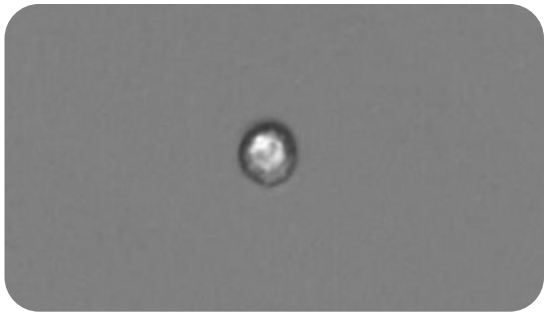
R&D systems by biotechne

SYNENTEC

Instruments

Accelerating Monoclonal Cell Line Development: *Validation of a High-Throughput Workflow*

Willms A[1], Shaaban A[2], Werdelmann B[1], McComb R[2], Vaghasiya A[1], Kollenda S[1], Sebens S[3], Geisen R[1], and Pirsch M[1]. [1]SYNENTEC GmbH Elmshorn, Germany, [2]Bio-Techne, Minneapolis, MN, USA, [3]CAU + UKSH Kiel, Germany



Abstract

The generation of monoclonal cell lines has become a fundamental process in biotechnology and biological research with applications ranging from the production of therapeutic proteins to stem cell-based therapies and the generation of disease models. Ensuring that colonies originate from a single cell progenitor is essential for reproducibility, regulatory compliance, and downstream data quality.

One of the most widely used methods for single cell deposition is the limiting dilution assay (LDA), as it is inexpensive and gentle on cells. However, LDA is inherently inefficient, resulting in a high number of empty wells and wells containing multiple cells, which increases time, labor, and consumable costs. Automated single cell dispensing technologies, such as the Pala™ Single Cell Sorter and Dispenser (R&D Systems™), promise to overcome these limitations by enabling targeted deposition of single cells with higher efficiency and speed.

In this study, we evaluated whether Pala fulfills this promise and whether it is comparable to, or superior to, LDA in terms of monoclonality, colony formation efficiency, and cell growth characteristics. Using SYNENTEC's automated high-throughput imaging platform and YT-SOFTWARE®, we directly compared Pala-based single cell dispensing with manual limiting dilution using CHO-K1 cells. Automated longitudinal imaging enabled objective assessment of monoclonality, growth kinetics, and final colony confluence.

Our results demonstrate that Pala significantly increases the number of colonies per plate without negatively affecting cell growth. Combined with SYNENTEC's imaging systems, it can lead to faster clone selection and decrease overall cost and resource consumption.

Pala and CELLAVISTA Key Benefits:

Pala Single Cell Dispenser

- ✓ Fast start up (5 min) and dispensing (96-well in <2 min)
- ✓ Gentle sorting for healthier single cells (<2 PSI/13.8 kPa)
- ✓ Fluorescence sorting with 2 lasers and up to 11 channels
- ✓ Simple interface with FACS-like gating for sorting cells of interest

CELLAVISTA 4K

- ✓ Monoclonality confirmation documentation (Day 0)
- ✓ Efficient clone tracking via image galleries
- ✓ Artifact discrimination through pre-imaging
- ✓ Validation of optimal growth conditions

Introduction

Monoclonal cell lines are fundamental to life science research and biopharmaceutical manufacturing. In particular, single-cell cloning (SCC) of genetically engineered cells is essential for the generation of stable, well-defined cell lines used as *in vitro* models and for the reproducible production of therapeutic proteins. Cultures derived from more than one progenitor cell may exhibit variability in product quality, stability and yield over time. High-producing clones are typically rare and can be outcompeted by low producers, leading to declining productivity. For these reasons, the identification and expansion of monoclonal, high-expressing cells is a critical step in cell line development (CLD), and regulatory authorities require documented evidence of monoclonal origin for production cell lines[1]. A central technical challenge of SCC is therefore the reliable deposition of exactly one viable cell per well and the proven detection thereof.

Limiting dilution assay (LDA) remains one of the most commonly used methods for single-cell isolation. In LDA, cells are diluted to low concentrations and distributed into multiwell plates such that wells statistically contain zero, one, or multiple cells. While this approach is inexpensive and minimally stressful to cells, its statistical nature results in low efficiency resulting in many wells remaining empty, whereas others contain multiple cells or aggregates. Consequently, LDA often requires the screening of large numbers of plates, increasing time, labor, and reagent consumption, especially in screening-intensive random integration workflows. Additionally, cell deposition with LDA cannot enrich for fluorescently labeled cells making certain cell line development workflows (i.e. complex genetic engineering) extremely challenging and requiring an onerous amount of plates to be dispensed and screened.

Fluorescence-activated cell sorting (FACS) represents an alternative strategy capable of accurate single-cell deposition. In addition to being high- throughput, FACS enables selective enrichment of target cells based on fluorescence markers associated with productivity or phenotype. However, exposure to high shear forces and pressure during sorting can impair cell viability and impact post-sorting outgrowth.

Moreover, conventional cell sorters are costly, require substantial maintenance, depend on specialized consumables, and demand skilled operators, which can complicate routine SCC workflows. In particular, lengthy instrument setup and decontamination procedures can substantially extend processing times, often necessitating extended operating hours or multiple work shifts, thereby increasing operational costs.

FIGURE 1

Experimental Workflow

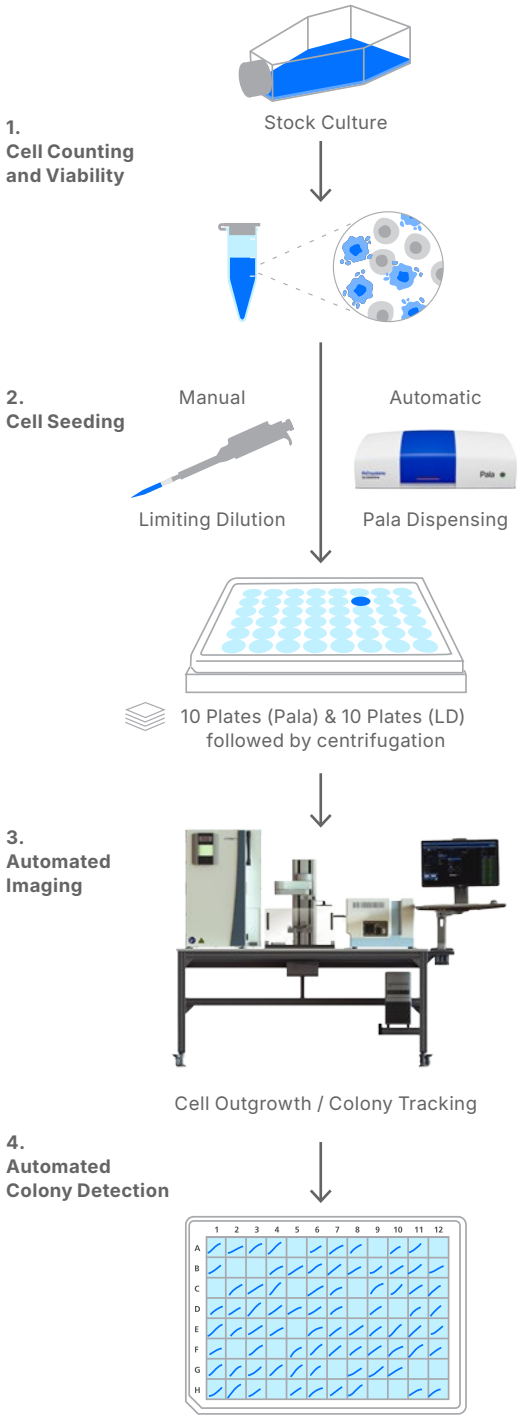


Figure 1. CHO-K1 cells were detached, counted and seeded into ten 96-well plates either by Pala dispensing or manually using limiting dilution (0.5 cells/well). After centrifugation, plates were immediately imaged using our automation system. Colony growth was monitored over time using YT-SOFTWARE.

Microfluidic cell dispensers have emerged as a promising alternative for single-cell seeding. These systems typically offer a compact footprint, simplified operation, reduced consumable costs, and rapid setup. Additionally, the sterile microfluidic disposable cartridges provide advantages in handling numerous samples, mitigating concerns related to cross-contamination between cell lines and waste cleanup. Image-based dispensers further enable direct visual confirmation of single-cell deposition, providing strong evidence of clonal derivation. Nevertheless, image acquisition and real-time decision algorithms can slow dispensing rates, potentially prolonging cell exposure to non-optimal conditions compared with high-speed FACS-based sorting.

Currently, only a limited number of microfluidic dispensers combine fluorescence-based cell enrichment with rapid and efficient single-cell deposition. One such system is the Pala microfluidic single-cell dispenser (R&D Systems), which integrates automated operation, low maintenance requirements, and multiparametric fluorescence analysis for target cell enrichment. These features suggest that Pala could represent a practical alternative to both FACS and LDA for SCC workflows[2]. However, the adoption of new dispensing technologies requires rigorous validation to confirm that monoclonality, growth characteristics, and overall cell performance are maintained.

In this study, we present a process validation comparing automated single-cell dispensing using the Pala system with classical limiting dilution. By coupling both seeding approaches with SYNENTEC's automated imaging and image analysis platform, we provide an objective, image-based assessment of single-cell deposition efficiency and subsequent colony outgrowth.

FIGURE 2

Pre-Sorting Viability Check on Pala

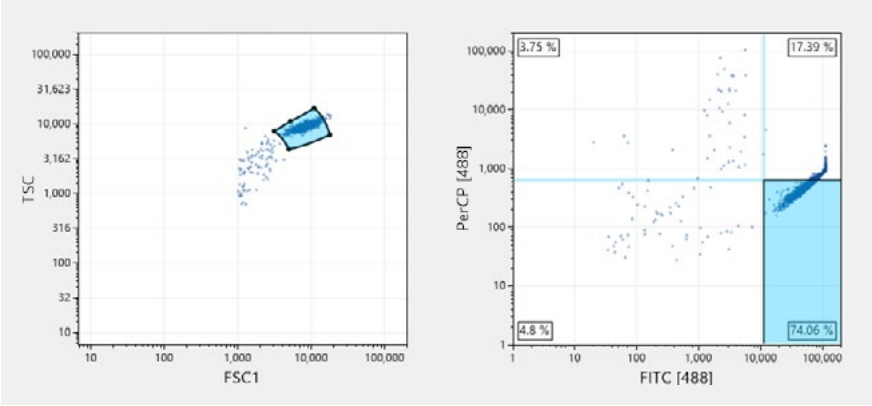


Figure 2. (Top) Pre-sorting viability check using Calcein-AM (live) and Propidium Iodide (dead) staining of CHO-K1 cells. Forward x Total scatter (FSC1 x TSC) used to differentiate cells from debris. FITC (Ex: 488, Em: 527/32) x PerCP (Ex: 488, Em: 692/80) used to differentiate Calcein-AM (live) and Propidium Iodide (dead) cells respectively. (Bottom) Representative screenshot from Pala software. After cell viability was confirmed, unstained CHO-K1 cells were loaded into a single cell cartridge and dispensed with only FSC1 x TSC gate across 10 × 96 well plates for single cell cloning experiments.

CHO cell Dispensing on Pala for Single Cell Cloning

Material

Cell Line

- Adherent CHO K1 cells, Chinese hamster ovary subclone K1

Devices and Software

- Pala™ single-cell sorter and dispenser (R&D Systems™) 488/561 Laser Configuration
- SYNENTEC automated imaging system (consisting of CYTOMAT™ 2 C-LiN incubator, SYBOT-1000® plate handler, CELLAVISTA 4K® imager)
- YT-SOFTWARE® for image processing, segmentation, and data analysis
- Centrifuge for multiwell plates with a swing-bucket rotor

Consumables

- 96-well SBS-format cell culture plates (e.g. CytoOne® from Starlab # CC7682-7596)
- Barcodes for plate identification
- CHO cell culture media with filtrated fetal calf serum (DMEM F12, 10% FCS, 2 mM GlutaMAX™, 1 mM Sodium pyruvate, Penicillin-Streptomycin 10 U/mL)
- Anti-clumping agent (e.g. Gibco # 0010057AE, 1:1000)
- Trypsin/EDTA (e.g. Pan-Biotech # P10-024100)
- 40 µm cell strainer caps (e.g. Corning Falcon® # 352340)
- DPBS, w/o: Ca and Mg (e.g. Pan-Biotech # P04-36500)
- Calcein-AM (e.g. Biolegend # 425201)
- Propidium iodide (e.g. Thermo Fisher Scientific # P3566)

Methods

Plate Preparation

Twenty plates were prepared two days before cell seeding by pipetting 100 µL (ten plates for LDA) or 200 µl (ten plates for Pala-dispensing) of medium into each well. Additionally, each plate was labeled with a barcode for identification. These empty plates were imaged before cell seeding (d-1) to identify plate artifacts & debris that interfere with single cell confirmation.

Cell Culture

CHO-K1 cells were routinely cultured in T75 flasks in cell culture medium (37 °C, humidified atmosphere, 5% CO_2). When ~ 90% confluency was reached, they were routinely passaged. Cells were harvested with trypsin and counted using SYNENTEC's Trypan Blue or Erythrosin B application. The required cell number was calculated based on the viable cell density (VCD).

Preparing a Single-Cell Suspension

To prepare a cell suspension, CHO-K1 cells were detached from the flask by trypsinization and collected in a 50 mL tube. The cells were pelleted by centrifugation (300 x g, 5 min), the supernatant was removed, and the pellet was resuspended in medium containing a 1:1000 dilution of anti-clumping agent. The suspension was then passed through a cell strainer to remove any clumps that could obstruct the microfluidic channels. Afterwards, cells were counted using the Trypan Blue application of NYONE® or CELLAVISTA® and YT-SOFTWARE®.

Limiting Dilution Assay

For the limiting dilution assay, a cell suspension at a density of 5 cells/mL was prepared in medium. Then, 100 µL of the suspension was manually dispensed into 96-well plates using a multichannel pipette leading to a statistical distribution of 0.5 cells/well. Each well reached a final volume of 200 µL, as 100 µL of medium had already been added during plate preparation (see above). A total of ten plates were prepared (see workflow in Fig. 1).

Cell Staining for Viability Confirmation Using Pala

To enable exclusion of dead cells and confirm cell health and viability prior to single cell cloning, cells were stained with 0.1 µM Calcein-AM (to label live cells) and 1 µg/mL propidium iodide (to label dead cells, Fig. 2). A cell suspension of 1 x 10^5 cells/ mL was prepared, and Calcein-AM was added at a 1:10,000 dilution, followed by a 20 minute incubation at 37 °C, humidified atmosphere, 5% CO_2. The suspension was then diluted to 5 x 10^3 cells/mL, and PI was added at a 1:1,000 dilution. Cells were analyzed using FITC and PerCP charts on Pala to ensure healthy, viable cells were prepped for experimentation (see Figure 2). This helped define the light scatter signal (FSC/TSC) gates for the unstained proportion of cells used for single cell cloning.

Pala Single Cell Sorting

For single cell cloning experiments, a cell suspension of unstained cells with a density of 7 x 10^3 cells/mL was prepared. Cells were seeded using light scatter detection (FSC/TSC) into ten 96-well plates which had been prepared with 200 µL medium before (see Figure 2).

Centrifugation

After seeding, all plates were centrifuged at 1125 x g for 5 min to position cells in the focal plane at the bottom of the wells.

Automated Imaging and Image Analysis

Automated imaging was performed on days -1, 0, 1, 4, & 8 followed by extended monitoring of colony outgrowth from days 9-14. Full-well brightfield images in 4K resolution and 10 x magnification were acquired using SYNENTEC's automated imaging systems. Autofocus was set to 'each image'. Images were processed with the Single-Cell Cloning application of YT-SOFTWARE®, applying standardized segmentation algorithms to detect outgrown colonies. Galleries enabled easy backtracking to the seeding day to analyze monoclonality (example in Fig. 3). Moreover, the confluence of each clone was assessed to analyze growth behavior. In addition, each well was manually checked to find single cells that did not grow into colonies. Graphs were generated using GraphPad Prism or Python 3.11 with Pandas[3], NumPy[4], and Plotly[5]. Statistical analyses were performed using SciPy[6].

FIGURE 3

Clone Gallery in YT-SOFTWARE®

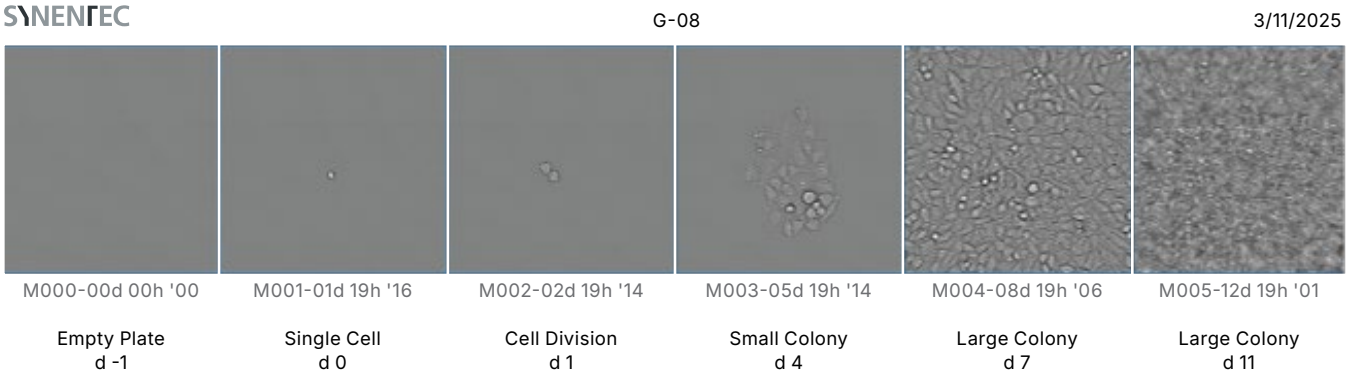


Figure 3. Clone Gallery in YT-SOFTWARE® Allows Quick Observation of Monoclonality. Plates were automatically imaged before (empty plate, d-1) and after cell seeding. YT-SOFTWARE® automatically detects colonies and generates galleries showing the same spot of the well in several measurements. This allows easy clone tracking and observation of monoclonality.

FIGURE 4

Categories of Different Wells

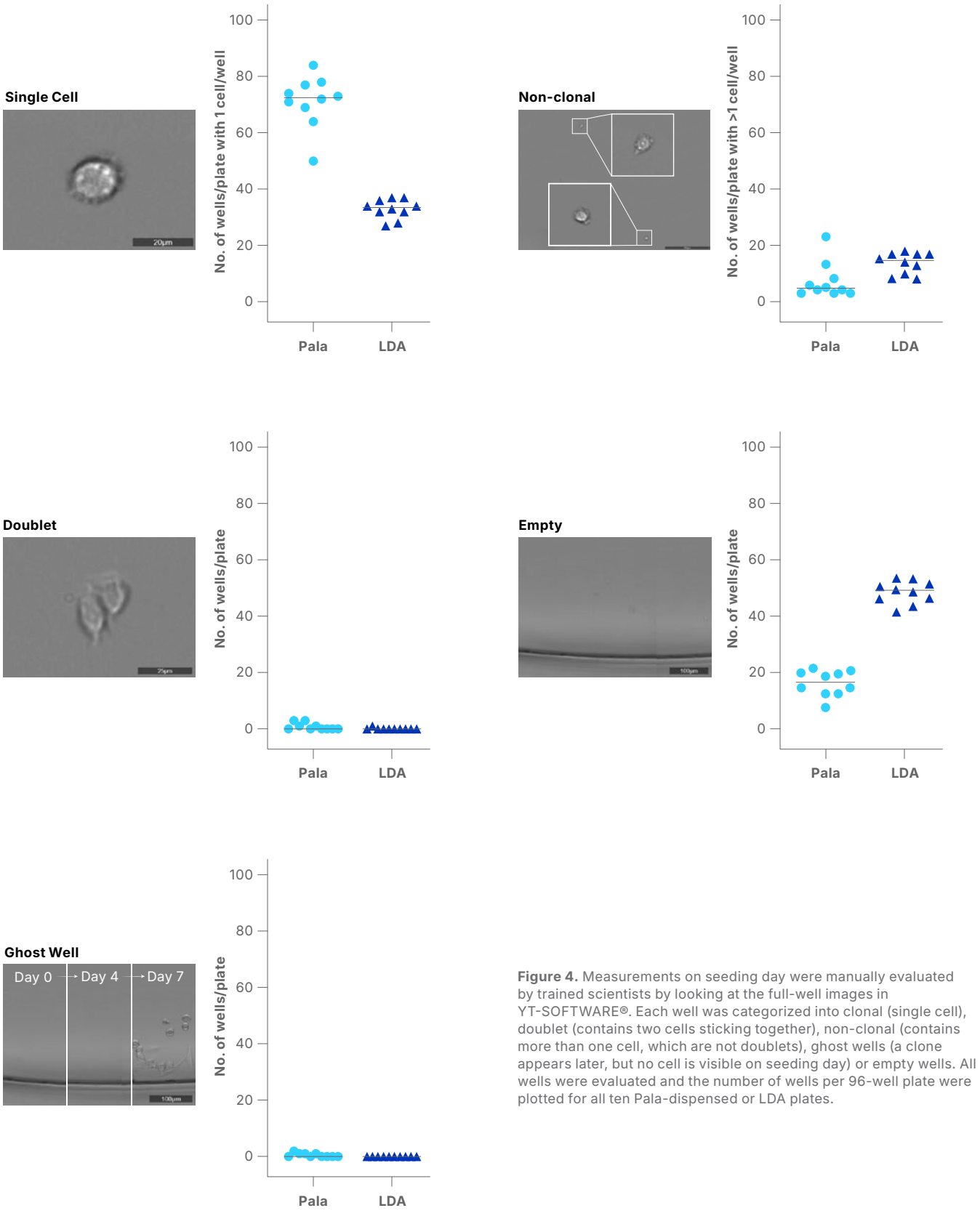


Figure 4. Measurements on seeding day were manually evaluated by trained scientists by looking at the full-well images in YT-SOFTWARE®. Each well was categorized into clonal (single cell), doublet (contains two cells sticking together), non-clonal (contains more than one cell, which are not doublets), ghost wells (a clone appears later, but no cell is visible on seeding day) or empty wells. All wells were evaluated and the number of wells per 96-well plate were plotted for all ten Pala-dispensed or LDA plates.

Results and Discussion

1. Seeding Efficiency and Monoclonality

At first, the monoclonal seeding efficiency of Pala dispensing compared to LDA was evaluated. To do so, the stitched whole well images of all 960 wells per method on seeding day were manually evaluated by trained scientists for single cells in YT-SOFTWARE® leading to the following classification (examples are shown in Fig. 4):

- Clonal (one cell per well)
- Doublets (two cells sticking together)
- Non-clonal (two or more cells not sticking together)
- Ghost wells (colony grows out even though there are no cells visible on seeding day, usually because they are on the well wall)
- Empty wells

As expected from the statistical nature of the approach, LDA resulted in a high number of empty wells. In contrast, Pala-based dispensing yielded a substantially higher number of wells containing exactly one detectable cell. Altogether, only four of all 1920 wells were classified as ghost wells demonstrating a good centrifugation process and the high image quality of CELLAVISTA. The 4K resolution of the imager enabled rapid visual verification of monoclonality, including identification of cells located at the well edge or ambiguous events that might otherwise be overlooked in manual inspection. Imaging the empty plates before cell seeding also helped in identifying plate artefacts that might otherwise be falsely identified as cells.

2. Colony Formation and Growth Behavior

Using the Single-Cell Cloning application, YT-SOFTWARE® automatically detected the colonies and determined their confluence. We analyzed how many monoclonal cells grew into colonies with a final confluence of 0.2 %. As expected, the overall number of wells with clonal outgrowth was higher for Pala dispensing compared to LDA. While limiting dilution yielded 234 colonies in total, automated single cell dispensing with Pala resulted in 636 colonies under identical culture conditions, and ~3-fold increase. This increase reflects the higher single-cell deposition efficiency of Pala and reduced loss due to empty or non-clonal wells.

Clonal cells eventually growing into colonies demonstrated that Pala dispensed single cells grew into colonies at a higher rate than LDA dispensed cells. This is likely due to the selective gating of viable cells using light scatter but determined through Calcein AM staining prior to sorting. This approach was used to identify where the highly stained, and viable, cells clustered in the FSC x TSC plots.

In line with this, confluence analysis of the longitudinal imaging data demonstrated that colonies derived from Pala-dispensed single cells grew slightly faster compared to those obtained by limiting dilution. This could be observed in the comparison of the final confluence (>300 h), the confluence in late stages (210-220 h) as well as in the growth rate and doubling time (Tab. 1 and Fig. 6). No negative influence on cell growth or morphology was observed.

3. Turning images into numbers

By converting images into quantitative data, SYNENTEC's imaging and analysis pipeline enabled an objective comparison of both methods. Key parameters such as colony growth over time and final confluence were extracted automatically, reducing user bias and variability.

FIGURE 5

Colony Detection and Clonal Outgrowth

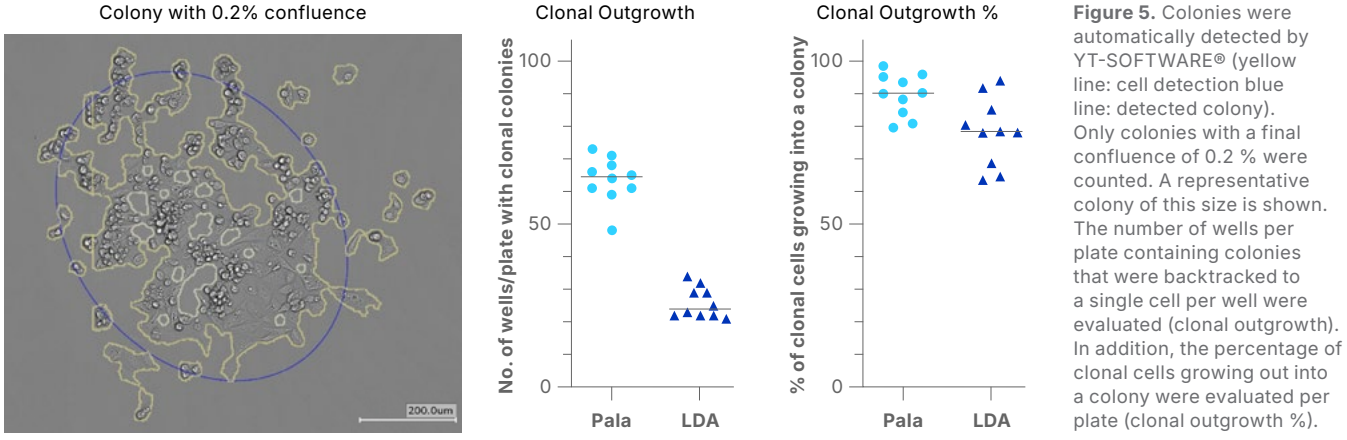


Figure 5. Colonies were automatically detected by YT-SOFTWARE® (yellow line: cell detection blue line: detected colony). Only colonies with a final confluence of 0.2 % were counted. A representative colony of this size is shown. The number of wells per plate containing colonies that were backtracked to a single cell per well were evaluated (clonal outgrowth). In addition, the percentage of clonal cells growing out into a colony were evaluated per plate (clonal outgrowth %).

FIGURE 6

Comparisons of Growth Characteristics

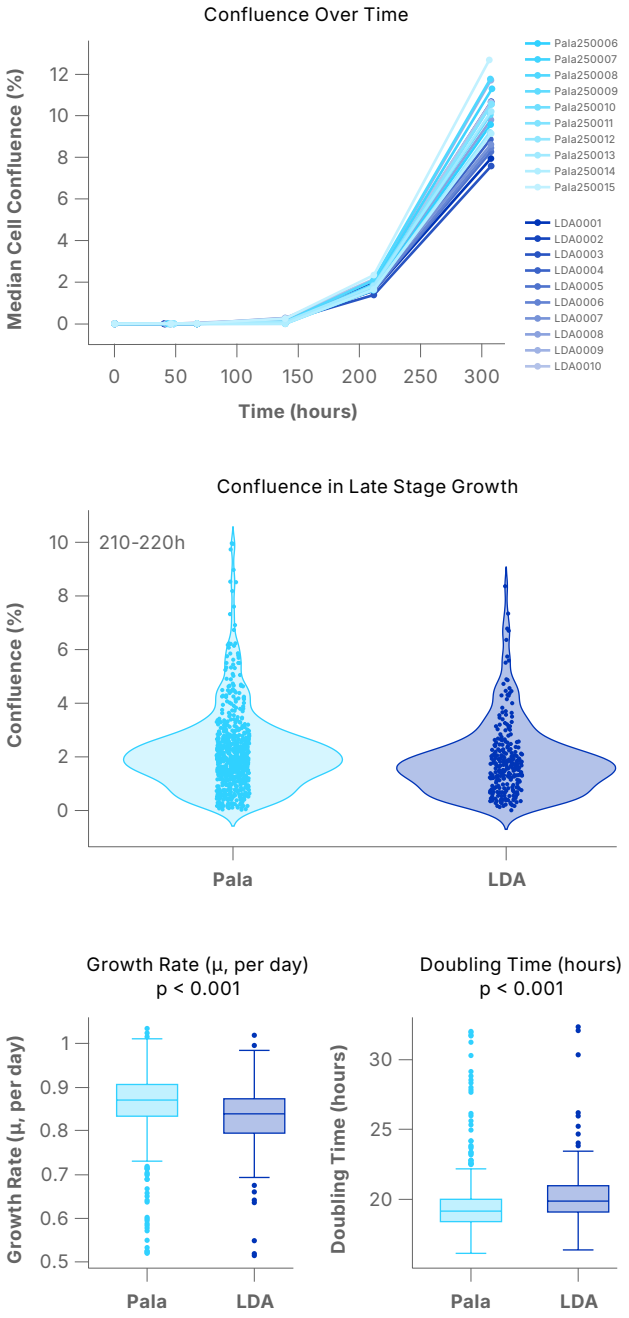


Figure 6. Confluence analysis allows comparisons of growth characteristics. The confluence of detected clonal colonies with a >0.2% final confluence was analyzed over time by YT-SOFTWARE. The median cell confluence for all colonies in each plate was calculated and plotted over time. Moreover, the confluence in late stage growth (210-220 h) was analyzed and plotted for each colony. Finally, using the over time data, growth rate as well as doubling time was calculated for each colony.

Conclusion

This study demonstrates that automated single cell dispensing using the Pala™ system is a robust and efficient alternative to classical limiting dilution. Using CHO-K1 cells, Pala significantly increased the number of clonal outgrowths without compromising cell growth or monoclonality. When combined with SYNENTEC's automated imaging and YT-SOFTWARE®-based analysis, the workflow provides end-to-end documentation of single cell origin and clonal outgrowth. This makes it well suited for method evaluation, process optimization, and qualification in regulated environments, as well as routine use in CLD, stem cell research, cancer research, and drug discovery.

Learn More About Pala

Scan the QR code or visit
rndsystems.com/single-cell-dispensers

Acknowledgements

We thank the Institute for Experimental Cancer Research, especially Prof. Susanne Sebens, for outstanding support, fruitful discussions and a great working atmosphere during this cooperation.

This technical paper is a collaboration of R&D Systems and SYNENTEC.

REFERENCES

1. A. E. S. Ammar, "Single-cell cloning and its approaches," Front. Cell Dev. Biol., vol. 13, Dec. 2025, doi: 10.3389/fcell.2025.1678157.
2. L. Chakrabarti, J. Savery, J. P. Mpindi, J. Klover, L. Li, and J. Zhu, "Simplifying stable CHO cell line generation with high probability of monoclonality by using microfluidic dispensing as an alternative to fluorescence activated cell sorting," Biotechnol. Prog., vol. 40, no. 3, p. e3441, 2024, doi: 10.1002/btpr.3441.
3. W. McKinney, "Data Structures for Statistical Computing in Python," SciPy 2010, May 2010, doi: 10.25080/Majora-92bf1922-00a.
4. C. R. Harris et al., "Array programming with NumPy," Nature, vol. 585, no. 7825, pp. 357–362, Sep. 2020, doi: 10.1038/s41586-020-2649-2.
5. Plotly Technologies Inc., "Collaborative data science," 2015, [Online]. Available: https://plot.ly
6. P. Virtanen et al., "SciPy 1.0: fundamental algorithms for scientific computing in Python," Nat. Methods, vol. 17, no. 3, pp. 261–272, Mar. 2020, doi: 10.1038/s41592-019-0686-2.

R&D systems by bio-techne

11136.0.0626