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Appraising the suitability of succinimidyl and lipophilic fluorescent dyes to track proliferation in non-quiescent cells by dye dilution

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ABSTRACT

Successful completion of the cell cycle usually results in two identical daughter progeny. This process of generational doubling is termed proliferation and when it occurs in a regulated fashion the benefits range from driving embryonic development to mounting a successful immune response. However when it occurs in a dis-regulated fashion, it is one of the hallmarks of cancer and autoimmunity. These very reasons make proliferation a highly informative parameter in many different biological systems. Conventional flow cytometry (CFC) is a high-throughput, fluorescence-based method for measuring the phenotype and function of cells. The application of CFC to measuring proliferation requires a fluorescent dye able to mark live cells so that when they divide, the daughter progeny receives approximately half the fluorescence of the parent. In measurement space, this translates into peaks of fluorescence decreasing by approximately half, each corresponding to a round of division. It is essential that these peaks can be resolved from one another otherwise it is nearly impossible to obtain accurate quantitative proliferation data. Peak resolution is affected by many things, including instrument performance, the choice of fluorescent dye and the inherent properties of the cells under investigation. There are now many fluorescent dyes available for tracking proliferation by dye dilution differing in their chemistry and spectral properties. Here we provide a method for assessing the performance of various candidate dyes with particular emphasis on situations where the cell type is non-quiescent. We have shown previously that even under optimised instrument and labelling conditions, the heterogeneity of non-quiescent cells makes it impossible to obtain an input width below the threshold for peak resolution without reducing the fluorescence distribution using a cell sorter. Moreover, our method also measures how the dye performs post-labelling in terms of loss/transfer to other cells and how the dye is inherited across the cytokinetic plane. All of these factors will affect peak resolution both in non-quiescent and primary cell types.

1. Introduction

Proliferation is a highly informative readout for many biological processes. Under normal conditions, a single round of the cell cycle should yield two identical daughter progeny, each with the potential to divide and give rise to four cells and so on. Proliferation is a positive process in the context of developmental biology but a negative process when linked to cancer through dis-regulated cell cycle and apoptotic controls [1,2]. In the context of an immune response, it can also be both positive for clearing an infection and negative with respect to auto-immunity and allergy [3]. Collectively, these factors necessitate the development of methods that can measure the proliferative potential of a cell as a biological readout for many different systems. Classically, one can measure proliferation using a number of "bulk" methods whereby the net response of the total cellular population can be measured. Examples of bulk methods include growth curves where the absolute number of cells is counted over time, measuring the incorporation of titrated thymidine into proliferating cells using a beta counter [4] and the colorimetric MTT assay whereby NAPDH-dependent cellular oxidase activity is used as a surrogate for determining the number of viable cells [5]. While these bulk methods will provide an answer, they fail to deal with the inherent heterogeneity at the single cell level. For example, a population of cells where few have divided many times may give the same bulk values as a population of cells where many have divided a few times. Conventional flow cytometer (CFC) is a powerful single cell analysis technique that uses laser excitation and photon counting

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to measure the phenotype and function of fluorescently labelled cells as they travel in comparative single file through a hydrodynamically focused suspension. The single cell, multiparameter nature of the platform means it is near perfect for dealing with the inherent biological heterogeneity of cellular populations. CFC became a mainstay in the measurement of proliferation after the serendipitous discovery that carboxy-fluorescein succinimidyl ester (CFSE) could fluorescently label live cells and track proliferation as the signal was diluted by division [6–8]. It now meant that there was a system that could cope with single cell heterogeneity and also allow for multiplexing with phenotypic surface markers and cytokines to look at "proliferentiation" [9]. There are two different classes of dyes; succinimidyl esters that bind to the amine groups of cellular proteins only becoming fluorescent after cleavage by intracellular esterases [10,11] and lipophilic dyes that label the cell membrane [11,12]. In each case the principle is highly elegant and simple, as the input population divides the initial fluorescence should be apportioned to each daughter cell so that individual rounds of division are represented in measurement space by sharp peaks with roughly half the intensity of the parental population. In order to succeed with this approach the input cell population must be labelled without significant toxicity so that they are fit to respond to stimuli. The staining intensity should be as bright as possible above background so as to facilitate the detection of as many rounds of division as possible before the dye dilutes into the area in measurement space occupied by unlabelled events and detector background. Finally the CV of the labelled input population should be within a twofold fluorescence range so as to ensure workable peak resolution [13]. The dye dilution technique has been heavily utilised in the immunological field to study the antigenic driven response of lymphocytes both *in vitro* and *in vivo* [10]. Resting lymphocytes are relatively easy to label with these tracking dyes, as they are highly uniform in terms of size and protein content. This normally results in well-resolved division peaks as long as no other significant sources of measurement error are present (extrinsic or intrinsic with respect to the cell). In contrast high resolution division tracking by dye dilution has been employed with minor success in the study of non-quiescent cells types including stem cells, T cell blasts and transformed cell lines [13–15]. The main reason for this is the inherent heterogeneity within these cells generates labelled population widths that far exceed the limits for division peak resolution. The only way to reduce this variation is to sort a narrowed population from within the peak channels of the fluorescent distribution [13–18]. However, determining the success of sort gate reduction is hampered by the fact that any measurement is always prone to error [19]. We have been able to determine detector specific sources of measurement error by sorting uniform fluorescent beads with similar spectral properties to the dyes under investigation and using the width of the re-analysed sorted population as a correction factor [13,20]. As such, we recently developed a method for assessing the suitability of tracking dyes and various cell lines to division tracking by dye dilution following cell sorting to reduce the input widths [20]. The criteria we set out for a good dye was it should label cells brightly, uniformly and with low toxicity. When the dye labelled population of cells is sorted to narrow the input width and re-measured to determine the measurement error as a function of population re-spreading, the dye should make a minimal contribution to any measurement errors. Finally, once in culture it should be well retained and inherited in a symmetrically fashion across the plane of cytokinesis [20]. Based on these criteria, we previously found that CellTrace Violet™ (CTV) was the favourable option for working with non-quiescent cells and that e-Flour Proliferation Dye 670™ (EPD) was the least favourable. We also found that certain cell lines were simply at the limit of this approach because the measurement errors associated with analysing such cells by flow cytometry meant that even sorting a narrowed input lead to spreading beyond the limits for peak resolution [20]. In this study, we have extended our appraisal to cover two lipophilic dyes PKH26™ and CellVue Claret™ (CVC) as well as a relatively new red excited version of CTV called CellTrace Far Red™ (CTFR). Once again, we assessed their performance using the Jurkat cell model due to the fact that is a more challenging system than primary resting cells as Jurkats are a transformed, highly heterogeneous cell line as opposed to a relatively uniform, resting primary T cell. We firstly judge the labelling efficiency by measuring the viability, intensity and uniformity of the dye labelled cells. Secondly we determine if we did indeed require cell sorting in order to resolve division peaks and whether cell viability was maintained for at least 48 h in culture. Thirdly we sorted each dye labelled population to equivalent fluorescence input width and determined the dye-specific contribution to any re-spreading error of the sorted population as if the division peak resolution was as expected based on these input widths. Lastly for all dyes, we determined the culture dependent sources of error, namely the propensity for dye transfer and the degree of symmetrical inheritance across the cytokinetic plane using imaging flow cytometry (IFC). All our findings were highly relevant to the use of each dye to track proliferation in resting primary T and B cells.

2. Materials and methods

2.1. Dye labelling

Dye labelling was conducted as outlined previously [16,20] and per the manufacturers recommendations [11,12]. Briefly 2× staining concentrations of CTV (C34557, Life Technologies, Paisley, UK), CTFR (C34564, Life Technologies), EPD (65-0840-90, eBiosciences, San Diego, USA), PKH26 (MINI26-1KT, Sigma, St Louis, USA) and CVC (MINCLARET-1KT, Sigma) were made up in either protein-free PBS (for succinimidyl dyes) or diluent C (for lipophilic dyes, provided with dyes). E6.1 Jurkat Cells (FHCRC-derived clone, Cell Services, CRUK) were cultured in RPMI media containing 10% FBS, penicillin/streptomycin, glutamine and 2-mercaptoethanol, counted and checked for viability using a Vi-Cell counter (Beckman Coulter Inc., USA), washed once and re-suspended in 1 ml of either protein-free PBS (for succinimidyl labelling) or diluent C (for lipophilic dyes) at a density of 4×10^6/ml. The 2× labelling solution (1 ml) was added to the cell suspension and incubated for 20 min at 37 °C (succinimidyl dyes) or 5 min (lipophilic dyes) at room temperature (RT) after which neat FBS was added to a final concentration of 10% (v/v) for 5 min to quench any free dye in solution. Cells were washed twice and re-suspended at a density of 1×10^7/ml in full culture media. Viability, staining intensity and uniformity was assessed immediately post-labelling using a BD LSRFortessa system (Becton Dickinson, Carlsbad, USA) configured with a 355 nm trigon, 405 nm octagon, 488 nm trigon, 561 nm octagon and 633 nm trigon laser excitation lines and associated detector arrays. Propidium Iodide (PI) was added to CTV labelled cells to determine viability (~0.5 μg/ml), and DAPI (~2 μg/ml) was added to EPD, CTFR, CVC and PKH26 labelled cells for the same purpose. Viable cells were gated based on forward scatter area (FSC-A) and exclusion of viability dye fluorescence. CTV fluorescence was measured in the Violet 450/50 channel. EPD, CTFR and CVC fluorescence was measured in the Red 670/30 channel. PKH26 fluorescence was measured in the yellow 585/15 channel. The median fluorescence intensity (MedFI) and co-efficient of variance (CV) was recorded for each. An example analysis is shown in Fig. 1A. PMT voltages were set based on the 10 μM labelled samples in order to achieve a channel median of ~50,000. The voltages set were 310v (CTV), 379v (EPD), 238v (CTFR), 370v (CVC) and 363v (PKH26).