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FLUORESCENT COMPOUND FORMED IN AND EFFLUX FROM YEAST CELLS STUDIED ON A FLOW CYTOMETRY MICROCHIP

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ABSTRACT

This paper describes an easy and simple method to perform yeast cell experiment on a flow cytometry chip. We are able to observe the influx-hydrolysis-efflux phenomenon of a fluorogenic substrate on yeast cells. As a study model, the fluorogenic substrate fluorescein diacetate (FDA) diffused into the yeast cell, and FDA was hydrolyzed in the cytosol to produce fluorescein. Then, fluorescein was effluxed from the yeast cell.

Keywords: Microfluidic Flow Assay Chip, Flow cytometry, Baker's Yeast, Fluorescein Acetate.

INTRODUCTION

In recent years, microfluidic lab-on-a-chip has widely been applied for biochemical analysis (Auroux *et al*., 2002; Landers, 2003; Li, 2010). In particular, various microchip techniques for cellular biochemical analysis have been developed recently (Gao *et al*., 2004; Li *et al*., 2008). For example, microchips have been used to study the intracellular activities of cancer cells (Li *et al*., 2009; Li *et al*., 2011).

Most cell studies were performed on groups of cells and only few studies were performed on a single cell (Roper *et al*., 2003; Li and Li, 2005a; Li and Li, 2005b; Li *et al*., 2007). Although it is useful to conduct single-cell experiments to elucidate cellular variations, it is easier to study a group of cells and when there is a need to understand cell-to-cell interactions.

For on-chip experiments, transport and selection of cells have been mainly achieved by liquid flow (Wheeler *et al*., 2003; Li *et al*., 2004). The flow cytometry chip is used to measure the selected characteristics of individual cells by determining fluorescence emission at wavelengths of interest (Breeuwer *et al*., 1995; Wittrup and Bailey, 1990).

In this study, a microfluidic cell chip (Fig. 1) is used in a commercial Bioanalyzer to perform a simple and fast flow cytometry experiment on yeast cells. The use of the chip saves money because less reagents and samples are used (Mitchell, 2011). Furthermore, it is easy to prepare the chip for experiments, and the results can quickly (in less than 30 minutes) and clearly are seen.

As a study model of a yeast metabolic process, a cell-permeable fluorogenic substrate fluorescein diacetate (FDA), normally used to determine cell viability (Jones and Senft, 1985), diffused into the yeast cell (Fig. 2A). FDA was then metabolized by a carboxylesterase (Degrassi *et al*., 1999) intracellularly to produce fluorescein (Fig. 2B). Then, fluorescein effluxed from the cell (Figs. 2C and 2D).

The movement of cells in the cell chip is controlled by a pressure-driven flow inside the interconnected networks of microfluidic channels. Cells are hydrodynamically focused in these channels before passing the fluorescence detector in single file.

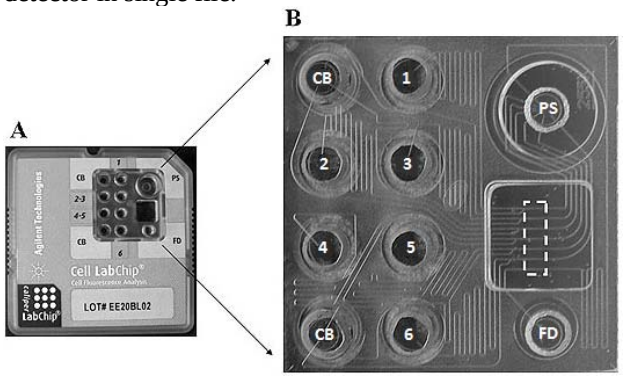

Fig. 1. Microfluidic cell chip is fixed in a plastic caddy for use in a commercial Bioanalyzer. The chip contains six sample wells (1-6), two cell buffer wells (CB), one well for a reference dye (FD), and one well for a vacuum interface and collection of the fluid waste (PS). A common buffer channel joins each sample channel in close proximity to the detection area (broken line box). Reprinted with permission from Elsevier Science (Kataoka *et al*., 2005).

The chip allows up to six cell samples to be analyzed and the cells are measured sequentially for 4 minutes each. For each event, the intensities of the red and blue

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fluorescent signals are recorded. The red light excitation is at 630 nm and emission is at 680 nm. The blue light excitation is at 470 nm and green emission is at 525 nm. The intensity of the fluorescent signal depends on the amount of fluorescent molecules present in the cell. The detector will collect the data and present them as dot plots and histograms (Nitsche, 2001).

MATERIALS AND METHODS

Preparation of FDA solution

2.5 mg of fluorescein diacetate (Sigma-Aldrich) was mixed with 500 µL of DMSO (Sigma-Aldrich, mol. biology grade) to make a stock solution of FDA. This solution was stored at -20°C and protected from light, as reported previously (Peng and Li, 2004a; Peng and Li, 2004b). A working FDA solution of 12 µM was prepared when needed.

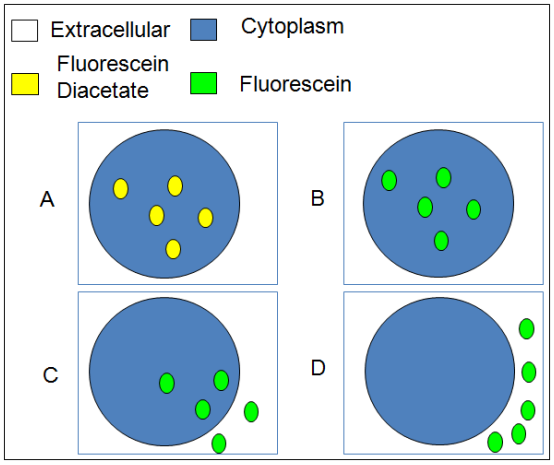

Fig. 2. A schematic diagram showing the fluorescent compound formed in a yeast cell and effluxed from it. A) fluorescein diacetate in the cell. B) fluorescein formed in the cell. C) Continuous efflux of fluorescein from the cell. D) Complete efflux of all fluorescein molecules.

Preparation of chip reagents

According to the vendor's protocol, the Cell Kit which contained the priming solution, the focusing dye, the cell buffer, and bead sample and FDA solution, were protected from light and equilibrated at room temperature for 30 min. before use. The bead vial was then vortexed for 15 s to resuspend the beads. Then, 95 µL of cell buffer were mixed with 5 µL of beads in a 0.5 mL microcentrifuge tube.

Preparation of the Cell Chip

On the glass cell chip, 10 µL of priming solution was added first in the well labelled PS. The priming solution filled the entire channel and displaced all the air in the microchannels after 1 min. The yeast solution and yeast-FDA solution were then quickly prepared (as described in detail in the next section) for sample wells 1-5. Yeast solution B (10 µL) was added to well sample 5 as the negative control. Then, 10 µL of yeast-FDA solution C was pipetted each to wells 1-4.

Then, 10 µL of focusing dye was added to the well labelled FC. This solution was used by the instrument to optically focus the detector on the channel. Buffer solution (30 µL) was then added to each of the wells labelled CB. This solution was used to hydrodynamically focus and align the cells before they passed the detection point. Lastly, 10 µL of bead solution (positive control) was pipetted to well sample 6. The chip was loaded onto the Bioanalyzer to initiate the flow cytometry experiment. After ~25 min., the experiment was completed and the results were presented either as dot plots or histograms. If the chip is needed to be reused, it will be cleaned as described previously (Chim and Li, 2012).

Preparation of Yeast and Yeast-FDA Solution

Baker's yeast (0.001 g) was mixed with 1000 µL of distilled water (solution A). 100 µL of solution A was diluted with 900 µL distilled water to give solution B (yeast-only solution), which was pipetted to sample well 5. Then, solution B was mixed with 1 µL of FDA solution to make solution C (yeast-FDA solution), which was pipetted to sample wells 1-4. Cell density for each of the solution B and C was ~1.25 x 10^6 cells per mL. All solutions were prepared at room temperature right after the channel priming step was completed.

RESULTS AND DISCUSSION

In figure 3, there are 2 dot plots on the left (a, c) which show red fluorescence intensity vs. the blue fluorescence intensity of the 2 types of particles (yeast cells and beads). The beads show high fluorescent intensity in the level of 10^2-10^3. The unstained yeast cells show the intensity below 10^0. As no red fluorescent molecules were present in the beads and cells, the intensities were all below 10^{-1}. There are 2 histograms on the right (b, d) which show the number of particles (events) vs. the fluorescence intensity. When there are fluorescein molecules inside the cell, the data from the cell would have fluorescence intensity greater than 10^0. An increase in the number of fluorescein molecules inside the cell would increase the fluorescence intensity.

Figures 3a and b are negative controls which are yeast cells without FDA. Both figures show there are no data above the fluorescent intensity of 10^0 which means there is no fluorescein molecule inside the cell. Figure 3c and d are positive controls which are the beads. Both figures show there are data above the fluorescent intensity of 10^0 which means there are fluorescein molecules inside the cell.

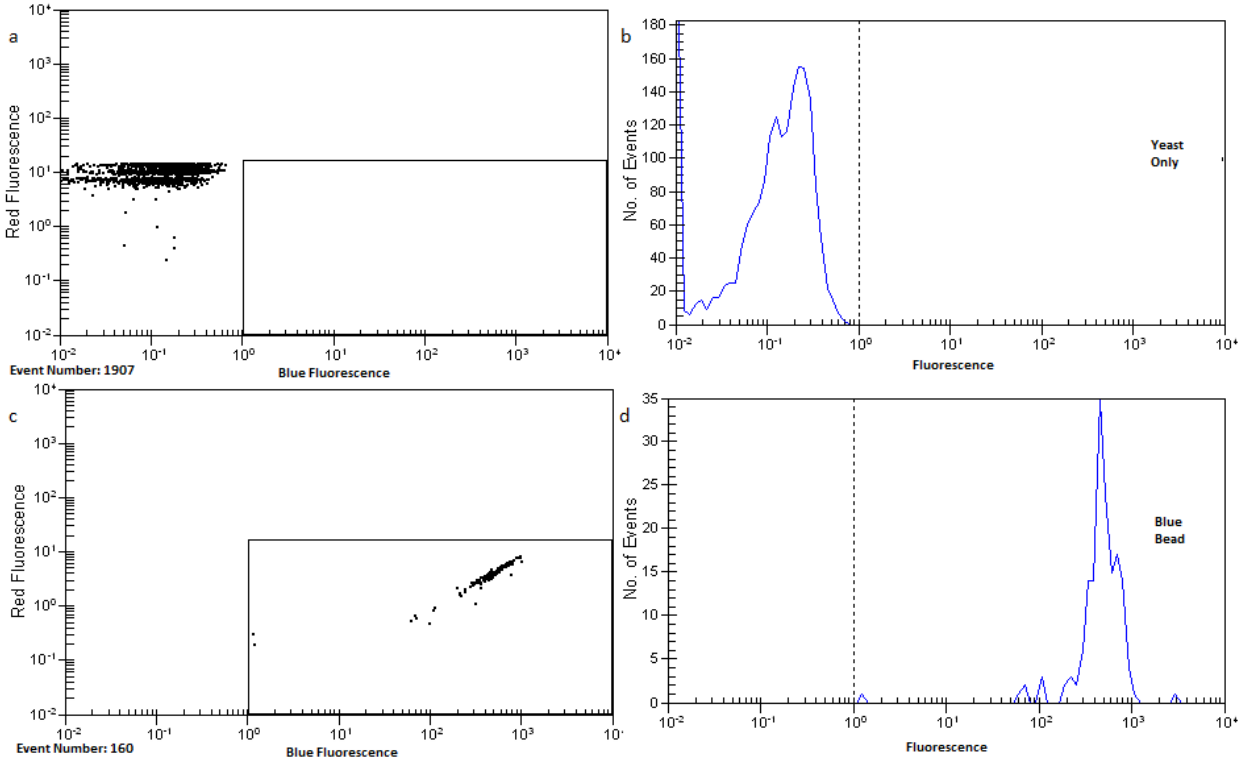

Fig. 3. Data of the negative control (unstained yeast cells) in (a) dot plot and (b) histogram, and the positive control (blue beads) in (c) dot plot and (d) histogram. The dot plots show the red fluorescence versus the blue fluorescence (should be green emission when excited in blue). The histograms show the number of events or cells versus the level of fluorescent emission only excited in blue.

The Bioanalyzer detected cells from each sample wells consecutively for 4 min. while a constant flow of cells was maintained in all channels. In figure 4, it shows the histogram of FDA-yeast cell collected consecutively every 4 min. In the cases of (a) 0-4 min. and (b) 4-8 min., the fluorescent intensity is higher than 10^0. When compared with the unstained cells, we know these cells are stained. However, from 8-12 min. (c) we begin to see fluorescent intensity lower than 10^0 which indicate the presence of an increasing number of unstained cells. Then from 12-16 min. (d), we only see unstained cells with fluorescent intensity lower than 10^0.

In order for the yeast cells to hydrolyze FDA to give fluorescein, the cell must have activated carboxylesterase. This intracellular enzyme broke the ester bond in FDA and released fluorescein, however, after a few min., some of the yeast cells started to pump fluorescein out of the cell. Moreover, some yeast cells produced more fluorescein as seen at 8-12 min. After a few minutes, all of the fluorescein inside the cells was pumped out by efflux as seen at 12-16 min. This is consistent with the time scale reported previously (Peng and Li, 2004b).

Results are also summarized in figure 5. There was no staining in the negative control and there was 100% staining in the positive control. At 0-4 min. and 4-8 min., the % stained cells are similar to that of the positive control, which indicates all the yeast cells are stained. At 8-12 min. there are both stained cells and unstained cells, resulting from the efflux of fluorescein from yeast cells. At 12-16 min., the histogram is similar to that of the negative control, which is resulted from complete fluorescein efflux .

CONCLUSION

This paper shows a simple flow cytometry experiment using readily obtained Baker's yeast. We also observed the influx-hydrolysis-efflux phenomenon of the fluorogenic substrate on yeast cells. This experiment can be completed in less than an hour, which saves time. Furthermore, it requires less samples and reagents, which save money. These experiments can be extended to measure multidrug resistance in cancer cells due to efflux, with proper biosafety precautions of working with cancer cell samples.

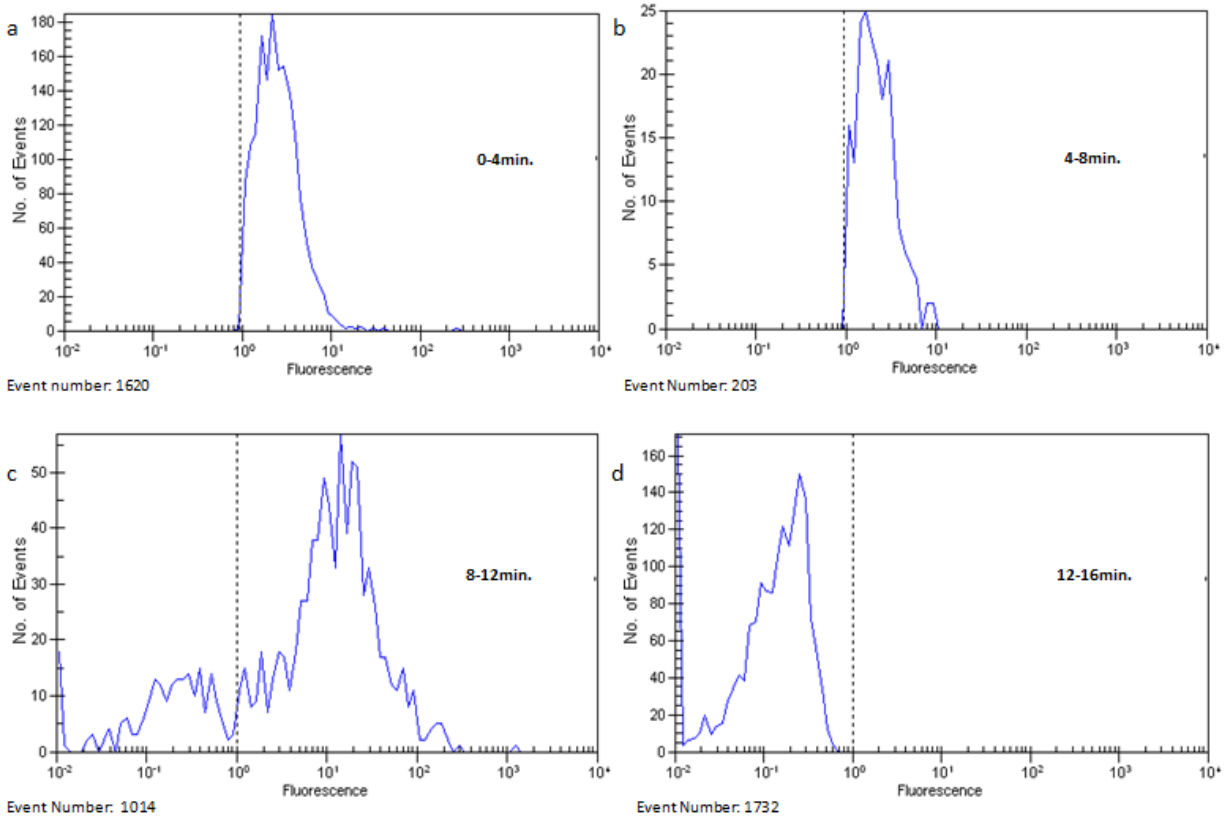

Fig. 4. The histograms of the influx-hydrolysis-efflux process in yeast cells. The data show the number of events versus the fluorescence intensity. The dotted line at 10^0 indicates the threshold level for stained cells to be recorded.

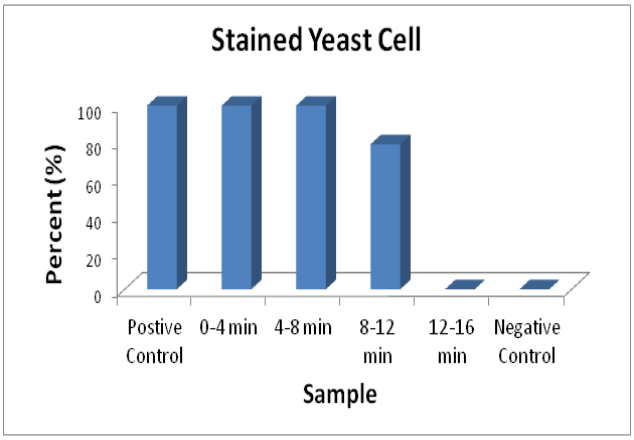

Fig. 5. The graph shows the percentage of stained yeast cells, as compared to the positive (beads) and negative (yeast cell only) control.

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