Optimizing Immunofluorescent Staining

Useful techniques
for producing optimal signal to noise ratios
during immunofluorescent staining

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TECHNIQUES

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A common problem encountered when performing immunofluorescent staining is a difficulty in distinguishing between the fluorescent staining of cells mediated by antibodies binding to their antigens and fluorescent staining of cells mediated by antibodies binding to cells via other molecular interactions. The binding of antibodies to cells, via interactions not involving the specific recognition of an epitope by the antigen binding region of an antibody, is often referred to as "non-specific" or "background" staining. This undesirable binding of antibodies to cells is particularly troublesome when the antigen being investigated is not highly expressed or if the antibodies being used are of relatively low affinity. There are a number of factors that can contribute to the presence and degree of background staining. These include: the condition and the types of cells being stained, the amount of time and temperature at which the cells are stained, the amount and condition of the antibody used for staining, and the type of fluorochromes used to detect antibody binding. Controlling these variables can make the difference between producing easy to interpret results and generating inconclusive data.

The following are useful techniques for producing optimal signal to noise ratios during immunofluorescent staining.

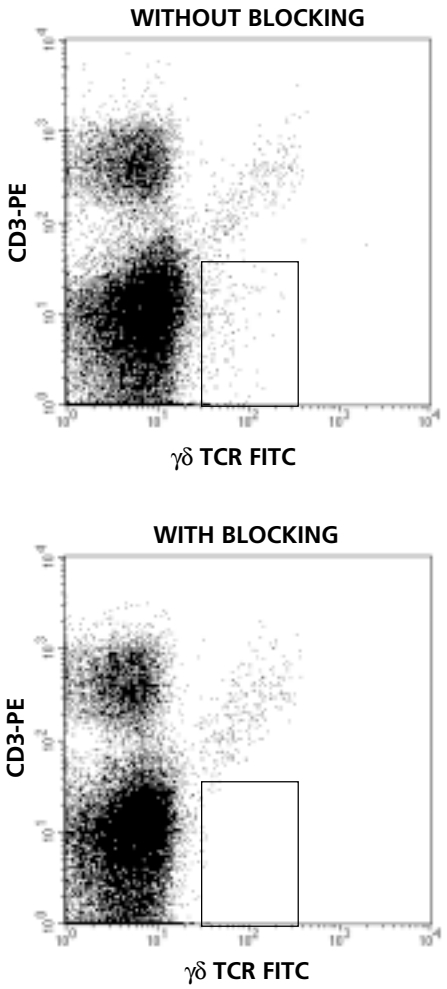
1. Eliminate antibody aggregates

Antibodies tend to form aggregates over time. Fc receptors bind these antibody aggregates with greater avidity than they have for antibody monomers. Improper handling and storage of antibody solutions can enhance the rate at which antibody aggregates form. Follow the manufacturer's instructions for handling and storage in order to prevent aggregates from occurring in antibody solutions. Antibody aggregates can be removed by centrifugation in a microfuge at high speed for ten minutes at 4° C. It is not recommended that either PE conjugates or IgM antibodies be centrifuged since these molecules are relatively large and may pellet out of solution along with antibody aggregates.

2. Block Fc receptors with purified IgG or serum

There are two different approaches commonly employed to block the binding of antibodies to Fc receptors. The first method involves incubating the cells with either serum or purified IgG taken from the same species from which the antibodies used for staining were derived. For instance, if a hamster anti-mouse CD3 antibody is being used to stain mouse splenocytes, then unlabeled hamster IgG or hamster serum can be used to competitively inhibit binding of the hamster anti-mouse CD3 antibody to Fc receptors on the mouse splenocytes. This can be done by incubating the cells for 10 minutes using 10 μg of purified IgG per one million cells. After 10 minutes, add the antibodies being used for immunofluorescent staining directly to the mixture of cells and blocking IgG and incubate for the appropriate amount of time. Figure 1. demonstrates blocking with purified IgG prior to staining.

FIGURE 1.



Blocking cells with purified IgG. *One million cells per sample from a single cell suspension of C3H splenocytes were stained with both .25 μg of PE conjugated anti-mouse CD3 (Cat # HM3404) as well as .5 μg of FITC conjugated anti-mouse γδ TCR (Cat # HM3801). The dot plot on the top shows the results of this staining when no blocking is performed. The dot plot on the bottom shows the results of this staining after Fc receptors are blocked using 10 μg of purified hamster IgG (Cat # HM00). Note the increased staining of CD3 negative cells by the FITC conjugated anti-mouse γδ TCR antibody in the unblocked sample.*

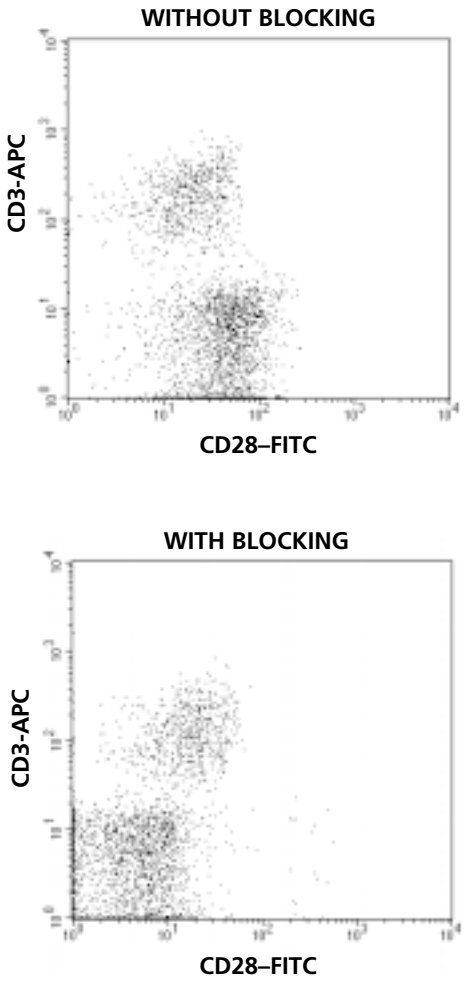


FIGURE 2.

Blocking Fc receptors with anti-mouse CD16/32 mAbs. *One million cells per sample from a single cell suspension of C57BL/6 splenocytes were stained with both .25 μg per sample of APC conjugated anti-mouse CD3 (Cat # HM3405) as well as .25 μg per sample of FITC conjugated anti-mouse CD28 (Cat # HM3501). The dot plot on the bottom shows the results of this staining after Fc receptors are blocked using 0.5 μg purified anti-mouse CD16/32 mAbs (Cat # MM7400). The dot plot on the top shows the results of this staining when no blocking is performed. Note the increased staining of CD3 negative cells by the FITC conjugated anti-mouse CD28 antibody in the unblocked sample.*

3. Block Fc receptors with monoclonal antibodies specific for Fc receptors

The second way to block Fc receptor mediated binding of antibodies incorporates the use of unlabeled antibodies directed against the Fc receptors. These antibodies prevent the Fc receptors from binding to other labeled antibodies either by inducing a conformational change in the Fc receptor or by binding to the portion of the Fc receptor involved with antibody binding. This method can only be used with antibodies specific for Fc receptors that are known to be capable of blocking. The procedure for blocking cells with antibody specific for Fc receptors is similar to the procedure used when blocking with purified IgG. Incubate the cells for 10 minutes in the presence of 0.5 μg of unlabeled anti-Fc receptor antibody per one million cells. Following the 10 minute incubation, add the appropriate primary antibodies and stain as usual. Note that this procedure can not be used for indirect immunofluorescent staining when the secondary antibody being used is directed against IgG produced by the same species from which the anti-Fc receptor antibody was derived. Figure 2. demonstrates the impact an anti-Fc receptor antibody can have on staining results.

4. Minimize the number of dead cells in a tissue sample.

Dead cells, whose membrane integrity has been compromised, non-specifically bind most antibodies. Samples containing excessive numbers of dead cells result in data that are difficult to interpret. This can be particularly troublesome when a rare cell population is being studied. There are several measures that can be taken to maintain a high percentage of viable cells throughout the staining process. These measures include: staining the cells promptly after they are harvested, keeping the cells at 4° C throughout the staining procedure, and including BSA or heat inactivated fetal bovine serum in the staining and wash buffers. It is also important to analyze unfixed cells as soon as possible following the staining procedure.

5. Distinguish dead cells by the use of a dead cell discriminator.

There will always be a small percentage of dead cells in any sample undergoing immunofluorescent analysis. A DNA binding dye such as propidium iodide (PI) or 7-amino-actinomycin D can be used to distinguish dead cells, which have lost their membrane integrity and are unable to exclude these dyes, from live cells whose cell membranes remain intact. If subsequently these cells are subjected to flow cytometric analysis any cells which have bound the dead cell discriminator can be excluded from the final data. CALTAG offers a convenient, prediluted and QC tested preparation of PI (Cat # DCD00) for use as a dead cell discriminator in flow cytometry. This product can be used in multicolor flow cytometry in combination with FITC, PE, and APC fluorochromes by adding a small amount directly to the

cell suspension immediately prior to flow cytometric analysis. PI is usually detected in the FL3 channel when it is being used as a dead cell discriminator. PI can not be used in conjunction with PE-Cy5 tandem fluorochrome or any other fluorochrome whose fluorescent emissions are also collected in the FL3 channel. PI does have broad spectral emissions that can be detected in the FL2 channel as well as in FL3. PI can still be used simultaneously with PE by adjusting the compensation to correct for PI emissions into FL2. Adjusting the compensation is not necessary when using PI as a dead cell discriminator because any cells that are positive for PI will be excluded from the cell population being analyzed. Thus the overlapping fluorescent emission from PI bound to dead cells is irrelevant. Figure 3 depicts the improved flow data generated after dead cells are excluded through the use of PI.

The following CALTAG products were described in the preceding examples.

CODE	CAT #	VOLUME	AMOUNT
Goat serum	10000	25.0 ml	
Goat IgG	10200	5.0 ml	15 mg
Normal mouse IgG	10400	5.0 ml	15 mg
Normal mouse serum	10410	10 ml	
Normal rabbit IgG	10500	5.0 ml	15 mg
Normal rabbit serum	10510	10.0 ml	
Normal rat IgG	10700	5.0 ml	15 mg
Normal rat serum	10710	10.0 ml	

Anti mouse CD16/32 (Fc receptors)

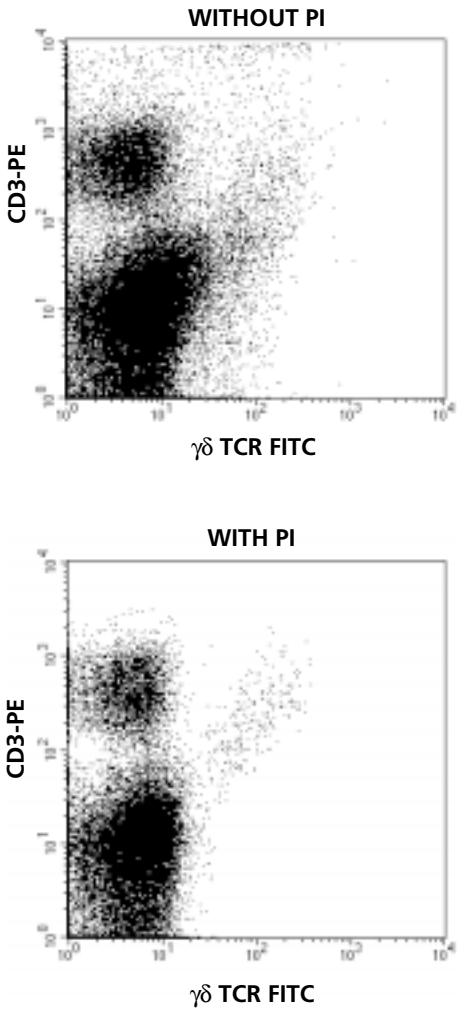
CAT #	VOLUME	AMOUNT
MM7400	1.0 ml	100 µg
MM7400-3	3.0 ml	300 µg

Propidium Iodide

CAT #	VOLUME	AMOUNT
DCD00	1.0 ml	200 determinations
DCD00-3	3.0 ml	600 determinations

To request a copy of the CALTAG 2002 Catalog, please call the toll-free number below.

FIGURE 3.



Excluding dead cells using propidium iodide. *A single cell suspension of C3H splenocytes were stored overnight at 4°C to increase the number of dead cells. The next morning one million cells per sample were stained with both .25 µg of PE conjugated anti-mouse CD3 (Cat # HM3404) as well as .5 µg of FITC conjugated anti-mouse γδ TCR (Cat # HM3801). The dot plot on the top shows the results of this staining when no PI is added to the sample. The dot plot on the bottom shows the results of the same staining after PI (Cat # DCD00-3) is added to the cells immediately before flow cytometric analysis. PI positive cells are excluded from this plot. Note the increased background when PI is not used to exclude the dead cells from analysis.*