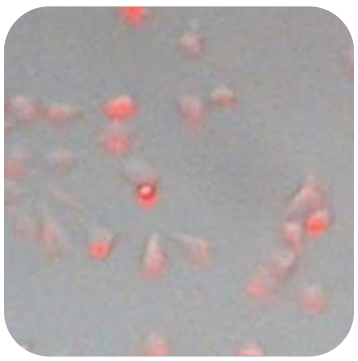
Reliable Detection of Single Cells (CHO) with CellTracker™ and the Cellavista System



Application Note
AN-B125-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™ Red (Life Technologies).

Improved Detection of Single Cells with CellTracker™

A staining protocol for CellTracker™ Red was optimized with the aim to improve the reliability of detecting single cells in 96- and 384-well plates. A final concentration of 2.5 µM of CellTracker™ Red was identified as suitable to stain CHO-K1 cells and obtain a reasonably high and stable 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 red fluorescent image basically consists of a dark background with one fluorescent object.

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.

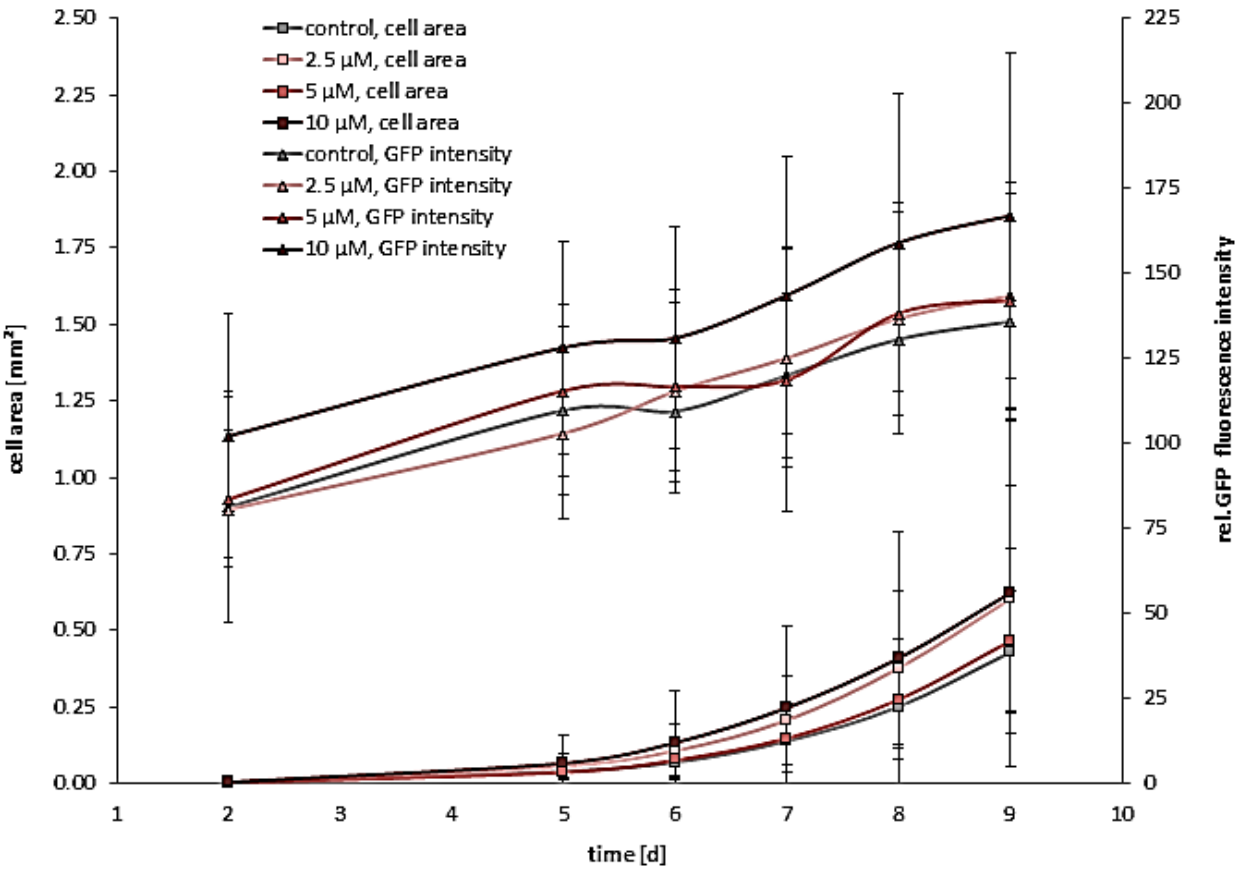
Fig.1 Comparison of brightfield (left) and fluorescence (middle) images using CellTracker Red staining (at 2.5 µM). In the brightfield image the single cells cannot be detected. On the fluorescence image the small green circles indicate the detection of the single cells with the Cellavista software. The right image shows an overlay of both channels.

Effect of CellTracker™ Red Staining on Protein Expression

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 in an appropriate dilution to obtain one cell per well.
In living cells the CellTracker™ 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 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 for each of them.
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 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 in the Cellavista image analysis 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 the dye CellTracker™ Red and tested it with regard to potential effects of the dye on cell growth and on heterologous protein production in CHO-K1 cells. 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.

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.

Materials

Reagents

- Confluent T-flask 25 cm² with 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
- Hemocytometer
- Automated micro plate reading microscope (e.g. Cellavista)

Procedure

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™ 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 hemocytometer
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
19. Incubate cells at 37 °C for 3-4 h and start first measurement

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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