Extraction of pure cellular fluorescence by cell scanning in a single-cell microchip†

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A 3-dimensional liquid flow control method has been developed to manipulate and retain a single yeast cell freely in a microchip. This method allows us to carry out single-cell experiments by selecting any desired single cell from a group, retaining the cell for cellular signal detection, and delivering reagents to the cell during continual detection and observation without any negative impact from the liquid flow on the live cell. The cell was scanned back and forth across an observation window in order to extract pure cellular fluorescent signals. Different scanning methods were discussed for effective collection of the cellular fluorescent signal. The cell scanning technique results in many advantages, such as distinguishing a small part of a cell, allowing for background correction and monitoring the switch of reagents. In addition, it is possible to evaluate the photobleaching effects on both the background and cellular fluorescence, with the latter found to be less significant in a restricted cellular environment.

Introduction

There have been numerous single-cell experiments recently performed using the microfluidic chip.[1–3] In particular, fluorescent measurements of suspension cells have been reported.[4–8] In most of the proof-of-concept experiments, the fluorescent signal represents both the cellular fluorescence and the background. In our opinion, to extract the genuine fluorescent signal of the single cell, it is essential to continuously measure the fluorescent signals from both the cell and the background. If the background level does not change, the changes in the total signal will represent the changes of the cellular fluorescence. However, if the background level changes during experiments, the total fluorescence will be too complex for us to extract the pure cellular fluorescence. In most cases, the background always changes in the live single cell experiment. There are at least three factors contributing to the changing fluorescent background in the microchip. First, the measured fluorescent substance may undergo chemical or photochemical reactions, which include photobleaching.[9,10] This will affect both the background and the fluorescent cellular signal. Second, a live cell normally exchanges (uptake or excretion) substances with the surroundings, thus altering the chemical composition of the background. Third, the liquid flow and reagent delivery in the microchip will alter the fluorescent background. In conclusion, to measure the real cellular signal, especially in the kinetic studies on a live cell, we need to simultaneously measure the total fluorescence and the background fluorescence in order to extract the pure cellular signal. We have developed the three-dimensional flow control method for single-cell experiments in which the cell scanning technique was applied to measure the total fluorescence and the background fluorescence.[11,12] In the present report, we demonstrate that this scanning technique allows us to correct for the background change, to monitor reagent switching, and to perform the photobleaching studies. In all experiments, the measured cellular fluorescent signal was derived from fluorescein, which was formed from a model fluorogenic substrate, fluorescein diacetate (FDA).

Experimental

A glass microchip was fabricated by wet HF etch at CMC (Canadian Microelectronic Corporation). The cell-scanning method has previously been described.[11,12] Briefly, a group of cells is introduced on the chip, and a desired cell is selected and retained on an arc slope opposite to a reagent channel. The single-cell microchip is shown in Fig. 1. The arc slope shown in Fig. 1 is the direct result of isotropic wet etch of the microchip fabrication. The flow of reagents is introduced from channel c. This flow, together with the flows from a and b, creates a

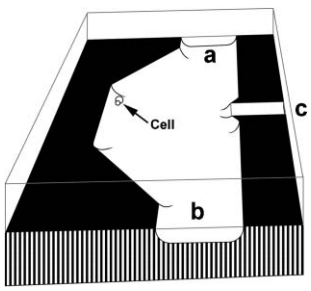

Fig. 1 The single-cell microchip .This is a 3-D illustration of the cell retention structure which contains two wide channels (a and b) and a reagent channel c.

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three-dimensional flow field as previously described.[11] Briefly, there is a zero-speed point (ZSP) in the three-dimensional flow field and it is located up the arc slope at which the cell remains stationary. By controlling the liquid pressures at a, b and c, the location of the ZSP or the cell can be varied, and thus the cell can be controlled either to lie stationary on the arc slope or to be scanned back and forth along the wall opposite channel c. In the process of cell scanning, reagents can be continually delivered or switched. Reagents from c actually flowed at a fast speed to very near the cell and transferred to the cell at a low speed, while keeping the cell freely lying on the arc slope.[11] The flows of a, b and c were provided either by electrical or pressure-driven means. The optical observation and fluorescent measurement systems are described in the Electronic Supplementary Information†.

The yeast cell (*Saccharomyces cereviase*) was selected for the single-cell experiments. Yeast cells were directly introduced into the chip and a single cell was selected to reach the detection region. Fluorescein diacetate (FDA) was delivered to the cell to perform the metabolism experiment, *i.e.*, formation of fluorescein after the intracellular hydrolysis of FDA by an intracellular carboxylesterase.[13] The cellular processes include FDA influx, formation of fluorescein, and the subsequent efflux of fluorescein.[12]

Results and discussions

Types of cell scanning

In fluorescence measurements, only when the background is measured at the same time as the total signal is the correction of background feasible. The measurement of these 2 parameters is carried out by cell scanning as follows. In Fig. 2A, the detection window, as depicted as a rectangle, remains stationary, and the cell is scanned, as shown at 2 locations (see ESI movie†). When the cell passes through the window, strong total fluorescence is detected and a fluorescent peak is generated. When the cell is out of the window, only background is measured. The double peak shape is caused by the yeast mother cell and its small bud, which has a weaker fluorescence than its mother cell. When the bud first enters the window, the small fluorescent peak due to the bud appears at first, followed by a higher peak of the mother cell (see the left double-peak of Fig. 2A). On the other hand, when the cell returns from right to left, the mother cell first enters the window, and the higher peak appears first (see the right double-peak of Fig. 2A). The use of a narrow window during cell scanning provides a means to measure the difference in cellular fluorescence of the yeast cell and its bud. The continual cell scanning process generates a pair of mirror peaks. When a wider detection window is used, the difference in the fluorescence of the mother cell and its bud cannot be distinguished, thus resulting in only a single peak (Fig. 2B).

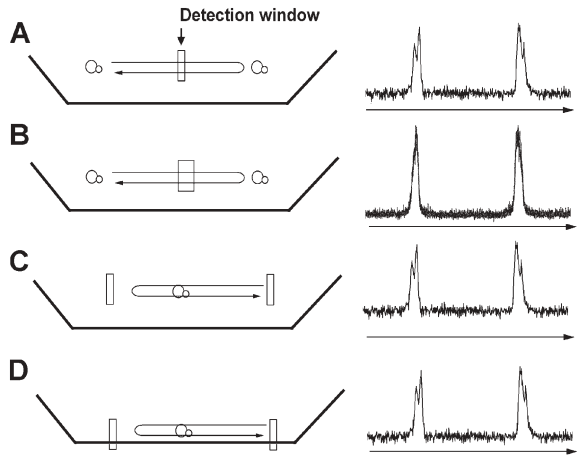

Fig. 2 The types of cell scanning. The left series of illustrations of **A–D** show the different scanning paths (the arrows indicates the moving cells in A and B or the moving detection windows in C and D) in the cell retention structure. The right series of illustrations of A–D show the measurement results.

In the use of a narrow window for cell scanning, the scanning speed is adjusted to reveal more details about the difference in the fluorescent intensities of the mother cell and its bud, see Electronic Supplementary Information†.

In some cases, the cell may become adherent to the microchannel bottom in a weak flow, and cannot readily be moved by the cell scanning procedure. In this case, scanning can be performed by moving the detection window, instead of moving the cell. When the detection window is scanned first from right to left, and then from left to right, double-peaks are obtained (see Fig. 2C), similar to those obtained by cell scanning. Background correction will still be performed, but this is based on the assumption that the background fluorescence near the cell is the same as where the cell lies. The detection window can also include the edge of the arc slope, resulting in a lower background measured during the scanning of the cell (Fig. 2D).

Advantages of scanning in an open region

There are a lot advantages of cell scanning for background correction, as previously described.[12] The scanning method is a way to get background and signal at the same time and a way to extract very weak signal out of noise. In the scanning procedure, the detection window does not include the wall of the cell retention structure, and this results in a similar background fluorescence over the entire scanning region (Fig. 3A). This procedure is dubbed as equal-background scanning. If the scanning window is moved down to include the horizontal wall, the background fluorescence is lower because of less reagent volume detected, although the background is still equal over the scanning region (Fig. 3B). The unchanging background is also essential to detect weak cellular fluorescent signal above the noise of the background. On the other hand, Fig. 3C shows the scanning in the vertical direction, in which the background is higher in the reagent region, and lower in the chip region, resulting in a valley-like signal. Any cellular fluorescent signal will only be superimposed on the sloping region of the valley, making is difficult to discern and extract pure cellular fluorescence.

Therefore, it is essential to design an open cell retention structure in the microchip, which not only provides the selection and retention of single cells of a wide range of sizes and shapes, but also provides a wide open space for

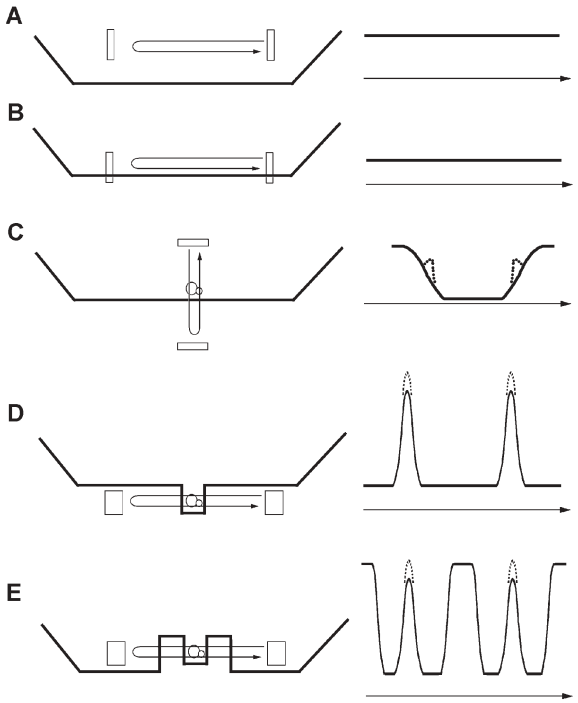

Fig. 3 The advantage of cell scanning in an open region. The left series of illustrations of A–E show the different scanning paths (the arrows indicates the moving detection windows) in different structures. The right series of illustrations of A–E show the measurement results (the broken lines in C–E indicate the possible cellular signals).

equal-background scanning. In addition, any changing composition due to reagent switching can be quickly homogenized in the open region to create a homogeneous background. However, if the cell retention structure has a similar size to the cell, as shown in Fig. 3D or Fig. 3E, the background signal appears as a peak. Worse still, the background peak occurs at the same location as the cell peak. Therefore, this scanning method is not useful for background correction. Moreover, the complex cell retention structure generates complex scattering light, and makes the extraction of the cell fluorescent signal even more difficult. It is also difficult to transfer reagents to distribute around the cell because of the small size of the structure. In the meantime, any substances excreted by the cell are also difficult to remove in the confined cell retention structure. The cell scanning procedure allows us to perform background correction, and only with this can we discard the background fluctuation even during buffer switching. In single-cell experiments, different reagents may have their own fluorescent backgrounds. Sometimes even the background of one reagent is not a constant, see Electronic Supplementary Information† for more information.

Photobleaching effect on background

Photobleaching is present when excitation radiation is used to excite a fluorophore for its emission detection and measurement. Although we use a xenon arc lamp instead of a high-power laser, the photobleaching effect is still present, albeit to a lesser extent. Before the photobleaching effect on the cellular fluorescent signal is studied, we should first examine the photobleaching effect on background fluorescence. Since there was no photobleaching when the excitation light shutter was shut off, and photobleaching was resumed when the shutter was opened again, this open–shut procedure was used to study the photobleaching effect in our fluorescent measurement system. In this study, the liquid flow was stopped in the microchip, and so there was no replenishment of FDA from the flow. Therefore, the measured fluorescence was dictated only by the processes of fluorescein formation (from FDA hydrolysis) and fluorescein photobleaching as follows.

First, the photobleaching effect is defined as follows:

$$dC/dt = -k_p C \quad (1)$$

where C is the concentration of fluorescein and k_p is the photobleaching rate constant.

By integrating equation (1), we have

$$\ln(C_T/C_0) = -k_p T \quad (2)$$

where C_0 and C_T are the concentrations of fluorescein when $t = 0$ and $t = T$, respectively.

Rearranging equation (2), k_p can be obtained as follows:

$$k_p = T^{-1} \ln(C_0/C_T) \quad (3)$$

Fig. 4A is the schematic diagram showing the fluorescent intensity during the two conditions of the excitation light, *i.e.*, open and shut. Since there is no photobleaching before $t = 0$, C_0 is F_0/m, where F_0 is the initial fluorescence intensity and m represents the instrumental factor relating the measured fluorescent intensity to fluorescein concentration. During $0 < t < T$, both photobleaching and formation of fluorescein from FDA hydrolysis occur. So the measured fluorescent intensity should be subtracted by an amount due to fluorescein formation, *i.e.*, $(F_2 - F_1)$, by assuming that the same extent of fluorscein formation occurred during $0 < t < T$ and $T < t < 2T$. This gives $C_T = [F_1 - (F_2 - F_1)]/m$. Together with C_0 and T, k_p can be determined using equation (3).

Fig. 4B shows the changes in the fluorescent background (*i.e.* no cell) during the open- and shut-cycles (in 100 s intervals) of the shutter for the excitation light. During each open-cycle, the fluorescence decreased due to photobleaching. However, after 100 s of excitation shut-off, the fluorescence became high again due to fluorescein formation, and so the overall fluorescence continued to increase due to ongoing FDA hydrolysis. Fig. 4B is actually a zoom-in region of the experiment over a much longer period, as shown in Fig. 4C. With this data set, more than 100 k_p values are calculated, and plotted in Fig. 4D. This results in an average k_p value of $0.001\,38 \pm 0.000\,22$ s^{-1}, as compared with a value of 0.038 s^{-1} reported for free fluorescein in an 0.01 M aqueous solution.[9]

Photobleaching effect on cellular signal

To study the photobleaching effect on the cellular signal, experiments were performed on the yeast cell using either the normal slide without liquid flow, or the microchip under a liquid flow.

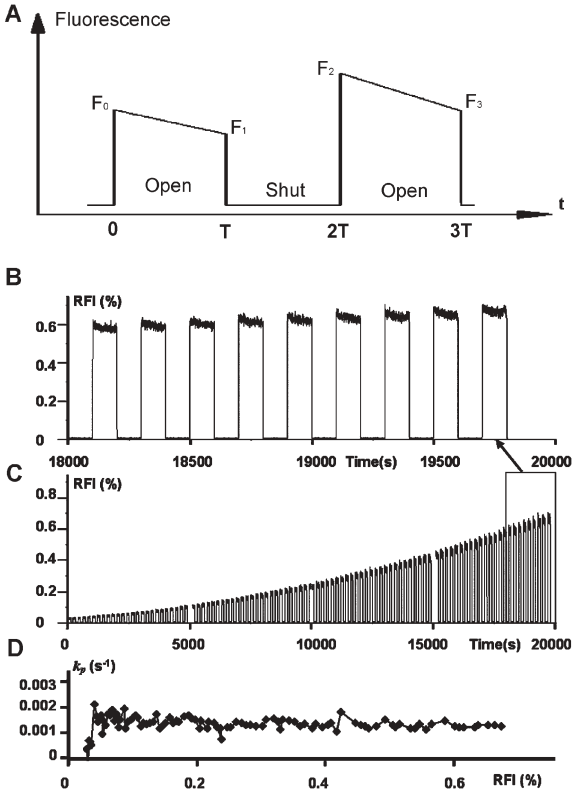

Fig. 4 (A) The parameters of the photobleaching model to separate the hydrolysis (which increases the fluorescent intensity) and the photobleaching effect (which decreases the fluorescent intensity). F_0, F_1, F_2 and F_3 are the fluorescence when $t = 0$, T, $2T$ and $3T$, respectively. When $0 < t < T$ and $2T < t < 3T$, the excitation light is on. When $T < t < 2T$, the excitation light is off. (B) Fluorescent intensity of fluorescein resulting from FDA hydrolysis without liquid flow. The shutter for the excitation light was opened and shut for an interval of 100 s. (C) The whole experiment, which lasted for 20 000 s, from which the data of (B) is derived.

In the normal slide experiment, there was no liquid flow, and so any free fluorescein was continually photobleached without replenishment. Fig. 5A shows the raw data. Following data extraction, Fig. 5B depicts the background and Fig. 5C shows the extracted cellular signal. It was clear that both the cellular and background fluorescein had photobleaching, as indicated by the gradual drop in fluorescent intensities during each excitation-on period, see Fig. 5B and 5C, respectively. In Fig. 5B, the fluorescent levels at the start and end of the shut-off period are compared. It is found that there is a slight recovery in the fluorescence level, although the general trend is decreasing. This general decrease trend is indicative of the absence of fluorescein replenishment. The recovery after the shut-off period is not obvious in the cellular fluorescence, as is shown in Fig. 5C. Moreover, the cellular fluorescence is overwhelmed by the general decreasing trend, possibly caused by the efflux of fluorescein. Since the efflux of fluorescein and its photobleaching cannot be separated in an experiment without flow, as conducted on the normal slide, we have conducted a microchip flow experiment. It was found that there is no fluorescein efflux and no photobleaching on the cellular fluorescence.

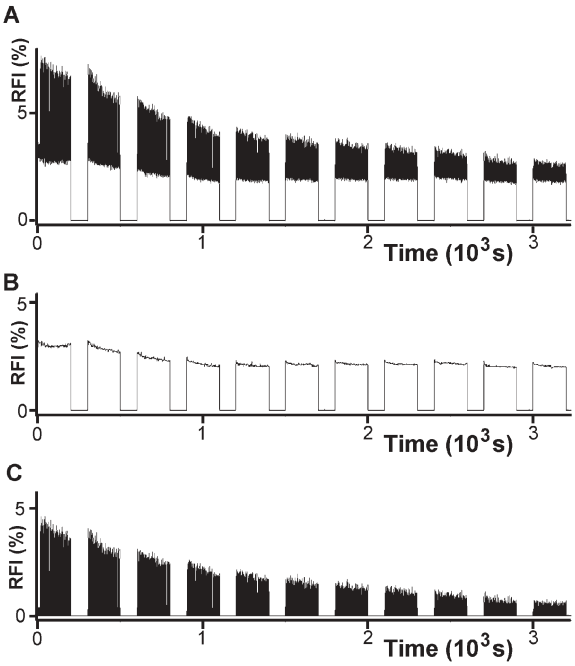

Fig. 5 The fluorescent measurement of a yeast cell embedded on a normal slide: the raw data (A), its separated background (B) and extracted cell fluorescence (C). FDA was used to generate the cellular fluorescence after hydrolysis. RFI denotes relative fluorescent intensity.

In the microchip experiment, the FDA-containing buffer was continually delivered to the cell, and the background level of fluorescein kept increasing because of FDA hydrolysis. The excitation shut-off period was set to 3 values, *i.e.*, 100 s, 200 s and 300 s, to evaluate the effect of shut-off time on photobleaching. Fig. 6A shows the raw data. After data extraction, Fig. 6B depicts the background, and Fig. 6C shows the extracted cellular signal. It is clear that there is photobleaching of fluorescein in the background, as shown by the sharp drop in the fluorescent intensity (Fig. 6B). Subsequently, the intensity became flat, mainly caused by the replenishment of FDA-containing buffer (and fluorescein) from the flow. After the excitation light was shut off, photobleaching no longer occurred, and the fluorescent intensity recovered to a higher value due to FDA hydrolysis. When the shut-off period was longer, the fluorescent intensity became higher because of more replenishment due to FDA hydrolysis for a longer time.

If the cellular fluorescence had a similar photobleaching effect, the signal should give rise to a characteristic drop during the excitation-on period. But no such drop is observed on the peak envelope after the shut-off period, and the intensity of the series of peaks remained roughly the same (series of peaks in Fig. 6C). This non-photobleaching effect in the yeast cell may be explained by the restricted environment of the fluorescein molecules inside the cell reducing the photobleaching effect.[10]

Conclusions

In this report, we have found that the cell scanning method is very useful for the extraction of pure cellular fluorescence,

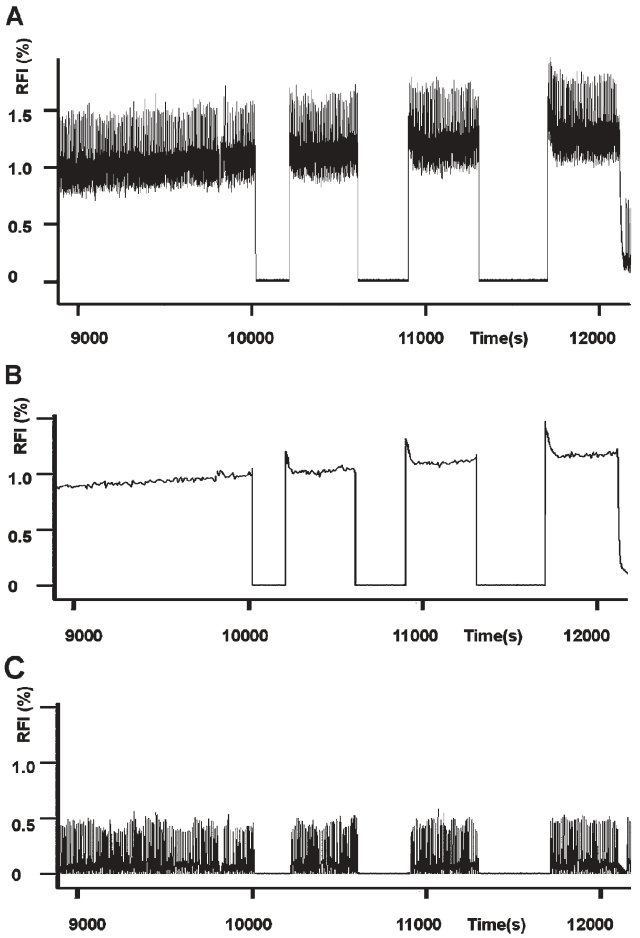

Fig. 6 The fluorescent measurement of a yeast cell under a flow within a microchip: the raw data (A), its separated background (B) and extracted cell fluorescence (C). FDA was used to generate the cellular fluorescence after hydrolysis.

which is unaffected by the presence of reagent switch and buffer fluctuations. We have demonstrated that the microchip should have a wide and flat region for effective cell scanning and successful background correction. With this procedure, even the photobleaching effect on out-of-cell fluorescent background and intracellular fluorescein can be studied, with the latter occurring to a lesser extent. Such a method for single-cell fluorescence measurement can be applied to the study of a wide range of cellular metabolites and cellular proteins.

The ESI† videoclips: A fluorescent yeast cell (mother and daughter) was scanned for signal collection; pdf file: supplementary figures including method diagram.

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