# IMMUNOFLUORESCENT STAINING THEORY

## WHAT IS IMMUNOFLUORESCENT (IF) STAINING?

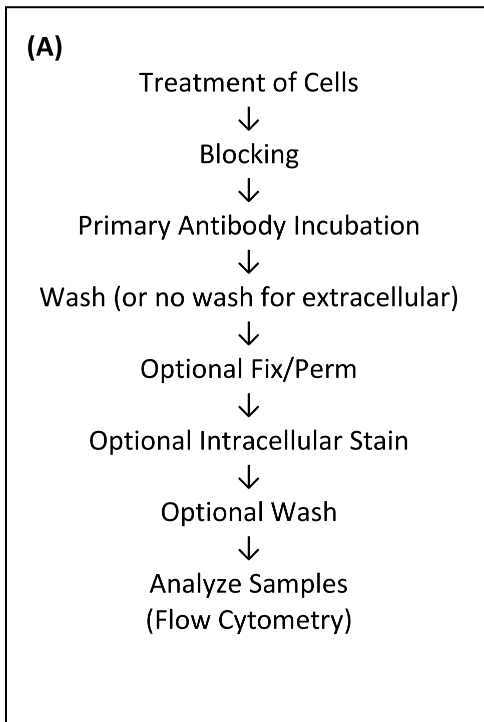
Immunofluorescent (IF) staining is a technique that utilizes the specificity of antibodies to their antigen in order to target fluorescent dyes to a specific biomolecule. In this technique a fluorophore is chemically conjugated to an antibody. The biomolecular targets, or antigens, are mostly proteins and polysaccharides, but less frequently may also be lipids. The fluorescent dye then allows for detection of the target molecule in the sample which can be used for morphological and/or quantitative measurements using light microscopy and/or flow cytometry. IF staining can be used on tissue sections, cultured cell lines, or cells in suspension. IF staining can also be used in combination with other, non-antibody methods of fluorescent staining; for example, use of fluorescent proteins (such as GFP), Hoescht to label nucleic acid, and fluorescent phalloidins to label F-actin.

## GENERAL STEPS OF IF STAINING: LIVE CELLS VERSUS FIXED CELLS

For this course we will be focusing on IF staining of cultured cell lines (typically monolayers) and cells in suspension. There are two general approaches to IF staining: labeling of live cells or labeling of fixed cells. These approaches are described below and a flow diagram of their steps is seen in Figure 1.

(A) In one method, **live** cells are stained using fluorescently conjugated antibodies that bind to extracellular antigens, such as surface proteins. At the end of the staining procedure the cells may or may not be preserved by fixation. The decision to fix will depend on many factors including, the compatibility of the fluorophore with the fixative method, the need to store the stained sample for prolonged periods (hours to days) and the type of detection (e.g. spinning of suspended cells onto slides for microscopic analysis). IF staining of live cells is quite popular for the preparation of cell suspension for flow cytometry for two main reasons: There are fewer steps involved thereby minimizing the amount of cells lost during centrifugation; and, flow cytometry is often used to detect molecules on the cell surface which are readily accessible to antibodies.

(B) In the second method, cells are **fixed** so that their morphology is maintained. The process of fixation kills the cells, and allows for subsequent detergent permeabilization of cell membranes without the loss of intracellular components. If intracellular molecules are being detected, the cell membrane must be partially permeabilized to allow antibody penetration into the cell. The fixed and permeabilized sample can then be incubated with fluorescently conjugated antibodies targeted to the molecule(s) of interest. This IF staining method is quite popular for the preparation of adherent cells to be visualized by microscopy since the process of fixation can preserve the cell morphology, and since the detection of the location of intracellular molecules is often desired.



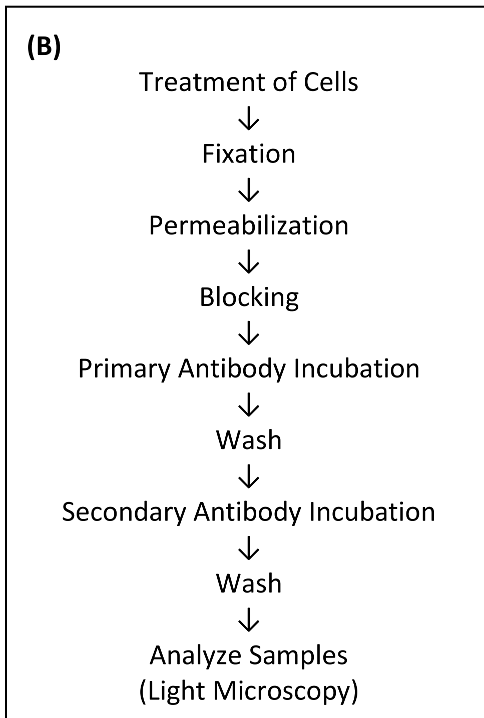




**Figure 1.** Work flow for IF staining for flow cytometry (A) or microscopy (B). Note: both flow cytometry and microscopy can be used in conjunction with live and fixed samples. Flow cytometry most often uses live cells and primary antibodies conjugated to fluorophores (no secondary) while microscopy most often utilizes fixed samples and primary/secondary antibody based staining.

## DIRECT VERSUS INDIRECT STAINING

The term primary antibody is used to indicate the antibody which binds to an antigen on your molecule of interest. If the primary antibody has a fluorophore directly conjugated to it, no further steps are required for detection and this is referred to as direct IF staining. Oftentimes, the primary antibody is unconjugated (so that it can be used for other techniques such as western blotting). In this scenario, a fluorescently tagged secondary antibody which binds to the primary antibody is required. This type of staining is caller indirect IF staining.

## DETAILED DESCRIPTION OF STEPS INVOLVED IN IF STAINING PROCEDURE

The following section will explain the various stages of IF staining and is modified from "Immunocytochemistry: A practical guide for biomedical research." by Richard W Burry. As you will notice there are several ways in which each step can be performed and optimized. The intent is to give you a solid understanding of the concepts and methods so that you may be able to design an experimental approach appropriate for your aim. In the following sections, several but not all of the steps are interchangeable for microscopy and flow cytometry. Any differences will be clearly indicated.

**FIXATION**

Fixation can be thought of as freezing cells or tissues at a desired point in time. Effective fixation should fulfill the following criteria: 1) Fixed cells should appear similar to living samples; 2) Fixation should be uniform throughout the samples (this is especially important for tissues) and; 3) There should be no loss or extraction of cellular molecules (e.g. proteins and lipids). Good sample fixation is essential to obtaining good results.

There are two kinds of fixatives: denaturing and crosslinking. Denaturing fixatives use heat or organic solvents. This type of "fixation" destroys protein secondary and tertiary structures and dissolves lipids into micelles. Because of this there is poor preservation of morphology and loss of cellular molecules. Therefore, this type of fixation is not recommended if the aim of the experiment is the detection of protein and the careful analysis of cell morphology. Of note, due to lipid micelle formation, samples fixed by these methods do not require a subsequent permeabilization step. The most common types of denaturing fixatives are cold methanol or acetone. Generally, methanol or acetone is cooled to –20˚C and then added to cells for 5-20 minutes on ice, followed by extensive washing. This type of fixation is used widely to look at intracellular (not membrane proximal) antigens and DNA quantitation in flow cytometry.

Crosslinking fixation utilizes chemicals with aldehyde groups. Aldehydes crosslink molecules within cells and tissues; specifically, 1˚ amines of Lys and Arg, sulfhydrol groups of Cys, hydroxyl groups and double bonds are crosslinked by aldehydes. The result is a meshwork of cellular molecules that are "stuck" in place (imagine a spider weaving a web which connects the molecules within a cell). This type of fixation better preserves the structure of the cell and is the recommended method for most fixations.

Generally, a 10-30 minute treatment at room temperature with a crosslinking fixative provides sufficient retention of cellular material. Importantly, over-fixation should be avoided as this can increase autofluorescence, cause crosslinking of epitopes (i.e. epitope masking) and more commonly can make it difficult for the relatively bulky antibodies to diffuse into the sample and reach their targets.

The most common crosslinking fixative is formaldehyde (see Figure 2). There are two main types of formaldehyde that can be purchased for IF: formalin and paraformaldehdye (PFA). Formalin contains several impurities, including 14% methanol, and because of this is not recommended. On the other hand PFA is very pure when prepared properly and is the recommended choice. Preparation of PFA from powder sources has some hazardous risks therefore most researches purchase dissolved pure 16% PFA. Generally, this is diluted to a final concentration of 4% PFA and used to fix cells for 10-30 minutes at room temperature. Samples should not be stored in 4% PFA for extended periods of time and it is recommended if you are storing samples to either wash off the PFA or dilute it to ≤1%.

**Safety Precaution:** Please be aware of the safety and disposal precautions for the fixative which is being used. PFA should be used in a chemical fume hood. PFA should be collected after use and stored in a labeled and sealed container suitable for chemical waste pick up.

**Note:** Formalin is often used in pathology labs. For IF procedures formalin fixation requires additional steps for antigen retrieval that may be difficult. These steps are omitted when PFA is used as a fixative.

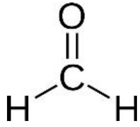



Figure 2. Chemical structure of formaldehyde

**Alternative Fixation Procedures for Flow Cytometry**

For flow cytometry it is common to prepare and store fixed samples for analysis at a later time point. In this case, it is recommended to stain for external (cell surface) antigens before fixation (as any fixative carries the risk of neutralizing an epitope). These samples can then be fixed and permeabilized (Saponin, Triton X-100, etc.) to stain for internal (cytosolic) antigens followed by a second fixation to immobilize the intracellular antibodies. This method allows one to store samples (light protected at 4 °C) for days to weeks. Done properly, one can stain for CD4+ T-cells from a blood sample in isolated parts of Africa and run the samples over a week later once the sample reaches a cytometer.

**Other Fixatives**

- Acrolein ($C_3H_4O$) is a fast penetrating fixative used together with PFA but it is extremely poisonous and carcinogenic and therefore not used.
- Glutaraldehyde is the absolute best for preservation of structure and is therefore used for electron microscopy. However for IF staining, it crosslinks too much and can hinder antibody penetration into sample. In addition, it is autofluorescent making it even less suitable for IF staining.
- PLP (periodate-lysine-paraformaldehyde) is used for paraffin embedded tissue to provide better fixation.
- Several other proprietary commercial fixatives (with and without permeants) are sold (i.e. FACS Lysing solution, CytoFix/CytoPerm, Phosflow buffers, etc. These are generally different combinatorial formulations of the above listed reagents.

**Diluents for crosslinking fixatives**

The diluents for the crosslinking fixatives should preserve the cellular morphology. For this reason they should be pH buffered (7.1 – 7.4) and isotonic (to avoid cell shrinking or swelling). Phosphate buffered saline (PBS) is often used as the diluent for PFA. For adherent cells the lack of calcium in this buffer may promote cell detachment, further if calcium is added to PBS the phosphates may form precipitates with the calcium. For this course we will use an isotonic saline solution for the adherent cells, which is HEPES buffered and contains physiological levels of calcium, magnesium and potassium (HBSS or IsoNaCl, see protocols).

**Delivery of fixative**

For adherent cells, treatment media can be gently aspirated and replaced with fixative. Importantly, if there are amino acids, phenol red or serum in the treatment media it should be washed away quickly and gently before fixation.

For cells in suspension, cells can be pelleted and subsequently resuspended in fixative (1mL of fixative per 50µL of packed cells). After washing off the fixative, by pelleting and resuspending in PBS, the cells can be attached to a poly-L-lysine coated surface or analyzed in suspension, depending on the downstream application.

**Fixative Removal and quenching**

At the end of fixation, the fixative should be thoroughly washed as it can damage antibodies, interfere with antibody-epitope interactions and/or be a source of autofluorescence. Typically this is done by several washes with PBS. In the case of crosslinking fixatives, such as PFA, a 10 minute incubation with 100mM glycine (in PBS) is included in the procedure since glycine will bind to free aldehyde groups.

## PERMEABILIZATION

The plasma membrane and intracellular membranes (e.g. mitochondrial, ER, golgi and nuclear membranes) are impermeable. If we want to localize molecules inside the cell we need to make the cell permeable to antibodies. Recall that denaturating fixatives (e.g. acetone or methanol) dissolve lipids into micelles; therefore permeabilization is not required for these types of fixation. However, for crosslinking fixatives the membranes need to be partially dissolved using detergents. Non-ionic detergents, such as Triton X-100 and Tween 20, are preferred over ionic detergents, such as SDS and DOC since they do not disrupt the tertiary structure of proteins. The permeabilization step can be done just before blocking or at the same time as blocking and should be performed at room temperature since detergents are less effective at dissolving lipids at colder temperatures. It is important to note that proteins that were embedded in cellular membranes are not lost upon detergent treatment since they are fixed in place due to crosslinking to other membrane and scaffolding proteins. Typically permeabilization is achieved by treating cells with 0.1% Triton-X 100 in PBS containing blocking agent (1% BSA and/or 1% serum) for 20 minutes at room temperature. For IF staining, it is not recommended that detergent be added to subsequent steps since it will cause further lipid extraction and may also interfere with antibody binding.

Importantly, conjugated antibodies can have bulky fluorophores. For example, PE (phycoerythrin) and PE tandem conjugate dyes (i.e. PE-Cy conjugates) are actually larger than the antibody itself and may restrict both the antibody getting into the cell as well as the removal of excess unbound antibody. Therefore, permeabilization (as well as antibody incubation and washing steps, see below) may need to be optimized to account for these bulky fluorophores.

Finally, you may not want to permeabilize your sample. For example, you may be interested in the surface expression of a transmembrane receptor protein. If you have an antibody that binds to an epitope on the extracellular surface of this protein you can monitor the amount of this protein that is in the plasma membrane alone by NOT permeabilizing your sample.

**Other methods of permeabilization**

For some procedures (e.g. EM-ICC or specific live cell studies) you may need to reversibly permeabilize you sample. This is done using saponin or digitonin. These detergents insert themselves into the plasma membrane next to cholesterol moieties in a concentration dependent manner, that is, they are reversible and can be washed away. As such it is critical that these detergents be included in both the staining and washing stages of IF.

**BLOCKING**

Blocking is an essential step to IF staining. Its purpose is to reduce the amount of non-specific binding of your antibodies (both 1˚ and 2˚) to your sample. Non-specific binding is any type of **non-epitope** binding of your antibody. These can be grouped into three main categories: charge binding, Fc receptor binding and, endogenous antibody binding. (Figure 3)

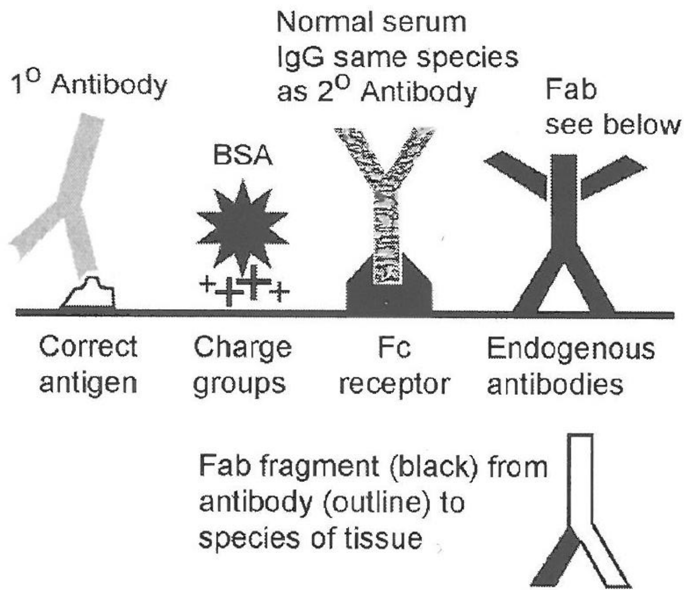



Figure 3. Types of non-specific antibody binding: Charge group binding, Fc receptor binding and Endogenous antibody binding. (Taken from "Immunocytochemistry: A practical guide for biomedical research." by Richard W Burry. Page 48)

**Charge blocking**

Antibodies binding to charged groups on protein or lipids is the most common type of non-specific binding. For example, histones contain several basic amino acids and are often the source of non-specific nuclear staining. In addition, charged groups can be introduced when using formalin or glutaraldehyde for fixation. For charged group non-specific binding the most common blocking agent used is bovine serum albumin (BSA) at 10mg/mL (or 2-5% w/v in flow cytometry) since BSA is not antigenic and binds to charged sites. It is important to purchase IgG-free BSA that has been purified and fractionated (usually *fraction V*) if you are using a secondary antibody (i.e. performing indirect IF staining).

**Fc Receptor blocking**

Fc receptors can be found in hepatocytes and circulating white blood cells such as macrophages, B-cells, neutrophils, mast cells, platelets, dendritic cells, eosinophils, monocytes and natural killer cells as well as at sites of high inflammation in tissue preparations or any sample including phagocytes. Fc receptors bind the Fc portion of antibodies, presenting a major problem since both the 1˚ and 2˚ antibodies can bind specifically (but undesirably) to this receptor. This binding is a highly specific interaction between the Fc and Fc receptor, which is independent of the desired

interaction between the CDR and the antigen of choice, leading to a strong "false" positive signal. Species-matched serum can be used to block this type of interaction since it contains IgG that will bind to and occupy the Fc receptors in your sample. Most often it is recommended to use 10% v/v serum, or alternatively a more costly (but often more effective) solution is the inclusion of an Fc blocking antibody (i.e. anti CD16/32). In staining Fc receptor enriched populations such as PBMCs (peripheral blood mononuclear cells) it is worth noting that Fc blocking is even more crucial when using activated or murine samples. When blocking it is important to use serum from the host species of the 2˚ antibody to eliminate the risk that the 1˚ and 2˚ antibodies will bind to the IgG present in the host serum. Note that serum contains albumin, nonetheless, additional BSA is often added when serum is used as a blocking agent. General-purpose recommendations are, for example 1-2% BSA *fraction V* and 5-10% serum.

**Endogenous antibodies**

This type of non-specific binding is most common in samples that have had an immune response (e.g. a macrophage that is reacting to invading bacteria). The antibodies that were produced as a result of the immune response can provide binding sites for 2˚ antibodies if they are of the same species. (For example, a mouse sample with endogenous antibodies and an anti-mouse 2˚ antibody) To block this type of interaction antibodies against the IgG of the species of the sample (i.e. anti-mouse IgG in our example) should be used. It is important to use the Fab fragment only as the Fc component may react with the 2˚ antibody.

So what is the best way to block your sample? The answer is that this depends both on the sample and the antibody and may require some optimization. As a starting point it is recommended that 3-5% BSA in PBS be used for non-immune cells (i.e. samples that do not have Fc receptors) or for immune cells/cells with Fc receptors, 5-10% serum (from the host species of the 2˚ antibody) plus 1-2% BSA in PBS. Finally, it is recommended that all subsequent steps contain blocking agent in them.

**Additional blocking agents**

Glycine- Binds free aldehyde groups that occur after formalin or glutaraldehyde fixation. (Also see fixative removal above.)

Gelatin-Binds charged groups.

Tx-100 or NP-40 (detergent)-Can be added to reduce non-ionic hydrophobic binding and H-bonding of antibodies, however, this will also permeabilize the cell membrane (see below).

Milk-Often used in Western blotting since it is a strong blocking reagent. It is not recommended for IF staining because it can reduce antibody binding.

**ANTIBODY TITRATION, BINDING & WASHING**

**Antibody Titration**

It is an excellent idea to do a titration of your antibody as a preliminary experiment. The goal of the titration is to determine the concentration of both 1˚ and 2˚ antibodies which give the best signal to noise ratio (SNR) and the lowest background staining. Randomly increasing antibody concentration is not a good solution since this often leads to both high background staining and high non-specific staining. The following chart (Table 1) is an antibody dilution matrix that can be used to set up an antibody titration experiment. There are eight samples in total. Further, this preliminary experiment will also serve as controls for your 2˚ antibody (i.e. the samples with no 1˚ antibody).

| | 1˚ antibody dilution | | | |
|---|---|---|---|---|
| 2˚ antibody dilution | 1 : 100 | 1 : 500 | 1 : 1000 | no antibody |
| 1 : 100 | | | | |
| 1 : 1000 | | | | |

Table 1. Antibody dilution matrix. Based on 1˚ antibody stock concentration of 1mg/mL and 2˚ antibody stock concentration of 2mg/mL.

**Antibody Binding**
Apart from antibody dilution, three other factors should be considered when incubating samples with antibody: time, temperature and buffer.

Time: Antibody binding can be done for 15 mins to several days. You want to allow sufficient time for the antibody to penetrate into the crosslinked sample. In microscopy, a one hour incubation at room temperature is usually sufficient for both 1˚ and 2˚ antibody incubation. However, in some cases longer antibody incubation times, overnight or longer times are required (if samples are thick or if antibody has weak penetration into the sample). In flow cytometry most antibody incubations are 15-30 minutes since penetration is not a general concern: That is, antibodies are often directed to external epitopes (no penetration required) and the sample is a cell suspension (only needs to penetrate into 1 cell).

Temperature: For microscopy, incubation at room temperature is ideal since membranes are less rigid. However, long incubation times may allow for bacterial growth. This can be minimized by either performing antibody incubations at 4˚C or by adding 0.02-0.05% sodium azide ($NaN_3$) to the antibody buffer.

In flow cytometry, staining of extracellular surface molecules is often done on live (non fixed, non-permeabilized) cells. For hardy cell types (e.g. ATCC cell lines), antibody incubations can be done at room temperature. However, incubation on ice limits apoptosis and prevents membrane fusion/fission events. It is also common to include sodium azide (0.1%) to prevent capping on motile cells. Capping is a phenomenon by which antigen is cross-linked by antibody, migrates to one end of the cell, and is cleaved in an ATP-dependent process. Antibody capping leads to antigen loss and cell death. Note that azide cannot be used when you are looking at ATP-dependent events such as cellular activation.

Buffer: The buffer that is used to dilute antibody should contain blocking agent. If cost is not an issue it is ideal to use the same concentration of blocking agent in your antibody dilutions, otherwise the concentration of blocking agent can be reduced. In some cases, detergent can be added to the antibody buffer, e.g. 0.01% Triton-X to help reduce non-specific antibody binding. However, this will lead to increased membrane extraction of your sample and may also decrease antibody binding to epitope, and is therefore only recommended as a last resort to improve antibody specificity.

**Antibody Washing**

Washing steps are performed in order to remove excess unbound antibody. Like the antibody buffer, decisions about whether or not to add blocking agent and or detergent must be made (see above). Usually, washing buffer is added to samples for a brief incubation time (5 - 10 minutes) and then removed. This is the repeated 4-7 times in order to both serially dilute and wash off your unbound antibody. Also important to note is that when using saponin or digitonin to permeabilize, these must be included in the washing steps or the temporary membrane pores can close and you will not be able to wash unbound antibody from the inside of the cell.

**CONTROLS**

Controls are an essential part of IF staining as they tell you whether or not your staining procedure is reliably labeling your molecule of interest. Controls can be grouped into three categories: 1˚ antibody controls, 2˚ antibody controls and labeling controls.

**Primary antibody controls**

Primary antibody controls are designed to show that your 1˚ antibody binds specifically to the epitope you are interested in. The best way to control for this is to use samples in which your epitope is absent and then verify that your staining procedure has no significant signal. If you are using primary cells or tissues, samples taken from a knockout animal provide the best control. If you are using cell lines (i.e. and knockouts are not available) siRNA can be performed to reduce the protein of interest. Of course, you must verify that your siRNA is effective.

If samples deficient in your protein of interest are not available it is useful to show that your antibody is reliable using another technique, such as ELISA, Immuno-precipitation (IP) or Western blotting. Keep in mind that IF staining (formaldehyde fixed) and IP interact with folded protein whereas Western blotting (WB) detects denatured protein; therefore it is conceivable that an antibody that is good for WB is not good for IF staining. In addition, the conditions of antibody binding during IP are quite different then IF staining (i.e. cell lysate versus fixed cells respectively), thus it is also possible that antibodies that are great for IP are not good for IF.

Another way to lend credit to your 1˚ antibody specificity is to show colocalization of your staining with a fluorescent-tagged version of your protein that is exogenously expressed. For example, show that anti-cortactin staining colocalizes with exogenously expressed GFP-cortactin. Keep in mind that exogenously expressed proteins do not always localize correctly. Similar to this type of control, showing that two antibodies against the same protein have colocalization can also lend credibility to your staining. (Note: The two different antibodies should be directed against different epitopes.)

Finally, for monoclonal antibodies adsorption controls using blocking peptides can also be performed to determine 1˚ antibody specificity. The blocking peptide should block signal completely at high concentrations and reduce signal at low concentrations. This type of control would not be useful to determine if the epitope exists on another protein. Finally, in some cases the blocking peptides can cause a false positive interaction, i.e. if the blocking peptide interacts with your sample.

**Secondary antibody controls**

Secondary antibody controls are designed to show that your 2˚ antibody binds specifically to your 1˚ antibody. For these controls the entire staining procedure is done with the 1˚ antibody either omitted or replaced with serum from the host species of the 1˚ antibody. If there is still signal in these controls then blocking solutions should be optimized and/or another 2˚ antibody should be tested. If multiple 1˚ and 2˚ antibodies are being utilized it is also important to show that each 2˚ binds only to its cognate 1˚ antibody and not to each other.

**Labeling controls**

Finally, labeling controls are performed to identify the amount of endogenous fluorescence (or autofluorescence) in your sample. Autofluorescence can be irregular (particulate) or uniform in pattern. The irregular pattern is often attributable to elastin, lipofusin, NADH, flavins, chlorophyl and hemoglobin while the uniform pattern is often due to the formation of aldehydes that are formed after formalin or glutaraldehyde fixation. Since fixative-induced autofluorescence tends to be more prominent in the green emission range, (i.e. higher energy, shorter wavelenghth), using red shifted fluorphores, such as Alexa 568, would be helpful.

In order to determine the amount of autofluorescence in your sample, all IF staining steps are performed except that both the 1˚ and 2˚ antibodies are omitted. These controls can then be examined (by flow cytometry or by fluorescent microscopy) using the same instrument settings that will be used for your specific staining. Importantly, the amount of allowable autofluorescence depends on what you are staining for. For example, if you are staining for a very abundant protein, the amount of signal (specific staining) to background (autofluorescence) ratio will be big, thus having low PMT gain or low exposure times will allow you to effectively remove the autofluorescent signal. On the other hand for low expressing proteins even a small amount of autofluorescence can be quite problematic. If a significant amount of autofluorescence is detected the following strategies can be aimed at minimizing this signal.

1. Increase signal by titrating antibody, i.e. increase the signal from your antibody.
2. Most autofluorescence is in the violet to green spectrum so switching to a red or far-red fluorophore may be helpful. Note that far-red signals are not visible to the human eye. Note. Autofluorescence also depends on your excitation source; a 405 excitation is the worst, followed by 488 excited samples in the Alexa 488 and PE channels.
3. Treatment with glycine or sodium borohydrate can be used to block free aldehydes generated during fixation.
4. Sudan black or $CuSO_4$ can be used to decrease the autofluorescence generated by lipofusin (i.e. a fluorescent byproduct produced by RBCs).
5. Some advanced confocal imaging systems allow you to perform spectral unmixing. (For more information on this technique please refer to:
   http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html)

In flow cytometry, the most often used controls are isotype controls, although considerable controversy is arising based on whether they are actually useful. In the case that you have a negatively staining population in the same tube, you decidedly do not need an isotype control. For example, in a CD3+ T-cell stain, all the non-T-cells in the sample serve as an appropriate antigen-negative internal control. The only time you unquestionably need an isotype control is when doing intracellular staining in flow cytometry since fixed, permeabilized cells are like little cages that can both specifically bind antibody and trap antibody non-specifically. When using

multiple colours, it is ALWAYS necessary to run single-stained controls and the most appropriate labeling control is the fluorescence minus one (FMO) control (also discussed in the “Flow Cytometry” section), which contains all antibodies and colours except for the channel that you are controlling for.

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