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Special Feature

Fluorescent dyes for lymphocyte migration and proliferation studies

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Summary Fluorescent dyes are increasingly being exploited to track lymphocyte migration and proliferation. The present paper reviews the properties and performance of some 14 different fluorescent dyes that have been used during the last 20 years to monitor lymphocyte migration. Of the 14 dyes discussed, two stand out as being the most versatile in terms of long-term tracking of lymphocytes and their ability to quantify lymphocyte proliferation. They are the intracellular covalent coupling dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and the membrane inserting dye PKH26. Both dyes have the advantage that they can be used to track cell division, both *in vitro* and *in vivo*, due to the progressive halving of the fluorescence intensity of the dyes in cells after each division. However, CFSE appears to have the edge over PKH26 based on homogeneity of lymphocyte staining and cost. Two other fluorescent dyes, although not suitable for lymphocyte proliferation studies, are valuable tracking dyes for short-term (up to 3 day) lymphocyte migration experiments, namely the DNA-binding dye Hoechst 33342 and the cytoplasmic dye calcein. In the future it is highly likely that additional fluorescent dyes, with different spectral properties to CFSE, will become available, as well as membrane inserting fluorescent dyes that more homogeneously label lymphocytes than PKH26.

Key words: calcein, carboxyfluorescein diacetate succinimidyl ester, flow cytometry, fluorescence microscopy, fluorescent dyes, Hoechst 33342, lymphocyte division, lymphocyte migration, lymphocyte positioning, PKH26.

Introduction

As an introduction to the Special Feature on the lymphocyte tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE), I think that it is appropriate for me to briefly describe the research at the John Curtin School of Medical Research in Canberra that led to the development of CFSE and, also, to review the considerable range of fluorescent dyes that are now available to immunologists for lymphocyte proliferation and/or migration studies.

Lymphocytes, the mediators of adaptive immunity, have the remarkable ability to continually migrate throughout the body and, depending on their differentiation state, position themselves in specific areas within lymphoid organs. Furthermore, following contact with antigen, there is a rapid proliferation of antigen-specific T cell and B cell clones, with a consequent change in the migration and positioning behaviour of the responding cells. In order to analyse this complex series of events, procedures are required that can simultaneously track the migration of lymphocyte into different tissues, the positioning of these lymphocytes within tissues and the proliferation history of the lymphocytes being studied.

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Early studies used radioactive markers to track lymphocyte migration.[1–3] Certainly, radiotracers provide a convenient means of gaining a general overview of the distribution pattern of injected lymphocytes throughout an animal. However, as well as being potentially biohazardous, they suffer from a number of drawbacks.[4] First, it is difficult to follow the migration behaviour of individual cells, except by tedious autoradiography of histological sections, and no information on the division history of the injected cells can be obtained. Second, gamma-emitting isotopes, such as ^{51}Cr and ^{125}I, can adversely affect cell viability and less damaging isotopes, like ^{3}H and ^{14}C, are difficult to detect *in vivo* due to their low emission energies and susceptibility to quenching. Third, it is difficult to determine whether the radioisotope is still associated with the injected lymphocytes or has been reused by bystander cells *in vivo*. Such reutilization is a well documented problem with the radioisotopes ^{125}I and ^{51}Cr.[4] Finally, due to their radioactive decay and cellular turnover, radioisotopes can only be used for relatively short-term migration studies.

In an attempt to overcome the numerous problems associated with radiotracers, during the last 20 years a number of fluorochromes have been used to track lymphocyte migration, positioning and proliferation. Initially, the migration behaviour of the fluorescent lymphocytes was usually assessed by fluorescence microscopy of sections of lymphoid organs or by the enumeration of fluorescent cells in lymphoid cell suspensions by fluorescence microscopy.[5–7] Quantification of fluorescent cells by microscopy is a very tedious procedure and was soon replaced by fluorescence flow cytometry methods of measurement.[8–10] The flow cytometry

Table 1 Fluorescent dyes used for lymphocyte migration and proliferation studies

Name	Abbreviation	Absorption	Emission	Detection
Hoechst 33342	H33342	355	460	FM, FC
Thiazole Orange	–	501	547	FM, FC
Calcein, AM	Calcein	496	520	FM, FC
2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester	BCECF-AM	505	545	FM, FC
Fluorescein diacetate	FDA	489	514	FM, FC
5-(and-6)-carboxyfluorescein diacetate	CFDA	489	517	FM, FC
5-(and-6)-carboxyfluorescein diacetate, acetoxymethyl ester	CFDA-AM	489	517	FM, FC
5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester	CFSE (CFDA-SE)	491	518	FM, FC
Fluorescein isothiocyanate	FITC	494	519	FM, FC
Tetramethylrhodamine isothiocyanate	TRITC	544	572	FM
3,3′-dioctadecyloxacarbocyanine perchlorate	DiO	484	501	FM, FC (?)
1,1′-dioctadecyl-3,3,3,3′ tetramethylindocarbocyanine perchlorate	DiI	549	565	FM, FC(?)
PKH 2	–	490	504	FM, FC
PKH 26	–	551	567	FM, FC

FM, fluorescence microscopy; FC, flow cytometry.

studies subsequently identified fluorescent dyes that also could be used to monitor the proliferation history of the labelled lymphocytes via progressive two-fold dilution of the dye in dividing cells.[11,12]

In the present paper, I will review the various fluorescent dyes that have been used to track lymphocyte migration and proliferation during the last 20 years and highlight some of the advantages and disadvantages of the different staining techniques. Table 1 lists the chemical names and acronyms of some 14 different fluorescent dyes that have been used to monitor lymphocyte migration. Most of the dyes listed have been used to track fluorescent lymphocytes by both fluorescence microscopy and flow cytometry. The only exception is tetramethylrhodamine isothiocyanate (TRITC), which has inappropriate spectral characteristics for detection by standard flow cytometers. The suitability of the lipophilic carbocyanine dyes, C18 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI), for flow cytometry tracking of lymphocytes has not been thoroughly investigated.

In order to facilitate discussion of the properties of the different dyes, they will be considered in groups according to their mechanism of staining.

DNA-binding fluorescent dyes

Two DNA-binding compounds that can label the DNA of viable cells, namely Hoechst (H)33342 and Thiazole Orange, have been investigated as potential fluorescent dyes for tracking lymphocytes *in vivo* (Table 2) and will be discussed below.

Thiazole Orange

Thiazole Orange is one of the few DNA dyes with similar spectral properties to fluorescein and therefore can be excited by the 488 nm argon ion laser found in most flow cytometers. It also labels viable lymphocytes to extremely high fluorescence intensities.[9] Unfortunately, Thiazole Orange is rapidly lost from lymphocytes and can only be used to track cells *in vivo* for a few hours.[9] Nevertheless, due to its convenient spectral properties and high labelling efficiency, the dye can be used in short-term *in vitro* cell adhesion assays.

Hoechst 33342

Hoechst 33342 is a bisbenzimide derivative that binds to AT-rich regions of DNA and, due to its membrane permeant properties, has been frequently used to label DNA in viable cells for cell cycle analysis. In the early 1980s, Mary Brenan and I investigated whether this dye could be used to follow lymphocyte migration.[6] We found it to be a very useful dye for such studies. It labels lymphocytes to high fluorescence levels, is well retained by lymphocytes over several days[6,9] and is particularly effective in determining the positioning pattern of lymphocytes in different lymphoid organs, the dye being well retained in either frozen or fixed tissues.[6,7,13–19] One of its major advantages is that labelled cells are remarkably resistant to quenching when viewed under the fluorescence microscope. In fact, the autofluorescence of tissue sections can be quenched by prolonged exposure to UV light without any apparent loss of H33342 fluorescence.[19] This

Table 2 Labelling properties of different fluorescent dyes used for lymphocyte migration and proliferation studies

Fluorescent dye	Staining properties	Staining persistence	Advantages	Disadvantages	Major use
H33342	Nuclear (DNA binding)	2–4 days	Highly fluorescent UV excitable Resistant to quenching *In vivo* labelling possible	Can inhibit cell proliferation Relatively short half-life Transfer to unlabelled cells	Migration and positioning studies
Thiazole Orange	Nuclear (DNA binding)	Hours	Highly fluorescent	Half-life too short	Better alternatives, e.g. H33342
Calcein	Cytoplasm	3 days	Highly fluorescent Identifies lymph node homing subset of lymphocytes	Relatively short half-life	Migration and positioning studies
BCECF-AM	Cytoplasm	3 days	Highly fluorescent	Relatively short half-life Can inhibit cell proliferation	Migration and positioning studies
FDA, CFDA	Cytoplasm	Hours	Moderate fluorescence	Half-life too short Transfer to unlabelled cells	Better alternatives, e.g. CFSE
CFDA-AM	Cytoplasm	Hours	Highly fluorescent	Half-life too short	Better alternatives, e.g. CFSE
CFSE	Cytoplasm* (covalent)	Weeks to months	Highly fluorescent Persistent staining Suitable for tracking cell division *In vivo* labelling possible	Toxicity at high labelling concentrations	Migration, positioning and cell division studies
FITC, TRITC	Membrane and cytoplasm (covalent)	Days to weeks	Persistent staining *In vivo* labelling possible	Toxicity Relatively low fluorescence TRITC unsuitable for flow cytometry	Migration and positioning studies
DiO	Membrane	Days to weeks(?)	Highly fluorescent Persistent staining *In vivo* labelling possible (?)		Migration and positioning studies Cell division?
DiI	Membrane	Days to weeks (?)	Highly fluorescent Persistent staining *In vivo* labelling possible (?)	Not suitable for flow cytometry (?)	Migration and positioning studies Cell division?
PKH2	Membrane	Days to weeks	Highly fluorescent Persistent staining *In vivo* labelling possible (?)	Toxicity	PKH26 a better alternative
PKH26	Membrane	Weeks to months	Highly fluorescent Persistent staining Suitable for tracking cell division *In vivo* labelling possible Red fluorescence	Toxicity at high labelling concentrations (?) Less homogeneous labelling than CFSE Expensive	Migration, positioning and cell division studies

*Dominant site of covalent attachment of dye to lymphocytes. H, Hoechst; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; AM, acetoxymethyl ester; FDA, fluorescein diacetate; CFDA, 5-(and-6)-carboxyfluorescein diacetate; CFSE, carboxyfluorescein diacetate succinimidyl ester; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; DiI, 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate.

resistance to quenching allowed us to detect extremely low numbers of H33342-labelled autoreactive T cells in the central nervous system of rats.[20] By counterstaining with ethidium bromide, the viability of H33342-labelled cells *in vivo* could also be determined.[14,21]

Hoechst 33342 is also an excellent dye for tracking lymphocyte migration by flow cytometry.[9,10,16] The dye absorbs in the UV range and therefore can only be excited by flow cytometers fitted with a UV light source. However, this limitation can be turned to advantage as, with UV light source instruments, H33342-labelled lymphocytes can be detected with all visible fluorescence channels still being available for the detection of other fluorochromes. Interestingly, H33342 also labels mouse splenic B lymphocytes more brightly than splenic T cells (A Manderson, unpubl. obs., 1999), a quite useful property when these two lymphocyte populations need to be tracked *in vivo*.

A novel use of H33342 has been to assess the distribution of tumour-infiltrating lymphocytes in relation to the blood supply of tumours.[22] When H33342 is injected intravenously into tumour-bearing animals, the dye diffuses slowly into the tumour via the tumour vasculature and labels the DNA of cells it encounters. In fact, the intensity of cellular fluorescence is inversely related to the distance of the labelled cells from the vascular supply. Using this approach, it has been shown by flow cytometry that NK cells, but not T lymphocytes, are concentrated in the vascularized areas of the preneoplastic lesions of a mammary adenocarcinoma.[22]

Despite H33342 being an excellent dye for following lymphocyte migration, it does suffer from a number of disadvantages. The most obvious is that it is retained by lymphocytes for only a few days and therefore its use is limited to relatively short-term migration studies. Studies by us[20] and others[23] indicate that the limit of reliable detection of H33342-labelled lymphocytes *in vivo* by immunofluorescence microscopy is approximately 4 days after injection of labelled cells. Flow cytometry studies have given a similar limit of detection.[9] However, undoubtedly the greatest problem associated with the use of H33342 is that it can inhibit lymphocyte proliferation, presumably due to its ability to bind to cellular DNA. A number of groups have reported this difficulty,[9,10,24] although it can be overcome if cells are labelled with concentrations of H33342 that are subsaturating for cellular DNA.[24] There is one report that H33342 labelling reduces the motility and migration pattern of lymphocytes,[24] although numerous studies in my laboratory over many years have failed to observe such an effect. It is also claimed that H33342 can elute from labelled lymphocytes and interact with the DNA of bystander cells.[10] Such a phenomenon may occur in sections from frozen or fixed tissues, where major cellular disruption has occurred,

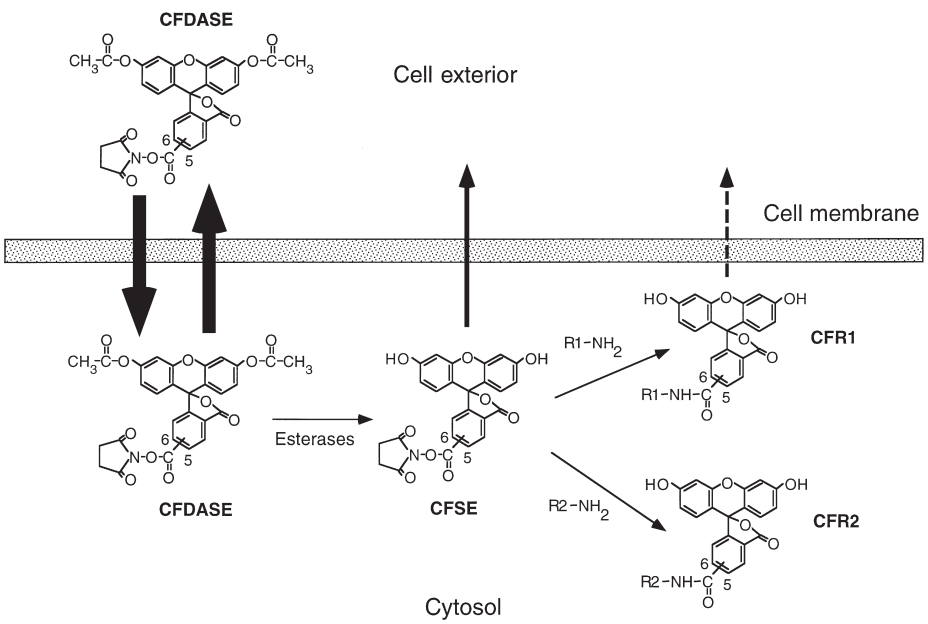

Figure 1 Schematic representation of the mechanism of fluorescently labelling cells with carboxyfluorescein diacetate succinimidyl ester. Initially, the non-fluorescent, highly membrane permeant, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE) is readily taken up by cells, although its high lipophilicity also allows it to freely exit from cells. Intracellular esterases, however, can remove the two acetate groups from CFDASE to yield fluorescent 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE), which is much less membrane permeant and, therefore, exits from cells at a much slower rate. The succinimidyl moiety of CFSE is highly reactive with amino groups and can covalently couple 5-(and-6)-carboxyfluorescein (CF) to intracellular molecules. In some cases, CF covalently couples to intracellular molecules ($R1\text{-}NH_2$) to form conjugates (CFR1) that can still exit from the cell or are rapidly degraded. However, a proportion of CF becomes coupled to long-lived intracellular molecules ($R2\text{-}NH_2$) to form conjugates (CFR2) that cannot escape from the cell and thus, stable fluorescent labelling of cells is achieved. Note that the size of the arrows in the figure is proportional to the rate of diffusion of the different molecules through the cell membrane.

but we have not observed H33342 transfer in *in vitro* coculturing experiments[6] or in *in vivo* flow cytometry studies. For example, when H33342-labelled lymphocytes are used that carry a CD45 allotype that differs from that of the recipient animal, flow cytometry studies have shown no evidence of dye transfer to recipient lymphocytes, even when lymphocyte suspensions prepared from the injected animals are stored overnight in the fixative paraformaldehyde before analysis (A Manderson, unpubl. obs., 1999).

Cytoplasmic fluorescent dyes

Clearly, H33342 is a valuable dye for studying lymphocyte migration. However, in the late 1980s Sue Weston and I made a concerted effort to identify new fluorescent dyes that exhibited more persistent staining properties and different spectral characteristics to H33342. In particular, I was keen to identify fluorescein-based dyes, which would be ideal for use with all standard flow cytometers. I was particularly impressed by the wide range of membrane permeant, fluorescein-based dyes that were becoming commercially available from Molecular Probes (Eugene, OR, USA). These dyes had been originally developed to measure intracellular pH, intracellular ion concentrations, intercellular adhesion and general cell viability, but in theory could be used to label lymphocytes for *in vivo* migration studies. A key feature of these dyes is that they are conjugated with acetate or acetoxymethyl side chains, which render the dyes much more membrane permeant. However, the side chains are esterase sensitive and, therefore, when the dyes enter cells the acetate/acetoxymethyl groups are removed by endogenous intracellular esterases and the dyes are trapped inside cells (see Fig. 1).

Initially we investigated nine membrane-permeant, fluorescein-based fluorescent dyes, of which six were found to be retained in the cytoplasm of lymphocytes, namely calcein, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), fluorescein diacetate (FDA), 5-(and-6)-carboxyfluorescein diacetate (CFDA), 5-(and-6)-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM) and CFSE.[9] One of these dyes, CFSE, also has the potential to become covalently coupled to cytoplasmic components and will be considered in detail later. The dyes FDA and CFDA, although labelling lymphocytes to relatively high fluorescence levels, were poorly retained by the cells,[9] an observation we had reported earlier based on fluorescence microscopy studies.[6] Furthermore, the rapid release of these dyes from cells resulted in considerable staining of bystander lymphocytes.[6] For these reasons, the two dyes have not been further investigated. The dye CFDA-AM labelled lymphocytes to extremely high fluorescence levels, due to it being highly membrane permeant, but, like FDA and CFDA, it was rapidly lost from labelled cells.[9] It was considered to be generally unsuitable for lymphocyte migration studies, particularly considering that better alternatives were available.

Calcein and BCECF-AM

Two of the membrane permeant fluorescent dyes, calcein and BCECF-AM, label lymphocytes to very high fluorescence intensities and are relatively well retained by the labelled cells.[9] It has been noted that *in vivo* there was a biphasic loss of the two fluorescent dyes from cells, namely an initial rapid loss of 80–90% during the first 15 h after the cells were injected, followed by a much slower release over the next 2–3 days.[9] One disadvantage with BCECF-AM is that mouse splenic T cells loaded with the dye exhibit a reduced proliferative response.[9] Such an effect has not been observed with human lymphocytes, although BCECF-AM does influence chemotaxis and superoxide production by human granulocytes.[25] Because BCECF-AM is designed to be used as an intracellular pH indicator, it is conceivable that the dye is buffering pH changes associated with cell signalling events. Nevertheless, BCECF has been successfully used by us to monitor lymphocyte migration by flow cytometry and to identify different polysaccharides and oligosaccharides that inhibit the entry of lymphocytes into murine spleen and lymph nodes.[26]

Calcein stands out as the most useful and interesting of the cytoplasmic fluorescent dyes we have so far studied. It labels cells to high fluorescence intensities and exhibits reasonably persistent labelling, particularly for a dye that does not covalently link to intracellular molecules or intercalate into cell membranes. Studies by us[9] and others[25] have also shown that the dye, unlike BCECF-AM and H33342, has no detectable effect on a range of cellular functions, even when cells are loaded with high dye concentrations. Recent reports have confirmed that calcein is an excellent fluorescent dye for relatively short-term *in vivo* migration studies.[27,28] However, the most remarkable feature of calcein is that it labels lymphocytes into two distinct subpopulations, based on fluorescence intensity, with the ‘calcein low’ population entering all lymphoid organs but with the ‘calcein high’ population being excluded from lymphoid organs where entry is high endothelial venule dependent.[29] The explanation for this phenomenon is unknown but one possibility is that calcein uptake is controlled by the same membrane properties, such as fluidity and plasticity, which are essential for the passage of lymphocytes through high endothelial venules.[29]

Covalent coupling fluorescent dyes

Fluorescein isothiocyanate and TRITC

Fluorescein isothiocyanate (FITC) and TRITC were the first fluorescent dyes to be used to track lymphocyte migration. Both compounds contain an isothiocyanate moiety, which is highly reactive with aliphatic amine groups and results in covalent attachment of the fluorescent dyes to cellular proteins. In early studies, FITC and TRITC were used to covalently couple fluorescein and rhodamine to lymphocytes *in situ*. For example, the intrathymic injection of FITC was used to label thymocytes and quantify the proportion of these cells that actually exit the thymus and enter peripheral lymphoid organs.[5] This was a classic study, which showed unequivocally that only 1% or less of lymphocytes in the thymus actually exit the organ, with the vast majority dying within the thymus.[5]

Conditions were soon established where lymphocytes could be labelled *in vitro* with FITC and TRITC[30] and then infused into animals and be readily tracked in lymphocyte

migration experiments.[8] Conjugating lymphocytes with high concentrations of FITC and TRITC, although having no effect on cell viability, did interfere with the ability of the cells to enter lymphoid organs.[8] However, coupling procedures were defined where the migration behaviour and immune responsiveness of the lymphocytes was unaffected, but where there was adequate fluorochrome labelling for cell tracking studies.[8,30] Both FITC and TRITC are suitable for detecting labelled cells in lymphocyte cell suspensions, but FITC-labelled cells are more readily detected in frozen sections of lymphoid organs.[8] An interesting feature of FITC labelling is that B cells are more brightly stained than T cells, a phenomenon not observed with TRITC labelling.[30] Fluorescence microscopy studies also suggest that both dyes label intracellular proteins as well as cell membranes.[30] However, FITC and TRITC are relatively membrane-impermeant dyes and therefore would be much less efficient at labelling cells intracellularly than the highly membrane-permeant dyes BCECF, calcein and CFSE. During the last 20 years there have been many studies using FITC, and to a lesser extent TRITC, to monitor lymphocyte migration. However, the emergence of dyes that label cells to much higher fluorescence intensities, without having adverse biological effects, has resulted in a decline in their use in recent years.

Carboxyfluorescein diacetate succinimidyl ester

As mentioned earlier, CFSE is one of the membrane-permeant, fluorescein-based dyes that was initially identified by my laboratory as having considerable potential as a long-term tracking dye for lymphocyte migration experiments.[9] There appears to have been some confusion regarding the correct nomenclature for this reagent. The compound used to label cells is carboxyfluorescein diacetate succinimidyl ester and therefore CFDA-SE would be a more appropriate acronym than CFSE. In fact, this is the acronym used for the dye in the current Molecular Probes catalogue. Paradoxically, when we first purchased the dye in the late 1980s it was listed in the Molecular Probes catalogue as CFSE and so this was the acronym we used in our first publication reporting the use of the dye.[9] In order to be consistent, we have continued to use the acronym CFSE in all our subsequent publications.

The dye has a number of important physicochemical properties that should be discussed. The two acetate side chains render the molecule highly membrane permeant. However, once inside cells, the acetate groups are removed by intracellular esterases and the resultant carboxyfluorescein exits from cells at a much slower rate (Fig. 1). The slow exit rate also provides ample time for the CFSE to covalently couple to intracellular molecules. Coupling is via the succinimidyl moiety, which reacts with intracellular amine groups, forming a highly stable amide bond. Succinimidyl esters have a number of advantages over isothiocyanates as covalent coupling reagents.[31] They have an intrinsically faster reaction rate, they form a more stable covalent linkage and they are highly reactive with amines at neutral pH. The latter point is particularly important for the intracellular labelling of cells.

CFSE can potentially react with a wide range of amine-containing molecules within a cell (Fig. 1). Many of the conjugates may be short-lived molecules or may be capable of passing through plasma membranes. Thus, much of the CFSE initially taken up by cells is lost during the first 24 h following labelling.[9] However, there are sufficient quantities of long-lived molecules labelled within cells to allow CFSE-labelled lymphocytes to be tracked *in vivo* for extraordinary lengths of time. In our early studies, we tracked non-dividing lymphocytes for 8 weeks,[9] but with more sensitive flow cytometers becoming available this has lengthened to 6 months.

As discussed in detail in other contributions to this Special Feature on CFSE, a major advantage of CFSE is that it can be used to track cell division, both *in vitro* and *in vivo*, due to the progressive halving of the fluorescence intensity of the dye in cells after each division.[12] With the current labelling protocols used, up to eight cell divisions can be tracked, an ample number for most immunological studies.[12] When Sue Weston and I first reported the use of CFSE as a long-term tracking dye,[9] we did not anticipate that it could also be used to monitor the division of labelled lymphocytes. Bruce Lyons spotted the effect, by chance, a few years later when he was using CFSE for long-term tracking studies of lymphocytes.

Since its discovery, CFSE has been used in a range of important immunological studies, which are extensively discussed by others in this Special Feature. For example, it has been shown that B cell isotype switching,[32–34] T cell cytokine production,[35,36] lymphocyte cell surface marker expression[37] and T cell apoptosis[38] are all highly division-dependent processes. CFSE has also been used to examine phenomena as diverse as the fate of autoreactive B cells,[39] the effects of different costimulatory signals on T cell proliferation,[40] the influence of pertussis toxin on B cell proliferation and survival *in vivo*,[41] the T cell blastogenic response of potentially immunodeficient patients to *Candida*[42] and the stimulatory requirements for the proliferation of human bone marrow stem cells.[43] Although most of the published reports have used CFSE to monitor lymphocyte proliferation, recent studies have used the dye to follow fibroblast division[44] and even bacterial proliferation![45]

CFSE has also been an extremely useful intracellular dye for conventional analyses of lymphocyte migration. We have found CFSE to be the fluorescein-based dye of choice for long-term lymphocyte migration experiments[9,18,41,46] and studies by others have supported this view.[47,48] CFSE has also been used, in a similar manner to FITC and TRITC, to label thymocytes[49] and embryonic cells[50] *in situ* for subsequent cell migration studies. In addition to fluorescence microscopy, the CFSE-labelled cells can be visualized in tissue sections by immunohistochemical means using antifluorescein antibodies.[49,50]

Based on the experience gained with CFSE, it should be possible to design a range of fluorescent dyes, with different spectral properties to fluorescein, for long-term tracking and cell division experiments. This would provide researchers with the capacity to simultaneously follow the migration and proliferation of differently labelled cell populations in the same animal. All that is required is the preparation of membrane-permeant, esterase-sensitive succinimidyl esters of the most popular fluorochromes used for fluorescence flow cytometry. As depicted in Fig. 1, the major advantage of such compounds is that high intracellular concentrations of the succinimidyl ester form of the dyes can be achieved at relatively low extracellular dye concentrations. This is a crucial

point, as in my experience the major reason for cellular toxicity with covalently coupling dyes is excessive substitution of the cell surface.

Membrane-inserting fluorescent dyes

Fluorescent lipophilic dyes have been used for many years to fluorescently label cell membranes, such dyes being particularly valuable in neuroscience for neuronal labelling and pathway tracing.[51] It appears that these dyes, when applied to cells, usually stain the entire cell due to the lateral diffusion of the dyes within cell membranes. In addition, with membrane-inserting lipophilic dyes the labelling of intracellular organelles often occurs.[52–54] The membrane-inserting fluorescent dyes that have been used to track lymphocyte migration are the C18 DiI and DiO dyes, manufactured by Molecular Probes, and the PKH series of dyes marketed by Zynaxis Cell Science Inc. (Malvern, PA, USA), all of these dyes being lipophilic carbocyanine dye derivatives. Each of these dye types will be discussed below.

C18 DiI and DiO

Molecular Probes manufactures and markets a wide range of lipophilic carbocyanine and aminostyryl dyes, but only two types of carbocyanine dyes, the yellow/red fluorescent DiI and the green fluorescent DiO, have been used for lymphocyte tracking studies. The DiI and DiO dyes are weakly fluorescent in aqueous solutions, but become highly fluorescent and reasonably photostable when incorporated into cell membranes. Forms of DiI and DiO are available with different hydrophobic sidechains. Sue Weston and I found that the dihexadecyl (C16) variants of DiI and DiO were poorly absorbed by lymphocytes,[9] but studies by others have shown that the more hydrophobic dioctadecyl (C18) forms are well absorbed by lymphocytes[55] and erythrocytes.[56] In fact, it is possible to include low concentrations of DiI and DiO in culture media and directly label proliferating cells in culture.[57] There is also clear evidence that the C18 carbocyanine dyes show little or no toxicity for human leucocytes.[25]

Despite the many advantages of the C18 carbocyanine dyes, they have been rarely used for lymphocyte migration studies. There is, however, one recent report,[55] which highlights the potential of the C18 carbocyanine dyes for leucocyte tracking experiments. In this study, dendritic cells (DC) were labelled with the green fluorescent dye DiO and TCR transgenic T cells with the red fluorescent dye DiI. The entry of the DiO-labelled DC from a subcutaneous site into a draining lymph node could be readily detected and clustering between green, peptide-pulsed DC and red, peptide-specific TCR transgenic T cells could be easily visualized in the node. However, I am unaware of any flow cytometry studies with lymphocytes labelled with C18 DiI and DiO, even though these two dyes have structural properties and spectral characteristics that closely resemble those of the PKH dyes (see Table 1 and later). In fact, it seems likely that the C18 DiI and DiO dyes could be used to monitor cell division in a similar manner to the PKH dyes (see later). Additional studies with the C18 carbocyanine dyes are clearly warranted.

PKH2, PKH3 and PKH26

During the late 1980s, Zynaxis Cell Science Inc. patented several membrane-inserting fluorescent dyes that could be used to monitor lymphocyte trafficking and proliferation. The first two dyes to be reported were the green fluorescent dye PKH2 and the red fluorescent dye PKH3.[11,58] Both dyes are excited by the 488 nm line of argon ion lasers, with the emission of PKH2 being detected in the fluorescein channel and PKH3 in the phycoerythrin channel of standard flow cytometers. Unfortunately, the exact structures of these two dyes and the later dye PKH26 are unclear, although a perusal of the relevant patents indicates that they are long chain carbocyanine dyes closely resembling the C18 DiI and DiO dyes discussed earlier. Early studies showed that both PKH2 and PKH3 are stably retained for weeks by erythrocytes[11,59] and lymphocytes.[60] However, PKH2 soon fell out of favour, because it was found to reduce lymphocyte viability,[10] to interfere with the entry of lymphocytes into lymph nodes, probably by down regulating CD62L expression,[10] and to be prone to dye transfer.[61]

PKH26, which replaced PKH3 as the red fluorescent dye marketed by Zynaxis, is now the dye of choice for migration and proliferation studies. Like CFSE, PKH26 has the advantage that a halving of cellular fluorescence is observed following each cell division, the reduction in fluorescence being readily quantified by flow cytometry.[61–63] The dye has been particularly effective in monitoring the *in vivo* homing and proliferation of haemopoietic stem cell.[64] PKH26 shows little or no toxicity,[10,65] except for some phototoxicity following prolonged exposure of PKH26-labelled cells to excitation light,[66] and can be used to track lymphocyte migration for weeks to months.[67–69] The dye has also been used to *in situ* label mouse spleen cells[70] and sheep peripheral blood neutrophils.[71]

Unfortunately, a drawback with PKH26 is that, due to the way in which it is currently marketed by Sigma (St Louis, MO, USA), it is much more expensive to purchase than CFSE. In addition, when we have compared PKH26 staining with CFSE we have observed that PKH26 gives less uniform staining of lymphocyte populations than CFSE. As a result, the profiles of proliferating cells are much less discrete than those observed with CFSE-labelled lymphocytes. In order to overcome this problem, Zynaxis markets, via Sigma, special ModFit software that deconvolves the flow cytometer data and estimates the number of cell divisions that the PKH26-labelled cells have undergone. Despite these drawbacks, however, PKH26 has the advantage that it has similar excitation and emission properties to phycoerythrin and therefore can be used to monitor cell division if the fluorescein channel of the flow cytometer is required for other fluorescence measurements. In fact, it is possible to label different lymphocyte populations with CFSE and PKH26 and simultaneously follow the proliferation of the two populations in the same recipient animal or in the same *in vitro* culture (A Manderson and C Parish, unpubl. data, 1999).

Concluding remarks

During the last 20 years a number of fluorescent labelling approaches have been used to track lymphocyte migration

and proliferation. At this time, two fluorescent dyes stand out as being the most versatile in terms of long-term tracking of lymphocytes and in terms of their ability to quantify lymphocyte proliferation, namely the intracellular covalent-coupling dye CFSE and the membrane-inserting dye PKH26. However, CFSE appears to have the edge over PKH26, based on homogeneity of lymphocyte staining and cost.

When contemplating using fluorescent dyes for lymphocyte migration and positioning studies, two other dyes should be seriously considered. In my experience, the DNA-binding dye H33342, due to its remarkable resistance to fluorescence quenching, is particularly suitable for short-term (up to 3 days) migration studies when the positioning of lymphocytes *in vivo* is being investigated. It has the added advantage of absorbing and emitting in the UV range, thus freeing visible fluorescence channels on flow cytometers for the detection of other fluorochromes. The other dye is calcein, a cytoplasmic dye with no reported toxicity, which has the remarkable property of differentially labelling the lymphocyte subpopulation that is capable of traversing high endothelial venules.[29]

In the future, it is highly likely that additional fluorescent dyes, with different spectral properties to CFSE, will become available for lymphocyte proliferation and tracking studies. Membrane-inserting fluorescent dyes that can more homogeneously label lymphocytes than PKH26 may also be identified. I am sure that many immunologists will follow developments in this area with considerable interest.

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