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Automated generation of human stem cell clones by Image-Activated Cell Selection (IACS™)

Clonal analysis represents the gold standard for assaying the differentiation potential of stem cells *in vitro*. Here we describe Elektra™, a system that permits efficient spotting of single cell clones in a coculture paradigm. Joining their expertise, Evotec Technologies and LIFE & BRAIN showed that neural precursors derived from human embryonic stem (ES) cells survive single cell spotting and continue to generate individual clones. This technology should be particularly useful for the high-throughput clonal analyses increasingly required for human stem cell applications.

Challenges in clonal analysis

Pluripotent stem cells are characterized by the potential of self-renewal and differentiation into any desirable cell type. The biomedical application of these cells depends critically on the ability to control their *in vitro* differentiation. An increasing number of growth factors, extracellular matrix components and master control genes have been shown to modulate stem cell fate, and standard cell culture paradigms are generally ill-suited for delineating the effects of compounds on individual cells and their progeny. For that reason, clonal analysis is becoming increasingly important for both fundamental and applied stem cell research.

Conventional methods for clonal analysis are mostly based on limited dilution. Cells are plated at very low density, and subsequently, single cells are identified and manually marked by careful microscopic examination. This process is tedious and time-consuming. Furthermore, the cultivation of single cells is complicated by the lack of cell-cell contact and paracrine factors frequently required for maintenance of the stem cell phenotype. Although these requirements could be met by co-culture systems, optical verification of single cells plated onto a recipient monolayer is even more challenging. Alternatively, low-titer retroviral infection could be used to label a small number of dividing cells. This system, too, is complicated by the tedious verification of single cell clones and the potential overlap of different clones. Thus, these technologies are not suitable for performing large-scale clonal analyses.

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Figure 1 | The Evotec Technologies Elektra system, for the IACS-based automated spotting of single cell clones.

The solution: Image-Activated Cell Selection (IACS)

Elektra (Fig. 1) substantially improves and expedites the cell cloning process using IACS, which provides high-content, single-cell information. This image-based cell sorting device is designed to select single cells from a cell population based on optical parameters, imaging and isolating them in one easy operation, and requires only a minimal number of cells and small volumes of reagents. Cells are characterized according to their subcellular fluorescence and morphology, and then placed into a microtiter plate. Elektra produces microtiter plates containing single viable clones and broadly characterized cells in each well, while maintaining an aseptic environment. Pure cell populations are obtained without the need for multiple iterations.

Elektra is based on Evotec Technologies' proprietary CellProcessor™ technology. It uses a microfluidic Sorter Chip, which uses microelectrodes to guide, trap and isolate cells via negative dielectrophoretic forces. Elektra is equipped with a Peltier-cooled CCD camera and a 40x objective lens for high-resolution imaging. Fluorescence is excited using a xenon arc lamp and up to eight different fluorescence filter sets. Cell viability and cell size are determined using phase-contrast illumination. Cells are analyzed online according to phase contrast, fluorescence and size while passing the Sorter Chip (Fig. 2a). Thresholds can be set for

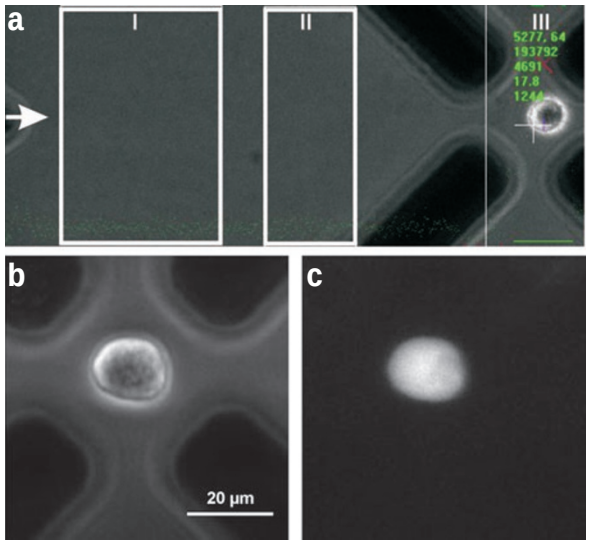

Figure 2 | Single cell analysis using IACS. (**a**) IACS permits real-time flow-through analysis of phase contrast, cell size (area I) and fluorescence intensity (area II) of single cells. Target cells identified by thresholds set for these three parameters are caged in the dielectric field cage (area III). (**b**,**c**) Phase contrast image (**b**) and fluorescence analysis (**c**) of a human neural precursor cell confirm single cell status, cell viability and expression of an EGFP reporter gene before clonal spotting.

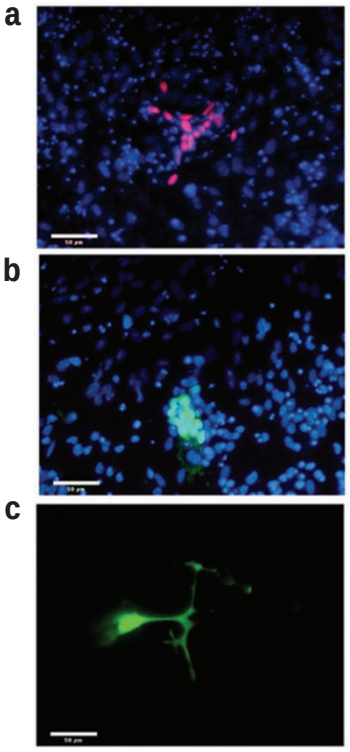

Figure 3 | Human neural precursor cell clones derived from single cells spotted on an astrocyte feeder layer. (**a**,**b**) Two weeks after IACS, human cells were identified by an antibody to human nuclei (**a**), or the expression of an EGFP reporter gene (**b**). Counterstain: DAPI. (**c**) Single differentiating neural precursor cell after isolation using IACS. Scale bars, 100 µm (**a**,**b**) and 50 µm (**c**).

target cell selection. For detected target cells an image series is taken while trapping the cells (Fig. 2b,c). Automated detection of aggregates, coincidence events, viability and single cell status ensures the ability to isolate only viable, single target cells. Target cells are placed into a microtiter plate within a closed box. Aseptic experiments are facilitated by a removable panel which contains all fluidic components and the microfluidic sorting chip. This system can be assembled and primed off-instrument in a laminar flow box. All fluidic components can be exchanged, and many of them can be autoclaved.

Automated generation of single human stem cell clones using IACS technology

The derivation of tissue-specific stem cells from pluripotent ES cells represents one of the breakthrough technologies of applied stem cell research. Validation of this process requires standardized methods for clonal analysis; IACS using the Elektra meets these requirements.

As a demonstration, Elektra was applied to human ES cell–derived neural precursors for the spotting of single cell clones in both isolated and coculture settings. For these studies, wild-type cells as well as neural precursors harboring an EGFP expression construct were triturated to a single cell suspension by treatment with trypsin and DNase (3 min at 37 °C). Cells were suspended at 5 x 10^5 cells/ml in Cytocon™ Buffer II, and 3–5 µl of the cell suspension were injected into the Elektra. Target cells were caged in the dielectric field of the Elektra Sorter Chip, then characterized by phase-contrast imaging, confirming single cell status (Fig. 2). Fluorescence imaging was used to confirm the status of EGFP expression.

The average diameter of selected cells was 12 µm. Cells were deposited either in conditioned medium or on mouse astrocyte feeder cells. Cells deposited on mouse astrocytes were detected by virtue of their EGFP expression and/or an antibody to human nuclear protein. Twenty-four hours after deposition, more than 90% of the plated single cells were viable based on calcein staining. Fourteen days after deposition on mouse astrocytes, 10% of the plated single cells had developed into clones encompassing ≥5 cells (Fig. 3a,b). IACS selection did not interfere with initiation of neural differentiation (Fig. 3c).

Conclusion

The results shown here demonstrate that IACS can be used for the automated isolation of single cell clones of human ES cell–derived neural precursors. After morphometric and immunogenic assessment, selected cells are gently and efficiently plated into individual wells where they give rise to clonal progeny. Elektra provides a powerful tool for the high-throughput analysis of compounds at clonal resolution, bypassing the limitations associated with the manual identification and labeling of single cell clones, and allowing for the first time the image-based clonal spotting of single stem cells in an automated, fully documented fashion.

Additional information is available online at company website (http://www.evotec-technologies.com; Products / Instruments and Systems).