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Bulk EV staining with CFSE protein binding dye

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ABSTRACT

Carboxyfluorescein Diacetate Succynidimil Esther (CFDA-SE) is a small molecule that has been used extensively for labeling and tracking cells by flow cytometry, along with other imaging techniques(Lyons, Blake, & Doherty, 2013; Perfetto, Ambrozak, Roederer, & Koup, 2004). CFSE irreversibly binds to positively charged functional groups, such as lysine and arginine in proteins, through a covalent bond.

The two diacetic groups in CFDA-SE's structure render the molecule highly permeable and non-fluorescent(Breeuwer et al., 1995). Therefore, it was believed that the presence of esterase, the main enzyme that catalyze the hydrolysis of the diacetic groups, is required to convert CFDA-SE to fluorescent CFSE(Wang, Duan, Liu, Fang, & Tan, 2005). However, the presence of the enzymes only accelerates the hydrolysis of CFDA-SE, due to being catalysts of this hydrolysis. Contrary to common misconceptions, esterases are*not required*for this hydrolysis, and other factors such as high CFSE concentration, long incubation times and high temperatures, can also promote hydrolysis of the diacetic groups(Bergsdorf, Beyer, Umansky, Werr, & Sapp, 2003; Hoefel, Grooby, Monis, Andrews, & Saint, 2003). Thus, the approach that is taken in this protocol has been optimized for staining cell culture-derived EVs with a fluorescent dye, regardless of the concentration of esterases in the EVs(Morales-Kastresana et al., 2017).

The following protocol was developed to bulk stain EV populations(Thery et al., 2001; Thery, Ostrowski, & Segura, 2009). The protocol was optimized for achieving good signal to noise ratios, while avoiding excessive loss of EVs(Morales-Kastresana et al., 2017). To maximize the fluorescent intensity of the EVs, the authors recommend using CFDA-SE aliquots prepared in DMSO rather than aqueous solutions, which minimize fluorescein quenching with light and loss of diacetic groups by hydrolysis, which will render the molecule less permeant(Banks et al., 2013). This protocol can be performed in non-sterile conditions, although appropriate handling of reagents to ensure the absence of contaminating particulates is highly recommended. Although this method is very useful for purified EVs, this method is not appropriate for use in the setting of highly heterogeneous samples such as for plasma or serum, where extracellular proteins (including lipoproteins and ribonucleoproteins) not associated with EVs would be labelled along with the EV-associated proteins.

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KEYWORDS

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PARENT PROTOCOLS

In steps of

Detection and Sorting of Extracellular Vesicles and Viruses using nanoFACS

MATERIALS TEXT

Reagents

·$Ca^{2+}Mg^{2+}$-free Dulbecco's Phosphate Buffer Saline, DPBS (Thermo Fisher Scientific, Cat. 14190250)
·EV sample in DPBS at a known concentration (particles/ml)
·CFDA-SE (Thermo Fisher Scientific, Cat. V12883)

Hardware

·1.7 ml microfuge tubes (Avanti)
·0.5 mL bed volume Sephadex G-25 size exclusion chromatography columns (or, equivalently, NAP-5 or PD-10 columns, GE Healthcare)
·Rack or similar to hold the chromatography columns
·Micropipettor and tips
·Incubator at 37ºC

DISCLAIMER:

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

ABSTRACT

Carboxyfluorescein Diacetate Succynidimil Esther (CFDA-SE) is a small molecule that has been used extensively for labeling and tracking cells by flow cytometry, along with other imaging techniques(Lyons, Blake, & Doherty, 2013; Perfetto, Ambrozak, Roederer, & Koup, 2004). CFSE irreversibly binds to positively charged functional groups, such as lysine and arginine in proteins, through a covalent bond.

The two diacetic groups in CFDA-SE's structure render the molecule highly permeable and non-fluorescent(Breeuwer et al., 1995). Therefore, it was believed that the presence of esterase, the main enzyme that catalyze the hydrolysis of the diacetic groups, is required to convert CFDA-SE to fluorescent CFSE(Wang, Duan, Liu, Fang, & Tan, 2005). However, the presence of the enzymes only accelerates the hydrolysis of CFDA-SE, due to being catalysts of this hydrolysis. Contrary to common misconceptions, esterases are*not required*for this hydrolysis, and other factors such as high CFSE concentration, long incubation times and high temperatures, can also promote hydrolysis of the diacetic groups(Bergsdorf, Beyer, Umansky, Werr, & Sapp, 2003; Hoefel, Grooby, Monis, Andrews, & Saint, 2003). Thus, the approach that is taken in this protocol has been optimized for staining cell culture-derived EVs with a fluorescent dye, regardless of the concentration of esterases in the EVs(Morales-Kastresana et al., 2017).

The following protocol was developed to bulk stain EV populations(Thery et al., 2001; Thery, Ostrowski, & Segura, 2009). The protocol was optimized for achieving good signal to noise ratios, while avoiding excessive loss of EVs(Morales-Kastresana et al., 2017). To maximize the fluorescent intensity of the EVs, the authors recommend using CFDA-SE aliquots prepared in DMSO rather than aqueous solutions, which minimize fluorescein quenching with light and loss of diacetic groups by hydrolysis, which will render the molecule less permeant(Banks et al.,

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2013). This protocol can be performed in non-sterile conditions, although appropriate handling of reagents to ensure the absence of contaminating particulates is highly recommended. Although this method is very useful for purified EVs, this method is not appropriate for use in the setting of highly heterogeneous samples such as for plasma or serum, where extracellular proteins (including lipoproteins and ribonucleoproteins) not associated with EVs would be labelled along with the EV-associated proteins.

1 Prepare 15 µL of DPBS containing between ~1x10^8 to ~2.5 x 10^9 DC2.4-derived EVs in a 1.7 ml microfuge tube.

Note: This number of EVs correspond to ~ 0.1-2.5 µL of EV stock, if prepared as described previously(Morales-Kastresana et al., 2017), starting with 60 ml of supernatant from cells cultured for 48h in EV depleted medium. The reaction volume and CFSE concentrations are optimized for staining approximately 1x10^9EVs. The use of different EV numbers may therefore result in suboptimal staining. When larger amounts of labeled EVs are required, this protocol can be scaled up with similar results.

2 In a separate 1.7 ml microfuge tube, prepare 15 µL of DPBS containing 80 µM of CFDA-SE, from a 10 mM stock solution of CFDA-SE in DMSO. For this, add 0.12 µL of 10 mM CFDA-SE in 15 µL of DPBS. Intermediate dilutions in DPBS may be prepared to facilitate the dilution process.

Note: It is recommended to store the CFDA-SE stock reconstituted in DMSO in small aliquots at -80ºC. Avoid using DPBS or other aqueous solutions for storing purposes, since aqueous solutions favor the hydrolysis of diacetic groups in CFDA-SE and therefore decrease the permeability of the dye and incorporation into EVs(Banks et al., 2013; Bergsdorf et al., 2003; Hoefel et al., 2003). Also due to this risk of hydrolysis, it is critical for the aliquots to be stored in an anhydrous manner. If the EV number and reaction volume are scaled up in step 1, scale up the CFDA-SE quantity, so as to achieve an 80 µM CFDA-SE solution.

3 Pipette the CFDA-SE solution on top of the EV solution. Mix the solution by pipetting and incubate for two hours at 37ºC in the dark. The incubated CFSE concentration is now 40 µM.

Note: The incubation time may be extended to increase CFDA-SE's incorporation into EVs. However, the authors have observed a decrease in the EV number after long incubation periods(Morales-Kastresana et al., 2017).

4 15 minutes before the incubation completion time, wash a NAP-5 size exclusion chromatography (SEC) column with 10 ml of DPBS. Never allow the column to dry.

Note: To automate the washing process, a pump can be setup to help add DPBS onto the column.

5 Prepare collection tubes for the collection of two fractions. To facilitate the visualization of eluted sample, use a marker pen to draw a line indicating the 500 µL mark on each collection tube.

Note: The first fraction is the "dead volume," of buffer alone, that elutes before fractions containing material from the loaded sample. The majority of DC2.4 EVs appear in fraction 2. Some EVs may however elute in fraction 3. Free dye elutes in fractions ~7-8.

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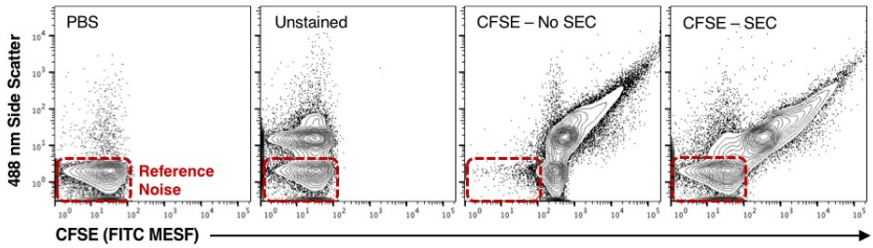
6 When the two-hour incubation period is complete, increase the CFSE-stained EV preparation volume to 100 µL by adding 70 µL of DPBS and mix by pipetting.

Note: If the staining is scaled up in step 1 to increase the number of EVs, then add DPBS to a final volume of 100 µL. If the total volume is higher that 100 µL, use multiple columns to wash the sample.

7 Pipette the 100 µL of CFSE-stained EVs on to the SEC column and immediately start collecting 500 µL fractions.

8 When the 100 µL of sample has completely entered the column bed, add 500 µL of DPBS and continue collecting fractions. ~80% of the eluted EVs will be collected in fraction 2.



Representative contour plots of unstained and CFSE-stained DC2.4 EV (before and after SEC). The number of fluorescein molecules incorporated in the EVs is shown in the plot (protocol for calculating the MESF is described in Basic Protocol 6). Red box indicates system reference noise.

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