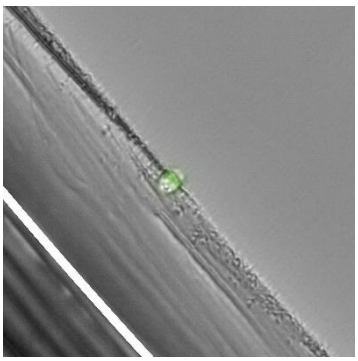
Detection of Single Cells with CellTracker™
Stainings and their Influence on Proliferation and Protein Expression

Application Note
AN-B125-XX-03

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 Fluorescence Activated Single Cell Cloning (FASCC) method using e.g. the non-toxic, synthetic dye CellTracker™ (Invitrogen™).

To ensure that the CellTracker™ has no negative effect on the single cell cloning process, SYNENTEC has tested the influence of the staining on cell proliferation and protein expression. To investigate the effect on proliferation, Molt-4 cells were stained with CellTracker™ Green CMFDA and the colony growth was observed. In order to check the effect on the production of proteins GFP was used as a model protein in stably transfected, monoclonal CHO-K1 cells which were stained with the CellTracker™ Red CMTPX dye. For both approaches, the CELLAVISTA® was used to measure and analyze.

KEYWORDS: SINGLE CELL CLONING, SCC, CELLTRACKER™, FLUORESCENCE ACTIVATED SINGLE CELL CLONING, FASCC, PROTEIN EXPRESSION, CELL LINE DEVELOPMENT, LIMITED DILUTION

Improved Detection of Single Cells Using the FASCC Operator

FDA requires more and more the image based proof of the clonality of cell lines. SYNENTEC's FASCC operator identifies potential clones from seeding day using a non-toxic fluorescent dye, in this case CellTracker™. FASCC consists of fast pre-scanning, high resolution imaging and exclusion of irrelevant wells to resolve potential clones for an unambiguous documentation without compromising throughput.

A FASCC experiment includes a three-step measurement on day 0. In the first step the fluorescent cells are measured in the pre-scan with a fast 4x lens scanning which determines the exact location of the potential clones inside the wells. The YT-software® automatically selects the wells with a potential single cell.

The second step, the Nanoview, spots the identified cells in one image with the detected cell in the center and reviews it in a higher resolution (e.g. 10x or 20x) in fluorescence and

brightfield. The image analysis is able to separate doublets (fig. 1) and exclude them for the following step.
In the last step, the third level, a full well confluence measurement in brightfield can be carried out and used as documentation for regulatory approvals (e.g. FDA) that there is no other e.g. non- or weak-fluorescent cell in the well and also as a starting point to monitor the colony growth and proliferation over time. This level will be continued on the following measurement days to generate growth curves for the clones.
For detailed practice information look at SYNENTEC's Technical Note for FASCC (TN-B123-XIV-11).

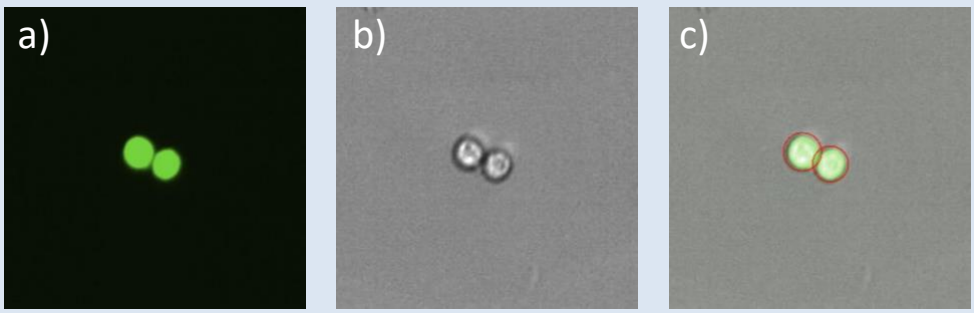


Fig. 1: Separation of doublets in the FASCC Nanoview with the YT-software®
a) CellTracker™ Green stained Molt-4 cells in the fluorescence channel. b) The same cells in the brightfield channel. c) Overlay image of fluorescence and brightfield with image analysis as red circles.

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

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 and an incubation time of 30 minutes were 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 YT-software®.

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 = 21) showed a survivability of over 90 %. Yet, it 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 21 different colonies derived from a stained single cell and 5 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 confluence operator of the YT-software® and are reported in mm^2. Variation in cell growth of CellTracker™ Green stained clones was similar to untreated control clones.

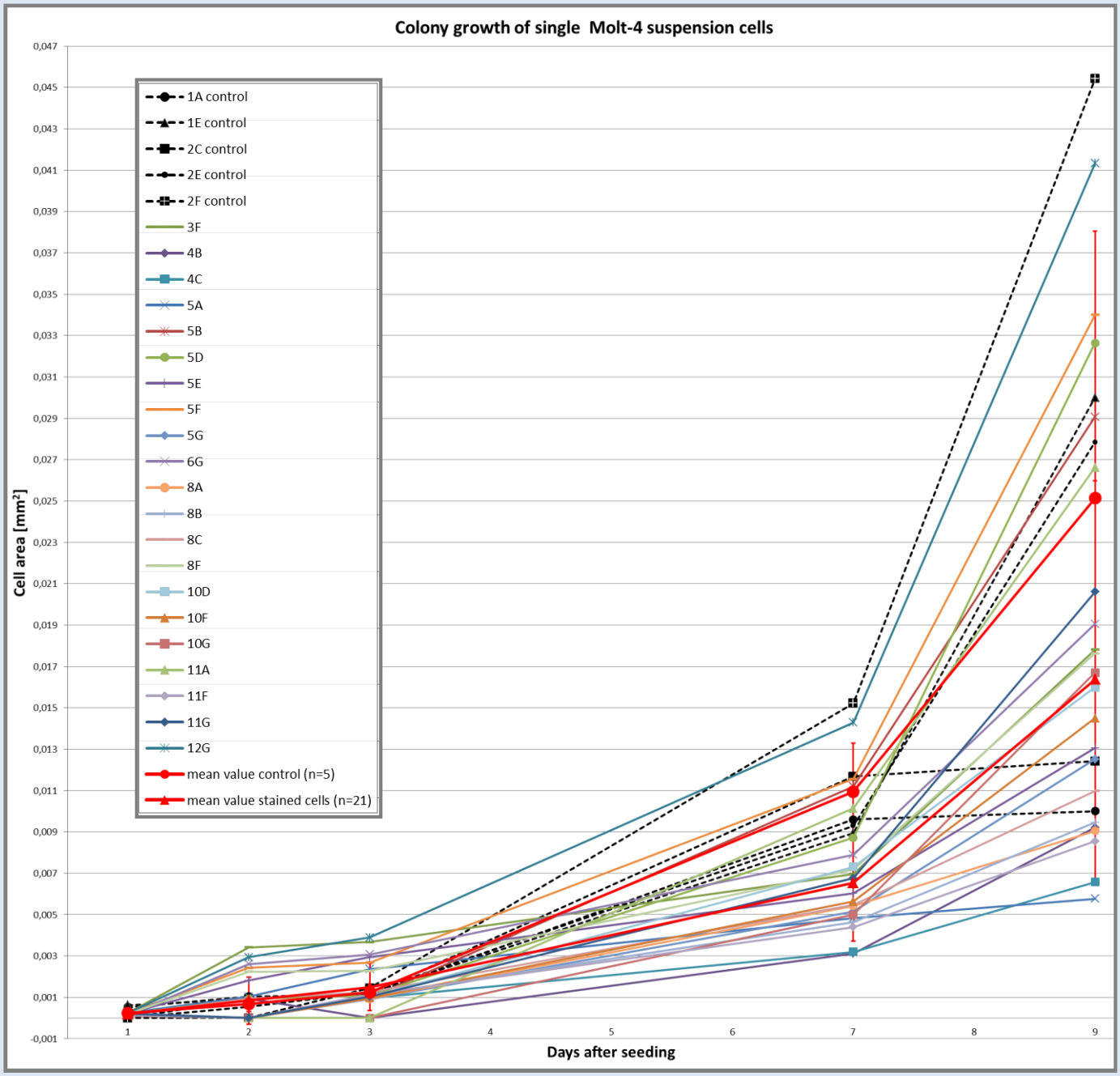


Fig. 2: Colony growth of stained Molt-4 cells over time

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 colonies 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 the values of stained cells.

Effect of CellTracker™ Red Staining on Protein Expression

For CellTracker™ Red a staining protocol was also optimized to improve the reliability of detecting single cells in 96- and 384-well plates. Here, a final concentration of 2.5 µM was identified as suitable to stain CHO-K1 cells and obtain a reasonably high and stable fluorescence signal for recognizing single cells by the YT-software®.

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

In order to check the effect of CellTracker™ Red staining on the production of proteins we used GFP as a model protein in stably transfected, monoclonal CHO-K1 cells with high GFP expression level and fast growth rate. These cells were preselected by an initial single cell cloning experiment in which a single cell was isolated and propagated. Subsequently, cells were stained with three different concentrations of CellTracker™ Red dye for 30 min and again seeded with limited dilution to obtain one cell per well.

In living cells the CellTracker™ Red passes freely through cell membranes, but once inside the cell, it will be transformed into a cell impermeant reaction product and passed down to the next generation [1].

To analyze long-term effects we measured colony size and fluorescence intensity over 9

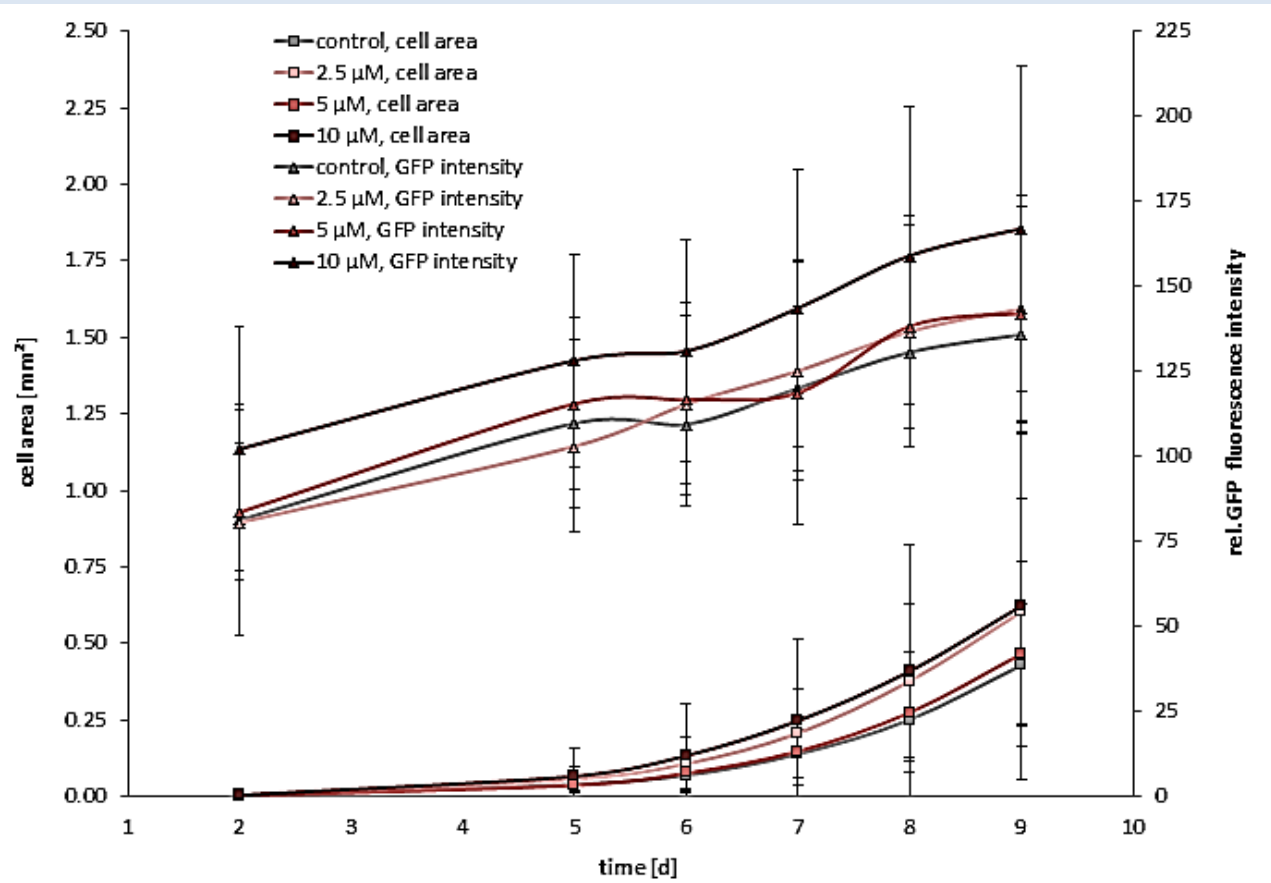


Fig. 3: Proliferation and fluorescence intensity of GFP transfected CHO-K1 cells over time
The lower four curves display the increase of the cell area in mm² (left axis) in 9 days. Each curve shows the average of one concentration. The upper curves represent the average GFP intensity per concentration. All points are shown with standard deviation.

days starting from day three. We tested four conditions (0/2.5/5/10 µM) and evaluated at least 13 colonies derived from a single cell each.

The overall fluorescence intensity of the GFP was taken as a measure for productivity. It has to be noted, however, that at later stages the measured GFP does not correctly reflect the amount of GFP produced as images are saturated and cells start to grow on top of each other.

Still the results (fig. 3) clearly demonstrate that the use of CellTracker™ Red does not negatively impact the production of GFP in CHO-K1 cells, even at CellTracker™ Red concentrations well exceeding those of the standard protocol (2.5 µM). The colony sizes were determined using the respective operator of the YT-software® and are reported in mm^2. The cell growth of the CellTracker™ Red stained clones was not different from the untreated control clones.

Conclusions

We have developed a method to improve single cell recognition with the CELLAVISTA® imager using CellTracker™ dyes and tested it with regard to potential effects of the dye on cell growth, survivability and heterologous protein production. As a result we could prove that CellTracker™ Red had neither an influence on the growth of CHO-K1 cells nor on the production of GFP and that 1 µM staining and lower concentrations of CellTracker™ Green had no influence on the cell growth of Molt-4 cells.

Thus, the combination of CellTracker™ with the FASCC Operator of our YT-software® 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.

Materials

Reagents

Proliferation Test

- 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™) 1 mM pre-dilution (0.5 µL 10 mM CT + 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
- One of SYNENTEC's imaging systems (here: CELLAVISTA® 3.1)

Reagents

Protein Expression Test

- Confluent T-flask 25 cm^2 with stable transfected CHO-K1
- Phosphate Buffered Saline (w/o Ca^{2+} & w/o Mg^{2+})
- DMEM/Ham's F12 1:1 with 5 % FBS and 1 % Penicillin/Streptomycin
- DMEM/Ham's F12 1:1 with 1 % Penicillin/Streptomycin
- Trypsin 0.25 %
- CellTracker™ Red CMTPX (Invitrogen™)

Equipment

- 96-well micro plate with transparent bottom (e.g. Nunclon™; Thermo Scientific)
- Centrifuge tubes 15 mL
- Centrifuge
- Micropipettes, Multipette and tips
- One of SYNENTEC's imaging systems (here: CELLAVISTA® 3.1)

Procedure

Proliferation Test

1. Harvest cells by centrifugation (5 min at 600 x g in centrifuge tube).
2. Aspirate supernatant.
3. Resuspend cells in serum free medium.
4. Pipet the volume of cells to obtain ca. 1x 10^6 cells/mL into a centrifuge tube and fill up to 900 µL with serum free medium.
5. Prepare 100 µL of staining solution of CellTracker™ Green in cold medium without serum (for a final concentration of 1 µM:1 µL 1 mM CT Green solution + 99 µL PBS).
6. Add the 100 µL CellTracker™ staining solution to the cells and mix thoroughly but carefully.

Protect stained cells from light during the following steps.

7. 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).
8. Mix 10 mL of warm serum free medium and the 1 mL of cell/staining solution and centrifuge cells 3 min at 300 x g.
9. Aspirate supernatant.
10. Resuspend cells in 1 mL normal growth medium.
11. Count cells via Suspension Cell Count Operator of the YT-software®.
12. 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.
13. Dilute cell suspension.
14. Seed cells into plate.
15. Spin cells down in a plate centrifuge at 30 x g for 1 min.
16. Incubate cells at 37 °C for 1-2 h and start first measurement.
17. Subsequent measurements should be done on day 2/3/7/12..

Protein Expression Test

1. Aspirate culture medium.
2. Wash confluent T-flask with PBS^{--}.
3. Aspirate PBS.
4. Detach cells with Trypsin.
5. Pipet cell suspension into a centrifuge tube.
6. Centrifuge 5 min at 600 x g.
7. While centrifuging prepare the dye solution: Add 1.13 µL of 10 mM CellTracker™ Red stock solution to 4.5 mL cold serum free medium (final concentration of 2.5 µM) and warm up to 37 °C.
8. After cell centrifugation aspirate supernatant.
9. Resuspend the pellet in 4 mL dye solution.
10. Incubate 10 min at 37 °C.
11. Centrifuge 5 min at 600 x g.
12. Aspirate supernatant.
13. Resuspend cells in 5 mL normal growth medium.
14. Count cells via Suspension Cell Count Operator of the YT-software®.
15. Calculate how to dilute suspension to obtain 1 cell per well in 200 µL medium.
16. Dilute cell suspension.
17. Seed cells into a 96-well plate (200 µL/well).
18. Allow the cell to settle down for half an hour and avoid agitation or spin cells down in a plate centrifuge at 30 x g for 1 min.
19. Incubate cells at 37 °C for 3-4 h and start first measurement.

Troubleshooting

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)

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