Chapter 8

Immunofluorescence

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Chapter Outline

Objectives

- Understand the basic principles of immunofluorescence.
- Develop the knowledge base to troubleshoot and optimize an immunofluorescence protocol.
- Describe the practical applications of immunofluorescence for clinical as well as research applications.
- Understand the limitations of immunofluorescence.

INTRODUCTION

Various techniques such as Western blotting, enzyme-linked immunosorbent assay (ELISA), reverse-phase protein array (RPPA), and others have been

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developed to examine protein expression. While these techniques are effective at determining protein expression, they do not provide any information regarding the cellular or subcellular localization of the protein being studied. Immunohistochemical techniques enable protein visualization based on antibody binding to the protein of interest followed by visualization of the antibody by either conjugating the antibody to an enzyme catalyzing a color-producing reaction or by conjugating the antibody to a fluorophore. Visualization of proteins by fluorophore-conjugated antibodies is a defining feature of immunofluorescence (IF). This technique can be used to visualize proteins in cells (both in suspension and adherent cells), tissues as well as 3D culture-derived spheroids.

Immunohistochemistry (IHC) studies are routinely used for pathological clinical diagnosis; however, when an experiment requires co-localization of proteins, IF technique is the method of choice. IF imaging depends on light emission from fluorophore-conjugated antibodies. A fluorescent chemical compound that can absorb light at a specific wavelength resulting in light emission at a longer or lower energy wavelength is referred to as a fluorophore [1]. For example, a commonly used fluorophore Alexa 488 has an excitation peak of 495 nm and emits light with an emission peak at 519 nm (green spectrum), while another fluorophore Alexa 594 has an excitation peak of 590 nm and an emission peak at 617 nm in the red spectrum. As distinct fluorophores have different excitation and emission wavelengths, multiple antigens can be visualized on the same biological sample by conjugating multiple antigen-recognizing antibodies to different fluorophores with distinct excitation and emission spectrums. Another important advantage of IF is the ability to capture images on a confocal microscope to determine the cellular localization of the protein of interest. Confocal microscopy enhances optical resolution by filtering out the light emitted from the out-of-focus planes [2]. To identify whether the antigen of interest is expressed in a particular intracellular compartment, confocal IF imaging of the antigen of interest along with a well-characterized protein known to be localized to the cellular compartment of interest is performed. Confocal imaging software also allows for image capture at multiple focal planes enabling 3D imaging of the specimen of interest. This application is particularly useful in studying the structures of various organs such as the mammary gland and the vasculature [3–5]. It is important to note that while traditional IHC staining can be preserved for a long time, IF staining is highly sensitive to photobleaching and therefore stained slides can be maintained in a −20°C freezer for a limited time.

IF was first described by Albert H Coons and colleagues who reported that antigen in mammalian tissues could be detected optically under ultraviolet (UV) light with an antibody chemically linked to the fluorophore fluorescein isocyanate [6,7]. By the mid-1960s, multiple studies described the application of IF techniques to study bacterial and viral proteins [8–10]. Recent advances have led to the development of more photostable fluorophores and currently IF is widely used for multiple applications in clinical medicine as well as in cell biology and pathology.

IN PRINCIPLE

Animals are equipped with an immune system whose primary purpose is to protect the organism from attacks by pathogenic bacteria and viruses. As a part of the immune response B-cells produce protein complexes called antibodies that can detect a target antigen and elicit an immune response [11]. An antibody contains the conserved Fc (fragment that crystalizes) domain and a variable Fab (fragment having the antigen-binding site) domain that contains the antigen-binding site (antibody structure reviewed by Edelman et al. [12]). The Fc region can be recognized by effector cells, immune proteins as well as by other antibodies. The Fc domain is conserved within a species and therefore a fluorophore-conjugated antibody against the Fc fragment of one species can be used to detect all primary antibodies generated in that species. IF protocols are classified into two groups depending on whether a single antibody or two antibodies (primary and secondary) are used for fluorophore labeling of the antigen of interest. Both methods have advantages and disadvantages, which must be considered prior to initiating an IF protocol.

DIRECT IMMUNOFLUORESCENCE

An IF protocol involving a fluorophore-conjugated antibody to the target antigen of interest is referred to as direct IF. Advantages of this method include a simpler protocol for labeling of multiple antigen as well as shorter incubation times. Additionally, staining with multiple antibodies generated in the same species does not pose a problem. On the other hand, there is no signal amplification and as a result the staining intensity may be low if the antigen of interest is expressed in low abundance. Also the experiments may be limited, based on the availability and cost of the fluorophore-conjugated antibodies. This method of immune-labeling is commonly used for flow-cytometry applications.

INDIRECT IMMUNOFLUORESCENCE

An IF protocol involving detection of an antigen by a fluorophore-conjugated secondary antibody that recognizes an unlabeled primary antibody bound to the antigen of interest is classified as indirect IF. As a consequence of multiple secondary antibodies bound to the primary antibody, indirect IF protocols results in signal amplification, which is extremely useful for detecting low-abundance targets. Commercially produced secondary antibodies are quality-controlled and are available conjugated to a multitude of fluorophores. As secondary antibodies can recognize all primary antibodies derived from the host species, indirect IF protocols are more flexible and cost-effective. However, while performing labeling of multiple antigen, the primary antibodies need to be raised in different species to prevent cross reactivity. Also samples with high expression of endogenous immunoglobulin may produce a high background interfering with fluorescence imaging.

The principles of direct and indirect IF are summarized in Fig. 8.1.

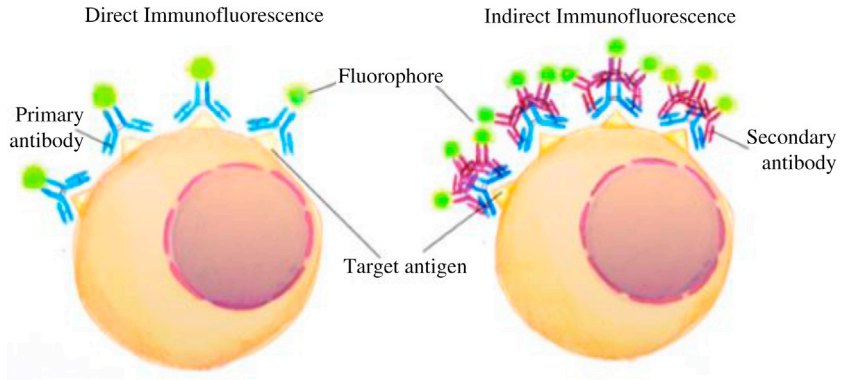

FIGURE 8.1 Direct and Indirect Immunofluorescence. Direct immunofluorescence utilizes a fluorophore-conjugated antibody to label the target antigen. An IF protocol involving detection of an antigen by a fluorophore-conjugated secondary antibody that recognizes an unlabeled primary antibody bound to the antigen of interest is classified as indirect IF.

IN PRACTICE

An IF protocol is a multistep procedure. Initially, the sample to be analyzed (cell lines, tissue samples, 3D cultures) are attached to a solid support such as a slide to facilitate visualization by a microscope. Next the sample is fixed to halt the biochemical cellular reactions to preserve cells and tissues and to enable the preparation of thin, stained sections. The samples may then be permeabilized to facilitate antibody binding to intracellular antigens. This is followed by incubating the sample with the primary antibody and multiple washes to remove the excess unbound antibody. If the primary antibody is bound to a fluorophore, the sample can then be mounted for imaging (direct IF). Alternatively, if the primary antibody is not bound to a fluorophore, the sample is incubated with a fluorophore-conjugated secondary antibody recognizing the primary antibody (indirect IF); and the excess unbound antibody is removed by multiple washes. The sample is then mounted for imaging.

It is important to note that there is no universal protocol for IF imaging. Testing and optimization is necessary to figure out the optimal conditions for every antibody, protein, and specimen combination. The sample preparation protocol may also need to be optimized for each experiment. The important steps and considerations for performing an IF experiment are discussed below.

Preparation of the Biological Sample for Immunofluorescence

IF can be performed on cell lines and primary cells grown in 2D culture, spheroids grown in 3D cultures as well as tissue samples. The main goal of this initial step is to facilitate the adherence of the specimen of interest to a solid optically suitable surface to facilitate imaging.

Primary Cells and Cell Lines Grown on a Petri Dish

Cells can be cultured on coverslip or on multiwelled chamber slides. If using coverslips, the thickness of the coverslip may have a significant impact on the image intensity and quality; it is important to note that #1.5 coverslip with a thickness of approximately 170 µm is generally compatible with most microscopes. The coverslips may be sterilized by exposure to UV light for 30 minutes prior to cell culture. For cells that do not easily attach to coverslips, the coverslip may be incubated in 50 µg/mL L-polylysine to facilitate cell attachment. Cells can also be directly cultured in multiwelled chambered slides. The advantage of using coverslips is the lower volume of antibody dilutions required for incubation with the primary or the secondary antibody, but, on the other hand, coverslips are fragile and therefore need to be handled with care during the multiple washes prior to imaging. The multiwelled chamber slides are sturdier and can accommodate multiple samples on a single slide but require a larger volume for incubation with the primary and secondary antibody. The cultured cells are then washed with 1X phosphate-buffered saline (PBS) to rinse off the cell culture media before proceeding to fixation. When using alcohol as a fixative, samples need to be air-dried prior to fixation.

Cells Grown in Suspension Culture

Cells cultured in suspension can be coated as a monolayer on a slide by the cytospin technique. About 150–200 µL of cells suspended in (1X) PBS are coated onto the microscope slide by spinning at low speeds to attach cells to the slide. The slides may be treated with L-polylysine to facilitate cell attachment. The slide is then air-dried before proceeding to fixation.

Cells Grown in a Semisolid Matrix

For IF imaging of cells grown in matrigel, the bottom of a multiwelled chamber slide is covered with a thin layer of matrigel to prevent cell attachment to the bottom of the plate. The cells are then cultured in matrigel-supplemented media to form polarized 3D structures. The liquid media is removed prior to fixation.

Tissue Samples from Humans or Animals

Prior to IF staining, tissue samples need to be cut into thin sections and attached to an imaging slide. The tissue is dissected immediately after euthanasia and is either flash-frozen or fixed prior to being embedded in paraffin. For frozen sections, the tissue is snap-frozen to avoid formation of water crystals. The freshly dissected tissue is placed on the prelabeled tissue mold and is covered with cyro-embedding media (i.e., OTC) and placed on dry ice. The samples can be stored either in liquid nitrogen or at −80°C. The blocks are then cut into approximately 6–8 µm sections and attached to a slide. For paraffin embedding, the freshly dissected tissue is fixed (typically in neutral buffered formalin overnight) and then embedded in paraffin. The paraffin blocks are then cut into 4–8 µm sections and attached to a glass slide. The section is deparaffinized by

multiple xylene washes followed by hydration through graded alcohol washes. As formalin fixation and paraffin embedding may mask antigen, the samples may be subjected to antigen unmasking by heat treatment (recommended) or pepsin or saponin treatment.

Fixation of the Sample

Fixation is an important step required for optimal IF imaging. Sample fixation is required to stop the degenerative processes resulting from loss of blood supply or nutrient media; and it helps to maintain cellular architecture as close to the native state as possible. A fixative may damage antigen sites and therefore the fixation process may need to be optimized for each antibody–antigen combination. Based on the sample to be analyzed there are different options for fixatives. The advantages and disadvantages of each are listed below:

Methanol

For methanol fixation, cells are covered with a thin layer of ice cold 100% methanol and incubated at −20°C for about 15 minutes. The methanol is then aspirated and the residual methanol is removed by three washes with (1X) PBS. This is the method of choice for staining cytoskeletal proteins. Additionally, methanol fixation disturbs hydrophobic bonding and is more effective for staining with monoclonal antibodies that recognize an epitope normally buried within internal protein structures. On the other hand, methanol fixation results in reduced protein solubility and therefore this method of fixation is not useful in staining lipid-associated proteins. Reduced protein solubility also results in cell flattening making it harder to stain proteins localized to the nucleus or the mitochondria.

Acetone

For acetone fixation, the sample is covered with a thin layer of ice-cold acetone and incubated at −20°C for 3–20 minutes. Acetone fixation is more effective at maintaining antigen integrity as compared to methanol and may be used as fixative if methanol fixation is ineffective. This is commonly used as a fixative for staining cytoskeletal proteins. Acetone fixation is very effective at cell permeabilization but has the same limitations as methanol fixation. Acetone fixation is generally the method of choice for frozen sections. Note: If neither methanol nor acetone is effective, the sample may be fixed with a 1:1 acetone/methanol solution by incubating the sample for about 10 minutes at −20°C. If using alcohols as fixatives, the permeabilization step may be skipped.

Paraformaldehyde

Paraformaldehyde (polymerized formaldehyde) fixation is commonly used for staining membrane-associated proteins. Paraformaldehyde fixation results in the chemical crosslinking of free amino groups, establishing a vast network of

interactions that better preserves cellular architecture. The sample is fixed in a freshly prepared 2–4% paraformaldehyde (in 1X PBS) solution for 15 minutes at room temperature. This is followed by three washes for 5 minutes each with (1X) PBS containing 100 nm glycine. Note: Paraformaldehyde is generally the fixative of choice while staining cells in 3D culture.

Formalin

Formalin is saturated 37% formaldehyde solution dissolved in water. It is important to note that a 10% formalin solution is equivalent to a 4% paraformaldehyde solution. The sample is fixed in 1% formalin in (1X) PBS for 10 minutes at room temperature followed by three washes for 5 minutes each with (1X) PBS.

Note: Fixation with paraformaldehyde or formalin may cause auto-fluorescence-mediated artifacts and therefore it is important to have a control sample that is not incubated with the primary antibody to determine nonspecific background signal while testing each antibody–antigen combination. For indirect IF, the control sample should be incubated with the secondary antibody only. Aldehyde-based fixatives do not effectively permeabilize the cell membrane and the samples need to be permeabilized prior to staining for intracellular biological molecules.

Cell Membrane Permeabilization

Permeabilization disturbs the cell membrane allowing the antibodies to bind to the intracellular antigen in the fixed cells. Fixatives such as acetone can fix as well as permeabilize cells while other fixatives are not very effective at permeabilization. IF staining for markers on the cell surface, permeabilization, will damage the cell membrane and is therefore not recommended. Detergents such as sodium dodecyl sulfate (SDS), Triton X-100, Tween-20, and saponin are commonly used for permeabilization. Different reagents, concentrations, incubation times may give different results and therefore the protocol must be optimized such that fixation and permeabilization results in minimal distortion of cellular morphology. Permeabilization with 1% triton X-100 for 1–5 minutes at room temperature followed by (1X) PBS washes is a commonly used methodology to permeabilize the sample. Note: The permeabilization step can be skipped if the protein to be stained is present on the cell membrane or if acetone is used for fixation. To achieve a specific signal it is recommended to use the mildest detergent that will allow antibody penetration. In order of increasing permeabilizing efficiency the commonly used detergents are saponin, Tween-20, triton X-100, and SDS.

Blocking to Limit Nonspecific Antibody Interactions

IF staining is enhanced by blocking nonspecific interaction of the primary and secondary antibodies with the biological sample. Nonspecific binding may result

from inappropriate binding of the antibody to nonantigen molecules by excess unreacted aldehyde, trapping of the antibody in hydrophobic structures or by low-affinity polyclonal antibody binding to nonspecific molecules. Incubating the sample in a protein solution prior to incubation with the primary antibody prevents these nonspecific interactions. The sample is incubated with blocking agents such as bovine serum albumin, milk, and serum. Note: Serum is generally the reagent of choice for IF staining. It is important to note that the blocking serum should be obtained from a species distinct from the species in which the primary antibody was raised. If performing indirect IF, the blocking serum should belong to the species in which the secondary antibody was developed. As a lot of the widely used secondary antibodies are raised in goat, goat serum is a common choice for the blocking step. For blocking, incubate the sample in a 5% serum solution (in (1X) PBS with 0.05% Tween-20 or 0.05% Triton X-100) for 30 minutes to an hour. Commercially available blocking buffers containing highly purified single proteins or proprietary protein-free compounds may also be used for blocking.

Incubation with Primary Antibody

After blocking, the sample is then ready for incubation with the primary antibody. A good primary antibody with a high affinity for the antigen and good specificity is essential to obtain high-quality trustworthy images and therefore it is worth testing multiple antibodies against the same antigen. The primary antibody is diluted in the blocking buffer and is then spread over the sample. The antibody is diluted 1:100–1:10000 (or outside this range) depending on antigen abundance, concentration of the antibody, and the affinity of the antibody to the antigen. The antibody dilution needs to be optimized for obtaining good-quality images. When staining for multiple proteins, the primary antibodies can be combined provided they are derived from different species and then added to the sample. The sample can be incubated with the primary antibody for an hour at room temperature or overnight at 4°C or a combination of both. The unbound primary antibody is removed by multiple washes with (1X) PBS containing either 0.05% Tween-20 or 0.05% Triton X-100. Care must be taken to see that the sample does not dry up between all the steps. If performing direct IF, proceed to mounting and imaging. Note: In order to avoid nonspecific binding, the primary antibodies need to be derived from a species distinct from the species being studied.

Incubation with Secondary Antibody

For this step, the fluorophore-conjugated secondary antibody is diluted (1:200–1:500) in the blocking buffer and added to the sample. The sample can be incubated with the secondary antibody for an hour at room temperature or overnight at 4°C. In our experience, Alexa Fluor secondary antibody conjugates are bright and stable and exhibit good specificity. Additionally,

secondary fluorophore-conjugated antibodies from Jackson ImmunoResearch and Rockland are also commonly used. Following incubation with the secondary antibody, the sample is washed three times with (1X) PBS containing either 0.05% Tween-20 or 0.05% Triton X-100 to remove the excess unbound secondary antibody. Care must be taken to ensure that the sample does not dry out between each step. Note: When staining with multiple primary antibodies, the corresponding secondary antibodies need to be conjugated to distinct fluorophores that do not overlap in their excitation and emission spectrum.

Mounting

After incubation with secondary antibody, the sample is dipped in distilled or deionized water to remove excess salts that may produce a residue after drying. After removing the excess water by capillary action with a Kim wipe, a small amount of mounting media just sufficient to cover the sample is added prior to covering the sample with a coverslip. The sample is then allowed to dry on a flat surface and the edges are sealed with nail polish. The mounting medium preserves the sample and increases the refractive index for obtaining high-quality images with an oil immersion lens. Additionally, commercially available mounting media such as Prolong Gold from Molecular Probes and Fluoromount-G(anti-fade) from Southern Biotech minimize photobleaching due to the presence of free radical scavengers. Mounting media also contains DAPI (4′,6-diamidino-2-phenylindole), a fluorophore that can interact with DNA emitting light in the blue spectrum and is commonly used to visualize nuclei. After mounting, the sample is ready for imaging. Note: It is important to note that after mounting the samples should be stored at −20° until imaging to preserve the fluorescence signal.

Imaging

After IF staining the antigen of interest can be visualized by a fluorescence microscope or confocal microscope depending on the research question being addressed. A regular fluorescence microscope is generally used to examine the expression but not the co-localization of different antigens. A study that aims to examine whether two or more proteins co-localize, a confocal microscope is the instrument of choice. Confocal microscopes utilize imaging techniques that capture images with high optical resolution in the confocal plane by eliminating out-of-focus light [2]. Images captured at multiple confocal planes can be integrated to produce a three-dimensional image.

Controls and Important Considerations

The quality of IF staining may be hindered by nonspecific binding of the primary or the secondary antibody resulting in a nonspecific signal. Use of appropriate

controls is essential to validate specific binding of the primary or the secondary antibody. To test the specificity of the primary antibody, IF staining should be performed either by blocking the primary antibody with the peptide/protein against which it was raised or by verifying a lack of signal in a sample with a targeted deletion or RNA interference-mediated silencing of the target of interest. To resolve issues with nonspecific staining with the secondary antibody, lack of signal needs to be confirmed in a secondary-only sample that is incubated only with the secondary antibody and is not incubated with the primary antibody. Problems with antibody specificity may be resolved by optimizing the fixation and blocking process or using different specific high-affinity antibodies against the target of interest.

Selections of fluorophore used for IF imaging is critical to obtaining good-quality IF images. An ideal fluorophore exhibits bright fluorescence, has high photostability, and its fluorescence is not altered by external factors such as antibody conjugation or changes in pH. Additionally, the fluorophore must have an excitation peak at an excitation wavelength available on the fluorescence microscope and a narrow emission spectrum that can be specifically detected by the fluorescence microscope.

APPLICATIONS

IF techniques are commonly used for both clinical and experimental pathology. For research purposes IF is used to determine organ-specific, tissue-specific, or cell-specific gene expression of both intracellular and cell membrane-associated antigen. Besides eukaryotic cells, IF techniques can also be used to identify the presence of bacteria, viruses, or other parasites in a given sample [8–10]. IF techniques are commonly used for the diagnosis of dermatological abnormalities such as bullous and connective tissue disorders, vasculitides, and conditions such as lichen planus, and also the scaling dermatoses, notably psoriasis [13]. Diagnosis of respiratory diseases resulting from viral infection can be diagnosed with direct IF-based assays [14]. Thus IF is a routinely used technique for both clinical and research purposes.

SCENARIO

A protein X is hypothesized to translocate from the nucleus to the cytoplasm in response to mitogenic stimulation. To test this hypothesis, cells are either treated with a vehicle control or with a mitogen. The cells are then fixed, permeabilized, blocked, and incubated with a primary antibody against protein X followed by incubation with a fluorophore-conjugated secondary antibody. The slide is then mounted with mounting media containing DAPI. Confocal imaging is then performed to determine whether DAPI and the fluorescent signal from protein X are colocalized. The hypothesis is supported if DAPI and the protein X are colocalized in the sample treated with the mitogenic stimulus.

KEY LIMITATIONS

As with all biochemical techniques, IF results are hindered by multiple limitations. The major issues are listed below:

Photobleaching

IF imaging requires excitation of the fluorophore at a specific wavelength resulting in light emission that can be detected by the fluorescence microscope. This process also results in the generation of reactive oxygen species (ROS) that can chemically interact with the fluorophore preventing optimal excitation of the fluorophore over time. Photobleaching may be prevented by optimizing the exposure time to the excitation wavelength or using ROS scavengers or using fluorophores specifically developed to minimize photobleaching.

Autofluorescence

The ability to obtain high-quality IF images is highly dependent on obtaining high-affinity antibodies highly specific to the target of interest. Biological samples may exhibit autofluorescence due to the presence of reduced pyridine nucleotides (NADH (nicotinamide adenine dinucleotide (reduced)): absorption 340 nm, emission 460 nm) and flavin coenzymes (FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide): absorption 450 nm, emission: 460 nm) that play an important role in regulating cellular metabolism. Therefore, detection of fluorophores that emit light in the green spectrum may result in a low signal-to-noise ratio. Often issues with autofluorescence can be eliminated by either using a different more specific high-affinity antibody or by optimizing the fixation process. Fixation methods using aldehydes such as glutaraldehyde may result in high autofluorescence that can be attenuated by washing with 0.1% sodium borohydride (a versatile reducing agent) in PBS prior to antibody incubation. Selection of appropriate probes and optical filters can minimize problems with autofluorescence.

Alternatively, for proteins that cannot be easily detected by IF, a fluorescent protein conjugated version of the protein such as GFP (green fluorescent protein) may be generated to address research questions.

Fluorophore Overlap

Fluorophore overlap is a common problem encountered while performing imaging for multiple targets in the same sample. This becomes a problem when the two or more fluorophores used emit light at similar wavelengths. To obtain IF images, fluorophore excitation by a light source results in light emission from the fluorophore at a narrow range of wavelengths. For example, Alexa fluor 430 has an excitation and emission of 434 nm and 539 nm, respectively, while another fluorophore Alexa fluor 514 has an excitation and emission of 518 nm

and 540 nm, respectively. If the two fluorophores are used to stain different antigen, the fluorescence microscope will not be able to differentiate between the light emitted from the two fluorophores due to a significant overlap between their emission spectrum. Alternatively, if Alexa fluor 434 is used in combination with another fluorophore such as Alexa fluor 594 (excitation peak: 590 and emission peak: 617 nm), the emission spectra of the two fluorophores will not overlap and therefore the proteins stained with the two dyes can be optimally distinguished.

TROUBLESHOOTING

Problem	Probable Cause	Solution
No staining	Problems with secondary antibody binding to primary	Verify that the secondary antibody was raised against the species in which the primary antibody was raised
	Not enough primary antibody is bound to the antigen	Increase the concentration of the primary antibody and/or increase incubation time
	Protein is absent or expressed at low levels	Use a positive control
	Improper storage of the primary antibody	Use positive controls
	Improper storage of secondary antibody	Use a new batch of secondary antibody and limit light exposure
	Problems with sample fixation	Fixation protocol may modify antibody epitope, use alternative fixatives
	Bacterial contamination of PBS	Use freshly prepared PBS
	Problems with membrane permealization	Use a more stringent detergent for permealization
High background	Ineffective blocking	Increase blocking time or use a different blocking agent
	Primary antibody concentration is too high	Use less primary antibody for incubation
	Nonspecific secondary antibody binding	Run a control incubated only with the secondary antibody, if secondary-only control is positive use a different secondary antibody
	Improper washing, fixative not properly removed	Increase PBS washes between each step

Problem	Probable Cause	Solution
	Fixative causes auto fluorescence	Formalin and paraformaldehyde may cause auto fluorescence in the green spectrum, use a different fluorophore
	Secondary antibody concentration is too high	Dilute the secondary antibody
	Excessive membrane permealization	Use a less stringent detergent for permeabilization
Nonspecific staining	The primary antibody was raised in the same species from which the biological sample of interest is derived	Use a different primary antibody that is raised in a species distinct from which the sample being studies was obtained
	Concentration of the primary and/or secondary antibody is too high	Decrease the concentration of the primary and or secondary antibody, may also decrease the incubation time
	The sample has dried out	Keep samples covered in liquid and prevent drying

CONCLUSIONS

In summary IF techniques involve the optical detection of the antigen(s) of interest with fluorophore-conjugated antibodies. This technique is widely used for the determination of protein expression and cellular localization of antigens. In the current age of high-throughput research, technological advances have led to the development of performing IF in a multiplex manner. The newly developed tyramide signal amplification system provides improved resolution and stability for multiplex studies [15,16]. Additionally, it is important to note that in addition to antibodies, nucleotide probes can also be conjugated to fluorescent probes to visualize DNA or RNA molecules and this technique is referred to as fluorescent in situ hybridization [17]. Thus, IF techniques will be used for research and clinical purposes for a long time in the future.

REFERENCES

[1] Liu JJ, Liu C, He W. Fluorophores and Their Applications as Molecular Probes in Living Cells. Curr Org Chem 2013;17(6):564–79.

[2] Kogata N, Howard BA. A whole-mount immunofluorescence protocol for three-dimensional imaging of the embryonic mammary primordium. J Mammary Gland Biol Neoplasia 2013;18(2):227–31.

[3] Johnson MD, Mueller SC. Three dimensional multiphoton imaging of fresh and whole mount developing mouse mammary glands. BMC cancer 2013;13:373.

[4] Saatchi S, Azuma J, Wanchoo N, Smith SJ, Yock PG, Taylor CA, et al. Three-Dimensional Microstructural Changes in Murine Abdominal Aortic Aneurysms Quantified Using Immunofluorescent Array Tomography. J Histochem Cytochem 2012;60(2):97–109.
[5] Coons AH, Creech HJ, Jones RN, Berliner E. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J Immunol 1942;45(3):159–70.
[6] Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. P Soc Exp Biol Med 1941;47(2):200–2.
[7] Cherrywbmoody MD. Fluorescent-Antibody Techniques in Diagnostic Bacteriology. Bacteriol Rev 1965;29:222–50.
[8] Hers JF. Fluorescent Antibody Technique in Respiratory Viral Diseases. Am Rev Respir Dis 1963;88(SUPPL):316–38.
[9] Kraft SC, Kirsher JB. Immunofluorescent Studies of Chronic Nonspecific Ulcerative Colitis. Gastroenterology 1964;46:329–32.
[10] Edelman GM, Gall WE. The antibody problem. Annu Rev Biochem 1969;38:415–66.
[11] Chhabra S, Minz RW, Saikia B. Immunofluorescence in dermatology. Indian J Dermatol Venereol Leprol 2012;78(6):677–91.
[12] Sadeghi CD, Aebi C, Gorgievski-Hrisoho M, Muhlemann K, Barbani MT. Twelve years' detection of respiratory viruses by immunofluorescence in hospitalised children: impact of the introduction of a new respiratory picornavirus assay. BMC Infect Dis 2011;11:41.
[13] Bobrow MN, Harris TD, Shaughnessy KJ, Litt GJ. Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. J Immunol Methods 1989;125(1–2):279–85.
[14] Mitchell RT, E Camacho-Moll M, Macdonald J, et al. Intratubular germ cell neoplasia of the human testis: heterogeneous protein expression and relation to invasive potential. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 2014;27(9):1255–66.
[15] Hu L, Ru K, Zhang L, Huang Y, Zhu X, Liu H, et al. Fluorescence in situ hybridization (FISH): an increasingly demanded tool for biomarker research and personalized medicine. Biomarker research 2014;2(1):3.
[16] Toda Y, et al. Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. Pathol Int 1999;49(5):479–83.
[17] Volpi EV, Bridger JM. FISH glossary: an overview of the fluorescence in situ hybridization technique. BioTechniques 2008;45(4):385–6. 388, 390.

SUGGESTED FURTHER READING

[1] Brelje TC, Wessendorf MW, Sorenson RL. Multicolor laser scanning confocal immunofluorescence microscopy: practical application and limitations. Methods Cell Biol 2002;70:165–244.
[2] Mysorekar VV, Sumathy TK, Shyam Prasad AL. Role of direct immunofluorescence in dermatological disorders. Indian Dermatol Online J 2015;6(3):172–80.
[3] Shakes DC, Miller III DM, Nonet ML. Immunofluorescence microscopy. Methods Cell Biol 2012;107:35–66.
[4] Willingham MC, Pastan IH. An atlas of immunofluorescence in cultured cells. Orlando: Academic Press; 1985.
[5] Caul EO, Great Britain. Public Health Laboratory Service Immunofluorescence: antigen detection techniques in diagnostic microbiology. London: Public Health Laboratory Service; 1992.

[6] Al-Mulla F. Formalin-fixed paraffin-embedded tissues: methods and protocols. New York: Humana Press; 2011.
[7] Lin F, Prichard J. Handbook of practical immunohistochemistry: frequently asked questions. New York: Springer; 2011.
[8] Beutner EH, State University of New York at Buffalo. Department of Microbiology Defined immunofluorescence and related cytochemical methods. New York, N.Y.: New York Academy of Sciences; 1983.
[9] Scheffler JM, Schiefermeier N, Huber LA. Mild fixation and permeabilization protocol for preserving structures of endosomes, focal adhesions, and actin filaments during immunofluorescence analysis. Methods Enzymol 2014;535:93–102.
[10] Stadler C, Skogs M, Brismar H, Uhlen M, Lundberg E. A single fixation protocol for proteome-wide immunofluorescence localization studies. J Proteomics 2010;73(6):1067–78.
[11] Stadler C, Rexhepaj E, Singan VR, et al. Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. Nat Methods 2013;10(4):315–23.
[12] Wick G, Traill KN, Schauenstein K. Immunofluorescence technology : selected theoretical and clinical aspects. Amsterdam; New York New York, N.Y.: Elsevier Biomedical Press ;Sole distributors for the USA and Canada, Elsevier Science Pub. Co.; 1982.
[13] Barbierato M, Argentini C, Skaper SD. Indirect immunofluorescence staining of cultured neural cells. Methods Mol Biol 2012;846:235–46.
[14] Chhabra S, Minz RW, Saikia B. Immunofluorescence in dermatology. Indian J Dermatol Venereol Leprol 2012;78(6):677–91.
[15] Knapp W, Holubar K, Wick G. Immunofluorescence and related staining techniques: proceedings of the VIth International Conference on Immunofluorescence and Related Staining Techniques held in Vienna, Austria on April 6–8, 1978. Amsterdam; New York New York: Elsevier/North-Holland Biomedical Press; Elsevier North-Holland; 1978.
[16] Kawamura A, Aoyama Y. Immunofluorescence in Medical Science. University of Tokyo Press; 1983.
[17] Pawley J. Handbook of Biological Confocal Microscopy. US: Springer; 2010.
[18] Muller M. Introduction to Confocal Fluorescence Microscopy. Society of Photo Optical; 2006.
[19] Klein RA, Storch WB. Immunofluorescence in Clinical Immunology: A Primer and Atlas. Birkhäuser Basel; 2012.
[20] Allan V. Protein Localization by Fluorescence Microscopy: A Practical Approach:. Oxford: OUP; 1999.
[21] WR Sanborn, Immunofluorescence, an Annotated Bibliography 4. Technical Procedures, Defense Technical Information Center, 1968.
[22] International IG Immunofluorescence: Webster's Timeline History. ICON Group International; 1960–2007.
[23] Russell J, Cohn R. Immunofluorescence: Book on Demand; 2012.
[24] Burry RW. Immunocytochemistry: A Practical Guide for Biomedical Research. Springer; 2009.
[25] Herman B. Fluorescence Microscopy. Bios Scientific Publishers; 1998.
[26] Gardner PS, McQuillin J, Grandien M. Rapid virus diagnosis: application of immunofluorescence, 2d ed. London; Boston: Butterworths; 1980.
[27] Lyerla HC, Forrester FT, Branch CfDCVT Immunofluorescence methods in virology. Dept. of Health, Education, and Welfare, Public Health Service, Center for Disease Control, Bureau of Laboratories, Laboratory Training and Consultation Division, Virology Training Branch; 1979.

GLOSSARY

Immunofluorescence (IF) IF is an immunohistochemistry technique used to visualize proteins with fluorophore conjugated antibodies.

Fluorophore A fluorophore is a chemical compound when excited with light at a specific wavelength, emits light as a higher wavelength.

Photobleaching Photobleaching is the chemical modification of a fluorophore that permanently inhibits light emission upon fluorophore excitation.

Phosphate-buffered saline (PBS) A water-based buffered solution containing sodium chloride, sodium phosphate, potassium chloride, and potassium phosphate (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4).

Spheroids A sphere-shaped collection of cells grown in a semi-solid 3D matrix.

LIST OF ACRONYMS AND ABBREVIATIONS

DAPI 4′,6-diamidino-2-phenylindole

IF Immunofluorescence

PBS Phosphate-buffered saline