A workflow combining single-cell isolation and microplate imaging improves cloning efficiency with higher assurance of clonality

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Benefits

- Isolate cells efficiently using label-free or fluorescence-based imaging
- Easily obtain high assurance of monoclonality with direct, image-based documentation of single cells captured pre- and post-cell sorting
- Improve clonal outgrowth compared to limiting dilution (4X to 8X improvement is typically observed) using a patented single-cell dispensing system
- Minimize the risk of cross-contamination between samples by using one-way disposable cartridge
- Efficiently track cell outgrowth over time
- Quickly generate monoclonality reports readily for submission to regulatory agencies

Introduction

The isolation of single cells has a broad spectrum of applications, from single-cell genomics to antibody discovery and cell line development. Current commonly used methods of single cell isolation present limitations in terms of reliability and efficiency. Limiting dilution, a traditional method for isolating single cells, relies heavily on statistical probabilities to assure monoclonality. It is sensitive to changes in protocols and day-to-day operations, which leads to variation in results between experiments. Furthermore, this technique is highly inefficient at isolating single cells because only a small fraction of wells within the microplate contain a single cell. Flow cytometry-based cell sorting is another common method for single cell isolation. However, the application is hindered by poor post-deposit cell growth, a steep learning curve, high maintenance cost, large amount of fluid waste generated, and the increased risk for cross-contamination among samples compared to manual dilution. Moreover, both methods do not provide image-based evidence of monoclonality.

The CloneSelect™ Single-Cell Printer™ Series (Figure 1) addresses the shortcomings of limiting dilution and flow cytometry-based cell sorting by offering image-based evidence for clonality, fast and efficient single-cell deposition based on white light (c. sight and f. sight systems) or optional fluorescence (f. sight systems only), excellent post-deposit cell growth, minimal risk of cross-contamination, and extremely low cost for maintenance. When coupled with the CloneSelect Imager (CSI), the Single-Cell Printer series demonstrates improvements over both of these workflows with both an increase in clonal outgrowth and additional image-based assurance of monoclonality.

In this proof of concept study, we compare single-cell cloning workflows using limiting dilution or a Single-Cell Printer to demonstrate the improved efficiencies that can be achieved with the Single-Cell Printer. We chose experimental conditions that maximized cell growth, rather than conditions that resemble real-world conditions in cell line development in order to more accurately assess the performance of the instrument. More specifically, we chose to work with adherent, non-transfected CHO-K1 host cells grown in culture media containing fetal bovine serum (FBS) instead of suspension, transfected CHO-K1, grown in animal component free (ACF) media because this minimized the impact of additional parameters such as the sequence of the gene-of-interest (GOI), the size of the GOI,
the expression vector, the host cell type, the transfection/sele-
cion conditions, and the culture conditions on clonal outgrowth. Furthermore, we controlled for these parameters by normalizing the outgrowth rate from cells deposited with the Single-Cell Printer to the outgrowth rate observed following limiting dilution since the experimental conditions were identical otherwise. Using this approach, we observed up to an 8X improvement in clonal outgrowth from the Single-Cell Printer compared to limiting dilution with non-transfected CHO-K1 cells. And although we did not undertake such testing under more real-world conditions in cell line development, we expect up to an 8-fold improvement in clonal outgrowth compared to limiting dilution to be observed under such conditions since this is a normalized measurement.

Materials and methods

SAMPLE PREPARATION

CHO-K1 cells were purchased from the American Type Culture Collection (ATCC, Cat# CCL-61™) and maintained in F-12K medium containing 10% FBS. To prepare for single-cell deposition, cells in the exponential phase were dissociated from the bottom of a T-75 flask (Cat# 430641, Corning) with 0.25% (w/v) Trypsin-0.53 mM EDTA solution, followed by neutralization and gentle washing, twice with plain F-12K medium, to eliminate the FBS effects on staining or deposition. A cell suspension with cell viability of greater than 90% was used for single cell deposition.

In a previous media study (data not shown), it was found that using XP CHO Growth A as the medium within the cartridge to isolate CHOK1 cells resulted in the highest single-cell deposition efficiency. Hence in this study, XP CHO Growth A is used as the only media within the cartridges while sorting. Due to the observations that adding FBS increases the viscosity of the medium and affects the stability of the droplets being generated at the nozzle which is critical for achieving successful single-cell deposition, FBS is not recommended to be used on the f. sight.

Sample preparation for deposit efficiency studies

Calcein AM (CAM) is a cell-permeant dye that can be used to label live cells fluorescently. To quantify deposition efficiency at the microplate level, 5 µM CAM (Part# R8343A, Molecular Devices) was applied to stain cells for 15-30 minutes at 37°C to enable fluorescence-based automated cell counting by a custom CloneSelect Imager (CSI) with fluorescent imaging capabilities. Before deposition, stained cells need to be washed twice with plain media to remove excess dye and resuspended in XP CHO Growth A medium (Cat# K8860, Molecular Devices) and adjusted to 0.5-1x 106 cells/ml. To eliminate clumping and achieve single cell suspension, cells were filtered through a 30 µm strainer (Cat# 435003003, pluriSelect) prior to loading into a cell cartridge. Cells were deposited into 96-well microplates (Cat# 3300, Corning) prefilled with 200ul medium per well or 384-well microplates (Cat#781182, Greiner) prefilled with 80 ul medium per well. Because cells stained with 5 µM CAM would not be used for

Figure 1: Working principles of the CloneSelect Single-Cell Printer Series. A) Microscopic droplets are generated by a piezo-driven actuator in a predictable sequence. In the default state, a vacuum mechanism (blue arrow) siphons away droplets. When single-cell events are identified, a shutter is triggered to inactivate the vacuum (burgundy X), allowing the droplet encapsulating the single cell to be delivered (blue arrow) to a well of the microplate. B) Evidence of clonality at the nozzle. Images 1–3 show the cell approaching the nozzle. Image 4 shows the detection of a single cell (inner circle) and the absence of any cells in its vicinity (outer circle). Finally, Image 5 shows the nozzle after droplet ejection to provide evidence that the single cell was expelled from the nozzle.
cell growth study, no FBS was added to the efficiency study medium.

Sample preparation for clonal outgrowth studies

For the outgrowth study, the sample preparation was largely the same as the efficiency study described above. However, in the outgrowth study, we used label-free cells or cells stained with much lower concentration of CAM (1 µM) to minimize the effects of fluorescence labeling and f. sight fluorescence-aided cell isolation (FACI) on post-deposit cell survival and growth. 96-well or 384-well microplates were prefilled with the F-12K medium supplemented with 10% FBS, which had been optimized for non-transfected CHO-K1 cells. Because cloning medium is critical for single-cell survival and colony forming after deposition, the optimization of cloning medium is highly recommended for each cell line before performing single-cell cloning.

To prepare for a limiting dilution, a cell count was obtained via the Countess™ (ThermoFisher Scientific), then serial dilutions were performed to dilute cells from 0.5-1x10⁶ cells/ml to a final concentration of 3.75 cells/mL using cloning medium. Finally, 80ul or 200 ul of diluted cells was added to each well of the 384-well plate or 96-well plate, respectively, to achieve a seeding density of 0.3 cell per well.

SINGLE-CELL DEPOSITION BY THE CLONESELECT SINGLE-CELL PRINTER F. SIGHT

The f. sight utilizes a disposable sterile cartridge, a laser, a novel dual camera system and a patented inkjet-like gentle dispensing system to isolate both label-free and green fluorescently-labeled cells from mixed populations. The laser system has varying laser power intensities, ranging from 1-100mW, and various camera exposure settings to achieve fluorescence excitation and detection within a region of interest (ROI) within the nozzle of the cartridge. The unique dual camera system captures and saves a total of 15 individual high resolution images of the end of the nozzle: five brightfield only, five fluorescence only, and five of the overlay between the two. This enables image-based assurance of clonality (Figure 2). Meanwhile, fluorophore bleaching and phototoxicity are minimized by focusing the laser beam at the ROI located at the nozzle.

Cells can be screened by the f. sight on the basis of three user-defined criteria: size, morphology, and fluorescence intensity. Screening for cells based on size may allow for isolating a specific cell type from a heterogeneous population of cells or may allow for screening of cells with higher viability, since dying cells tend to swell and then shrivel. Selecting cells based on cell roundness provides another parameter to screen for healthy cells by ensuring that asymmetrical structures like dividing or unhealthy cells are not deposited into the wells. Finally, selecting cells based on fluorescence provides a unique tool to

**Figure 2**: Instruments overview and optimized workflow. A) The f. sight system is used for the gentle and precise isolation of single living cells from a liquid sample with optional green fluorescence sorting function. B) The single-cell image captured at the nozzle under fluorescence-aided cell isolation. C) This system empowers fast and consistent brightfield imaging of microplates (90s per 96-well plate or 384-well plate). An on-the-fly label-free analysis algorithm allows objective and label-free assessment of confluence and can be used to plot cell growth. D) Multiple day scanning on CSI allows an individual well history from a single cell to a colony to be documented and generated into a report. E) A day-7 microplate is imaged after the sorting. Green pseudo coloring show the segmentation of colonies imaged in brightfield.
screen cells for viability with fluorescent dyes like Calcein AM or CellTracker Green.

Furthermore, the f. sight has a cell population profiling function. By running this function, the user can quickly gain knowledge about the distribution of cell size, morphology, and fluorescence intensity of a sample and select the suitable gating parameters to screen the cells.

PLATE IMAGING

Plates were centrifuged at 200g for 3 mins and imaged immediately after cell deposition by f. sight or limiting dilution.

Plates containing cells stained with 5uM CAM for the efficiency study were scanned on the CSI system with fluorescence channel customization. Exposure settings for the green fluorescent channel were optimized to minimize artifact effects which could lead to false cell counts. The CSI imaging algorithm automatically analyzed images from the green fluorescent channel and generated the cell count data (Figure 3).

For the outgrowth study, plates containing non-fluorescent, label-free cells were imaged on the standard CSI system at day 0. After the day 0 scan, plates were incubated in a tissue culture incubator at 37°C and 5% CO2 with humidity control. Plates were scanned on the CSI on the following days: 1, 4 and 6, and returned back to the incubator after each scan. Visible colonies could be seen by eye on day 7 and plates were scanned on the CSI to complete cell outgrowth tracking.

GENERATION OF MONOCLONALITY REPORT

The Monoclonality Report Generator software wizard enables the rapid set up, customization, and delivery of a Monoclonality Report. In the optimized workflow we describe here, the growth of clones from single-cell deposition were tracked over 6-7 days with the CSI. On the CSI, in the Plate Thumbnails tab, green overlays show the segmentation of colonies originating from single cells. When one switches to the View Image tab, clicking on individual wells will allow visualization of the colony at the whole well level. The Report Generator tab can be utilized to generate reports for individual wells using images from day 0 onwards. Here, a cell region (yellow green box) can be defined for each time point. The example cell shown contains four time points. The user can demonstrate the presence or absence of cells and how many cells in a well by showing images of all regions of a well over the time course or by marking the debris/artefacts regions from day 0 onward. Since cell shaped debris makes it difficult to conclusively distinguish single cells at day 0, the designation of debris regions adds additional confidence by showing that debris does not divide over time. Furthermore, the f. sight offers a fluorescence-aided cell isolation (FACI) function, which enables single-cell isolation based on fluorescence intensity and provides additional evidence to ensure monoclonality.

Figure 3: CSI-FL automated cell counting for efficiency analysis. A, B) Screen shot captured on CSI-FL showing the same well to be imaged on both brightfield channel and fluorescence channel to validate the single-cell deposition on the 384-well microplate after sorting. C) After images were captured and analyzed, the CSI-FL fusion software automatically generated the cell count on each well that can be exported to excel file for deposition efficiency analysis. The red box represents well with more than one cell; the green box represents well containing single cell while the white box means no cell detected.
Results

EFFICIENCY

Cells were fluorescently labeled and loaded into a cartridge. After single-cell deposition, microplates were immediately spun down and scanned on a CSI customized to include both white light and fluorescent imaging. The fluorescence-based cell counting algorithm on the CSI was used to assess deposition efficiency after manual verification. Wells with deposited cells that cannot be confirmed by the white light channel image were excluded from data analysis.

Single cells were isolated and deposited into two 384-well microplates using the same cell cartridge. An average single-cell deposition efficiency of 92.6% ±0.4% (n=761 wells) was observed. Two-cell and null events were observed at 7.0% and 0.4%, respectively. The average single-cell deposition time for one 384-well microplate was 21.02 mins.

OUTGROWTH

The capability of post-deposit single cells to form colonies is a key parameter because it indicates the viability of the cells after deposition. It also reflects how the entire workflow will affect the cell growth. In this study, the viability of cells post-deposit was measured by the clonal outgrowth following deposition. For non-transfected host CHOK1 cells, it typically takes 6-7 days to form colonies that can be consistently detected and measured by CSI.

Multiple factors can affect clonal outgrowth, including sample preparation, culture and media conditions, gating parameters, and other external factors. Since outgrowth can be variable between different experiments, we used limiting dilution as a control for the outgrowth measurement. The seeding density for limiting dilution was 0.3 cells per well. We calculated the outgrowth percentage as the number of wells with a single colony divided by the total number of wells within a microplate.

An overall single colony outgrowth percentage of 84.6% ±2.3% (n=759 wells) was observed in label-free CHOK1 cells based on the data analysis from cells deposited into two 384-well microplates using the same cartridge (Figure 4). The average time per 384-well plate was 18:43± 01:09 mins.

When comparing the single colony outgrowth percentage of the microplates filled with the f.sight to that of limiting dilution (10.5%±2.9%, n=1272 wells), we saw a 8.1-fold increase in single colony outgrowth from single-cell isolation on f. sight. This led to higher throughput and an increased potential number of clones for downstream analysis. The distribution of colonies throughout the plate and the lack of skipped wells indicate that following a successful cloning process, the f. sight can effectively deliver single cells to individual wells with a high level of efficiency and viability.

One of the concerns of using laser enabled FACI is phototoxicity, which will affect post-deposit cell viability and growth. To assess the effects of fluorescence labeling and laser-enabled FACI on the growth of post-deposit cells, we labeled CHOK1 cells with a low concentration of viability dye CAM (1µM), which we previously showed had no significant effects on cell growth when used with the limiting dilution method (data not shown).

Using one cartridge, five 96-well microplates were filled with single cells and imaged across multiple time points. The average deposition time for 96-well microplates was 05:16 ±00:46 mins per plate. On day 7, an overall single-colony outgrowth percentage of 89.1% ±3.5% (n=478 wells) was observed, which was comparable to that of the unlabeled CHOK1 cells deposited into the two 384-well plates (84.6% ±2.3%, n=759 wells) on the same f. sight instrument but without using laser enabled FACI function. This allows us to conclude that there is no significant impact of the exposure to the laser in the f.sight on clonal outgrowth.
Conclusion

We have demonstrated that the f. sight significantly improved single-cell derived clone throughput by 8-fold compared with limiting dilution. Single cells were deposited and imaged and growth was tracked over time on the CSI platform, which made documentation and the generation of a monoclonality report easy. High assurance of clonality is achieved with direct, image-based evidence provided pre- and post-cell deposit.

Overall, this optimized workflow saves time, labor and reduces consumable and reagent costs by increasing outgrowth efficiency, while providing higher assurance of clonality and rapid documentation that is ready to use for submission to regulatory agencies.