motility assay. It is a combinational measurement of cellular motility and directionality, both of which could be affected by cell density.

Keywords: Cell Culture, Microscopy, Wound healing, Cell motility
Abstract

Wound-healing assay is frequently used in the analysis of cell motility (1-3). However, it is not known whether such an assay is a pure measurement of cell motility or it reflects a combinational effect of cell motility and other factors. Neither has it been described that how individual cells migrate during the wound-healing process. In this study, we first examined the effects of the size of the flanking area and the density of cells on the rate of wound closure. We found that the rate of wound healing was faster in experiments with higher initial cell densities, whereas the sizes of the areas flanking the linear wounds did not affect the wound-healing rate. We further monitored the process of wound healing with our long-term, time-lapse recording system. The migration path of each cell during the wound-healing process could be traced, plotted, and calculated. We found that only the first few rows of cells in the wound regions contributed to the wound closure, which explained why the size of the flanking area was not important in a wound-healing assay. This observation was in agreement with the general notion that wound healing was a local event (4). With increasing row numbers, cells spent less time moving in the direction of wound healing and lower percentage of cells contributed to the closure of the wound. Interestingly, the total migration lengths of cells in different rows were essentially the same. Cells in the first rows showed better directionality in wound closure, while those in the 5th or 6th rows tended to roam back and forth. The lengths of the migration paths were greater in experiments with higher initial cell densities, especially for the first row of cells, indicating an increase in cell motility when cell densities were higher. The first row of cells also showed better directionality in experiments with higher initial cell densities. These results demonstrated that wound-healing assay was not a pure motility assay. It is a combinational measurement of cellular motility and directionality, both of which could be affected by cell density.

Keywords: Cell Culture, Microscopy, Wound healing, Cell motility
Scratch-wound assay is frequently used in monitoring and analyzing cell motility in vitro (1-3). A wound is introduced to a monolayer of confluent cells by appropriate instruments, and the rejoining of the wound edges is measured and quantified by microscopy. Although the rate of wound closure is generally accepted as a measurement of cell motility, other factors may also affect the healing rate. In this study, we first examined the effects of the size of the flanking area of the wound and the cell density of the monolayer on the rate of wound closure. A long-term time-lapse recording system was used to monitor the wound-closure process. With this system, we were able to investigate not only the closure of the wound edges, but also the behavior of every single cell during wound healing. The migration path of each cell during the process could be traced, plotted, and calculated. We observed and calculated the movements of cells in different rows behind the initial wound edges with different initial cell densities. We found that wound-healing assay was not a pure motility assay. Cell density and directionality of cell motility also affected the rate of wound closure.


**Material and methods**

**Cell line and cell culture**

T24 cell is an epithelial cell line derived from high-grade human bladder cancer. Cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were cultured at 37°C in a 5% CO₂ atmosphere.

**Mini-culture system**

The mini-culture system consists of a stage chamber, a temperature controller, appropriate tubing, a perfusion pump, a gas pump, and a conventional CO₂ incubator to supply 5% CO₂. The chamber is composed of 1) an insert disk which has a Teflon wall for insulation and an indium tin oxide (ITO) coated glass as the bottom which will generate heat when electric current is applied to the electrodes attached to the glass; 2) heated upper glass plate to prevent condensation; 3) heated outer wall; 4) inlet and outlet for CO₂ circulation; 5) a humidifying device; and 6) inlet and outlet for medium perfusion. The insert dish can hold 2-3 ml of medium. An air pump will draw 5% CO₂ from the conventional CO₂ incubator, pump the gas through the mini-culture chamber, and return it to the conventional CO₂ incubator to complete the closed CO₂ circulation. The insert disk in the chamber can also be used in the conventional CO₂ incubator. We plated our cells onto the insert dish with or without a cover slip inside and keep them in the conventional CO₂ incubator for 3 hours before assembling the insert disk to the mini-culture chamber on the microscope stage for experiments.

**Time-lapse recording:**

Equipments and software for time-lapse recording included: 1) a microscope (Nikon TMD); 2) a CCD camera (Cohu 4910 color CCD camera); 3) a computer (Pentium 100 running Windows NT3.51); 4) a video capture card (Truevision Targa 2000, Elsa bus); 5) a video capture program (Vidcap); 6) a shutter (Uniblize D-122); and 7) a custom made shutter control program (written by Mr. Kuo-Ho Yan in Department of Computer Science, NCKU). After plating the cells onto the insert disk and keeping them in the conventional CO₂ incubator for 3 hours, we assembled the insert disk into the mini-culture chamber on the microscope stage. Series of phase-contrast images of the cells were captured every 20 minutes and transformed into video files by video editing programs such as MediaStudio 6 (Ulead Systems, Inc.). The images could be further analyzed and each cell in the images could be traced using an ImageTool script written by Mr. Shung-Min Lan who was a medical student in NCKU. First we marked the position of the nucleus of the cell we wanted to trace in the first frame, then we went to the next frame (which would be 20 minutes apart) and marked the new position of the same nucleus. The distance would be calculated and a line would be automatically drawn from the previous position. With passing of
time, the positions of the nucleus we traced were marked and recorded on consecutive frames until the scratch wound was healed. The marked distances on these consecutive frames could be linked as a track of the moving cell. The records of the positions and distances were used to calculate the segmental and total migration lengths and the direction of the movements.

The movements of the first 5 rows of T24 cells in the wound regions were observed with two different cell-density ranges at the start of the wound healing. High densities ranged from $1.5 \times 10^5$ to $1.6 \times 10^5$ cells/mm$^2$, and low densities ranged from $6 \times 10^2$ to $7 \times 10^2$ cells/mm$^2$.

For the percentage of cells that moved forward in each row, the number of the cells that moved closer to the midline of the wound at the end of 24 hours was calculated and divided by the total cell number at that row.

For time spent on moving forward, the direction of the cell movement was determined for each successive frame (forward or backward, i.e. in the direction of wound closure or the opposite), and the number of frames with forward movement was subtracted by the number of frames with backward movement. The result was further divided by the total frame number. The final number represents the percentage of the time that the cells purely spent on moving forward.

For frequency distribution of the migration distance of each cell in each 20-minute interval, the distances that a cell migrated in successive frames were measured and classified into different groups.

The length of the moving path was the summation of the migration distance of each cell in each 20-minute interval.

Directionality was defined as the total length of moving path divided by the distance along the x-axis from the original position to the end position.

Cell wounding

For controlling the density of each experiment, approximately $12 \times 10^5$ cells or $9.5 \times 10^5$ cells were plated in 3 cm dish with cover slips, the cell density was controlled in the range of $1.5-1.6 \times 10^3$ cells per mm$^2$ area and $5-7 \times 10^3$ cells per mm$^2$, designated higher cell density and lower cell density, respectively. Using a yellow tip with the aid of a ruler across the plate and a paper with a drawn line representing the wound position under the plate produced a middle-wound or an asymmetric wound. For producing different area, cells were plated on cover slips with $24 \times 40$ mm$^2$ or $40 \times 40$ mm$^2$ area with a middle wound, or an asymmetric wound on a round cover slip.

Results

The rate of wound closure was not affected by the size of the flanking area, but was affected by the cell density.

Wound-healing assay was performed on 2 different sizes of cover slips with 2 different cell densities at confluence. The linear wound was made at the midline of the cover slips. The result of the 24-hour wound closure showed that the size of the flanking area did not affect the rate of wound healing.
whereas higher cell density resulted in faster wound closure (Fig. 1A).

![Distance vs. Time Graph](image)

**Fig. 1A** Healing of scratch-wounds in T24 monolayers during a 24-hour period. \(12 \times 10^5\) cells or \(9.5 \times 10^5\) cells were plated on cover slips with 24*24 mm\(^2\) (small) or 24*40 mm\(^2\) (large) in area, designated as 12-large, 12-small, 9.5-large, and 9.5-small.

The cover slips with different densities of monolayers were introduced into our long-term time-lapse recording system for further investigation. The same phenomena were observed with the live cell recording system. An asymmetric wound was introduced to the monolayer in the culture chamber, which resulted in a linear wound with 2 different sizes of flanking areas. There was no significant difference between the migration rates of the 2 wound edges with different flanking areas (Fig. 1B).
**Fig. 1B** Time-lapsed recording of the migration distances of wound edges with different sizes of flanking areas. The wound was made 4mm away from the midline to produce 2 different sizes of flanking areas. “Major”: larger flanking area. “Minor”: smaller flanking area.

Nevertheless, the rate of wound closure with higher cell density was significantly faster (Fig. 1C).

![Graph showing velocity (um/hr) for high and low density](image)

**Fig. 1C** The rate of wound closure with high and low density in live-cell recording. The wound closure with high density was significantly faster than low density. (P value = 0.0011)

**The percentage of cells that moved forward in 24 hours decreased from row one to row five.**

The movements of the first 5 rows of T24 cells in the wound region were observed in monolayers with high and low cell densities at the start of the wound healing. For each row, the number of the cells that moved forward (in the direction of wound closure) were calculated and divided by the total cell number at that row. The percentage of cells that moved forward decreased from row 1 to row 5. There was no significant difference between experiments with different cell densities (Fig. 2).
The percentage of cells that moved in the direction of wound closure decreased as the distance from the wound edge increased.

The time purely spent on forward movement decreased from row one to row five. Time-lapse recording of the wound-healing process was performed with an interval of 20 minutes. The direction of the cell movement was defined as "forward" when the position of the nucleus of one cell in one frame was closer to the midline of the wound compared to its position in the previous frame. The number of frames with forward movement was subtracted by the number of frames with backward movement. The result was further divided by the total frame number. The final number represents the percentage of the time that the cells purely spent on moving forward. The percentage decreased from row 1 to row 5 (Fig. 3).
was calculated for cells in different rows behind the wound edge.

Every cell participating in the wound-healing process could be traced individually with our long-term, time-lapsed recording system.

To look into the movements of the cells contributing to the wound-healing process in more details, we used our long-term time-lapsed recording system to trace individual cells with a script written for Image Tools (Fig. 4).

![Image](image.png)

**Fig. 4** The cells in each row could be traced separately.

We marked the position of the center of the nucleus in each frame in series. The script would calculate the position shifts between successive frames and draw a path of the cell migration.

The frequency distribution of the migration distance of each cell in each 20-minute interval and the total migration length did not show any difference between cells in different rows.

The frequency distribution of the migration distance of each cell in each 20-minute interval was not
significantly different between cells in different rows (Fig. 5A).

![High density diagram]

![Low density diagram]

**Fig. 5A** The frequency distributions of the migration distances of each cell in each 20-minute interval. Y-axis is the percentage of the count in each group against the total count.

The distances that cells moved in every 20 minutes were recorded and classified into 14 groups. When experiments with the same cellular densities were considered, the total lengths of moving paths of cells in different rows did not show any significant differences, neither in experiments with higher densities (gray bar) nor in those with lower densities (dotted bar) (Fig. 5B).

**The total migration lengths of row 1 cells were longer with higher cell densities.** When comparing between experiments with different cell densities, the lengths of moving paths of cells in row one in the higher density experiments were greater than those in the lower density ones (P value < 0.0001). The differences in row 3 and row 5 were not statistically significant (Fig. 5B).
The cells in row 1 showed better directionality in experiments with higher cell densities.

The cells in the wound edges in experiments with higher cell densities showed better directionality than those in experiments with lower densities (Fig. 6; P value = 0.0104, n=20). The directionality was defined as the total length of moving path divided by the distance along the x-axis from the original position to the end position.
Fig. 6 Directionality of the forward movements of cells at the wound edges.
Gray bar: high density. Dotted bar: low density.

Summary

1) Only the first 5 to 6 rows of cells in the wound region participated in the scratch healing process.
2) Since wound healing was a local event confined to the first 5 to 6 rows of cells, the sizes of the flanking areas would not affect the rate of wound closure.
3) The cells in the first few rows spent more time on moving forward, whereas the cells positioned farther away from the wound edge tended to move back and forth.
4) However, cells in different rows did not differ in total migration lengths and frequency distribution of segmental migration rates, indicating that cells in different rows had essentially the same overall motility if directionality was not taken into account.
5) Cell densities affected the rate of wound closure. Monolayers with higher cell densities showed faster wound healing.
6) Two factors contributed to the faster healing rates with higher cell densities: a) the total non-directional migration lengths of the cells at the wound edges (row 1) were longer; and b) the directionality of the cells in the first rows was better with higher cell densities.

In conclusion, wound-healing assay is not a pure motility assay. It is the combination of the motility and directionality of the moving cells that determine the rate of wound closure. In a wound-healing assay, cell densities at the time of wounding must be carefully controlled, since cell densities can affect both motility and directionality of the moving cells. Parameters such as the lengths of the moving paths, the distances from the origins to the end positions of the cells, and the directionality of cell migration should be calculated carefully. We believe that the total length of the migration path is the better indicator of cell motility in a wound-healing assay, rather than the healing rate.
計劃成果自評

我們先前已經發展出一種新的顯微鏡台細胞培養器，其效能與傳統細胞培養箱相若。我們已申請台灣專利，至於美國及歐盟的專利申請，目前正由校方評估之中。在過去一年，我們將這項研發成果，應用到傷口愈合的研究方面，看到了一些過去無法觀察到的現象，未來本細胞培養系統也可以應用到其它方面，例如癌症的研究。

感謝詞

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參考文獻