

A Method of Estimation of the Cell Doubling Time on Basis of the Cell Culture Monitoring Data

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Investigation with time-lapse microscopy of the clonal growth of stem cells provides to the analysis of image sequences, which document development of a single clone in constant time increments. The cell doubling time (T_2) determines the dynamics of the cell culture development as average time taken for a cell to complete the cell cycle and is typically estimated with cytometric measurements within several days. However our monitoring of the cell culture lasts shorter, so the dependence of T_2 from measurements available from image sequences has been found and applied to the collected data. The results are compared with the cell doubling time estimated and published for the cell line by the lab, in which it has been established.

K e y w o r d s: cell culture, cell doubling-time, time laps microscopy

1. Introduction

Stem cells are a potential source of cells for use in the regenerative medicine. Monitoring and analyzing of quantity of the cell culture is crucial for reliable optimization of culturing methods [1]. Monitoring of the living cell cultures by human visual inspection and examination is widely used in the labs as the fastest and cost free method [2]. Even the very experienced lab-worker captures some subjective information and, from time to time, supports it by the objective information using immunohistochemistry and flow cytometry methods [3]. So an unexpected growth of the cell culture (e.g. differentiation instead of self-renewal of the cells) may be noticed. The continuous monitoring of the culture condition with the computer supported microscopy, will

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allow a quick response for changing of culture growth and become a tool for investigation and optimization of reliable and fast culturing methods.

The automation of the cell culture growth observation and evaluation is a general idea of investigation which can be achieved solving several problems separately. One of these problems is estimation of one of typically evaluated parameters of the cell culture growth from data captured with time-lapse microscopy [4], it means from a sequence of images made in constant time intervals and in several image planes.

A single sequence documents the development of cells within a single area of microscopic view. It carries a lot of information about the culture condition that must be extracted and used to verify if the cell culture develops in the expected direction.

The length of time-lapse observation of living cells in the culture is influenced both by limitations of the equipment (computer memory size and data transmission speed) and physiological limitations of the cell type under examination (cell ability to form clones or disperse and to be in a good condition in perfusion chamber without changes of the medium). So duration of the time-lapse examinations is usually limited to 2–4 days between two following changes of a medium; it means environment in which cells live.

The important parameter used for description of dynamics of the cells clone development, called the cell doubling time T_2 , which shows average time between successful cell divisions, is measured typically by observation of changes of the cell density in time [8] or by the measurement of fractions of cells in various cell cycle stages [9, 10] using the flow cytometry method [3, 5, 6, 7].

In this paper it is proposed to estimate the T_2 value processing the sequence of images.

In case of growth of the stem cells culture, the aim is to increase a number of cells with the regenerative potential [1], what is called the cell self-renewal. A sample of the stem cells acquired from mobilized blood of a donor or umbilical cord blood of a newborn child is small. So it is important not to employ the flow cytometer which damages cells under observation. While using time-lapse microscopy to evaluate cells in a sample and observe changes of a number of cells in time is more suitable in the situation of shortage of the cells [15, 16].

Despite the knowledge that T_2 slowly changes during the cell culture development [11, 12], it is treated as a constant value in this short time window of observation (hardly 2 or 3 days, as it has been mentioned earlier).

The correlation of the value of T_2 calculated using the flow cytometry with the value obtained with the proposed method will be investigated in the future work [11]. It is planned to examine other cells, e.g. stem cells of hepatocytes, keratynocytes, than the neural stem cells used in the preliminary study.

In the next section the related research is presented, while in two subsections of the following section two methods of estimation of the T_2 value: (1) the first one, based on a number of cells in division and (2) the second one, based on the plot of

cell growth in time, are presented. The next section contains the experimental data used in verification of both methods. In the last two sections there are results, discussion and conclusions of the proposed methods of the T_2 estimation as well as results of comparison of the T_2 values, evaluated for the neural stem cells using the two proposed methods, with the T_2 values published by the lab which has established the cell line.

2. Related Research

T_2 is a measure of the population growth and has come to cell investigations from demography [12]. T_2 for the cell growth in culture *in vitro* is measured for various types of cells, e.g. for cancer or embryonic cells [11, 14], bacterial or fungal cells [8]. In the case of cancer cells T_2 demonstrates either a prognostic value or a predictive value for the efficiency of certain treatment strategies, e.g. radio- and/or chemotherapy, while in the case of pathogens T_2 allows to evaluate drugs efficiency used in therapy.

In the above-described studies, a single sample of cells from a patient is used in the methods of the T_2 evaluation. A fraction of cells in a particular state of the cell cycle makes the additional information important in such cases, information which is available if markers of cellular DNA are used in the flow cytometry. Torricelli and others use BrdUrd to label a fraction of proliferating cells in the S phase [3].

It is reported that the T_2 time needed to double the cell population in the culture, is about 24–72 h (1–3 days) for various types of cells [2]. However, for the neural stem cells, which are the subject of our preliminary experiments described in this paper, the T_2 is reported to be about 4 days [1, 13], according to the lab where this cell line has been established.

3. The Methods of Estimation of T_2

Experimental data, gathered through the analysis of sequence of images, allow to assess three important parameters: time between subsequent cell divisions (time between the moment when the cell is born and the moment of its division into two daughter cells), number $N(t)$ of cells in the clone as the function of time and number $N_p(t)$ of divisions of cells in time. They are collected for time window of two to tree days.

Two methods are proposed to estimate the value of T_2 : first, in which the calculation is done and the second, in which a plot of the cell number in time is used. Both the methods base on the following assumptions which are true in time window of some days before cells form a monolayer:

- every division is an independent random event,

- its probability distribution is the same for all cells and doesn't change in time,
- probability of the division is described by an exponential distribution [8]:

$$N(t) = N_0 e^{pt} \quad (1)$$

and for both methods the doubling time ($T2$) for the selected time t and for the assumed growth curve is calculated.

3.1. Method I

If the number of cells is equal to N_0 at $t=t_0$, then

$$N(t) - N_0 = N_0(e^{pt} - 1). \quad (2)$$

Let ΔN denote $N(t) - N_0$. Now equation (2) can be rewritten as

$$\frac{\Delta N}{N_0} + 1 = e^{pt}. \quad (3)$$

Finding a logarithm of both sides the following equation

$$\ln\left(\frac{\Delta N}{N_0} + 1\right) = p\Delta t. \quad (4)$$

becomes true. Let $T2$ be an interval after which the number of cells doubles, i.e.

$$2N_0 = N_0 e^{pT2}. \quad (5)$$

Dividing by N_0 and applying a logarithm to the both sides we obtain

$$\ln 2 = pT2. \quad (6)$$

From (4) and (6) it can be written that

$$p = \frac{1}{\Delta t} \ln\left(\frac{\Delta N}{N_0} + 1\right) = \frac{\ln 2}{T2}. \quad (7)$$

and hence

$$T2 = \frac{\Delta t \ln 2}{\ln\left(\frac{\Delta N}{N_0} + 1\right)}, \quad (8)$$

or in terms of a binary logarithm

$$T2 = \frac{\Delta t}{\log_2\left(\frac{\Delta N}{N_0} + 1\right)}. \quad (9)$$

In the equation (9) N_0 is the number of cells at the beginning of observation (on the first image of the sequence) and ΔN is the increase in the number of cells during the period of time of the length Δt . Note that since each division increases the number of cells by adding 1, ΔN is also the number of cell divisions during the same period.

3.2. Method II

It is also possible to assess the value of $T2$ using the method of linear regression [11], applied to the plot of the binary logarithm of the number of cells against time, see Fig. 1. To obtain a one $T2$ value for the cell line, when several area of observations and/or several experiments have been performed, the values of $T2$ calculated separately for each observation should be averaged.

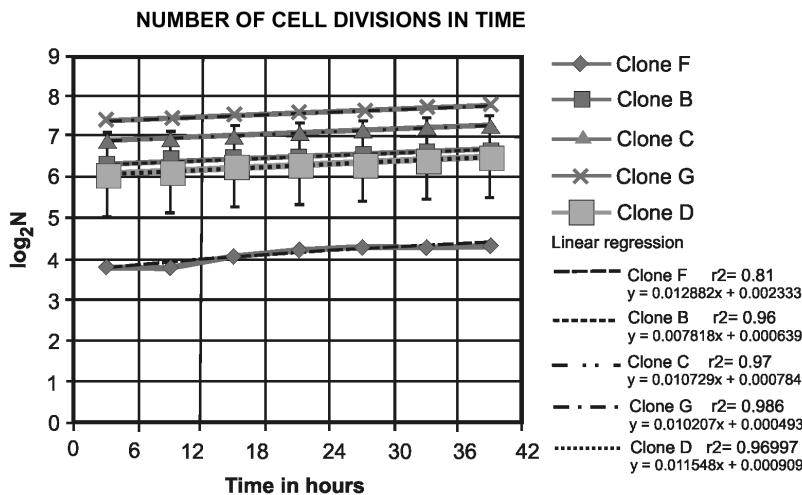


Fig. 1. $T2$ has been estimated using the linear regression: plots of $\log_2 N$ (number of cells) vs time (in hours) for various clones supported by the linear regression, calculated for each of five plots individually

4. Experimental Material

The neural stem cells from the HUCB-NSC line [1, 13], established in NeuroRepair Dpt. Lab., MRI, PAS, were used to obtain experimental data about the cell growth to verify the $T2$ value in the proposed method of evaluation. The observations were done using an incubation chamber with perfusion [Zeiss] and a confocal microscope with

red colour laser [Zeiss]. The cells' behaviour in standard conditions (37°C , 5% CO_2 and 95% humidity) incubated in DMEM/F12 medium (Gibco) with low fetal calf serum (FBS; Gibco), ITS (Gibco) and AAS (Sigma) addition was observed. The cells were seeded in density 1000 cells/1 ml, after few hours of culturing (from 2 to 10) were monitored every 15–20 minutes for the next 46 hours. A chosen observation plane covering only $120 \times 120 \mu\text{m}$ space on the microscopic slide was acquired by the camera to the computer as a digital image of 2048×2048 pixels. Fragments of the observation plane at the beginning and end of experiments was shown in the top and the bottom image of the right column in Fig. 2. Careful visual inspection of sequences allowed to find the moment of separation of the cells, which was regarded as the cell division. The fragments of two consecutive images: the first one, just before the mother cell's division, and the second one, just after the daughter cells' birth, were presented in Fig. 2 for three divisions of related cells. The database of cell divisions in experimental material was constructed. Then the lineage (parent's trees), similar to that presented in Fig. 2, and the time between the cell division were calculated based on this database.

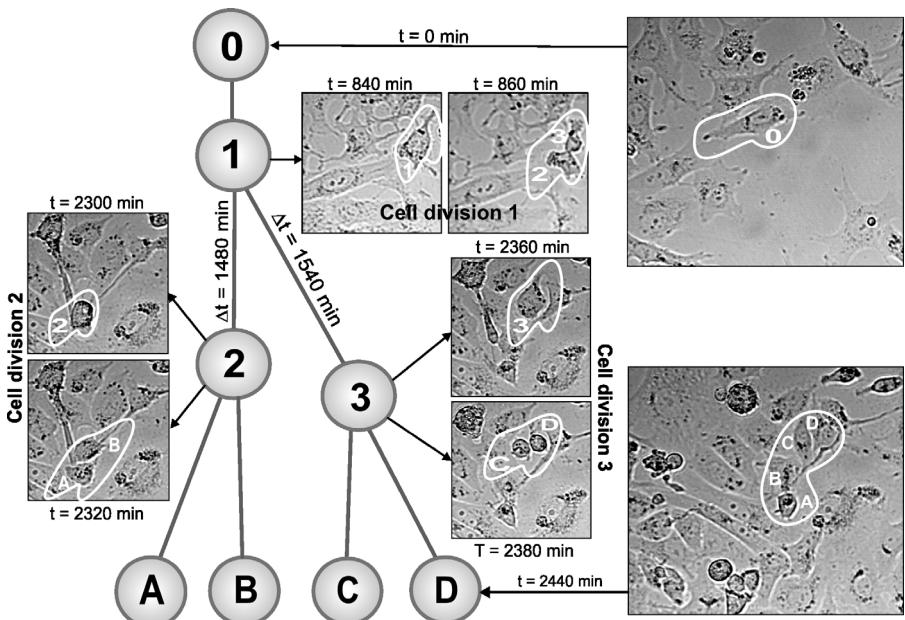


Fig. 2. The estimation of the time between the neural stem cells divisions and the lineage (parents) trees constructed from images sequences captured from laser scanner confocal microscopy. The lineage tree of the cell, marked in the first image of sequence (top right), shows the process up to births of four daughter cells marked A, B, C, D in the last of sequence image (bottom right). There are three cell divisions in this tree: the first, marked 1, it is the oldest cell division and two their daughters' divisions, marked 2 and 3. All divisions are presented with fragments of two consecutive frames in the image sequence ($\Delta t = 20 \text{ min}$), which show the mother cell, just before its division, and the daughter cells, just after their births

Carrying out six experiments, only five of them contain the visual area in which the cells were divided in such a way that they formed the clenched or dispersing clones. For each of them the number of cells and the number of the events of cell division were counted using software for interactive cell counting, whilst trees of lineage were outlined using specially prepared software [17]. It was done only for the cell, that itself and all its daughters were located within the visual area during the whole time of observation.

The doubling time of the individual cell, i.e. the time between mother cell appearing and its daughters cells births, were recorded separately, as the time between subsequent cell divisions, while the population doubling time of the cells in the culture was estimated according to the methods presented above.

5. Results

The presented methods of the T_2 value estimation were used for analysis of the experimental data on the neural stem cells of the HUCB-NSC line [1, 13]. Based on the trees of lineage, the average time between cells divisions was estimated as 25.5 ± 5 h.

For analysis of the T_2 value according to the equation (9), the time increment $\Delta t = 12$ h was used to make sure that the increase of the cell population in 12 h provides the stability of the element $\Delta N/N_0$. $T_2 = 94 \pm 60$ h (3.9 ± 2.5 days) was obtained. Next the estimation of T_2 from the graph of the linear regression was performed. The estimated T_2 conforming the relation $\text{tg}(\alpha) = 1/T_2$ to the directional coefficient of the linear regression. It was calculated for each sequence separately and values: 127.9 h; 93.2 h; 86.6 h; 77.6 h and 97.97 h were obtained (see Fig. 1). The mean value of T_2 calculated for each observed clone was equal to 96.17 ± 19 h ($4 \pm 2/3$ days).

The T_2 values calculated from the experimental data in both ways were not statistically different ($p < 0.05$). Both obtained results were just about 4 days, similarly to the T_2 value reported by the Buzanska and others [1, 13] calculated by the other method (from monitoring changes in cells number in time). But in mansion publications there were not confidence range, so any evaluation of this difference was impossible.

6. Conclusions

Both the proposed methods allow, on the base of gathered experimental data from time-lapse microscopy observation, to assess T_2 which is the time of doubling the number of cells in the culture. The experimental data of the HUCB-NSC cells allow to compare the values of T_2 calculated using the proposed methods and the standard method. These values are enough close to enable evaluation dynamic

of cell culture grows using proposed method of T_2 estimation instead of T_2 obtained using conventional methods. This preliminary verification shows that this parameter can be used to the cell culture grows evaluation but it must be verified using large samples and various cell types. In the close future it is planned to examine the new proposed method on early hepatocytes and early keratynocytes cultures sown in various cells density.

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