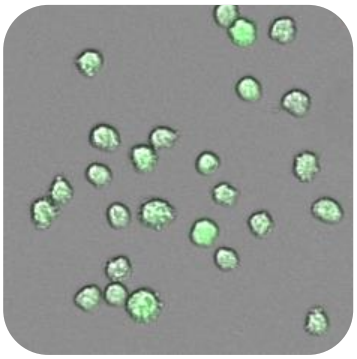
Detection of Single Cells with CellTracker™ Green and the Cellavista

Application Note
AN-B126-XIV-04

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 used 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. Thus, SynenTec has developed an improved single cell cloning method using the non-toxic, synthetic dye CellTracker™ Green CMFDA (Life Technologies).

Procedure

A staining protocol for CellTracker Green was optimized with the aim to improve the reliability of detecting single cells in 96- and 384-well plates. A final concentration of 1 µM of CellTracker Green was identified as suitable to stain Molt-4 suspension cells and obtain a reasonably high fluorescence signal for recognizing single cells with the automated image analysis of the Cellavista.

Fig. 1 demonstrates the improvement that can be obtained by comparing brightfield and combined brightfield/fluorescence imaging. The reason for this is that the green fluorescent image basically consists of a dark background with one fluorescent object.

The measurement was done with both brightfield and fluorescence using a 4x objective (4 images per well) and included highest quality images with individual autofocus for each image.

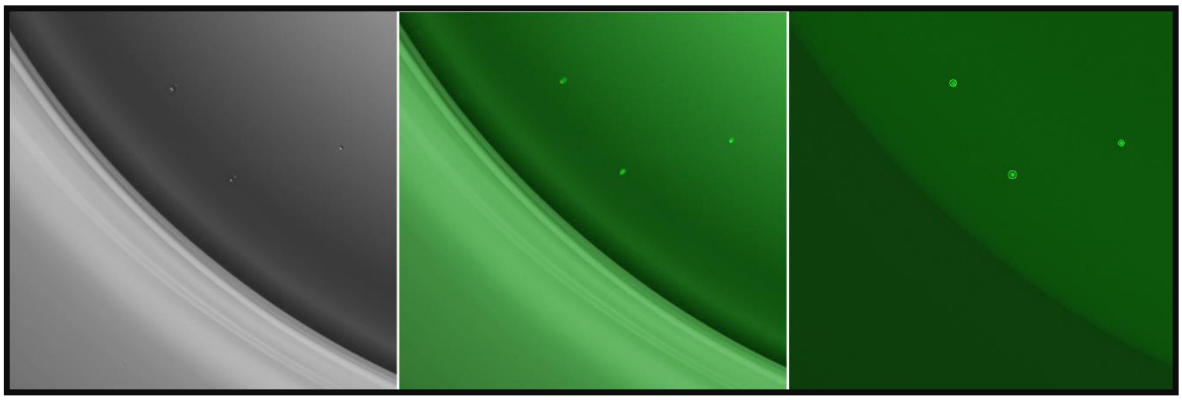


Fig.1 Comparison of brightfield (bf, left) and fluorescence + bf (overlay, middle) images using CellTracker Green staining (at 1 µM). In the brightfield image the single Molt-4 cells cannot be detected reliably. The small white circles in the fluorescence image (right) indicate the detection of single cells with the Cellavista software.

Effect of CellTracker™ Green on Single Cell Survivability and Colony Growth

Molt-4 cells were stained with CellTracker Green for 30 min on a slowly rotating device to allow uniform staining, washed and then pipetted in an appropriate dilution to obtain one cell per well.

In living cells CellTracker Green passes freely through cell membranes, but once inside the cell, it is fluorescent activated by intracellular esterases and transformed into a cell impermeant reaction product[1]. Low staining concentrations usually disappear after 1-2 days.

In order to check survival of individual, stained cells we spotted all cells at day 1 and determined proliferation. Consistent with control cells stained cells (n = 22) showed a survivability of over 90 %. Yet, is has to be noted that survivability and growth rate can vary with different cell lines, staining concentrations and cell handling procedures.

To analyze staining effects on colony growth we measured colony size over 9 days starting from day one (Fig. 2). We evaluated 22 different colonies derived from a stained single cell and 6 control cell colonies. For all of them a mean value with standard deviation was calculated.

Still the results clearly demonstrate that the use of CellTracker Green does not negatively impact the proliferation of Molt-4 suspension cells. The colony sizes were determined using the respective operator in the Cellavista image analysis software and are reported in mm^2. Variation in cell growth of CellTracker Green stained clones was similar to untreated control clones.

Conclusions

We have developed a method to improve single cell recognition with the Cellavista imager using the dye CellTracker Green and tested it with regard to potential effects of the dye on cell growth and survivability in Molt-4 cells. As a result we could prove that 1 µM staining and lower concentrations of CellTracker Green had no influence on cell growth.

Thus, the combination of CellTracker with the Cellavista System provides a method for reliable single cell detection from the first day of cultivation using fluorescence. The method is free of labels from animal origin and leads to unambiguous results when monoclonality needs to be proved and documented for regulatory agencies.

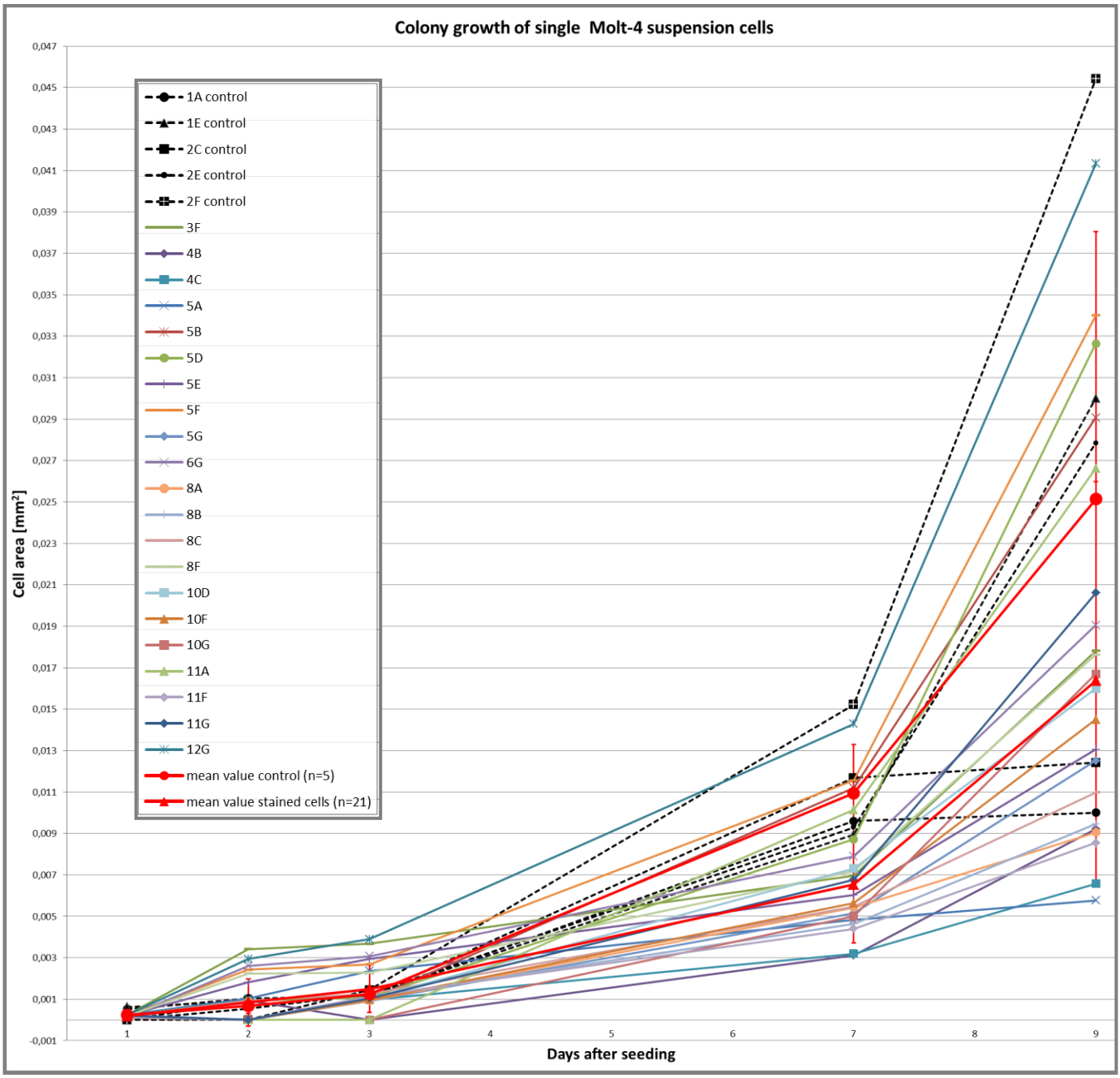


Fig.2 Colony growth of 21 stained, individual single Molt-4 cells labeled with CellTracker™ Green and 5 unstained control cells (dashed lines). Standard deviation is given for mean values of stained (n=21) and control (n=5) cells (bright red lines). Some cell lines show only minor growth rates (e.g. 4C, 11F) while for other colonies growth is arrested from day 7 onwards (5A, 1A, 2C). Due to the small sample size of control cells, the mean growth values of control cells are higher than of stained cells.

Materials

Reagents

- Log phase Molt-4 cells
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+})
- RPMI medium with 5 % FBS and 1 % Penicillin/Streptomycin
- RPMI medium with 1 % Penicillin/ Streptomycin
- CellTracker™ Green CMFDA (Invitrogen), 10 mM in DMSO
- Diluted Cell Tracker Green solution 1:10 in PBS^{--} to obtain a small volume of 1 mM stock solution (0.5 µL dye + 4.5 µL PBS^{--})

Equipment

- micro plate with transparent bottom (e.g. 96er Nunclon™; Thermo Scientific), especially black plates are beneficial
- Centrifuge tubes 15 mL
- Centrifuge
- Micropipettes, Multipette and tips
- Hemocytometer
- Automated micro plate reading microscope (e.g. Cellavista)

Procedure

1. Harvest cells by centrifugation (5 min at 600 x g in centrifuge tube)
2. Aspirate supernatant
3. Resuspend the pellet in serum free medium to reach a cell concentration of at least 1 x 10^6 cells/mL
4. Pipet the appropriate volume of cells to obtain ca. 1x 10^6 cells/mL and transfer to a 2 ml reaction tube and fill up to 900 µL with serum free medium.
5. Prepare 100 µL of staining solution of CellTrackerGreen in cold medium without serum: e.g. for a final concentration of 1 µM dilute 1 µL of 1 mM CTGreen solution in 99 µL PBS, mix well. Add 100 µL of the CellTracker staining solution to the cell solution and mix thoroughly but carefully.
6. Protect stained cells from light during the following steps. Incubate cells 30-45 min at 37 °C in the dark (rather longer staining with less staining concentration). Invert centrifugation tube 3-4 times during incubation or incubate on a slowly rotating device (30 rpm).
7. Wash cells 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.
8. After cell centrifugation aspirate supernatant.
9. Resuspend cells in 1 mL normal growth medium
10. Count cells via hemocytometer
11. Calculate how to dilute suspension to obtain 1 cell per well in e.g. 200 µL medium, depending on the well format you are using.
12. Dilute cell suspension
13. Seed cells into plate
14. Allow the cells to settle down for half an hour and avoid agitation, then spin cells down in a plate centrifuge at 30 x g for 1 min.
15. Incubate cells at 37 °C for 1-2 h and start first measurement
16. Subsequent measurements should be done on day 2/3/7/12..; always avoid agitation of plate otherwise cells might drift.

Troubelshooting

issues	possible reason	help
• High fluorescent background (BG) • Low BG/Signal ratio	Cells are in a high metabolic state and affect transfer of exogenous substances to the cellular outside	Incubate CellTracker at room temperature instead of 37° C to decrease the metabolism of the cells (in the dark)

References

[1] Molecular Probes, CellTracker™ Probes for Long-Term Tracing of Living Cells, 1, (2006) http://tools.invitrogen.com/content/sfs/manuals/mp02925.pdf

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