Cell count determination of aggregated HEK293 suspension cells and Vero cells on microcarriers

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Introduction

Nuclei counting was first described by Sanford et al. in 1951[1] and modified by van Wezel[2]. Using this method, cells are permeabilized using hypotonic solutions and nuclei are stained and counted using a dye solution, e.g. crystal violet. This often leads to nuclei overestimation as nuclei cannot be distinguished accurately from debris[3]. To overcome this problem, the image cytometers by Chemometec A/S offer nuclei counting using fluorophores, enabling a clear distinction between nuclei and cellular debris and ensuring a fast and reliable method for nuclei determination. Vero cells are anchorage dependent cells[4] and can be cultivated on microcarriers[5] for scaling up the production to achieve yields of several million cells per milliliter[6] in large-scale fermenters[7]. Monitoring microcarrier cultures is challenging and time-consuming as the cells need to be detached in a multistep process from the microcarriers. HEK293 cells are forming cell aggregates of up to 3 mm in diameter[8]. Using automated cell counters, many algorithms are not able to determine the correct amount of cells due to the three dimensional appearance, leading to imprecise results. For these applications, counting nuclei provides a fast method for cell count determination.

We have tested and validated the nuclei counting method using the inbuilt protocols in the NucleoCounter NC-3000™ for aggregated HEK293 cells and Vero cells on microcarriers.

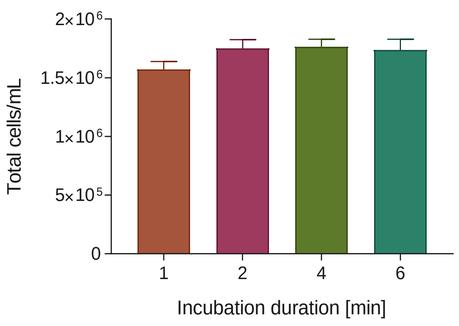
Materials and Methods

HEK293 cells, (adapted to grow in suspension in chemically defined, animal component-free growth medium) were seeded at 3x10^5 cells/mL in 40 mL working volume in a 125 mL shake flask. Cells were incubated at humid, 5% CO_2- atmosphere at 37°C on a shaker at 140 rpm with a shaking radius of 2.5 cm. After 96 hours post seeding, homogenous samples were drawn and cell count was determined using the NC-3000™ and Via1-Cassettes™ (Chemometec A/S). Samples were analyzed using the protocols "*Viability and Cell Count Assay*" and "*Count of Aggregated Cells – A100 and B Assay*".

Vero cells (*Vero (AC-free) (ECACC 08011101)*) were obtained from the ECACC (European Collection of Authenticated Cell Cultures) and adapted to growth in animal component free medium. Cells were seeded at 4x10^4 cells/cm² in 40 mL serum free growth medium, VP-SFM (Gibco) supplemented with 4 mM L-Alanyl-L-Glutamine (Gibco), containing 3 g/L swollen Cytodex 1 microcarriers (GE) in a 125 mL shake flask (Corning). Cells were incubated until 24 hours post seeding at humid, 5% CO_2- atmosphere at 37°C on a shaker at 90 rpm with a shaking radius of 2.5 cm. The shaking speed was then increased to 100 rpm, to keep microcarriers in suspension. Microcarriers were previously swollen in Ca^{2+}, Mg^{2+}-free PBS (Thermo Fisher Scientific) and autoclaved in a glass bottle coated with Sigmacote (Merck) according to manufacturer instructions. Afterwards microcarriers were washed twice with cultivation medium to remove residual buffer solution. For determination of the incubation time of Reagent A100 using the NC-3000™ and Via1-Cassettes™, homogenous microcarrier samples of microcarrier/cell suspension were drawn 96 hours post seeding and mixed with the same amount of Reagent A100 by pipetting. After 1, 2, 4 and 6 minutes, samples were stabilized by mixing with an equal amount of Reagent B. After the microcarriers settled, the Via1-Cassette™ was loaded with the sample by inserting it halfway into the liquid and pressing down the piston. The cell count was analyzed using the NC-3000™ using the protocol "*Count of Aggregated Cells – A100 and B Assay*". Additional microscopic images at 100 x magnification were taken after the stabilization of the different incubation steps to visually control the microcarrier appearance.

Results

Vero cells on microcarriers



Incubation duration [min]	Total cell concentration [x 10^6 cells/mL]	Std Dev [x 10^6 cells/mL]
1	1.57	0.07
2	1.75	0.08
4	1.76	0.07
6	1.74	0.09

Figure 1: Incubation time of Vero cells on microcarriers. Already after 1 minute incubation time, the majority of Vero cells could be detached from the microcarriers using Reagent A100. After 2 minutes incubation time, the total cell concentration increased by approximately 11%, reached a maximum and did not further increase when incubating 4 and 6 minutes. (Graph: Mean and error bars represent the standard deviation of 3 measurements; Table: Mean cell concentration and standard deviations of 3 measurements).

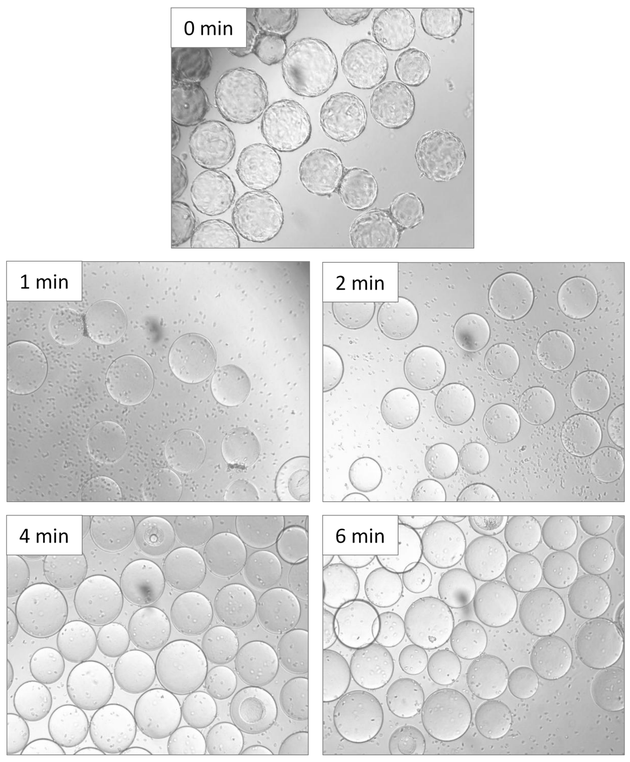


Figure 2: Microscopic images of Vero cells on microcarriers. Untreated Vero cells appeared as a confluent cell layer on the microcarriers. Already after 2 minutes incubation time, the cell layer was dissolved. Reagent A100 was not able to dissolve all aggregates, noticeable in non lysed cell clumps between remaining aggregated microcarriers after 1 minute of incubation. After 2, 4 and 6 minutes incubation time, aggregated microcarriers and cell clumps were not visible anymore (100 x magnification).

HEK293 suspension cells

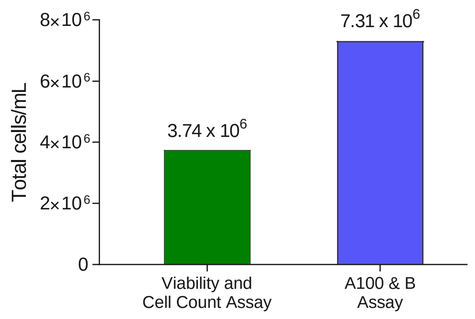


Figure 3: Total cell count of HEK293 cells. Using the protocol "*Count of Aggregated Cells – A100 and B Assay*", almost the double amount of total cells could be determined in comparison to the protocol "*Viability and Cell count Assay*".

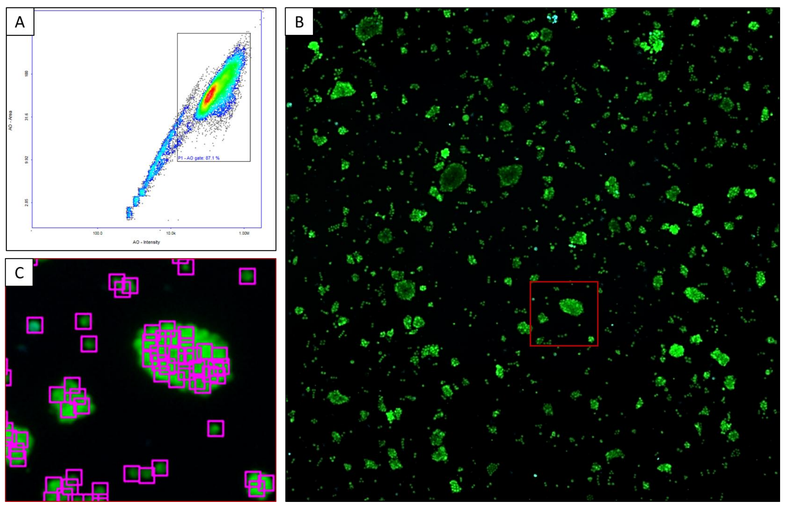
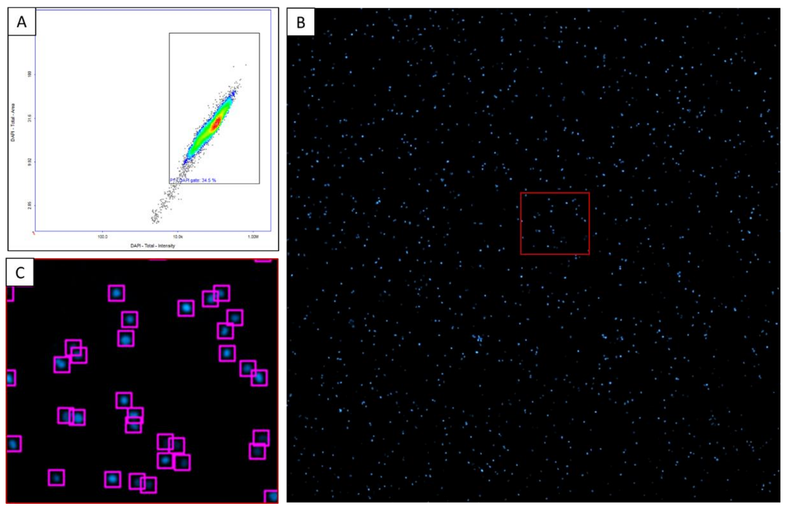


Figure 4: HEK293 cells counted using the "*Viability and Cell count Assay*". The uneven distribution and aggregate size led to numerous non-distinguishable overlapping cells. The algorithm, visualized by the pink square image overlay, was not able to identify all individual cells in the aggregates. This resulted in an underestimation of the cell count. The obtained images show (A) the raw data scatter plot of acridine orange (AO) area over intensity of stained cells in green with the standard counting gate. Multiple acquired images (one displayed in (B)) were used for automated cell count determination. Image in (C) displays in detail a stained aggregate and the image overlay used to determine individual cells.

Figure 5: HEK293 cells counted using the "*Viability and Cell Count – A100 and B Assay*": The lysis using Reagent A100 led to a uniform distribution of nuclei and the algorithm was able to distinguish and count the single nuclei. The obtained images show the (A) raw data scatter plot of DAPI area over intensity of stained cells in blue with the standard gating strategy. Multiple acquired images (one displayed in (C)) were used for automated cell count determination. Image in (C) displays in detail single nuclei and the image overlay for highlighting individual cells.



Summary

For counting Vero cells grown on Cytodex 1 microcarriers, the A100 & B assay was used. Already after 2 minutes incubation time with reagent A100, the maximum number of cell nuclei could be detected and the total cell count could be determined. Furthermore, we´ve tested the same protocol for highly aggregated HEK293 cells. The A100 & B assay was able to dissolve the HEK293 aggregates, resulting in a single cell suspension. Stained cell nuclei formed a uniform distribution which doubled the amount of counted total cells in comparison to counting unlysed cells. We are stating that counting nuclei using the NC-3000™ is a precise and fast way to determine total cell count and viability of highly aggregated HEK293 cells and Vero cells on microcarriers.

[1] Stanford et al., *J Natl Cancer Inst,* ***1951***
[2] van Wezel, *Tissue Cult*, **1973**
[3] He et al., *BioTechniques,* ***2017***
[4] Nor et al., *Mater Sci Appl*, **2010**
[5] Groot, *Cytotechnology,* **1995**
[6] Clark et al., *Biomaterials*, **1999**
[7] Barrett et al., *Expert Rev Vaccine,* **2009**
[8] Côté et al., *Biotechnol Bioeng,* **1998**