Chapter 10 | Immunofluorescence

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Immunofluorescence (IF) is a common laboratory technique used in almost all aspects of biology. This technique based on pioneering work by Coons and Kaplan (1, 2), and later by Mary Osborne (3), has been widely used both in research and clinical diagnostics. Applications include the evaluation of cells in suspension, cultured cells, tissue, beads and microarrays for the detection of specific proteins. IF techniques can be used on both fresh and fixed samples. In IF techniques, antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). These labeled antibodies bind (directly or indirectly) to the antigen of interest which allows for antigen detection through fluorescence techniques. The fluorescence can then be quantified using a flow cytometer, array scanner or automated imaging instrument, or visualized using fluorescence or confocal microscopy.

The two main methods of immunofluorescent labeling are direct and indirect. Less frequently used is direct immunofluorescence whereby the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect immunofluorescence, the antibody specific for the molecule of interest (called the primary antibody) is unlabeled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) is tagged with the fluorescent dye (Figure 1).

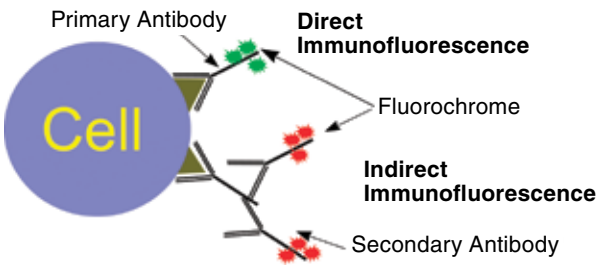

Figure 1. Schematic of direct and indirect immunofluorescence.

Advantages of direct immunofluorescence include shorter sample staining times and simpler dual and triple labeling procedures. In cases where one has multiple antibodies raised in the same species, for example two mouse monoclonals, a direct labeling may be necessary.

Disadvantages of direct immunofluorescence include lower signal, generally higher cost, less flexibility and difficulties with the labeling procedure when commercially labeled direct conjugates are unavailable.

Advantages of indirect immunofluorescence include greater sensitivity than direct immunofluorescence. There is amplification of the signal in indirect immunofluorescence because more than one secondary antibody can attach to each primary (see Figure 1). Commercially produced secondary antibodies are relatively inexpensive, available in an array of colors, and quality controlled.

Disadvantages of indirect immunofluorescence include the potential for cross-reactivity and the need to find primary antibodies that are not raised in the same species or of different isotypes when performing multiple-labeling experiments. Samples with endogenous immunoglobulin may exhibit a high background.

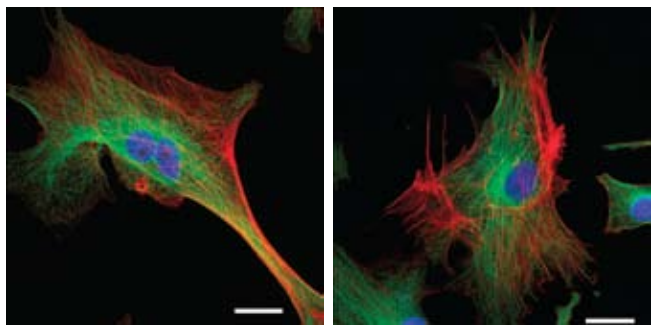

Figure 2. Cultured pulmonary artery endothelial cells stained for tubulin (red), actin (green) and DNA (blue). The dual immunofluorescence procedure used rabbit anti-actin IgG and mouse anti-alpha tubulin IgG as primary antibodies. The secondary antibodies used were Texas Red-conjugated goat, anti-rabbit IgG and FITC-conjugated goat, anti-mouse IgG. The sample was also stained with the DNA-specific dye Hoechst 33342. Scale bar is equal to 20 microns.

Principle of Fluorescence

Fluorescence and phosphorescence are both types of luminescence. When molecules with luminescent properties absorb light, they emit light of a different wavelength. With fluorescence the emission of light occurs extremely rapidly after the absorption of excitation light, whereas with phosphorescence emission continues for milliseconds to minutes after the energy source has been removed. Fluorescent materials give off light because of their atomic structure. Electrons are arranged in discrete energy levels surrounding the atom's nucleus with each level having a predetermined amount of energy. When an electron absorbs the energy from a photon of light (Figure 3) it becomes "excited" and jumps to a higher, less stable energy level. The excited state does not last long. The half-life of the excited state is generally less than 10 (8) seconds. The electron loses a small amount of energy as heat and the remainder of the extra energy is given off in the form of a photon. The emitted fluorescence has a lower energy than the absorbed light, so the wavelength of the emitted light is longer than that of the excitation light (except in the case of multi-photon excitation).

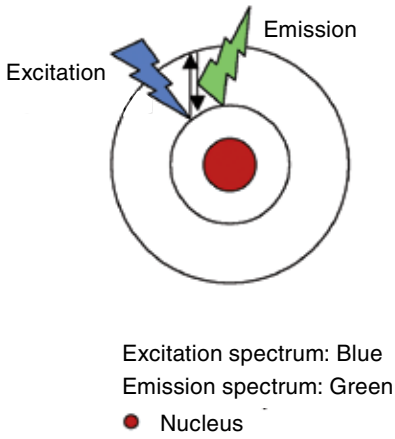

Figure 3. Principle of fluorescence.

A range of wavelengths of light can excite the electrons of a fluorochrome. For example, fluorescein will fluoresce when hit by light with any wavelength between 450 nm and 520 nm. However, the closer the excitation wavelength is to 495 nm, the more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly, the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein ranges from 490 nm to 630 nm, and the emission peak is approximately 515 nm. Since the phenomenon of fluorescence was first explained by a British scientist, Sir George Stokes, in 1852, the shift in wavelength from short to long during fluorescence is called "Stokes shift" (Figure 4).

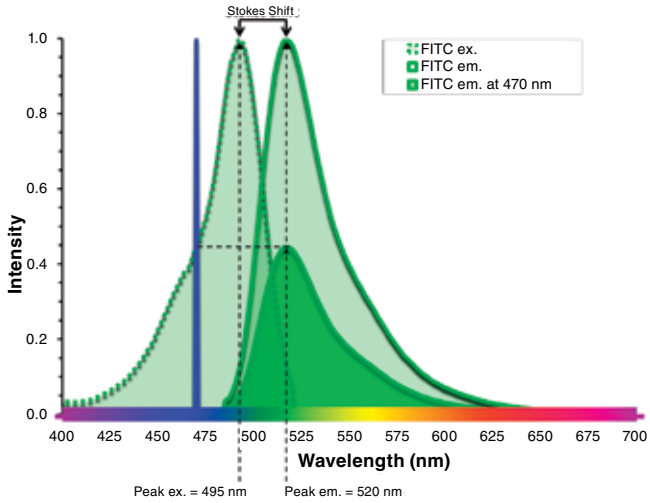

Figure 4. Excitation and emission spectrum of fluorescein. When fluorescein is excited at a wavelength other than its peak excitation (470 nm in this example), the shape of the emission curve (darker green) remains the same, but the relative intensity is reduced. The efficiency of the excitation at 470 nm is 45% of peak excitation.

Some fluorochromes have a small Stokes shift while other fluorescent compounds have large Stokes shifts. For example, the fluorochrome fluorescein can be excited by blue-green light, and its Stokes shift is only about 20 nm, which means that the light emitted is green. This contrasts with another fluorochrome, phycoerythrin, which also can be excited by blue-green light, but has a large Stokes shift. Thus, the light emitted is yellow-orange. In immunofluorescence, a single wavelength can be used to excite several fluorochromes with different Stokes shifts and thereby produce a variety of fluorescent colors as shown in Figure 5.

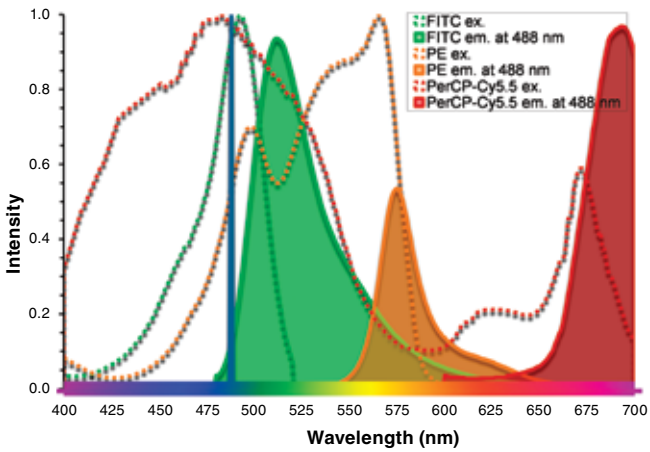

Figure 5. Excitation of three spectrally distinct fluorochromes using a single laser line.

The example in Figure 5 shows a single wavelength at 488 nm (blue line) exciting three different fluorochromes identified by their absorption curves on the left of the figure (blue line). Each fluorochrome is excited at a different efficiency and, therefore, the resulting emission will be at different intensities for equivalent fluorochrome concentrations. Knowing the excitation and emission properties of fluorescent compounds makes it possible to select combinations of fluorochromes that will work together. However, for a fluorochrome to be useful in a biological application it must attach to or be contained within a structure of biological significance.

Fluorochromes can be attached to antibodies which will then bind to specific chemical structures on or inside cells. Many other chemical and physical properties of fluorochromes determine when and where these dyes are useful in various biological assays. For example, some of the fluorochromes that bind to DNA, such as Hoechst 33342, can get into living cells, but most DNA-binding fluorochromes cannot get past the cell membrane. Those fluorescent dyes that cannot get past an intact cell membrane, such as propidium iodide (PI), are often used to distinguish live from dead and dying cells.

The ideal fluorochrome would be a molecule with the following properties:

- An absorption peak at an excitation wavelength available on the fluorescence detection instrument (large extinction coefficient at the wavelength of excitation)
- Bright fluorescence (high quantum yield)
- A narrow emission spectrum that falls within one of the instrument's detection bands
- Good photostability and
- Fluorescence properties that are not significantly altered by conjugation to an antibody or by the local environment of the sample

Limitations of IF Techniques

Photobleaching

As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching (and phototoxicity). Photobleaching is the photochemical destruction of a fluorophore due to the generation of reactive oxygen species in the specimen as a byproduct of fluorescence excitation (Figure 6). Although the exact mechanism of photobleaching is unknown, it is thought that the primary causative mechanism appears to be photosensitization of singlet oxygen ($^1O_2$) (see glossary for details) generation by the dye triplet-excited state and reference (4) for details). Photobleaching can be minimized by: (a) decreasing the excitation light in both intensity and duration, (b) reducing the availability of singlet oxygen ($^1O_2$) by the addition of singlet oxygen scavengers (= antifade reagents), and (c) using a low concentration of a fluorochrome with high-quantum efficiency.

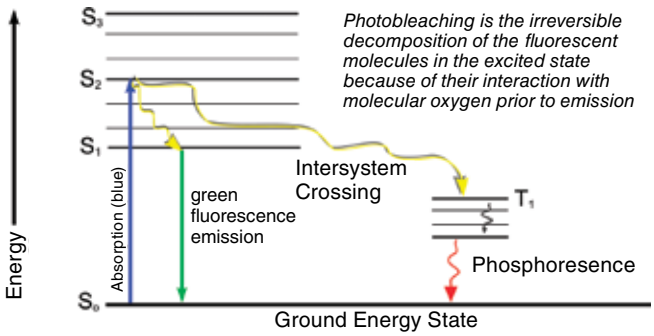

Figure 6. Illustration of how a singlet-excited state can convert to a triplet-excited state.

Autofluorescence

Biological autofluorescence in mammalian cells due to flavin coenzymes (FAD and FMN: absorption, 450 nm; emission, 515 nm) and reduced pyridine nucleotides (NADH: absorption, 340 nm; emission, 460 nm) can be problematic in the detection of fluorescence probes in tissues and cells. Fixation with aldehydes, particularly glutaraldehyde, can result in high levels of autofluorescence. This can be minimized in fixed cells by washing with 0.1% sodium borohydride in phosphate-buffered saline (5) prior to antibody incubation. Problems due to autofluorescence can be minimized by selecting probes and optical filters that maximize the fluorescence signal relative to the autofluorescence. Other factors that limit IF include the performance of the detection instrument (i.e. how well the microscope has been calibrated and set), the specificity of the antibodies, and the specimen preparation.

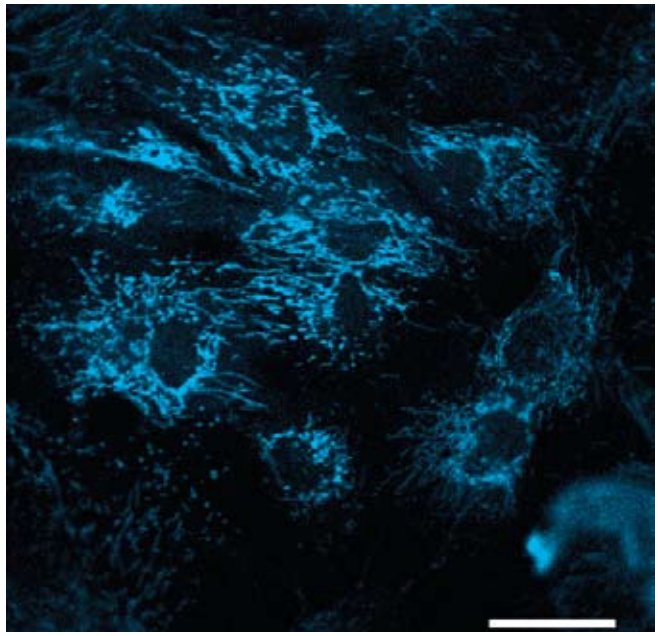

Figure 7. NADH autofluorescence in a human colon carcinoma cell line (HCT116). Ultra-violet excitation at 363 nm was used and the emitted fluorescence greater than 440 nm was collected. Scale bar is 10 microns. Credit: Giselle M. Knudsen, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA.

Fluorescence Overlap

One of the problems that must be dealt with when measuring fluorescence of more than one color is the possibility that the emission signals overlap. It is necessary to remove the overlapping signal or it will give a false level for one or more colors. For example, as shown in figure 8, there is significant overlap when using FITC and PE. A range of wavelengths will be collected for each detection channel. In the figure, these are identified as the fluorescein detector bandwidth and the PE detector bandwidth. These band-pass optical filters will allow photons within this wavelength range to reach the detector. However, as can be seen in figure 8, there is a very small amount of PE fluorescence which is within the FITC band and similarly a small amount of FITC fluorescence within the PE band. These must be electronically removed or the measurement for each detector will overestimate the actual signal. This process is called fluorescence

compensation and can be automatically calculated in many detection systems using single positive controls.

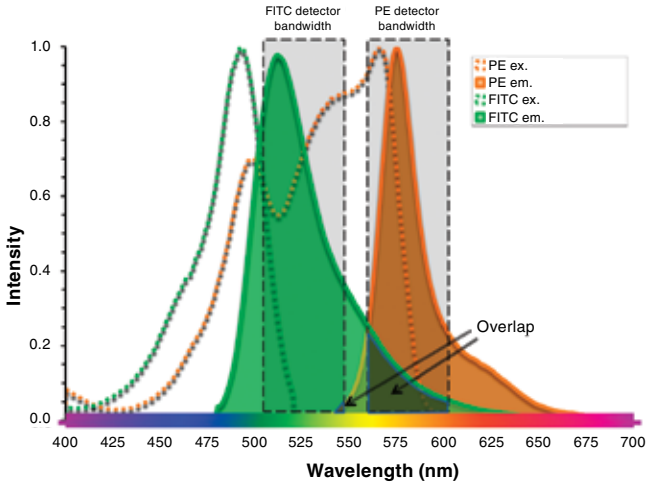

Figure 8. Fluorescence overlap of FITC and PE.

Applications of Immunofluorescence in Pathology

Some practical applications of immunofluorescence in diagnostic pathology are:

- Analysis of antigens in fresh, frozen or fixed tissues; sub-cellular localization of antigens in tissue culture monolayers; observation of bacterial or parasitic specimens;
- Detection and localization of the presence or absence of specific DNA sequences on chromosomes; and
- Defining the spatial-temporal patterns of gene expression within cells/tissues.

The most widely used method of IF in pathology is indirect IF. However, in some very specialized applications direct IF has been used for localization of IgG in immune complexes along the dermal-epidermal junction of skin biopsies from patients suffering from systemic lupus erythematosus (6).

In summary, immunofluorescence is the visualization of antigens within cells using antibodies as fluorescent probes. The benefits of immunofluorescence are numerous, and the technique has proven to be a powerful tool for determining the cellular distribution of known antigens in frozen tissues or in the localization of specific DNA sequences on chromosomes. The method has achieved the status of combining high sensitivity with high resolution in the visualization of antigens and will be a major tool for many years to come that any pathologist studying cells or molecules cannot afford to ignore.

For a methodology article on immunofluorescence labeling of formalin-fixed, paraffin-embedded tissue, see reference 7.

References

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