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UNCERTAINTY ANALYSIS OF CELL COUNTING BY METABOLIC ASSAYS

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Abstract: Cell counting is an important procedure in cell biology and its uncertainty evaluation is fundamental to improve cell-based measurement comparability which is currently variable. In this study, for the first time, the uncertainty of a metabolic assay for cell counting based on fluorescence intensity measurement has been evaluated. Results, in 2D cell culture, revealed that three are the main components: repeatability and reproducibility of the measurement system and a contribution due to the sample loading step procedure. This study represents the basis of a 3D cell culture in which metabolic methodologies are the most suitable method to monitor cell growth.

Keywords: 2D and 3D cell culture, scaffold, cell proliferation rate, CellTiter Blue, resazurin, uncertainty analysis.

1. INTRODUCTION

Cell counting is a fundamental procedure in living cell culture-based experiments and protocols in which the cell number determination is required. Cell number quantification is one of the parameters to investigate several cell culture features such as cell viability, proliferation, growth, fitness and metabolism which require a monitoring as function of time [1]. To quantify cells number is considered a measurement service requested by NMIs related to the quantification of cells in cells adhered and 3D cells measurement in tissues [2]. Cell counting is the quantification of the quantity of organism in a bioreactor. A bioreactor is any limited volume in which a cell culture evolves.

Cell number can be determined by removing cells from the bioreactor and counting them with either a hemocytometer or an electronic cell counter, or by cell image analysis, or by assaying a metabolic activity of the cells within the bioreactor (e.g. intracellular enzymes activity) [3], when the activity is proportional to the number of cells explicating that activity.

The measurement of cell viability and proliferation *in vitro* provides important information about the bioreactor biocompatibility and its influence on cells state, including cell fitness, cell metabolism and cell differentiation. In addition, by monitoring cell proliferation an differentiation *in vitro*, many assumptions and predictions about cell behaviour in *in vivo* situations, can be made.

The possibility to perform sequential measurement in the same cell population is extremely important in cell proliferation analysis. However, cell counting methodologies are mostly destructive or invasive. In addition, in the last few years, three-dimentional (3D) cultures, mimicking the cells native microenvironment better than the traditional bi-dimensional (2D) culture [4], are being developed and used. However, often in 3D cultures the detachment of cells as well as the observation of cells are not possible, thus indirect assays are the only applicable methods to assess the cell number quantification.

In the present study a metabolic, indirect, non destructive method has been used to assess cell proliferation.

Metabolic assays measure a metabolic activity accounting for vital cells only and quantify the number of individuals under the hypothesis that all the active individuals show a metabolic activity and that the mean of these individual activities is stable.

The metabolic activities of a cell vary with the cell phenotype (e.g. undifferentiated and differentiated phenotype) and the phenotype changes depend on several phenomena (influence parameters) occurring in cell culture environments, such as glucose limitation or pH modification of the medium, which influence the metabolic activity and introduce artefacts into the metabolic assays [5]. Hence, when the cell number estimation is based on a cell metabolic activity it is extremely important to consider that metabolic assays are affected by various influence parameters and their entire characterization requires a deep and intense analysis of the expressed phenotype, aiming to evaluate the uncertainty of the methods. In a 3D system (also called scaffold) the metabolic activity measurement is obtained by measuring the concentrations of a substrate or a specific metabolic product over time.

There are at present some metabolic reaction-based methods in the literature for monitoring animal cell number (giving information on cell proliferation) over time [6, 7]. However, most of them requires the use of substances that are cytotoxic [6], or results in cell lysis to measure the metabolic product [6,7,8]. The method chosen to be analyzed in this work is based on the reduction of resazurin into the end product resorufin. It is not cytotoxic and do not require any cell damaging steps [9].

This work aims to emphasize the procedures for the uncertainty evaluation of a metabolic method for measuring the cell number. The innovative contribution of this work is because metabolic methods to determine the cell number,

although widely used, have never been metrologically characterized, neither in 2D nor in 3D cell cultures.

This study is applied to human mesenchymal stem cell (hMSC) 2D cultures, using the CellTiter-blue® method (CTB), a metabolic assay based on the resazurin conversion to resorufin to evaluate the cell number and estimate the cell proliferation.

2. MATERIALS AND METHODS

CellTiter-blue® proliferation assay: The CTB proliferation assay is a fluorimetric and metabolic assay to determine the number of living cells in a biological *in vitro* system. It is based on a redox reaction: the reduction of resazurin (dark blue in color, redox dye with a slight intrinsic fluorescence) into the end product resorufin (pink, highly fluorescent molecule, $579_{Ex}/584_{Em}$), made by redox enzymes. Only living cells can perform this reaction because nonviable cells rapidly lose metabolic capacity and do not generate any fluorescent signal [9]. O'Brien et al. (2000) proposed the resazurin dye, the original name of the Alamar Blue dye, to assess the mammalian 2D cell cultures cytotoxicity [10]. Resazurin solution is added to the bioreactor as 10-20% in volume. Fluorescence intensity has been measured after a contact time (0.5 to 6 h).

Fluorescence measurement system: The GloMax®-Multi Microplate Multimode Reader (Promega) was used to measure fluorescence intensity. Excitation was performed at 525 nm wave length while emission was measured in the range 580-640 nm. 96 well plates were used.

Cell seeding and cell culture medium: hMSCs were purchased from Lonza (Basel, Switzerland). They are bone marrow derived-hMSCs from donor. hMSCs were expanded and maintained in a complete non differentiating growth medium (aMEM, Listarfish) supplemented with 10% fetal bovine serum (FBS, Listarfish), 1% L-glutamine, 1% antibiotics (kanamycin). Cells were grown in monolayers, until 80-85% of confluence, they were washed once with 1X Phosphate Buffer Saline (PBS), detached with 0.05% trypsin, counted by means of a hemocytometer and suspended at several concentrations (cells/ml) in 100 µl complete non differentiating growth medium to be seeded and cultured statically in 96 well plates. Then, cells are posed in the incubator at 37°C with 5% CO_2 over night to let the cells to adhere to the bottom of the wells.

3. PROTOCOL ANALYSIS

CellTiter-Blue reagent is a buffered solution containing highly purified resazurin (substrate of the reaction). An aliquote of the reagent is added directly to cells in culture into the bioreactor. The resaruzin molecule can penetrate cells by passing the cell membrane and into the cytoplasm is reduced by cytosolic, microsomal and mitochondrial redox enzymes producing the fluorescent resorufin (reaction product). Resorufin diffuses out of cells back to the culture medium which alone does not reduce resazurin [11].

The number of cells in the bioreactor is considered proportional to the total metabolic activity of cells. The metabolic activity is the reaction rate and can be calculated by the substrate consumption rate or by the product formation rate. The substrate can have a limiting effect, thus is usually supplied in excess. The formation of the product is measured. The product concentration in the culture medium is detected by measuring the fluorescence intensity due to an adequate excitation. The reaction rate can be calculated as the variation of product concentration over time. Usually, the measure of product concentration at a standard time point provides a measure of the mean reaction rate from the starting time point to the standard time point of measurement.

Under the hypothesis that the metabolic activity of the cells is stable, i.e., the measuring time is very lower than duplication characteristic time, the number of cells N in the bioreactor is proportional to the product molar concentration in the liquid phase $C_{Prod,t}$ available in the bioreactor at time t and to the volume of the liquid phase in the bioreactor V_{LF}.

$$N = K_1 C_{Prod,t} V_{LF} \qquad (1)$$

The product concentration at time t in the liquid phase is proportional to the fluorescence intensity emitted by the molecules of product $I_{f,Prod,t}$.

$$C_{Prod,t} = K_2 I_{f,Prod,t} \qquad (2)$$

The cell culture medium and the substrate could emit in the same spectrum region and lead to modifications of the fluorescence intensity. It is, hence, necessary to measure the blank (b) in absence of cells but with times and treatments analogous to those of the samples (s) with cells.

$$I_{f,Prod,t} = \left(I_{f,s,t} - I_{f,b,t}\right) \qquad (3)$$

Hence, the measurand equation can be written as:

$$N = K\left(I_{f,s,t} - I_{f,b,t}\right)V_{LF} \qquad (4)$$

The fluorescence intensity is determined by the instrument used for the measure. An influence quantity is the sample volume loaded in the well. Thus, an analysis of the fluorescence intensity sensitivity with respect to the sample volume loaded in the well needs to be carried out to evaluate the sensitivity coefficients.

The cell culture medium can interfere also on the reaction rate. Hence, two different media added each one with two different sierum quantities have been analyzed. The difference of fluorescence intensity of the different media as function of the contact time τ_c, i.e., contact between resazurin and cells, for two different cell concentrations (cell number/ml) seeded on wells, has been evaluated.

Several measurement instruments are available for measuring the fluorescence intensity. Results coming from different systems are not easily comparable to each other. In this work it has been used one single measurement system,

which is routinely used for diagnostic measures due to the possibility to perform up to 96 analysis at the same time.

The fluorescence measurement needs to be characterized with respect to the adopted measurement system. In the specific case, the fluorescence measurement can be influenced by: the repeatability of the measurement, the position of the well within the plate, the plate re-positioning into the measuring system and the sample volume loaded in the well.

The slope of calibration curve (K) can be calculated by regression analysis of experimental data given by cell culture with known cell number. The cell number can be or estimated from the cell seeding concentration or measured by detaching and counting the cell by means of an hemocytometer, e.g., Neubauer chamber.

4. INFLUENCE QUANTITIES

Fluorescence. The repeatability of the fluorescence measure in a single well has been evaluated lower than 0.5% over the whole fluorescence intensity range of interest. The reproducibility evaluated among the 96 wells on the same plate has been found lower than 1%, even though results from 5-10% of wells were outliers. Thus, it is necessary to work in triplicate in order to eventually identify outliers. The plate placement into the plate reader could contribute to the reproducibility. Hence, the standard deviation of repeated measurements has been calculated. The reproducibility does not make the repeatability of the measurements worse (data not shown).

The system measures the fluorescence intensity due to excitation produced by an incident ray entering the well. The effective optical path length of the incident beam is influenced by the liquid level in the well. The fluorescence intensity referred to the maximum measured value, for the considered solution, as function of the volume filling the well V_W has been measured. The fluorescence intensity is highest in the range around 200 µl. The intensity fluorescence dependence trend can be described by a fifth-degree polynomial and by the latter the sensitivity coefficient of fluorescence intensity with respect to the filling-the-well volume, can be calculated by the derivatives. The volume corresponding to the maximum fluorescence intensity minimizes the contribution to the uncertainty of the filling-the-well liquid volume fluorescence intensity, taken into account as influence quantity. To summarize, the fluorescence intensity uncertainty has a component of repeatability of instrument measure (0.5%) and a component of reproducibility due to the well positions in the plate (1%). A further contribution is due to the volume of liquid filling the well and can be evaluated separately as function of the available sample volume.

Liquid Volumes. The volume of the liquid phase in the bioreactor V_{LF} and the volume filling the well V_w have been measured by a calibrated micropipette. V_{LF} is affected by the residual volumes during liquid replacements, evaporation during the contact time and micropipette uncertainty. In the specific tests the bioreactor was the well itself, thus V_W and V_{LF} are the same volume. A 3% total uncertainty has been calculated.

Slope. The slope of calibration curve K have been calculated by regression analysis of experimental data measured by detaching and counting the cell by means of a Neubauer chamber.

The linear regression minimizes the objective function at the estimated K value [12]:

$$K = \frac{\sum_{i=1,n} \frac{N_i \left(I_{f,t,i} - I_{f,b,t,i}\right) V_{LF,i}}{\sigma_{y,i}^2}}{\sum_{i=1,n} \frac{\left(\left(I_{f,t,i} - I_{f,b,t,i}\right) V_{LF,i}\right)^2}{\sigma_{y,i}^2}} \qquad (5)$$

While the uncertainty of K is [12]:

$$u(K) = \sqrt{\frac{1}{\sum_{i=1,n} \frac{\left(\left(I_{f,t,i} - I_{f,b,t,i}\right) V_{LF,i}\right)^2}{\sigma_{y,i}^2}}} \qquad (6)$$

where $\sigma_{y,i}$ is the uncertainty of each experimental point and combines the uncertainty of all the measured quantities, i.e., number of cells, volume and fluorescence intensities. Cell counting relative uncertainty has been calculated to be 4%.

The K uncertainty decreases when the ratio between cell number N and available liquid volume V_{LF} increases and depends on the number of experimental points. The conditions in which the experiments are made to calculate the calibration curve constant should be as similar as possible to the measurements conditions of the cell number. The K uncertainty has been calculated to be 2.2% for time of contact larger than 1 h and 3% at 0.5 hours of contact.

Dilution. The fluorescence intensity measurement instrument has a detection limit of $5x10^5$ fluorescence units. Consequently, the maximum ratio between the number of cells and the volume of available liquid has a limit. The limit value for the different contact times has been analyzed. For values above the detection limit a dilution of the samples is necessary. The optimal dilution is with the blank solution. However, the available volumes of the blank solution are rarely sufficient for each dilution. The water has produced a non linear reduction to the interfering quantities effect. Hence, it has been necessary to dilute also the black solution to obtain a correct linearity of the calibration curve. The dilution of sample and black solutions with a solvent similar to the blank, i.e., fresh medium, has given a good response.

5. RESULTS

The uncertainty budget of the CTB metabolic assay is reported in table 3.1. It has been calculated for a contact time of 2.5 h and low cell number at the experimental conditions of the tests. The Significance Index (SI) reported in the budget is defined as the ratio between the contribution of the influence quantity analyzed and the maximum contribution to the uncertainty provided by one of the influence quantities. SI lower than 1% indicates that the

contribution to the uncertainty is negligible, SI upper that 10% indicates that the contribution is relevant, SI=100% indicates the most relevant contribution.
As a result, all contributions are relevant unless the blank intensity. V_W contribution can be reduced dosing around 200 µl of liquid in the well. The V_W contribution to the uncertainty become negligible. Larger bioreactors, i.e., larger V_{LF}, allow to reduce liquid volume uncertainty to around 1%. SI would result 21% for volume contribution and the calibration constant would be the most relevant contribution with a total uncertainty of 2.7%.The calibration constant uncertainty can be reduced by increasing the number of calibration points. While the fluorescence intensity may be enhanced calibrating the system by fluorophores standard solutions.

Table 3.1 CTB metabolic assay uncertainty budget.

X	[X]	x	u(x)	u(x)/x	c(x)	$[uc]^2$	SI
$I_{f,S,2.5h}$	-	$3.8x10^5$	$3.8x10^3$	1,0%	$3x10^{-2}$	$1x10^4$	15%
$I_{f,B,2.5h}$	-	$5x10^4$	$5.0x10^2$	1,0%	$3x10^{-2}$	$2x10^2$	0%
V_W	L	$1x10^{-4}$	$3.0x10^{-6}$	3,0%	$-3x10^6$	$1x10^4$	18%
V_{LF}	l	$1x10^{-4}$	$3.0x10^{-6}$	3,0%	$9x10^7$	$7x10^4$	100%
K	cell/l	270	$5.9x10^0$	2,2%	$3x10^1$	$4x10^4$	54%
N	cell	8900	350	4,1%		$1x10^5$	

6. CONCLUSIONS

The cell counting methods based on metabolic activities are indirect methods and require a high reproducibility in experiments to calibrate the method and in experiments with samples. The identification of standard measurement units for fluorescence intensities and absorbance are certainly a significant prerogative for methods unification in order to increase their traceability, regardless of the adopted measurement instrument.

Results, in 2D cell culture, revealed that three are the main components: repeatability and reproducibility of the measurement system and a contribution due to the sample loading step procedure. Uncertainty is around 4% in the tested experimental condition. All the influence quantities give a relevant contribution to the total uncertainty. It means that to reduce uncertainty by one order of magnitude, the uncertainty of all the quantities must be reduced. However the use of appropriate volumes for bioreactor and well filling allow to reduce uncertainty to 2.7%. Increasing the number of calibration points on the curve reduce calibration constant uncertainty and gives an opportunity to further reduce uncertainty.

The metabolic methods to the 3D cell cultures requires a deep knowledge of the scaffold-cells and scaffold-metabolites interactions to ensure the independence from transport limitation and to foresee the effective activity of the cells attached to the scaffold. For a 3D system, several other component can be influence parameters.

For example, in the most difficult 3D culture conditions, due to internal porous architecture of the 3D system, usually only the peripheral environment of the 3D system is available for collecting the culture medium in which the metabolic product is released. Only in that liquid volume the metabolic product concentration can be measured. In addition, in a 3D system, limitation factors of the cell activity are the diffusion of nutrients, such as oxygen and glucose, from the cell culture medium surrounding the scaffold to the cells within the scaffold pores and the diffusion of metabolic products from the inner part of the scaffold to the outer environment. Limitations of the cellular activity influence rate and yield of the cell growth.

This justifies the need to evaluate the uncertainty in a 3D cell culture system. This work is a first approach to the uncertainty evaluation of a metabolic assay in conventional 2D cell cultures and can be the basis for a 3D system in order to increase the traceability of those methods, regardless of the adopted measurement instrument.

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