Carboxyfluorescein Diacetate Succinimidyl Ester Fluorescent Dye for Cell Labeling

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Abstract  
Our objective was to study the properties of the carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and the methodology of cell labeling using CFDA-SE fluorescent dye. First, we analyzed the kinetics of CFDA-SE fluorescent dye intensity over time. Second, we determined the optimal concentration of CFDA-SE fluorescent dye for cell labeling. Third, we tested the toxicity of CFDA-SE fluorescent dye on labeled cells. Finally, we determined the optimal staining time of CFDA-SE fluorescent dye for cell labeling. The results show that the optimal concentration of CFDA-SE fluorescent dye for cell labeling varies according to different cell types. CFDA-SE fluorescent dye is non-toxic to cells as the cell death rate caused by CFDA-SE labeling is below 5%. The optimal cell labeling time was determined to be 8 min of incubation with CFDA-SE fluorescent dye. We concluded that the advantages of using CFDA-SE fluorescent dye for cell labeling are as follows: (1) the binding of CFDA-SE fluorescent dye to cells is stable; (2) CFDA-SE fluorescent dye is not toxic and does not modify the viability of labeled cells; and (3) CFDA-SE fluorescent dye is a suitable fluorochrome for cell labeling.

Key words  
carboxyfluorescein diacetate succinimidyl ester (CFDA-SE); carboxyfluorescein succinimidyl ester (CFSE); flow cytometry

The carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is a lipophilic molecule that is only minimally fluorescent until it is transported inside cells, where esterase cleaves the acetyl groups and the molecule becomes markedly fluorescent. The succinimidyl ester group covalently binds to amino groups on intracellular macromolecules, anchoring the dye [1]. CFDA-SE is a non-fluorescent compound that diffuses passively into cells. Within the cells, esterases remove the acetyl moieties, leaving carboxyfluorescein succinimidyl ester (CFSE) that binds covalently to proteins and is well retained within the cells (Fig. 1) [2].

Since its introduction in the flow cytometric analysis of lymphocyte proliferation by serial halving of the fluorescence intensity in 1994, the dye CFDA-SE has become widely used in immunological laboratories around the world [3,4]. It has also been applied to the quantitative analysis of in vitro natural killer cell proliferation detected by flow cytometry [5]. Research workers even use CFDA-SE-labeled NK cells to evaluate several class I MHC receptors for their ability to activate or inhibit NK cell division [6]. Although most of the published reports cover the use of CFDA-SE to monitor lymphocyte proliferation, some studies have been carried out using the dye to follow fibroblast division [7] and even bacterial proliferation [8]. CFDA-SE is progressively used as an indicator of bacterial activity and CFDA-SE labeling offers a means for the rapid detection of other kinds of cells by flow cytometric analysis. Researchers have reported using CFDA-SE to label hepatocytes for localization following intraportal transplantation. Researchers have also used CFDA-SE to localize labeled human Schwann cells in the spinal cord of nude rats in vivo. It allows in vitro identification and in vivo localiza-

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Another study describes a rapid, reproducible and simple method for the fluorescent labeling of murine blood cells in situ using intravenous injection of CFDA-SE [10]. Recently, an investigation was carried out to analyze what happens to CFDA-SE in cells undergoing apoptotic or necrotic cell death [11]. CFDA-SE is also suitable for analyzing the cytolytic activity of cytotoxic T lymphocyte (CTL) [2,6,12]. So far, however, the conditions for the CFDA-SE labeling of cells have not been made clear and the effect of CFDA-SE on cells is largely unknown.

In this study, we investigate the properties of CFDA-SE and explore its application in in vitro cell labeling.

Materials and Methods

Cell lines and cell culture

Four tumor cell lines were labeled with CFDA-SE and subjected to analysis by a flow cytometer. These were the human erythroleukaemic cell line K562, mouse lymphoma cell line YAC-1, human mammary cancer cell line MCF-7 and human melanoma cell line A375. All cells were cultured in a complete medium composed of Iscove’s modified Dulbecco’s medium (IMDM), containing 3024 µg/ml NaHCO₃, 584 µg/ml L-glutamine, 25 mM HEPES and 10% fetal calf serum.

Cell labeling

The 5 mM CFDA-SE stock (Cat. No. C-1157, Molecular Probes, California, USA) in DMSO (Fisher Scientific, Fair Lawn, NJ, USA) was diluted to different concentrations (2 µM, 3 µM, 4 µM, 5 µM, 10 µM and 20 µM) in PBS with a total volume of 1 ml. After each cell line was harvested and washed three times with PBS, 1×10⁶ cells were added to equal volume of CFDA-SE with different concentrations and incubated at 37 °C for 5, 6, 7, 8, 10 and 15 min with agitation. The labeling reaction was stopped for 1 min by adding an equal volume of heat-
inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) [13]. The CFDA-SE labeled cells were washed twice with PBS and recounted, and the cell concentration was adjusted to 6×10⁴ cells/ml in IMDM containing 10% FCS.

**Assay of fluorescence intensity and spontaneous cell death**

The cells were incubated in a final volume of 200 µl of complete medium (CM) at 6×10⁴ cells per well on 96-well U-bottomed plates in triplicate (Becton Dickinson, Franklin Lakes, New Jersey, USA) in a humidified 37 °C, 5% CO₂ incubator with seven different time points (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h). The cells were then put in an ice water bath and incubated with 50 µl of 50 µg/ml propidium iodide (PI; St. Louis, MO, USA) for 5 min, which was followed by flow cytometric analysis within 60 min.

**Flow cytometric analysis of the percentage of cell death**

All cells were analyzed using a FACSCalibur (BD Bioscience, Franklin Lakes, New Jersey, USA) equipped with an air-cooled 15 mW argon laser emitting at a fixed wavelength of 488 nm. The fluorescent filters and detectors used were all standard with green fluorescence collected in the FL1 channel (530±30 nm). Events for each well were acquired at a fixed speed (high setting) for 1 min to measure an equal volume from each sample, thereby providing a basis for the relative comparison of data collected for different samples. Samples were gated on forward scatter (FS) versus side scatter (SS) to exclude debris and clumps. The cells were analyzed using a logarithmic amplifier to determine the percentage of stained cells and their mean fluorescence intensity.

**Data and statistical analysis**

The data were analyzed using CellQuest acquisition and analysis software (BD Bioscience Franklin Lakes, New Jersey, USA). The total number of events (cells) were determined by analyzing the data using a dot plot and rectangular regions to define the cell populations. During data acquisition, a “live gate” was set on the CFSE-stained cell population using an FL1 histogram [Fig. 2(A)], and 1000 cell events were collected. For data analysis, the CFSE-stained cells were further gated in an FL1/FL3 dot plot [Fig. 2(B)], including all living cells and dead cells. An FL3 histogram [Fig. 2(C)] was used to determine the cell death rate.

Analysis of variance of multiple factors was used to compare the fluorescence intensity and spontaneous cell death with different concentrations of CFDA-SE and different incubation times. P<0.05 was considered statistically significant.

**Results**

**Mean fluorescence intensity and percentage of CFSE-positive cells**

The peak fluorescence intensity was reached within about 0.5 h, stabilized within 4 h and then declined at a very slow rate. The pattern is the same among all the four tumor cell lines tested (Fig. 3).

**Efficiency and optimal concentration of fluorescent dye labeling**

Table 1 shows that 2.5 µM CFDA-SE stained more...
than 95% of the cells on all cell lines tested ($P > 0.05$). Fig. 4 shows the dose-dependent increase of CFSE fluorescence intensity. As the histogram of fluorescence intensity on the flow cytometer showed a normal distribution, we determined the optimal labeling concentration based on a fluorescence intensity exceeding 2.0. The optimal concentration for K562 and YAC-1 was found to be 2.5 $\mu$M, while the optimal concentrations for A375 and MCF-7 were found to be 5 $\mu$M and 10 $\mu$M, respectively.

**CFDA-SE cytotoxicity**

The cells were labeled with CFDA-SE using the optimal concentration for each cell line, and incubated for different durations. The cell death was detected by PI staining. The cell death rate was found to be less than 5% within 1 to 6 h of incubation [Fig. 5(A)]. The spontaneous cell death was also determined by PI staining, and was found to be 2.74% (data not shown). There is no cytotoxicity related to CFDA-SE as no increase in PI staining was detected with 1–10 $\mu$M CFDA-SE compared with control [Fig. 5(B)].

**CFDA-SE optimal labeling time**

The four tumor cell lines were labeled using optimal CFDA-SE concentrations at 37 °C and shaken for 5, 6, 7, 8, 10 and 15 min, respectively. The cells were washed 3 times with PBS and the fluorescence intensity was measured after 4 h. The optimal labeling time was determined based on cell death rate and fluorescence intensity. There was no change in fluorescence intensity after 8 min of CFDA-SE labeling [Fig. 6(A)] and the percentage of cell death for each duration of shaking was below 5% [Fig. 6(B)].

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**Table 1  Percentage of CFSE-positive cells for concentrations ranging from 1 to 10 $\mu$M**

<table>
<thead>
<tr>
<th>CFDA-SE concentration</th>
<th>CFSE-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K562</td>
</tr>
<tr>
<td>1 $\mu$M</td>
<td>74.28±1.67</td>
</tr>
<tr>
<td>1.5 $\mu$M</td>
<td>82.29±3.45</td>
</tr>
<tr>
<td>2 $\mu$M</td>
<td>91.18±2.54</td>
</tr>
<tr>
<td>2.5 $\mu$M</td>
<td>97.40±2.97</td>
</tr>
<tr>
<td>5 $\mu$M</td>
<td>96.30±3.12</td>
</tr>
<tr>
<td>10 $\mu$M</td>
<td>98.80±3.20</td>
</tr>
</tbody>
</table>

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**Fig. 3  Variation of fluorescence intensity with time after labeling cells**

The mean fluorescence intensity versus time was plotted. Flow cytometer settings were identical for all tumor cell lines analyzed.

**Fig. 4  Fluorescence intensity of CFSE at different concentrations with 4 h incubation**

A dose-dependent increase in the fluorescence intensity was observed from 1 $\mu$M to 10 $\mu$M.


Discussion

Over the last 20 years, fluorescent labeling has become widely available and is now the preferred method for labeling cells, thus avoiding some of the pitfalls of radioactive isotopes. Non-radioactive methods, including the use of a range of fluorochromes, have been used for measuring the proliferation and tracking the migration of lymphocytes *in vivo*. Among fluorochromes, CFDA-SE stands out as the most versatile cell labeling dye in terms of long-term cell tracking and quantifying proliferation either *in vivo* or *in vitro* [13]. CFDA-SE, a membrane-permeant dye that covalently links to free amines of cytoplasmic proteins, has been reported to be effective in labeling hepatocytes, lymphocytes, CD34+ cells, neural stem cells, fetal CNS cells and human intervertebral disc cells [14–16]. Fluorescent labeling, in combination with flow cytometry, has permitted the comparative analysis of many samples.

CFDA-SE persists in cells 24 h after labeling, allowing the long-term detection of labeled cells. However, during the first 4 h after labeling, there is a 50% decline in fluorescence intensity, as illustrated in Fig. 3. The likely reason for this is the rapid degradation of a fraction of CFSE-protein conjugates. Some of the intracellular proteins labeled with CFSE are turned over rapidly, while others are retained for prolonged periods [10]. Another
possible reason is that the dye, which is not associated with intracellular macromolecules, can escape from the cell during the first hours of incubation, resulting in a drop of fluorescence intensity which reaches a stable level after 4 h [11]. Another explanation is that CFSE can potentially react with a wide range of molecules containing amine 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 hour after labeling. However, there are still sufficient amounts of long-lived CFSE conjugates within cells.

CFSE-SE is competent enough for labeling cells. The percentage of CFDA-SE-positive cells is over 95% 4 h after labeling using a concentration of at least 2.5 µM. The two acetate side chains of CFDA-SE render the molecules highly membrane-permeant. However, once inside the cells, the acetate groups are removed by intracellular esterase and the resultant carboxyfluorescein exits cells at a much slower rate. The slow exit rate also provides ample time for the CFSE to covalently couple to intracellular molecules. Coupling is via the succinimidy moiety, which reacts with intracellular amine groups, forming a highly stable amide bond. Succinimidy esters have a number of advantages over isothiocyanates as covalent coupling reagents. They have an intrinsically faster reaction rate, they are highly reactive with amines at neutral pH, and they form a more stable covalent linkage. The latter is particularly important for the intracellular labeling of cells [4]. So CFDA-SE is very efficient in labeling cells.

The optimal concentrations are different among the four tumor cell lines in our studies. Although all cells are labeled with relatively high fluorescence intensity by CFSE-SE, the efficiency of labeling is highly variable. We speculate that the variable labeling efficiency with different types of cells in vitro may be caused by differences in cell size and different esterase expression. Uncleaved dye can diffuse out of cells; therefore, the extent of esterase expression and the resultant efficiency of dye cleavage may determine the labeling efficiency of cells [11].

Toxicity of CFDA-SE on cells was tested 6 h after the staining procedure and analyzed with PI incorporation. PI is an intercalative DNA dye that detects the loss of plasma membrane integrity, an early event associated with cell death. Its level of incorporation depends on the extent of membrane alteration during the apoptotic process [12]. Fig. 5 shows the lack of toxicity of CFDA-SE on labeled cells, as no increase in PI-positive cells was detected after incubation with 2.5–10 µM CFDA-SE and there was no difference between the experimental group and control group. However, within 1 h of CFDA-SE labeling, the percentage of cell death determined by PI staining was higher than the percentage of spontaneous cell death. This may be a result of the overlapping of CFSE emission and PI emission.

The optimal labeling time was determined by the fluorescence intensity of CFDA-SE-positive cells with a reasonable percentage of cell death. There was no change in mean fluorescence intensity and cell death after 15 min of CFDA-SE labeling. So we decided to use 8 min as the optimal labeling time.

In our study, we have shown that CFDA-SE is suitable for cell labeling. This labeling process is stable and nontoxic to cells. However, optimal labeling conditions may vary with specific cell types.

References

5. Khil LY, Kim JY, Yoon JB, Kim JM, Keum WK, Kim ST, Yoon Y et al. Insulin has a limited effect on the cell cycle progression in 3T3 L1 fibroblasts. Mol Cells 1997, 7: 742–748


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