New technologies for automated cell counting based on optical image analysis 'The Cellscreen'

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Abstract

A prototype of a newly developed apparatus for measuring cell growth characteristics of suspension cells in micro titre plates over a period of time was examined. Fully automated non-invasive cell counts in small volume cultivation vessels, e.g. 96 well plates, were performed with the Cellscreen system by Innovatis AG, Germany. The system automatically generates microscopic images of suspension cells which had sedimented on the base of the well plate. The total cell number and cell geometry was analysed without staining or sampling using the Cedex image recognition technology. Thus, time course studies of cell growth with the identical culture became possible. Basic parameters like the measurement range, the minimum number of images which were required for statistically reliable results, as well as the influence of the measurement itself and the effect of evaporation in 96 well plates on cell proliferation were determined. A comparison with standard methods including the influence of the cultured volume per well (25 μ l to 200 μ l) on cell growth was performed. Furthermore, the toxic substances ammonia, lactate and butyrate were used to show that the Cellscreen system is able to detect even the slightest changes in the specific growth rate.

Introduction

Mammalian cells are widely cultivated in 96 well plates for different purposes e.g. inhibition studies, testing of additives or evaluation of new media components and their influence on cell growth and specific productivity.

Commonly, the cells are cultured in a 96 well plate and after a growth period of a few days the cultures are analysed by adding different types of stains or dyes. The cells are destroyed by the measurement and there is no possibility to get any information about the time course or the growth characteristics of this individual culture. The results obtained by a MTT assay for example correspond to the number of viable cells. There is no information whether the cells are actually in the stationary phase, still in the log phase or already in the dying phase. Most of the assays commonly used only give information about the physiological status

or activity of the culture at one certain point of time. Also the cell concentration at the beginning of the experiment can not be determined. But even small variations of the cell number per well at the beginning of the experiment yield considerable differences of this parameter after a few days of cultivation. This leads to results of high statistical variance and the necessity of multiple parallel experiments to get sound information.

Contrary to other methods, the prototype of the Cellscreen system measures cell numbers in 96 well plates without effecting the culture process in any way. Cell growth can be monitored by daily measuring the total cell concentration in a 96 well plate. This offers the opportunity to get the whole growth curve and therefore much more information about growth characteristic and the influence of additives on cell proliferation. An additional activity test, e.g. a MTT assay, at the end of the cultivations is also possible.

Figure 1. Schematic view of the prototype Cellscreen system.

A 96 well plate, filled with cell suspension is positioned on the motor stage to the microscope. The images of the microscope are stored in a personal computer. For microscopic monitoring, the cells have to be on the same focus level. In fact sedimentation of the cells depends on the cell size and media composition (approx. 15–30 min). So image recording takes place at the base of the well.

The Cellscreen system quantifies the cell density of a cell suspension in selected and defined sections of the well base.

This new method of automatically counting cells in 96 well plates using digital image analysis with pattern recognition strategies (Guderman et al., 1996) was evaluated. Case studies with inhibiting substances proved the versatile use of this prototype Cellscreen system.

Material and methods

Cell culture

Chinese hamster ovary cells (CHO) were cultivated in a commercially available protein free medium (MAMPF, BioConcept, Switzerland). These cells were adapted for growth in suspension. A mouse mouse Hybridoma and a human leukaemia cell line

Precision of the Cellscreen system

Figure 2. To evaluate the precision of the Cellscreen system a comparison with a MTT-assay was done. Twelve different cell densities of K562 cells (four wells each) from 0.03×10^5 up to 7.0×10^5 cells mL^{-1} were seeded into the MT plate. R^2 Cellscreen: 0.993; R^2 MTT-assay: 0.916.

(K562) were cultivated in a serum free standard medium based on a 1:1 mixture of DMEM/F12 with the protein supplements human albumin (0.5 g l^{-1}), human transferrin (10 mg l^{-1}) and bovine insulin (10 mg l^{-1}) (Jäger et al., 1988). The cultivation was done in T-flask (Costar) and spinner systems (Belco) (Büntemeyer et al., 1991). The micro titre (MT) plates used have been purchased from Nunc.

The Cellscreen system

The basic components of the prototype Cellscreen system (Figure 1) are: an inverse microscope (IX 50, Olympus) equipped with a 10x objective, a CCD

Figure 3. The accuracy of the Cellscreen system was evaluated by comparing it with other counting methods. Four different cell concentrations were seeded (A–D). Up to a cell density of 8.7×10^5 cells mL^{-1} (K562) and 1.1×10^6 cells mL^{-1} (CHO) the Cellscreen showed a good correlation to the other methods. The counting error was 20% for the manual count (Trypan blue), 5% for Cedex and only 4% for Cellscreen.

camera (KPF100, Hitachi) with 1024×1024 pixel, a motorised x-y. The Cellscreen system is operated by a special application software. Configuration of experiments and result representation, image acquisition, storage of results and images in the systems data base are handled via the graphical user interface.

After inserting the 96 well plate into the x-y stage a calibration procedure starts to detect the precise position of the well plate. This guarantees that exactly the same area of the well plate is analysed with each measurement. If the plate has been removed and is then inserted again, the Cellscreen system is able to find the same position of the culture area analysed before with an error of less than $1 \mu\text{m}$. The positioning of the culture is followed by an auto focus process and, if necessary, a modulation of light intensity. Using an objective with a $10\times$ magnification each image represents a culture area of $680 \mu\text{m}^2$. Usually measurements are performed taking six images per well. The images are stored in a database and analysed by an adapted version of the Cedex image recognition software afterwards. Viable and dead cells are automatically detected, counted and their geometry (diameter and eccentricity) is analysed.

Figure 4. The same starting culture was used, but only varying the volume per well from 25 to $200 \mu\text{l}$ resulting in a variation of volume to plate area and therefore led to a bigger range for the cultivation. Using a volume of $25 \mu\text{l}$ only the specific growth rate μ declined drastically.

The general procedure using the Cellscreen system was the following: 100 or $200 \mu\text{l}$ of cell culture were seeded in selected wells of the 96 well plate.

All pattern recognition calculations were done after taking all pictures images of the plate, therefore the MT plate could be placed back in the CO_2 incubator after a short period of time.

Analytical methods

Manual cell density estimation

The cell suspension was stained by diluting $100 \mu\text{l}$ of cell suspension with $100 \mu\text{l}$ of Trypan blue dye solution (Sigma). Cell density and viability of the sample were determined using a hemacytometer (Neubauer chamber) (Tennant, 1964).

Screening of the well base

Figure 5. The well base of a MT plate was divided in 88 squares (=images). With this prototype it was not possible to analyse the edge squares (24). Six different patterns are shown, indicating the area and the number of images (blue squares) taken for the cell count. 64 images were taken from the well base for the pattern 'complete' but only 3 images for the pattern '3'.

Statistical reliability

Figure 6. To explore the statistical reliability three different cell concentrations (A, B, C; 4 wells each) were analysed. Taking images from different areas and taking different numbers of images per well (pattern see Figure 5) the results were compared. All patterns led to a comparable result even if only 3 images per well (3) were analysed. The small standard deviation bars indicated a high reliability.

Classification of MT plate

Figure 7. Definition of four different areas on a MT plate to analyze evaporation and its effect on cell growth.

Automated cell density estimation

A volume of 1 ml of the cell suspension was processed by the Cedex system (Gudermann et al., 1997). Sample preparation, image acquisition and analysis were performed automatically with the standard routines that were implemented. During each measurement 20 images were analysed utilising the standard operator version 3.0 for image analysis.

Viability determination via MTT assay

The 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay was performed according standard procedures (Mosman, 1983; Berridge and Tan, 1993). The colourless salt of tetrazolium was converted by physiological active cells to the dark blue coloured formazan. The measurement was done at 570 nm using a 96 well plate reader (Elx 808, Bio-Tek).

Results

The aim of the investigation was to show whether the prototype version of the Cellscreen system was able to determine cell densities in 96 well plates reliably. The constraints and conditions which have to be taken

into account to get valid results were tested. Therefore, the basic parameters like handling of the MT plates, filling volume and evaporation effects were investigated. A comparison with other commonly used assays and counting methods should prove the accuracy of the system. Different case studies should show the versatility of the system.

Precision of the Cellscreen system

To prove the precision of this new method the results of the Cellscreen system were correlated with the established MTT Assay. Therefore, MT plates were prepared with cell suspensions of different cell densities. Twelve different cell densities from 0.03×10^5 up to 7.0×10^5 cells ml^{-1} were seeded into the MT plate. Four wells were prepared with the same cell density to carry out four parallel experiments. The mean value of these four results were used for further analysis. First, the MT plates were measured with the Cellscreen system. Afterwards, the same plate was measured using the MTT assay. The resulting cell densities of both methods were compared to the calculated values and plotted in Figure 2.

The results of the Cellscreen system correlated to the calculated cell densities of the respective ex-

Figure 8. Evaporation effects in micro titre plates. All wells of the MT plate were filled with $200\ \mu\text{l}$ Hybridoma cell suspension starting with $0.9 \times 10^5\ \text{cells mL}^{-1}$. Cells seeded in the edge and exterior wells (see Figure 8) showed a drastical reduction in growth.

periments while the data of the common MTT assay showed greater deviations. In comparison to the MTT assay the results of the Cellscreen were more precise – closer to the calculated values – and had less variability.

Counting accuracy

To evaluate the counting accuracy of the Cellscreen system, a comparison with other standard counting methods were carried out using the K562 and the CHO cell line (Figure 3). Four different cell densities from 1×10^5 up to $16 \times 10^5\ \text{cells mL}^{-1}$ were counted manually (Trypan blue) and automatically (Cedex). For the Cellscreen system a MT plate was prepared with four wells for each cell density. All the wells were filled with $100\ \mu\text{l}$. Up to a cell density of $8 \times 10^5\ \text{cells mL}^{-1}$ (K562) and $11 \times 10^5\ \text{cells mL}^{-1}$ (CHO) the Cellscreen system showed a good correlation to the other methods used.

Figure 9. To analyse the lower limit of cell concentration detectable by Cellscreen. Six different cell densities of CHO cells from 0.012×10^5 up to $1.4 \times 10^5\ \text{cells mL}^{-1}$ were seeded into the MT plate. The growth behavior was monitored for four days (numbers in the legend ($\times 10^5\ \text{cells mL}^{-1}$), $\mu\ (\text{d}^{-1})$).

Cell concentration upper limit

The upper counting limit was given by the measurement principal of the Cellscreen itself. It detected the cells lying on the base of the well. The upper limit was attained, when the whole area of the well was covered by cells. The system was only able to detect and count one layer of cells. Depending on the cell diameter, the number of cells necessary to fill the whole base, varies. For bigger cells like K562 (average diameter of $17\ \mu\text{m}$) the system detected only 8×10^4 cells. Depending on the volume in the well ($100\ \mu\text{l}$), this corresponded to $8 \times 10^5\ \text{cells mL}^{-1}$ (Figure 3), for CHO cells (average diameter of $13\ \mu\text{m}$) more than 10×10^4 cells ($10 \times 10^5\ \text{cells mL}^{-1}$) were counted.

Figure 10. Influence of measuring time on cell growth. Starting with 0.9×10^5 cells ml^{-1} three MT plates were prepared with CHO cells. The cell growth was monitored for four days while measuring the plates for different periods of time (0.5, 1.0 and 1.5 h).

Volume variation and limits

To overcome the limitations given by the measurement principle, the volume of cell suspension and therefore the ratio between volume and base area was varied. Portions of 25 to 200 μl of identical CHO cell suspension were filled into the wells and analysed by the Cellscreen system for four days (Figure 4). Comparing the courses of the cell growth and the specific growth rate μ , cultivation of CHO cells from 50 to 200 μl was possible.

In 50 μl cell suspension there were only half of the cells compared to 100 μl culture of the same density, only half of the well plate was occupied by the cells. Therefore, using 50 μl per well up to a cell density of 20×10^5 cells ml^{-1} could be counted.

Figure 11. Inhibiting effect of different NH_4^+ concentrations (2, 5, 8 mM) on cell growth of a hybridoma cell line was analysed. The cell density at the beginning of the cultivation was 0.7×10^5 cells ml^{-1} , 200 μl cell suspension in five wells each were analysed.

Statistical reliability

The next step was to prove, that the results of the Cellscreen system were independent of the number of images taken per well and also independent of the position of the area of the well plate.

The complete digitalisation of a well plate means taking 52 images (without the edge of the well). To reduce this number and to prove the statistical reliability, different selected areas and different number of images were used. These 'pattern' are shown in Figure 5. For this experiment three different cell concentrations were tested, four wells in each case. The same wells were counted with the different pattern. Neither the counting of different areas nor the reduction of the number of images to only three per well led to deviation in cell count from the seeded cell number (Figure 6). The small standard deviation indicates a high precision of the system. Four wells per experi-

Figure 12. The influence of different lactate concentrations (5, 10, 15, 20 mM) on cell growth of a hybridoma cell line was monitored. The cell density at the beginning was 0.7×10^5 cells mL^{-1} , 200 μl cell suspension in five wells each were analysed.

ment were enough to ensure a precise result. It would have been enough to take only three images per well, but to be on the safe side in all other experiments the pattern with six images per well was used.

Evaporation effect

Using MT plates in a humidified CO_2 incubator evaporation leads to an increase in osmolarity in the culture medium which can influence the growth of mammalian cells drastically. To evaluate evaporation effects in different areas of the 96 well plate, the plate was divided into four different areas (Figure 7). The four edge wells, the exterior wells, the middle wells and the interior wells. 200 μl cell suspension with a cell density of 0.9×10^5 cells mL^{-1} was seeded into the wells. Figure 8 shows a good growth of the cells only in the middle and interior wells, the growth rate of the cells in the exterior wells and especially in the edge wells was significantly reduced. To avoid wrong

Figure 13. The influence of different butyrate concentrations (0.5, 3 mM) on cell growth of a hybridoma cell line was monitored. The starting cell density was 0.17×10^5 cells mL^{-1} for all the cultures.

results in subsequent experiments only the middle and interior wells were seeded and the edge and corner wells were filled with culture medium only.

Cell concentration lower limit

The Cellscreen system was able to detect very low cell densities. But depending on the cell line and the culture medium a certain seeding cell density was necessary for cell growth. Ten different cell densities of CHO cells from 0.012×10^5 up to 1.4×10^5 cells mL^{-1} were seeded into the MT plate. For this CHO cell line the lower limit which led to a good growth rate was only 0.02×10^5 cells mL^{-1} (Figure 9). For the Hybridoma cells at least ten times more cells per ml were necessary (0.16×10^5 cells mL^{-1} , data not shown).

Influence of measuring time on cell growth

The Cellscreen system needs a certain amount of time for measuring a MT plate. About 6 min for calibration per MT plate and 6 sec per image. Depending on the number of wells analysed, the plate was out of the incubator for several minutes. During this time the plate was not kept on 37 °C and there was no CO₂ atmosphere. To monitor a possible negative effect the cooling down of the cultures may have the MT plates were repeatedly measured using different measuring times. Figure 10 shows growth curves for the Hybridoma cell line. 100 µl cell suspension was seeded in three different plates. One plate was measured 0.5, 1 and 1.5 h per day, respectively. The cells in the plate which was kept out for 0.5 h per day showed nearly the same specific growth rate ($\mu = 0.75 \text{ d}^{-1}$) compared to the best results obtained in controlled bioreactors ($\mu = 0.8 \text{ d}^{-1}$). At $8.3 \times 10^5 \text{ cells ml}^{-1}$ the cell cultures reached the upper limit of the Cellscreen system. With a specific growth rate of 0.65 d^{-1} the other cells grew significantly slower. Therefore, to avoid growth reduction the measuring time should not exceed half an hour. Alternatively a heating device could be used for the system.

Application examples

Inhibiting effect of ammonia and lactate

Well-known inhibiting substances like ammonia and lactate were tested to inhibit cell growth of the Hybridoma cell line. Cultures with an addition of 2,5 and 8 mM ammonia were compared with an untreated reference culture. The cell density at the beginning was $0.7 \times 10^5 \text{ cells ml}^{-1}$. Figure 11 shows the reduction of cell growth according to the increasing ammonia concentration. The same type of experiment was carried out with lactate (5, 10, 15 and 20 mM) as inhibiting substance. Figure 12 shows the inhibiting effect of increasing concentration of lactate to cell growth.

Toxicity of butyrate

The effect of butyrate on cell growth of Hybridoma cells was analysed (Figure 13). Starting with a cell density of $0.17 \times 10^5 \text{ cells ml}^{-1}$ for all the cultures, butyrate was added after four days. Both butyrate concentrations resulted in a significant reduction of cell proliferation. 24 h after the addition of butyrate the specific growth rate of the Hybridoma cell line decreased to zero.

Summary and discussion

The Cellscreen prototype tested follows the growth progression of suspension cell cultures in micro titre plates. It had a high precision and counting accuracy. The familiar effects of evaporation on the edges these plates and its influence on cell growth could be verified. The influence of inhibitory and toxic substances on cell growth of three different cell lines within 96 well plates were examined and demonstrated for an applied example. Approximately 15% of the culture area was analysed in these spot-test experiments. The results show that the Cellscreen was capable of detecting even the slightest changes in growth behaviour of cells. This method was sensitive and could be applied to cell cultures with a cell density as low as $10\,000 \text{ cells ml}^{-1}$. A drawback to this system was the limitation to measure high cell densities due to a fully covered well base. This could be partly prevented by reducing the volume in the wells and therefore enhancing the volume to base ratio. Another important issue to be addressed for the next system generation is the possibility of cell counts near the well edges. This prototype system fulfills the expectation of a versatile tool for cell-based assays in micro titre plates.

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