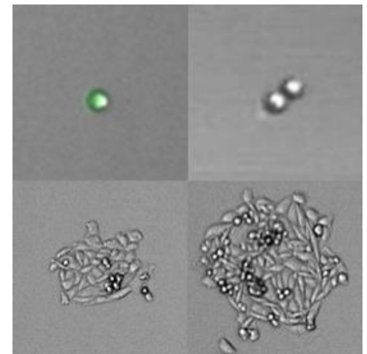
SYNENTEC

Testing the Toxicity of Calcein-AM under Fluorescence Activated Single Cell Cloning (FASCC) Conditions

Application Note
AN-B128-XX-08

Introduction



For an easy identification of an individual (single) cell at the date of seeding, we use Calcein-AM for the Fluorescence Activated Single Cell Cloning (FASCC) application. Calcein-AM is a cell permeable dye which does not fluoresce outside the cell. Only if Calcein-AM enters a cell, the AM (Acetoxymethyl) gets separated by esterases and the dye begins to fluoresce (excitation maximum 495 nm, emission maximum 515 nm) (fig. 1). Due to this enzymatic reaction only the cytoplasm of vital cells is stained.

Several toxicity studies have shown that Calcein-AM is a non-toxic dye e.g. for lymphocytes using 5 µM Calcein-AM for 15 min at 37 °C[1] or for DLD-1 cells using 50 nM for 2 h[2]. Nevertheless we carried out this toxicity study to exclude an influence of our used Calcein concentration of 0.1 µM on the cell viability and growth. Therefore we stained CHO-K1 cells with Calcein concentrations of 0.05 up to 5 µM followed by an incubation period of 30 min and subsequently watched and evaluated the growth of cells over 8 days.

For our study we decided to use a calculation of 10 cells per well to ensure that this test is as close as possible to a 'Single Cell Cloning' experiment regarding the number of cells but achieves a higher efficiency than 1 cell per well. Furthermore the toxicity of a substance greatly depends on the number of neighboring cells.

A DMSO control has been measured in parallel to preclude an influence of the DMSO concentration in which the Calcein is dissolved.

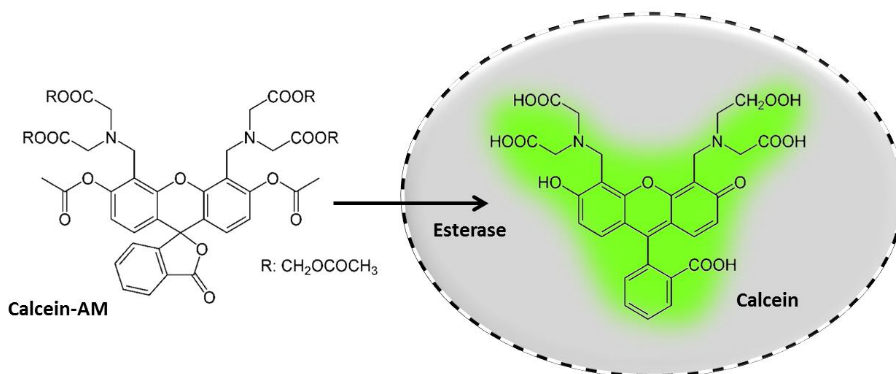


Fig. 1: Simplified scheme of the change from Calcein-AM to the fluorescent Calcein

If Calcein-AM gets into a viable cell with active esterase, this esterase separates the acetoxymethyl from the Calcein. The Calcein starts to fluoresce.

After the evaluation of growth of the individual samples, the statistical significance and the IC50-value was determined to allow a reliable statement at which concentration of Calcein an inhibitory effect occurs.

The LD50-value was not defined for this toxicity study as for the user of Single Cell Cloning applications it is far more interesting and relevant if a substance has an inhibitory effect before a toxic/lethal effect occurs. According to the FDA the EC50-value for a half-maximal effective dose of agonists is used *in vivo*. But as we evaluate an inhibitory effect working *in vitro*, we use the IC50-value[3].

Materials

Cells/Reagents

- E.g. CHO-K1 cells
- Phosphate Buffered Saline (w/o Ca2+ & w/o Mg2+ (PBS--))
- Trypsin 0.25 %
- DMEM/Ham's F12 1:1 medium with 5 % FBS and 1 % Penicillin/Streptomycin
- DMEM/Ham's F12 1:1 medium (serum-free)
- Calcein-AM (2 mM in DMSO)

Equipment

- Black microplate with transparent bottom (e.g. 384 well, low volume, Corning #3542)
- Swing out plate centrifuge
- One of SYNENTEC's automated microplate imaging microscopes (e.g. Cellavista® or NYONE®)

Procedure

1. Aspirate culture medium
2. Wash confluent T-flask with PBS--
3. Aspirate PBS
4. Detach cells with Trypsin
5. Suspend cells in 5 mL serum-free medium
6. Dilute Calcein-AM stock solution 1:10 in serum-free medium (e.g. 90 µL medium + 10 µL Calcein stock solution)
7. Prepare 10 tubes with 0.5 mL cell suspension and 1.5 mL serum-free medium
8. Add different volumes of Calcein predilution (resp. DMSO for controls)

1)	0 µL	Calcein	(0 µM)
2)	0.5 µL	"	(0.05 µM)
3)	1 µL	"	(0.1 µM)
4)	2.5 µL	"	(0.25 µM)
5)	5 µL	"	(0.5 µM)
6)	7.5 µL	"	(0.75 µM)
7)	10 µL	"	(1 µM)
8)	50 µL	"	(5 µM)
9)	1 µL	DMSO	(≙ 1 µM Calcein)
10)	5 µL	"	(≙ 5 µM Calcein)

9. Incubate cells 30 min at 37 °C in the incubator on a slowly rotating device (30 rpm) or invert tube 3-4 times during incubation

Protect stained cells from light during the following steps!

10. Centrifuge cells 5 min at 300 x g
11. Aspirate supernatant
12. Resuspend cells in 1 mL serum-free medium
13. Count cells of one tube e.g. with the suspension cell count operator of the NYONE® System
14. Calculate how to dilute suspension to obtain 10 cells per well in e.g. 40 µL medium (depending on the well format you are using)
15. Dilute cell suspension in normal growth medium
16. Seed cells into the microplate e.g. 40 µL/well for 384-well plates
17. Centrifuge plate 3 min at 200 x g to spin down the cells and to remove air bubbles
18. Image cells immediately after staining (latest 2 - 3 h after staining)
19. Monitor growth of the cells with e.g. daily measurements - here:

    Measurement 1 / Day 0 / after 0 h
    Measurement 2 / Day 1 / after 26 h
    Measurement 3 / Day 3 / after 76.5 h
    Measurement 4 / Day 4 / after 97.5 h
    Measurement 5 / Day 5 / after 124 h
    Measurement 6 / Day 6 / after 145.5 h
    Measurement 7 / Day 8 / after 192 h

Results

Regarding the analysis of toxicity of Calcein-AM we could prove with our conducted experiments that the used standard concentration of 0.1 µM at an incubation period of 30 min did not have any influence on the growth of our CHO-K1 cells in our experiment setup.
From a concentration of 0.25 µM Calcein upward a smaller detected cell density is already visible to the naked eye in the plate overview after 145.5 hrs of cultivation (fig. 2). The covered areas are marked yellow. At a concentration of 5 µM Calcein the yellow cell area is no longer apparent, which shows that no cells are grown.

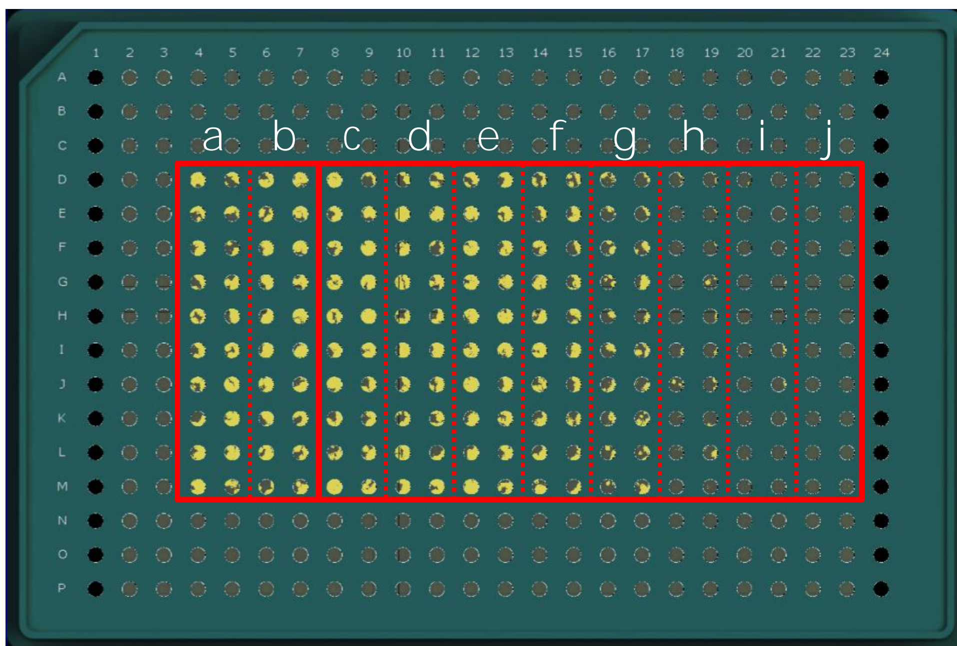


Fig. 2: Plate overview of measurement 6 (after 145.5 h).
The detected cell area is shown in yellow. a) - b) DMSO control. a) DMSO like in the 1 µM Calcein sample b) DMSO like in the 5 µM Calcein sample. c) Unstained/untreated cells. d - j) Adherent CHO-K1 cells stained with Calcein. d) 0.05 µM Calcein-AM. e) 0.1 µM Calcein-AM. f) 0.25 µM Calcein-AM. g) 0.5 µM Calcein-AM. h) 0.75 µM Calcein-AM. i) 1 µM Calcein-AM. j) 5 µM Calcein-AM.

Fig. 3 shows the averaged cell area of each concentration over time. It becomes clear that the curves of the concentration of 0, 0.05 and 0.1 µM hardly differ from one another, the growth of cells of these three concentrations is thus very similar. Our used standard concentration might seem to have no inhibitory effect at first glance. The curve of 0.25 µM lies however much clearly lower and the higher the concentration of Calcein the lower the growth curves proceed. For the highest curve there is no more growth recorded at all. At measurement 7, after 192 hrs growth period, the curve noticeable levels off because the confluence is reached at the first wells. The second diagram (fig. 4) compares the untreated sample with the also measured DMSO controls to preclude that the DMSO, in which Calcein is dissolved, has an effect on the cell growth. The DMSO concentrations as for the 1 µM and 5 µM Calcein sample were used and correspond to an absolute DMSO concentration of 0.05 % and 0.25 %. It is seen that the DMSO curves have a very similar curve progression like the one for untreated cells, whereas the lower DMSO concentration shows a slightly lower growth than the higher concentration.

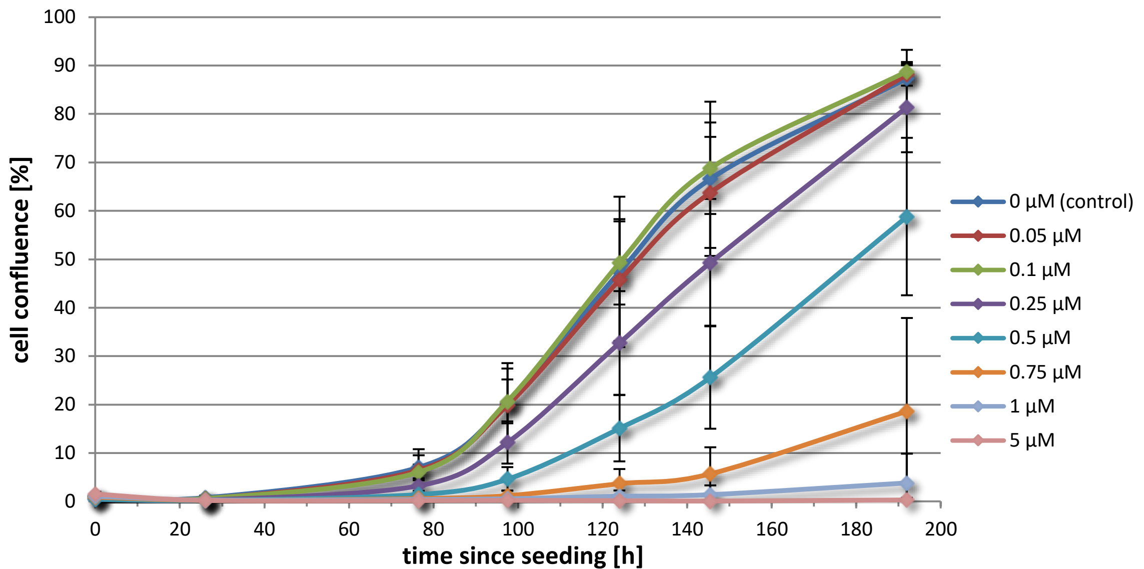


Fig. 3: Growth of CHO-K1 cells over time stained with different Calcein-AM concentrations

Represented is the increase of the cell confluence in 192 hrs. Different Calcein concentrations were used to show the influence of the staining on the cell growth rate as a marker of cell health. The higher the Calcein concentration the lower the growth curve.

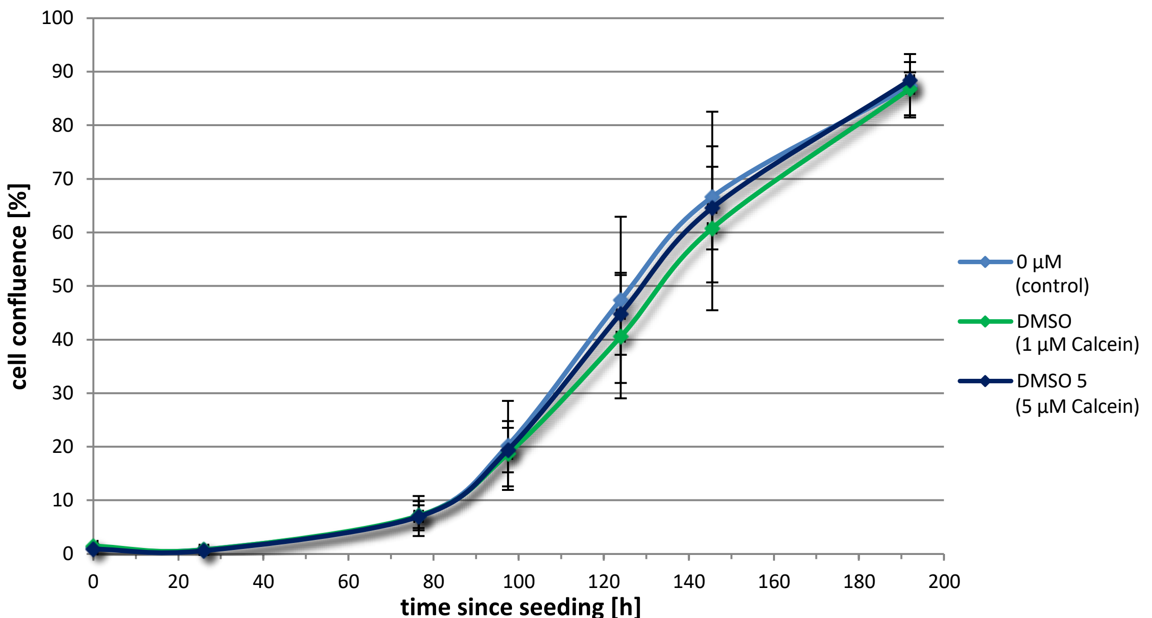


Fig. 4: Growth of CHO-K1 cells over time, treated with different DMSO concentrations.

Represented is the increase of the cell confluence in 192 hrs. Two different DMSO concentrations were tested compared to an untreated sample.

Because of the fairly big error bars both in fig. 3 and fig. 4, yet there is a high variance between the several wells of one sample, we carried out a t-test to figure out which differences are significant and which are not. Respectively if the difference of the 0 sample to the 0.05 and 0.1 µM Calcein sample and the DMSO concentrations is not significant as expected and the difference from 0 to 0.25 µM Calcein and all higher concentrations is highly significant. Therefore measurement 6 was used as this is the last measurement in which all wells are still in the log-phase. The carried out t-test demonstrated that the deviation to the 0 µM sample as from a concentration of 0.25 µM Calcein is highly significant (fig. 5).

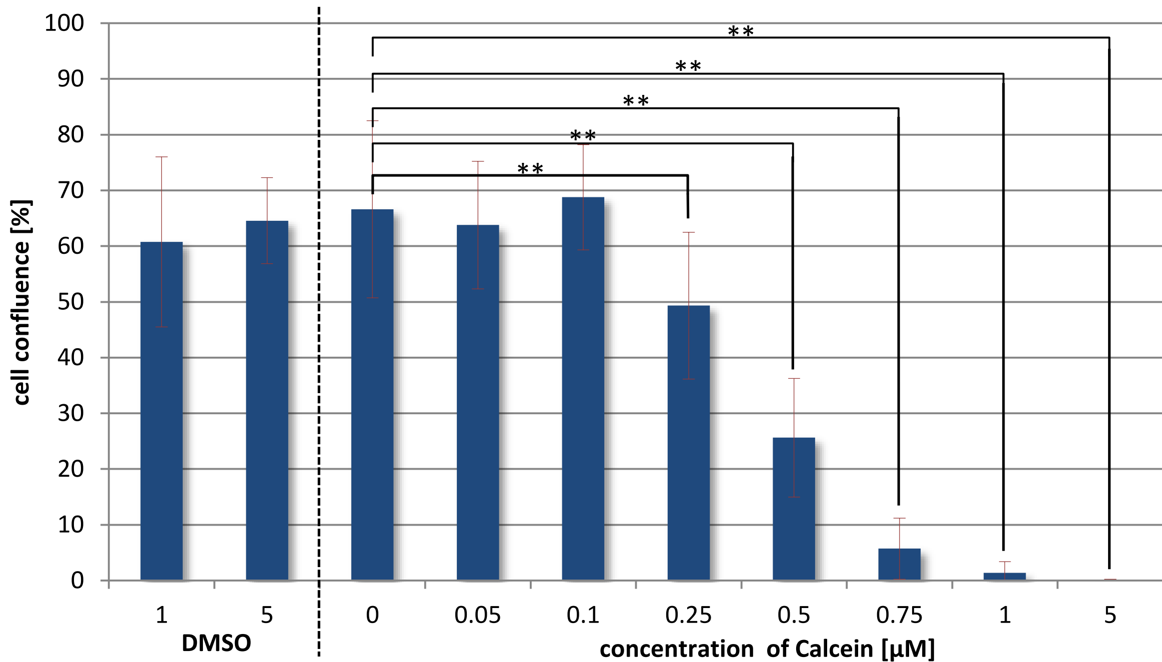


Fig. 5: Averaged cell confluence of all samples in measurement 6 with t-test results
This chart shows the confluence of the DMSO treated cells (left) vs. the Calcein stained cells (right) and the statistical significance of the variation between the sample without any treatment and all other samples. The variation from 0 µM Calcein to DMSO 1 and 5, 0.05 and 0.1 µM Calcein is not significant but the variance from 0 to 0.25 µM Calcein and to all higher concentrations is highly significant (T-test: p = level of significance; $p \leq 0.01$ = highly significant = **).

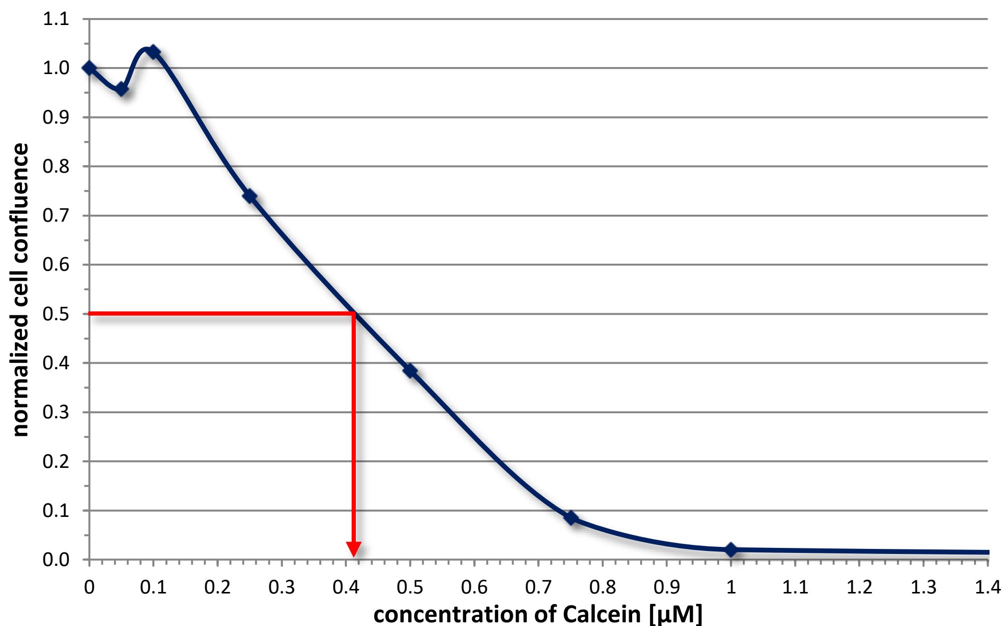


Fig. 6: Normalized cell confluence for all Calcein concentrations to find the IC50 value (measurement 6).
At the normalized cell confluence of 0.5 50% of the confluence of the 0-sample were reached. The red arrow shows the located IC50 value: 0.415 µM Calcein-AM.

Here the p-value is at 0.0006 and is getting continually smaller at higher Calcein concentrations. At 5 µM Calcein e.g. the p-value is at 9.32×10^{-21}. Between 0, 0.05, 0.1 µM Calcein and the two DMSO controls visual differences are apparent but without significance with a p-value between 0.25 and 0.61.

Finally we defined the IC50-value in order to make a statement about the inhibitory effect of Calcein-AM. Therefore we standardized the values of measurement 6 to 0-sample=1 and applied the concentration to the standardized confluence (fig. 6). Afterwards the x-value to the normalized confluence of 0.5 was determined with the help of the axis labeling, hence the Calcein concentration where only 50% confluence were achieved. The IC50-value for Calcein for our experimental setup (CHO-K1 cells, 30 min incubation, 37 °C, 5 % CO_2) is at 0.415 µM Calcein-AM.

Conclusions

1. The used Calcein concentration of 0.1 µM in our experimental setup did not have any influence on the growth of our CHO-K1 cells.
2. The DMSO in which the Calcein is dissolved has no effect on the cells not even when high Calcein concentrations are used.
3. The IC50-value in our experimental setup is at 0.415 µM Calcein-AM.

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References

[1] Luc S. De Clerck, Chris H. Bridts, Annemie M. Mertens, Marleen M. Moens, Wim J. Stevens. Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. *J Imm Met* 172 (1994) 115-124.

[2] Joshua E. Allen1,2, Lori S. Hart1, David T. Dicker1, Wenge Wang1, and Wafik S. El-Deiry. Visualization and enrichment of live putative cancer stem cell populations following p53 inactivation or Bax deletion using nontoxic fluorescent dyes. *Cancer Biol Ther* 8(22) (2009) 2194–2205.

[3] FDA: „IC50 versus EC50"
http://www.fda.gov/ohrms/dockets/ac/00/slides/3621s1d/sld036.htm

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