1 Title: Single-cell-based breeding: Rational strategy for the establishment of cell
2 lines from a single cell with the most favorable properties
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4 Running title: SINGLE-CELL-BASED BREEDING
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Abstract

For efficient biomolecule production (*e.g.*, antibodies, recombinant proteins), mammalian cells with high expression rates should be selected from cell libraries, propagated while maintaining a homogenous expression rate, and subsequently stabilized at their high expression rate. Clusters of isogenic cells (*i.e.*, colonies) have been used for these processes. However, cellular heterogeneity makes it difficult to obtain cell lines with the highest expression rates by using single-colony-based breeding. Furthermore, even among the single cells in an isogenic cell population, the desired cell properties fluctuate stochastically during long-term culture. Therefore, although the molecular mechanisms underlying stochastic fluctuation are poorly understood, it is necessary to establish excellent cell lines in order to breed single cells to have higher expression, higher stability, and higher homogeneity while suppressing stochastic fluctuation (*i.e.*, single-cell-based breeding). In this review, we describe various methods for manipulating single cells and facilitating single-cell analysis in order to better understand stochastic fluctuation. We demonstrated that single-cell-based breeding is practical and promising by using a high-throughput automated system to analyze and manipulate single cells.

Mammalian cells have been utilized for long time as a resource in the manufacture of biomolecules and biopharmaceuticals. Hybridomas and Chinese hamster ovary (CHO) cells are the most common cells used in this field (1,2). For efficient production of biomolecules using these cells, it is essential to select cells from cell libraries based on their high expression rate, propagate the cells while maintaining a homogeneous expression rate, and stabilize the high expression rate for long-term culture. Cell screenings for conventional methods of addressing these issues (mass production, cellular homogeneity, and cellular stability) have so far only been executed at the colony level (*i.e.*, single-colony-based breeding; **FIG. 1A**). Candidate colonies are initially isolated using selection markers (*e.g.*, drug resistance (3–5), auxotrophy (6,7), reporter gene expression (8–10)), subjected to limiting dilution (11), and then evaluated by immunoassays and/or functional assays to identify the single colonies with the highest expression. Recently, the detection and retrieval of those colonies with the most favorable properties have been performed automatically using the robotic system ClonePix FL (12). This system uses fluorescence-linked immunosorbent assay (FIA) to detect colonies that have the highest expression rates in groups of candidate colonies that are grown in a semi-solid medium. The system then uses a metal capillary to retrieve the colonies. Cell lines are established from the selected colonies by propagation over several passages and further evaluation of the stability of the higher rate of expression.

Two major problems of conventional cell breeding

After hybridomas established by the conventional ways are grown by fed-batch culture for more than 80 days, their expression decreased in a culture time-dependent manner (13). In addition, when isogenic IgG-producing CHO cells were separated into two groups (higher and lower IgG production) by fluorescence-activated cell sorter (FACS), the expression levels of both groups converged to that of parental cells after long-term culture (10). These spontaneous changes are considered to be due to the “cellular heterogeneity” in a selected single colony or “stochastic fluctuation” that occurs in each cell during long-term culture. Moreover, when single cells from isogenic hybridomas grow into single colonies of more than 4 cells, each colony has distinct levels of antibody expression because of stochastic fluctuation during the early phase of cell growth (14). Therefore, for effective establishment of cell lines, it is crucial to isolate single cells that have the highest expression rate as quickly as possible, and then propagate them homogeneously while minimizing the stochastic fluctuation (*i.e.*, single-cell-based breeding; **FIG. 1B**). However, single cells cannot be manipulated in a high-throughput manner using conventional techniques. This makes it difficult to minimize cellular heterogeneity and determine the molecular mechanisms that underlie stochastic fluctuation in specific single cells. On the other hand, substantial numbers of mammalian cell lines that were created by single-colony-based breeding for industrial production of biomolecules have been incidentally found to possess excellent mass productivity, cellular homogeneity, and cellular stability. This strongly suggests that the stochastic fluctuation was successfully suppressed in spite of the lack of precise knowledge about its mechanism. An automated system for manipulating single cells in a high-throughput manner that could identify single cells with the most favorable

properties from huge number of candidate cells and isolate single cells of interest automatically would facilitate the refinement of cell breeding. This innovative approach would solve the problem of cellular heterogeneity, lead to the deciphering of the mechanisms of individual cell stochastic fluctuation, and facilitate the effective establishment of biomolecule-producing cells with high expression, homogeneity, and stability.

Here, we will provide an overview of the current techniques for single-cell analysis and isolation, and introduce an automated single-cell analysis and isolation system that we developed for single-cell-based breeding. We will also describe how to refine cell breeding using this system, and discuss the potential of this rational and reliable methodology for cell-based research and industry.

Current techniques for identifying single cells with the highest secretion rate

To select single cells that secrete relatively large amounts of biomolecules, candidate cells in a cell library were often fluorescently labeled according to their secretion rates, and then subjected to fluorescent measurement, and single-cell isolation. This process requires nascent biomolecules to be retained within cell bodies by treating them with brefeldin A and labeling them with fluorophores, which severely affects cell viability and prevents the isolated cells from producing biomolecules. To accomplish single-cell-based breeding in a non-destructive, high-yield, and high-throughput manner, a reporter gene that encodes a fluorescent protein is introduced into the biomolecule gene using an internal ribosome entry site (IRES) (8). This allows the fluorescent measurement of the expression rate of each cell (**FIG. 2A**). However, this “bicistronic

reporter assay” requires additional genetic modification and directly evaluates the synthesis rate of biomolecules, not their secretion rate. Gel microdrop assays have been used to measure secretion. For this assay, candidate cells are grown in a solid-medium containing biotinylated agarose, streptavidin, and biotinylated capture antibody. The cells are then formulated into gel microdrops, and placed in contact with fluorescence-labeled detection antibodies. The secreted biomolecules of each cell are then immobilized in the agarose matrix of the gel microdrop and form fluorescence-labeled immunocomplexes (**FIG. 2B**) (15). This method can be adapted to high-throughput isolation of efficient biomolecule-producing cells in a secretion-dependent manner, but both low encapsulation efficiency of cells (<15% of gel microdrops) and the physical stress caused by the solid medium remain to be addressed. Moreover, to avoid the encapsulation of cells into gel microdrops, the cell surface is initially coated with semi-solid medium, chemically labeled with biotin, and then mixed with neutravidin and biotinylated capture antibody. Secreted biomolecules immobilized as an immunocomplex in the vicinity of cells are detected by fluorescence-labeled detection antibody. This assay is known as the matrix-based secretion assay (**FIG. 2C**) (16). Although this assay is more efficient than the gel microdrop assay, the cells still suffer from chemical stress caused by direct biotinylation. More recently, a cell-surface fluorescence-linked immunosorbent assay (CS-FIA) was developed to measure the amount of nascent biomolecules secreted from single cells in a non-destructive manner (17). After the surface of candidate cells is modified with non-toxic lipid-labeled capture antibodies, nascent secreted antibodies are captured on the cell surface and subsequently detected with fluorescence-labeled detection antibodies (**FIG. 2D**). This method can be used to measure the amount of captured

antibodies with extremely high sensitivity (from 6.25 fg/cell to 6.40 pg/cell). CS-FIA is a promising and reliable non-destructive and high-throughput method for identifying the individual cells that most efficiently secrete biomolecules.

Conventional techniques for single-cell analysis and isolation

The “limiting dilution method” has long been used to generate single colonies from single cells in order to isolate single cells with favorable properties (*e.g.*, the highest synthesis and secretion rate of biomolecules). Candidate cells are serially diluted in multi-well plates so that each well contains approximately 1 cell. The cells then undergo long-term culture (3–7 days) for colony formation. Either supernatant or cell lysate of each colony is then subjected to enzyme-linked immunosorbent assay (ELISA) or to a functional assay in order to **rank the colonies by according to the degree of favorable properties (*e.g.*, productivity of biomolecules) (*see* Nos. 1-8 with circle in **FIG. 1A**)**. Because the cells within a colonies are often heterogeneous, limiting dilution should be repeated until a colony with low cellular heterogeneity is obtained that also has the most favorable properties (14,18). The limiting dilution method is laborious, time-consuming, and unsuitable for high-throughput screening.

FACS, which uses fluorescence as an indicator for the most favorable cell properties, has been widely used for a high-throughput single-cell analysis and isolation in biological and biomedical fields (1). In this method, cells in sheath solution are introduced at high flow velocity ($\sim 10^4$ cells/sec) in a high-pressure flow path (~ 70 psi) to align them in a laminar flow formation. Target cells are identified using an optical system based on their fluorescent intensities and are then sorted into reservoir wells

using high voltage ($\sim 6 \times 10^3$ V). At least 10^5 cells at a density of $10^5 \sim 10^6$ cells/ml (19,20) are necessary for FACS analysis. Positive cells should make up more than 0.1% of the candidate cells (21), which is the threshold for discriminating them from background cells spectrophotometrically. Before cell sorting, a portion of the cell sample must be discarded in order to optimize the measurement conditions. Additionally, single cells isolated by FACS generally suffer from chemical stress derived from the sheath solution, mechanical stress from laminar-flow formation, and electrical stress from sorting (22,23). These issues render FACS unsuitable for rare cells (less than 10^5 cells), diluted cells (less than 10^5 cells/ml), samples containing less than 0.1% positive cells, and vulnerable cells.

As an alternative to FACS, on-chip flow cytometers have been developed that use microfluidics (24–26). Single cells in a culture medium are flowed at a moderate flow velocity ($\sim 5 \times 10^2$ cells/sec) under atmospheric pressure through a microchannel that is ~ 80 μ m in diameter. The cells of interest are identified by their fluorescent intensities, and are sorted to reservoir wells by weak voltage (~ 6 V). Using this method, the processing rate can be increased by increasing the number of microchannels in parallel. This system is suitable for rare cells and vulnerable cells. However, probably due to the failure of cells to align in the microchannels, this system is only able to detect 70% of positive cells in samples (27).

Cell array systems

In cell array systems, each cell is accommodated into a microchamber (10 $\sim$ 30 μ m in diameter, approximately 2×10^5 microchambers/chip) made of various polymers, such

as, polystyrene, polymethylmethacrylate, polydimethylsiloxane, or silicon substrates (21,28,29). These cell arrays are subjected to large-scale analysis of single cells under mild conditions (atmospheric pressure, culture medium). The cells of interest can be detected at the single-cell level with a high signal to noise ratio, which excludes contamination by pseudo-positive cells in the same microchamber. However, because this method involves manual cell isolation using a micromanipulator, a semi-automated colony isolation system, CellCelector, has been optimized as a single-cell isolation system (30). In this system, one or a few antibody-secreting cells are accommodated into cuboid microchambers (50 μm wide $\times$ 50 μm height $\times$ 50 μm depths) and covered with a glass slip coated with capturing molecules (anti-IgG antibodies). After removing the glass slip, the secreted antibodies captured on the slip are visualized with fluorescence-labeled secondary antibodies or specific antigens for identifying the cells that secrete antigen-specific antibodies. After matching the positions of the glass slip with those of microchambers, the cells of interest are automatically retrieved from the respective microchambers using a glass capillary. A similar automated single-cell isolation system, Cellporter, has been recently developed (31). When cells in microchambers (10 μm in diameter, 15 μm depths) were stimulated by oxidative or acidic stress, the responsive cells are manually identified by an independent fluorometric system and then each cell is automatically retrieved using a metal capillary. Both CellCelector and Cellporter systems facilitate the isolation of single cells from rare samples (less than 10^5 cells), diluted samples (less than 10^5 cells/ml), samples containing less than 0.1% positive cells, and vulnerable samples. However, both systems still require an additional device for identifying the cells of interest, which significantly reduces their processing capabilities. Therefore, the

development of a fully automated system for single-cell analysis and isolation has been eagerly awaited.

Automated single-cell analysis and isolation system

We recently developed a stand-alone automated system for high-throughput single-cell analysis and isolation (**FIG. 3A**) (32). This system consists of a motorized stage (for xy movements, <1- μ m movement accuracy), a motorized micromanipulator (for z movement, <0.1- μ m movement accuracy), a pencil pump, a glass capillary (40.5 μ m inner diameter), fluorescence microscopy equipped with a CCD camera, a reservoir plate (6-, 12-, 24-, 48-, 96-, or 384-well format; 48- or 96-well PCR plate), a microchamber array chip (polystyrene, 30 μ m in diameter, 22 μ m in depth, 23 $\times$ 23 microchambers/subarea, 16 $\times$ 10 subareas/chip, 84,640 microchambers/chip; *see* also **FIG. 3B**), and a control computer. After fluorometrically scanning all cells in a cell array (excitation at 405 nm, 488 nm, and 633 nm; 7 s/subarea), a histogram of fluorescent intensity from each cell is generated that is used to rank cells in order of fluorescent intensity. Simultaneously, the system generates a list of addresses, fluorescent intensities, and images, including a transmission image of each cell. The cells of interest are identified automatically or manually based on their fluorescent intensities. Microchambers containing more than 2 cells are automatically excluded. Selected cells are promptly retrieved one-by-one using a glass capillary, and are transferred to assigned reservoir wells (10 s/cell). The retrieval of selected cells is confirmed by the elimination of fluorescence in each microchamber. Upon failure, the system automatically repeats the isolation process for the cell. The automated single-cell

analysis and isolation system maximizes the usability of cell array systems. Moreover, as the parts that come in contact with samples (*i.e.*, microchamber array chips, glass capillaries, reservoir plates) are essentially disposable, the system significantly reduces the risk of contamination and infection, and is therefore more applicable than conventional cell analysis and isolation systems to research conducted in accordance with good laboratory practices (GLP) and production performed under good manufacturing practices (GMP).

Single-cell-based breeding of hybridomas and embryonic stem cells

We used the automated single-cell analysis and isolation system to select 362 cells that exhibited higher fluorescence than parental hybridomas from $\sim 5.0 \times 10^4$ hybridomas that were established by conventional methods and subjected to CS-FIA (**FIG. 2D**). Eight cells with the highest level of fluorescence were isolated by the system, and were further propagated to about $\sim 5.0 \times 10^5$ cells over 2 weeks. Finally, after 21 days of culture, 7 hybridomas were found to secrete higher amounts (up to 10-fold) of antibodies than parental hybridomas (**FIG. 4A**) (32). Hybridoma clone No. 1 was able to maintain the high secretion rate without any decrease for at least 35 days. The combination of the automated single-cell analysis and isolation system with CS-FIA was thus demonstrated to be effective and practical for improvement of the productivity of biomolecules that are secreted from various cells.

To establish mouse embryonic stem cells (ESCs) with the highest pluripotency, parental ESCs were firstly modified to incorporate the *EGFP* (enhanced green fluorescent protein) gene under the *Rex1* gene (a pluripotent marker) using a bicistronic

reporter system (**FIG. 2A**). About 1.0×10^5 cells of parental ESCs were subjected to the automated single-cell analysis and isolation system and 25 single cells showing higher fluorescence were isolated. These cells were subsequently cultured for 7 days to form colonies. About 64% (16/25) of daughter cells formed more spherical colonies and exhibited more uniform fluorescence than parental cells (**FIG. 4B**). Furthermore, after 50 days, some portions of daughter cells were retained stable and high expression of the *Rex1* gene, indicating that the stochastic fluctuation of pluripotency was successfully suppressed by single-cell-based breeding (**FIG. 4C**). Taken together, the automated single-cell analysis and isolation system may resolve cellular heterogeneity; one of the two major problems that occur during conventional single-colony based breeding (*see above*). However, it is an indispensable tool for solving the problem of stochastic fluctuation, fully establishing the single-cell-based breeding.

Management of stochastic fluctuation for single-cell-based breeding

Even among isogenic cells, stochastic fluctuation is often found in epigenetic modification (33,34), protein expression (35–37), transcription (38,39), and cell-cycle progression (40). Single-cell omics analyses of isogenic cells have recently revealed that these stochastic fluctuations occur at the level of individual cells. When cellular heterogeneity is successfully excluded from cell breeding using our system, the established cell lines often have reduced productivity of biomolecules during long-term culture. This might be caused by the stochastic fluctuation that occurs in each cell. To establish single-cell-based breeding, it is necessary to decipher the molecular basis underlying stochastic fluctuation and to thereby identify the master regulators that

govern this fluctuation. These postulated regulators could be found using global analyses (*e.g.*, genomics, epigenomics, transcriptomics, proteomics, and metabolomics) of cells with relatively high expression, stability, and homogeneity and using isogenic cells as controls. The use of the regulators as a marker in an automated single-cell analysis and isolation system would significantly increase the likelihood of isolating those cells with relatively low stochastic fluctuation. Furthermore, stochastic fluctuation could be minimized by managing the expression level of the regulators. Indeed, through the single-cell-based breeding of ESCs, Nanog, a pluripotent marker, has been identified as one of the master regulators that can be managed to maintain high pluripotency by suppressing stochastic fluctuation (41).

Conclusion

In order to obtain the efficient secretion of biomolecules, cell lines should be established that avoid both cellular heterogeneity at the colony level and stochastic fluctuation at the single-cell level. The combination of the automated single-cell analysis and isolation system with CS-FIA allowed us to obtain single cells that efficiently secrete biomolecules, thereby excluding the influence of cellular heterogeneity in the colonies bred from these cells. To establish “single-cell-based breeding,” global analysis of these cells would be an effective method for identifying master regulators that govern stochastic fluctuation.

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Legends to Figures

FIG. 1. Comparison of single-colony-based breeding (conventional) method with single-cell-based breeding (new) method. (A) Single-colony-based breeding. Candidate cells were divided into single cells by the limiting dilution method, and allowed to form colonies. Each colony was ranked based on the degree of favorable properties (light green cell showed the most favorable properties). Numbers in circles indicate the rank order of each colony. Top-ranked colony was further propagated to establish master cell bank, which was still contaminated with cells with non-favorable properties. The breeding process should be repeated “n” times to maximize the abundance of cells with favorable properties. The process is time-consuming and laborious. (B) Single-cell-based breeding. By using an automated single cell analysis and isolation system, cell showing the most favorable properties was isolated as a single colony. The cell was further propagated to establish master cell bank, which was completely devoid of the contamination of cells with non-favorable properties. Theoretically, it is not necessary to repeat the breeding process.

FIG. 2. Fluorescence-labeling techniques for single cells in a biomolecule secretion level-dependent manner. (A) Bicistronic reporter assay. A fluorescent protein gene was placed under the biomolecule gene of expression cassette by using IRES (internal ribosome entry site). This assay allows real-time measuring the transcription level of biomolecule gene in each cell, not always reflecting the secretion level. (B) Gel microdrop assay. Each cell was encapsulated in a solid

medium microdrop containing biotinylated agarose, avidin, and biotin-labeled capture molecule. Biomolecules secreted from each cell were immobilized on the agarose matrix, and then sandwiched with fluorescence-labeled detection molecules. Fluorescence intensity of each cell increases in proportion to the secretion rate of biomolecules from each cell. (C) Matrix-based secretion assay. Surface of each cell was chemically labeled with biotin, and then each cell was encapsulated in a semi-solid medium containing avidin and biotin-labeled capture molecules. Secreted biomolecules were immobilized with biotin-labeled capture molecules at cell surface, and then sandwiched with fluorescence-labeled detection molecules. Fluorescence intensity of each cell increases in proportion to the secretion rate of biomolecules from each cell. (D) Cell-surface fluorescence-linked immunosorbent assay (CS-FIA). Each cell was incubated with lipid-PEG (polyethylene glycol)-labeled capture molecules for displaying the moiety of capture molecules onto cell surface spontaneously. Secreted biomolecules were immobilized with capture molecules at cell surface, and then sandwiched with fluorescence-labeled detection molecules. Fluorescence intensity of each cell increases in proportion to the secretion rate of biomolecules from each cell.

FIG. 3. Automated single-cell analysis and isolation system. (A) Overview of the robot (top). The robot consists of a motorized micromanipulator equipped with a glass capillary, a motorized stage, a reservoir plate (*e.g.*, 6-, 12-, 24-, 48-, 96-, or 384-well format; 48- or 96-well PCR plate), and a microchamber array chip (middle & bottom). (B) Microchamber array chip. A microchamber array chip ($1 \times$

25.6 × 44 mm) made of polystyrene (PS) consists of 16 × 10 subareas, each of which contains 23 × 23 microchambers (30 μm in diameter, 22 μm in depth).

FIG. 4. Single-cell-based breeding of hybridomas and embryonic stem cells. (A)

Fluctuation of antibody secretion rate of hybridomas after the propagation from single cells (21 days). Parent hybridomas (antibody secretion was optimized by single-colony-based breeding method) were subjected to CS-FIA (cyan bars), and then single hybridomas showing highest secretion rate (daughter hybridomas) were isolated by the automated single-cell analysis and isolation system. After these hybridomas were propagated for 21 days, culture media were analyzed by ELISA for the determination of secretion rate of antibodies (magenta bars). The antibody secretion rate of clone No. 1 was found stable during at least 21-day culture from single cell isolation, while those of other clones were found to be decreased. *Error bars, $p < 0.05$, $n = 6$.* (B) Colony formation of single ES cell showing highest expression of pluripotent marker *Rex1* gene. Mouse ES cells harboring *Rex1-IRES-EGFP* gene (parent cells) were subjected to the automated single-cell analysis and isolation system. Single cell showing highest *Rex1* expression (daughter cell) was automatically identified and subsequently isolated by the robot, and cultured to form colony for 7 days. *Bar = 50 μm.* (C) Fluctuation of pluripotent marker *Rex1* gene expression of daughter ES cells after the propagation from single cells (30 and 50 days). Parent ES cells (*Rex1* gene expression was optimized by single-colony-based breeding method) were subjected to the robot, and then two single cells showing highest *Rex1* gene expression (daughter cells; #1 and #2) were

494 isolated by the robot. After the propagation for 30 and 50 days, parental, daughter
495 #1, and daughter #2 cells were analyzed by FACS for the determination of *Rex1*
496 gene expression. The *Rex1* gene expression of daughter #2 was found stable during
497 at least 50-day culture from single cell isolation, while that of daughter #1 was
498 found to be decreased.

A Single-colony-based breeding

B Single-cell-based breeding

FIG. 1

FIG. 2

A

B

FIG. 3

A

B

C

FIG. 4