In the development of biopharmaceutical products, the expectation of regulatory agencies is that the recombinant proteins are produced from a cell line derived from a single progenitor cell. A single limiting dilution step followed by direct imaging, as supplemental information, provides direct evidence that a cell line originated from a single progenitor cell. To obtain this evidence, a high-throughput automated imaging system was developed and characterized to consistently ensure that cell lines used for therapeutic protein production are clonally-derived. Fluorescent cell mixing studies determined that the automated imaging workflow and analysis provide $\geq 95\%$ confidence in accurately and precisely identifying one cell in a well. Manual inspection of the images increases the confidence that the cell line was derived from a single-cell to $> 99.9\%$. © 2017 American Institute of Chemical Engineers Biotechnol. Prog., 000:000–000, 2017 Keywords: single-cell cloning, clonally-derived cell line, automated high-throughput imaging, cell line development

**Introduction**

Biopharmaceuticals are predominantly produced by recombinant gene expression in mammalian cells. The cell of choice for the majority of monoclonal antibodies (mAb) production has been Chinese hamster ovary (CHO) cells as they are easy to genetically manipulate, grow to high cell densities, and can produce an abundance of recombinant mAb in chemically defined media. Guidance documents from the United States Food and Drug Administration (FDA) and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) state that “the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single-cell progenitor.” The World Health Organization (WHO) provided additional input to the ICH statement: “the cloning procedure should be fully documented, with details of imaging techniques and/or appropriate statistics. For proteins derived from transfection with recombinant plasmid DNA technology, a single fully documented round of cloning is sufficient, provided that product homogeneity and consistent characteristics are demonstrated throughout the production process and within a defined cell age beyond the production process.”

The WHO Expert Committee of Biological Standardization also states that “Cloning by one round of limiting dilution will not necessarily guarantee derivation from single-cells; additional
subcloning steps should be performed.” However, multiple rounds of cloning are time- and labor-intensive and at odds with the clinical drivers to deliver drugs quickly to patients. Therefore, we sought to show that one round of cloning—along with appropriately characterized supplemental data—provides ample evidence that the cell line was derived from a single progenitor.

There are several different methods of cloning that can be used to generate a clinical cell line. Some of these techniques include fluorescence-activated cell sorting (FACS), CloningPix, and the traditional method of limiting dilution cloning (LDC). While FACS and ClonePix use sophisticated instrumentation to isolate cells from cell populations, LDC is a straightforward process that requires seeding plates with dilute culture to provide a statistical assurance of a clonally-derived cell line. Industry cell line developers use LDC to select cell lines that have increased productivity, desired product quality attributes, and to provide assurance that the cell line was clonally-derived. The LDC process is complicated by cell recovery rates, cell clumps, and the statistical analysis used to support the assurance of a clonally-derived cell line.

Regulatory agencies have indicated that calculating Poisson survival statistics from LDC would not be an acceptable statistical analysis to support that a cell line is clonally-derived. Recent presentations from the FDA stated that “prospective probability” is to be used to ensure that cell lines are clonally-derived. We interpret “prospective probability” as designing a process that ensures that cell lines are clonally-derived using data established prior to the cloning process and independent of survival statistics. With current technologies, the only direct evidence of the cloning event is imaging of the cell culture plate immediately after plating. However, the timing, throughput, and accuracy needed to image single-cells after seeding required extensive optimization. Furthermore, the imaging workflow must be well characterized and consistent to prevent artifacts in the imaging data.

In this work, we developed and characterized an automated imaging workflow to compensate for the inherent problems of LDC and provide additional assurance that our cell lines are clonally-derived. This workflow combines brightfield and fluorescent detection of cells to provide direct evidence of single-cell deposition during cell line development. Transient fluorescent labeling increased sensitivity of detection and allowed for automated well analyses. Automation of the entire single-cell cloning (SCC) workflow reduces time and labor, allowing increased throughput, consistency, and accuracy. Characterization of the automated single-cell cloning imaging workflow determined that the automated imaging workflow alone provided ~95% confidence in accurately and precisely identifying one-cell in a well. Furthermore, manual inspection of the images increases the confidence that the cell line was derived from a single-cell to >99.9%.

Materials and Methods

Titration of live-cell dye to determine concentration for staining CHO-K1 cells

Lyophilized CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (Thermo Fisher, Waltham, MA) powder was reconstituted in 100% dimethyl sulfoxide (DMSO) to a final concentration of 215 μM and sterile filtered through DMSO-compatible syringe filters. Aliquots sufficient for one single-cell cloning experiment were stored at −20°C protected from light. To determine a usable concentration range for CMFDA with our CHO-K1 cells, a titration was performed in which working concentrations of 50, 100, 200, 300, and 400 nM CMFDA were used to stain CHO-K1 cells. The dye and cells were allowed to incubate for 45 min, protected from light at room temperature, and then washed twice with media (Genentech proprietary media). Cell pellets were resuspended in media and counted with a Vi-Cell (Beckman, Brea, CA) and cell culture was diluted to 30 cells/mL (1.2 cells/well; each well contains 40 μL) in single-cell cloning media (Genentech proprietary media). Diluted cell culture was plated into Corning 3542 plates (VWR, Visalia, CA) with a bulk liquid dispenser. Plates were incubated for 3 weeks in a humidified, CO₂ incubator at 37°C. The Celigo imager (Nexcelom, Lawrence, MA) was used to determine percent cell growth per well by confluence measurements after 14 days of incubation. Percent wells with growth >15% confluence was compared between the different dye concentrations used to stain the cells.

Effect of CMFDA dye and imaging on random mutagenesis of CHO-K1 cells

The HPRT forward mutation assay was used to determine random mutagenesis rate. Three days prior to the assay, 17 clones of mAb A which were derived through CMFDA dye staining prior to single-cell cloning with imaging and 13 clones of mAb A which were derived without exposure to CMFDA dye prior to single-cell cloning (thus not imaged), were each seeded into separate shake flasks containing seed media (Genentech proprietary media) without selective agent. On the day of seeding, cells were passed through a 40 μm strainer and counted on a Vi-Cell. The cell density was adjusted to 0.5 × 10⁶ cells/mL in seeding media. Thirty milliliters of selective media (50 μM 6-thioguanine (6-TG) (Sigma, St. Louis, MO) in seed media) was added to 150 mm petri dishes (three petri dishes per clone) and 2 mL of 0.5 × 10⁶ cells/mL cell suspension was added to each respective petri plate. Plates were incubated for 3 weeks in a humidified, CO₂ incubator at 37°C. After incubation, media was aspirated and plates were washed with PBS. Cells were fixed for 10 min with the addition of 5 mL of cold methanol. The methanol was aspirated from the plates and 5 mL of crystal violet solution (0.5% crystal violet in methanol) was added to each plate and incubated at room temperature for 10 min. The crystal violet solution was aspirated and plates were washed with water and allowed to dry before stained colonies were counted.

Determination of error rate of high-throughput automated imaging method in documenting single-cell cloning

Development of Fluorescent CHO-K1 Hosts. Dasher (GFP) and Rudolph (RFP) were purchased from DNA2.0 under their IP-Free Protein Paintbox reagents agreement (ATUM, Newark, CA). The DNA was used to transfect CHO-K1 cells and bulk selected with 5 or 10 μg/mL puromycin. Once cultures recovered from selective pressure, the cells were single-cell cloned by staining with 322 nM CellTracker Green CMFDA and imaged at the time of plating according to the Genentech platform cell line development (CLD) protocol. The Genentech platform CLD protocol uses fluorescent dye staining of CHO cells to determine cell number during the single-cell cloning step. Fluorescence
from stably expressed fluorescent proteins is not used to count cells during single-cell cloning. The Celigo software was used to count fluorescent cells after imaging at the time of plating. Plates were incubated in a humidified, CO2 incubator at 37°C for 21 days and then clones were picked that were day 0 = 1 at the time of plating and were fluorescent for the correct color after incubation. Fluorescent CHO-K1 hosts were cultured under puromycin selective pressure.

Characterizing Single-Cell Cloning Recovery Rate of CHO-K1 Fluorescent Hosts. CHO-K1 Green (Dasher) and CHO-K1 Red (Rudolph) clones were characterized by cell growth and single-cell cloning efficiency. Cell growth was tabulated to identify clones with similar doubling times. Individual CHO-K1 Green and CHO-K1 Red clones were single-cell cloned according to the Genentech platform CLD protocol to determine the clone recovery rate. Plates were incubated in a humidified, CO2 incubator at 37°C for 21 days and confluence was determined by imaging. Clone recovery defined as >25% confluence was used to count how many wells grew from a single-cell (day 0 = 1 cell) at the time of plating. These data were used to select clones that were best matched for single-cell cloning recovery for cell mixing experiments.

Fluorescent Cell Mixing Experiments with Fluorescent CHO-K1 Host Cells. Fluorescent CHO-K1 host cells with similar doubling times and single-cell cloning recovery rates were mixed at the appropriate ratio to compensate for their differences in doubling time and co-cultured for two passages. The co-culture was used in a single-cell cloning experiment using standard conditions as listed previously. A total of thirty-four 384-well plates were plated. The Celigo image analysis software was used to determine Day 0 cell counts and Day 21 confluence in brightfield and fluorescence. Overlays of brightfield, green and red fluorescence were used to determine wells in which there were mixed populations.

Fluorescent Cell Mixing Experiments with Fluorescent CHO-K1 Hosts Transfected with mAb B. The same fluorescent CHO-K1 hosts used in the host cell mixing experiment were used as host cells to transfect mAb B. Both CHO-K1 Green and CHO-K1 Red expressing CHO-K1 hosts were transfected with the same DNA preparation of mAb B and carried forward as a standard CLD according to the Genentech platform CLD protocol. Briefly, postelectroporation via MaxCyte (Gaithersburg, MD), transfected cells were plated into 384-well plates containing 25 μM methionine sulfoximine (MSX) in media and incubated in a humidified, CO2 incubator at 37°C for 3 weeks. Plates were imaged with the Celigo imager for confluence and the results were used to drive automated hit-picking where 1,056 wells with growth were picked at random into 96-well plates. Supernatants were collected after three days of culture and assayed for antibody titer. The top 48 wells according to titer were subsequently pooled and cocultured for two passages prior to single-cell cloning. Single-cell cloning was performed as previously described above with fluorescent dye staining immediately prior to Day 0 imaging followed by Day 21 confluence imaging. A total of forty 384-well plates were plated for each seeding condition. After 21 days of culture, plates were imaged in brightfield, green, and red fluorescence confluence and analyzed for mixed populations.

Figure 1. Cell line development flow chart. Heavy green outline highlights single-cell cloning workflow and heavy blue outline highlights the automated workflow. Automated plate handling allows for consistent plate movement and timing to minimize variability between cell culture plates that are imaged.

Results and Discussion

We have developed an accurate and precise automated imaging workflow to document single-cell cloning during cell line development. Considerations for imaging that were addressed included quality of the images, characterization of the imager, accuracy and precision of identifying cells from the images, error rate of the imaging workflow, and robustness of the workflow to varying input number of cells. Through this work, we concluded that imaging the cell culture plate at the time of single-cell cloning provides direct evidence that the cell line was clonally-derived.

Cell line development (CLD) workflow

The current Genentech platform CLD protocol was optimized to balance cell line productivity and product quality with a short, consistent timeline. The workflow has been automated to carry out the tedious and highly repetitive steps, including single-cell cloning (Figure 1). Our automated workflow was also optimized to ensure consistent and accurate plate movement via a robotic arm so that each plate is treated similarly to ensure precise imaging results. The only manual step during single-cell cloning is fluorescently staining cells with a fluorescent live-cell dye to prepare a
dilute cell culture targeting 0.5–1 cell/well. The automated workstation seeds imaging quality 384-well plates by limiting dilution and these plates are immediately centrifuged to ensure that all cells are at the bottom of the well prior to imaging. The plate is precisely moved by the robotic arm from the automated centrifuge to the Celigo imager and full-resolution brightfield and fluorescence images are acquired. After imaging, each plate is immediately moved to an automated incubator. Plates are processed in series until all plates are completed to support the single-cell cloning of the cell line. Optimized parameters were determined for the Celigo image analysis algorithm and are used to identify and count fluorescent cells within each well at the time of plating. This cell count data at the time of plating is combined with cell growth data after plate incubation, to determine which wells will be picked by our automated hit-picking work cells. The clones are scaled up for clone selection in fed-batch production cultures.

**Cell imaging development and optimization**

Brightfield image analysis algorithms across multiple vendors are unable to accurately identify cells from plate artifacts with high accuracy or precision. Therefore, the brightfield imaging, single-cell cloning workflow relies on either a time-course of images or manual image verification by a researcher, which is time-consuming, low-throughput, and can be subjective. To avoid these issues, we developed a brightfield and fluorescent imaging process to identify single-cells.

The quality of the images during single-cell cloning is paramount and is directly associated with the imager that is used and the plate which holds the cells. Different imager vendors were evaluated and the Celigo imager was chosen for its high sensitivity in fluorescence imaging and clear, high-quality brightfield imaging. The Celigo imager is capable of 1 micron/pixel resolution. The Celigo uses a fixed magnification of 3.5× with a custom objective lens that has a high numerical aperture (NA) of 0.25. While the quality of the images is the foremost consideration, imaging speed is also critical as our CLD workflow carries numerous cell lines simultaneously and projects are tightly scheduled. The Celigo achieves its fast imaging speeds by using mirrors mounted on high-speed galvinometers to change the path of light instead of moving the plate to every well. The Celigo imager can acquire full resolution brightfield and fluorescence images for an entire 384-well plate in 2.5–5 min depending on the acquisition settings. The Celigo is one of the fastest imagers available for this purpose and we exploit the speed of the imager to identify more clones to be screened. The second critical part to ensure image quality is the cell culture plate. It is important that an imaging quality plate is used to ensure that clear images can be acquired. We have tested many different cell culture plates and selected Corning 3542 plates as they provided consistent, high-quality images with a well size that can be captured in one image on the Celigo imager.

After we were able to precisely acquire high-quality images, optimization of the single-cell cloning imaging analysis algorithm was carried out to ensure that selected parameters would accurately and precisely identify cells within the well. Within the algorithm, we set a 25 μm cutoff for cell size during automated image analysis to eliminate any large clumps of cells. However, a small number of doublets and small clumps of cells up to 35 μm have been observed to pass through this software analysis filter. The cell size filter algorithm was not set closer to the 15–20 μm cell size because it would dramatically reduce the number of wells that the analysis software would recognize as a cell in fluorescence image analysis and therefore would limit the throughput of the system. We also implemented the use of a 45 μm cell strainer immediately prior to single-cell cloning and use a disaggregation agent in our proprietary media throughout cell line development to minimize the formation of clumps during cell culture.

To demonstrate the impact of high-intensity light on cell culture performance, a 384-well plate containing cell culture was subjected to 200 ms of white, blue or no light. The 200 ms exposure that was tested is 10 times the exposure that is used within our single-cell cloning workflow. The results showed that there was no significant effect on clone recovery for CHO-K1 cells exposed to high-intensity white or blue light (data not shown).

To ensure cells to be imaged are at the bottom of the plate and in the focal plane, which is critical to the success of this process, the plates were centrifuged immediately after plating. We surveyed a wide range of g-force (275–1000g) and found no differences in clone recovery rates or cell counts (data not shown). Stokes’ Law can be used to calculate the duration and force required to sediment a cell in a fluid. Even with conservative assumptions for the density of the cell and media, Stokes’ law indicated that 300g force for 3 min in duration was sufficient to sediment a cell in a 384-well plate. In contrast, Evans et al. suggested that much higher centrifugation force and duration are required when flow sorting cells into media containing plates. Note that in our LDC single-cell cloning workflow, the cells are already in suspension culture as a dilute cell population just prior to single-cell cloning, rather than introduced in a droplet which must coalesce with the medium surface as occurs during flow sorting. The exact media composition and cell type may also affect the required sedimentation duration and force. In agreement with the Stokes’ Law estimates, we generated data that confirmed that higher centrifugation forces or durations are not required when using limiting dilution (data not shown).

**Identification of a suitable fluorescent live-cell cell stain**

To identify a suitable live-cell dye, we tested numerous fluorescent dyes and selected a green fluorescent live-cell dye produced in an animal-component-free process to reduce the risk of adventitious agents. A criterion for all dyes tested was that none were expected to localize to the nucleus thus reducing the chance of DNA damage. The selected live-cell green dye, CellTracker Green CMFDA was effective in making CHO-K1 cells fluoresce at a broad range of concentrations. CMFDA is sequestered in a cell via a reaction between the chloromethyl group of CellTracker Green CMFDA and thiol-containing biomolecules within the cell. It is believed that glutathione trans-merases mediates this reaction. CMFDA is nonfluorescent until acetate groups are cleaved by intracellular esterases. Importantly, the staining of cells did not impact the survival of cells. Figure 2 shows the clone recoveries as a percentage of confluent wells with different dye concentrations (50–400 nM) after single-cell cloning. A separate experiment comparing clone recovery between no dye with concentrations up to 50 nM showed that there was no effect to clone recovery (data not shown).
we measured one mutation in the clones derived without dye staining during single-cell cloning. This analysis showed no significant difference in mutation Green CMFDA dye staining and subsequent imaging. All the mAb A which were single-cell cloned with CellTracker clones expressing mAb A, which were single-cell cloned without dye, were compared against 17 clones expressing triplicate, 13 clones expressing mAb A, which were single-cell cloned with CellTracker Green CMFDA dye to stain $2 \times 10^6$ CHO-K1 cells for single-cell cloning experiments which is twice the amount of dye that is needed from our staining experiments.

The CellTracker Green CMFDA dye is retained for several hours in the cells and to determine if there is a risk of mutagenesis in the presence of the dye, we used the HPRT forward mutagenesis assay as a surrogate estimate of random mutagenesis. In triplicate, 13 clones expressing mAb A, which were single-cell cloned without dye, were compared against 17 clones expressing mAb A which were single-cell cloned with CellTracker Green CMFDA dye staining and subsequent imaging. All the cell lines for mAb A that were evaluated in the HPRT assay were developed in the same conditions prior to single-cell cloning. This analysis showed no significant difference in mutation rate between cell lines derived with dye staining followed with imaging or without dye staining and without imaging. For clones derived without dye staining during single-cell cloning, we measured one mutation in the hgprt gene in $1.3 \times 10^6$ cells plated. For clones derived with dye staining during single-cell cloning, we measured one mutation in the hgprt gene in $2.9 \times 10^6$ cells plated. These results demonstrate that neither the CellTracker Green CMFDA dye nor imaging increase the rate of random mutagenesis in our CHO-K1 cells.

**Image review and analysis workflow**

The well images were manually evaluated by researchers who were trained to identify plate and well artifacts that interfere with the visualization of the cell. We have implemented three levels of manual image review by trained researchers at different stages of the CLD to ensure that the clone that is taken forward has high assurance of being clonally-derived. The first manual image review is completed by the CLD researcher responsible for the cell line. A second manual image review step is completed by a member of the automation group who is a subject matter expert (SME) in imaging. The second manual image review ensures that all clones to be evaluated in fed-batch production cultures have high-quality images. The third level of review is executed by a group leader and SME in imaging prior to cell banking.

**Determination of error rate of high-throughput automated imaging method in documenting single-cell cloning**

A series of experiments were completed to characterize the accuracy and precision of our high-throughput automated imaging workflow. As our current workflow employs LDC, it was important to gather supporting information to demonstrate that one-round of LDC, with supplemental imaging data, was fully sufficient to provide assurance that the cell line was clonally-derived. As LDC follows the Poisson distribution, a seeding density of one cell/well maximizes the number of wells with one cell/well. Different seeding densities between 0.2 and 1 cell/well demonstrated that the input number of cells has little bearing on the level of assurance that the cell line was clonally-derived.

Cell mixing studies are appropriate to measure the error rate, defined as the frequency of obtaining a mixed culture from wells classified as containing a single-cell after both automated image analysis and manual image verification. Cell populations with traceable characteristics—i.e., fluorescence—were purposely mixed and single-cell cloned in our automated workflow to determine the error rate from the number of mixed populations after cell growth to confluence.

Fluorescent cell mixing experiments were also used to address false negative results such as when the Celigo imager does not see a cell at the time of plating but the well contains cell growth after incubation. It would be inappropriate to use the false-negative rate as the error rate in our fluorescent live-cell staining workflow because only cultures derived from wells with one-cell at the time of plating and developed growth after incubation are carried forward in our automated CLD workflow. The false-negative rate is an important concern for workflows that only rely on brightfield imaging, as there is no direct way to distinguish live or dead cells versus artifacts within the well. Therefore, with brightfield imaging only, all the wells are taken forward until there is a subjective evaluation as to whether the well contained a single-cell at the time of plating.

A false-negative result can occur when a cell is outside the focal plane of the imager, either due to insufficient plate centrifugation or improper imager focus. Our centrifugation experiments show that false-negative rates do not change between $g$ forces of 275–1000g (data not shown). Therefore, a false-negative result is most likely due to images being out-of-focus. The Celigo imager in our workflow is operated at its fastest acquisition speed in which the imager must focus, acquire high-resolution brightfield and fluorescent images, process the image data, store the image data and move to the next well in $\sim 0.75$ s per well. In addition, automated focus registration can be challenging when there are very few events in the well to focus on, such as the situation during single-cell cloning.

The CHO-K1 fluorescent hosts—CHO-K1 Green and CHO-K1 Red—were characterized for doubling time and clone recovery after single-cell cloning to find matching pairs (data not shown). A matching pair of CHO-K1 fluorescent hosts was co-cultured for two passages to mimic cell-to-cell interactions during our platform CLD process.

![Figure 2. Clone recovery data after staining with different concentrations of CMFDA dye.](image-url)
The co-cultured cells were then used in a single-cell cloning experiment according to our standard fluorescent dye staining procedure followed by imaging. The seeding density of this fluorescent CHO-K1 mixing experiment was one cell/well, which represents the highest seeding density of our standard single-cell cloning workflow. The Celigo image analysis algorithm determined which wells contained fluorescent cells and the number of cells deposited in the well at the time of plating and which have growth after incubation are picked by our automated hit-picking robots. Of the wells which were determined to be one-cell at the time of plating by the automated image algorithm, 0.3% of the wells had a mixed green and red fluorescent population, 10.7% were green, and 7.5% were red. All wells with cell growth contained fluorescent cells.

According to the Celigo image analysis algorithm, there were 4,403 wells that were determined to have one-cell in the well at the time of plating. Of those 4,403 wells, 2,407 wells (18.4% total recovery, 54.7% recovery from wells with one-cell) were determined to be positive for green and/or red fluorescence (Figure 3). All wells that showed growth were manually inspected. Manual inspection confirmed that 1,400 wells (10.7% total wells) were green fluorescence positive, 974 wells (7.5% total wells) were red fluorescence positive, 33 wells (0.3% total recovery) were positive for both green and red fluorescence and zero wells were non-fluorescent. Further inspection of the images at the time of plating of the 33 red and green fluorescent wells showed that 32 wells would not have passed the criteria that there was only one-cell in the well by manual inspection. The only well that could have passed manual inspection was of unusual morphology with flat sides versus a round shape (Figure 4). We have since updated our criteria during manual inspection to discard clones with this type of cell morphology. Most of the 32 wells with mixed populations after incubation started off as two or three cells due to the image analysis settings that were applied that can allow clumps up to 35 μm to be counted as one-cell. The results of this experiment demonstrate that our host cells do not have a propensity to clump as only 0.3% of the total wells had cell clumps which grew.

This fluorescent cell mixing experiment cannot distinguish if there are two cells in the well of the same color at the time of plating. Using binomial probability and the recovery rate of green only and red only wells in this specific experiment, we calculated the incidence of having two green cells or two red cells at the time of plating is double the observed error rate. Therefore, from 2,407 wells, the one observed instance of a mixed culture represents the average number of wells that would originate from the growth of one red and one green cell, then we would expect on average another well passing both image inspections to originate from two cells of the same color.
To derive numerical data on the assurance of our automated imaging workflow to accurately and precisely identify that our cell lines are clonally-derived, the following calculations were performed from the data acquired. For our automated hit-picking, we rely on the Celigo image analysis software to count fluorescent cells and do not evaluate the images manually at this point. We have 33 wells that had mixed fluorescent populations even though the software counted one-cell at the time of plating. To compensate for the inability to see wells that contain cells of the same color, the number of wells in error would be doubled to 66 wells. To compensate for the cloning efficiency of wells with one-cell, the number of wells which had growth after culture, and were counted as one-cell after plating, was divided by the total number of wells which were counted to have one-cell after plating. The efficiency of clone recovery for wells with 1 cell was determined to be 54.7% (2,407 wells grew/4,403 = 54.7%). To account for wells that potentially had more than one-cell at the time of plating but did not grow due to cloning efficiency, the total wells in error is divided by the cloning efficiency which yields 121 wells (66/0.547 = 121). The confidence for our automated imaging and hit-picking workflow to identify wells with one-cell without manual inspection is calculated by taking the potential number of wells with more than one-cell divided by the total number of wells in error. When clone recovery efficiency is compared, where well recovery percent is calculated for wells which contain cells, not the total number of wells, we do not see a large difference in clone recovery efficiency for different seeding densities if the same number of input cells are considered (Figure 6). These results suggest that there is no synergy for clone recovery. Wells which contain more than one-cell do not have greater than twofold increase in clone recovery as compared to wells which only contain one-cell. As expected, wells with >1 cell/well have a higher clone recovery.

When analyzing the 0.2 cell/well seeding density, 1,777 wells were identified to contain one-cell at the time of plating by the Celigo image analysis algorithm. Of those wells, 385 had cell growth after incubation to a confluence cutoff greater than 25% (Table 1). After manual inspection of the 385 wells, 167 (43.4%) wells had green fluorescence, 214 (55.6%) wells had red fluorescence and four wells (1.0%) had mixed fluorescent cell populations. Upon manual inspection of the images at the time of plating for the four wells with a mixed fluorescent cell population, none of the wells would have passed manual inspection as they contained more than one cell.

When analyzing the 0.4 cell/well seeding density, 3,049 wells were identified to contain one-cell at the time of plating by the Celigo image analysis algorithm. Of those wells, 835 had cell growth after incubation to a confluence cutoff greater than 25% (Table 1). After manual inspection of the 835 wells, 214 (43.4%) wells had green fluorescence, 214 (55.6%) wells had red fluorescence and 17 wells (2.7%) had mixed fluorescent
cell populations. Upon manual inspection of the images at the time of plating for the 17 wells with a mixed fluorescent cell population, none of the wells would have passed manual inspection as they contained more than one cell.

When analyzing the 0.8 cell/well seeding density, 4,594 wells were identified to contain one-cell at the time of plating by the Celigo image analysis algorithm. Of those wells, 796 had cell growth after incubation to a confluence cutoff (>25% (Table 1). After manual inspection of the 796 wells, 380 (47.7%) wells had green fluorescence, 403 (50.6%) wells had red fluorescence and 13 wells (1.6%) had mixed fluorescent cell populations. Upon manual inspection of the images at the time of plating for the 13 wells with a mixed fluorescent cell population, none of the wells would have passed manual inspection as they contained more than one cell.

Performing the same calculations to determine confidence numbers as with the CHO-K1 fluorescent host cell mixing experiment, we have 97.9%, 94.6%, and 96.7% confidence of the automated imaging and hit-picking workflow for 0.2, 0.4, and 0.8 cells/well seeding densities, respectively (Table 1). However, after manual inspection by trained researchers, none of the mixed well populations would have passed inspection; therefore, we have strong confidence that we can accurately and precisely identify a cell during single-cell cloning.

From our cell mixing experiments at different seeding densities, we can conclude that different input number of cells does not change the accuracy and precision of a well-characterized automated imaging workflow. The accuracy and precision in counting cells in a well of our imaging workflow is independent of the number of cells in the well. The requirement is that the imaging data allows for the recognition of all cells.

Combining the data from the fluorescent CHO-K1 host cell mixing experiment with the fluorescent mAb B expressing cell mixing experiment, we can conclude with strong confidence that our automated imaging workflow with manual image verification can accurately and precisely identify monoclonal wells with one round of LDC and that seeding at densities up to one cell/well does not influence the accuracy and precision of the imaging workflow.

Conclusions

We have developed an automated single-cell cloning workflow which incorporates plate imaging at the time of cell seeding to provide assurance that the cell line was clonally-derived. We have optimized the conditions to use a fluorescent live-cell dye to increase throughput and accuracy of identifying cells in a well. We have optimized fluorescent dye concentration staining, image acquisition/analysis, and centrifugation force to enable precise acquisition of high-quality images to support that our cell lines are clonally-derived. Characterization of our workflow by CHO cells with a traceable phenotype (fluorescence) was carried out to provide confidence that our automated imaging workflow along with manual researcher inspection of the images can accurately and precisely identify cells in a well with confidence levels >99.9%.

We have demonstrated a method of direct imaging which can dramatically improve the results obtained from limited dilution cloning. When applied together, the likelihood of obtaining a cell line originating from more than one cell is reduced to the order of one in a thousand.

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