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REVIEW

Single-cell-based breeding: Rational strategy for the establishment of cell lines from a single cell with the most favorable properties

Nobuo Yoshimoto and Shun'ichi Kuroda*

Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi 464-8601, Japan

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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.

[**Key words:** Automated single cell analysis and isolation system; Cellular heterogeneity; Biomolecule production; Stem cells; Hybridomas; Chinese hamster ovary 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

* Corresponding author. Tel./fax: +81 52 789 5227.
E-mail address: skuroda@agr.nagoya-u.ac.jp (S. Kuroda).

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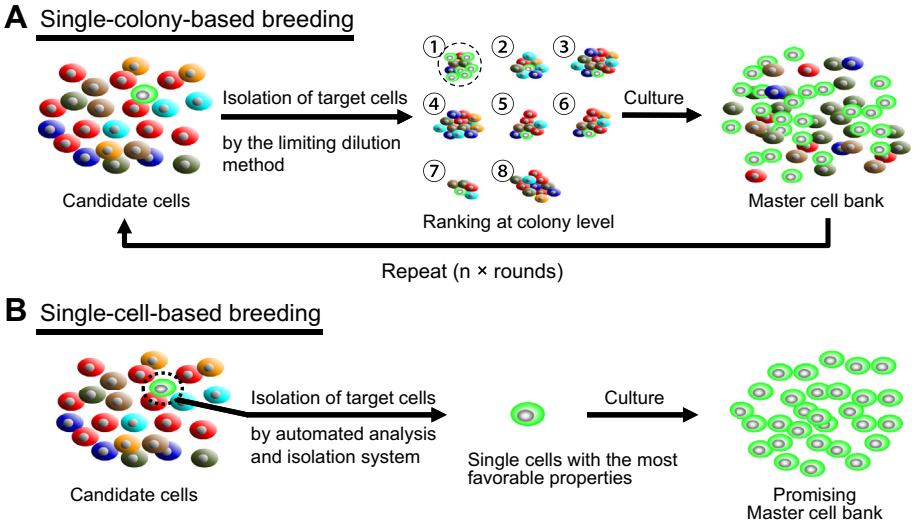

FIG. 1. Comparison of single-colony-based breeding (conventional) method with single-cell-based breeding (new) method. (A) Single-colony-based breeding. Candidate cells were divided into single cells by the limiting dilution method, and allowed to form colonies. Each colony was ranked based on the degree 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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.