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# Use of SNARF-1 to measure murine T cell proliferation *in vitro* and its application in a novel regulatory T cell suppression assay

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ABSTRACT

The green fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) has been used to track the proliferation of T cells *in vitro*. Such assays often incorporate more than one population of cells, but the paucity of alternative, spectrally distinct dyes suitable for measuring proliferation has hampered the simultaneous tracking of multiple cell populations; furthermore, CFSE is not compatible with green fluorescent protein (GFP), used to identify T cells in various transgenic mice. We have therefore validated the use of the far red dye seminaphthorhodafluor-1 (SNARF)-1 – originally developed to measure intracellular pH – to track murine T cell proliferation *in vitro*, demonstrating its ability to distinguish multiple cycles of proliferation over three days in a similar fashion to CFSE. The small changes in fluorescence emission attributed to intracellular alkalinisation of proliferating T cells have minimal impact on the ability of SNARF-1 to track cell division and this dye induces minimal cell death at the concentration used in this application. On the basis of these results, we have developed a novel *in vitro* murine T cell suppression assay, in which the proliferation of both conventional T cells (Tcons) stained with SNARF-1 and regulatory T cells (Tregs) stained with CFSE can be measured simultaneously. We have also demonstrated that SNARF-1 may be used to stain Tcons in assays of suppression involving 'designer' Tregs, generated by the transduction of $CD4^+$ T cells with constructs encoding the $Foxp3^{gfp}$ fusion protein.

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## 1. Introduction

While *in vitro* assays are rarely able to reproduce the cellular complexities of *in vivo* microenvironments, they have nevertheless made an important contribution to the advancement of immunology [1–3]. Several key discoveries in the field of Treg immunology have been made as a consequence of *in vitro* studies, including the initial characterisation of naturally occurring Tregs [4], the elucidation of key suppressor-effector molecules [5,6], and the identification of defects in Treg function in various diseases [7,8]. Read-outs of these assays have included the measurement of both Tcon proliferation and the concentrations of cytokines such as IL-2 and IFN-γ in culture supernatants.

The proliferation of T cells may be measured in various ways, including the widely employed technique of tritiated thymidine ($^3$H-TdR) incorporation [9,10]. This technique provides an indication of proliferation by measurement of the incorporation of $^3$H-TdR into replicating DNA during the S phase of the cell cycle [10,11]. However, this technique has several shortcomings, including the need to handle and dispose of a radioactive isotope, a variable signal-to-noise ratio, and the inability to assign responses to a particular cell type in co-cultures of viable cells; moreover, the cells cannot be used for downstream applications and proliferation is measured over a short period of time, rather than the duration of the culture. These shortcomings have spurred the development of alternative techniques, such as colorimetric ELISA assays [12] and the measurement of 5-bromo-2-deoxyuridine (BrdU) [13], PKH26 [14,15] and CFSE [16–19] dilution by flow cytometry. Of these dyes, the most widely used for monitoring cell proliferation *in vitro* is CFSE, which fluoresces at 518 nm and is thus detected in the 'green' channel of the flow cytometer [14,19]. CFSE reacts covalently with intracellular proteins and undergoes only limited leakage from the cell, partitioning equally among daughter cells and yielding a sufficiently bright signal to allow the distinction of up to eight to ten cycles of division [14,19]; moreover, it may also be used to track cells *in vivo* [18,20,21]. However, the paucity of reliable alternative dyes with distinct spectral characteristics has challenged both the simultaneous measurement of proliferation of multiple T cell

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populations *in vitro* and the design of assays incorporating cells labelled with GFP – for example, Tregs derived from the transgenic Foxp3$^{gfp}$ mouse [22].

The dye SNARF-1, which emits light at a wavelength of 670 nm, was originally developed for the measurement of intracellular pH ($pH_i$) [23–25]. It diffuses passively into cells in its nascent acetomethyl ester form; the ester is then hydrolysed by ubiquitous intracellular esterases, releasing a fluorescent, proton-sensitive, membrane-impermeant probe that becomes trapped within the cell [23–25]. In addition to its well established use to measure $pH_i$, more recent studies have employed SNARF-1 to label antigen-presenting cells in mixed lymphocyte reactions containing CFSE-labelled responder cells [26] and to track the proliferation of human CD4$^+$ T cells *in vitro* [27,28]. In the current study, we have validated the use of SNARF-1 to monitor the short-term proliferation of murine T cells *in vitro*, demonstrating its ability to distinguish multiple cycles of proliferation in a similar fashion to CFSE. On the basis of these results, we have developed a novel *in vitro* murine T cell suppression assay, in which the proliferation of both Tcons stained with SNARF-1 and Tregs stained with CFSE can be measured simultaneously. We have also demonstrated that SNARF-1 may be used to stain Tcons in assays of suppression involving 'designer' Tregs, generated by the transduction of CD4$^+$ T cells with constructs encoding the Foxp3$^{gfp}$ fusion protein.

## 2. Materials and methods

### *2.1. Mice*

Mice were purchased from Harlan Olac (Loughborough, UK) or bred in-house and housed in specific pathogen-free conditions at the Central Biological Services, Hammersmith Campus of Imperial College London (ICL). C57BL/6 mice were used for all experiments. All mice were humanely killed by asphyxiation in carbon dioxide, according to UK Home Office and local institutional regulations.

### *2.2. Purification of CD25$^+$ and CD25$^-$ CD4$^+$ T cells*

Spleens and superficial inguinal lymph nodes from freshly euthanized mice were macerated through a 70 μm cell strainer, before washing, second passage through a cell strainer and treatment with red cell lysis buffer (8.3 g/l ammonium chloride, 37.0 g/l disodium-ethylenediaminetetraacetic acid and 1.0 g/l potassium bicarbonate, maintained at pH 7.5). Negative selection of CD4$^+$ T cells was performed with sheep anti-rat DynaBeads® (Invitrogen Dynal; Oslo, Norway) to capture cells labelled with monoclonal antibodies (mAbs) against MHC class II (M5/114.15.2), CD8 (53.6.72) and CD32 (2.4 G2), all added as hybridoma supernatants. CD4$^+$CD25$^+$ T cells were positively selected by means of an autoMACS™, using Streptavidin MicroBeads® (Miltenyi Biotech; Bergisch Gladbach, Germany) to capture CD4$^+$ T cells labelled with biotinylated anti-CD25 mAb (clone 7D4; BD Biosciences, Heidelberg, Germany). The purity of CD25$^+$ and CD25$^-$ CD4$^+$ T cells was >90%.

### *2.3. Staining of cells with CFSE and SNARF-1*

Cells ($10 \times 10^6$/ml in PBS) were stained with freshly prepared 2 μM CFSE (Invitrogen Ltd.; Paisley, UK) for 15 min, or 10 μM SNARF-1 (Invitrogen) for 30 min, before washing twice in PBS containing 10% (v/v) fetal calf serum – all performed at room temperature. In some experiments dual-staining was performed, first with SNARF-1 and then with CFSE, with a single washing step in between the respective staining protocols, as described above.

### *2.4. Proliferation and suppression assays*

Cells were cultured in round-bottom, 96-well plates ($1–2 \times 10^5$/well) in the presence of Epoxy DynaBeads® coated with anti-CD3 and anti-CD28 mAbs, at a ratio of one bead per two total cells. In some experiments, cells were also stained with Annexin-V conjugated to allophycocyanin (eBioscience Ltd.; Hatfield, UK), according to manufacturer's recommendations. For the suppression assays, a varying number of CD4$^+$CD25$^+$ T cells were cultured with a constant number ($1–2 \times 10^5$/well) of CD4$^+$CD25$^-$ T cells, establishing co-cultures of CD25$^+$:CD25$^-$ CD4$^+$ T cells of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32; monocultures of CD25$^+$ or CD25$^-$ CD4$^+$ T cells were also established. After three days, co-cultured cells were pulsed with $^3$H-TdR (0.5 μCi per well; Amersham Biosciences, UK), before measuring the incorporation of $^3$H-TdR by means of a cell harvester and liquid scintillation counter 16 h later (Wallac Trilux; Perkin Elmer, UK).

### *2.5. Flow cytometry*

CD4$^+$ T cells were stained with CFSE and SNARF-1 as described above; all experimental conditions were examined in triplicate. Samples were acquired on a BD FACSCalibur™ or BD FACSCanto™ II (BD Biosciences, NJ, USA), exciting both dyes with the 488 nm argon laser and measuring emission by means of the 530/30 nm band-pass filter (FL-1 channel) for CFSE and the 650 nm long-pass filter (FL-3 channel) for SNARF-1. Instrument setting were selected to ensure that autofluorescence of both stimulated and unstimulated cells fell in the first decade, while fluorescence signals for stained, but unstimulated, cells fell in the third or fourth decade. Cell proliferation data were analysed using Flow Jo™ (Tree star Inc.; Ashland, OR, USA) software. In experiments involving the admixture of cells respectively stained with CFSE and SNARF-1, both FSC-A *versus* SSC-W parameters and distinct staining gates were drawn around single-positive events for the purposes of analysis to avoid the confounding influence of double-positive (CFSE$^+$SNARF-1$^+$) events – which could represent doublets, triplets or larger micro-aggregates of interacting Tregs and Tcons, or passive back-leakage of dye into the culture medium and partial staining of the co-cultured population. Proliferation data were presented either as the proportion of events at the time of data acquisition representing cells that had divided zero, one, two or *n* times, often summarising the data as the proportion of cells that had divided one or more times; or as the division index, calculated by Flow Jo™ as the product of the proliferation index (mean number of cell divisions for the whole population) and precursor frequency (proportion of cells in the original sample that had undergone division). Suppression mediated by Tregs was calculated as the decrement in the proliferation metric – either the percentage of cells at the time of data acquisition that had divided one or more times, or the division index – expressed as a proportion of the proliferation of Tcons cultured without Tregs.

### *2.6. Transduction of T cells*

CD4$^+$ T cells isolated by negative selection were activated by means of anti-CD3/CD28 Dynabeads® and 50 U/ml IL-2 (Roche, UK) for two days prior to transduction. On day 3, CD4$^+$ T cells were transduced by retrovirus produced by lipofectamine-mediated transfection of Phoenix™ eco packaging cells (Orbigen Inc.; San Diego, CA, USA) [29] with a control construct encoding the islet-cell specific T cell receptor (TCR) BDC2.5 and GFP (pMIG II/BDC2.5; 'CD4$^{BDC2.5}$') [30] or a construct encoding both BDC2.5 and the fusion protein FoxP3$^{gfp}$ (pMIG II/BDC2.5/FoxP3$^{gfp}$; 'CD4$^{BDC2.5+F}$'); an additional experiment was performed with similarly prepared CD4$^+$ T cells transduced with a control construct encoding the islet-

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