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Molecules and Cells

Synchronization of Cell Cycle of *Saccharomyces cerevisiae* by Using a Cell Chip Platform

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Cell synchrony is a critical requirement for the study of eukaryotic cells. Although several chemical and genetic methods of cell cycle synchronization are currently available, they have certain limitations, such as unnecessary perturbations to cells. We developed a novel cell cycle synchronization method that is based on a cell chip platform. The budding yeast, *Saccharomyces cerevisiae*, is a simple but useful model system to study cell biology and shares many similar features with higher eukaryotic cells. Single yeast cells were individually captured in the wells of a specially designed cell chip platform. When released from the cell chip, the yeast cells were synchronized, with all cells in the G1 phase. This method is non-invasive and causes minimal chemical and biological damage to cells. The capture and release of cells using cells chips with microwells of specific dimensions allows for the isolation of cells of a particular size and shape; this enables the isolation of cells of a given phase, because the size and shape of yeast cells vary with the phase of the cell cycle. To test the viability of synchronized cells, the yeast cells captured in the cell chip platform were assessed for response to mating pheromone (α-factor). The synchronized cells isolated using the cell chip were capable of mediating the mating signaling response and exhibited a dynamic and robust response behavior. By changing the dimensions of the well of the cell chip, cells of other cell cycle phases can also be isolated.

INTRODUCTION

Eukaryotic cells must divide to proliferate. The cell cycle is an ordered sequence of events in cell division and replication. Typically, eukaryotic cell cycles are divided into 4 phases: G1, S, G2, and M. In a bulk population, cells generally exist at different phases of the cell cycle (Krylov et al., 2003; Nasmyth, 1995; 1996; Novak et al., 1998). Synchronization of cell cycle is critical for many biological experiments because heterogeneity in cell cycle of the experimental cell population often leads to misinterpretation of the experimental data. The methods used to achieve cell synchrony are classified into chemical, physical, and genetic types (Davis et al., 2001; Lee et al., 2011; Margolis, 1970; Merrill, 1998). Chemical methods involve the use of drugs to arrest cells at a specific phase of the cell cycle (Mitchison and Creanor, 1971) or the induction of nutritional deprivation of cells to arrest them at the G1 phase (Austin and Warren, 1983). However, the use of drugs is harmful to the cells (Cho et al., 1998; Park et al., 2006a; Spellman et al., 1998; Wyrick et al., 1999). Physical methods employ techniques such as centrifugal elutriation, which separates cells according to cell density and sedimentation velocity (McEwen et al., 1968; Wahl et al., 2006), by means of specially designed centrifuges and rotors. Genetic methods use strains with specific genetic backgrounds to produce temperature-sensitive cell division cycle (*cdc*) mutants (Hartwell et al., 1970; Reid and Hartwell, 1977).

The budding yeast, *Saccharomyces cerevisiae*, is a simple but very useful eukaryotic model system because it shares many characteristics with higher eukaryotic cells. Further, the division of yeast cells progresses throughout the typical eukaryotic cell cycle (Reid and Hartwell, 1977). These properties make *S. cerevisiae* one of the most extensively analyzed eukaryotic model system in molecular and cellular biology (Choi et al., 2008; Hur et al., 2008; Jeong et al., 2001; Park et al., 2003). It is used to study many topics such as the mechanisms of the cell cycle (Hanrahan and Snyder, 2003; Nasmyth, 1996; Tanaka et al., 2011) and the variations in gene expressions at a single-cell level (Colman-Lerner et al., 2005; Dong et al., 2011; Raser and O'Shea, 2004). Cell synchronization is important for these studies. Synchronization of the cell cycle is typically achieved by arresting them at a certain phase by treatment with α-factor (Breeden, 1997), hydroxyurea (HU) (Day et al., 2004; Elledge et al., 1993), or nocodazole (Day et al., 2004; Jacobs et al., 1988) or by collecting small G1 cells from the bulk population by using centrifugal elutriation (Futcher, 1999; Walker, 1999).

Here, we describe the development of a novel, but efficient, method for cell synchronization by using a cell chip platform. *S. cerevisiae* cells are known to differ in size and shape according to the phase of the cell cycle (Alberghina et al., 2003; Rupes, 2002). We have previously shown that yeast cells can be captured by using cell chips with microwells of different sizes and shapes (Park et al., 2006b; 2011). Yeast cells are the smallest at the G1 phase and could be separated from the bulk population by capturing them in microwells with circular cavities of diameter 8 μm. When the captured yeast cells were released

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from the cell chip, they were synchronized in cell cycle phase with a uniform distribution at the G1 phase. The degree of synchronization by the cell chip was comparable to that achieved by HU treatment. The cells synchronized by the cell chip platform were perfectly viable and capable of mediating biological responses such as the mating signaling. The capture and release process using a cell chip platform provides for a simple but efficient means to synchronize yeast cells with no biological perturbations and/or damages to cells.

MATERIAL AND METHODS

Yeast strains and materials

The yeast strains used in this study were SO992 (wild type) and SH129 (fus1::yEGFP-SpHIS5, gpd1::Tdimer2-CaURA3). Cells were grown at 30°C in YPD medium, containing 2% (w/v) dextrose as the carbon source, to an A_{600} of ~0.5. A peptide corresponding to α-factor was chemically synthesized using F-moc chemistry and was purified by HPLC. All chemicals and reagents used in this study were of the highest grade commercially available.

Rhodamine-phalloidin and DAPI staining of yeast cells

Cultured cells were harvested by centrifugation at 1,935 × *g* for 5 min at 4°C and were fixed by resuspending in 100 μl of PBS containing 4% (v/v) formaldehyde for 1 h at room temperature (RT). Fixed cells were washed 2 times with PBS and resuspended in 50 μl of PBS containing 0.1% (v/v) Triton X-100 for permeabilization. Cells were treated with 2.4 μl of rhodamine-phalloidin solution (14 μM in methanol) and incubated for 30 min in dark at 4°C with gentle rocking. The cells were then washed 3 times with 1 ml of PBS and reconstituted in 50 μl of DAPI solution (400 nM in PBS) followed by incubation for 3 min and washing once with PBS. The stained cells were monitored and photographed using an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss).

Capturing yeast cells in the cell chip platform

For optimal docking and capturing, yeast cells treated with or without HU were concentrated to a density of ~5.0×10^9 cells/ml by centrifugation. For the patterned microfluidic channel, a PDMS microfluidic mold and a PUA microwell-patterned glass slide were plasma-cleaned (60 W; PDC-32G plasma cleaner; Harrick Scientific Products Inc.) simultaneously for 45 s and then irreversibly bonded under slight pressure. The PUA microwells contained hollow cylindrical cavities with both diameter and depth of 8 μm. A small amount (~0.7 μl) of cell suspension was introduced into the microfluidic channel by capillary action, and the solution plug was carefully swept by finger-applied pressure. As the meniscus of the cell solution receded over the microwells, cells were spontaneously captured into the microwells at a single-cell level. The uncaptured cells in the microchannel were removed by washing them with synthetic complete (SC) medium. The microfluidic channel filled with the SC medium was monitored using an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss).

Analysis of cell size

The cells captured in the microwells were retrieved by washing them with SC medium introduced using a micropipette. The cultured and released cells were resuspended separately in 2 ml of fresh SC medium. The cultured cells were sonicated for 3 s to remove cell clumps. The size of cells from the 2 samples was determined by measurement of the forward scatter (FSC) (Hoffman and Hansen, 1981) by using a FACSCanto flow cytometer (Becton Dickinson) equipped with a 488-nm blue laser. Measurements were made for 10,000 cells per sample.

Cell cycle arrest using hydroxyurea

To synchronize cells in the G1 phase, cultured cells were treated with 0.2 M HU for 2 h. HU was washed away, and the cells were released from the cell cycle arrest by resuspending them in SC medium prewarmed at 30°C.

Analysis of cell cycle

Samples of bulk cells, cells synchronized by HU, and cells retrieved from the cell chip were harvested by centrifugation at 1,935 × *g* for 5 min and washed once in 2 ml of PBS. All samples were resuspended in 1.5 ml of distilled water and incubated with 3.5 ml of 100% ethanol for 1 h at RT or overnight at 4°C for fixation. Fixed samples were harvested by centrifugation at 1,935 × *g* for 5 min and washed 2 times with 1 ml of distilled water. Samples were resuspended in 0.5 ml of RNase solution (2 mg/ml RNase in 50 mM Tris-Cl, pH 8.0, containing 15 mM NaCl) and incubated for 1 h at 37°C with gentle rocking. Samples were resuspended in 0.2 ml of pepsin solution (5 mg/ml pepsin and 4.5 μl/ml HCl in distilled water), incubated for 15 min at 37°C, subjected to centrifugation at 16,100 × *g* for 30 s, and resuspended in 0.5 ml of propidium iodide (PI) solution (3 μM PI in 50 mM Tris-Cl, pH 7.5). The fluorescence of the PI-stained cells was monitored using a FACSCanto flow cytometer (Becton Dickinson) equipped with a 488-nm blue laser. A 670-nm-long pass filter was used to acquire PI fluorescence. Fluorescence was recorded for 10,000 cells per sample (Haase and Reed, 2002). Analysis of DNA contents and the relative population of cells in each phase were performed as described previously (Zou and Bi, 2008).

Analysis of mating response in yeast cells

The cells captured in microwells filled with SC medium were visualized using a live-cell imaging system (DeltaVision, Applied Precision Inc.), which allowed for automated multi-position time-course fluorescence imaging. To initiate mating signaling, the 20 nM α-factor solution was introduced into the inlet of the cell chip and drawn inside, by absorbing the initial solution with a tissue paper from the outlet of the reservoir. For live imaging, 20 spatial points, each covering 49 microwells, were arbitrarily selected, and the fluorescence from the mating response was traced for 2 h with a ×60 oil-immersed objective. The green (GFP) images were obtained every 15 min (9 time points) for all the 20 spatial points. The 20 sets of captured images, each of which contained 9 GFP images, were scaled for adjustment with a reference time point and respective spatial point intensity. Green fluorescence intensity of the stimulated cells was quantified and analyzed using a commercialized image processing software (ImagePro, Media Cybernetics Inc.).

For monitoring the mating response from bulk cells, cultured yeast cells were harvested by centrifugation at 1,935 × *g* for 5 min and resuspended in 2 ml of SC medium. The resuspended cells were treated with α-factor at a concentration of 20 nM and were grown at 30°C. Cells (200 μl) were harvested at fixed time intervals of 15 min for 2 h and were sonicated for 3 s to remove cell clumps. The green fluorescence from the cells was quantitatively monitored using a FACSCanto flow cytometer (Becton Dickinson) equipped with a 488-nm blue laser. A 530/30 nm band-pass filter was used to acquire the green fluorescence and florescence was recorded for 10,000 cells per sample.

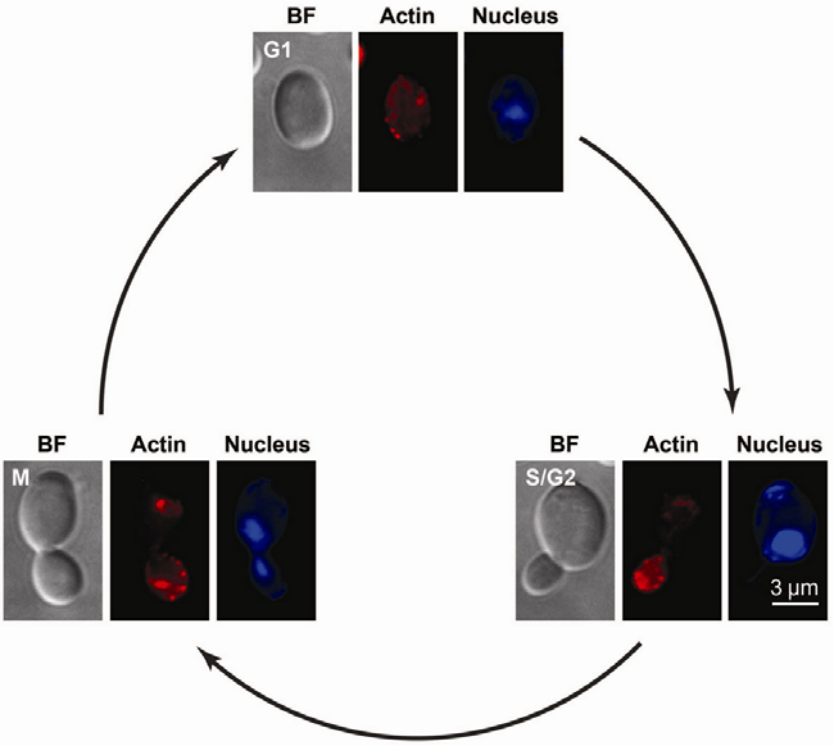

Fig. 1. The size and shape of yeast cells at different phases of the cell cycle. Yeast cells at different phases of the cell cycle were stained with DAPI for nucleus and rhodamine-phalloidin for filamentous actin, including actin patches (round bright spots). Cells at G1 phase were the smallest in size and most uniform in shape. Cells were observed using an inverted fluorescence microscope. BF indicates the image from the bright field.

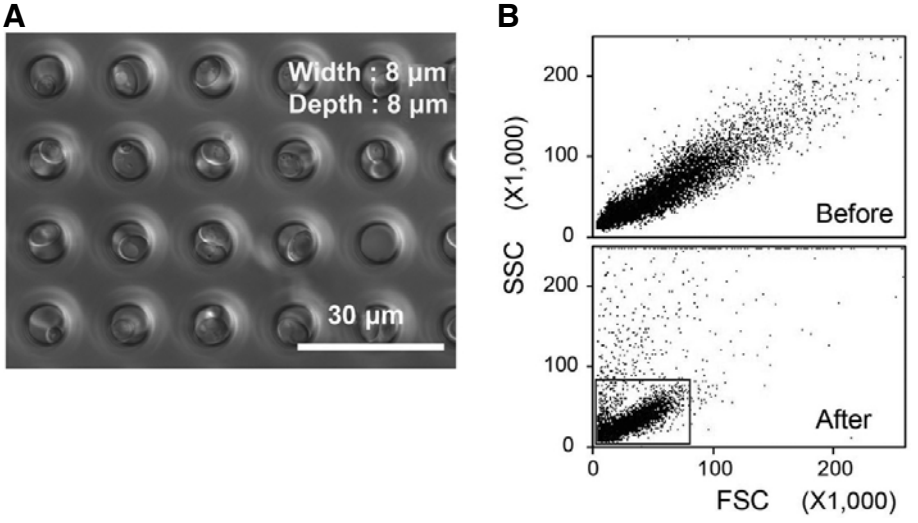

Fig. 2. Capturing of yeast cells in microwells. (A) Yeast cells were captured into the microwells of the cell chip. The microwells are hollow cylindrical holes (diameter and depth of 8 μm). Images were acquired under white light by using an inverted microscope. Over 90% of the microwells were filled with cells. (B) The size distribution of yeast cells before and after capture. The forward scatter (FSC) on the x-axis and the side scatter (SSC) on the y-axis indicate the cell size and granularity, respectively.

RESULTS

Different sizes and shapes of yeast cells

The phases of the cell cycle of *S. cerevisiae* can be monitored by staining with DAPI for the nucleus and rhodamine-phalloidin, for filamentous actin and formation of the bud tip (Karpova et al., 1998). The nuclear division and the polarity of the actin patches toward the bud tip are indicative of cell division. A typical yeast cell at the G1 phase is ovoid in shape and about 6 μm in diameter. In the early S phase, cells start to form a bud, and their shape changes to resemble that of a snowman and increase in diameter to ~9 μm. To complete cell division, they return to a small size and ovoid shape. The size and shapes of yeast cells at different phases of the cell cycle are shown in Fig. 1.

Capturing of yeast cells in microwells

The PUA-based microwells in the cell chip are hollow cylindrical cavities of diameter and depth 8 μm. The efficiency of the capturing process using the receding-meniscus approach was about 90%. Most of the captured cells were non-budding and small (< 6 μm in diameter) (Fig. 2A). The size of the cells before and after capturing was determined by measuring the FSC that is proportional to cell size and the side scatter (SSC) that is proportional to cell granularity derived mainly from the vacuoles. The captured cells were clearly smaller than the bulk cells (Fig. 2B). This result indicated that the capturing of cells using the cell chip efficiently and selectively filtered cells of a certain size and shape from the bulk population.

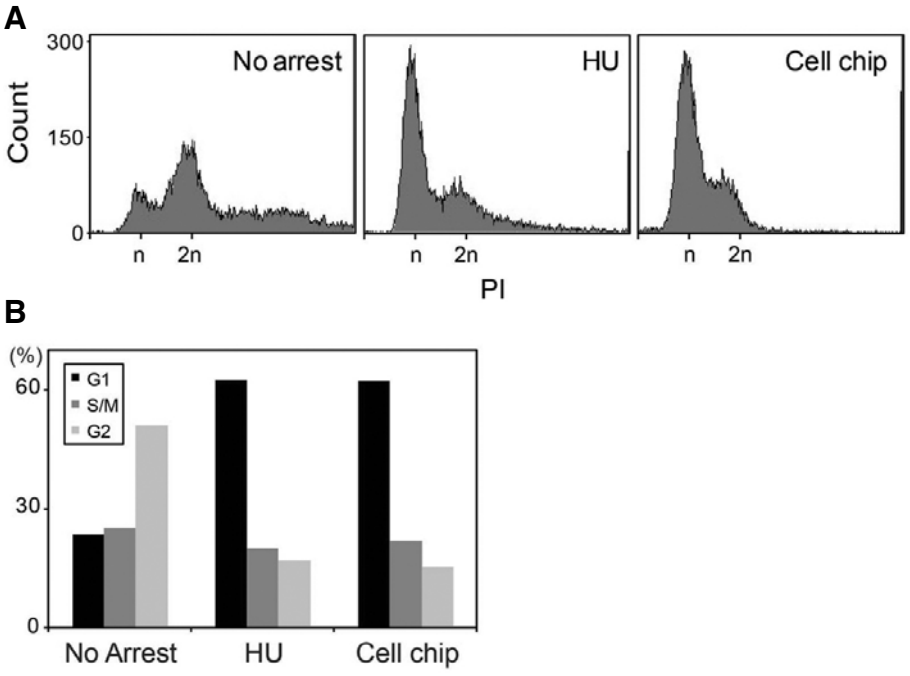

Fig. 3. Analysis of synchronized cells. (A) Yeast cells were synchronized either by treatment with HU or capturing in the cell chip. Cells were stained with propidium iodide (PI) for analysis of DNA content, and the PI fluorescence was monitored by flow cytometry. (B) Relative populations of cells in each phase were compared among the HU-treated, captured, and bulk cells. The effect and efficiency of cell cycle synchronization were similar in both HU-treated and captured cells.

Analysis of cell cycle of captured cells

To determine the cell cycle phase of the captured cells, they were stained with PI to measure DNA content by using flow cytometry (Fig. 3A). HU is a popular agent used to arrest cells in the G1 phase by blocking progression into S phase via inhibition of the enzyme ribonucleotide reductase that synthesizes deoxyribonucleotides (Elledge et al., 1993). About 62.4% of the cells captured in the cell chip were arrested in the G1 phase, while 62.6% of HU-treated cells were arrested at G1 phase (Fig. 3B). Thus, the efficiency of cell cycle synchronization by capturing cells in a cell chip was similar to that achieved by HU treatment.

Viability of captured cells

The maintenance of the biological integrity and viability of synchronized cells is crucial. To test whether the cells captured in the cell chip platform were capable of processing biological responses, they were treated with the mating pheromone (α-factor), and their mating responses were analyzed by monitoring the transcription of a well-known mating gene *fus1*. Stimulation of haploid yeast cells with the mating pheromone is known to initiate MAP kinase signaling responses (Park et al., 2006b; 2011). By using a yeast strain carrying the *fus1-EGFP* reporter, we monitored and compared the mating responses of the captured, HU-treated, and bulk cells. Cells were treated with α-factor at a concentration of 20 nM and the green fluorescence from the stimulated cells was monitored using a live-cell imaging system. The average green fluorescence intensity from the captured cells was similar to that from the cells of the bulk population, whereas the HU-treated cells showed significantly low fluorescence intensity (Fig. 4). Thus, HU treatment appeared to interfere with the mating signaling response, thereby suggesting that chemical arrest is not always compatible with biological application. The mating response of the captured cells was as robust and dynamic as that of the bulk population. This indicates that cell cycle synchronization by capturing them in microwells does not affect cell viability or disturb cell-signaling processes.

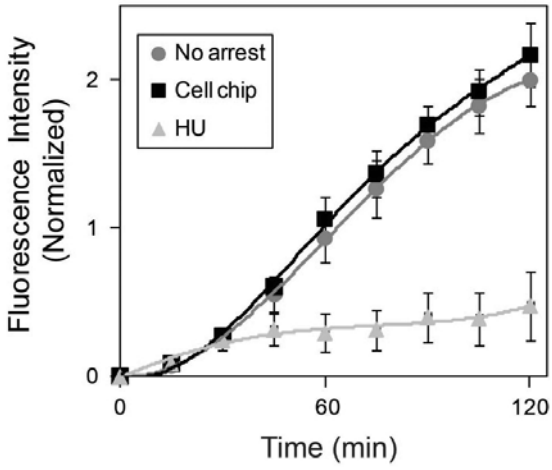

Fig. 4. Analysis of mating signaling responses. Cells treated with or without HU were captured into the cell chip and treated with 20 nM of α-factor, and the green fluorescence intensity of cells was analyzed using a live-cell imaging system. The bulk cells were harvested and stimulated with 20 nM of α-factor and the green fluorescence intensity was quantitatively monitored using flow cytometry. Cells synchronized by capturing a response as robust as that of the bulk cells, while HU-treated cells failed to generate a significant response.

DISCUSSION

Several chemical, genetic, and physical methods have been developed and used widely for the synchronization of the cell cycles of eukaryotic cells (Austin and Warren, 1983; Breeden, 1997; Day et al., 2004; Futcher, 1999; McEwen et al., 1968; Merrill, 1998; Reid and Hartwell, 1977; Wyrick et al., 1999). However, currently established methods are known to have certain limitations, including induction of stress responses or

physiological perturbations (Cho et al., 1998; Spellman et al., 1998; Wyrick et al., 1999) and the requirement of cells with specific genetic backgrounds (Hartwell et al., 1970) and specialized equipments (McEwen et al., 1968; Walker, 1999; Wyrick et al., 1999). Often, these limitations hinder the biological analyses.

We developed a simple yet efficient method for the isolation of yeast cells at the G1 phase from bulk populations. Since the size and shape of yeast cells change during the progression of the cell cycle, we utilized a cell chip platform with microwells having specific dimensions (diameter and depth of 8 μm) that are optimal for capturing of small yeast cells at the G1 phase. The synchronization by capturing of cells in microwells seems to be as effective as HU treatment (Fig. 3). Furthermore, unlike the HU-treated cells, the cells synchronized by capturing retained their biological integrity and viability, which was proven by their ability to generate mating signaling responses. The cell chip-based capturing of yeast cells described in this study provides for a simple, efficient, and non-invasive means for cell cycle synchronization with minimal biological perturbations.

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