Scanning Ion-Conductance Microscopy

Variable Temperature Live Cell Morphological Studies

We report on the development of a simple heating device integrated into a scanning ion-conductance microscope (SICM) allowing the investigation of the morphology of live cells, maintained at different temperatures. The shape and volume of the cells could then be measured with nanoscale precision. With SICM, the live cells are not typically physically compressed, which can be the case for atomic force microscopy (AFM) methods [1].

SICM [2] can be used to produce high-resolution topographic images of live cells in their respective physiological buffer [3] in a noncontact regime - topographic features with lateral dimensions less than 14 nm have previously been resolved using SICM [4]. For this scanning probe microscopy (SPM) technique, the sample is immersed in an electrolyte and a nanoscale pipette is used as the probe, as shown in figure 1a. The ion-conductance through the aperture of the nanopipette is used as the feedback parameter in order to generate topographic images of a surface, thus electrolytic cell culture medium is ideal for SICM imaging. Hopping probe ion-conductance microscopy (HPICM) [5] has further improved the imaging of live cells (where there are large variations in topography several micrometers in height over nanometer lateral dimensions), by 'hopping' the probe over the surface, rather than rastering over the target area, thus minimizing any damage to the surface. However, SICM imaging of live cells is typically only performed at temperatures of 22-28°C. [2,6] Under these conditions the cells are typically still alive, provided with the required nutrients and maintained at physiological pH. However, as the cells are not maintained at human body temperature (37°C), their behavior may not be a true representation of in vivo conditions.

This is in contrast to optical techniques such as confocal microscopy, which are commonly used to image live cells at 37°C over many hours. However, confocal microscopy cannot achieve the same nanoscale-resolution as SPM techniques, and imaging cells with confocal techniques generally requires the use of exogenous fluorophores.
SICM Instrumentation and Temperature Control

Our ICnano S scanning ion-conductance microscope (Ionscope Ltd., Herts, U.K.) allowed a maximum (lateral) sample scan size of 100 μm x 100 μm, with a 25 μm range in the Z-axis (height).

Two Ag/AgCl electrodes were used for ion current measurements, with the reference electrode positioned in the sample electrolyte, as shown in figure 1a. A high-gain amplifier connected to the working electrode inside the nanopipette provided a bias (+200 mV for all measurements detailed) and also monitored the resulting ion current (typically 0.5-1.5 nA in magnitude) flowing through the pipette aperture. Nanopipettes were produced from borosilicate single capillaries with filament, pulled using a laser-based pipette puller to achieve approximately 50-100 nm diameter apertures.

The ICnano SICM was mounted on an Olympus IX71 inverted microscope, allowing bright field optical imaging of transparent samples. A controlled heating unit was designed and constructed to fit directly into the SICM sample holder to allow localized heating of the sample whilst the sample surface was imaged, as shown in figure 1b. The heater element was constructed from a 1.1 mm thick glass slide with a 15-30 nm indium tin oxide (ITO) thin film covering one side of the slide. This heater element was resized to 22 mm x 22 mm to fit into the heater base. Electrical contacts were made to the ITO thin film and connected to the power supply unit, which was used to control the electrical current that passed through the heating element and adjust the temperature. The temperature was then monitored using a thermocouple.
A Petri dish could be positioned inside the heater unit so the bottom of the sample Petri dish was then in contact with the top of the ITO heater element, thus the heater provided localized temperature control rather than heating a substantial amount of the SICM set-up.

The response of the heater was found to be very rapid, with the temperature reaching equilibrium within 10 min, at which point there were no recordable image artifacts caused by thermal fluctuations and the heater provided an absolute temperature variation of ~0.5°C over several hours. A temperature feedback loop was not employed for the heater current in order to avoid image artifacts due to thermal fluctuations, but instead the heater current was set at a fixed current output, determined by a prior calibration, for any required temperature value.

**Cell Morphology Studies**

The MG-63 cells, derived from human osteosarcoma, were immersed in a complete CO2-independent medium to allow SICM imaging of the cells at physiological pH level. Figure 2 shows the development of an MG-63 cell maintained at 37°C over 12 hours. Initially quasi-spherical in shape, the cell flattens over time and spreads over the surface. From figure 2, the greatest rate of change of morphology occurs during the first 4 hours after seeding and visually the variation in morphology after the cell has been seeded for 8 hours is less significant. A fast rate of cell adhesion is implied, as the cell rapidly spreads over the surface and decreases in thickness [7].

In comparison, MG-63 cells that were maintained at 22°C (no current supplied to the heater element) did not spread over the surface as rapidly as those maintained at 37°C, as shown in figure 3c and 3d, which compares the SICM images of two MG-63 cells after 12 hours, one at 22°C and one at 37°C. The corresponding bright field optical images (10x magnification) of cells at 22°C and 37°C after 24 hours are also shown in figure 3, revealing how the cell behavior at each temperature is consistent for many cells maintained at that specific temperature.

This implies that the true behavior of certain cells may not be understood by imaging them with SICM at temperatures less than 28°C. It appears that the morphological changes of the cell are particularly affected by temperature at the early stages (less than 2 hours) of attachment.
Figure 4 shows the variation of MG-63 cell thickness, footprint and volume over time for live cell temperatures of 22°C, 28°C and 37°C. This was calculated by performing a series of image processing operations on the SICM topographic data using a MATLAB algorithm. The maximum cell thickness was the distance from the Petri dish surface to the point of the cell furthest from the Petri dish surface, whilst the cell footprint was the projected cross-sectional area of the cell in the vertical direction. The cell thickness and footprint data shown in figure 4 were ascertained from consecutive SICM images of a single live cell for each temperature. Due to the high lateral and vertical resolution capability of SICM, the cell volume could then be precisely calculated from the same thickness and footprint data, [8,9] without requiring any fluorescent labelling.

The results in figure 4a show quantitatively how the thickness of the cell reduces at a faster rate when the live cells are maintained at higher temperatures. Figure 4b also shows how the cell footprint increases over time, with greater rates of increase for temperatures greater than 28°C. However, the experiments performed at 22°C show a very different behavior with a linear decrease in thickness and linear increase in cell footprint over time.

The variation in cell volume for cells at different temperatures is shown in figure 4c and illustrates how the cells attempted to maintain an optimal cell volume. The cells at 22°C and 28°C only varied from a mean value by a maximum of ~5%, as the cell volume was corrected by the cell to maintain an approximately constant volume. The cell maintained at 37°C was initially only 65% the volume of the optimal-volume MG-63 cells and increased in volume towards the optimal value of ~1900μm3. This smaller initial cell volume is attributed to the point at which the cell was trypsinised in the cell cycle. Initial cell volumes from a selection of other cells are included in figure 4c as a guide to the range of cell volume post-seeding and the increase in cell volume was correlated to initial cell volume rather than sample temperature.

In summary, a bespoke heater, constructed with an ITO thin-film as the heater element, has been integrated into an SICM system to allow the accurate control of the temperature of samples during imaging, in order to image live cells at human body temperature and in a physiological medium. The cells were found to attach to and spread over the surface of the Petri dish more readily at human body temperature (37°C) and were revealed to spread over the substrate at a slower rate at room temperature [10]. These results show that although an SICM can be used to non-invasively investigate the morphology of live cells at high resolution, some form of temperature control is required to study cells at their optimal conditions.
where their natural mechanisms are revealed, and may be necessary depending on the characteristics of the cell that are being studied.

**Acknowledgements**
This work was funded by the National Measurement Office. The author wishes to thank colleagues at the National Physical Laboratory (NPL) who contributed to this work; Dr Debdulal Roy and Dr Charles Clifford, Mr Michael Shaw for MATLAB analysis and Mrs Nilofar Faruqui for providing the live cells.

**References**

**Contact**
**Dr. Andrew Pollard**
National Physical Laboratory
Surface and Nanoanalysis Group
Teddington, UK
www.npl.co.uk/nanoanalysis

---

**Contact**
**National Physical Laboratory - Surface & Nanoanalysis Group**
**Hampton Road**
**Teddington, Middlesex**
**United Kingdom**