal high-contrast topography information, as well as position and distribution of internal structural features of cells. This allows for fast cell screening based on morphology. Fluorescence microscopy has proven to be a powerful tool for selective and specific visualization of labelled molecules down to the single-molecule level. This allows for discriminating cells according to their protein expression levels or even following cellular processes and monitoring the dynamics of living cell components. But the resolution of optical imaging is hundreds of nanometer and not in the sub-nanometer range like with AFM.

The advantages of both techniques complement each other however. Therefore, combining an AFM with an optical microscope enables to quickly find the region of interest using the light microscope and subsequently position the AFM tip there for in-depth structural imaging or looking for biological activity. To this date, a combined device of an AFM with an optical microscope has limited the capabilities of each single unit and full integration has been lacking on the market with a unified software and control unit. Agilent has developed the 6000 ILM AFM which seamlessly integrates a high resolution AFM with inverted light microscopes (ILMs), including the Zeiss AxioObserver, and Olympus IX series. The integration does neither compromise the performance of the AFM nor does it interfere with optical imaging.

The 6000 ILM supports contact mode and AAC (acoustic AC) mode AFM imaging techniques, plus the very powerful Agilent-only imaging modes - MAC (magnetic AC) mode and

topography and recognition imaging (PicoTREC). MAC mode is a dynamic, oscillating probe (AC) imaging technique that is the most gentle and precise AFM imaging mode available on any AFM, that is particularly well suited for imaging biological samples. With the 6000 AFM in place, the ILM can be operated in all the popular optical imaging modes, including bright field (Kohler) illumination, differential interference contrast (DIC), phase contrast and fluorescence imaging, so even highly opaque or translucent samples can be located with the ILM and further investigated with the AFM. Also, it is fully compatible with the highest quality, off the- shelf 0.5/0.55 NA OEM condensers and objectives.

Here we demonstrate the ability of Agilent's 6000 ILM to easily position the AFM tip in a region of interest using a contrast enhanced optical image or/and a fluorescence image, and precisely overlay images of topography and fluorescence.

Experimental

One of the most desirable topics in life science and bio-nanotechnology is the investigation of living cells and the organization and function of proteins in the cell membrane. Cell signalling, communication to neighbouring cells and transport to adjacent tissue is all organized through membrane proteins. Here we present two examples of important protein systems for cell signalling and cell membrane organization.

Organization of cell signalling proteins

Store-Operated Calcium Entry (SOCE) is important for many cellular signalling processes in proliferation, apoptosis, secretion and gene expression. The best studied SOCE current is the Calcium Release Activated Calcium (CRAC) current in T-cells and mast cells. Recognition of pathogenic antigens triggers a signaling cascade that leads to depletion of intracellular Ca^{2+} stores and finally causes activation of plasma membrane located CRAC channels. Proper function of the CRAC channel is vital, since it is involved in promoting the immune response to pathogens. Severe combined immunodeficiency (SCID, "Bubble boy syndrome") is an inherited immune disease, where patients are extremely vulnerable to infections due to the loss of CRAC channel function (Feske, *et al.* 2006). The essential pore forming subunit of CRAC channels is the plasma membrane protein Orai1 (Prakriya, *et al.* 2006). In the resting state, Orai1 is homogeneously distributed throughout the plasma membrane. Upon CRAC current stimulation, Orai1 forms clusters in the membrane and transmits calcium. Therefore studying Orai1 localization is beneficial for understanding CRAC signalling.

The distribution of the Orai1 protein was investigated in T24 cells (human bladder carcinoma cells). For this, T24 cells were transfected to express green fluorescent protein (GFP) fused to Orai1. Such cells were fixed and imaged using fluorescence microscopy and AFM. The fluorescence image (Figure 1A) illustrates the cellular distribution of Orai1-GFP, which was found to be located mainly in the plasma membrane.

The topography image (Figure 1B) gives information about cell dimensions. Typical T24 cells are about 40-60 µm wide and about 3 µm high in the nucleus region. Topographically distinct membrane features like lamellipodia, and filaments of the cytoskeleton were resolved at high resolution. The overlay of the fluorescence and topography image (Figure 1C) allows for correlating the distribution of Orai1 with structural and topographical features. Not all T24 cells express the GFP labelled Orai1 proteins. This allows studying the influence of the Orai1 expression level on the cell morphology. In this example it is interesting to note that Orai1 expressing cells exhibited a flat and extended morphology, while cells which did not express Orai1 were round shaped and higher.

Lipid rafts on living cells

In the plasma membrane of cells cholesterol- and sphingolipid-enriched microdomains, termed "lipid rafts", are found. These lipid rafts serve as organizing centres for the assembly of signalling molecules, influence membrane fluidity and membrane protein trafficking, as well as receptor trafficking. For example, upon specific triggering, membrane receptors such as T-cell receptors or B-cell receptors translocate into lipid rafts, which is the prerequisite for efficient receptor-mediated signal transduction.

The Agilent 6000 ILM allowed for investigating the morphology, the distribution of lipid rafts and the topography of T24 cells. For this, T24 cells, which were transfected to express GPI anchor derived from DAF fused to GFP [termed GPI-(DAF)-GFP], which is an highly effective lipid raft marker (Weghuber, *et al.* 2010, Legler, *et al.* 2004), were grown on glass bottom Petri dishes. The motorized sample stage of the 6000 ILM allowed for fast and easy screening of the Petri dish for proper

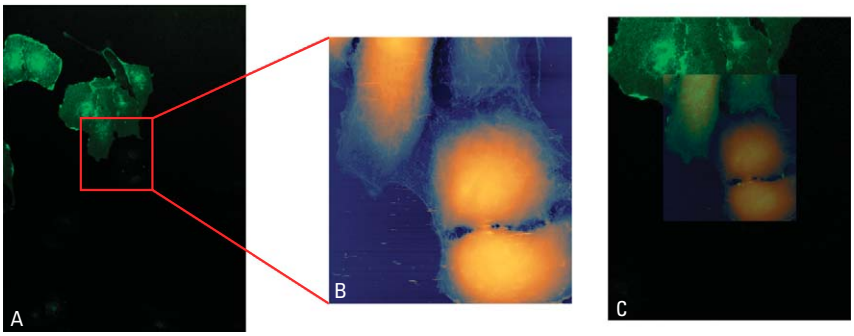

Figure 1. Fluorescence and atomic force microscopy images of fixed T24 cells expressing GFP fused to Orai1. (A) The GFP fluorescence represents the distribution of the Orai1 protein in the cells. The pattern shows that Orai1 is mostly located in the plasma membrane. (B) The topography of the indicated cell cluster revealed several features like lamellipodia and the cytoskeleton (contact mode, 80 x 90 µm). (C) The overlay of topography and fluorescence image allows comparing the expression of Orai1 with the cell morphology. In this example the two cells not expressing Orai1-GFP exhibited a round shape, while Orai1 expressing cells were more flat in their appearance.

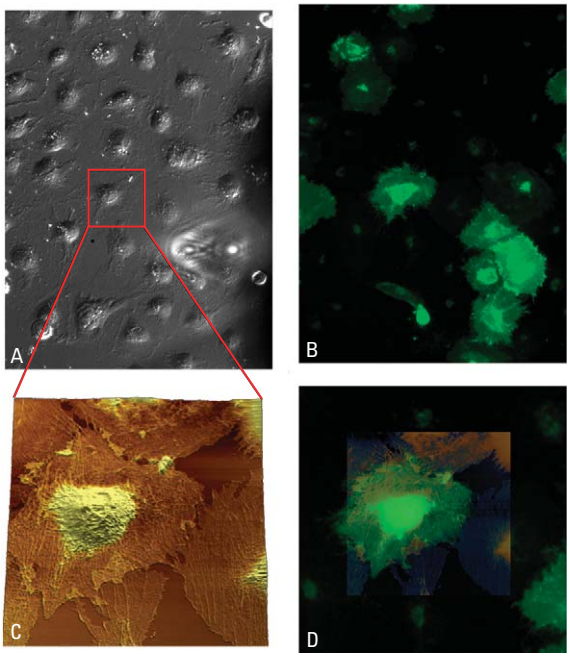

Figure 2. Optical and atomic force microscopy images of living T24 transfected to express GPI-(DAF)-GFP anchored to DAF cells. (A) The morphology of the T24 cell sample using DIC imaging. (B) Lipid raft distribution measured by GFP fluorescence of the same area as in panel a. From the fluorescence it can be seen that the expression level varies from cell to cell. (C) AFM topography (AAC mode, 90 x 90 µm) of an area where a highly GPI-(DAF)-GFP protein expressing cell interacts with cells expressing hardly any GPI- (DAF)-GFP. The cytoskeleton was clearly resolved. In addition several connections between individual cells were found. (D) Overlay of topography and fluorescence image.

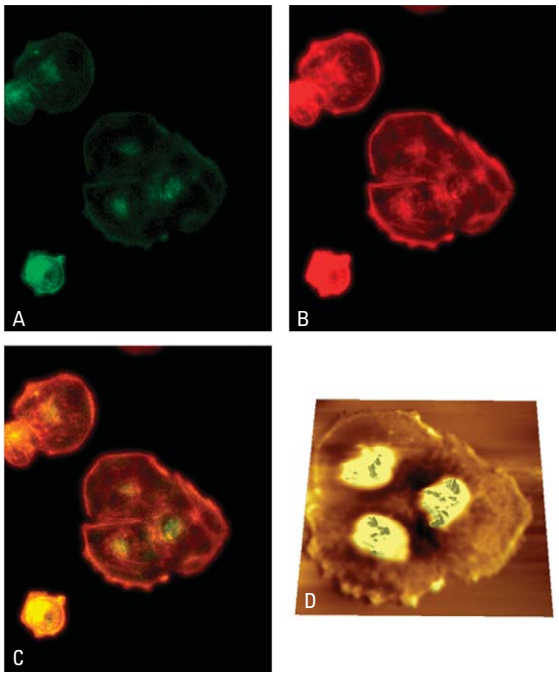

Figure 3. Investigation of phalloidin-rhodamine stained GPI-(DAF)-GFP T24 cells. (A) The GFP fluorescence showed the distribution of the lipid rafts in the cell. Most of the proteins are found around the nucleus. (B) The rhodamine fluorescence allowed determining the location of actin filaments, several of them were found to point from the nucleus to the cell border. (C) The overlay of the previous fluorescence image was used to cross correlate the GPI-(DAF)-GFP position with the cytoskeleton. (D) Topography image of the same cell cluster (MAC mode, 90 x 90 µm).

cells: First, the vitality of the cells was judged using differential interference contrast imaging (DIC) imaging (Figure 2A). Next, the level of GPI-(DAF)-GFP expression in the cells was tested by obtaining GFP fluorescence images (Figure 2B). Bright spots indicated cells which indeed expressed GPI-(DAF)-GFP protein. It was found that approximately only one third of the cells expressed the protein. Additionally, the distribution of the lipid rafts can be determined from the fluorescence image (by changing the focal plane on the optical microscope and analyzing the different sections). As expected, it was found that the protein locates into the plasma membrane. For topographical imaging a protein expressing cell which was surrounded by non-fluorescent cells was selected (black square in Figure 2A). The topographical image of the living cells was obtained using AAC (acoustic AC) mode. The filaments of the cytoskeleton were clearly resolved, as well as presumably lamellipodia at the cell border. In addition several small connections between individual cells were found. The overlay of the fluorescence and topography image (Figure 2D) allowed to allocate certain filaments to cells as well as to cross-correlate the position of GPI-(DAF)-GFP with topographical features, like cytoskeleton filaments or lamellipodia.

Co-localization of lipid rafts and the cytoskeleton

The filaments of the cytoskeleton can be visualized by staining of F-actin, which is one of the most abundant proteins in cells and important for building the cytoskeleton. The cells were fixed and F-actin was stained using phalloidin-rhodamine, a red fluorescent stain for filamentous actin. The GFP fluorescence image of such a sample (Figure 3A) represents the distribution of lipid rafts. In addition, the rhodamine fluorescence image (Figure 3B) indicates the position of the actin filaments. It can be seen that a lot of filaments are reaching from the nucleus to the cell border where a higher concentration of actin was found. Overlaying the GFP fluorescence image with the rhodamine fluorescence image (Figure 3C) showed a high spatial correlation of lipid rafts with the cytoskeleton, at least on this large scale of a full cell. This fact is supported by a cross-correlation coefficient of 0.85, which is significantly different from zero and indicates a linear relationship between lipid rafts and F-actin concentration. The topographical image (Figure 3D) of the cell cluster showed that the height is approximately 4 µm and the size of a single cell is about 60 µm.

Conclusion

We demonstrated that our new and fully integrated AFM – inverted light microscope combination enables an easy and quick investigation of the distribution of cell membrane proteins as well as resolving structural features of the cell itself down to the molecular level. The ease-of-use and convenient handling of this device makes it *THE*

tool for biologists and biophysicists in numerous future applications in life science at the nanoscale, and thereby bridges the world of optics with the world of atomic resolution under physiological conditions.

Material and Methods

T24 cell culture

All T24 cell lines (human urinary bladder carcinoma cells) were a kind gift of Julian Weghuber from the Institute for Biophysics at the University of Linz, Austria. T24 cells were cultured in RPMI 1640 medium (E15-848), containing 10% fetal calf serum, supplemented with penicillin/streptomycin. (all: PAA Laboratories, Pasching, Austria). Cells were passaged twice a week using a dilution of 1:2 and were maintained under a humidified atmosphere with 5% CO_2 at 37° C. For AFM experiments, cells were seeded on glass bottom micro well dishes (MatTek, Ashland, USA) with three different dilutions to obtain an average of 40% confluence after 1 day. Cell were either imaged alive or used after fixing them.

Cell fixation

Before fixation cells were rinsed in HBSS (Hank's buffered saline solution) to remove media components. Cells were then fixed with 4% paraformaldehyde in HBSS over a period of 20 min and washed again in HBSS twice. To quench excess aldehyde cells were kept in 10 mM ethanolamine in HBBS for 5 min. This step is necessary to reduce cell auto fluorescence.

Actin staining with phalloidin-rhodamine

Fixed cells were permeabilized in 0.1% Triton-X100 in PBS for 5 min for subsequent incubation with phalloidin-rhodamine (Molecular Probes, Eugene, USA) diluted 1:100 in PBS over a period of 15 min. Afterwards the cells were washed with HBSS (3 times, 5 min/wash).

Atomic Force Microscopy

AFM experiments were performed using an Agilent 6000 ILM AFM mounted on a Zeiss AxioObserver A1 equipped with DIC and fluorescence options. Glass bottom micro well dishes containing the cells were mounted on the 6000 sample holder. All images were acquired in HBSS buffer at room temperature using contact, AAC or MAC mode as indicated in the figure legends. Contact mode images were acquired using cantilevers with 0.01 N/m. For AAC imaging cantilevers with 0.1, 0.2, or 0.5 N/m spring constant were used. For MAC mode imaging Type VI MAC levers were used.

References

Daniel F Legler, Marie-Agnès Doucey, Pascal Schneider, Laurence Chapatte, Florent C Bender, and Claude Bron. "Differential insertion of GPI-anchored GFPs into lipid rafts of live cells." *The FASEB Journal,* 2004: 03-1338fje.

Julian Weghuber, Stefan Sunzenauer, Birgit Plochberger, Mario Brameshuber, Thomas Haselgrübler, and Gerhard J Schütz. "Temporal resolution of protein-protein interactions in the live-cell plasma membrane." *Anal Bioanal Chem* 397 (2010): 3339-3347.

Stefan Feske, *et al.* "A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function." *Nature* 441 (May 2006): 179-185.

Murali Prakriya, Stefan Feske, Yousang Gwack, Sonal Srikanth, Anjana Rao, and Patrick G Hogan. "Orai1 is an essential pore subunit of the CRAC channel." *Nature* 443 (September 2006): 230-233.

AFM Instrumentation from Agilent Technologies

Agilent Technologies offers high-precision, modular AFM solutions for research, industry, and education. Exceptional worldwide support is provided by experienced application scientists and technical service personnel. Agilent's leading-edge R&D laboratories are dedicated to the timely introduction and optimization of innovative and easy-to-use AFM technologies.

www.agilent.com/find/afm

Americas

Canada	(877) 894 4414
Latin America	305 269 7500
United States	(800) 829 4444

Asia Pacific

Australia	1 800 629 485
China	800 810 0189
Hong Kong	800 938 693
India	1 800 112 929
Japan	0120 (421) 345
Korea	080 769 0800
Malaysia	1 800 888 848
Singapore	1 800 375 8100
Taiwan	0800 047 866
Thailand	1 800 226 008

Europe & Middle East

Austria	43 (0) 1 360 277 1571
Belgium	32 (0) 2 404 93 40
Denmark	45 70 13 15 15
Finland	358 (0) 10 855 2100
France	0825 010 700* *0.125 €/minute
Germany	49 (0) 7031 464 6333
Ireland	1890 924 204
Israel	972-3-9288-504/544
Italy	39 02 92 60 8484
Netherlands	31 (0) 20 547 2111
Spain	34 (91) 631 3300
Sweden	0200-88 22 55
Switzerland	0800 80 53 53
United Kingdom	44 (0) 118 9276201

Other European Countries:
www.agilent.com/find/contactus

Printed in USA, October 25, 2010
5990-6703EN

Agilent Technologies