DRUG DISCOVERY
AND GENOMIC TECHNOLOGIES

Short Technical Report

Use of CMFDA and CMTMR Fluorescent Dyes in FACS®-Based Antibody Screening

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ABSTRACT

Cell-based immunizations are often used when membrane antigens are difficult to purify. To confirm that an antibody binding to the surface of a cell line is, in fact, binding to the desired antigen, FACS® can be performed independently on two cell lines, a transfected cell line expressing the antigen of interest and a control cell line not expressing the antigen. Antibodies binding only to the transfected cell line are then selected for further analysis. This approach can be challenging if a large number of antibodies need to be screened and the antibody quantities are limited. Here we describe a novel method that combines the above two steps of FACS screening into a single step, based on the use of two fluorochromes, CMFDA and CMTMR, to stain transfected and control cell lines, respectively. Antibodies conjugated to a third fluorochrome are then added to the combined cells, followed by three-color FACS analysis. The newly modified FACS method is simple, sensitive, and high throughput. It can be used for antibody screening in multiple cell lines simultaneously.

INTRODUCTION

Fluorescence-activated cell sorter (FACS®) is a powerful technology to measure biological properties of cells by scanning single cells as they pass through a laser beam. Since the amount of fluorescence emitted is proportional to the amount of fluorescent probe bound to the cell surface, antibodies conjugated to fluorochromes such as R-phycoerythrin and fluorescein isothiocyanate (FITC) are routinely used as reagents to measure the antigen distribution both qualitatively and quantitatively on the cell surface (5). In addition, FACS is widely used to screen antibodies for desired binding specificities to membrane-bound antigens.

The recently developed XenoMouse® technology (Abgenix, Fremont, CA, USA) allows human antibodies to be generated by traditional methods from a transgenic mouse. While deficient in producing murine immunoglobin (Ig), XenoMouse strains contain germline-configured yeast artificial chromosomes carrying portions of the human IgH and Igκ loci where the majority of the human variable region repertoire is represented (3). The resulting mouse strain can generate a diverse primary immune repertoire similar to that in adult humans (3). The high affinity and specificity of these antibodies make them suitable candidates for therapeutic modalities.

Typically, XenoMouse strains are immunized with either soluble human antigens or peptides. Because of the difficulty of purifying membrane-associated antigens, a cell immunization method can be applied as an alternative, based on the use of a transfected cell line expressing an antigen of interest. However, this complicates the screening for antigen-specific antibodies because of the high frequencies of antibodies binding to cell-surface molecules other than the desired antigen. To confirm that an antibody binding to the surface of a recombinant cell line is, in fact, binding to the desired antigen, a cell-based FACS screening is usually divided into two steps. First, a recombinant cell line expressing an antigen of interest is used to screen a pool of antibodies. Next, the positive antibody candidates are used for screening against a control cell line that does not express the specific antigen. The second step is necessary to filter out the false-positive antibodies that recognize cell-surface molecules that are non-antigen-like. However, it requires additional assay time and reagents. This particularly becomes a problem when a large number of antibodies need to be screened from primary hybridoma cultures that contain relatively small quantities of antibody.

Here, we describe a new and reliable flow cytometry method that identifies antibodies binding specifically to antigens expressed on the cell surface. This method is based on the application of 5-chloromethylfluorescein diacetate (CMFDA) and 5-([4-chloromethyl)benzoyl]amino) (CMTMR), two fluorescent dyes commonly used in cell-cell electrofusion (4). Both CMFDA and CMTMR are cell permeable dyes, and they pass freely through the membranes into the cytosol. Once they are in the cytosol, both dyes undergo a GST-mediated reaction that renders the dyes membrane-impermeable (1,4). The

transfected cell line expressing a tumor-specific antigen was labeled with CMFDA, and the control cell line was labeled with CMTMR. The two labeled cell lines were then mixed together. Antibodies to be screened were incubated with this mixed cell population in a normal FACS staining procedure. A secondary antibody conjugated with biotin was added, followed by the addition of Streptavidin-conjugated fluorochrome Cy-chrome. The specificity of antibody binding to antigen was determined by three-channel FACS analysis.

MATERIALS AND METHODS

Immunization

Two different recombinant forms of the tumor-specific antigen, an Ig fusion protein and a His-tagged protein, were purified from the 293 cells following transient transfection. The B300.19/antigen cell line was generated by transfecting B300.19 cells with a plasmid derived from cloning the cDNA of the antigen into pCR3.1 (Invitrogen, Carlsbad, CA, USA), selecting in the presence of 2 µg/mL puromycin. Six human IgG2 XenoMouse mice in each group were immunized with the IgG fusion protein (10 µg/animal) or antigen-transfected B300.19 cells (5×10^6 cells/animal) through injection at the base of the tail and into the peritoneal cavity (6). Seven days after the first injection, the animals were boosted with the same antigen. Four weeks after the second injection, mouse blood was taken, and serum titers were measured by standard ELISA.

ELISA

ELISA plates were coated with 100 µL/well of antigen-IgG fusion or antigen-His fusion protein at 1 µg/mL in coating buffer (0.1 M carbonate buffer, pH 9.6). Plates were incubated at 4°C overnight or 37°C for 2 h, followed by washing three times in PBS. Blocking buffer (100 µL/well; 0.5% BSA, 0.1% Tween® 20, 0.01% Thimerosal in PBS) was added, and the plates were incubated at room temperature for 2 h. After washing the plates three times with PBS, 50 µL/well of serum were added in various dilutions in blocking buffer and incubated at room temperature for 2 h. The plates were washed three times with PBS, followed by the addition of 100 µL/well of 1:2000 diluted goat anti-human IgG-conjugated with HRP (Caltag, Burlingame, CA, USA) and then incubated at room temperature for 1 h. After the plates were washed three times with PBS, 100 µL/well of TMB (Invitrogen) were added, and samples were incubated at room temperature for 7 min. Stop solution (50 µL/well; 2 M sulfuric acid) was added, and the samples were read at A_{450} by using SPECTRAmax® 250 (Molecular Devices, Sunnyvale, CA, USA). Titers were calculated based on the A value at least 2-fold above background.

Quantitative Real-Time PCR

Total RNA from B300.19 and B300.19/antigen cells was isolated by using RNeasy® kits (Qiagen, Valencia, CA, USA). Quantitative real-time PCR was performed using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described (2). The antigen-specific primers and probe and human 18S rRNA primers and probe were designed using Primer Express™ (Applied Biosystems). Primers were used at a concentration of 200 nM and probes at 100 nM in each reaction. Relative quantitations of gene expression were calculated using standard curves and normalized to 18S.

Cell Staining

B300.19 and B300.19/antigen cells were collected and suspended at 1.5×10^6/mL in culture media. CMFDA (emission 530 nm; Molecular Probes, Eugene, OR, USA) was added to B300.19 cells at a final concentration of 0.25 µM, and CMTMR (emission 585 nm; Molecular Probes) was added to B300.19/antigen cells at a final concentration of 3 µM. Cells were incubated separately at 37°C for 30 min. Cells were washed twice with cell culture media and suspended in the same media. Stained cells were incubated at 37°C for 60 min, washed three times with PBS, and then suspended in FACS

buffer (PBS + 2% FBS). Cells were then examined under a fluorescence microscope to confirm the staining.

FACS

CMFDA- and CMTMR-stained cells were mixed together, and an anti-antigen antiserum was added at 1:200 in FACS buffer. After incubation on ice for 40 min, cells were washed twice with 200 µL FACS buffer. Biotin-labeled goat anti-hIgG (Caltag) was added at 1 µg/100 µL for each sample, followed by incubation on ice for 40 min. Cells were washed twice with 200 µL FACS buffer. Streptavidin-conjugated Cy-chrome (BD Biosciences, San Jose, CA, USA) was then added at 2 µg/mL for each staining, followed by incubation on ice for 20 min and washing twice with 200 µL FACS buffer. Cells were then suspended in 200 µL fixation buffer for each sample and transferred to tubes with an additional 200 µL fixation buffer. The stained cells were shielded from light. FACS analysis was performed by using FACScalibur™ (Becton Dickinson, San Jose, CA, USA) according to the published protocols (4,5).

RESULTS AND DISCUSSION

Generating Transfected Cell Lines Expressing Antigen

To generate an antigen-expressing cell line, a plasmid encoding a human tumor-specific antigen was transfected into a murine B cell line B300.19. The stable transfected cell line B300.19/antigen was generated by growing cells in the presence of puromycin. Antigen expression was examined by quantitative real-time PCR. There was an approximately 17-fold increase in antigen RNA expression compared with the parental B300.19 cell line, as estimated by quantitative real-time PCR (data not shown). Antigen protein expression was later confirmed by FACS analysis using polyclonal sera.

To produce antigen-specific human antibodies, XenoMouse animals were immunized with either purified antigen-IgG protein or a transfected B300.19 cell line expressing the antigen. The mice were bled four weeks after immunization. Serum titers were measured by standard ELISA (see Materials and Methods). Only high-titer serum 313.2 and 313.8 were used in the following experiments.

Developing a One-Step FACS Method

To develop a one-step FACS screen method, we reasoned that we would label two cell types with two different fluorescent dyes, one with CMFDA and the other one with CMTMR. Two cell types were then mixed, and a primary antibody was added. After binding, excessive primary antibodies were washed off. A biotin-labeled secondary antibody was then added, followed by Streptavidin-conjugated Cy-chrome staining. The specificity of antibody binding could be determined by three-channel FACS staining.

To test our hypothesis, B300.19 parental cells were labeled with CMFDA, whereas B300.19/antigen-expressing cells were labeled with CMTMR. Stained cells were incubated at 37°C for 60 min. As shown in Figure 1, A and B, almost 100% of cells were stained with either CMTMR or CMFDA fluorescent dyes. Cells were then mixed, and sera were added, followed by secondary antibody biotin-labeled goat anti-human IgG and, subsequently, SA-CYC. Based on our three-channel FACS data, both cell populations were stained equally with two fluorescent dyes (Figure 2, left panels). Control serum did not show much staining (FL1-H positive and FL3-H negative; FL2-H positive and FL3-H negative; Figure 2A). When serum from animal 313-2 was added, only B300.19/antigen cells were stained (FL2-H and FL3-H double positive) but not B300.19 parental cells (FL1-H positive and FL3-H negative; Figure 2B), suggesting that 313-2 was an antigen-specific serum. By contrast, 313-8 was a nonspecific serum, since it stained both B300.19/antigen expressing cells (FL2-H and FL3-H double positive) and B300.19 parental cells (FL1-H and FL3-H double positive; Figure 2C). The FACS results were further confirmed by immunofluorescence staining, where specific cell staining was observed with 313.2 on B300.19/antigen expressing

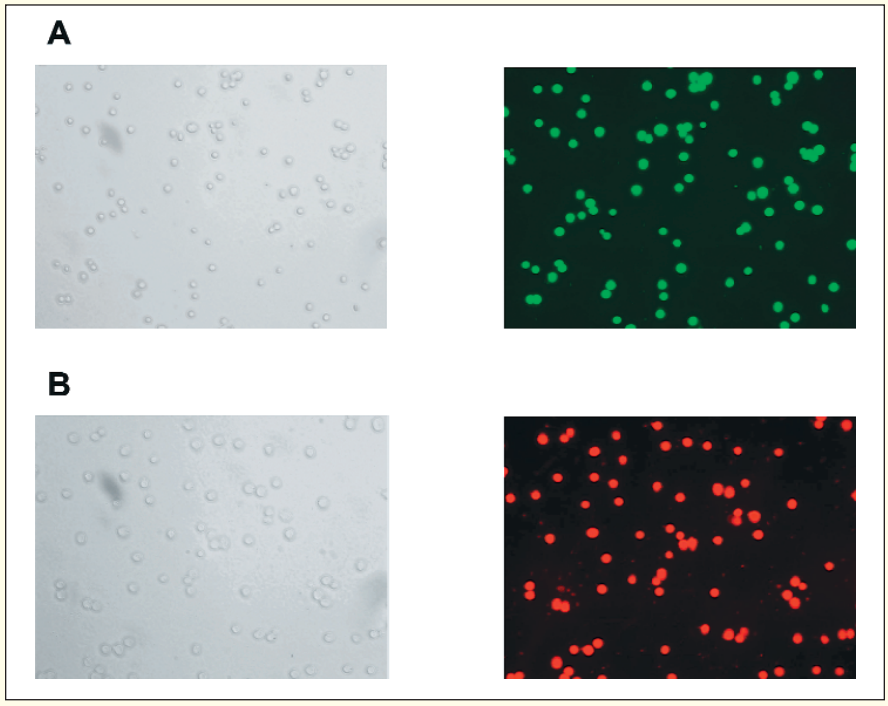

Figure 1. Cell staining with CMFDA and CMTMR. CMFDA and CMTMR fluorescent dyes were added to B300.19 parental cells and B300.19/antigen cells, respectively. Cell staining was examined under the microscope. (A) B300.19 phase contrast picture was shown on the left, and CMFDA staining was shown on the right (20×). (B) B300.19-transfected cells expressing antigen were shown on the left, with CMTMR staining shown on the right (20×).

cells but not on B300.19 parental cells. Consistent with FACS data, 313.8 serum stained both B300.19 cells and B300/antigen cells (data not shown).

The two-cell staining approach described here provides a useful tool for rapid and selective antibody screening. Instead of screening two cell lines independently by FACS, the use of CMFDA and CMTMR dyes allows us to mix two cell populations together and perform FACS analysis in one step. Because cell staining efficiency of CMFDA and CMTMR was high (close to 100% based on the microscopic examination), the specificity of antibody binding to cells would be easily revealed by identifying which stained cell population bound to a tested antibody, as in the case of 313.2 (specific) and 313.8 (nonspecific). A similar approach has been used successfully to identify specific hybridoma cell lines that bind to membrane-associated antigens (data not shown). We did observe some background staining of 313.2 antibody to negative cell line B300.19, although the background has not been an issue in other tumor cell lines that we have tested. This could be due to the nonspecific binding of antiserum from XenoMouse animals to Fc receptors expressed in the mouse B cell line B300.19. The use of an Fc blocker helped to reduce background (data not shown). Since Cy-chrome has a tendency to bind to cells nonspecifically, another approach to improve the signal-to-noise ratio is to replace Cy-chrome with peridinin chlorophyll protein. Our current experimental procedure can be further simplified by staining only one of two cell types with a fluorescent dye and leaving the other one unstained. Therefore, a two-channel FACS analysis would be sufficient to reveal the specificity of antibody binding. This approach can also be applied to antibody screening in three cell types: one unstained, one stained with CMFDA, and one stained with CMTMR. A single antibody could be added to the mixed cell population. The specificity of antibody binding to cells could be determined by three-channel FACS analysis.

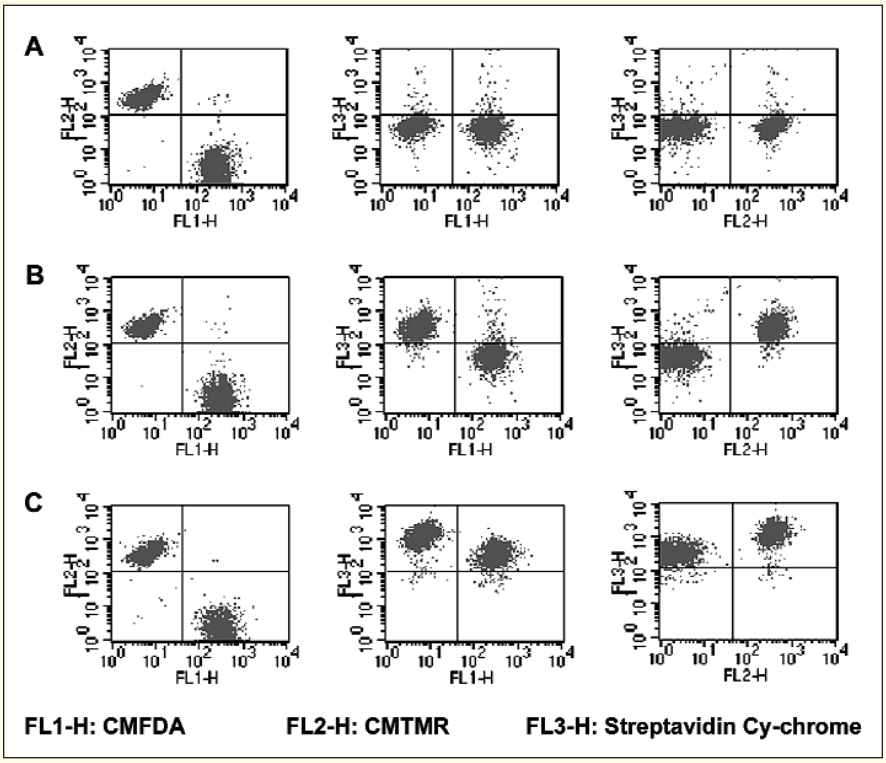

Figure 2. FACS analysis of sera. Control XenoMouse serum, 313.2 serum, and 313.8 serum were added to the mixed B300.19 and B300.19/antigen cells. A biotin-labeled secondary antibody was added, followed by the addition of Streptavidin-labeled Cy-chrome. FACS analysis was performed by using FL1 (detecting CMFDA), FL2 (detecting CMTMR), and FL3 (detecting Cy-chrome). The three-color FACS results were shown in three separate panels: (A) control XenoMouse serum, (B) 313.2 serum, and (C) 313.8 serum.

In summary, the use of cell staining dyes CMFDA and CMTMR in FACS analysis has been proven to be sensitive and selective. This approach can be widely used in FACS-based antibody screening and other fluorescence-based methods such as fluorometric microvolume assay technology when multiple cell lines are required for screening.

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