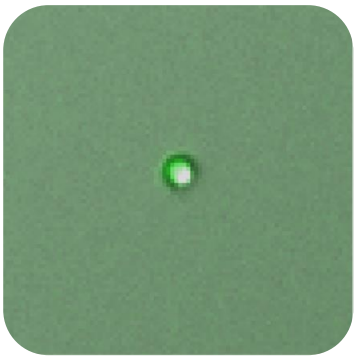
Single Cell Cloning of Mammalian Cells with Calcein-AM and the Cellavista System



Application Note
AN-B122-XIV-05

Introduction

Single cell cloning (SCC) represents a critical step in cell line development for the production of biopharmaceuticals. The aim of SCC is to identify and isolate the most productive monoclonal cell populations after transfection or hybridization. Usually limited dilution or fluorescence activated cell sorting (FACS) is employed for seeding single cells into microplates. The number of cells per well and the subsequent growth of the colonies is determined by microscopic analysis.

Reliable identification of single cells with brightfield imaging can be difficult and time consuming, even with high quality microscopes, and requires a very clean and particle free environment. Dirt, scratches and bubbles in microplates can lead to ambiguous results so that some valuable, high producing colonies need to be discarded.

Fluorescence detection would, as in FACS sorting, greatly simplify the identification of single cells. However co-transfection with e.g. GFP is undesirable in many cases as it may decrease productivity and furthermore labeled antibodies are critical because of their animal origin.

Here we present a study that uses the non-toxic, synthetic dye Calcein-AM to stain suspension cells for subsequent identification and visualization of single cells with the Cellavista System. Colony growth was followed and quantified over 13 days. Based on our experience, Calcein is a superior alternative regarding cell survival and growth for a number of cell lines compared to the widely used CellTracker™ dyes.

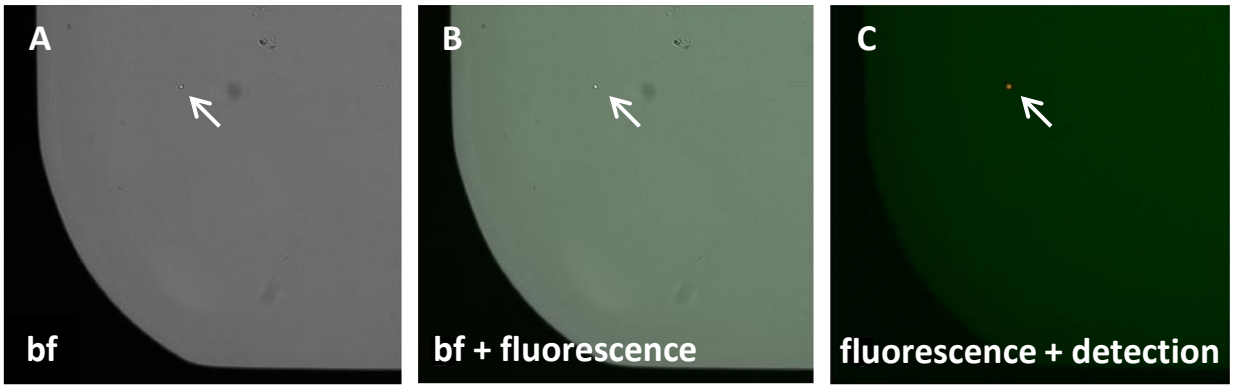


Fig. 1 A-C: Automated detection of Calcein stained cells with the Cellavista System

A, B: Comparison of brightfield (bf, A) and fluorescence + bf (overlay, B) images using Calcein-AM staining (0.1 µM). In the brightfield image the single Molt-4 cells cannot be detected reliably. **C:** The small red circle (arrow) in the fluorescence image indicates that the single cell was detected by the Cellavista software.

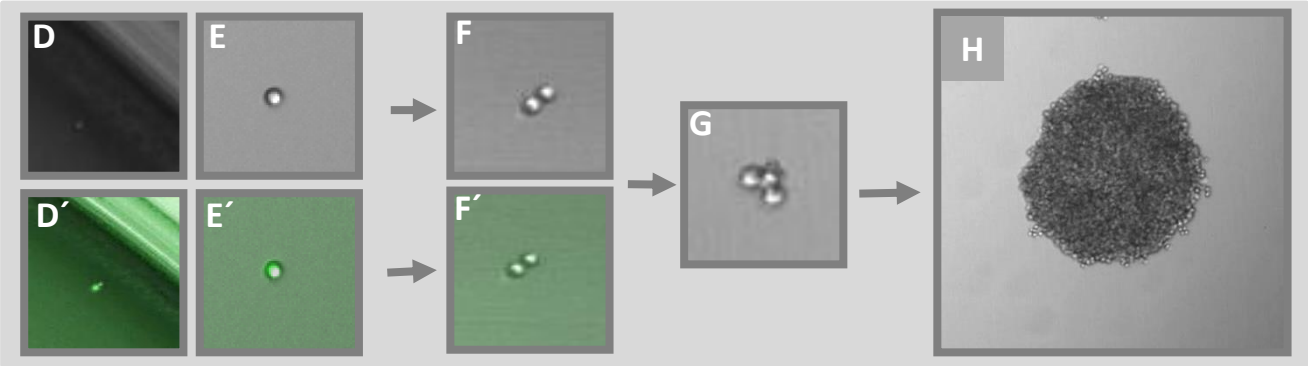


Fig. 1 D-H: Automated detection of Calcein stained cells with the Cellavista System
D, D´: Detection of cells located at the edge of the well is improved by fluorescence staining of cells. **E´, F´:** Calcein staining disappears after the first cell division (also see fig. 3). **E-H:** Proliferation of single cells can easily be visualized in the brigthfield channel with the Cellavista System.

Transient staining with Calcein

Calcein-AM has been commonly known and used as a live cell dye for more than 30 years. The non-fluorescent, cell permeable acetoxymethyl ester (AM) derivate of Calcein is cleaved by nonspecific intracellular esterases and produces the green fluorescent Calcein. In vital cells with intact plasma membranes Calcein is retained in the cytoplasm over several hours depending on cell types and staining concentration (Fig. 3). Damaged or dead cells can neither hydrolyse nor retain the dye. For suspension cells like Molt-4 staining concentrations between 0.1 and 0.5 µM were sufficient to reliably detect cells via the Cellavista System.
Fig. 1 (A-E´) demonstrates the improvement that can be obtained by using fluorescence imaging. The reason for this is that the green fluorescent image basically consists of dark background with one fluorescent object. The measurement was done using a 4x objective in a 384-well microplate (1 image per well) with an individual automatic autofocus for each image for best image quality.

Effect of Calcein on colony growth

Molt-4 (human T-cell leukemia) cells were stained with Calcein-AM for 30 min on a slowly rotating device to allow uniform staining. Afterwards cells were washed, transferred into normal growth medium and then diluted to obtain 0.8 cells per well into a 384-well microplate.

To analyze staining effects on colony growth we measured colony sizes over 13 days starting from day one (Fig. 2). We evaluated 19 different colonies derived from a stained single cell and 11 control cell colonies. These colonies were chosen as they showed the highest cell area [mm^2] at day 13. Colony sizes were determined using the respective operator in the Cellavista image analysis software. The cell growth of Calcein stained clones and its variation was similar to untreated control clones. For all of them a mean value was calculated.

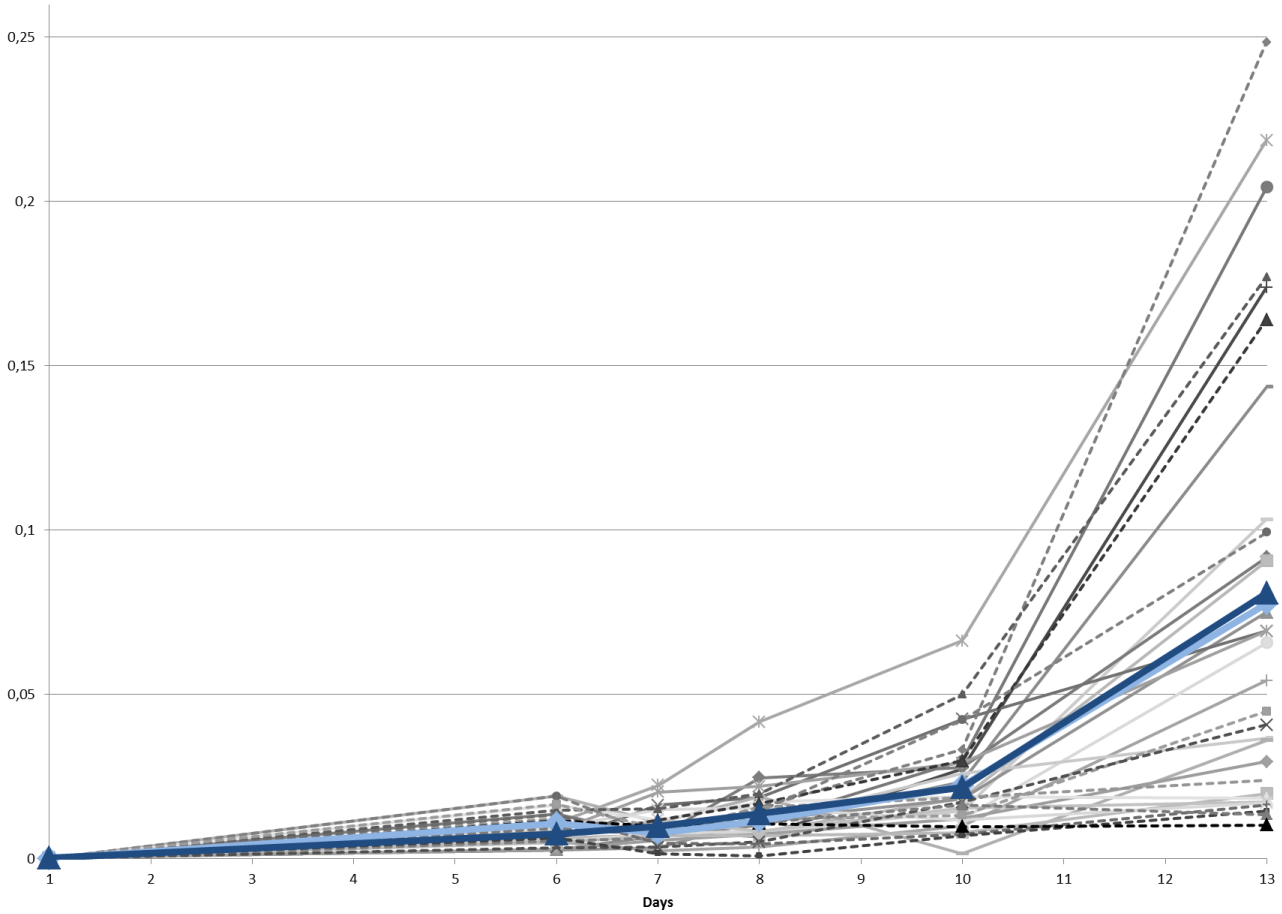


Fig. 2: Growth rates of single cell Molt-4 clones
Cells stained were measured over 13 days in a 384-well microplate. 19 curves of single cells from Calcein-AM stained cells (0.25 µM, solid lines) and 11 control, unstained cells (dashed lines) were plotted. Mean values of stained cells (dark blue) and control (light blue) cells are shown. Standard deviation increases with colony growth.

Conclusions

We could demonstrate that Calcein stained cells can be easily and reliably detected with the Cellavista System. Therefore, the combination of both fluorescence staining and automated detection of single cells is highly beneficial to gain unambiguous results when monoclonality needs to be proved and documented for regulatory agencies. Growth rates of surviving Calcein stained cells were comparable to untreated control cells (Fig. 2). In general, staining times between 10 and 30 minutes, and Calcein-AM concentrations in the range of 0.1 µM to 0.5 µM should be employed. However, the staining protocol should be optimized according to the cell line in use to make sure that every cell can be detected.

Fluorescence intensity of individual cells mainly depends on dye concentration, staining time and cell proliferation state. As Calcein is a non-toxic, transient dye, we recommend to image cells shortly after staining (30 min to 3 hours) to guarantee automatic and accurate cell detection.

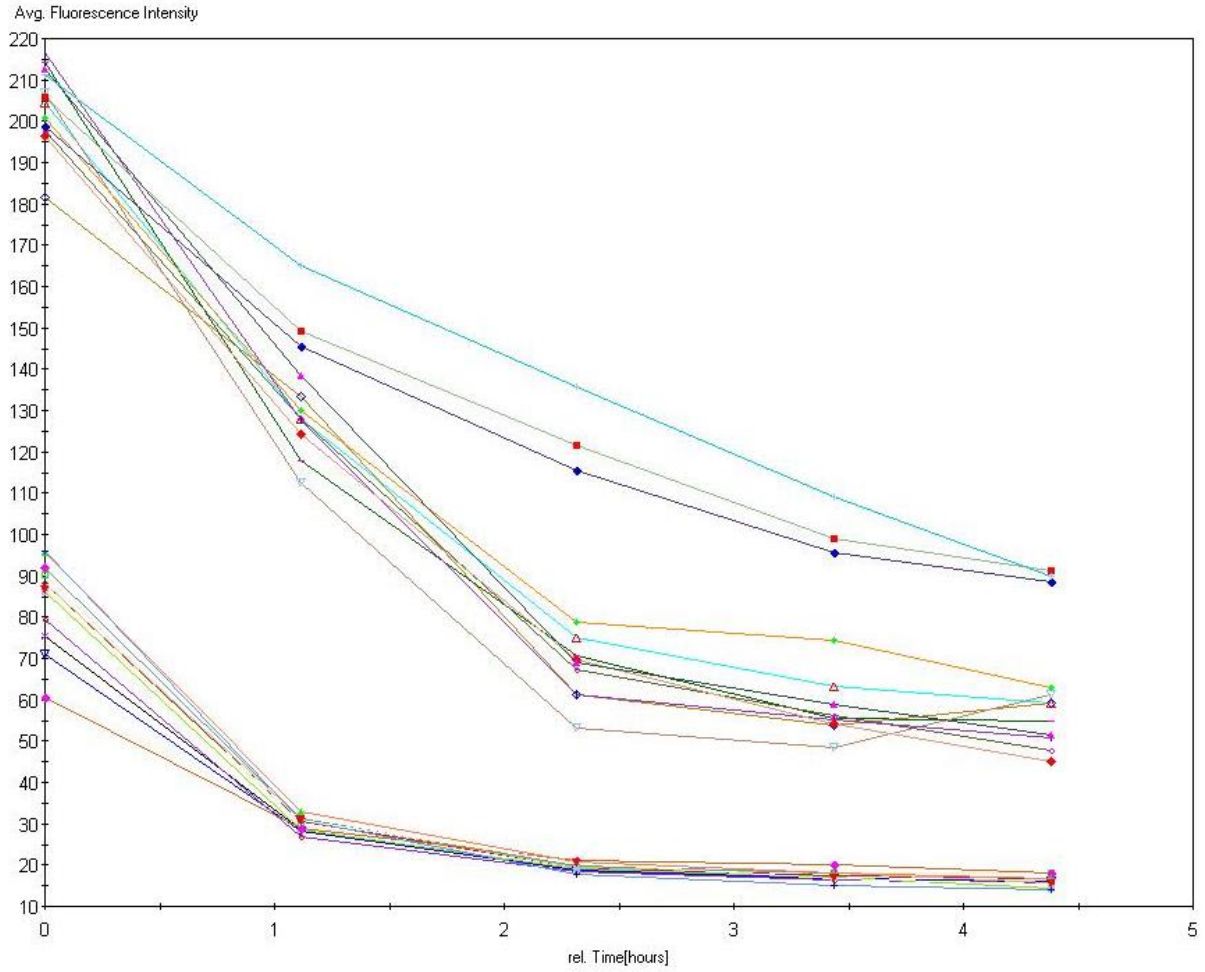


Fig.3: Decline of mean Calcein fluorescence signal in individual wells.
Time chart of average fluorescence intensities of cells per well generated by the Cellavista software. Representative cells were stained for 30 min with 0.1 µM and 0.5 µM Calcein-AM, respectively, and seeded into wells at about 30 cells/well. Cells were detected in brightfield via the suspension cell cytometry 1F operator. Fluorescence intensities of all cells within individiual wells was averaged and plotted by the Cellavista software. Measurements were performed 50, 117, 180, 256 and 313 minutes after staining. At 0.5 µM Calcein-AM staining concentration the signal variation among cells is higher than at 0.1 µM Calcein-AM.

Materials

Reagents

- Log phase Molt-4 cells
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+} (PBS^{--}))
- RPMI medium with 10 % FBS and 1 % Penicillin/Streptomycin
- RPMI medium with 1 % Penicillin/Streptomycin
- Calcein-AM, 2 mM in DMSO
- Diluted Calcein-AM solution 1:10 in PBS^{--} to obtain a small volume of 200 µM stock solution (0.5 µL dye + 4.5 µL PBS^{--})

Equipment

- microplate with transparent bottom (e.g. 384-well, black, flat bottom # 3712, Corning; black plates are optimal for fluorescence imaging)
- Centrifuge tubes 15 mL
- Swing out plate centrifuge
- Pipettes and tips
- Hemocytometer
- Automated microplate imaging microscope (e.g. Cellavista)

Procedure

1. Harvest cells by centrifugation (5 min at 600 x g in centrifuge tube) and aspirate supernatant

   [for adherent cells: harvest cells via trypsinisation and centrifugation]
2. Resuspend the pellet in serum free medium to reach a cell concentration of at least 1x 10^6 cells/mL
3. Pipet the appropriate volume of cells into a 2 mL reaction tube to obtain ca. 1x 10^6 cells
4. Fill this tube with serum free medium to obtain a final volume of 900 µL
5. Prepare 100 µL of Calcein-AM staining solution in cold medium without serum: e.g. for a final concentration of 0.25 µM dilute 1.25 µL of 200 µM Calcein-AM solution in 98.75 µL PBS^{--}, mix well
6. Add 100 µL of the Calcein-AM staining solution to the 900 µL of cell solution and mix thoroughly but carefully
7. Protect stained cells from light during the following steps. Incubate cells 30 min at 37° C in the dark (rather longer staining with less staining concentration).
8. Invert centrifugation tube 3-4 times during incubation or incubate on a slowly rotating device (30 rpm)
9. Wash cells and mix 10 mL of warm serum free medium and the 1 mL of cell/staining solution in a 15 mL sterile centrifugation tube and centrifuge cells 5 min at 600 x g
10. After cell centrifugation aspirate supernatant
11. Resuspend cells in 1 mL normal growth medium
12. Count cells via hemocytometer
13. Calculate how to dilute suspension to obtain 1 cell per well in e.g. 70 µL medium (depending on the well format you are using)
14. Dilute cell suspension in normal growth medium and seed cells into plate
15. Allow the cells to settle down for half an hour at RT and avoid agitation, then spin cells down in a plate centrifuge at 30 x g for 1 min
16. Incubate cells at 37° C
17. Image cells shortly after staining (30 min to 3 h, depending on staining concentration); subsequent measurements should be done on day 2/3/7/12...;

Always avoid agitation of plate otherwise cells might drift

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