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Single-Cell Migration as Studied by Scanning Electrochemical Microscopy

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ABSTRACT

Scanning electrochemical microscopy (SECM) was used to study the migration of single live head and neck cancer cells (SCC25). The newly developed graphite paste ultramicroelectrode (UME) showed significantly less fouling in comparison to a 10 μm Pt-UME and thus could be used to monitor and track the migration pattern of a single cell. We also used SECM probe scan curves to measure the morphology (height and diameter) of a single live cancer cell during cellular migration and determined these dimensions to be 11 ± 4 μm and 40 ± 10 μm, respectively. The migration study revealed that cells within the same cell line had a heterogeneous migration pattern (migration and stationary) with an estimated migration speed of 8 ± 3 μm/h. However, serum-starved synchronized cells of the same line were found to have a non-heterogeneous cellular migration pattern with a speed of 9 ± 3 μm/h. Thus, this non-invasive SECM-based technique could potentially be expanded to other cell lines to study cellular biomechanics for improved understanding of the structure-function relationship at the level of a single cell.

INTRODUCTION

Cell migration occurs both collectively and at the single-cell level. Collective migration, also called the migratory stream, plays a crucial role in embryonic morphogenesis[1] and tissue homeostasis[2] and contributes to several important pathological processes such as cancer invasion and metastasis formation[3]. The study of collective migration, however, provides no information about the migratory mechanism of an individual cell. The study of the mechanism(s) of cellular migration at the single-cell level is therefore important for improved understanding of the basic biology underlying both homeostasis and pathological conditions. Single-cell migration is a complex, multistep process that is generally initiated by protrusion of the cell membrane, driven by actin polymerization, and further stabilized by adhesion to the extracellular matrix[4], depending on the cell's morphological, structural, and functional characteristics.

Several techniques, including time-lapsed microscopy, have been used extensively to study cell migration[5,6,7,8]. A number of studies have reported the use of fluorescence microscopy to image a monolayer of cells and then identify individual cells to quantify single-cell migration[9]. A major drawback of these fluorescent-based techniques is prolonged exposure to a high-intensity light source, which causes irreversible bleaching of the dyes and damages live cell samples. Several other techniques, such as transwell migration and wound closure, have also been reported for the study of cellular migration[10]. However, these techniques use phase contrast microscopy and lack the resolution to monitor a single cell. In addition, live cells are translucent, making it difficult to locate and track their exact location in a dish with a phase contrast microscope. Other scanning probe techniques such as atomic force microscopy and scanning ion conductance microscopy

(SICM) have good resolution and were previously used to study single cells, but they have a small working range and limitations in tracking cell migration. [11,12,13]

Scanning electrochemical microscopy (SECM) provides an ideal scanning probe technique that can be used to overcome all of these analytical challenges. This technique is noninvasive and has a large working range (10 to 1,000 μm), which is suitable for tracking a relatively large cell with a wide migration range. Several studies have reported[14,15,16] the investigation of single live cells with SECM. Koley and Bard[17] reported using SECM to measure the permeability of a single cell to highly hydrophilic molecules such as ferrocyanide, in addition to measuring the morphology (height and diameter) of a single live cell. Live cell imaging has also been reported for alternating current mode SECM without using a redox couple. Diakowski and Ding[18] were able to measure the change in the height of a cell and to monitor cellular activity by the addition of ethanol and phorbol-1,2-myristate-acetate-3. Schuhmann's group [19] combined an inverted optical microscope with SECM to facilitate tip positioning and used SECM in shear-force feedback mode. This Bio-SECM has also been able to detect catecholamine-releasing chromaffin cells among single secretory vesicles. Li and Bard[20] reported using ferrocene methanol as a redox molecule indicator to study the viability of a live HeLa cell. Bergner et al.[21] reported the use of SECM to determine the passive transport of hydrophilic redox mediators such as ruthenium hexamine chloride or lipophilic ferrocene methanol molecules across a monolayer of epithelial cells. SECM has also been used to monitor the respiration of a single cell in the presence and absence of potassium ferrocyanide and has further been reported to have good correlation with fluorescent assays that use fluorescent calcein-AM dye.[22] To the best of our knowledge, however, no

study has reported the use of SECM to quantitatively monitor single-cell migration under physiological conditions.

Herein, we report the use of SECM to study isolated single-cell migration in the presence or absence of an external stimulus such as serum that contains different growth factors for improved viability and cell proliferation. We also used a graphite-paste-packed ultramicroelectrode (UME) for the first time to study the biomechanics and morphology of a single live cell. These electrodes show significantly less biofouling than a similarly sized Pt-UME in the presence of complex cell culture media. SECM imaging and scanning probe techniques were used to track and quantify cellular migration, in addition to monitoring morphological changes in the cells in terms of height and diameter.

EXPERIMENTAL

Chemicals: Graphite powder (7 to 11 μm size), potassium ferrocyanide, and potassium chloride were purchased from Alfa Aesar. Silicone oil was obtained from Sigma Aldrich. All purchased chemicals were of highest purity and were used without further purification. The solutions were prepared by using 18 MΩ deionized water (ELGA water system). All electrochemical experiments were performed with SECM (CHI 920D, CH Instruments, Austin, TX).

The head and neck squamous carcinoma cell line (SCC25) was obtained from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium, Ham's F12 medium, fetal bovine serum, hydrocortisone, antibiotic/antimycotic (Cnt-ABM10/ABM20), and dithiothreitol were all purchased from Sigma Aldrich (St. Louis, MO). Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN).

Cell Culture: The SCC25 (#30-2006; head and neck squamous cell carcinoma) cell line was used in this study. Cells were cultured as a monolayer in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% fetal bovine serum (Gibco-Invitrogen Corp., Waltham, Massachusetts, USA), hydrocortisone (final conc. 0.4 μg/ml), and 1× antibiotic/antimycotic (Cnt-ABM10/ABM20). When the cells reached confluence of 75-80%, they were split and used for further analyses. All cells were grown and maintained in polystyrene-coated culture flasks in a humidified atmosphere with 5% CO_2 at 37 °C for all experiments.

SECM Experiments.

Graphite-packed UME: The graphite powder and paraffin oil were weighed in a 70:30 w/w% ratio on a watch glass. A glass rod was used to mix the components thoroughly to obtain the graphite paste. The graphite paste was stored in a glass bottom dish in a desiccator. A platinum UME (Pt-UME; 10 μm diameter) was made as described in reference.[23] A Pt-UME of RG 5 was then electrochemically etched in a solution of 60:36:4 (v/v) $CaCl_2$:H_2O:HCl to obtain a 15 μm cavity by applying 5 V AC. The UME cavity was further cleaned by sonication in water followed by ethanol, and then dried under a stream of nitrogen gas. The UME cavity was then packed with graphite paste by repeatedly tapping the UME on the graphite paste. The extra paste sticking on the glass surface of the UME was wiped off with a wet Kim wipe. The packing was confirmed by observing graphite-packed UME under an optical microscope and by measuring its resistance in 0.1 M KCl solution vs. Ag/AgCl. Electrochemical characterization of the 10 μm graphite-packed UME was performed before each SECM experiment by recording the cyclic voltammogram in a 2 mM ferrocyanide and 0.1 M KCl solution.

SECM imaging: A petri dish containing the cells was taken out of the incubator when the appropriate cell coverage was obtained. For all single experiments, cell coverage on the petri dish was maintained at approximately 30% coverage at the center of the dish to increase the chance of finding an isolated single cell. The petri dish was mounted on the SECM stage, along with a counter (0.5 mm Pt wire) and a reference electrode (Ag/AgCl), as shown in Figure 1A. The petri dish was first incubated at 23 °C for 1 h and then the cell culture medium was replaced carefully with the same cell culture medium containing 2 mM ferrocyanide. Cell viability was unaffected in the presence of a 2 mM ferrocyanide solution. The cell culture medium contained 110 mM NaCl and no additional supporting electrolyte was added during the electrochemical measurements.

A graphite-packed electrode or a 10 μm diameter SECM tip was used as a working electrode to obtain the approach curves and to perform all single-cell imaging. A diffusion-controlled potential at +0.4 V vs. Ag/AgCl was applied to the working electrode for all SECM single-cell experiments. Assigning an initial position to the cell is important for calculating the total linear distance traveled by a single cell within a specified period. Since the cell is constantly moving, the only way to fix the initial position of such a moving target is to fix its position with respect to time. Since the present sample did not move fast enough in comparison to the imaging time, we fixed the position of the cell w.r.t time. This method may not be suitable, however, for a fast-moving cell type or when a fast scanning speed is required so that the cell does not change its position during the scanning period.

High-speed SECM images were first recorded in a large working range of 500 μm × 500 μm to identify individual cells. After the single cell was located, a smaller working range of approximately 150–200 μm was used to zoom in on a single cell. The tip-dish distance was always maintained between 16 and 20 μm to avoid any accidental damage to the live cell. After locating a single cell, we recorded time-lapse SECM images of it over a 4 h period at 25 μm/s in a 150 × 150 μm or 200 × 200 μm X-Y distance window to study its migration. Before we performed the SECM experiments, the substrate (petri dish) tilt was always fixed over a distance of 5,000 μm.

Probe scan curves: Besides identifying and tracking a single live cell by using the SECM imaging technique, we also performed a probe scan to quantitatively estimate the speed of migration of a single cell, as well as its morphology (height and diameter). Both X-direction and Y-direction probe scan curves were performed at fixed tip-substrate (dish) distances over a single cell to estimate the morphology. The position at the top of the cell was obtained by choosing the X and Y coordinates from the peak position in the X and Y probe scan curves, respectively. This procedure was repeated to find the coordinates of the top of the cell as the cell migrated over time. This technique provided the information about the change in cell morphology

with respect to cell migration. The height of the cell was estimated from the percentage of current drop observed against the background current in the probe scan curve.

RESULTS AND DISCUSSION

Characterization of graphite-packed UME: The 10 μm diameter graphite-packed SECM tip (UME) was prepared (Figure 1B) before each SECM experiment. The electrode was characterized by running a cyclic voltammogram in a freshly prepared 2 mM ferrocyanide and 0.1 M KCl solution (Figure S1A). The calibration of a graphite-packed UME with ferrocyanide is shown in Figure S1B. To obtain a time-lapse SECM image of a single cell over 4 h, it is essential to obtain a working SECM tip that shows less drift in the background current to record a better-contrasted image. Figure 1C shows the negative feedback approach curve on an insulated petri disk containing 2 mM ferrocyanide solution. The experimental approach curve (Figure 1C black line) shows good fit with the theoretical curve (Figure 1C, red line).[23] This result confirms that the newly developed graphite-packed UME has similar behavior to that of Pt-UMEs or Au-UMEs. Therefore, the graphite-packed UME was used for subsequent SECM studies.

Migration of a single live cell: After the initial electrochemical characterization of the graphite-packed UME, we performed SECM imaging in negative feedback mode to obtain the topographical features of the live cells, as shown in Figure 1D. In Figure 1D, the current over the surface of the cell was lower (red color) than the background current (blue color) because of the blocking of redox species (Figure 1B) by the topographical features of the cell. For comparison, a similar experiment was performed with a 10 μm diameter Pt-UME, as shown in Figure S2. Because of the fouling of the Pt surface, the tip was deactivated and hence the gradient in the background current was observed, resulting in a low-contrast image. On the other hand, good electrical conductivity and less fouling

of the electrode surface of the graphite-packed UME gave a stable background current (Figure 2) during scanning, even in the presence of a protein-containing complex cell culture medium, which allowed us to obtain sharper contrasted images and hence the exact morphology and position of the single cell. The 3D graphical representation of the single-cell SECM image is represented in Figure 1D, from which we measured the diameter of a single cell as 23 μm.

In the current experimental setup, the cells were typically 20 μm in diameter and 10 μm in height and the working window had to be at least 150 ×150 μm to monitor cell migration. In addition, constant height mode SECM imaging allowed us to monitor the morphology at the same time. Therefore, to track the path and speed of migration of a single cell, we recorded time-lapse SECM images in negative feedback mode at a constant tip-substrate/petri-dish distance over a fixed window of X and Y distances. Figure 2A shows a representative image of a single cell at t = 0 and t = 4 h. The cell migrated up to 24.5 μm from its initial position. Figure 2B, on the other hand, represents a case in which a cell migrated only about 7.5 μm from its initial position, which is negligible compared with the cell's diameter. This observation demonstrates that significant variation in biomechanical properties (such as migration) occurs at the single-cell level between cells of a given cancer cell line and suggests the existence of cellular heterogeneity within a given cell line. This is an important observation from a biological perspective, as most of the existing analytical techniques for the study of cellular migration involve a collection of cells rather than single isolated cells. The representative 3D SECM images of a migrating cell are shown in Figure 3. In Figure 3B, a large change in current over the cell is observed because of a change in cellular height of 3 μm. This

result shows that the technique can be used to monitor small changes in cellular morphology because the signal is amplified (due to the nonlinear relationship of the SECM approach curve) by a small change in tip-substrate distance. From Figure 3, we calculated that the cell migrated 13.4 μm within 55 min.

We further investigated whether random cellular migration occurs in well-synchronized cells of the same cell type. The cells were subjected to serum starvation for 10-12 h and then the culture medium was replaced with regular cell culture medium that contained serum. We performed similar time-lapse SECM imaging experiments at a constant height to track single-cell movement. We observed uniform migration for all of the synchronized cells, confirming our hypothesis that cellular migration is heterogeneous within the same cell line unless the cells are synchronized to be in the same phase of the cell cycle. A set of time-lapse 2D images recorded on the same cell over 4 h is represented in Figure S3. Figures S3A and B show the set of SECM images for non-serum-starved and serum-starved synchronized cells over 4 h, respectively. These results confirm that changes in cellular migration behavior at the single-cell level can be observed and studied quantitatively by using the SECM technique.

To obtain a more quantitative estimation of migration speed, we performed a series of time-lapse X and Y probe scan curves over a single cell and also recorded SECM images. After obtaining the SECM image, we performed a series of X and Y direction scans to identify the exact coordinates of the top of the cell. A typical X-direction probe scan curve is shown in Figure S4. The position of the maximum dip in the curve represents the maximum topographical height of the cell or the top of the cell. The calculated X_{top} and Y_{top} coordinates were then plotted, as shown in Figure 4A and B, to estimate the total

linear distance traveled and hence to calculate the speed of migration of a single live cell. In addition, we observed that the migratory path of a single cell follows a zigzag pattern instead of a straight-line trend (Figure 4A and B). Therefore, we used the total linear distance traveled by the cell divided by the total time to calculate the speed of migration. As observed in Figure 4B, the non-migrating cell also traveled in a zigzag pattern, but for a relatively short distance (3 μm over 4 h). Thus, it is essential to assign a threshold value to differentiate between migrating and non-migrating cells. K-means clustering was used to distinguish between migrating and non-migrating cells (see Table S1 for more details). A speed of 5 μm/h was determined as the threshold limit, above which the cell was designated as migrating. Table 1 shows the migration speeds of migrating, non-migrating, and serum-starved synchronized single cells. It is worth noting that the serum-starved synchronized cells showed similar migratory behavior compared with that of the non-synchronized migrating cells. Both cell types migrated at an average speed of 8 to 9 μm/h, whereas the speed of the non-migrating cells was 3 μm/h.

Morphology of a single live cell: To determine whether any correlation exists between morphological changes and migration pattern, we studied the morphology of a single SCC25 cell by using the probe scan curve in negative feedback mode at a constant probe-substrate distance. We calculated the height of the cell from the percentage of the current drop (peak current) against the background current during a probe scan curve (Figure S4) by using the theoretical negative feedback approach curve (Figure 1C). The cell diameter was calculated from the peak width of the probe scan curve (Figure S4). Table 1 indicates the average height and diameter of the cell for migrating (11 ± 4 μm, 40 ± 10 μm,

respectively), non-migrating (16 ± 2 μm, 47 ± 4 μm, respectively), and serum-starved synchronized cells (13 ± 4 μm, 40 ± 12 μm, respectively). As shown in Table 1, no significant correlation exists between migration pattern and cellular height and diameter. It is well-known that cellular migration steps include drag, roll, and pull, which might involve a change in the morphology of the cell. However, no such observation was made in the present study. This could be due to the limitations of the SECM probe size (10 μm diameter), which is unable to record such small changes in cellular morphology during migration. A smaller SECM probe of submicron diameter might improve the resolution and thus be able to monitor small changes. This technique is currently under investigation in our laboratory. Additional systemic studies will be necessary to establish the correlation between cellular morphology and functions.

CONCLUSIONS

We have fabricated a graphite-packed UME (10 μm diameter) and used it as an SECM tip to image a single live cell. This graphite-packed electrode showed significantly less biofouling than that of a 10 μm diameter Pt-UME and thus increased the capability of observing a live cell over a period of 4 h. Hence, this modified electrode might be a good alternative to the carbon fiber electrode (given the challenge of fabricating a 7 μm carbon fiber electrode). We also developed a new SECM-based method to study single-cell migration and morphology in a model cell line (SCC25). Thus, SECM might be a powerful tool to study single cell biomechanics under varied chemical gradients and is currently under investigation in our laboratory.

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FIGURES AND TABLES

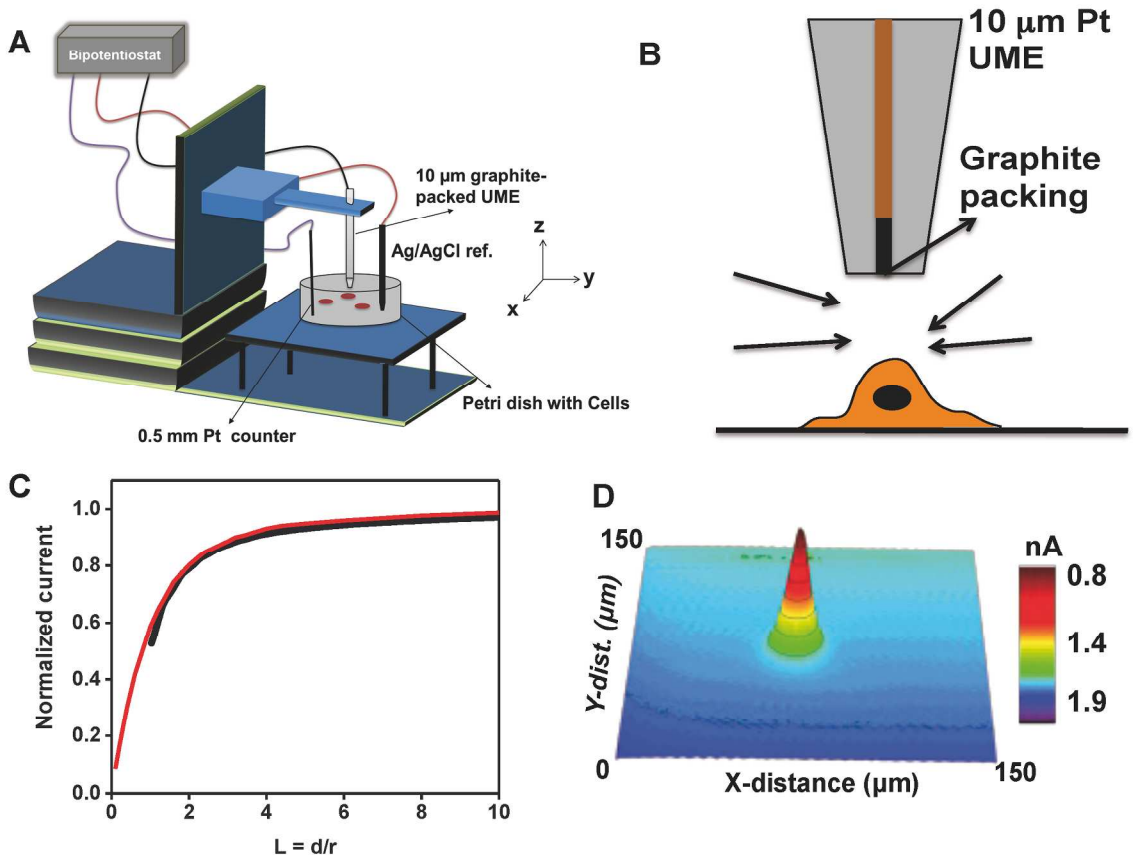


Figure 1: (A) Schematics of the SECM setup used for single-cell imaging and morphology measurements. (B) Schematics of the graphite-packed ultramicroelectrode (UME) used with SECM. The schematic also shows the diffusion blockage of the $[Fe(CN)_6]^{4-}$ redox couple as it approaches the cell surface. (C) Probe approach curve (black line) over the petri dish using a 10 µm diameter graphite-packed UME (RG = 5). The red line represents the theoretical approach curve of the same RG. (D) SECM image showing the topographical features of a single live cell. The image was taken at a constant distance of 17 µm above the dish using a 10 µm diameter graphite paste UME.

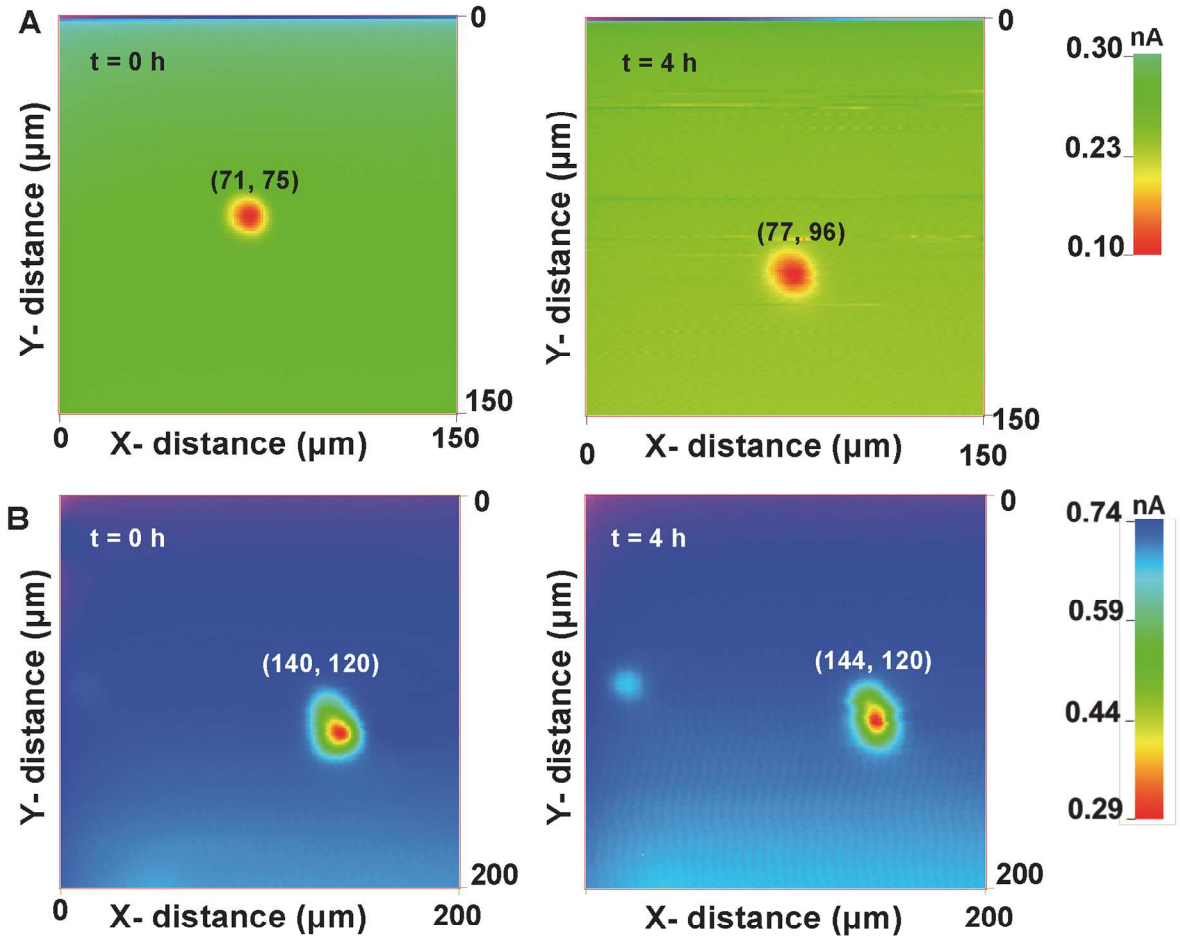


Figure 2: Time-lapse 2D SECM images of a single cell over 4 h using a graphite-packed UME. Tip-substrate distance: 17 µm. (A) A migrating single cell. (B) A non-migrating cell.

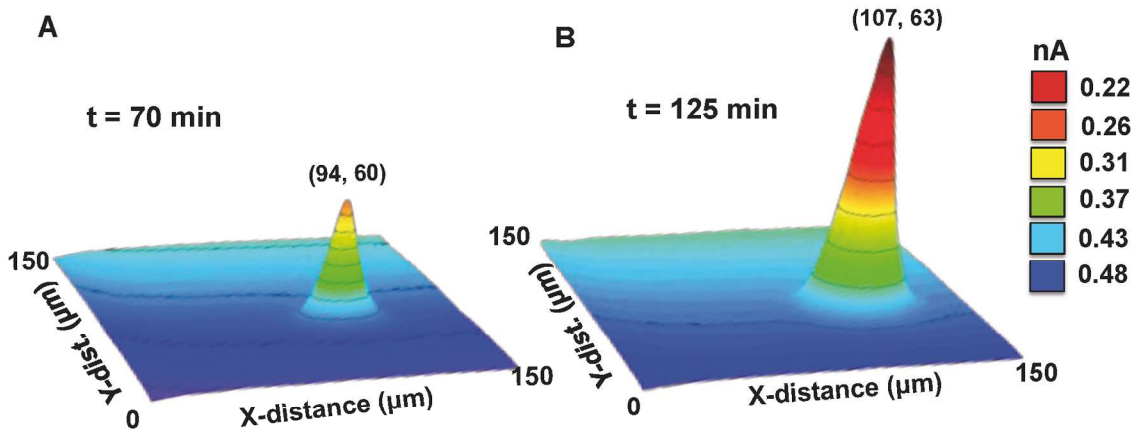


Figure 3: Representative SECM images for simultaneous monitoring of morphology and migration pattern of a single live cell. (A) t = 70 min; position: (x: 94 and y: 60); height and diameter are 12 and 32 μm, respectively. (B) t = 125 min; position: (x: 107 and y: 63); height and diameter: 15 and 50 μm, respectively.

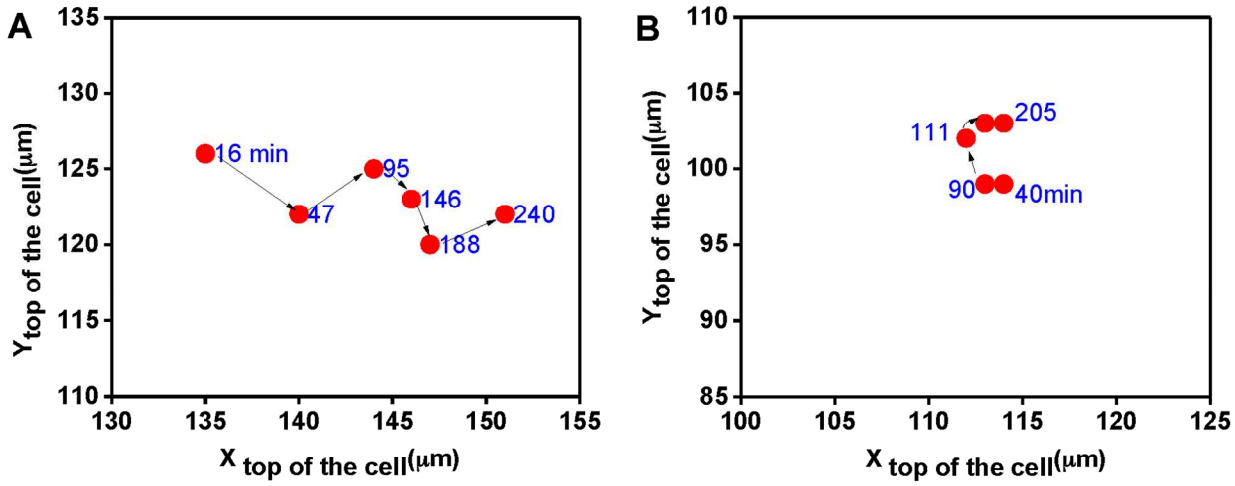
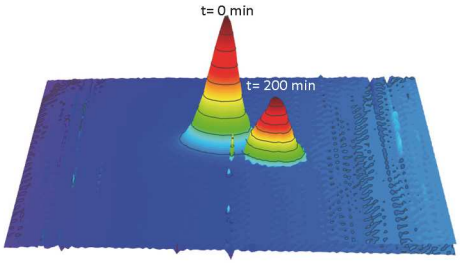


Figure 4: (A) Single-cell migration in the X-Y plane of a 25 × 25 μm window. The blue legend indicates time in minutes. The migrating single cell travelled a distance of 25 μm in 240 min. (B) A stagnant single cell does not change position in 205 minutes. Both (A) and (B) are in the same distance range of 25 μm.

	Migrating cells	Non-migrating cells	Serum-starved synchronized cells
Speed of migration (μm / h)	8 ± 3	3 ± 1	9 ± 3
Height of a single cell (μm)	11±4	16±2	13±4
Diameter of a single cell (μm)	40±10	47±4	40±12

Table 1: Quantitative estimation of the speed of migration and the height and diameter of a single cell.



SECM based analytical methods to study single-cell biomechanics and is reported to differentiate between migrating and stationary cancer cells.