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Research paper

New and improved methods for measuring lymphocyte proliferation *in vitro* and *in vivo* using CFSE-like fluorescent dyes

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ABSTRACT

The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to measure lymphocyte proliferation by flow cytometry has become one of the most widely utilised assays for assessing lymphocyte responses. The properties of CFSE make it ideal for such a task, covalently labelling cells with a long-lived fluorescence of high intensity and low variance with minimal cell toxicity. No dye in the last 20 years has been capable of replicating CFSE in these respects. However, currently CFSE is limited to following a maximum of 7 cell divisions and is not compatible for use with ubiquitously available fluorescein conjugates or other fluorescent molecules with spectral properties similar to fluorescein, such as EGFP. Here we characterise two new fluorescent dyes for measuring lymphocyte proliferation, Cell Trace Violet (CTV) and Cell Proliferation Dye eFluor 670 (CPD), which have different excitation and emission spectra to CFSE and, consequently, are compatible with fluorescein conjugates. We found that while both CTV and CPD can label cells to a high fluorescence intensity, which is long-lived and has low variability and low toxicity and makes them ideal for long-term tracking of non-dividing lymphocytes *in vivo*, CTV offers possibly the best available alternative to CFSE in the analysis of cell divisions. We also describe how intercellular dye transfer and cell autofluorescence can affect division resolution with the three different dyes and describe labelling conditions for the three dyes that produce ultra-bright lymphocytes for *in vivo* tracking studies and allow up to 11 cell divisions to be detected when using CFSE and CTV as the fluorescent dyes.

1. Introduction

The development of an adaptive immune response relies on a rapid expansion in the number of lymphocytes specific for a foreign antigen. Therefore, measurement of lymphocyte expansion using sensitive but convenient methods is critical for the progress of immunological research. The measurement of lymphocyte proliferation, both *in vitro* and *in vivo*, took a dramatic leap forward following the discovery of the cell labelling properties of the dye carboxyfluorescein diacetate succinimidyl ester (CFSE) which was ideal for flow cytometry assays (Weston and Parish, 1990; Lyons and Parish, 1994). Since then CFSE has been used in numerous studies on lymphocyte function (Hodgkin et al., 1996; Kurts et al., 1997; Bird et al., 1998; Gett and Hodgkin, 1998; Nordon et al., 1999; Marzo et al., 2000; Jedema et al., 2004; Stambas et al., 2007). In fact, since its initial description as a lymphocyte tracking and proliferation dye, CFSE has been used in over 20,000 scientific publications and has been cited in almost 1000 patents.

The use of CFSE in cell division analysis relies on the simple premise that when a dye-labelled cell divides, the CFSE fluorescence intensity is halved in the two daughter cells. However, in order to achieve high resolution of each cell division, it is essential that the labelling dye meets several important characteristics, namely (i) high initial staining intensity

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; CTV, Cell Trace Violet;CPD, Cell Proliferation Dye eFluor 670

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which allows numerous cell divisions to be detected before the dye dilutes to levels approaching cellular autofluorescence, (ii) low fluorescence variance, in order to maintain discrete division peaks, (iii) minimal and uniform dye leakage over long periods, so that fluorescence intensity and variance are not compromised, and (iv) low cell toxicity to ensure that cell function is not altered by the labelling procedure. Although numerous dyes have met some of these labelling characteristics, none have been able to match the unique properties of CFSE (Parish, 1999).

The remarkable success of CFSE is based on two main chemical attributes of the dye (Parish, 1999; Quah and Parish, 2010). The first is the presence of two acetate groups, which allows the dye to rapidly cross the plasma membrane of cells. However, removal of the acetate groups by intracellular esterases lessens the membrane permeability of the dye, thus allowing the dye to concentrate within cells. The second important chemical attribute of CFSE is the amino-reactive succinimidyl side chain of the dye, which allows CFSE to covalently couple to numerous intracellular proteins, including those with a low turnover rate. This results in a high degree of stable fluorescence, with CFSE typically allowing up to 8 peaks of fluorescence (or 7 cell divisions) to be measured by flow cytometry before the dye dilutes to cellular autofluorescence levels (Quah et al., 2007). When labelling is performed quickly, the rapid reactivity of CFSE also results in a population of lymphocytes with a low variance of fluorescence, typically with a standard deviation of each division peak being ~20% or less of the mean fluorescence intensity (Quah et al., 2007). This allows clear visualisation of discrete fluorescence peaks following each cell division. Although CFSE labelling under some conditions can affect cell behaviour (Quah et al., 2007; Last'ovicka et al., 2009; Parish et al., 2009), when used to label cells in appropriate buffering conditions it has very low cellular toxicity (Quah et al., 2007; Parish et al., 2009).

One of the main disadvantages of CFSE is that it removes the use of one of the most commonly used fluorescence channels in flow cytometry, *i.e.*, the channel used for fluorescein detection. An additional disadvantage of CFSE is that, based on current labelling procedures, a maximum of ~7 cell divisions can be measured. An added incentive to develop proliferation dyes with different spectral properties to CFSE is that it would allow the proliferation of different populations of lymphocytes to be simultaneously measured in the same culture or animal. Recently, two new fluorescent dyes have become available that seem to have the potential to work as well as CFSE but possess different fluorescence excitation and emission spectra. Cell Trace Violet (referred to here as CTV), produced by Molecular Probes, is excited by violet emissions (405 nm) commonly used in violet laser-equipped flow cytometers. It emits at a peak wavelength of ~450 nm and thus can be used in flow cytometers equipped with detectors for Pacific Blue. It is claimed to be capable of detecting up to 8–10 cell divisions, although this is based on results from flow cytometers equipped with acoustic focusing, this procedure being capable of generating better fluorescence peak resolution than standard flow cytometers (Molecular Probes, Invitrogen; Applied Biosystems Life Technologies). The second dye, Cell Proliferation Dye eFluor 670 (referred to here as CPD), produced by eBioscience, has a peak excitation of 647 nm and can be detected using the APC fluorochrome detectors. It is claimed by the manufacturer to be capable of detecting up to 5 cell divisions. Although these dyes appear to be promising candidates for measuring lymphocyte proliferation, there are no available reports comparing them side by side with CFSE.

In this report we have examined the ability of CTV and CPD to detect the proliferation of mouse lymphocyte *in vitro* and *in vivo* in comparison with CFSE, focusing on the key virtues of CFSE, namely, fluorescence intensity, low fluorescence variance, high stability of cell labelling and low cellular toxicity. It was found that CTV is comparable to CFSE in monitoring lymphocyte proliferation but CPD was a less effective dye, although both new dyes stably label lymphocytes and are potentially as useful as CFSE for long-term tracking of non-dividing lymphocytes *in vivo*. During this comparative study, we also identified intercellular dye transfer and cell autofluorescence as two potential causes of reduced resolution of cell division peaks using CFSE and CFSE-like dyes. In addition, we describe an optimised methodology for cell labelling with all three fluorescent dyes that results in ultra-bright lymphocytes for cell migration studies and increases the number of cell division detectable, potentially up to 11, when employing commonly used flow cytometers.

2. Methods

2.1. Animals

Mice were obtained from the Australian National University (ANU) Bioscience Services, ANU. Mice were housed and handled according to the guidelines of the ANU Animal Experimentation Ethics Committee. Mouse strains used were C57BL/6 (B6), B6.CD45.1 (B6 congenic for CD45.1) and the transgenic (Tg) mouse strains MD4 (B cell receptor (BCR)-Tg expressing hen egg lysozyme (HEL)-specific-IgM^a and IgD^a on a B6 background (Goodnow et al., 1988)), OT-II (T cell receptor (TCR)-Tg specific for I-A^b-ovalbumin (OVA)$_{323–339}$ peptide on a B6 background (Barnden et al., 1998)), P14.CD45.1 (TCR-Tg specific for D^b-GP$_{33–41}$ peptide (lymphocytic choriomeningitis virus-derived) on a B6.CD45.1 background (Pircher et al., 1989)), F5 (TCR-Tg specific for D^b-NP$_{366–374}$ peptide (influenza virus-derived) on a B6 background (Mamalaki et al., 1993)) and OT-I (TCR-Tg specific for K^b-OVA$_{257–264}$ peptide on a B6 background (Hogquist et al., 1994)). Male mice were used at 6–12 weeks of age.

2.2. Lymphocyte preparation

Lymphocytes were obtained from spleen and/or lymph nodes as previously described (Quah et al., 2004). CD4$^+$ T cells and CD8$^+$ T cells were enriched from pooled lymph nodes as well as spleen, and B cells were enriched from spleen via magnetic-activated cell sorting (MACS) (Miltenyi Biotec) as previously described (Quah et al., 2004).

2.3. Labelling lymphocytes with fluorescent dyes

CFSE, CTV (both from Molecular Probes, Invitrogen) and CPD (eBioscience) fluorescent dyes were dissolved in DMSO as 10 mM stock solutions (stored at −20 °C). For dye labelling lymphocytes were resuspended to 1×10^8/mL in 20 °C RPMI 1640 medium (Invitrogen) supplemented with 10%