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APPLICATION NOTE

Combined Single-cell Dispensing and 3D Full Well Imaging for Cell Lines with >99.99% Probability of Monoclonality

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Abstract

Clonal cell line development is a crucial step for biopharmaceutical generation (e.g., the production of monoclonal antibodies). To ensure a consistent product, the cell lines used in production must originate from a single cell. Common cloning workflows consist of one to two rounds of single cell cloning followed by imaging of the well plate to confirm single cells were dispensed. However, since there is no confirmation of a single cell dispensed in methods such as limiting dilution or FACS, the detection of errors in the dispensing process is solely dependent on the accuracy of well-plate imaging. Plate imaging has its own error in cell detection and is dependent on centrifugation to ensure all cells are in the imaging focal plane. In combination, these errors impact the probability of monoclonality that can be achieved with a given workflow.

The UP.SIGHT™ addresses these problems by providing double assurance that a single cell was dispensed into a well. Single-cell isolation is documented through a series of nozzle images. Directly after dispensing, the novel technology 3D Full Well Imaging captures images of the liquid volume as a second verification that a single cell was dispensed. As 3D Full Well Imaging captures the full liquid volume, centrifuging after cloning is not required. The UP.SIGHT also performs colony growth monitoring post cloning. The UP.SIGHT is well-suited for high-throughput workflows since it is fast, compatible with 96-, 384- and 1536-well plates and has a high single-cell dispensing efficiency of >98%. In this study, 4000 single cells and almost 1,000 growing colonies were analyzed, and the data demonstrates that the combination of nozzle images and 3D Full Well Imaging results in cell lines with a probability of monoclonality of >99.99%, without the need for fluorescent cell staining or imaging. Since the cell settling time is longer than the 3D Full Well Imaging, we show that performing 3D Full Well Imaging only on the top half of the liquid volume results in the same probability of monoclonality, while drastically reducing the workflow time. Thus, more than fifteen 384-well plates can be generated and imaged in a single day with minimal hands-on time. The UP.SIGHT provides single-cell cloning, verification and colony tracking in a single device.

Figure 1. *The UP.SIGHT provides multiple levels of assurance that a single cell is dispensed during single-cell dispensing and in-well verification. The UP.SIGHT also performs colony growth imaging post cloning.*

Introduction

Cell line development (CLD) is a critical part of creating biopharmaceutical products. To ensure the consistency, quality and safety of the biologics, producing cell lines must be derived from a single-cell progenitor as specified by several regulatory authorities (Plavsic, 2018; European Medicines Agency, 2012). When companies submit their biological products for review, they must provide evidence that their cell lines are monoclonal. CLD is time and resource intensive as large numbers of clones must be screened for productivity, stability and product quality before finding promising candidates for scaling up and production. Therefore, there is a continuous need for improvements to the CLD workflows for faster clone generation while providing assurance of monoclonality.

While there is the overall guideline that cell lines must be monoclonal, the workflows and tools used to generate these clones vary. Most workflows involve single-cell isolation with verification of the isolated cell in the well plate. Even gold standard methods of single-cell cloning have several limitations, including low throughput with limiting dilution or harsh isolation and the need for cell staining for fluorescence-activated cell sorting (FACS). Additionally, neither method provides a visual confirmation of a single cell being dispensed. Therefore, well plate imaging after dispensing is required for single-cell confirmation in the well. Well plate imaging exclusively can result in errors such as "ghost wells," where a generated colony has no evidence of a single cell on Day 0 of dispensing. This error often arises when the cell is not in the imaging focal plane even after centrifuging the plate, and this can affect the overall probability of monoclonality that can be achieved with the workflow. Features of the plate, such as edge effects, shadows or artifacts, can also hinder the ability to evaluate if a well has a single cell. Therefore, the accuracy of the analysis is highly dependent on the plate's quality.

Here, we establish a high-throughput single-cell cloning workflow with the UP.SIGHT, where single-cell isolation, verification and colony growth monitoring are all performed on a single device. The UP.SIGHT uses the same gentle single-cell isolation technology that has already been proven by previous devices, like the single-cell printer™ and f.sight™, which have been rapidly adopted for CLD workflows and are now widely used. However, the UP.SIGHT provides both single-cell isolation confirmation through images from the dispensing nozzle and in the well through the novel 3D Full Well Imaging that occurs directly after each cell is dispensed. Since the entire well liquid volume is imaged, centrifugation is not required. Additionally, the plate bottom quality has minimal impact on the ability to distinguish a single cell in the liquid volume. The UP.SIGHT provides fast and gentle isolation while achieving a high single-cell dispensing efficiency, and EASY.ON disposable cartridges ensure no cross-contamination between samples. The UP.SIGHT is readily compatible with 384- and 1536-well plates for high-throughput workflows, but it also works with 96-well plates. After dispensing, the UP.SIGHT can image colony growth, and a clonality report is generated for each experiment for the easy tracking and verification of colonies. Thus, the UP.SIGHT enables fast, single-cell cloning with verification for a high probability of monoclonality of generated clones.

Materials and methods

Cell culture conditions and single-cell isolation preparation

The single-cell cloning experiments were performed with red and green fluorescent CHO-K1 cells (Innoprot). CHO-K1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high-glucose (Sigma-Aldrich) that was supplemented with 10% fetal bovine serum (Gibco) and 1% of 10 mM Non-essential Amino Acid (NEAA) (Gibco) and 10,000 U/mL Penicillin-Streptomycin (Gibco). Prior to isolation, 384-well plates were prefilled with media filtered through a 0.22 µM strainer to remove any debris. Cells were harvested with TrypLE (Gibco), washed with phosphate-buffered saline (PBS) and resuspended in DMEM high glucose at a concentration of 0.8 x 10^6 cells/mL. For the probability of monoclonality studies, equal concentrations of red and green CHO-K1 cells were mixed together. Using a pipette, 50 µL of the cell suspension was added to an EASY.ON cartridge and placed on the UP.SIGHT for cell dispensing.

Single-cell cloning workflow

The UP.SIGHT dispensed single cells into 384-well plates where they were imaged with 3D Full Well Imaging. For comparison studies, a subset of plates was centrifuged and plate bottoms were imaged using a conventional imager (NyONE, Synentec). Plates were imaged on Days 0, 1, 4, 7 and 14 to track growth with both the imager and the UP.SIGHT. For the probability of monoclonality studies, the colony images were evaluated to determine wells that had mixed (red and green) colonies. Wells that contained bubbles or other possible debris were excluded from analysis.

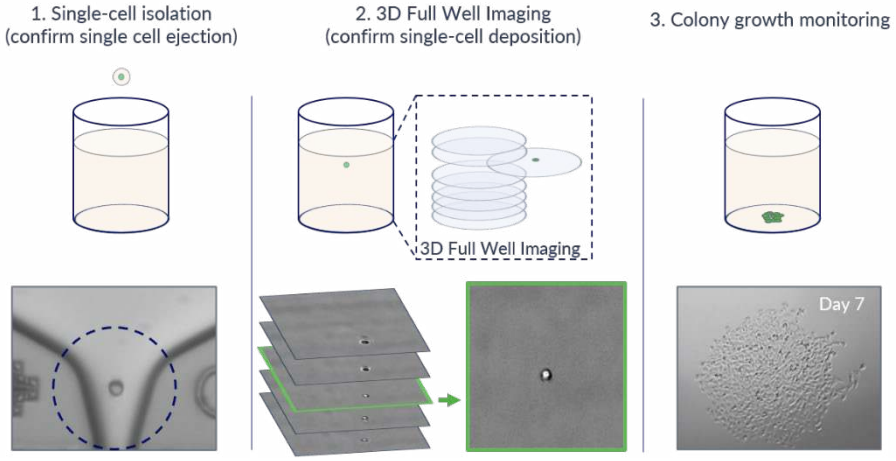

Figure 2. *For every well, the UP.SIGHT goes through a workflow where 1) the UP.SIGHT dispenses a single cell within a droplet, which is verified by nozzle imaging, 2) immediately after dispensing, a series of images are captured in the well through the volume of the media as a second verification of a dispensed cell, and 3) the UP.SIGHT is used to image colony growth over time with multiple assurances of clonality.*

Probability of monoclonality determination

As with any method, there is an error rate associated with the nozzle and 3D Full Well Imaging images. To evaluate this error rate and determine the probability of monoclonality, both sets of images were analyzed. For the nozzle images, the error was determined by evaluating the instances where the nozzle images suggest one cell was selected and ejected but, actually, two cells sticking together were dispensed. Verification of cells in well plate was assessed using both modalities (3D Full Well Imaging and NyONE) to offset any additional error that could occur from using a single device.

$$\text{Nozzle detection error} = (\#\text{ of wells with } >1 \text{ cell} / \#\text{ of wells where nozzle image} = 1 \text{ cell}) \times 100$$

The second error is associated with the 3D Full Well Imaging images. Here, we estimate this error by comparing the wells that had a single cell, according to 3D Full Well Imaging, but resulted in a mixed (red and green) colony.

$$\text{3D Full Well Imaging error} = (2 \times \#\text{ of mixed colonies/colony outgrowth}^2) / (\#\text{ of wells where 3D Full Well Imaging} = 1) \times 100$$

To determine the probability of monoclonality, the nozzle image error was multiplied by the 3D Full Well Imaging error.

$$\text{Probability of monoclonality} = (1 - \text{nozzle image error} \times \text{3D Full Well Imaging error}) \times 100$$

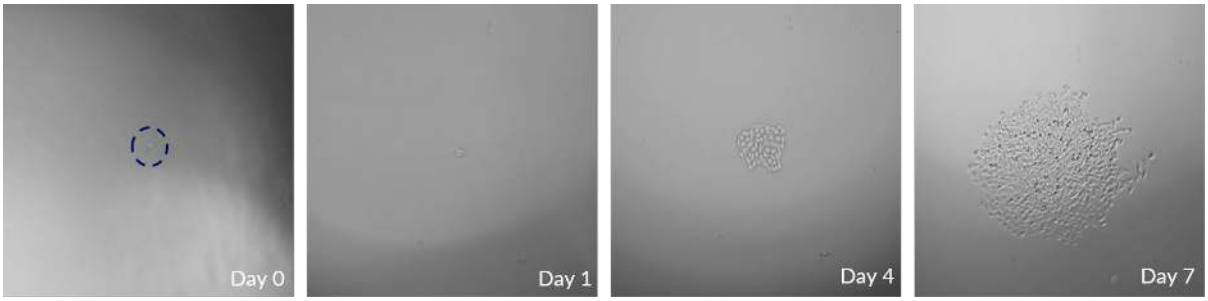

Figure 3. *The UP.SIGHT can provide post-cloning imaging for colony growth tracking. A colony was imaged at Days 0, 1, 4 and 7.*

Results and discussion

The UP.SIGHT provides single-cell dispensing, in-well verification and colony tracking in one device

The UP.SIGHT provides a complete workflow for cloning, clone verification and tracking (Figure 2). The process begins with loading a cell suspension into a cartridge that is placed in the device. For single-cell dispensing, miniature, free-flying droplets are generated from the nozzle, which is continuously imaged (Figure 2.1). An image-processing algorithm evaluates the occupancy of the upcoming droplet to determine if it will have 0, 1 or multiple cells. If the upcoming droplet is determined to have no cells, multiple cells or a single cell that does not match the criteria (size, roundness or, if applied, fluorescence intensity), the droplet is discarded. If a target single cell is detected, and cell size, roundness and fluorescence intensity (if applied) meet the preset setting, then it is dispensed into the well plate. For each single cell dispensed, a series of five images is generated to confirm single-cell isolation and ejection.

After dispensing, a second camera underneath the well plate is used to capture a series of images through the well (3D Full Well Imaging) (Figure 2.2). This imager provides a second confirmation that a single cell was dispensed in the target well. The number of images captured by 3D Full Well Imaging is dependent on the plate type and media volume in the well. The full well imaging eliminates the need to centrifuge plates. Since

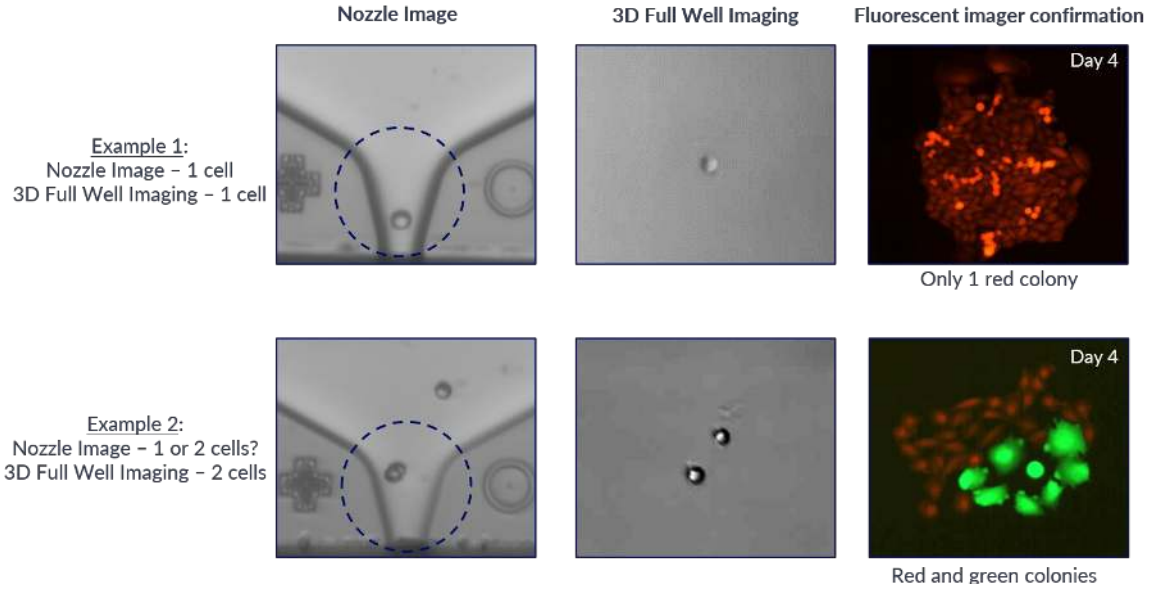

Figure 4. *The UP.SIGHT provides multiple assurances of clonality with nozzle and 3D Full Well Imaging. In Example 1, both the nozzle and 3D Full Well Imaging confirm that a single cell was dispensed and thus clonal. This is confirmed by the development of a single-color colony. In Example 2, it is unclear if one or two cells were dispensed by reviewing the nozzle image only. However, 3D Full Well Imaging confirmed two cells were dispensed, which resulted in nonclonal, two-color colonies.*

Probability of a single cell dispensed

Total number of wells where nozzle image = 1 cell	Total number wells where 1 cell is present (true positive)	% of wells with >1 cell (nonclonal)	% of wells with 1 cell (clonal)
4003	3962	1.0%	99.0%

Probability 3D Full Well Imaging determines a single cell

Total number of wells where 3D Full Well Imaging = 1	Number of wells with outgrowth	Number of wells with mixed colonies	% of wells with >1 cell (nonclonal)	% of wells with 1 cell (clonal)
1822	991	2	0.7%	99.3%

Probability of Monoclonality = 1 – (cell cam error x 3DVI error) x100 =
1 – (0.01 x 0.007) x 100 = **99.992%**

Table 1. *Probability of monoclonality as determined by nozzle and 3D Full Well Imaging data obtained in this study The UP.SIGHT achieves a probability of monoclonality >99.99%.*

the well imaging is performed while the cell is settling in the media and the cell is usually in the upper portion of the volume, distortions from plate or edge effects will not affect cell detection and are greatly minimized as compared to only imaging the bottom of the well plate for Day 0 imaging.

Post cloning, the UP.SIGHT can image and track colony growth for a selection of clones that have the ideal growth curves (Figure 2.3). Figure 3 shows the growth of a colony that was verified as derived from a single cell by nozzle images and 3D Full Well Imaging on Day 0. Here, we show the cell after settling at the bottom with subsequent images captured on Day 1, Day 4 and Day 7 after the dispensing event. The imaging time is fast and takes ~3 minutes to process a 384-well plate, leading to high-throughput processing for subsequent colony tracking.

Multiple assurances of monoclonality are achieved with the combination of nozzle images and 3D Full Well Imaging

The combination of the nozzle images with 3D Full Well Imaging provides an additional confirmation that the cell is in the well plate. Compared to other methods, like limiting dilution or FACS, the UP.SIGHT provides verification of single-cell isolation during the dispensing process. The UP.SIGHT achieves a high single-cell dispensing efficiency of >98%. However, errors can still arise where the algorithm predicts only one cell is selected but, actually, two cells sticking together are dispensed. Thus, the addition of 3D Full Well Imaging provides a second method to confirm the dispensing was successful and to confirm that there was only one cell. Figure 4 shows two examples of nozzle images and their corresponding 3D Full Well Imaging. Here, red and green CHO cells were mixed and dispensed. The resulting colonies were also captured by a fluorescent imager. In Figure 4.1, it is clear that only one cell was selected, and this is verified by a single cell shown in the 3D Full Well Imaging and only a single-color colony arising. Thus, this is a monoclonal cell line. In Figure 4.2, it is difficult to confirm by evaluating the nozzle image alone that only a single cell was selected. However, the 3D Full Well Imaging confirms that two cells were dispensed, which resulted in red and green colonies. In this example, the combination of nozzle and 3D Full Well Imaging would have ruled this well as nonclonal and excluded it from further analysis.

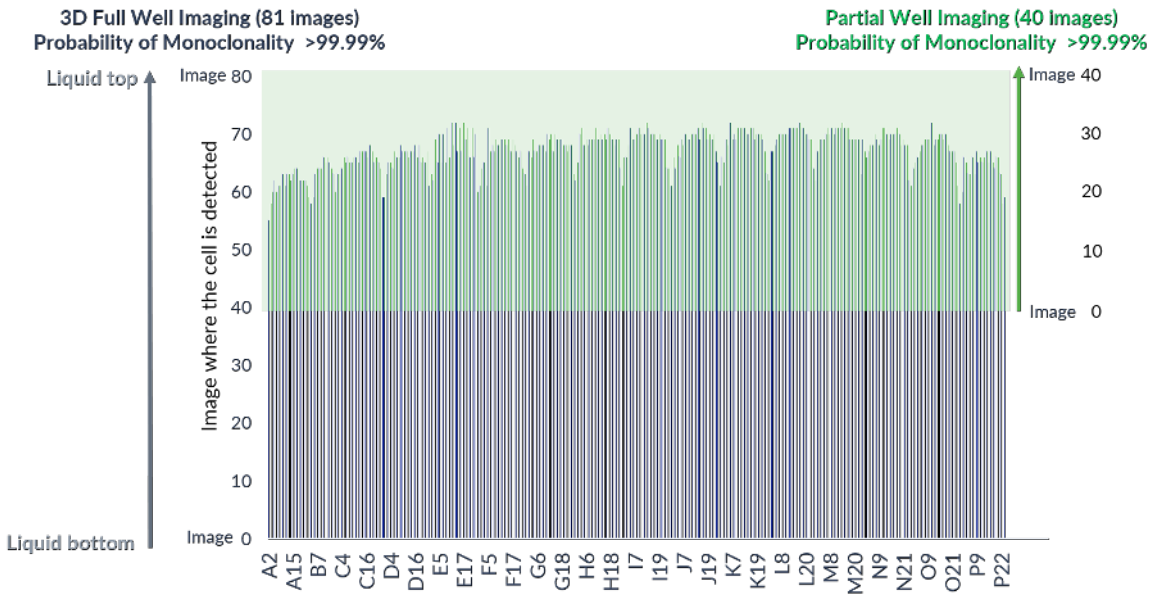

Table 2. *Imaging from the center of the liquid volume still captures the cells in 3D Full Well Imaging and maintains a high probability of monoclonality. 81 images were used to capture the full volume in a well, and here on average the cell was visible between Images 55 and 70. Due to the slow settling rate of cells, using only 40 images starting from the center of the volume was also sufficient, and no cell was missed by evaluating only the top half of the well.*

UP.SIGHT achieves a probability of monoclonality of >99.99%

To determine the probability of monoclonality achieved by the UP.SIGHT, the error rate associated with the nozzle images and 3D Full Well Imaging were calculated. Here, the validation study used a 1:1 ratio of mixed red and green CHO cells to determine the error rate of single-cell dispensing and 3D Full Well Imaging as similarly performed by Yim et. al with cytena's SCP and a fluorescent imager. To determine the nozzle image error rate, over 4,000 cells were dispensed into plates that were classified as single cells by the nozzle images. After verification of the cells in the well plate, it was confirmed that 41 wells actually had two cells, which resulted in an error rate of 1.0%. For the 3D Full Well Imaging error rate, a subset of the plates was kept for colony evaluation, and the wells that were classified as containing a single cell by 3D Full Well Imaging were compared to resulting colonies. Both red and green cells were deposited so wells that contained multicolor colonies indicate the 3D Full Well Imaging error. Over 1,800 wells were evaluated which resulted in 991 colonies. In total, there were two mixed color colonies that were classified as having been derived by a single cell via 3D Full Well Imaging. Using the calculation,

$$\text{3D Full Well Imaging error} = (2 \times \#\text{ of mixed colonies/colony outgrowth}^2 \,/\, \#\text{ of wells where 3D Full Well Imaging} = 1) \times 100$$

the estimate for the 3D Full Well Imaging error rate was 0.7%. Multiplying the two error rates results in the UP.SIGHT providing a probability of monoclonality >99.99%. Although the two mixed color colonies could not be identified as non-clonal in the 3D Full Well Images, both wells could be identified as non-clonal in the respective nozzle images.

Partial 3D Full Well Imaging also delivers >99.99% probability of monoclonality with faster imaging

The image stack position where the cell is in focus during 3D Full Well Imaging is consistent across plates. Here, 81 images were used in 3D Full Well Imaging to capture the full well volume. Over 1,500 wells were evaluated and on average, the cell was located at image stack position 65. (Image 1 is the well bottom and Image 81 is the top of the liquid.) The standard deviation of the image stack where the cell was located was 4.2, and the minimum value was Image 45. The variations could be attributed to inconsistencies in the well bottom height in addition to errors in pipetting. However, the speed at which the cell settles makes it highly unlikely that the cell will be in the lower part of the well plate right after dispensing. Therefore, it is possible

Workflow	384 well Time per plate	Probability of Monoclonality
Single cell dispensing + no 3D Full Well Imaging	~8 min	99.0%
Single cell dispensing + partial 3D Full Well Imaging	~30 min	>99.99%
Single cell dispensing + 3D Full Well Imaging	~50 min	>99.99%

Table 3. *A comparison of the workflows with processing times.*

to forgo imaging the lower portion of the well and start from the middle to the top of the liquid level. This greatly reduces processing time while maintaining a high probability of monoclonality. Table 2 shows the image stack position where the cell was in sharp focus for each well in one example 384-well plate. A total of 81 images were captured, however even 40 images starting from the center of the liquid volume were sufficient to capture all the cells and achieve the same probability of monoclonality as in the 3D Full Well Imaging. Table 3 compares the different workflows with the corresponding processing time and the probability of monoclonality.

Conclusions

- The UP.SIGHT is an all-in-one device that performs single-cell isolation, verification and colony growth monitoring.
- 3D Full Well Imaging is a novel technology that provides full liquid volume imaging of each well to verify single-cell dispensing without the need to centrifuge well plates.
- The UP.SIGHT offers double assurances of clonality by imaging and documenting both single-cell isolation and dispensing into well plates.
- Our extensive validation study with over 4000 individual cells has shown that with this workflow we obtain cell lines with a probability of clonality of >99.99 %.
- Partial 3D Full Well Imaging also delivers >99.99% probability of monoclonality with faster imaging.

References

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