

Flow Cytometry

Flow cytometry (also termed FACS) has been widely used for a number of years and is an increasingly sophisticated tool for the analysis of intracellular and surface expressed antigens. It is useful, for example, for investigating populations of cells, changes, purity and viability. The methodology requires the presence of single cell suspensions which are then incubated with antibodies (fluorescently labelled directly or indirectly) and analysed on the flow cytometer. Laser light is then directed onto individual cells and a series of detectors employed to determine forward scatter (FSC), side scatter (SSC) or fluorescence (Fig 1). FSC will determine cell size whereas SSC depends on the density of the cell. This leads to the ability to differentiate different cell populations in some circumstances (Fig 2). The different fluorochromes used in the staining emit light when excited by the laser at different wavelengths and this ensures that a number of parameters can be measured at once. Direct staining is the most often employed method. This is where a directly conjugated antibody is used and this will often decrease the amount of non-specific binding seen. Indirect staining is used when there are no directly conjugated antibodies available and this can either be by the use of a secondary antibody specific to the immunoglobulin of the first or it can utilise the avidin-biotin system.

As stated above it is possible to stain the intracellular contents of the cell and the methodology for this often requires an amount of optimisation. A fixation step to stabilise antigens in their current position followed by a cell permeabilisation procedure to allow penetration of the detection antibody is engaged. The permeabilisation step leads to changes in the light scattering properties of the cell. It is usual for any surface staining to be performed prior to fixation in case it alters the antigenicity of the target. There are a number of methods available; Formaldehyde and detergent – this uses 0.01% formaldehyde then detergent, usually Triton X-100 or NP-40 for nuclear staining or Tween 20, saponin, digitonin or leucoperm as a gentler alternative, cold methanol plus detergent or cold acetone. If the antigen is close to the plasma membrane or soluble in the cytoplasm a mild permeabilisation is required and fixation can be omitted. If the antigen is within intracellular organelles a harsher method may be needed. The precise optimal conditions for staining of intracellular antigens may be influenced by the nature of the antigen and its localization. It might be necessary to modify the length of the incubation with the antibody and/or the temperature of the reaction. Keeping the antibody in the detergent solution during the incubation step has also been described as a measure to improve penetration of the reagent to the reaction site. Antibodies from different manufacturers directed at the same antigen can display different properties when they are used for intracellular staining. All reagents should be titrated for optimal internal staining and may require different amounts than those used for detection of surface antigens. The localisation of the fluorescent reaction should always be verified by fluorescence microscopy.

As mentioned above flow cytometry can be used to determine cell death such as apoptosis. There are a variety of methods used but the most reliable ones are those which maintain these fragile cells as close as possible to their natural state. The rapid 7-AAD staining method uses unfixed cells and thus permits the detection of changes in light scatter parameters and their correlation with other indicators of programmed cell death. However, one drawback of using any live staining method for measuring apoptosis is the variability of dye uptake in different cells and its possible change through certain treatment conditions. Furthermore, reagents which affect membrane permeability (e.g. calcium ionophores) cannot be used with this technique. Many staining methods for flow cytometry use either fixed cells or treat cells with a hypotonic solution to permit DNA staining by non-vital dyes. The apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented in so-called "sub-G1" peaks on DNA histograms. Many different DNA dyes produce similar distributions of "sub-G1" peaks irrespective of their binding mode. However, dissimilar sample preparation methods (e.g. live staining with HO342 vs. hypotonic PI) can lead to dramatic differences in the ability to detect apoptotic cells. Rapid flow cytometric staining methods that use Annexin V for detection of phosphatidylserine exposure on the cell surface as a marker of apoptosis are commercially available. For this staining method it is essential to add a dead cell discrimination dye like propidium iodide or 7-amino-actinomycin D to the stained cells, because late apoptotic or necrotic cells can also express phosphatidylserine and have to be distinguished from the early apoptotic cells by fluorescence. The newest flow cytometric assays measure Caspase-3 activity, an early marker of cells undergoing apoptosis and kits for performing these assays are again commercially available. It is best to keep in mind that not all methods for detection of apoptosis in cells may be equally sensitive and techniques must be assessed critically with respect to their applicability to a particular cell type or system.

Of all of the procedures in flow cytometry the one that generates the most controversy is that of compensation in the use of multiple fluorophores. Proper compensation is absolutely crucial for some aspects of flow cytometry, one of which is antigen density measurements. Unfortunately, because

compensation is often misunderstood, and because there is so much incorrect mythology about it floating around, many laboratories do not set compensation properly. The goal of compensation is to remove the spill over fluorescence of a particular probe from the "wrong" channel. I.e., fluorescein fluorescence is primarily green, which is measured in the FL1 (FITC) channel. But fluorescein also has a significant yellow component to the fluorescence, which appears in the FL2 (PE) channel (Fig 3). Proper compensation occurs when, on average for a population, there is no contribution of FITC fluorescence in the PE channel (and so on for other combinations of fluorescent molecules). Therefore a population of cells stained with fluorescein (but not PE) should have the same median PE fluorescence as a population of cells that is unstained for fluorescein. The compensation is increased until the centre of the positively stained population lines up with the centre of the negatively stained population. Because of a compounding measurement error, this means that some of the cells will be above a quadrant line set on the negatives! Indeed, note that if compensation is set so that the positive population lines up underneath the quadrant line, then the cells are actually over-compensated: on average, most cells have too much signal subtracted, and the mean PE fluorescence of this population is less than that of the negative. In flow cytometric analyses on logarithmic axes, the median is generally a much better estimate of central tendency than the mean. The mean can be significantly skewed higher by a few outliers. As well, we do not know the real fluorescence of an event appearing on the lower axis (it could be any value below the minimum); thus the mean will be artificially inflated by this as well. However, neither process significantly affects the accuracy of the median. Therefore, if possible, use the median fluorescence when doing compensation: adjust compensation until the median of the positives is equal to the median of the negatives (be sure to include all cells in a population in the analysis gates). For the most part, autofluorescence does not affect compensation. Autofluorescence is present in all channels, and it will still be present after compensation (although the absolute value will be altered by the compensation). However, note that the autofluorescence of the positive and negative populations must be the same in order to achieve proper compensation. Using a fluorescence probe specific to monocytes and lining them up with lymphocytes, whose autofluorescence is significantly lower than monocytes, will fail to yield proper compensation. In general the best compensation is attained by mixing, in roughly equal proportions, unstained cells with an aliquot of the same cells stained with the appropriate antibodies.

As with most immunologically-based techniques background can be a problem. Try diluting the fluorochrome-conjugated antibody further. If it is too concentrated, background may increase due to an increase in non-specific interactions. Prior to applying the primary antibody, incubate it with excess protein such as BSA, non-fat dry milk, or normal serum from the same host as the labelled secondary antibody. This step may reduce background by blocking non-specific interactions between the primary antibody and the cell surface or intracellular structures. After applying the primary antibody, incubate with 5% to 10% normal serum from the same host as the labelled secondary antibody. This step may reduce unwanted interactions between the secondary antibody and the primary antibody, the cell surface, or intracellular structures. It is possible to avoid this step by diluting the labelled antibody with normal serum from the same species. This works well in many cases, but occasionally it can lead to the formation of immune complexes between the labelled secondary antibody and immunoglobulins in the normal serum. These complexes may bind to some cellular structures preferentially, or they may eventually lead to a loss of desired antibody activity. Use F(ab')₂ fragments where background may be due to binding of primary or secondary antibodies to Fc-receptors. F(ab')₂ fragments of most secondary antibodies are readily available. F(ab')₂ fragments of primary antibodies, however, are either unavailable or difficult to produce. Alternatively, incubate fresh tissues or cells with normal serum in the presence of sodium azide prior to addition of the primary antibody. Under these conditions, background due to Fc-receptor binding is not significant even if whole antibody molecules are used in subsequent steps. Background also can arise from unwanted cross-reactivities between labelled antibodies and any other immunoglobulins inherent or added to the experimental system. To reduce this background, all labelled antibodies should be absorbed against other species to reduce binding to those immunoglobulins introduced from culture fluids, present on cell surfaces or in tissues, or added as primary and other secondary antibodies during multiple labeling. Cross-reactivities between multiple primary and secondary antibodies during multiple labeling also can be reduced by using labelled antibodies which have been derived from a single host species as well as absorbed against other host species. If your cells have many Fc-receptors on the cell surface (in particular monocytes, macrophages) or they have been cultured in serum free medium, it is advisable to block non-specific binding of monoclonal antibodies by pre-incubation of cells with human AB serum (HAB). Note that for staining of whole blood this is not necessary, because serum in high concentration is present during staining.

The differentiation of positive from negative signal can often depend on which fluorochrome is used. Generally intensities are as follows PE, APC > AF47 > FITC AF488. The lower the anticipated concentration of the antigen the brighter the fluorophore used should be.

Some general tips are as follows. Cells should be spun down hard enough that the supernatant can be removed but not so hard as the cells become difficult to resuspend. Ice cold reagents and sodium azide are often used for surface staining to avoid internalisation of the antigens. Around 0.1-1ug/ml of antibody is a good starting range and this can be in up to 3% BSA/PBS. A concentration of $1-5 \times 10^6$ cells/ml and 100ul per sample is usual. Always run calibration beads on a daily basis. The limits of sensitivity of the technique somewhat depend on how many cells you run. In theory it is possible to detect one cell in a million but you would have to run through at least 1000 million cells to achieve statistical significance which would take a very long time.

Some statistical definitions - The arithmetic mean is the "best" measure of central tendency for true normal (gaussian) distributions, the geometric mean is the same for distributions that are made normal if they are transformed logarithmically (log-normal distribution). The mean is calculated by summing the values of all measurements and then dividing by the number (n) of measurements, whereas the geometric mean is found by multiplying all measurements and finding the nth root of the product. If a "log" sample is normally distributed (i.e. Log-normal), then the geometric mean would indicate the centre better than the arithmetic mean. The median (50th centile) is the value that corresponds to the middle item in a ranked list (i.e. sorted by magnitude) of all measurements. It's robust in that it doesn't necessarily move in response to small numbers of outliers, or to skewing of the tails of a distribution, whereas the mean is tugged by both. One situation where the median is probably the only valid measure is where data pile up at one extreme of measurement, as long as more than 50% of the cells are clear of the sides you get a valid median, but either type of mean will be way off. So if you've got normally distributed data, and want to be able to reflect small changes, use the arithmetic mean, if you've got lognormal data use the geometric mean, and if you want a robust indicator use the median (but this will fail to indicate subtle changes).

A few good links

<http://www.protocol-online.org/cgi-bin/prot/search.cgi?query=facs>

<http://www.cyto.purdue.edu/class/>

<http://flowcyt.salk.edu/sitelink.html>

http://www.sciencedirect.com/science?_ob=PublicationURL&_tockey=%23TOC%234931%232000%23997569998%23209389%23FLA%23&_cdi=4931&_pubType=J&_auth=y&_acct=C00010083&_version=1&_urlVersion=0&_userid=122868&md5=ab146e0c89c390f0fc496951f2684725

<http://www.isac-net.org/content/view/82/43/>

Figures

Fig 1

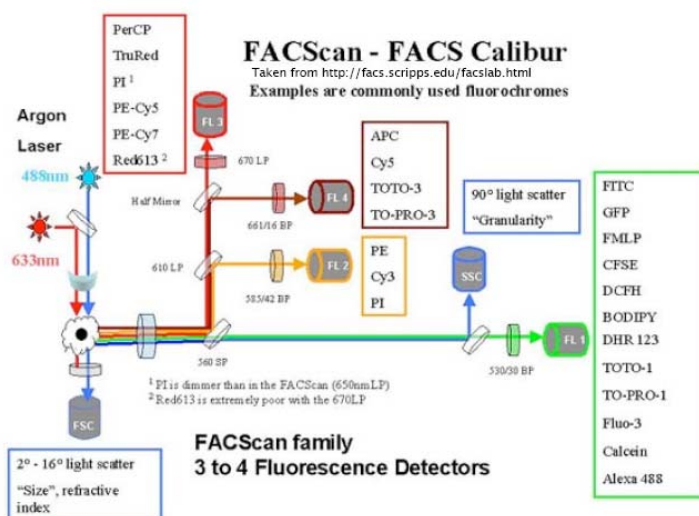


Fig 2

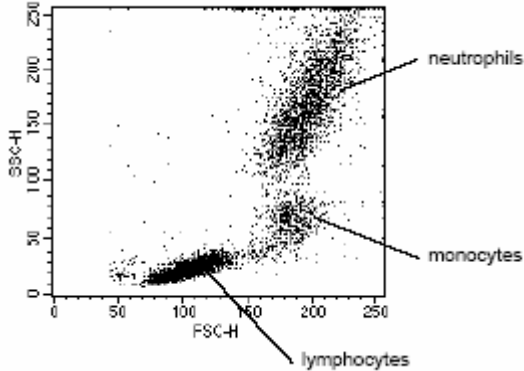
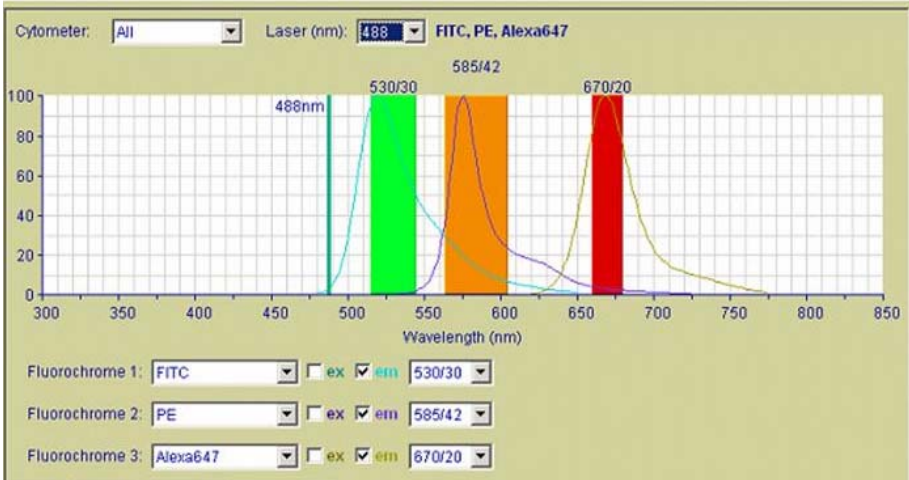


Fig 3



Happy FACS'ing!

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