Theoretical Basis for the Measurement of Small Differences in the Length of the Cell Cycle between Two Cell Populations

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Abstract: The length of the cell cycle (T_C) is a tight regulated process and is important for proper development and homeostasis. Although several methods are available for estimating the duration of the cell cycle, it is difficult to determinate small differences of T_C between two different cell populations due to biological and/or experimental variability. A novel strategy based in co-cultivation of two cell strains followed by a series of dilution and propagation of the culture will allow the quantification of very small differences in the length of two cell populations at resolution levels not possible at present with current methods. This is achieved by a separation of the endpoint variable measured to compare between two cell populations. The theoretical basis of this approach is discussed in the context of published experimental data and simulation of idealized experiments using virtual strains of different cell cycle length.

Keywords: Cell cycle length, method, co-cultivation, cell population, single cell measurement, proliferation, cell cycle kinetics.

INTRODUCTION

Alterations in the length of the cell cycle (T_C) might confer different growth properties. For unicellular organisms, a shorter time to divide gives cells growth advantage. In theory, the faster growing strain can overgrow the slower growing strain that would eventually disappear. For multicellular organisms a fine regulation of the length of the cell cycle is required for proper development and homeostasis. Indeed, progressive lengthening of the cell cycle during development has been observed in several organisms [1-5]. The most remarkable example occurs during Drosophila development where the T_C varies from few minutes to 24 hrs [5]. Evolutionary modifications of T_C have also been reported among species [6, 7]. Dysregulation of the T_C might be related to several diseases. For instance, it was recently reported that glioma stem cells have elongated cell cycle compared to non-stem glioma cells. This difference might confer differential properties including increased radioresistance [8]. Therefore, accurate measurement of the length of the cell cycle as well as accurate determination of cell cycle differences between cell types are important parameters for a broad range of applications and have important implications for biology and medicine.

There are several methods to establish T_C that rely on cell cycle synchronization and/or population doubling time using either cell count or DNA content and direct observation of single cells by time lapse video microscopy [9-13]. Each method has theoretical and practical limitations and a detailed description of each assay is beyond the scope of this manuscript. For convenience, methods are divided into two categories: a) **M**ethods **B**ased in **C**ell **P**opulation (**MsBCP**; e.g. cell culture, *in vivo* labelling with DNA precursors) and, b) **M**ethods **B**ased in **S**ingle **C**ell (**MsBSC**; e.g. time lapse video microscopy).

MsBCP have the advantage to averaging the small variation in the T_C of individual cells and the T_C measured should be a very good average estimation. However, experimental variability is an important limitation and makes MsBCP not suitable for comparing cell types with a small cell cycle length difference (ΔT_C). One example analysing published data illustrates this point: Table **S1** shows the T_C of the retinal cells by published experiments ([1]; data from Table **3**). The RT_C (See glossary for the nomenclature used in this manuscript) for two consecutive days can be calculated by dividing the T_C at one particular day over the T_C from the next day. As shown in Table **S1**, the RT_C varies daily (range 0.904 to 0.830). The data from Alexiades and Cepko, demonstrate a daily variation in the T_C of retinal cell during development indicating that there are factors controlling (elongating) the T_C in a daily basis. The conclusion of this experiment is that cells from E14 have a T_C shorter than E15. However, by comparing only two cell populations, it will be very difficult to find a statistical significant difference between E14 *vs*. E15 (RT_C =0.904) but it will be easy for E14 *vs*. E21 (RT_C =0.442). Thus, the closer the RT_C to 1, the more difficult will be to reach statistical significance (See Table **S1** and accompanying figure). This evaluation suggests that MsBCP are not suitable for estimating small ΔT_C.

Historically, time lapse video microcoscopy (TLVM - probably the only MBSC suitable to estimate T_C in single cell-) was introduced to study variability in generation time and showed that the T_C of individual cells varies from generation to generation and of course from cell to cell [10]. Analysis of the original data relevant to this manuscript is shown in Table **S2** and accompanying figure. The T_C of

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different strains obtained by TLVM [10] shows a considerable standard deviation. It is important to point out that due to the random variation of the T_C [14] the standard error in this assay is due to an inherent property of the cells and not to experimental variability. The limitation of single cell measurement of the T_C might be solved by technological improvement that will allow to measure huge number of events to reach statistical significance of the ΔT_C between two cell populations. The accompanying figure in Table **S2** shows that if we plot the T_C (media ± SD) for three different strains (real experimental data from [10], Table **IV** therein) it will be difficult to determinate whether those differences are real or not. Thus, even measurement of T_C in single cell gives a significant standard error [10] that limits the resolution of the method . Therefore, analysis of individual cells does not solve the limitations of MsBCP.

In principle, the main limitation to measure small ΔT_C between two cell populations is the standard error of the measured endpoint due to inherent properties of the cell and/or experimental variability. To overcome this limitation, this manuscript proposes a strategy based in **C**o-**C**ulture + **D**ilution and **P**ropagation (**CCDP** method) of two unsynchronized cell strains that would allow the detection of minimal ΔT_C between two cell types. In this approach, the number or percentage of cells for each strain is measured as the endpoint variable. Conceptually, this strategy (due to the use of cell populations) minimizes the biological variability (by averaging a significant number of cells) and simultaneously (due to the series of dilution and propagation) "separates" the difference of the measured endpoint used to discriminate between two cell populations. This "separation" is made by progressively increasing the ratio N_E/N_C (number of the experimental cell/number of the control cells) after each round of dilution and propagation. In statistical terms, it increases the distance between the media of two different groups and makes it easy to be able to reach statistical significances even when the actual ΔT_C of two strains are minimal regardless of the expected experimental variation (Standard error). The only requirement for this strategy to be used in the laboratory is to quantitatively discriminate the percentage (or number) of cells (endpoint measured) of each sub-populations when cells are co-cultivated.

METHODS

Simulation of an Idealized Experiment

Assumptions

1) Cell number in co-cultures increases independent of each other following classical exponential growth (See glossary),

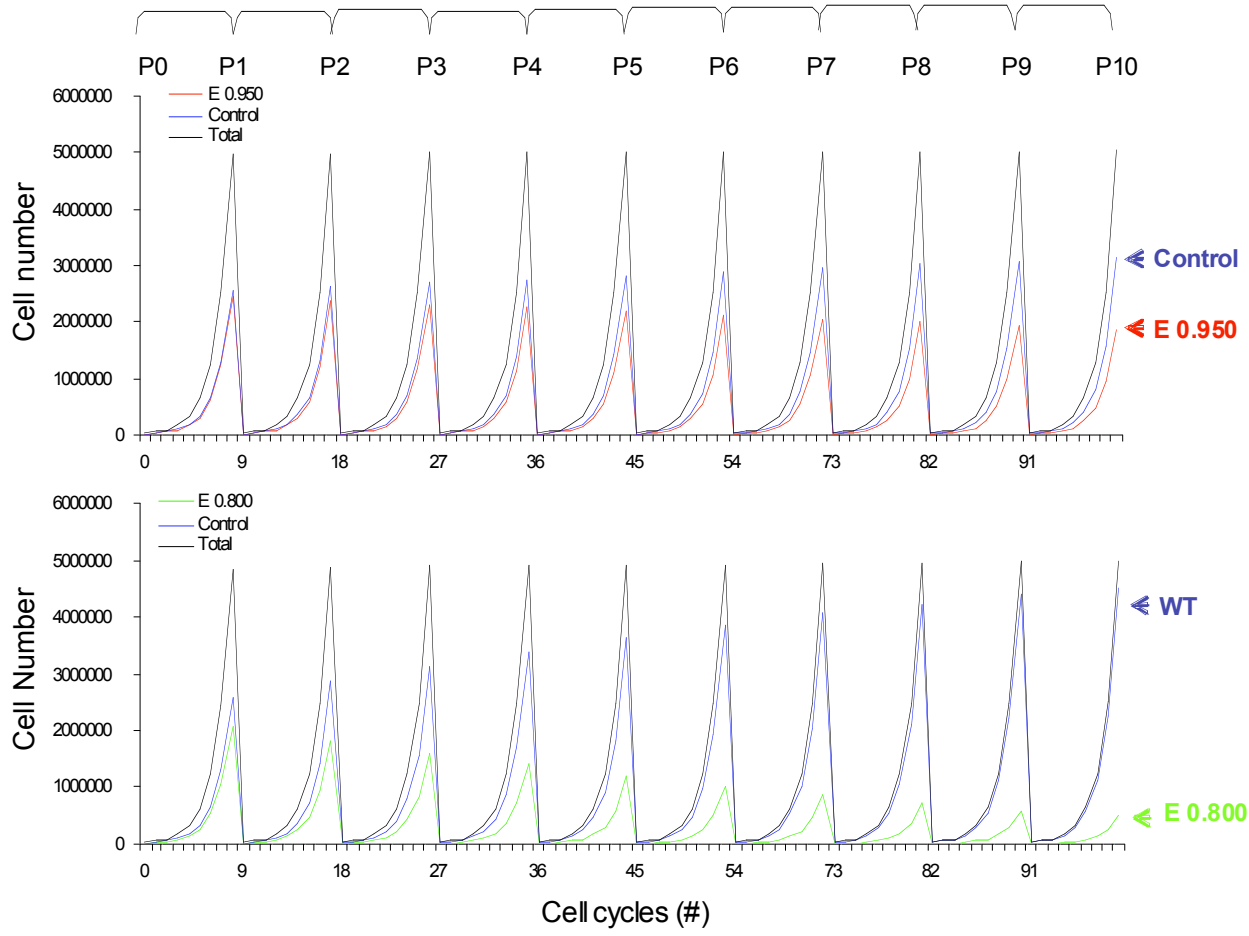


Fig. (1). Simulation of the CCDP method comparing Control *versus* E 0.950 strain (Top) and control *versus* E 0.800 strain (Bottom). P0 - P10 denote the number of passages. Increase in cell number at each passage was simulated using classical exponential growth equation (See Glossary).

2) No loss of cells occurs during the assay.

The CCDP method is based in 1) Initial co-cultivation of both strains (Passage 0) at a 1:1 ratio ($N_E/N_C = 1$), 2) at least 10 cycles of dilution and propagation for 6-10 cell divisions (Passages 1-10). 3) Sample collection and storage at each passage before dilution. 4) Determination of the number of control and experimental cells for each collected samples (Passage 0-10).

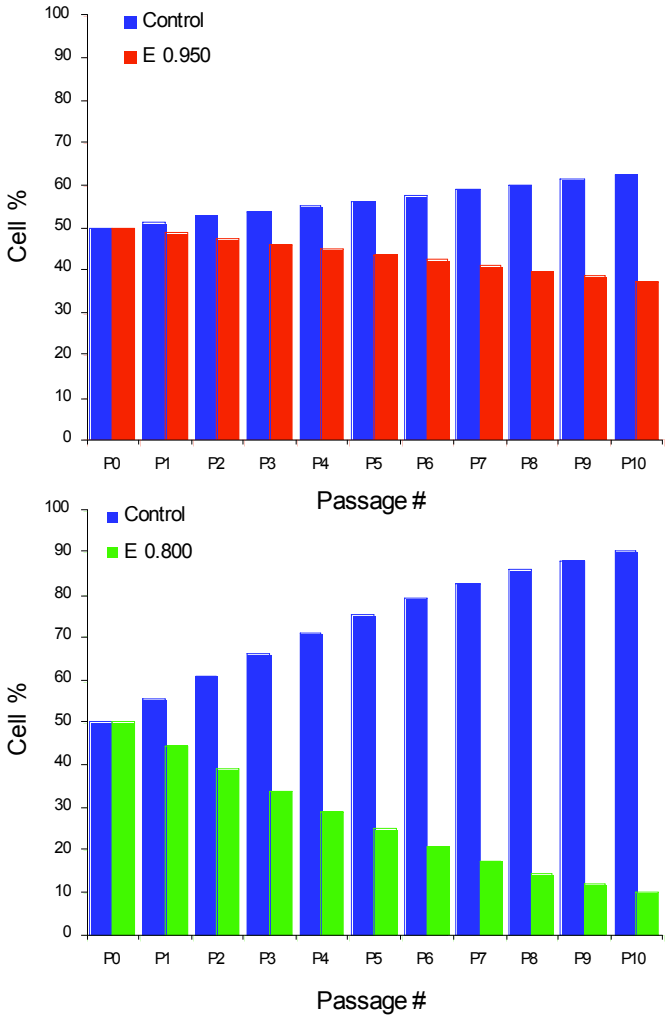
Virtual experiments were performed using a virtual control strain with a T_C =3 hr (180 min) and experimental strains (E 0.999 – 0.800, T_C ranging from =180.180 - 225 min). For all simulations 2,500,000 cells of each strain were mixed in a final 10 ml volume, 2) a 40 µl aliquot (20,000 cells) was immediately diluted into a final 10 ml volume and allowed to propagate while the rest of the cells were collected and stored (Passage #0). When the cell density reached ~ 500,000 cells/ml (close to the initial mixing - approximately 8 cycles-), an aliquot of 20,000 cells were diluted in fresh 10 ml culture media and allowed to propagate, the rest of the cells were collected and stored (passage #1). This cycle was repeated to collect 10 passages.

RESULTS

A simulation of the above experiment with strain E 0.950 is shown in Fig. (**1**) top. It can clearly be appreciated that after each passage the cell number of the control strain (N_C) increases gradually while at the same time, the cell number of E 0.950 strain (N_E) decreases proportionally. The same experiment using E 0.800 shows more dramatic changes in the relative number of cells present in the culture (Fig. (**1**) bottom).

The changes in the N_C and N_E after each passage modify the N_E/N_C ratio from 1:1 (P0) to 0.598 (P10) (Fig. **2** top). The progressive modification in the N_E/N_C ratio after each passage allows to estimate the proliferation kinetics of both subpopulations. The same experiment using E 0.800 showed a more pronounced change in the N_E/N_C ratio from 1:1 (P0) to 0.107 (P10) (Fig. (**2**) bottom).

The N_E/N_C ratio is shown for all the E strains (E 0.999 to E 0.800) in Fig. (**3**). The N_E/N_C ratio progressively decreases for each strain as the number of passage increases. Fig. (**4**) shows the N_E/N_C ratio at passage # 10 for all the experimental strain (E 0.999 to E 0.800). By comparing to the



Passage	Ne/Nc
P0	1
P1	0,95
P2	0,9025
P3	0,857375
P4	0,81450625
P5	0,77378094
P6	0,73509189
P7	0,6983373
P8	0,66342043
P9	0,63024941
P10	0,59873694

Passage	Ne/Nc
P0	1
P1	0,8
P2	0,64
P3	0,512
P4	0,4096
P5	0,32768
P6	0,262144
P7	0,2097152
P8	0,16777216
P9	0,13421773
P10	0,10737418

Fig. (2). Quantitative determination of the percentage of cell after each passage for the virtual experiments from Fig. (**1**): Control *versus* E 0.950 (Top) and control *versus* E 0.800 strains (Bottom). The table at the right of each figure shows the progressive changes in the N_E/N_C ratio after each passage. See text for details.

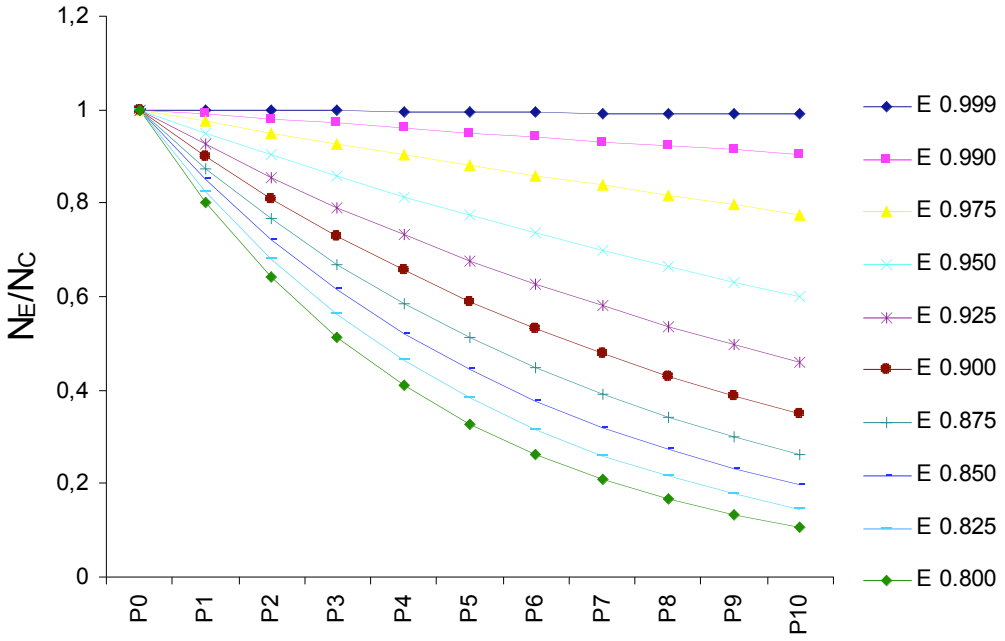


Fig. (3). N_E/N_C ratio for control versus 10 different experimental strains with different RT_C (E 0.999 to E 0.800) plotted against passage number.

N_E/N_C ratio at passage # 1 it can easily be appreciated that the CCDP strategy "modifies" the N_E/N_C ratio (endpoint variable measured), that depends on small differences in the length of the cell cycle, at values that can be quantitatively measured with sensitivity enough to detect minimal differences. Comparing control *vs.* all different strains (E 0.999 - E 0.800) and assuming an experimental variation of 10 % for all measured endpoint the CCDP method shows that it will sensitive enough to resolve cell with $RT_C \leq 0.975$ (Fig. **4**). Higher resolution can be obtained by increasing the number of passages.

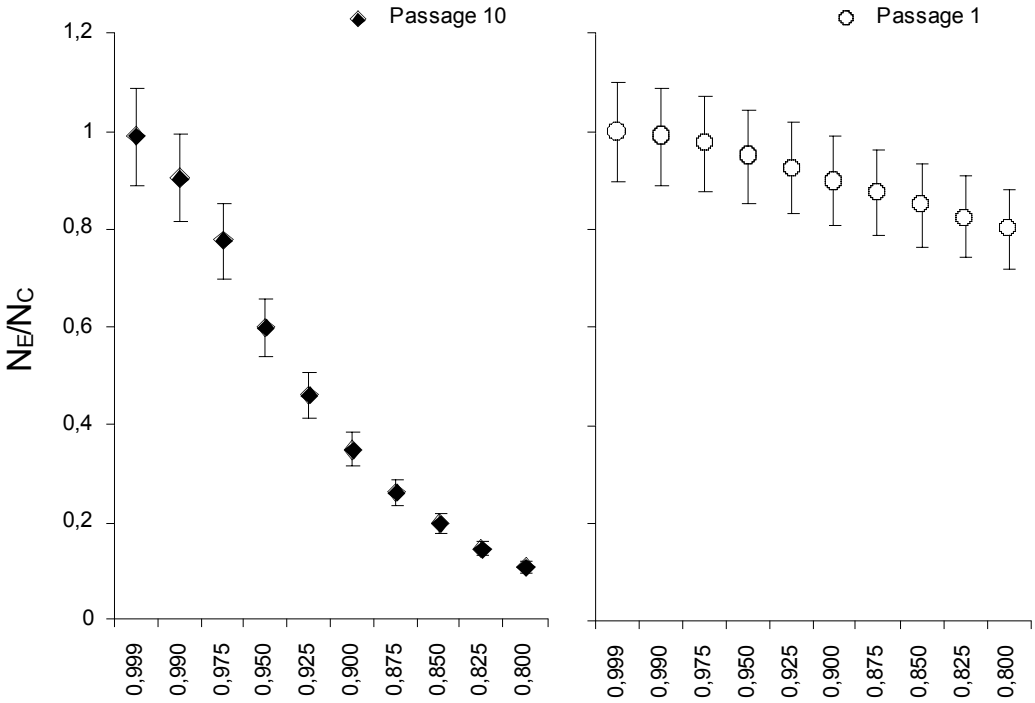


Fig. (4). Theoretical resolution of the CCDP method at passage 10 compared to passage 1 (similar to the resolution of current alternative methods. N_E/N_C ratio at passage #10 for all experimental strains with different RT_C (0.999 to 0.800) (Left panel). N_E/N_C ratio at passage #1 for all experimental strains with different RT_C (0.999 to 0.800) (Right panel).

DISCUSSION

The CCDP strategy depends on the accurate quantification of each subpopulation (absolute numbers or percentage) present in a cell culture. The ideal variable for the quantification of the percentage of control and experimental cells present in a co-culture system (endpoint variable measured) must fulfil few essential requirements: 1) should be proportional to the number of cells in the culture 2) should be able to faithfully discriminate the percentage of each cell subpopulation. In practice, it is reasonable to assume that the ideal marker is DNA with a polymorphism (restriction site, insertion or deletion) that can be accurately measured by quantitative PCR to distinguish both subpopulations. In nature, components of the cell cycle are often mutated in cancer cells and experimentally, cells lines are transformed in order to recapitulate these mutations (or create others) to understand the mechanism of cell cycle control in normal and neoplastic cells. These mutations can affect the T_C but at present are difficult to measure using current methods. The CCDP strategy can be used in available –transformed- cell lines that already have DNA polymorphisms compared to the parental strain to detect small differences in the T_C caused by a particular mutation. When required, cell lines can also be specifically transformed creating polymorphism for this specific purpose to study genes of interest. The CCDP approach is ideal for non-adherent cells with relatively short T_C such as yeast and ciliates. These model organisms are very useful to dissect genetic determinant of the cell cycle. The CFSE staining method [13] is the only one that allows the monitoring of cultures for multiple cell cycles and detects slower or faster growing sub-populations in culture [15]. The method is based in initial labelling of cells and quantification of CFSE for up to ten generations by flow cytometry. Since the CFSE method uses only one cycle of propagation the resolution is comparable to the CCDP method at passage #1 and will not allow the detection of small ΔT_C (Fig. **4**).

In summary, a method to evaluate small differences in the T_C will be an important tool to gain mechanistic insight into the regulation of the length of the cell cycle and would have broad applications in biology and medicine. The application of the CCDP strategy to cell lines carrying spontaneous or experimentally-induced polymorphism will help to identify factors (e.g. genes, pathways) important for the fine regulation of the length of the cell cycle and it will expand our knowledge on the mechanism of several diseases and development.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

GLOSSARY

Cell cycle length (T_C)	=	Time to complete a cell cycle.
Relative T_C, (RT_C)	=	The ratio between the T_C of a control and a "experimental" strain. For example if a control strain has a T_C = 3h and the experimental strain T_C =3.5 hr, RT_C = 0.857. For this paper the RT_C will be used to denote the T_C of experimental (E) strains: e.g., E 0.857 will be a strain that compared to a normal strain with a T_C = 3h has a T_C =3.5 hr. The advantage of using RT_C is that one becomes independent of the T_C value (in hours or minutes) of the strain.
Cell cycle length Difference (ΔT_C)	=	The difference in time of the cell cycle length (T_C) between two strains.
Resolution	=	The minimal difference between the length of the cell cycle (ΔT_C) of two strains that can be detected by a method.
Exponential growth equation	=	$N_T=N_0\ (2^{Tx\ RTC})$ where N_T= Cell number at time T; T = Time expressed as cell cycle number; N_0 = Initial number of cells in the culture; RTC = relative T_C (1 for control strain).

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