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Rapid selection of single cells with high antibody production rates by microwell array

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ABSTRACT

Selection of single cells capable of producing target proteins at high rates is crucial for the development of protein manufacturing processes. Here, we present the rapid selection of single cells producing immunoglobulin antibodies at high specific rates by microwell array and microengraving. Chinese hamster ovary (CHO) cells secreting chimeric antibodies were deposited in a microwell array in a manner such that each microwell contained a single cell. Secreted antibodies in the microwells were transferred onto a glass slide by microengraving, followed by interrogation using fluorescence-based immunoassay. Single cells displaying high signal intensities were selected, retrieved, and clonally expanded to assess their specific antibody production rates. Three successive rounds of the process resulted in the selection of single cells showing significantly increased antibody production rates. The present approach can be applied to the selection of single cells for producing other therapeutic proteins in a high-throughput manner.

1. Introduction

Immunoglobulin antibodies are widely used in many areas as molecular binders and therapeutic agents, constituting a major portion of approved protein-based drugs (Walsh, 2005). Due to the necessity of proper glycosylation, antibodies are currently produced using mammalian cell systems such as Chinese hamster ovary (CHO) cells or murine lymphoid cell lines (e.g., NS0, SP2/0) (Birch and Racher, 2006; Butler, 2005). One of the key issues in the manufacture of antibodies using mammalian cells is the selection and establishment of adequate cell lines capable of producing antibodies at high specific production rates. Hence, much effort has been made to develop methods for selecting mammalian cell lines with desired production capacity. Such cell lines are generally obtained through multiple rounds of selection from heterogeneous populations followed by interrogation of the selected clones (Browne and Al-Rubeai, 2007). Genetically engineered cells are heterogeneous in terms of their growth rates and protein production rates even though they are clonal cells (Browne and Al-Rubeai, 2007; Pilbrough et al., 2009). Heterogeneity of cells has been shown to influence the performance of the overall protein manufacturing process.

There have been reported many approaches for selecting single cells with high protein production capacities. A microtiter plate-based method is commonly used in conjunction with enzyme-linked immunosorbent assay (ELISA). However, this method is laborious and time-consuming since it requires handling of many well plates at a time to obtain sufficient throughput (Puck and Marcus, 1955). In addition, the assay should be conducted only after the concentration of secreted proteins reaches a measurable level. Fluorescence-activated cell sorting (FACS) was shown to allow the selection of single cells in a high-throughput manner (Brezinsky et al., 2003; Sleiman et al., 2008). However, its application is limited to the cells secreting the target proteins. In an effort to overcome these drawbacks, gel microdroplets encapsulating cells or designed affinity matrixes mounted upon cell surfaces were employed to capture secreted proteins (Holmes and Al-Rubeai, 1999; Weaver et al., 1997). Nonetheless, these methods are technically complex, causing changes in the physiology and production capacity of cells. Recently, a microengraving method to assay secreted proteins from single cells in array of microwells was reported (Jin et al., 2009; Love et al., 2006; Park et al., 2010; Story et al., 2008). In this approach, the secreted proteins in individual microwells are transferred to a glass surface modified with capturing molecules having specific affinity for the secreted proteins in a form of protein microarray. We previously applied this approach to the selection of single CHO cells producing glycoproteins with desired glycosylation (Park et al., 2010).

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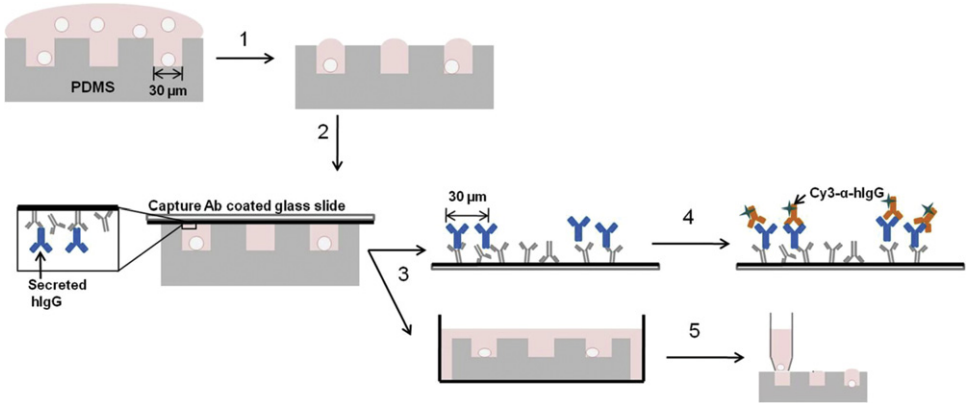

Fig. 1. Schematic of selecting single cells with high antibody production rates using a microwell array. (1) A solution of hIgG-producing CHO cells is deposited on a PDMS microwell array, and the cells are left to settle down into the microwells. (2) A glass slide coated with anti-hIgG antibody is placed over the microwell array, pressed, and incubated for 10 min to capture hIgG secreted by individual cells in the microwells. (3) After incubation, the cell-containing microwell array is separated from the glass slide and immersed in a reservoir of culture medium for retrieval after sorting. (4) The resulting slide is then treated with dye-labeled secondary anti-hIgG antibody to estimate the amount of secreted antibodies in the individual microwells. (5) Based on the analysis of the fluorescent spots, corresponding single cells are selected, retrieved, and expanded for the production of hIgG.

We here present the rapid selection of single CHO cells showing high antibody production rates by array-based analysis of secreted antibodies in individual microwells (Fig. 1). The chimeric antibody-secreting CHO cells were deposited in an array of microwells in a manner such that each well contained a single cell. Secreted antibodies in the microwells were then transferred onto a glass slide by microengraving, followed by interrogation using fluorescence-based immunoassay. Selected single cells were retrieved, clonally expanded, and tested in terms of specific antibody production rates. Three rounds of the process were conducted, resulting in the selection of single cells showing significantly increased antibody production rates.

2. Methods

2.1. Cells and culture

CS13*-1.0 cell line expressing a chimeric antibody with human constant and mouse variable regions against the S surface antigen of hepatitis B virus was used as the starting antibody-producing cell type (Kim et al., 1998). CS13*-1.0 cell line was derived from a parental cell through multiple rounds of screening under selection pressure by methotrexate (MTX) at different concentrations. Cell lines were grown in IMDM media (Gibco) supplemented with 5% d-FBS (Gibco), 1% antibiotic–antimycotic solution, and 1.0 μM MTX at 37 °C (5% CO_2). The cells were sub-cultured every 3 days for long-term culture by trypsinization.

2.2. Probing of purified hIgG

Purified human immunoglobulin G (hIgG, Sigma) was used as a standard for the assay. Standard hIgG was serially diluted and spotted on an ultrathin nitrocellulose-coated glass slide (PATH slides, GenTel Bioscience, Madison) in nine spots with a 3 × 3 format using a robotic microarrayer (OMNIGRID, Genomics Solutions), followed by incubation for 1 h at 37 °C. Following washing with TBS/Tween20 (0.05%, w/v) (TBST) and deionized water, a solution of anti-human IgG (Fc-specific)-Cy3 antibody (Sigma) (Cy3-α-hIgG) was loaded on the surface of the slide and incubated for 30 min at room temperature. The resulting slide was thoroughly rinsed with TBST and deionized water, dried with nitrogen gas, and scanned with a GenePix 4100A scanner (Molecular Devices) using a 532 nm laser. The fluorescence intensities from Cy3 were measured and analyzed using GenePix Pro 6.0 software (Molecular Devices) in order to correlate them with the levels of hIgG. The intensities of all of the spots were corrected considering the background level.

2.3. Cell deposition in a microwell array

The microwell arrays were fabricated with poly (dimethylsiloxane) (PDMS) using a photolithographic process as described in our previous report (Park et al., 2010). The arrays were composed of blocks of microwells with 30 μm diameter, 35 μm depth, and 100 μm inter-microwell distance. A single array set contained 45 × 45 microwells. The fabricated PDMS microwell array was sterilized by an autoclave under 1 kg/cm^2 pressure for 15 min. To facilitate cell adhesion, the inside wall and bottom of the microwells were coated with fibronectin by incubation with fibronectin solution (50 μg/mL in PBS, Sigma) for 1 h. The surface of the array was washed with sterile PBS by pipetting several times, and fibronectin absorbed on the inter-well region was swept carefully using a cotton swab soaked in acetone. A suspension of cells was diluted to 5 × 10^5 cells/mL in culture media, and 20 μL of the cell suspension was pipetted onto a set of arrays and left to settle down into the microwells by gravitational force. After 10 min, residual cells on the surface between the microwells were removed by aspiration and moderate washing with culture media. The resulting microwell array containing deposited cells was submerged in a reservoir of culture media and incubated for 12 h at 37 °C (5% CO_2) to allow attachment of cells.

2.4. Capture and probing of secreted hIgG in a microwell array

The ultrathin nitrocellulose-coated glass slides (PATH slides, GenTel Bioscience) were modified with goat anti-human IgG antibody (Sigma) and used to capture hIgG secreted by single cells in the microwells. A solution of anti-human IgG antibody (0.5 mg/mL in PBS buffer, pH 7.4) was loaded onto the surface of the slides and incubated at room temperature for 2 h in a humidity chamber (75% humidity). After incubation, the slides were immersed in TBST containing 1% bovine serum albumin (BSA, Sigma) for blocking at 4 °C overnight. The slides were washed with deionized water immediately before use. The cell-loaded microwell array was washed with fresh culture media, followed by vacuum aspiration while tilting the array to remove excess medium on the surface of the array. To capture the secreted hIgG, the capture glass slide was placed on the microwell array containing individual cells, pressed with the appropriate weight, and incubated for 10 min at 37 °C (5% CO_2).

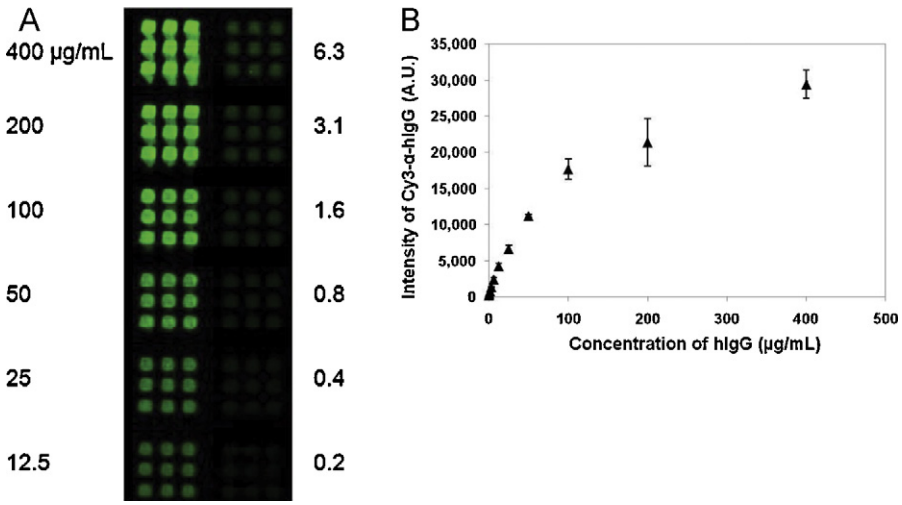

Fig. 2. Fluorescence image and intensity of purified hIgG spots. The levels of hIgG were probed by Cy3-α-hIgG, followed by scanning with a fluorescence scanner. The hIgG was serially diluted and spotted on the glass slide in nine spots with a 3 × 3 format using a robotic microarrayer, followed by probing with Cy3-α-hIgG. (a) Fluorescence image of the spots. (b) Changes in the fluorescence intensities of the spots.

Following incubation, the glass slide was removed from the microwell arrays and washed with TBST and deionized water. The slide was then treated with Cy3-α-hIgG (2.5 μg/mL) following the same procedure as that used in the probing of purified hIgG described above. The resulting slide was thoroughly washed with TBST and deionized water, dried with nitrogen gas, and scanned with a GenePix 4100A scanner (Molecular Devices). The fluorescence images were analyzed using the GenePix Pro 6.0 software (Molecular Devices). Background intensities were subtracted using median values measured for the empty wells of each sub-array. The spots showing obvious defects were excluded from analysis.

2.5. Retrieval and expansion of single cells

Selected cells were manually retrieved from the corresponding microwells using a micropipette with a tip diameter of 50 μm as previously reported (Park et al., 2010). Briefly, micropipettes were drawn using a Flamming/Brown micropipette puller (Sutter Instrument). To retrieve the cells, the array of microwells was positioned under the 10× lens of an inverted microscope (DMI 3000 B, Leica) and treated with culture media containing 10% trypsin (TrypLE™ Express, Gibco). The micropipette tip was positioned over a cell-containing microwell, and the media was pulled out from the microwell by capillary force. The culture media containing the detached cell in the micropipette were transferred into a 96-well plate with 200 μL of media supplemented with 5% d-FBS. Deposition of the cells into a 96-well plate was visually verified. Retrieved cells were expanded to approximately 10^6 cells in 75-cm^2 T-flasks according to standard procedures and stored at −70 °C for banking.

2.6. Determination of antibody production rate by a single cell

To determine the specific production rate of hIgG by a single cell, exponentially growing cells were seeded at a density of 1×10^5 cells per well in a 6-well plate, followed by incubation at 37 °C (5% CO_2). The culture media in each well were sequentially sampled every 12 h and stored at −20 °C until the assay was conducted. The number of cells in each well was estimated with respect to culture time using a hematocytometer after trypsinization. The amount of produced hIgG was determined by conventional ELISA method. Briefly, the microtiter plate wells were coated with mouse anti-human IgG antibody (2 μg/mL in sodium carbonate (pH 9.0), Sigma) overnight at 4 °C, followed by treatment with blocking buffer (2% BSA in TBST) for 1 h at room temperature. Peroxidase-conjugated anti-hIgG specific to Fc (Sigma) was used for the immunoassay. The color was developed by treatment with 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma), and the optical density of the reaction solution was measured at 655 nm using a spectrophotometer (Infinite M200, Tecan). The specific production rate is defined as the amount of hIgG produced by a single cell per day. The concentration of hIgG was estimated from the standard curve obtained with purified hIgG, and the specific production rate was calculated by plotting the hIgG concentrations against the time integral values of the viable cell growth curve as described elsewhere (Renard et al., 1988).

3. Results and discussion

3.1. Analysis of hIgG spotted on a glass slide

We first tested whether the amount of hIgG can be determined with Cy3-labeled polyclonal anti-hIgG antibody (Cy3-α-hIgG) as well as whether Cy3-α-hIgG intensity can be correlated with the amount of hIgG. For this, purified hIgG was spotted on a slide at various concentrations, followed by probing with Cy3-α-hIgG. Fig. 2a shows typical fluorescence images of the spots of purified hIgG, and the correlation between the signal intensity of Cy3-α-hIgG and concentration of hIgG is shown in Fig. 2b. The Cy3-α-hIgG intensity was linearly proportional to the hIgG concentration up to 100 μg/mL. Further increase in hIgG concentration resulted in the saturation of signals. This result indicates that the amount of hIgG was correlated with the fluorescence intensity of Cy3-α-hIgG.

3.2. Microengraving and analysis of hIgG secreted by single cells in microwells

To test the feasibility of the microengraving method for analysis of hIgG secreted by single cells in microwells, CS13*-1.0 cells producing hIgG were deposited on the surface of a PDMS microwell array. The microwell diameter, depth, and inter-well distance were fixed at 30 μm, 35 μm, and 100 μm, respectively, for adequate occupancy based on our previous report (Park et al., 2010). Similar to the previous results, the occupancy ranging from one to two cells per microwell was approximately 65% of the total microwells. The cell-containing microwell array was submerged in a reservoir of culture media for 6 h, and washed with fresh media

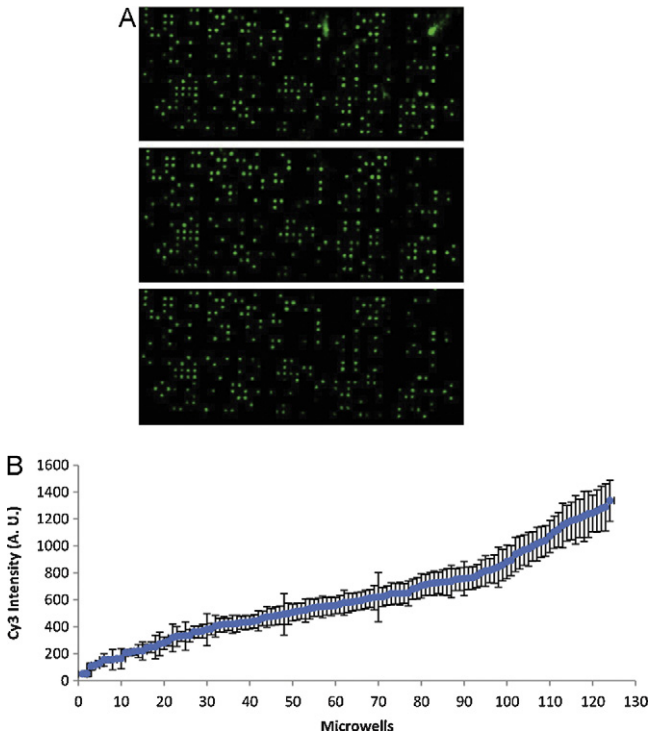

Fig. 3. Fluorescence images and distribution of the Cy3-fluorescence intensities from the same microwell array containing the cells in three successive microengraving experiments. (A) Fluorescence images of the three replicas. Top: first replica from the first microengraving experiment. Middle: second replica. Bottom: third replica (B) Distribution in the Cy3-fluorescence intensities in each well shown by the mean and standard deviation. Individual microwells were sorted in order of increasing fluorescence intensity.

three times. For microengraving, the microwell array was dewetted and placed in contact with a glass slide coated with anti-hIgG monoclonal antibody, followed by compression with an appropriate weight and incubation for 10 min. The resulting slide was probed with Cy3-α-hIgG and scanned using a scanner to correlate fluorescence intensities with the amounts of secreted hIgG in the individual microwells.

We checked the reproducibility and reliability of the experimental procedure by analyzing the standard deviation of the Cy3-fluorescence intensity measurements in individual microwells in three successive microengraving experiments. We first measured the Cy3-fluorescence intensities from the microwells containing the cells as described above. For the second measurement, same microwells containing the cells were incubated in reservoir with fresh culture media for 1 h at 37 °C in 5% CO_2 incubator. Following a washing with fresh media three times, the microwell array was placed in contact with a glass slide coated with anti-hIgG monoclonal antibody for 10 min for producing and capturing the antibodies. The resulting slide was treated with Cy3-α-hIgG and scanned to measure the Cy3-fluorescence intensities. The third fluorescence measurement was carried out using the same procedure as the second measurement. Fig. 3A shows the fluorescence images of three successive measurements, and distinct variations in the Cy3-α-hIgG intensities were observed from spot to spot. This result strongly implies that individual cells in microwells produced the different amounts of hIgG. We calculated the mean and standard deviation of the Cy3-fluorescence intensity measurements in three successive experiments shown in Fig. 3A, and sorted individual microwells in order of increasing average fluorescence intensity. As can be seen in Fig. 3B, the fluorescence intensity measurements of individual microwells were shown to be reproducible and reliable, verifying the robustness of the experimental procedure.

Table 1
Specific production rates of hIgG by selected cell lines derived from different cell lines. Values represent means and standard deviations in two independent experiments.

Maternal cell lines	Cell lines (signal intensity[a])	Specific production rates (pg hIgG/cell/day)
CS13*-1.0	r1-23 (L)	19.5 ± 3.2
	r1-117 (L)	19.6 ± 0.7
	r1-17 (H)	15.1 ± 0.3
	r1-67 (H)	20.0 ± 1.4
	r1-169 (H)	22.9 ± 1.9
	r1-227 (H)	20.3 ± 1.7
r1-169	r2-105 (L)	21.2 ± 2.0
	r2-223 (L)	23.3 ± 1.0
	r2-4 (H)	28.1 ± 0.4
	r2-48 (H)	28.9 ± 1.2
	r2-72 (H)	24.4 ± 2.1
	r2-178 (H)	23.1 ± 1.4
	r2-189 (H)	25.8 ± 0.8
	r2-193 (H)	20.7 ± 1.9
	r2-196 (H)	21.7 ± 1.1
r2-48	r3-68 (L)	24.2 ± 0.8
	r3-113 (L)	26.6 ± 1.1
	r3-19 (H)	28.8 ± 0.8
	r3-90 (H)	29.3 ± 3.1
	r3-122 (H)	29.2 ± 1.9

[a] Fluorescence intensities of selected single cells in microwells. L and H stand for low and high signal intensities, respectively.
The specific production rate of the starting cell line (CS13*-1.0) was estimated to be 18.9 ± 1.6 pg/cell/day.

3.3. Selection, retrieval, and expansion of single cells

In order to select single cells with high specific production rates of antibodies, approximately 1000 wells were screened first. Of them, we selected around 200 wells displaying single-cell occupancies and S/N ratios above 3 for further analysis. Fig. 4A shows the typical fluorescence image of the slide comprising the arrays of secreted hIgG, and distribution of the Cy3-α-hIgG signal intensities were observed as in Fig. 3A, confirming the reproducibility of the experimental procedure. The fluorescence intensities from individual microwells showed a distinct distribution (Fig. 4B), implying that the individual cells were heterogeneous in their specific antibody production rates despite being a clonal population. To analyze the difference between the high- and low-producing single cells, we selected the upper and lower 5% of the cells. The average fluorescence intensity was 1640. The fluorescence intensities of the lower group ranged from 152 to 353, whereas those of the upper group were within the range between 3598 and 5152. The selected cells were recovered from the array using micromanipulation techniques for further examination after clonal expansion as described in our previous report (Park et al., 2010). Selected cells were established as new cell lines by following standard protocol. Two cell lines (r1-23 and r1-117) with low Cy3-α-hIgG intensities and four cell lines (r1-17, r1-67, r1-169, and r1-227) showing high Cy3-α-hIgG intensities were grown for production of hIgG. To validate our approach, we determined the specific antibody production rates of selected cells by measuring the amounts of produced hIgG in microwells using conventional ELISA. As shown in Table 1, the specific production rates of the six cell lines (r1-23 to r1-227) were estimated to be 19.5 ± 3.2, 19.6 ± 0.7, 15.1 ± 0.3, 20.0 ± 1.4, 22.9 ± 1.9, and 20.3 ± 1.7 pg/cell/day, respectively. The specific production rate of the starting cell line (CS13*-1.0) was estimated to be 18.9 ± 1.6 pg/cell/day. Interestingly, most cell lines displayed comparable productivities to the starting cell line (CS13*-1.0), implying

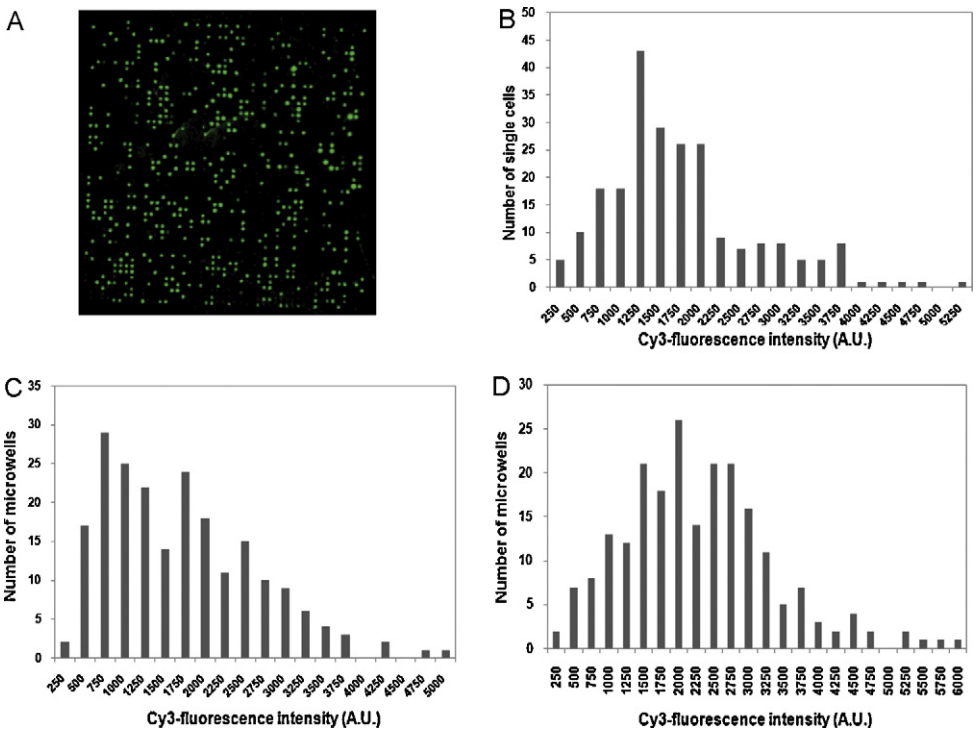

Fig. 4. Distribution in the Cy3-fluorescence intensities by hIgG-producing cells derived from different cell lines in microwells. Secreted hIgG was captured using a glass slide coated with anti-hIgG antibody, probed with Cy3-α-hIgG, and scanned using a fluorescence scanner. (A) Distribution in the Cy3-fluorescence intensities by the cells derived from CS13*-1.0 cell line. (B) Distribution in the Cy3-fluorescence intensities by the cells derived from r1-169 cell line. (C) Distribution in the Cy3-fluorescence intensities by the cells derived from r2-48 cell line.

that antibody production capacities of these cell lines returned to their original levels during clonal expansion. Similar to the secretion of protein using *Pichia pastoris* (Love et al., 2010), our result also suggests that fluctuations in the production rates stemmed from a short-lived, epigenetic event which became diluted over a number of cell divisions. It was previously shown that lower production capacities of cell lines derived from a single cell can be restored during clonal expansion (Love et al., 2010; Pilbrough et al., 2009; Sleiman et al., 2008).

3.4. Second round of single cell selection

In order to select single cells producing hIgG at increased specific production rates, we repeated the same selection procedure starting with the r1-169 cell line, which showed the highest specific production rate in the first round. Based on the fluorescent signal intensities on a glass slide, approximately 200 cells were screened and analyzed (Fig. 4C). Of them, the upper and lower 5% of the cells were selected, retrieved, and expanded for banking. The average intensity for the second round was 1875, and fluorescence intensities for the lower and upper groups were 227–421 and 3418–4939, respectively. Two cell lines (r2-105 and r2-223) with low signal intensities and seven cell lines (r2-4, r2-48, r2-72, r2-178, r2-189, r2-193, and r2-196) showing high signal intensities were grown for the production of hIgG, and the specific production rates of selected single cells were determined. The specific production rates of nine cell lines (r2-105 to r2-196) were shown to range from 20.7 ± 2.0 to 28.9 ± 1.2 pg/cell/day (Table 1). Of the seven cell lines exhibiting high fluorescence intensities, four cell lines (r2-4, r2-48, r2-72, and r2-189) displayed improved production rates compared to the starting cell line (r1-169). The r2-48 cell line exhibited the highest specific production rate of 28.9 ± 1.2 pg/cell/day. It was expected that new cell lines derived from a single cell that were selected in the first round would have higher production rates than the maternal cell line (r1-169). However, the four cell lines showed improved specific production rates compared to the maternal cell line. It was previously suggested that heterogeneity in the production rates of cell lines is caused by two major factors (Pilbrough et al., 2009). One is heritable clonal variation, and the other is non-heritable expression instability. Expression instability is typically caused by random fluctuation in chromatin folding and can be restored during repetitive cell division. In such case, the production rates of cell lines would return to their original levels during clonal expansion. On the other hand, heritable clonal variation arises from the rearrangement of transgene copies, and it is preserved during cell proliferation, resulting in the maintenance of stable productivity. Four selected cell lines retained their high specific antibody production rates, implying that their production capacities were due to a heritable genetic modification. But, the specific production rates of the other cell lines were restored to that of the maternal cell line even though they displayed high fluorescence intensities in the microwell assay, and this seems to be due to non-heritable expression instability. A stringent selection threshold is expected to increase the selection of single cells maintaining high production rates during cell proliferation.

3.5. Third round of single cell selection

In an effort to select single cells having improved production rates, we repeated the same selection procedure starting with the r2-48 cell line, which had been selected in the second round. Based on the fluorescent signal intensities on a glass slide (Fig. 4D), we screened for the upper and lower 5% of the single cells to establish new cell lines. As for the third round, the average intensity was 2153. The ranges of fluorescence intensities for the lower and upper groups were 222–517 and 4414–5889, respectively. As a result, two cell lines (r3-68 and r3-113) showing low signal intensities and three cell lines (r3-19, r3-90 and r3-122) with high signal intensities

were selected, and their specific production rates were determined after clonal expansion following the same procedure as described above. The specific production rates of the single cell lines were estimated to be 24.2 ± 0.8, 26.6 ± 1.1, 28.8 ± 0.8, 29.3 ± 3.1, and 29.2 ± 1.9 pg/cell/day, respectively (Table 1). As mentioned above, the cell lines, which showed low production rates mainly due to non-heritable expression instability, recovered their production capacities to that of the maternal cell line (r2-48). On the other hand, three cell lines (r3-19, r3-90, and r3-120) displaying higher signal intensities maintained productivities comparable to that of the maternal cell line. The r3-90 cell line displayed the highest specific production rate of 29.3 pg/cell/day, which corresponds to a 55% increase in the specific production rate compared to the starting cell line (CS13*-1.0). This increase is significant when one considers the increment that is usually achieved by the LDC method, which relies on a gradual increase in MTX concentration. In the case of the CS13* cell line used in this study, only a 5.8% increase in the specific production rate was obtained even though the MTX concentration increased up to 4 μM (Kim et al., 1998). The specific production rates of the selected cell lines in the third round were almost the same as that of the starting cell line (r2-48). Therefore, it is likely that the production capacity of the selected cells already reached saturation, and cell lines with higher production rates were no longer selected. Repeated rounds of the procedure allowed for the selection of single cells showing improved production rates at a given selection pressure. Thus, to further improve the production rate, it may be necessary to perform the selection process under enhanced selection pressure such as higher MTX concentration. Higher selection pressure might result in the survival of cells with increased transgene numbers.

4. Conclusion

We have demonstrated the selection of single cells showing high specific production rates of antibodies in a high-throughput manner using a microwell array. The present approach offers several advantages over conventional selection methods. First, a few thousand cells secreting target proteins can be screened at the same time in a high-throughput manner using microwell arrays. Second, proteins produced by single cells in microwells can be assayed after a short period of cultivation since the protein concentrations rapidly reach sufficient levels due to the small volume of the microwells (around 25 pL). Based on our results, the total time for selecting single cells can be shortened to less than 10 h from about 1 week by the conventional LDC method. An automated cell retrieval system will significantly improve the efficiency of the present approach. It is anticipated that our approach can be widely applied to the selection of single cells producing various therapeutic proteins at high specific rates.

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