Scanning ion conductance microscopy–a tool to investigate electrolyte-nonconductor interfaces

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The scanning ion conductance microscope is a tool to investigate non-conducting surfaces in electrolyte solutions. It is based on the measurement of the change in access resistance arising when an electrolyte-filled glass electrode approaches a non-conducting surface. The resistance changes before a mechanical contact between electrode tip and sample surface occurs and thus allows one to image single living cells repeatedly. Its range of applications ranges from high resolution images of cell surface subsections – allowing one to observe outgrowth and retraction of cell membrane protrusions like microvilli – to lower resolution images of entire cells offering the opportunity to quantitatively determine cell motion, cell swelling and in general changes in cell shape. Here we provide an overview of different modes of application of scanning ion conductance microscopy and a more detailed picture of its application as a tool for the observation of changes in cell shape.

1. Introduction

1.1 Scanning ion conductance microscopy

The scanning ion conductance microscope (SICM), introduced by Hansma in 1989 [1], monitors the topography of nonconducting surfaces covered with electrolyte solution. In contrast to other scanning probe microscopes, SICmicroscopy does not depend on solid surfaces of the scanning sample, but is able to image solid as well as soft samples [1,2].

The working principle of the SICM is as follows: The scanning probe, consisting of an electrolyte filled glass micropipette connected to an electrical amplifier via an Ag/AgCl-electrode is approached through the electrolyte solution towards the nonconducting surface of the scanning sample as shown in figure 1. The different shades of grey indicate the sequence of the approach, lighter ones representing earlier steps. During the approach steps the conductance between the Ag/AgCl-electrode inside the glass micropipette and a reference electrode at a distant location in the electrolyte is monitored. The inset in figure 1 shows the conductance as a function of time. When the electrode tip has closely approched the electrolyte nonconductor interface area (black and dark grey step in figure 1a), the resistance for the ion flux through the electrode opening increases (see the curved arrows in figure 1b) and therefore the conductance decreases (black and dark grey dots in the insert in figure 1b). When the conductance decreases below a previously selected threshhold level (dotted line in figure 1b) the approach of the electrode is stopped and the *z*-position is digitized and stored. The acquisition mode of the next data point depends on the operation mode of the SICM, as explained later in this chapter.

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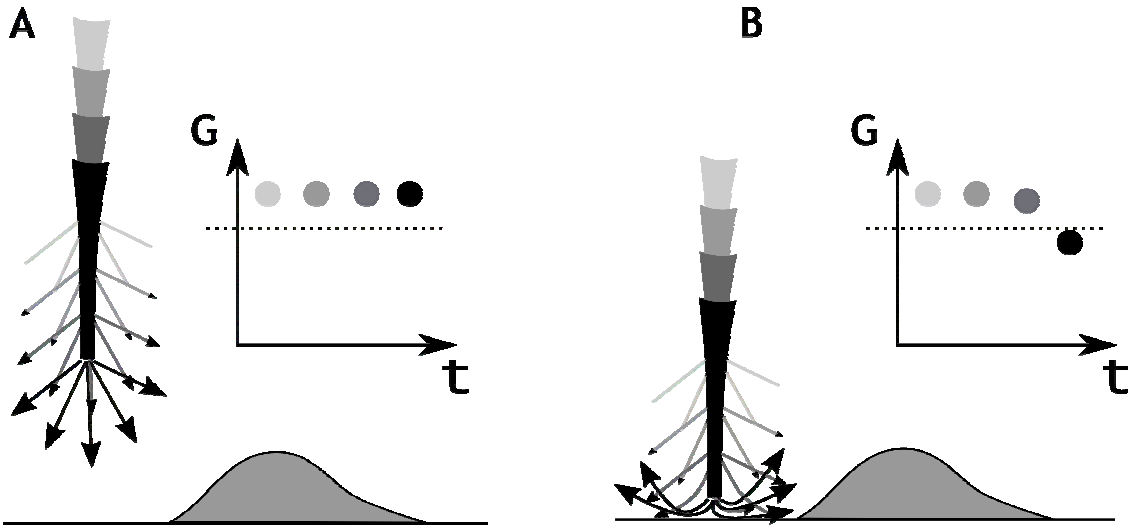

Fig. 1: Principle of operation of a scanning ion conductance microscope. (A) An electrolyte filled glass electrode is approached towards a nonconducting surface and the conductance is monitored. As long as the electrode opening is distant from the electrolyte non-conductor interface the conductance of the system is constant (diagram in A). (B) When the electrode tip is close to the non-conductor the ion flux is disturbed (indicated by the distorted arrows in B) and thus the conductance decreases (inset). When the conductance drops below a previously selected threshold (dotted line in insets in A und B) the advancement of the electrode ist stopped (from Happel, 2007).

Figure 2 shows the mechanical assembly of an SICM. The sample to be scanned is submerged with electrolyte solution in a measurement chamber, in the most simple version the Pertri dish in which the cells had been cultured. The Ag/AgCl reference electrode is inserted at a distance from the sample in the chamber. The scanning electrode is mounted on a *xyz*-axis feedback controlled piezoactor positioned by a personal computer. The resistance of the electrolyte solution between electrode tip and sample surface is monitored using an amplifier as used for electrophysiological measurements connected additionally to the personal computer for monitoring the conductance and data acquisition. The measurement chamber is mounted on an object translation stage of an inverted microscope that offers visual control of the raw positioning of the glass microlelectrode with respect to the scanning objects.

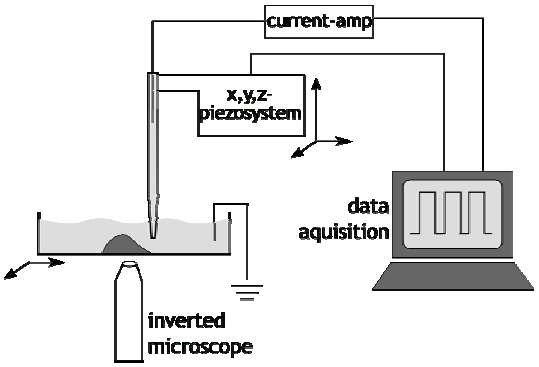

Fig 2: Schematic of the assembly of an SICM. The scannig chamber is mounted on the *xy*-translation stage of an inverted microscope (lower left). The object for scanning (dark grey) is submerged with electrolyte solution (light grey). The tip of the glass microelectrode penetrating into the electrolyte solution is connected to the amplifier headstage which in turn is mounted on a 3-axis piezo system. Amplifier and piezo system are computer controlled.

We introduced a *pulse-mode* SICM [3] that uses current pulses to determine the ion conductance therefore avoiding potential drifts on one of the Ag/AgCl electrodes during longer times of measurement. The computer screen in figure 2 displays these pulses.

Scanning ion conductance microscopy is especially suited to investigate surfaces of living cells, since physical contact of probe and the soft surface of cells, as occurs in atomic force microscopy is avoided. Furthermore, in contrast to most other scanning probe microscopical techniques no preparation of the sample is needed, thus enabling the investigator to study the surfaces of living cells surrounded by electrolyte solution. The first successful recordings of cell surfaces from living cells with an SICM were obtained from mouse melanocytes by Korchev in 1997 [4,5]. In the last years SICmicroscopy has been used to image a vast variety of cell types, including rat neurons [3], rat oligodendrocytes [6,7], mouse myocytes [8] and renal tubular cells from the A6 cell line [9].

Since SICmicroscopy uses electrodes and amplifiers similar to those employed for electrophysiological recordings the SICM is a feasible tool to combine patch-clamp measurements and topographic mapping. Combined measurements revealed the distribution of sodium channels in the cell membrane of cardiac myocytes [10] and offer the opportunity to perform single channel patch-clamp recordings at well defined locations on the cell membrane – even allowing one to investigate specific subcellular structures – and further offers the opportunity to perform patch-clamp recordings on non-transparent samples as intact tissues or cells grown on non-transparent material [11].

Since its first description SICmicroscopy has been combined with different other microscopes to increase its range of applications. Proksch et al. [12] constructed a combination of tapping and contact atomic force microscope with an SICM to obtain high resolution images of topography and conductance of synthetic membranes in ionic solutions. The distance control of the SICM has been used to apply fluorescent molecules close to a biological surface to provide a light source for optical or scanning near-field optical miscroscopy (SNOM) [13,14]. Since the fluorescent molecules in the bath electrolyte are continuously renewed from the inside of the micropipette this light source is not subject to fotobleaching.

1.2 Operating modes of the SICM

Scanning ion conductance microscopes can be assembled in various ways and operate in different modes. Since the conductance is simply given by Ohm's law as $G = R^{-1} = I \cdot V^{-1}$ changes in conductance can be determined by applying a constant voltage and measure the resulting current or measure the voltage required to apply a constant current. These modes are both commonly used in electrophysiological recordings termed current-clamp or voltage-clamp mode respectively.

Voltage or current can either be applied constantly or in pulses. Electrode drifts occurring due to the application of continuous currents can be avoided by the application of current pulses leading to stable recordings. The disadvantage of this method is, however, that due to the electrical capacitance of the electrodes the charging of the capacitor has to be completed before the height of the pulse can be detected. Due to the fact that a capacitor's time constant τ linearly depends on the resistance R of the electrode, the acquisition of high resolution images is slower than using a constant current mode.

Another solution to the problem of stability, especially applied for high resolution images consists in the modulation of the z-position of the electrode. The conductance versus time then becomes sinusoidal after the electrode tip closely approaches to the scanning object and changes in conductance can be detected via a lock-in amplifier. Due to the high signal to noise ratio of the lock-in amplifier this method can detect very small changes in conductance. For high resolution images, furthermore, eletrodes with small opening diameters are required [1]. Since the conductance starts to change at a distance of approximately the opening diameter of the electrode tip from the sample surface [4] detecting conductance changes via a lock-in amplifier is extremely helpful when microelectrodes with small opening diameters are used.

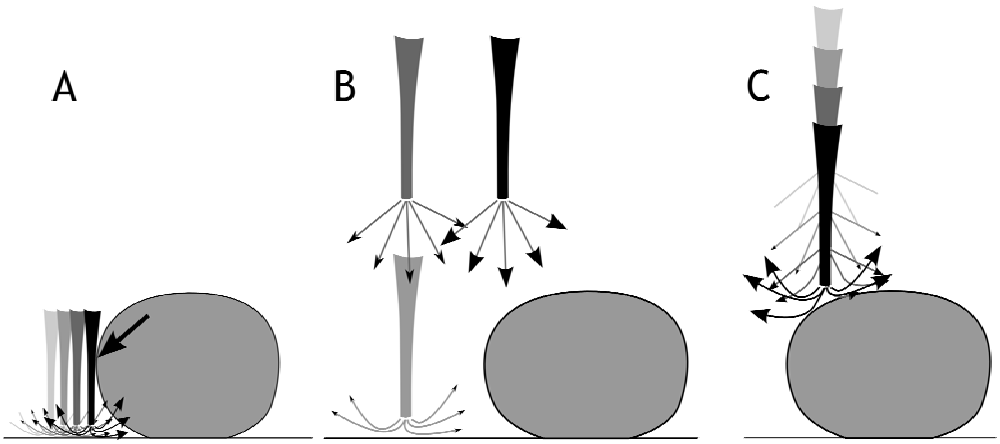

Fig. 3: Comparison of constant distance and backstep mode. (A) In constant distance mode the probe is not able to detect overhanging membrane areas und thus can run into the scanning sample. (B) In backstep mode the electrode (light grey) is dragged back by a predefined step after the approach (mid-grey) and then moved laterally (black electrode scheme). (C) Following the backstep the electrode is approached again detecting the scan sample avoiding damage (from Happel, 2007)

Following the first successful approach to the sample surface, lateral scanning can be performed in various ways. Most scanning probe microscopes use a constant-distance mode that moves the electrode laterally and then modulates the *z*-position to achieve the same conductance as one step before. This is a very fast method providing a high resolution. Lateral steps have to be small to avoid crashes of the electrode into steep slopes of the scanning sample. The small step size in combination with tip sizes, which can be as small as 25 nm [4], results in high resolution images. For obtaining images of whole cells the constant distance mode does not allow one to detect overhanging membrane sections at larger distances from the bottom of the cell culture dish and thus causes damage to the cell and ruptures of the delicate tips of the glass electrodes (see figure 3A). We thus introduced a backstep-mode [2] that allows us to detect these areas prior to collisions of the electrode shaft with steep membrane slopes. After recording a *x*,*y*,*z*-position the electrode is dragged back by a predefined distance (backstep), then moved laterally (figure 3B) and finally approached again to detect the next point of conductance change (figure 3C). This method takes much longer since the electrode tip has to be moved over significanly larger distances. To improve the temporal resolution we further refined our backstep mode by introducing a "floating backstep" [6] mode, which uses a pre-scan of a low spatial resolution of 3 µm. By comparing the *z*-values of consecutive data points the program automatically detects areas of high slopes in the *z*-axis, where larger backsteps are needed. In the following scan with a higher spatial resolution larger backsteps are only used in areas with larger slopes and time-saving smaller backsteps in areas with smaller changes in height. This procedure reduces the area of large backsteps – depending on the topography of the scan sample – to about 30 % of the complete scan area.

2. Investigation of changes in cell shape

We now provide a more detailed view of how SICmicroscopy can be used to investigate changes in the shapes of living cells. While light microscopy is suited to monitor changes in cell shape in a 2D-representation, SICmicroscopy provides quantitative 3D-information at a cellular or even subcellular level.

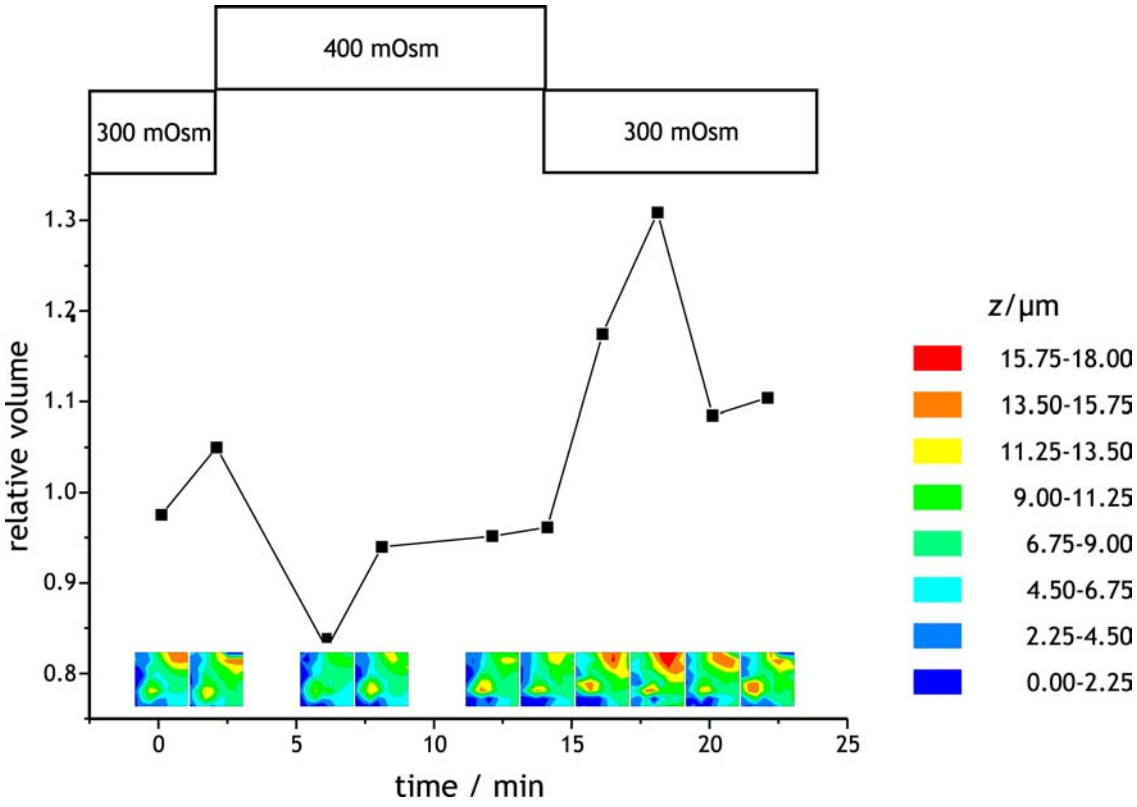

Fig. 4: Volume changes of hepatic cells following fast changes of the osmolarity of the extracellular fluid. To obtain a higher temporal resolution the spatial resolution of the scans was reduced to 64 points in an area of 30 µm × 30 µm (see row of colour coded insets at bottom of the diagram, red areas representing higher *z*-values) With these settings typically about 2 minutes are required to complete a scan. This is fast enough to quantitatively measure regulatory volume changes following osmotic stress. Cell preparation and culture as described in Wehner et al. 1995 [16] and Wehner and Tinel, 1998 [17].

Example 1: The maintenance of a constant cell volume is important for cellular homeostasis. Changes in cell shape thus induce various signalling cascades and induce manifold regulatory reactions (for a review see Wehner et al. 2003 [15]). Figure 4 shows changes in the cell volume of rat hepatocytes in response to a change in extracellular osmolarity from 300 mOsm to 400 mOsm. A superfusion chamber as described in [16] was mounted on the translation stage of the SICM's inverted microscope. Cells were superfused at a rate of approximately 3 mL/min with tyrode solution with an osmolarity of 300 mOsm or 400 mOsm, respectively [16]. Since cellular volume regulation occurs in the range of minutes we chose a spatial resolution of 64 points for the SICM images to reduce scanning time (see lower colour coded images in figure 4).

Figure 4 shows the results of an experiment in which 10 scans were performed consecutively. During the first two scans cells were superfused with a bath solution of 300 mOsm. From scans 3 to 6 tyrode with increased osmolarity was applied (by adding NaCl). The last four scans show the recovery after reperfusion with control medium. The data points in the graph of figure 4 represent the normalized volume, calculated as desribed in Happel et al, 2003 [6], versus time. After application of the medium with high osmolarity an initial shrinkage of the cell down to about 80 % of the control volume occurred, followed by a recovery to about 90 % of the control. This process is known as "regulatory cell volume increase" (RVI). Cell volume then stayed constant until control medium was applied again. After a reactive swelling of the cell to 130 % of control volume the cell volume was downregulated again to

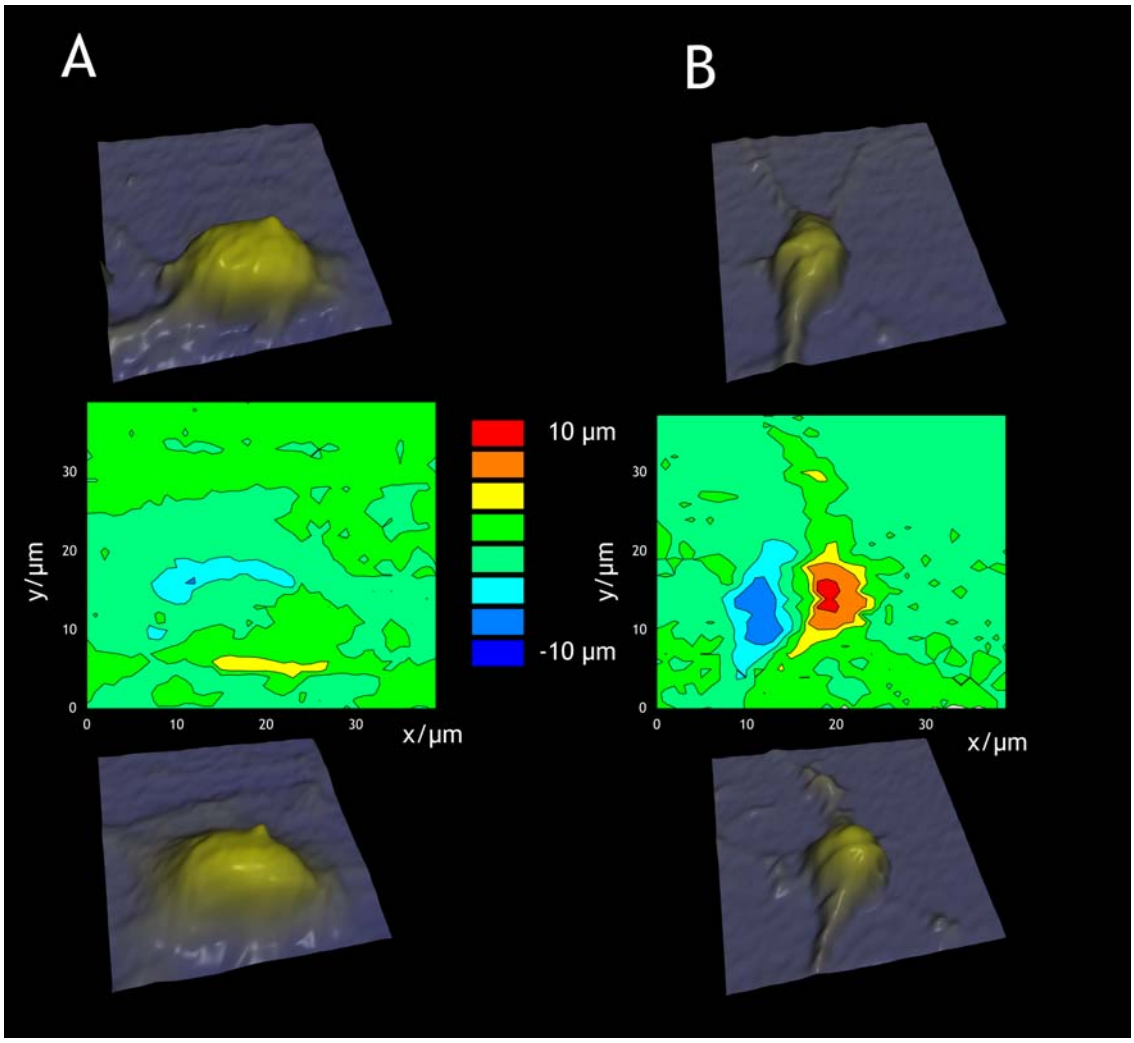

Fig. 5: Comparison of mobilities of a mature (A) and an immature oligodendrocyte (B). Scan area: 40 µm × 40 µm, Step sizes: 1 µm (lateral) and 100 nm (vertical), resistance of the scanning microelectrode: ~5 MΩ. Time between the scans: ~ 70 minutes, scanning time for each image ~ 20 minutes. Colour coded difference images, calculated by subtracting data points of consecutive scans (see [6]) show changes in cell position between both scans, areas of height increase labelled in red and areas of height decrease labelled in blue. Data smoothed using surface subdivision [19] and images were obtained via POVRay-raytracing tool (http://povray.org). Cells prepared and cultured as described in [20].

about 110 % of control volume (regulatory volume decrease, RVD). This example shows, that even with a low spatial resolution the SICM can be applied to monitor cell volume changes reliably in the range of minutes.

Example 2: We now tested whether the SICM can be employed in long-term recordings to observe quantitative changes in cell shape during migration, a characteristic feature of many living cells. As an example we chose to monitor the movement of mature and immature oligodendrocytes from rat brain. Figure 5 shows the comparison of the mobility of a mature oligodendrocyte (figure 5A) and an oligodendrocyte precursor cell (figure 5B). Time between the displayed scans was about 70 minutes.
The colour coded difference images shown in the centre between the two images obtained from each cell reveal that the immature cell had a higher mobility than the differentiated cell. The yellow and red areas

represent points in space, where new membrane has appeared during the time between the two scans, light blue areas represent spaces of lower height, from which the cell has moved away. Green areas represent spaces in that no change in height had occurred. While the immature cell (figure 5B) moved about 5 µm towards the positive *x*-direction, the mature oligodendrocyte (figure 5A) moved only 1 µm towards the negative *y*-direction corresponding to velocities of 4.29 µm/h and 0.86 µm/h, respectively. This example confirms the expectation, that immature cells, which *in vivo* migrate from their sites of origin into distant tissues are much more mobile than differentiated cells.

3. Discussion

Scanning ion conductance microscopy is a recently developed tool to investigate the soft membrane topography of living cells. Due to its contactless height control it allows one to image the delicate soft surfaces of biological samples. Its applications range from the observation of the outgrowth of microvilli to the quantitative determination of cellular volume changes allowing additionally the combination with other techniques, such as patch clamp recording and atomic force microscopy. In contrast to conventional light microscopy, which only provides 2D-data the three dimensional information offered by the SICM permits a more precise estimation of cell volume, even when it is applied at a resolution of more than 500 nm. Unlike fluorescence methods, like confocal laser microscopy, this method avoids photobleaching. Furthermore, no complex reconstructions of the cell shapes from serially scanned images are needed. In addition to other cell volume measurements possible under phase contrast optics [18] it offers a higher resolution thus allowing the investigator to additionally differentiate between soma and neurite volumes and surface areas in cultured cells [7].

In this chapter we have presented two examples that extend the known applications of the SICM in two respects. The first example illustrates that the SICM is fast enough to be applied for the investigation of regulatory volume changes induced by osmotic challenges within minutes. The second example shows that the SICM can also be employed to observe changes in the mobility of cells. As expected, after two successive scans at a time interval of 70 min, the differentiated oligodendrocyte showed a smaller displacement while the immature cell had moved by several µm. Since the SICM offers full 3D-information of cellular shapes during migration on planar substrates it could thus help to unravel hitherto unknown aspects of membrane surface changes during cellular migration.

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