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INSTITUTE OF BIOTECHNOLOGY

Project report

Mechanical stress analysis of transfected CHO suspension cells in different pumps

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1 Introduction

Pumps are commonly used in the biotechnology industry for various applications in up- and down-streaming processes. In all these processes, the cells are exposed to different kinds and magnitudes of mechanical forces. In most cases, mechanical stress is generalized as shear stress, but cells can also be damaged by normal gradients [Langer & Deppe (2000)]. Especially animal cell cultures are believed to be sensitive to mechanical stress due to the cell size and their morphology [Chisti (1993)]. Besides loss in biological activity, cell disruption can lead to the release of cell components and enrichment of cell debris in the culture media. Hence, numerous studies have been described to obtain critical shear values for different cell lines. In most cases, simple test systems, such as capillaries [McQueen *et al.* (1989); Mollet *et al.* (2004); Thomas (1993)] or viscometers [Mardikar *et al.* (2000)], have been used to find correlations between shear force and animal cell viability.

Few studies have been carried out to study the effect of pumps and valves on cells [Vandanjon *et al.* (1999); Alias *et al.* (2004); Jaouen *et al.* (1991)]. Jaouen *et al.* (1999) investigated the shear stress of microalgal cell suspensions caused by different pumps. The authors could show that a loss of microalgal motility can be used as an indicator for cell damage. It was demonstrated that centrifugal and rotary vane positive displacement pumps cause cell damage, but no effect was observed with eccentric rotor pumps or a peristaltic pump [Jaouen *et al.* (1999)]. Kamaraju *et al.* (2010) studied the effect of different pump rotational speeds and slip conditions on CHO suspension cells in a rotary positive displacement pump. They could show that the slip flow rate more severely impacts the viability of the CHO cells than the pump speed. Furthermore, a novel mathematical modeling approach was presented which predicts cell damage based on an analytical solution of the Navier-Stokes equation for laminar flows [Kamaraju *et al.* (2010)].

A novel continuous-flow, centrifugal-type rotary pump named CentriMag was developed by Levitronix in the last years. It was designed for pumping blood providing hemodynamic support for patients with reversible ventricular failure. The CentriMag blood pump utilizes an impeller that is suspended by magnetic forces avoiding the existence of shafts and seals used by traditional blood pumps. Hence, associated problems such as heat generation, thrombus formation around the seals and infection are effectively eliminated [Chua *et al.* (2006)]. The inner fluid flow was investigated by the aid of computational fluid dynamics (CFD). A clean and streamlined flow field in the main components of the CentriMag blood pump was demonstrated [Zhang *et al.* (2006)]. Furthermore, hemolysis studies were performed for flow rates of 5.0 L/min [Zhang *et al.* (2006)] and 3.4 L/min [Hahn (2010)]. Both, the hemolysis tests and the CFD simulation showed no significant high shear stress regions.

The aim of the present project was the evaluation of mechanical stress caused on CHO suspension cells by the magnetically driven centrifugal pump Levitronix BPS-200. For this purpose, different rotational speeds in the range of 1500 to 6000 rpm were investigated. Two peristaltic pumps were used as reference systems (see section 2.5). Additionally, CHO cells were incubated in non-shaken shake flask (called static culture), in which shear stress can be assumed as negligible.

2 Materials and Methods

2.1 Cell expansion in a wave-mixed system

The CHO suspension cells (*CHO XM111-10*) were cultivated in the single-use wave-mixed system BioWave® 20L with a maximum working volume of 10 liter (see Fig. 1). Chemically defined culture media (HP-1, Cell Culture Technologies, Switzerland) was used. The bioreactor was inoculated with cells from shake flasks at an initial working volume of 2.0 L and a cell density of 0.5 x 10^6 cells/mL. The cultivation was realized in fed-batch mode (addition of fresh medium depending on cell growth as well as substrate and metabolite concentration). When the maximum working volume of 10 liters and a total cell density of 3.5 x 10^6 cells/mL were reached, cell suspension for the stress experiments was harvested. However, a small fraction (about 0.5 L) was remained as inoculum for the next cultivation cycle in the BioWave®. A detailed procedure is described by Eibl and Eibl (2007) in the book chapter "Disposable bioreactors for inoculum production and protein expression" (in Methods in Biotechnology, Vol. 24: Animal Cell Biotechnology: Methods and Protocols, 2nd Ed., edited by: R. Pörtner, Humana Press, Inc., Totowa, NJ).

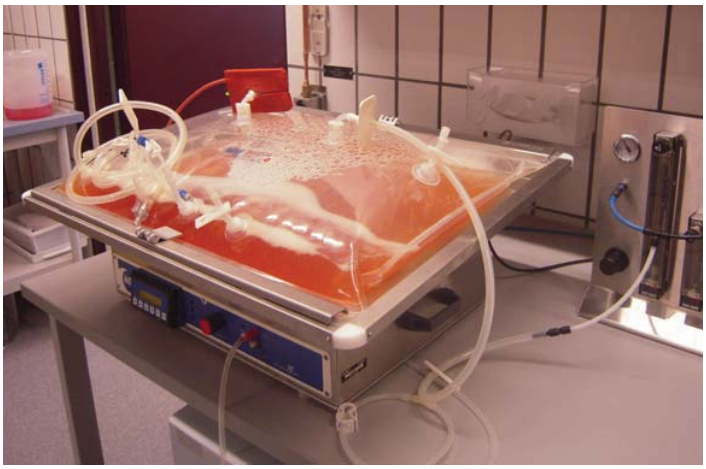

Fig. 1: Picture of the cultivation system BioWave® 20L for cell expansion.

For in-process control samples of about 2 mL were taken with a sterile syringe at least twice a day, depending on cell growth and medium addition. Viable and total cell density was determined as described in section 2.3. Besides, concentrations of glucose, lactate, glutamine, glutamate and ammonium as well as the pH value were quantified using a multi biosensor analysis system BioProfile 100 Plus (Labor-Systeme Flükiger AG, Switzerland).

2.2 Preliminary test in a shake flask

An experiment in a 250 mL shake flask was realized to validate the suitability of the vitality assay provided by Chemometec as described in the project proposal. The vitality assay enables to detect changes in intracellular level of (reduced) thiols which occur in apoptotic cells or cells undergoing other pathological processes. The cultivation was realized at a working volume of 80 mL and the initial cell concentration was set to 1.0×10^6 cells/mL. The shake flask was incubated at 37 °C in an incubator with 7.5 % CO_2 supply. The shaking frequency was set to 120 rpm. Cell growth and profiles of substrate and metabolite concentrations were analyzed with the automated cell counting device Cedex HiRes (Roche Innovatis AG) and the multi biosensor analyzer Bioprofile 100 Plus (Labor-Systeme Flükiger AG, Switzerland).

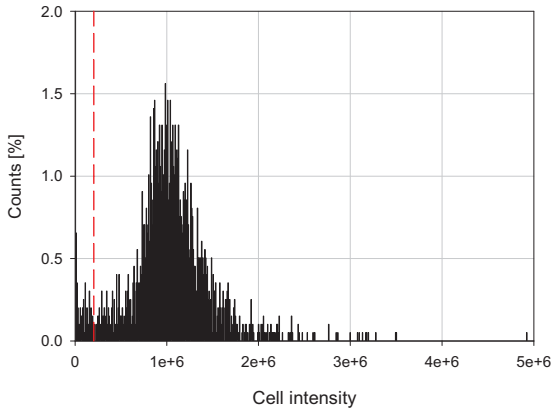

Fig. 2: Result of the vitality assay using NC-3000. VitaBright-48 intensity is displayed in the histogram. Cells with low thiol level (apoptotic cells) are found at the lower end of the histogram. The red marker is placed by the user and divides the cell population into two subpopulations.

The vitality assay was done as described in the application note 3005 provided by Chemometec (see appendix). A representative aliquot of the cell suspension was mixed with reagent solution 5 (VB-48·PI·AO, VitaBright-48) and 30 µL of this solution was loaded into the chamber of a NC-Slide A2™. The cellular fluorescence is quantified by the automated cell counter NC-3000 and the VitaBright-48 intensity is displayed by the accompanying software. The cell population can be divided into two (or more) subpopulations by placing a marker in the histogram (see Fig. 2).

2.3 Determination of cell concentration and viability

Total and viable cell densities were measured automatically with the cell counting devices NucleoCounter® NC-100 (ChemoMetec, Denmark) and Cedex HiRes (Roche Innovatis AG, Germany) (see Fig. 3). The NucleoCounter® is based on the fluorescence detection of the fluorescent dye, propidium iodide (PI) which bound to DNA. Results from the NucleoCounter® represent either total or non-viable cell concentration, depending on the sample preparation. In the Cedex HiRes device, the common trypan blue exclusion method is applied. Besides the densities of viable and dead cells, aggregation rate, cell morphology, and cell debris are detected by high-resolution digital image recognition.

a b

Fig. 3: Automatic cell counting devices. (a) Cedex HiRes (Roche Innovatis AG, Germany) [source: http://www.research-ins.com/] and (b) NucleoCounter NC-100 (Chemometec, Denmark) [source: http://www.licor.com/].

The viability is defined as the ratio of living (viable) cell density to the total cell density as indicated by Eq. 1, where LCD, DCD and TCD represent the living, dead and total cell densities.

$$\text{Viability}\,[\%] = \frac{\text{LCD}}{\text{TCD}} = \frac{\text{TCD} - \text{DCD}}{\text{TCD}} \qquad \textbf{(1)}$$

2.4 Determination of cell death kinetics

For quantification and evaluation of mechanical stress, the kinetics of the viable cell density decay was modeled assuming first order kinetics, which can be described by the following relation, where LCD and k_D are living cell density and cell death coefficient (h^{-1}).

$$\frac{dLCD}{dt} = -k_D \cdot LCD \tag{2}$$

By integration of Eq. 2 with LCD(t=0) = LCD_0 one obtains:

$$LCD(t) = LCD_0 \cdot e^{-k_D \cdot t} \tag{3}$$

If this function is linearized, as given by Eq. 4, the cell death coefficient k_D can be obtained as the slope when plotting the logarithmic viable cell density against the time (see Fig. 4).

$$\ln(LCD(t)) = \ln(LCD_0) - k_D \cdot t \tag{4}$$

The quality of the predicted cell death coefficients was evaluated by the determination coefficient R^2 defined by Eq. 5, where y_i and $\bar{y}$ are the measured data and their mean value and f_i are the predicted values.

$$R^2 = 1 - \frac{\sum_N (y_i - f_i)^2}{\sum_N (y_i - \bar{y})^2} \tag{5}$$

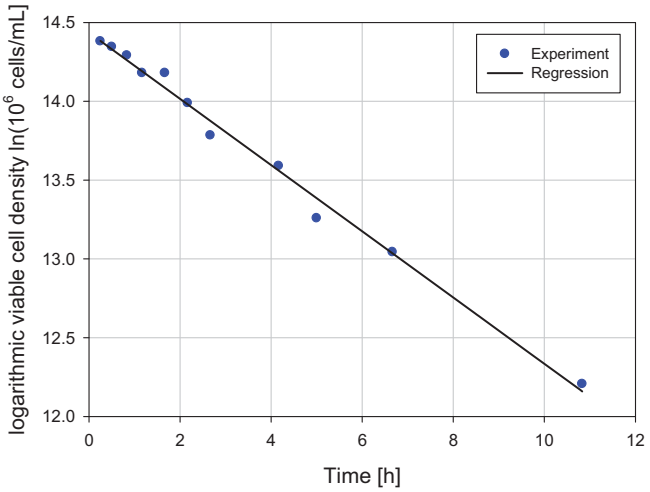

Fig. 4: Regression model for cell death kinetics. The cell death coefficient k_D is obtained by plotting the logarithmic viable cell density against the time and determining the slope of the obtained function.

2.5 Test setup and components

In the pump tests, three different pumps were used, as shown in Fig. 5 and 6. In addition to the Levitronix BPS-200, the two peristaltic pumps Masterflex® I/P Easy Load and Masterflex® L/S Cole-Parmer were investigated as reference systems. In the biological experiments, the rotational speed of the Levitronix BPS-200 was varied in a range between 1500 and 5000 rpm. To guarantee a constant flow rate of 3.4 L/min, the back-pressure was varied from 5 to 465 mmHg (corresponding to 6.5 and 567 mbar) by constricting the tubing with a hose clip (see Tab. 1). The cell suspension was pumped in closed loops from three beakers with filling volumes of 3.3 liters, which were tempered by a water bath. Aliquots of 2 mL were taken with a pipette from the three cell suspension reservoirs and the static culture. For controlling the flow rate and pressure, ultrasonic clamp-on flow sensor and pressure sensor (provided by Levitronix) were used respectively. All components with additional data are listed in Tab. 1.
The parameters used in the four test cases are summarized in Tab. 2. As mentioned above, the rotational speed of the Levitronix centrifugal pump was set to four different values while keeping the flow rate constant at a value of 3.4 L/min. During the pump tests, cell viable and total densities as well as cell morphology and nutrient concentrations were determined periodically.

Tab. 1: Components of the pump setup

Pos.	Part name	Description
1a	Levitronix BPS-200	- Re-usable pump head - Bearingless motor - Controller LPC-200.3 (firmware C7.48 R05) - Manual panel
1b	Masterflex® I/P Easy Load	- PSF housing/CRS rotor - Masterflex tubing 06435-26 PharmaPure®
1c	Masterflex L/S Cole-Parmer	- Digital drive 600 rpm - Platinum-cured silicone tubing, L/S® 36
2	Pressure sensor	Netech Digimano 1000
3	Clamp-on flow sensor	Transonic systems Inc, H9XL, ultrasonic type
4	Steel pipe coil	-
5	Hose clip	Only used in test cases 2, 3 and 4
6	Cell suspension reservoir	Plastic beaker
7	Water bath	-

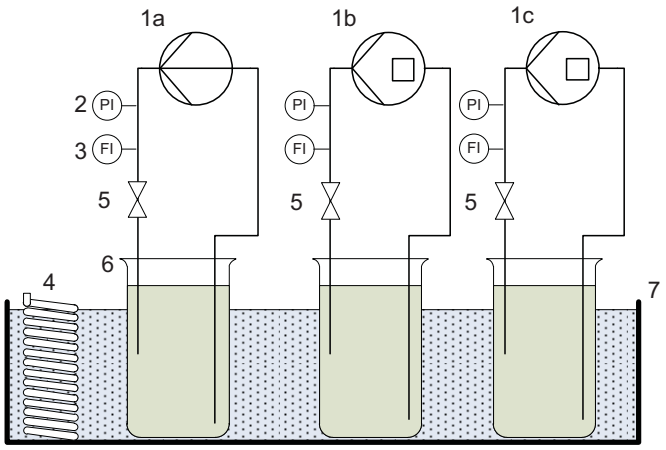

Fig. 5: Schematic of the test setup. The cell suspension is pumped with the three pumps Levitronix BPS-200 (1a), Masterflex® I/P Easy Load (1b) and Masterflex® L/S Cole-Parmer (1c) in closed loops from beakers (6) placed in a water bath (7) which is tempered by a pipe coil (4) connected to a thermostat. The flow rate and pressure were monitored by ultrasonic clamp-on flow sensor (2) and pressure sensor (3). The restriction of the tubing was realized by a hose clip (5) in the test cases 2, 3 and 4.

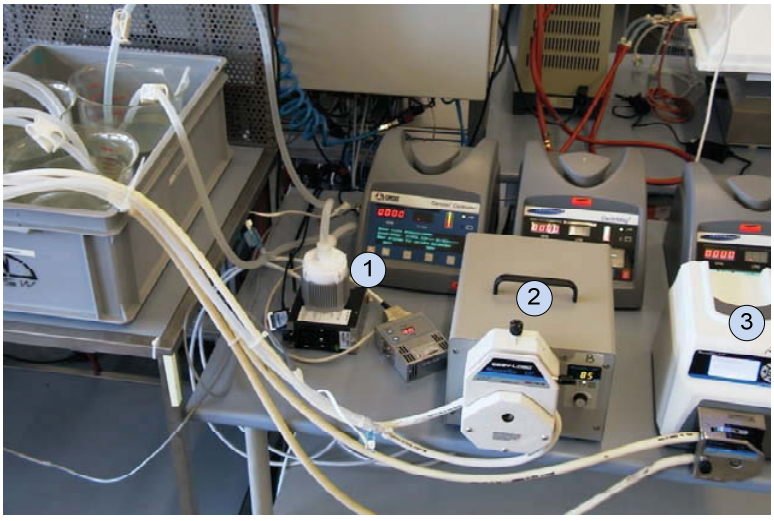

Fig. 6: Picture of the test setup. The Levitronix BPS-200 (1) was tested against the two reference peristaltic pumps Masterflex® I/P Easy Load (2) and Masterflex® L/S Cole Parmer (3).

Tab. 2: Descriptions and parameters of the investigated test cases

Characteristics	Dimension	Test1	Test2	Test3	Test4	Notes
Flow rate	L/min	3.4	3.4	3.4	3.4	- target flow rate; flow rate was controlled periodically during the experiment with clamp-on flow sensor - small variations (about ±5 %) occurred
Back pressure	mmHg mbar	5 6.5	190 253	288 385	465 567	- pressure adjustment by constricting the tubing with a hose clip
Levitronix BPS-200 speed	rpm	1500	3500	4200	5000	- values were kept constant
Tubing constriction	--	none	hose clip	hose clip	hose clip	- constriction of the tubing was readjusted periodically in order to keep pressure and flow rate constant
Cell culture volume in the beaker	L	3.3				- culture volume and volume inside the tubing circuit sums to about 3.4 liter
Cell culture temperature	°C	37				- kept constant by steel pipe coil connected to a thermostat
Measured characteristics	--	cell densities, viability, cell morphology (diameter, compactness, aggregate rate), substrates & metabolites (glucose, glutamine, glutamate, lactate, ammonium)				- cell densities and viability were estimated by Cedex HiRes and NucleoCounter® NC-100; not all measurements were done for each sample - concentrations of substrates and metabolites were analyzed by multi biosensor analyzer Bioprofile 100 Plus; not all measurements were done for each sample

3 Results and discussion

3.1 Flow rates

The flow rates depending on the pumping parameters were evaluated by taking the time needed to pump 4 liters out of the beaker and by measuring the flow rate with the clamp-on flow sensor. As shown in Fig. 7, differences of about 10 to 15% between the two measurement techniques were observed for all investigated cases. These differences can be a result of both the measuring technique and the flow sensor. The flow meter is fabricated and custom-calibrated for a wide range of tubing sizes and materials [Transonic systems (2010)], but no specific information could be found about measuring accuracy in use with the applied tubing. Due to the obtained differences, the values of the flow rate measured by the clamp-on sensor were corrected with the obtained factors during the stress experiments.

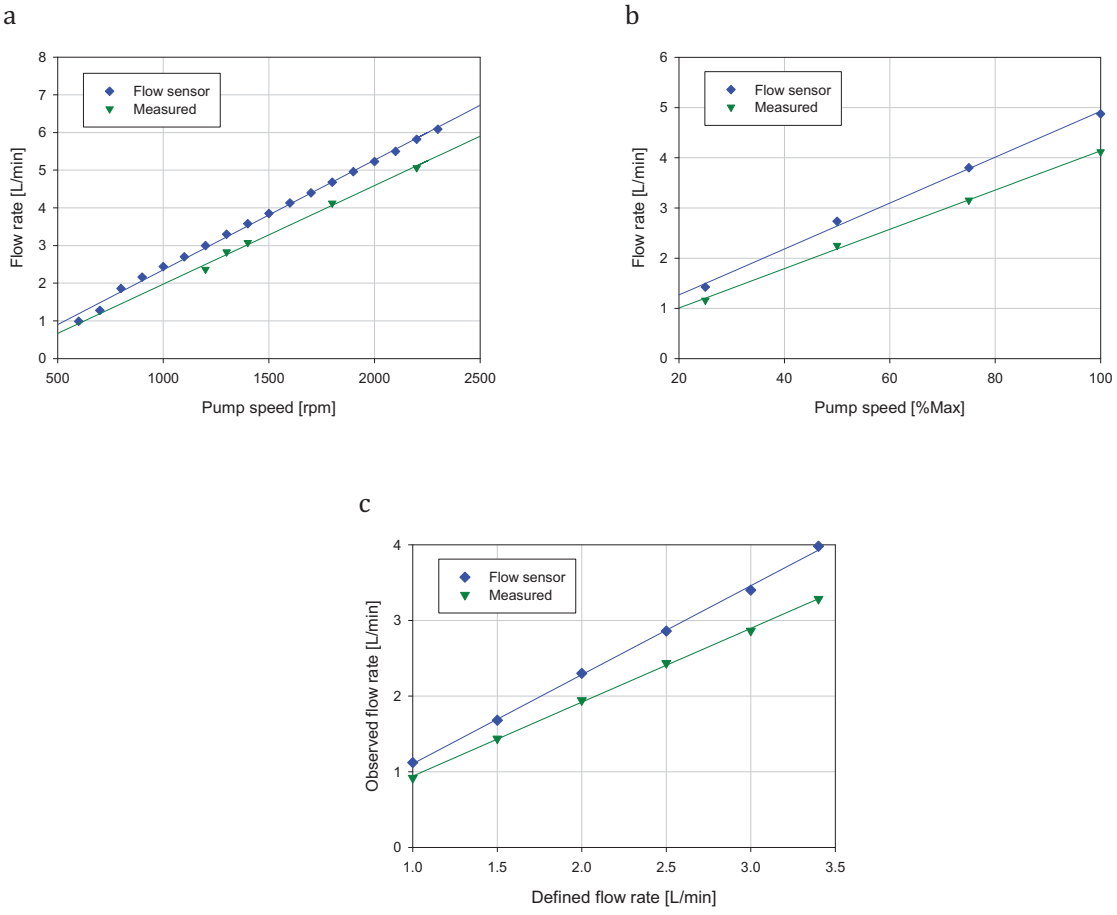

Fig. 7: Measured flow rates depending on pumping settings for the Levitronix BPS-200 (a), Masterflex® I/P Easy Load (b) and Masterflex® L/S Cole-Parmer (c).

3.2 Pump characteristic of the BPS-200 pump

For the determination of the Levitronix BPS-200 pump characteristic, the flow rate was measured with the clamp-on flow sensor at varying rotational speeds in the range between 1500 and 6000 rpm. Variation of the pressure was realized by constricting the tubing with a hose clip as in the stress experiments. Due to the limited measurement range of the flow sensor, only flow rates below 10 L/min could be measured. As shown in Fig. 8, a clear decrease of the flow rates for higher back-pressures was obtained for the realized pump speeds, which is typical for impeller-driven pumps.

For higher rotational speeds of the pump it was more difficult to adjust the pressure by the used hose clip, because minimal screwing led to significant changes in pressure. Thus, the pump speed was not set above 5000 rpm in the biological stress experiments.

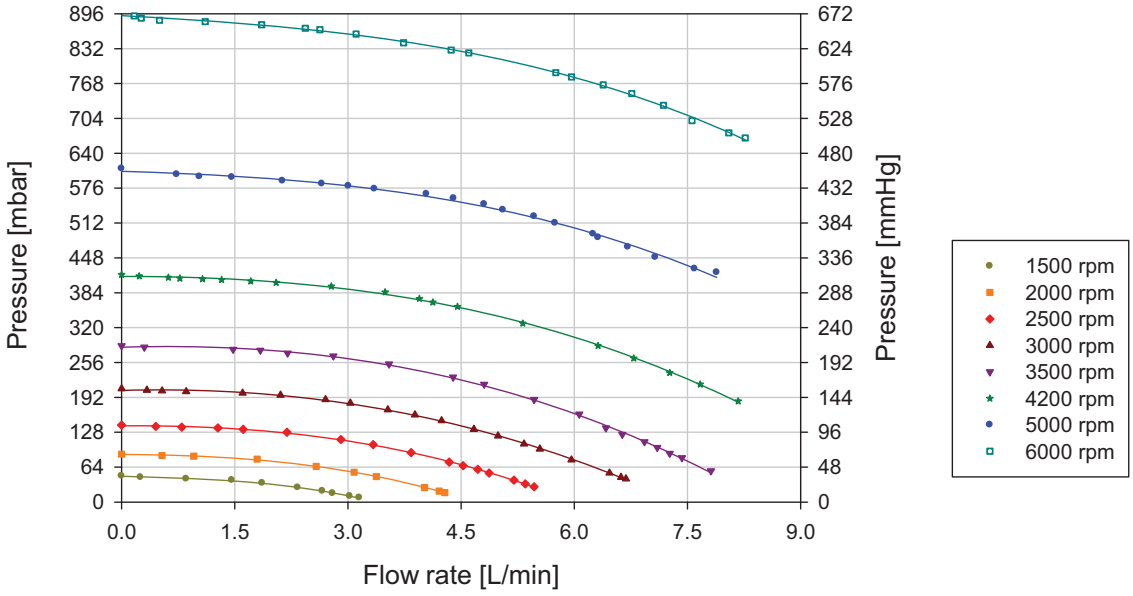

Fig. 8: Pump characteristic of the Levitronix BPS-200 pump estimated for different rotational speeds between 1500 and 6000 rpm. Symbols indicate measured data. The lines were obtained by non-linear regression.

3.3 Evaluation of vitality assay

As described in section 2.2, the vitality assay provided by Chemometec is designed to detect changes in the intracellular level of (reduced) thiols which are an indicator of apoptosis (programmed cell death) or other pathological processes [see appendix]. For insect cells (High Five™), it was demonstrated in our working group that the vitality assay can indicate early intracellular changes, even when the determined viability is still high [Ries *et al.* (2010)]. Such intracellular changes are regarding as cell stress responses. Thus, it was investigated whether the vitality assay is also suitable for the CHO suspension cells, which were used in the stress experiments.

As shown in Fig. 9a, a typical growth curve was obtained. The cell density increased exponentially from the initial value of 1 x 10^6 cells/mL to about 2.6 x 10^6 cells/mL within 23.5 h. The specific growth rate and the doubling time for the growth phase were calculated to be 0.0378 h^{-1} and 18.3 h, respectively. These values are very typical for the used cell line (CHO XM 111-10). Doubling times between 18 and 24 h are obtained routinely in our laboratory. After about 28 h, the stationary phase is introduced, in which the cell density became stagnant. Remaining glucose was consumed without an increase in cell density (see Fig. 9b). In the same time, concentration of lactate and ammonium, as main waste products, increased to a maximum concentrations of 1,96 g/L and 1.58 mmol/L respectively. Due to the complete consumption of substrates and high metabolite concentrations, the viability dropped below 40% after 87 h.

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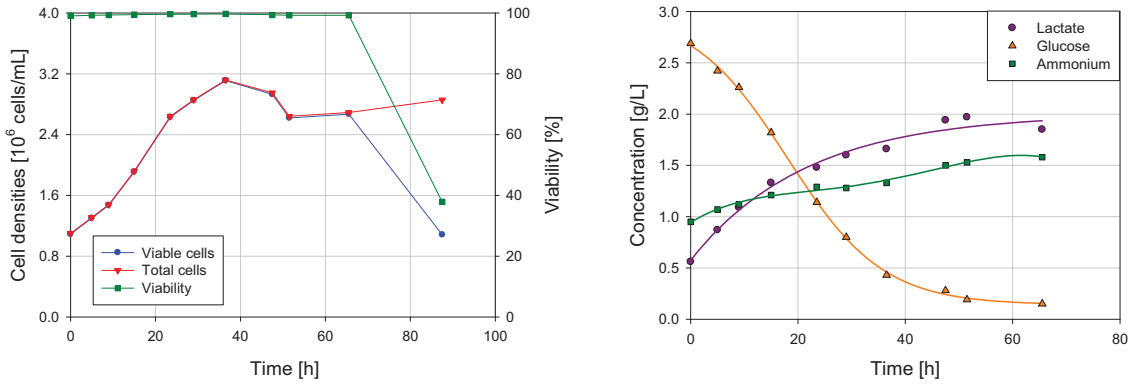

Fig. 9: Growth kinetics of CHO suspension cells in single-use 250 mL shake flask. (a) Cell densities measured with NucleoCounter NC-100. (b) Glucose and lactate concentration measured with Bioprofile-100. Symbols indicate measured data, the lines are regression curves.

Despite the typical growth curve, no early indication of intracellular changes of the CHO cells were observed (Fig. 10). After a decreasing vitality in the exponential phase from 92 % to 86 %, an increase to almost 95 %, which is higher than the initial value, was achieved during the stationary phase. Even the last sample had a vitality of 84 %, although the viability already dropped to 40 %. This behavior should be reinvestigated, but was not part of this study. However, due to these results the vitality assay was not used for the characterization of cell culture health during stress experiments.

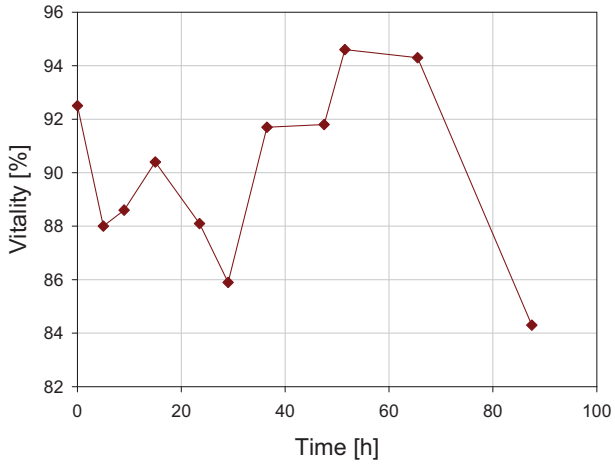

Fig. 10: Time course of the vitality obtained by the vitality assay. For the evaluation of vitality, the cell population was divided in two subpopulations (see section 2.2). The first sample was used as reference.

3.4 Results of the mechanical stress experiments

3.4.1 First test case

In the first test case, the rotational speed of the Levitronix BPS-200 was set to 1500 rpm without the use of a hose clip. The initial living cell densities before start of pumping were between 2.38 and 2.73 x 10^6 cells/mL at a viability of about 97%. The glucose, lactate and ammonium concentrations were 0.45 g/L, 1.89 g/L and 1.36 mmol/L respectively (see Tab. 3).

Tab. 3: Initial values for cell densities and concentrations before start of pumping

Component	Unit	Value
Living cell density	10^6 cells/mL	2.57 ± 0.17
Total cell density	10^6 cells/mL	2.64 ± 0.17
Viability	%	97 ± 0.3
Glutamine	mmol/L	0
Glutamate	mmol/L	0.02
Glucose	g/L	0.45
Lactate	g/L	1.89
Ammonium	mmol/L	1.36

As shown in Fig. 11, the viable cell density of the cultures pumped by the two peristaltic pumps decreased from the initial value of 2.5 x 10^6 cells/mL within 400 minutes to 0.46 and 1.2 x 10^6 cells/mL respectively. A sharp decrease of the viable and total cell densities of up to 30 % was observed with the peristaltic pumps directly at the beginning of the experiment.

A certain fraction (about 5 to max. 10 %) can be explained by a dilution of the cell suspension by remaining water inside the tubing. Additionally, it is known, that suspension cells react to mechanical stress by cellular adhesion to surfaces [Boccafoschi et al. (2010)]. If the cells attached to the wall, they are not detected by sampling. This assumption would be supported by the higher decline in cell density for subsequent test cases, where higher mechanical stress is expected (compare sections 2.4.2 to 2.4.3). Nevertheless, no specific information about the cell adhesion of CHO under the investigated conditions was found. Summarizing, the significant loss of cell density at start could not be completely clarified. Thus, further investigations are required.

In contrast to the peristaltic pumps, the viable cell density for the centrifugal pump remained rather constant with some variations which are probably a result of errors in the measurement and sampling. The viable cell density for the BPS-200 centrifugal pump was 2.2 x 10^6 cells/mL at the end of the experiment, which is only about 7.5 % lower than the initial value, compared to 82 % and 44 % for the two peristaltic pumps.

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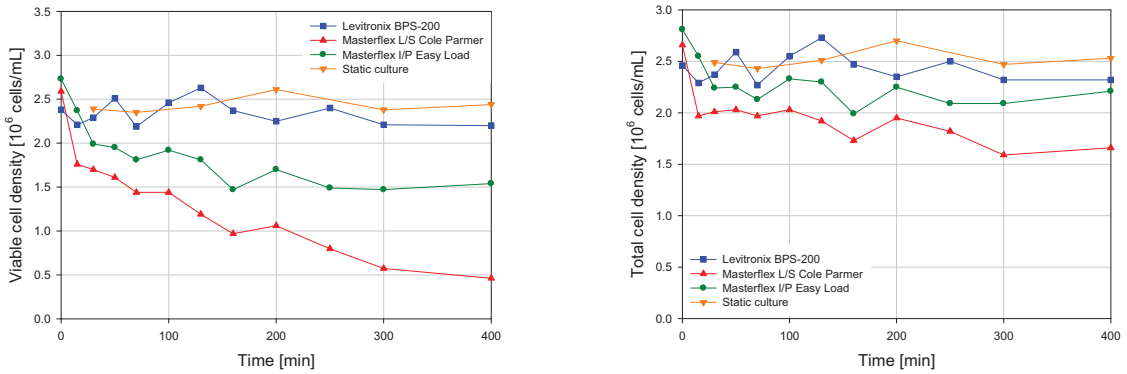

Fig. 11: Time-dependent viable (a) and total (b) cell densities of the cell suspension pumped with 3.4 L/min without back-pressure. Additional, values for the static culture are given.

Interestingly, also decreasing total cell densities were found for the peristaltic pumps. The effect was more significant for the Masterflex® L/S Cole-Parmer, where the total cell density declined to 1.6 x 10^6 cells/mL, compared to the Masterflex® I/P Easy Load, where 2.2 x 10^6 cells/mL were measured at the end of the experiment. As described in section 2.3, the cell density measurement in the NucleoCounter NC-100 is based on the fluorescence detection from the fluorescent dye propidium iodide (PI) which bounds to DNA. Therefore, a loss in total cell density means a reduction of the overall DNA content, which could be a result of proteolysis caused by the activity of proteinases released by damaged cells. A decrease in cell density due to sedimentation can be excluded because of the high flow rate and the resulting low mean residence time of the cell suspension inside the reservoir (about 1 min).

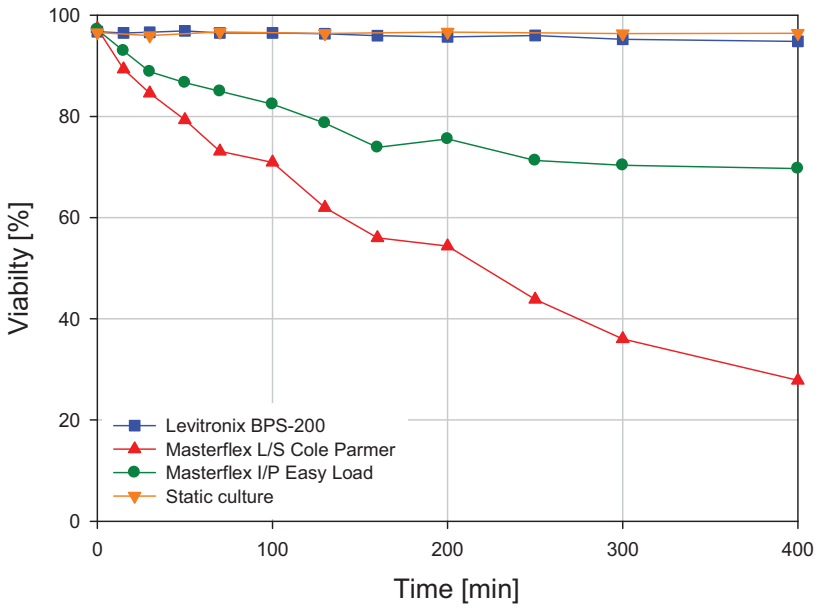

Fig. 12: Time-dependent viability of the cell suspension pumped with 3.4 L/min without back-pressure. Additional, values for the static culture are given.

Due to the decreasing viable cell density, the viability of the cultures pumped by the two peristaltic pumps decreased from the initial value of 97 % to values between 70 and 28 % within 400 min (equal to 400 passages through the pump). In contrast, the viability of the culture pumped by the Levitronix BPS-200 remained high with values of about 95 % over the complete process (see Fig. 12). A small decrease in viability can be explained by the depletion of substrates, as glucose and glutamine, and the high lactate concentration of 1.9 g/L present at the end of the cultivation. This was also observed in the static culture.

3.4.2 Second test case

In the second test case, the rotational speed of the Levitronix BPS-200 was set to 3500 rpm with a back-pressure of 190 mmHg (253 mbar) which leads to the same flow rate of 3.4 L/min as in the first experiment. The initial living cell densities before start of pumping were between 1.95 and 2.22 x 10^6 cells/mL at a viability of about 93 %. The glucose and lactate concentration were 0.18 g/L and 1.96 g/L respectively (see Tab. 4).

As shown in Fig. 13, the viable cell densities decreased from the start value of about 2.1 x 10^6 cells/mL within the first 30 minutes to values about 1.5 x 10^6 cells/mL for all cultures. This is equal to reductions of up to 25 % and 36 % in total and viable cell density. In this experiment, short overshoots of the back-pressure occurred when the restriction of the tubing was adjusted by the hose clip, but it is doubtful that these pressure overshoots had such a significant influence. To avoid these problems, the tubing restriction was adjusted before the start of pumping in the subsequent test cases.

Tab. 4: Initial values for cell densities and concentrations before start of pumping

Component	Unit	Value
Living cell density	10^6 cells/mL	2.08 ± 0.14
Total cell density	10^6 cells/mL	2.24 ± 0.13
Viability	%	92.8 ± 0.7
Glutamine	mmol/L	0.02
Glutamate	mmol/L	0.11
Glucose	g/L	0.18
Lactate	g/L	1.97
Ammonium	mmol/L	1.65

Between 30 and 360 minutes, the living cell density decreased to values between 0.7 and 1.44 x 10^6 cells/mL, whereby the highest reduction again was achieved with the Masterflex® L/S Cole Parmer and the lowest was found for the Levitronix BPS-200. Almost no differences between the pumps were obtained for the total cell density, as shown in Fig. 13b. As in the first experiment, the viability of the cells pumped with the Masterflex® L/S Cole-Parmer pump showed the highest decrease, as given in Fig. 14. For the Levitronix BPS-200 only a slow decrease of the viability was found after a more rapid decline to about 80 % in the first two hours. At the end of the experiment, the viabilities were between 43 % and 77 %, which is significantly lower than in the first experiment and in agreement with the expectations.

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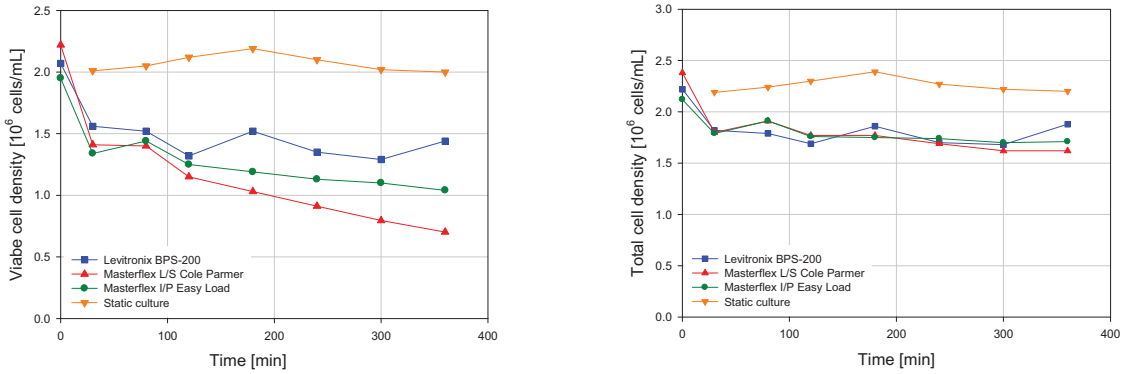

Fig. 13: Time-dependent viable (a) and total (b) cell densities of the cell suspension pumped with 3.4 L/min with a back-pressure of 190 mmHg.

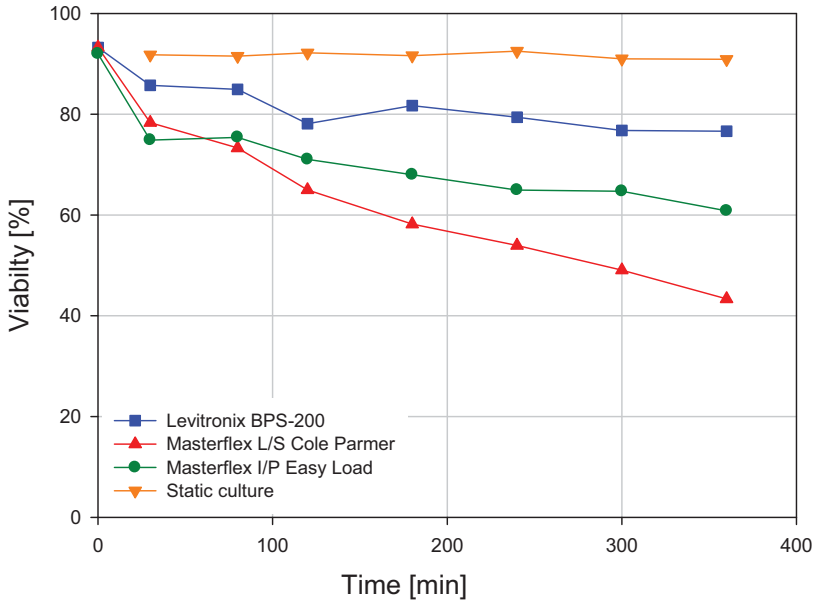

Fig. 14: Time-dependent viability of the cell suspension pumped with 3.4 L/min at a back-pressure of 190 mmHg. Additional, values for the static culture are given.

3.4.3 Third test case

In the third test case, the rotational speed of the Levitronix BPS-200 was set to 4200 rpm with a back-pressure of 288 mmHg (385 mbar), which leads to the same flow rate of 3.4 L/min as in the first two experiments. The initial living cell densities before start of pumping were higher compared to the previous experiments with values between 2.68 and 3.02 x 10^6 cells/mL at a viability of about 97 %. In contrast to the previous experiments, the glucose concentration of the medium at the end of cultivation was rather high (1.24 g/L). The lactate concentration was similar to the previous tests with a value of 1.96 g/L (see Tab. 5).

Tab. 5: Initial values for cell densities and concentrations before start of pumping

Component	Unit	Value
Living cell density	10^6 cells/mL	2.83 ± 0.18
Total cell density	10^6 cells/mL	2.92 ± 0.13
Viability	%	96.7 ± 0.1
Glutamine	mmol/L	0.1
Glutamate	mmol/L	0.09
Glucose	g/L	1.24
Lactate	g/L	1.96
Ammonium	mmol/L	1.06

As shown in Fig. 15, the viable cell densities decreased from the start value of about 3.0 x 10^6 cells/mL within 400 minutes to values between 1.6 x 10^6 cells/mL for centrifugal pump and 0.8 x 10^6 cells/mL for the Masterflex® L/S Cole-Parmer peristaltic pump (see Fig. 15). Compared to the first two experiments, the decrease of the viable cell density was sharper at the beginning, but with longer pumping time it decreases in a similar manner as in the second case, although the mechanical stress is expected to be much higher. This is also indicated by the viability (see Fig. 16). It is well-known that cell cultures are able to adapt to higher shear stress, which is for example utilized to transfer cells from static culture to agitated systems. However, this phenomenon is only observed over several cell generations and is not to be expected as a reason for the higher resistance against the shear stress.

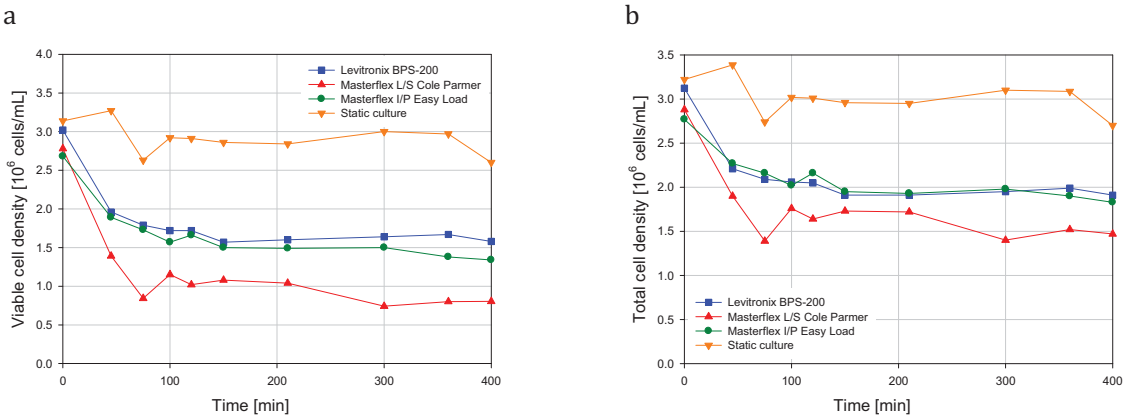

Fig. 15: Time-dependent viable (a) and total (b) cell densities of the cell suspension pumped with 3.4 L/min at a back-pressure of 288 mmHg.

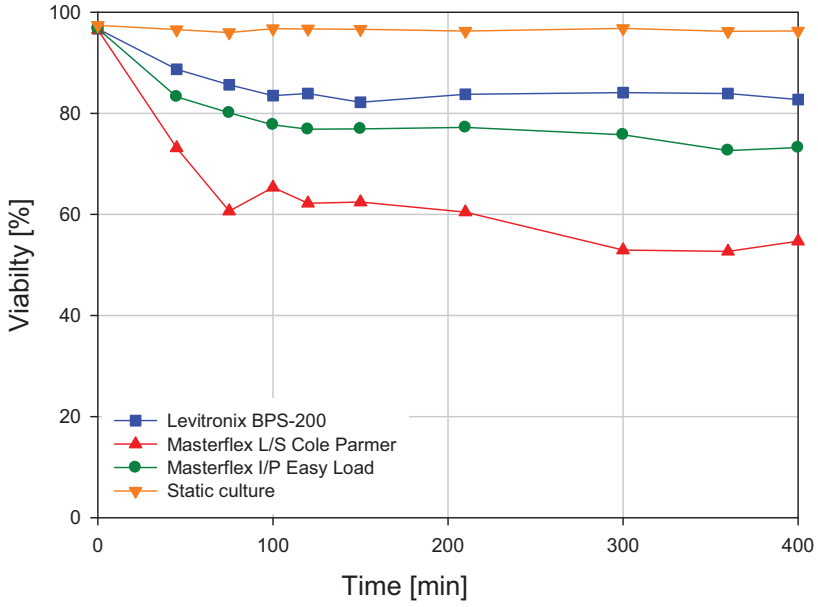

Fig. 16: Time-dependent viability of the cell suspension pumped with 3.4 L/min with a back-pressure of 288 mmHg. Additional, values for the static culture are given.

a b

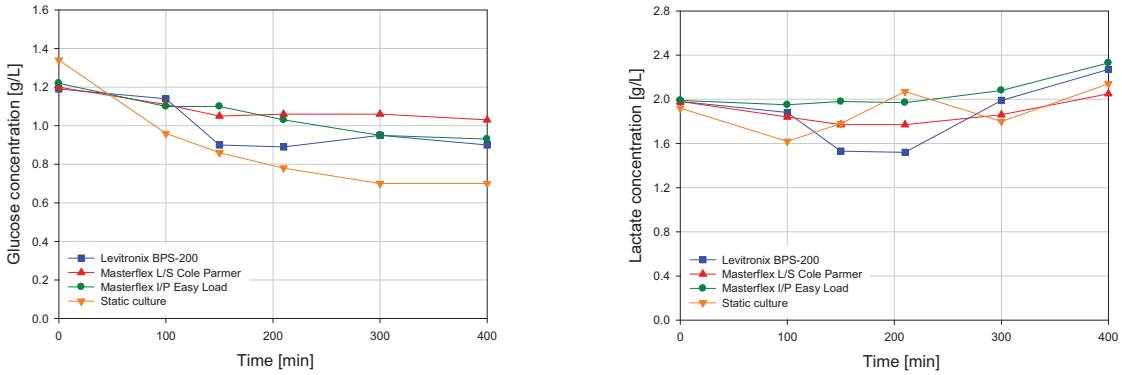

Fig. 17: Time-dependent glucose (a) and lactate (b) concentrations.

As mentioned before, the culture medium contains glucose of about 1.2 g/L at the beginning (see Fig. 17). A reduction of glucose concentration over the complete process was observed in all samples with final glucose concentrations between 0.7 and 1.03 g/L. The specific substrate consumption rate was determined to be about 4 mg/(10^3 viable cells * h) for the BPS-200 centrifugal pump which is only one-tenth of the consumption rate calculated for the cultivation in shake flask described in section 3.3 (data not shown). The lactate concentration increased in the same time from the initial value of 1.96 g/L to values between 2.05 and 2.33 g/L. Nevertheless, no cell growth was found (neither in the static culture).

3.4.4 Fourth test case

In the fourth test case, the rotational speed of the Levitronix BPS-200 was set to 5000 rpm with a back-pressure of 425 mmHg (567 mbar). The initial living cell densities before start of pumping were comparable to the third test case with values between 2.88 and 3.26 x 10^6 cells/mL at a viability of about 97 %. Comparable to the previous case, the remaining glucose concentration was 1.09 g/L, but the lactate concentration was less with a value of 1.39 g/L (see Tab. 6).

Tab. 6: Initial values for cell densities and concentrations before start of pumping

Component	Unit	Value
Living cell density	10^6 cells/mL	3.16 ± 0.1
Total cell density	10^6 cells/mL	3.27 ± 0.1
Viability	%	96.7 ± 0.1
Glutamine	mmol/L	0.17
Glutamate	mmol/L	0.11
Glucose	g/L	1.09
Lactate	g/L	1.39
Ammonium	mmol/L	1.24

As shown in Fig. 18, both viable and total cell density dropped significantly by about 60 and 50 % for the Levitronix BPS-200 and Masterflex® I/P Easy Load within the first hour. For the Masterflex® L/S Cole Parmer an even higher decrease of about 75 % was observed. Afterwards, the cell densities declined rather slowly. No significant difference between the Levitronix BPS-200 and the Masterflex® I/P Easy Load could be found.

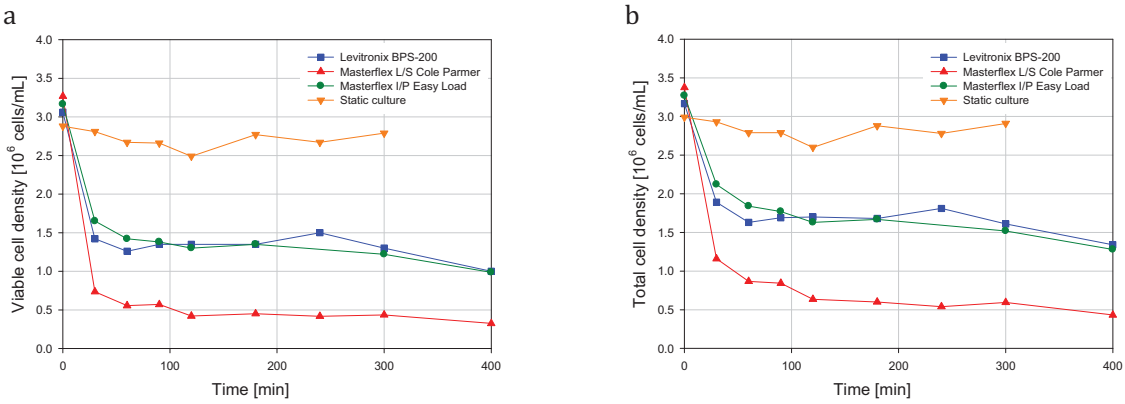

Fig. 18: Time-dependent viable (a) and total (b) cell densities of the cell suspension pumped with 3.4 L/min at a back-pressure of 425 mmHg.

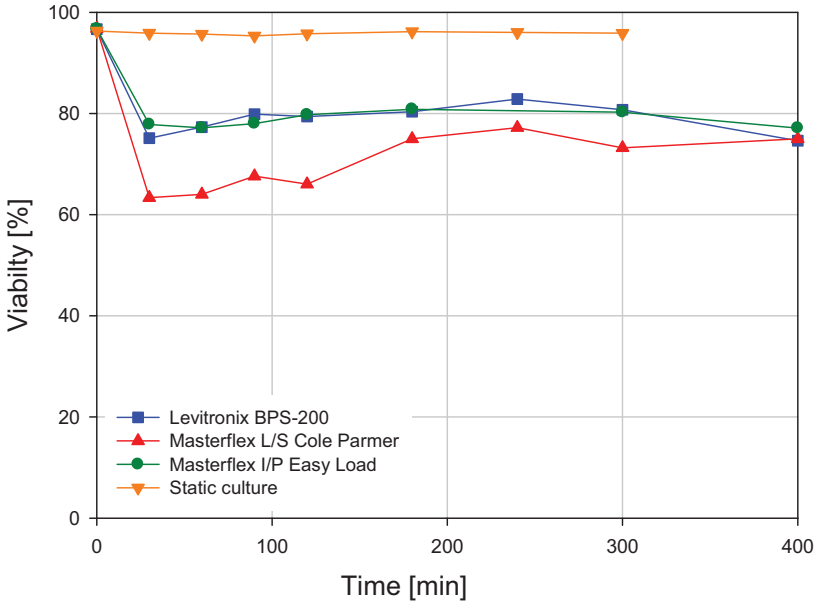

Fig. 19: Time-dependent viability of the cell suspension pumped with 3.4 L/min with a back-pressure of 425 mmHg determined with NucleoCounter® NC-100. Additional, values for the static culture are given.

The time-dependent course of the viability determined with the NucleoCounter® NC-100 device is in clear contrast to the previous test cases. Starting from the a value of 97 % for all samples, the viability for the Masterflex® L/S Cole Parmer dropped down to 64 % within the first 30 minutes. Afterwards, it increased to about 75 % and remained nearly constant until the end of the experiment. This behavior distinguished significantly from the previous experiments and seems not logical, but no reasonable explanation could be found. For the Masterflex® I/P Easy Load and the Levitronix BPS-200 nearly identical curves were obtained. In the first 30 minutes the viability decreased to about 80 % for both pumps, remained nearly constant over 6 hours and showed a slight reduction until the end of the test.

Due to the unexpected results of the viability with the NucleoCounter® NC-100, the viability was additionally determined with the Cedex HiRes cell counting device. As shown in Fig. 20, the viabilities determined by the Cedex HiRes distinguish from the previous described results. The initial viability was somewhat lower compared to the NucleoCounter® NC-100 result with values of about 90.5 %. It is known from cultivations in our laboratory that viabilities determined with the Cedex HiRes® are approximately 5 to 10 % lower than for the NucleoCounter® NC-100. Comparable to the aforementioned data, the viabilities dropped within the first 30 minutes to about 80 % for the Levitronix BPS-200 and the Masterflex® I/P Easy Load and to 66.4 % for the Masterflex® L/S Cole Parmer peristaltic pump. Afterwards, all samples reveal a slow reduction of viability until the end of the experiment.

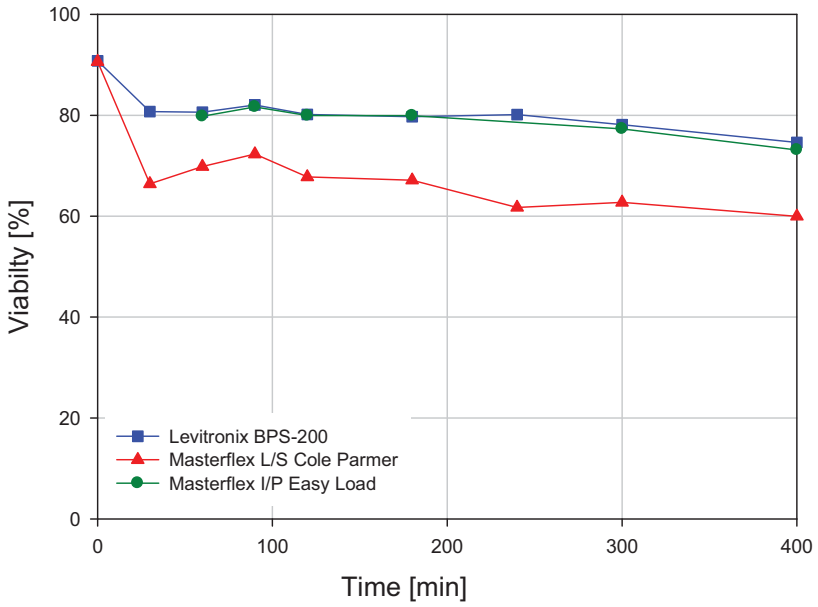

Fig. 20: Time-dependent viability of the cell suspension for the fourth test case determined with Cedex HiRes. For the static culture no additional measurements were realized.

3.4.5 Additional indicators for mechanical stress

As described in section 2.3, besides viable and total cell density additional parameters, such as aggregation rate, cell compactness and average cell diameter, are determined by the automated cell counting device Cedex HiRes. Hence, it was evaluated whether these parameters are suitable to indicate mechanical stress.

a b

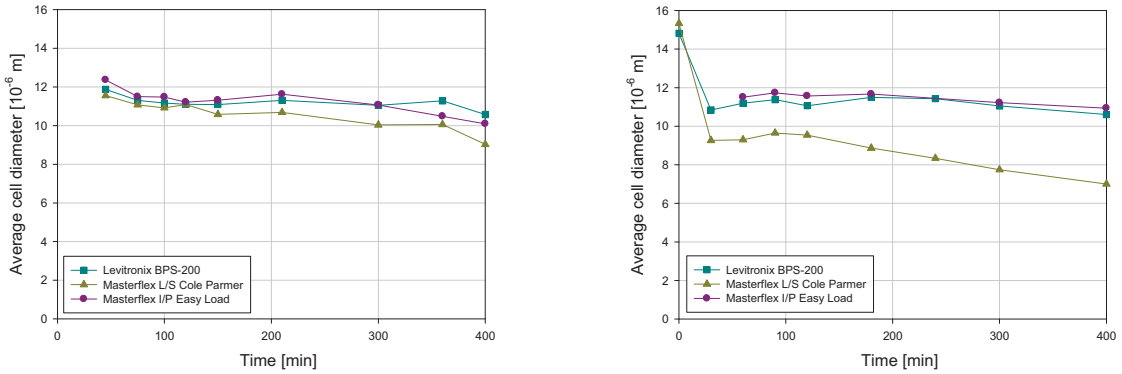

Fig. 21: Time-dependent average cell diameter determined by Cedex HiRes® for the third test case (a) and the fourth test case (b).

Typical graphs for the time-dependent average cell diameter are given in Fig. 21. The average cell diameter at the end of the cultivation was about 15 µm, which is routinely found in our laboratory depending on the growth phase and the cultivation system. For all investigated cases, it was found that the cell diameter decreased with longer pumping time. In the third test case, the average cell diameter declined to about 9 µm for the Masterflex® L/S Cole Parmer pump. At the highest pressure, even values below 7 µm were obtained (see Fig. 21b). In contrast, for the Levitronix BPS-200 only small changes were found in the first test case (data not shown), where also no significant differences in the cell viability were determined. In other words, decreasing cell diameter means increasing shear stress for the cells. This phenomenon is also evident when the used CHO cells are chemically stressed, as observed in previous studies realized in our laboratory.

As shown in Fig. 22, for mechanical stressed cells a second peak in the histograms appeared at lower cell diameter, which means that two cell diameters were frequently found. This can be explained by cell breakage and the formation of cell debris. However, no clear correlation between mechanical stress and the resulting cell diameter was found. For this reason, the cell diameter should only be used as a qualitative criterion for shear stress.

Moreover, changes in the cell compactness and aggregation rate were found, but in contrast to the cell diameter no general trend could be determined for these two parameters (data not shown). Hence, they do not seem to be suitable for the evaluation of mechanical stress.

a b

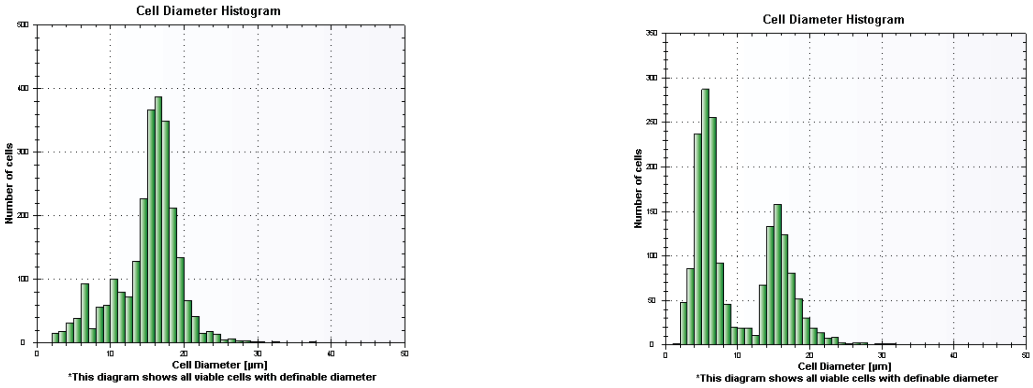

Fig. 22: Typical histograms of the cell diameter for (a) non-stressed and (b) mechanical stressed cells determined by cell counter Cedex HiRes.

3.5 Evaluation of mechanical stress by cell death kinetics

As discussed in the previous sections, mechanical stressed caused on CHO suspension cells was evaluated by the time-dependent viable cell density and the viability, which is the ratio of living to total cell density. Nevertheless, it is difficult to use only the time course for quantitative evaluation and comparison of different mechanical stress values. Hence, the cell death rate was calculated for each experiment assuming first order cell death kinetics, as explained in section 2.4.

The calculated cell death coefficient k_D and the determination coefficients R^2 are summarized in Tab. 7. In general, it can be seen that the death constants for the BPS-200 centrifugal pump are significantly lower than for the peristaltic pumps over the complete range of investigation. Due to the low values of k_D (which is represented by the slope of the logarithmical viable cell density over time), the model regressions are rather uncertain resulting in low values for the determination coefficient (between 0.162 and 0.544). In contrast, excellent approximations were found for the peristaltic pumps ($R^2 > 0.85$), which means that the decrease of viable cell density is well predicted by first order kinetics.

Tab. 7: Results of the regression analysis for the cell death kinetics.

Flow rate	Pressure	Levitronix BPS-200		Masterflex® L/S Cole Parmer		Masterflex® I/P Easy Load	
[L min^{-1}]	[mbar]	k_D [h^{-1}]	R^2 [-]	k_D [h^{-1}]	R^2 [-]	k_D [h^{-1}]	R^2 [-]
3.4	6.5	0.009	0.203	0.141	0.903	0.052	0.946
3.4	253	0.020	0.272	0.133	0.982	0.046	0.996
3.4	385	0.017	0.544	0.077	0.894	0.045	0.876
3.4	567	0.013	0.162	0.072	0.769	0.075	0.933

The cell death coefficient determined for the Levitronix BPS-200 increased from the first to the second case (k_D circa doubled from 0.009 to 0.02 h^{-1}). Two reasons for the higher mechanical stress are possible. On the one hand, the higher mechanical stress can be caused be the pump itself. With increasing pump speed the fluid velocities inside the pump increase as shown by CFD simulations [Zhang *et al.* (2006)]. The higher fluid velocities (respective the higher tip speed of the pump blades) result in higher turbulence, which can be quantified by the Reynolds number. Chua *et al.* (2006) give a Reynolds number of about 78'500 for a rotational speed of 2000 rpm, which is much higher than typical Reynolds number observed during cell cultivation processes. Turbulence, which is defined as three-dimensional stochastic temporal fluctuations of the fluid velocities, is often attributed to mechanical stress caused on cell cultures [Chisti (1993); Christi(2000); Wollny (2000)].

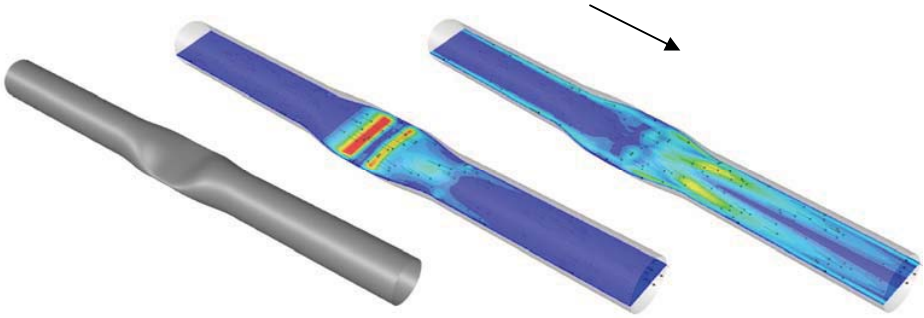

Fig. 23: CFD predicted distribution of normal (middle) and shear gradients (right) inside a tube with a restriction similar to the geometry used in the biological experiments. Red color represents highest values, blue color means low values. The main flow direction is indicated by the arrow and by the fluid velocity vectors.

On the other hand, the higher cell damage in the second case could be explained by the restriction of the tubing. Realizing the same flow rate of 3.4 L/min, the tubing was restricted by a hose clip resulting in a higher back-pressure of about 250 mbar. In doing so, an additional turbulence region with enhanced shear and normal gradients was introduced to the closed loop (see Fig. 23). In addition to turbulence, high velocity gradients are considered as main reason for hydrodynamic stress, although it is discussed in literature whether cells are stronger influenced by normal or shear gradients [Wollny (2010)]. However, the cell death coefficients obtained with the two peristaltic pumps do not differ significantly between the two pressures (compare Fig. 23). Thus, it can be argued that the higher hydrodynamic stress obtained for the Levitronix BPS-200 pump in the second case was not a result of the restriction respective the pressure drop, but a result of the higher rotational speed inside the pump.

Contrary to expectations (but already indicated by the time course of the vitality), the cell death coefficient did not increase in the third and fourth test cases (which means with higher rotational speed respective pressure). The calculated k_D value for the Levitronix BPS-200 was 0.017 h^{-1} and 0.013 h^{-1} for 4200 rpm and 5000 rpm respectively. For the Masterflex® I/P Cole Parmer even a decrease of k_D was observed for the higher pump speed. Here, the cell death coefficient was only about 0.075 h^{-1}.

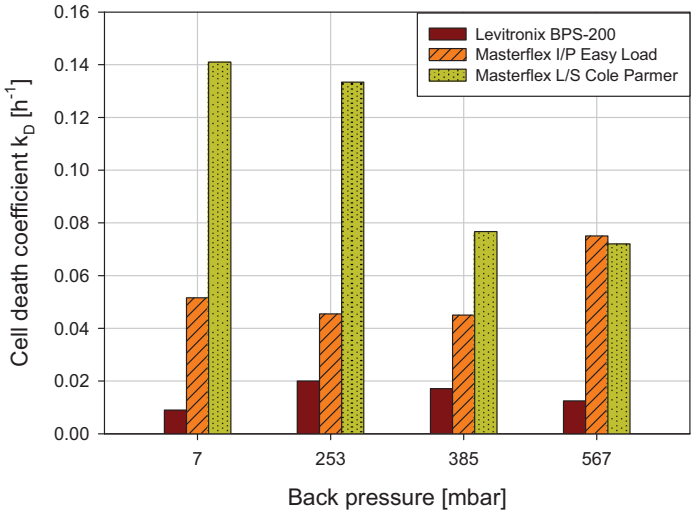

Fig. 24: Determined cell death coefficients for the three pumps against the rotational speed of the Levitronix BPS-200.

As described in section 3.4.3 and 3.4.4, the main difference regarding to the cell culture was the rather high concentration of remaining glucose at the end of the cultivation respective the start of the pumping experiment. The slightly higher cell density of 3.0 x 10^6 cells/mL compared to 2.0 and 2.5 x 10^6 cells/mL is not expected to have a significant influence on the results. As mentioned in section 3.4.3, no cell growth was observed although remaining glucose was consumed. Hence, it could be argued that the substrate was consumed for maintenance metabolism, which has potentially a positive effect on the resistibility of the cell culture against hydrodynamic stress. Additionally, the cells used during the first two experiments were probably chemically stressed due to the low glutamine and glucose concentration. Glucose is the essential carbon source for most cell lines and glutamine as an important source for the synthesis of purines, pyrimidines and amino sugars [Kretzmar (2000)].

It is well-known, that apoptosis can be induced by chemical stress, such as low substrate concentrations or suboptimal pH value, resulting in mechanically more sensitive cell cultures. However, no literature data was found regarding to a combination of chemical and mechanical stress. Hence, these phenomena have to be further validated.

4 Conclusion and outlook

The aim of the project was to evaluate mechanical stress caused on CHO suspension cells by the magnetically driven centrifugal pump Levitronix BPS-200. For this purpose, different rotational speeds in the range of 1500 to 5000 rpm were investigated.

Different parameters for the evaluation of mechanical stress were tested. In a preliminary test, the vitality assay provided by Chemometec was investigated for its suitability in the pump tests as offered in the project proposal. Contrary to expectations, no relation between cell growth respective death and the vitality (represented by the reduced thiol content of the cells) was found. Better results were obtained with the viable cell density and viability determined by two different automated cell counting devices, NucleoCounter® NC-100 and Cedex HiRes. Clear

decreases of the viable cell density were measured, if the cells were mechanically stressed. Additional parameters, such as average cell diameter and cell compactness, were evaluated. It was shown, that the cell diameter and its distribution are suitable as an indicator for mechanical stress, but no quantitative correlations could be found.

In general, the mechanical stress on CHO suspension cells caused by different pumps could be validated. The first two experiments showed that a higher pumping speed (respectively pressure) results in higher cell damage for the centrifugal pump expressed by higher cell death rate. The third and fourth test cases indicated that further increase of the pump speed do not lead to higher cell damage which is probably a result of remaining substrates in the medium. As described before, pathological processes (apoptosis), which are known to be induced by chemical stress, are expected to play a role. The metabolism of animal cells and their responses to shear stress are rather complex [Kretzmer (2000)]. Thus, these phenomena have to be further validated.

For quantification of mechanical stress and quantitative comparison between the different pumps and test conditions, regression models were developed assuming first order cell death kinetics. Good approximations (represented by the determination coefficient) were obtained for the peristaltic pumps. The cell death coefficients found for the Levitronix BPS-200 centrifugal pump showed significantly lower determination coefficients (R^2), but this is reasonable as the viable cell density decreased not or only slightly, depending on the adjusted rotational speed. Therefore, it can be argued that the proposed regression model can be used in further investigations to evaluate mechanical stress.

In further investigations, the influence of the different flow rates should be considered. All tests in this study were realized for a flow rate of 3.4 L/min. At a total liquid volume of 3.4 liter inside the cell reservoir and tubing the cells were pumped through the complete loop within one minute. In experiments with blood it was demonstrated that the hemolysis level depend both on the magnitude of the mechanical stress and the exposure time [Bludszuweit (1995); Giersiepen *et al.* (1990); Yeleswarapu *et al.* (1995)]. The exposure time respective the residence time of the cell suspension inside the pump could be varied by different flow rates and working volumes.

Moreover, investigations with non-biological test systems should be conducted. In this study, variations in the response to mechanical stress could be attributed to the conditions of the used cell culture (composition of the culture broth etc.). In literature, different test systems, such as oil-in-water emulsion or clay–polymer particle, are described [Wollny (2010), Stintzing *et al.* (2008)]. It has been demonstrated that the particle or drop sizes (distributions) depend on the hydrodynamic forces and can be used as an indicator of mechanical stress. The main advantages of these systems are the high reproducibility and the reduced costs compared to biological experiments.

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6 Appendix

Application Note – Chemometec vitality assay

Technology that counts

chemometec

Application note No. 3005. Rev. 1.1

NucleoCounter® NC-3000™

Vitality assay: Analysis of the level of cellular thiols using the NucleoCounter® NC-3000™ system

Product description

The NucleoCounter® NC-3000™ system enables the user to perform automated cell counting and analyses of a broad range of eukaryotic cells.

Application

This protocol for the NucleoCounter® NC-3000™ system enables the user to detect changes in the intracellular level of (reduced) thiols. Such changes may occur in apoptotic cells or cells undergoing other pathological processes. As the intracellular reducing power available to the cell is an indicator of overall health status, this assay provide a very easy and fast way to evaluate cell vitality.

Introduction

This application note describes a method for investigating apoptosis and cell health by determining the level of free thiols such as reduced glutathione. The tripeptide glutathione exists in two forms, a reduced state (GSH) and in an oxidized state; glutathione disulfide (GSSG). In the reduced state the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to unstable molecules such as free radicals. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form GSSG. GSH can be regenerated from GSSG by the enzyme glutathione reductase. (See ***Figure 1***.)

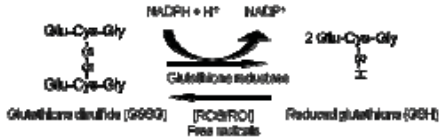

Figure 1. *Glutathione redox cycle. The flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase reduces GSSG to GSH. When the cell encounters free radicals, the antioxidant GSH reduces the free radicals, thereby itself becoming oxidized to GSSG.*

GSH is the most abundant low molecular weight thiol in animal cells; thus, its oxidation status largely determines the thiol-disulfide status of the cell by thiol-disulfide interchange reactions. Moreover, GSH is involved in many cellular processes including quenching of free radicals, drug detoxification, cell signaling, and cell proliferation. Alterations in the concentration of intracellular GSH have been demonstrated as a common feature of many diseases including AIDS, neurodegenerative diseases, and cancer.

A decrease in cellular GSH concentration is an early hallmark in the progression of cell death in response to different apoptotic stimuli. Studies have shown a correlation between cellular GSH depletion and the progression of apoptosis. The decrease in GSH level in connection to apoptosis seems to be attributed to two mechanisms; A) direct GSH oxidation promoted by radicals and B) export of GSH through an ATP-dependent plasma membrane transport system which is triggered by the initiation of apoptosis. When GSH is depleted, the cytosol is shifted from a reducing to an oxidizing environment, which may lead to a further depletion of GSH.

This assay provides a very easy method to quantify the amount of free thiols at the single cell level. The stain VitaBright-48 (which is a component of Solution 5) immediately reacts with thiols forming a fluorescent product. By quantifying the fluorescence it is possible to determine the level of cellular thiols, and thus determine cell health.

Principle

In this application note, a method for measuring the cellular level of thiols is described. The cells to be investigated are mixed with Solution 5. The solution contains three different reagents: a stain staining all nucleated cells, a stain staining dead cells only and VitaBright-48™ which stains viable cells in an intensity-dependent manner depending on their level of thiols. A high fluorescence intensity of a particular cell indicates that the cell has a high level of thiols such as GSH.

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Technology that counts

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The stained cells are immediately loaded into a NC-Slide: either the 2-chamber NC-Slide A2™ or the 8-chamber NC-Slide A8™. Samples are analyzed using the NC-3000 system. A fluorescence intensity histogram showing the distribution of thiol levels in all cells are displayed on the PC screen. By comparing histograms of treated cells to controls the fraction of cells with low vitality (e.g. apoptotic or stressed cells) can be determined.

Procedures

If the cell line to be investigated is adherent or semi-adherent, then start by getting all cells into suspension using the preferred method of your laboratory (e.g. trypsin/EDTA treatment). Although the NucleoCounter® NC-3000™ is able to count aggregated cells, the accuracy is higher for single cell suspensions.

Materials needed

- Cells to be stained [1, 2]
- Solution 5 (VB-48·PI·AO)
- **NC-Slide A2™** or **NC-Slide A8™**

[1] Cells provided by the user.
[2] An untreated control should be included. Preferable, use logarithmically proliferating cells as control.

1. Pipette a representative cell sample from the cell suspension into a microcentrifuge tube. Add one volume of Solution 5 into 20 volumes of the cell suspension. E.g., if the volume of the cell suspension is 190 µL then add 10 µL Solution 5. Mix by pipetting.
2. Engage NucleoCounter® NC-3000™ by starting the accompanying software.
3. Depending on the number of samples a 2-chamber slide (**NC-Slide A2™**) or an 8-chamber slide (**NC-Slide A8™**) can be used.
    a. **NC-Slide A2™**: Load 30 µl of each of the cell suspensions into the chambers of the NC-Slide. Place the loaded NC-Slide on the tray of the NucleoCounter® NC-3000™ and select "**Vitality protocol**" and sample unit **NC-Slide A2™** and press RUN.
    b. **NC-Slide A8™**: Load 8 µl of each of the cell suspensions into the chambers of the NC-Slide. Place the loaded NC-Slide on the tray of the NucleoCounter® NC-3000™ and select "**Vitality protocol**" and sample unit **NC-Slide A8™** and press RUN.

Cellular fluorescence is quantified by NC-3000 and the VitaBright-48 intensity is displayed in a histogram. Cells with a low level of thiols (e.g. apoptotic cells) also have a low intensity score, and are thus found in the lower end of the histogram. By placing the marker in the histogram, the user can divide the cell population into two (or more) subpopulations; e.g. divide the cell population into a fraction of cells with a low thiol level and another fraction with high thiol level, and if desired, an intermediate thiol level cell subpopulation. Typically the histogram shows two distinct populations, and in this case the marker is placed in between the two peaks (See example in ***Figure 2***). The markers can be stored and retrieved in a new histogram. Thus, the position for the markers can be determined using an untreated control, and then retrieved for the treated cell sample. As nonviable cells always have a very low level of thiols, only the fluorescence intensity of viable cells is represented in the histogram.

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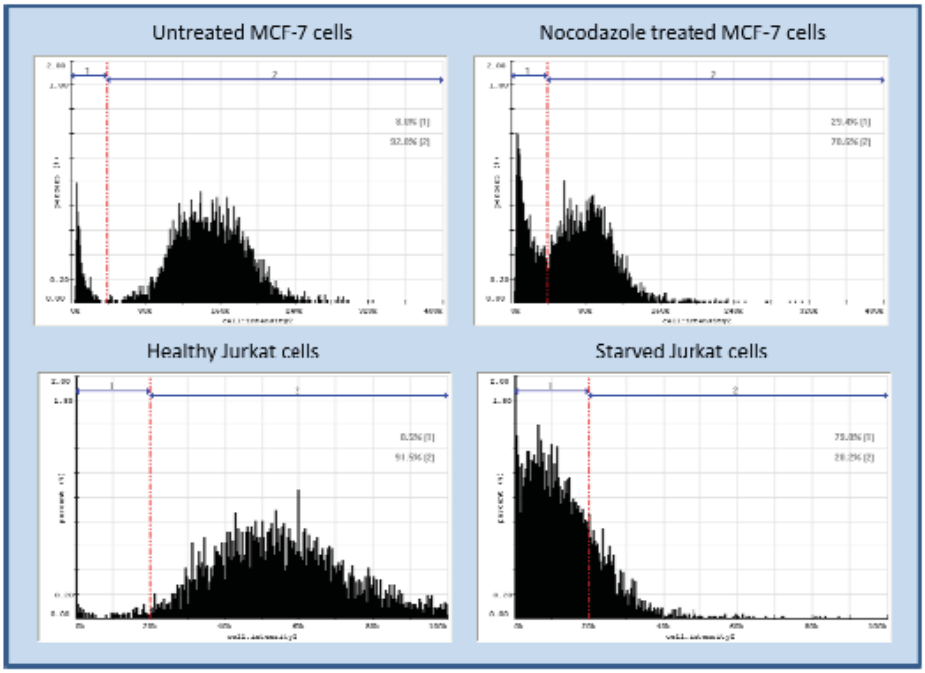

Figure 2. *Fluorescence intensity histogram for untreated MCF7 cells (upper left panel) and nocodazole treated MCF7 cells (upper right panel). Nocodazole treatment causes a decrease in the level of thiols in a subpopulation of cells (cells with low fluorescence intensity). The marker is placed in between the two populations in the first histogram (untreated MCF7 cells). Storing and retrieving the stored marker in the second histogram (nocodazole treated MCF7 cells) makes quantitative comparison possible. Below is fluorescence intensity histogram for healthy Jurkat cells (lower left panel) and starved Jurkat cells (lower right panel). Starvation causes a decrease in the thiol level of the cell population seen as a decrease in fluorescence intensity.*

Note

To assure reliable results, it is recommended that the total cell concentration of the cell suspension should be in the range of $5{\cdot}10^4$ cells/mL to $5{\cdot}10^6$ cells/mL. If the concentration of cells is below $5{\cdot}10^4$ cells/mL then the cell concentration may be increased by centrifugation followed by resuspension of the pellet using growth media or PBS. The resuspended cell sample is then treated as described above.

If the total cell concentration is above $5{\cdot}10^6$ cells/mL, the cell suspension can be diluted with growth media or PBS to achieve the desired concentration. The diluted cell sample is then treated as described in the procedure.

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