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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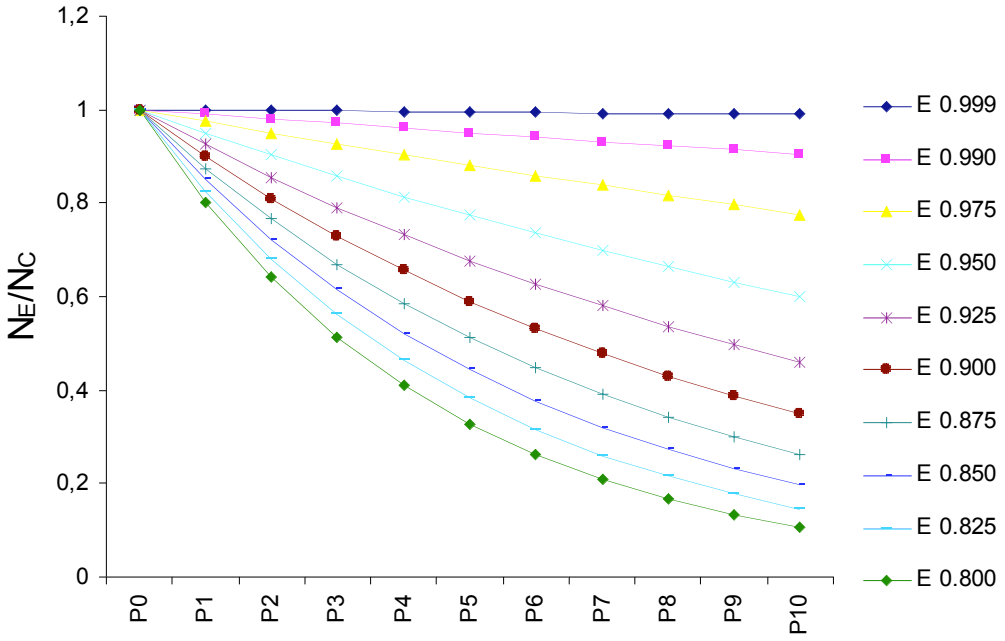

Fig. (3). N_E/N_C ratio for control versus 10 different experimental strains with different RT_C (E 0.999 to E 0.800) plotted against passage number.

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.

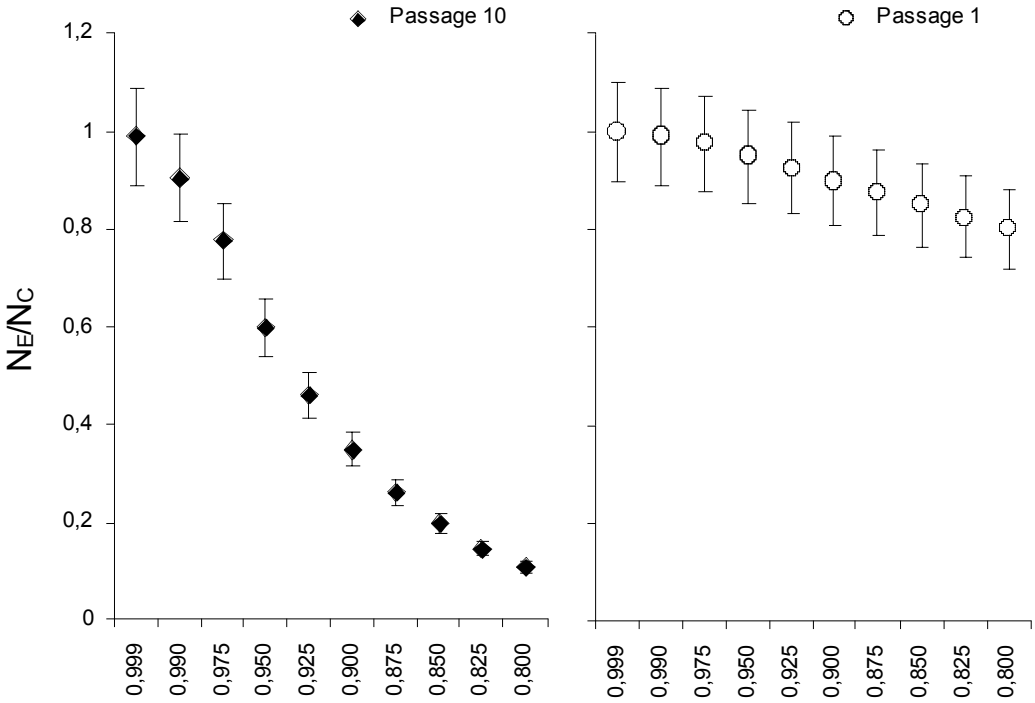

Fig. (4). Theoretical resolution of the CCDP method at passage 10 compared to passage 1 (similar to the resolution of current alternative methods. N_E/N_C ratio at passage #10 for all experimental strains with different RT_C (0.999 to 0.800) (Left panel). N_E/N_C ratio at passage #1 for all experimental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