Scanning Ion Conductance Microscopy of Live Keratinocytes

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Scanning Ion Conductance Microscopy of Live Keratinocytes

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ABSTRACT: Scanning ion conductance microscopy (SICM) is perhaps the least well known technique from the scanning probe microscopy (SPM) family of instruments. As with its more familiar counterpart, atomic force microscopy (AFM), the technique provides high-resolution topographic imaging, with the caveat that target structures must be immersed in a conducting solution so that a controllable ion current may be utilised as the basis for feedback. In operation, this non-contact characteristic of SICM makes it ideal for the study of delicate structures, such as live cells. Moreover, the intrinsic architecture of the instrument, incorporating as it does, a scanned micropipette, lends itself to combination approaches with complementary techniques such as patch-clamp electrophysiology: SICM therefore boasts the capability for both structural and functional imaging. For the present observations, an ICnano S system (Ionscope Ltd., Melbourn, UK) operating in ‘hopping mode’ was used, with the objective of assessing the instrument’s utility for imaging live keratinocytes under physiological buffers. In scans employing cultured HaCaT cells (spontaneously immortalised, human keratinocytes), we compared the qualitative differences of live cells imaged with SICM and AFM, and also with their respective counterparts after chemical fixation in 4% paraformaldehyde. Characteristic surface microvilli were particularly prominent in live cell imaging by SICM. Moreover, time lapse SICM imaging on live cells revealed that changes in the pattern of microvilli could be tracked over time. By comparison, AFM imaging on live cells, even at very low contact forces (<nN), could not routinely image microvilli: rather, an apparently convolved image of the underlying cytoskeleton was instead prevalent. We note that the present incarnation of the commercial instrument falls some way behind the market leading SPMs in terms of technical prowess and scanning speed, however, the intrinsic non-obtrusive nature of SICM imaging leads us to advocate its use for monitoring the most delicate living structures with attendant high spatial resolutions.

1. INTRODUCTION

Investigating the structural and functional character of living cell membranes is a highly challenging process. Scanning probe microscopy (SPM) represents one approach for imaging live cells with nanoscale resolution (Driscoll et al., 1990; Arkawa et al., 1992, Campbell et al. 2001). However, due to the necessary contact the probe makes with the fragile surface of the cell, this invariably leads to some deformation, sometimes damaging the cell, and almost certainly affecting the local reaction of the cell to the probe environment. The risk of damage in such circumstances is greatly reduced by the use of atomic force microscopy (AFM) utilizing appropriately low contact forces, however even then, understanding the image generation mechanism, and its interpretation, remains complicated (Dufrene, 2008). One emerging solution to this challenge is scanning ion conductance microscopy (SICM), which facilitates cell surface imaging under physiological conditions, without physical contact (Hansma et al., 1989, Korchev et al., 1997) but importantly retaining a capability for nanometer scale resolution under optimal conditions.
SICM uses a glass micropipette as the sensitive distal probe, and relies on a [constant] ion current for feedback controlled imaging of even insulating surfaces, submerged within an electrolyte bath. Briefly, as topography sweeps below the scanning pipette, the active ion flow through the aperture will be partially occluded, so that by direct comparison with a set-point ion current, electronic feedback can then control the displacement of the micropipette relative to the target surface and this adjustment is interpreted as target topography. Previously, this technique was restricted to imaging flat surfaces, as the probe had a propensity to collide with the specimen upon encountering vertical surfaces (Novak et al., 2009). Nowadays, a countermeasure has been developed that dispenses with the continuous feedback and raster scan methodology, and where instead the pipette undergoes approach to the surface in order to measure its relative height difference, whereupon it is quickly withdrawn to a position well above the sample, scanned laterally, and the process repeated. This protocol, which is now known as ‘hopping mode’ in SICM parlance (Novak et al., 2009), has proved to be a useful tool for the study of the structures on live cell membranes, and also for studying the pattern evolution of microvilli on cell membranes by time lapse imaging (Gorelik et al., 2003, Novak et al., 2009). The study presented here highlights some of the preliminary data that we have achieved on live HaCaT cells by both SICM and AFM, where the focus for our attention was directed to the appearance of surface microvilli under both imaging regimes. For comparison, chemically fixed cells were also analysed by both methods, as described below.

2. METHOD & RESULTS

A keratinocyte cell line (HaCaT cells), as well as Xenopus laevis kidney epithelial cell line, A6 cells, were cultured separately in Dulbecco’s Modified Eagle’s Medium (DMEM), this solution having been supplemented with 10% (v/v) fetal bovine serum at 37 °C and 5% CO₂ in a humidified atmosphere. These cells were cultured in 35 mm dishes and used for scanning when they were 90 – 95% confluent. For live cell imaging, HaCaT cells were bathed with an iso-osmotic solution similar to the growth medium with pH of 7.4 and an osmolarity of 300 milliosmolar/kg containing (in mM): 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 11.8 HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) and 5.56 glucose before the imaging. To image the fixed cells, 90 – 95% confluent cells in 35 mm dishes were washed with Dulbecco’s Phosphate Buffered Saline (DPBS) solution, followed by fixing with 4% paraformaldehyde in DPBS for 20mins before rinsing and storing in DPBS. The glass micropipette probes were pulled from borosilicate glass on a laser puller (P-2000, Sutter Instrument Co., Novato, CA, USA). Micropipettes had resistances in the range 100 - 200 MOhm when filled with phosphate buffered saline (PBS). Initial studies employed silicon gratings (normally used as calibration objects for atomic force microscopy) to gain familiarization with the technology by optimising scan parameters on a somewhat less challenging target object, but also to validate absolute topography measurements downstream.

![Figure 1: Initial SICM images in (a) birdseye, and (b) 3D perspective topography of A6 cells fixed using 4% paraformaldehyde in DPBS. Note the appearance of microvilli across the cell membrane.](image-url)
In operation, the user-declared reference current is continually measured while the pipette is retracted, and upon extension towards the target, the current is then reduced by some predefined amount based on the user experience with the subject under study, but which might typically be 0.25–1%. The position of the $z$-dimension actuator during this process is then recorded and interpreted as the topographic height differences across the sample. Notably, even a 1% reduction of the current leads to a situation where the pipette is still at a distance of about one inner pipette radius from the surface. The software then forces the $z$-actuator to withdraw the pipette away from the surface and the sample is moved laterally to the next imaging point. By continuously updating the reference current while the pipette is away from the surface, this procedure automatically adjusts for any slow drifts in the pipette current.

Figure 2: (a) SICM observations on live HaCaT cells, imaged under physiological buffer and showing characteristic microvilli on the surface. (b) SICM image of a paraformaldehyde-fixed HaCaT cell - the delicate microvilli structures are preserved somewhat, but evidently not with the clarity of (a) [Note that both images (a) and (b) are presented with the same z-scale.] (c) AFM of a live monolayer of HaCat cells imaged under physiological buffer solution. The intercell junctions are comparable to those observed using SICM however microvilli are not obvious at all. Image windows in (a) and (b) are 30μm x 30μm, whereas (c) has exhibits an image field of 50μm x 50μm.

Figure 2 above illustrates the strength of the SICM approach for live cell imaging: to the left in (a) two live HaCaT cells are imaged under physiological buffer whereas in (b) the same monolayer has been reimaged after chemical fixing according to the prescription detailed earlier. The extraordinary sensitivity of SICM to capture the native structure on live cells, in
terms of, for example, the microvilli which stand proud of the membrane surface, is clearly evident. On fixed cells (b) and where the z projection is identical to that in 2(a), it is clear that the fixing process, whilst ‘freezing’ in the macroscopic cell topography, appears to alter [flatten] the microvilli from their natural state. Most notably however, is the difficulty in obtaining accurate membrane surface structure on such biological targets when regular AFM is employed. Figure 2(c) shows a typical AFM image on live HaCat cells under the same physiological conditions used to capture the image in Fig 2(a) – notably, microvilli can not be distinguished at all, and the topography arising appears to represent a convolved image of the underlying cytoskeletal architecture, this despite the use of sub-nN set-point forces. On fixed cells however (data not shown, but for example highlighted in the paper by Ali et al (2010)) the enhanced rigidity of the chemically modified cells does allow for high resolution imaging of microvilli by AFM. Here, we note that spatial resolutions are typically higher on fixed cells, and that AFM with nominal tips, when compared with SICM, will lead to superior resolution for that circumstance. Moreover, with alternative imaging schemes, such as environmental SEM, it must be noted that the attractiveness of achieving a much wider areal coverage is somewhat undermined by the diminished z-resolution, so that detection of subtle sub-micron topography alteration, as we intend to measure, is unlikely. It would therefore seem that SICM has much to offer those seeking to quantitative measurements on such delicate, sub-micron sized target structures. Previously, the development of distinct topographical signatures on HaCat cells in response to biochemical stimulus has been observed, for example, PAR-2 was seen to upregulate phagocytosis in HaCat cells, resulting in a specific morphological change in podia (Sharlow et al. (2000)) that was characterized by fixing and reimaging in SEM. We anticipate that with SICM’s enhanced facility to image delicate natural topography on live cells, then similar expectations might be achievable in response to [potentially therapeutic] physical stimuli, but with the significant advantages that surface alteration could be monitored in real time, with quantitative and high spatial resolution (in z as well as x-y), and on live target cells.

3. CONCLUSIONS

In summary, we presented SICM images of HaCaT keratinocytes in the live state. We note that intrinsic native structure is captured without interference, and without the need for chemical fixing, which itself alters the appearance of the true topography. Furthermore, we demonstrate that this sensitivity outperforms AFM for routine live cell imaging.

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