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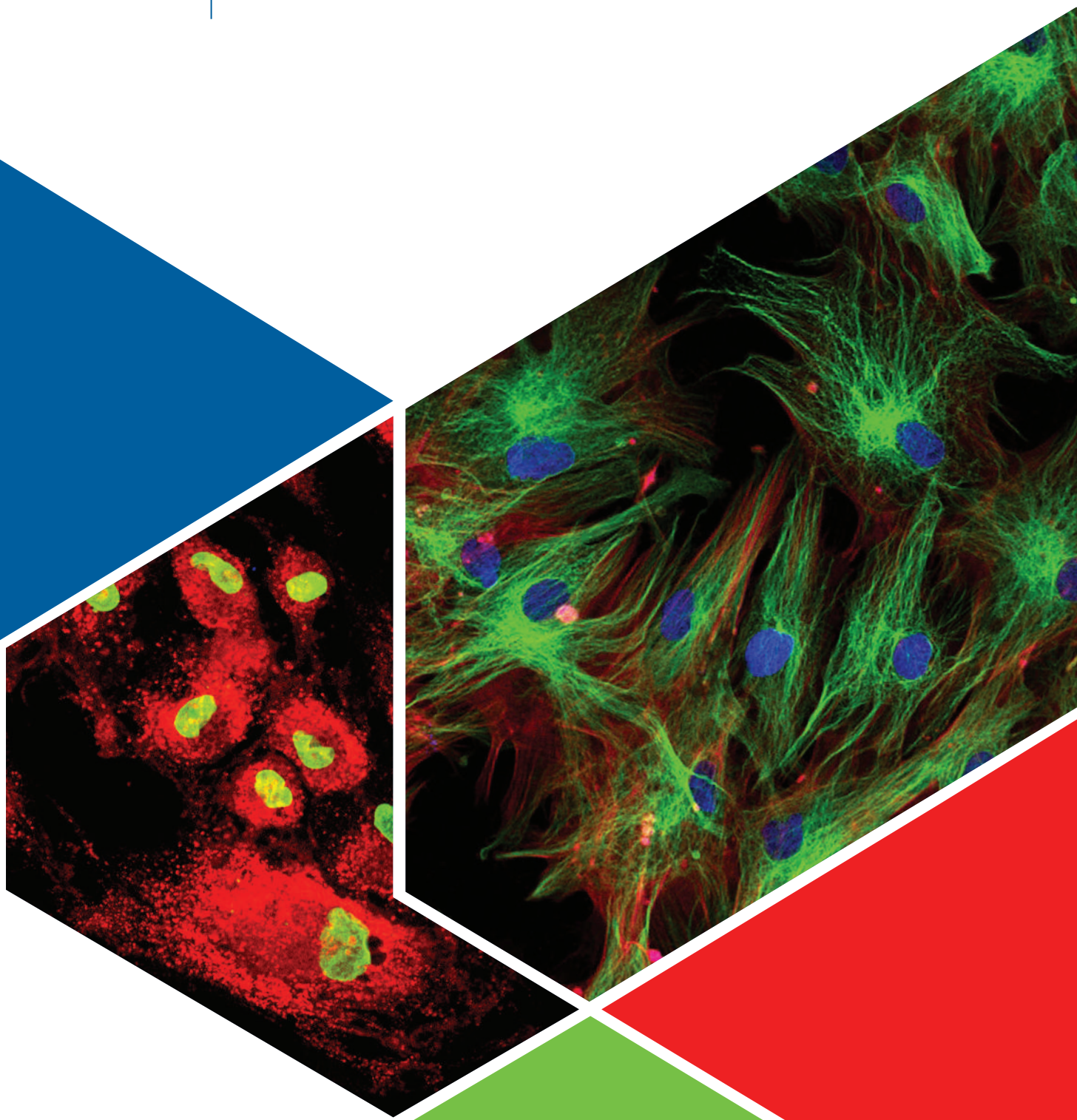
Immunocytochemistry (ICC) Handbook



Conjugate	Excitation/ Emission	Emission Color	Laser (Excitation Source)	Notes
DyLight™ 405	400/420	Violet	Violet (405 nm)	Bright and photostable
Alexa Fluor® 405	401/421	Violet	Violet (405 nm)	Best when used with more abundant targets
DyLight™ 350	353/432	Violet-Blue	Ultraviolet (355 nm)	Can be used with DyLight™ 488, 594 and 647 in multiplexing
Alexa Fluor® 350	346/442	Violet-Blue	Ultraviolet (355 nm)	Often used with Alexa Fluor® 488, 594 and 647 in multiplexing, best for high-abundance targets
DyLight™ 488	493/518	Green	Blue (488)	Brighter, photostable replacement for FITC; not suitable for use with GFP
Alexa Fluor® 488	495/519	Green	Blue (488)	Photostable over a broad pH range; replaces FITC
FITC	495/519	Green	Blue (488)	Small organic fluorophore; cannot be used with DyLight™ 488, Alexa Fluor® 488 or GFP
DyLight™ 405LS	397/572	Yellow	Violet (405 nm)	Superior alternative to Pacific Orange; good choice for multicolor applications on the violet laser
Alexa Fluor® 546	556/573	Yellow	Yellow-Green (561 nm)	Photostable over a broad pH range; brighter than Cy3
DyLight™ 550	562/576	Yellow	Yellow-Green (561 nm)	
PE	565/578	Yellow	Yellow-Green (561 nm)	Subject to photobleaching; can be excited by the 488, 532, and 561nm lasers on flow cytometers
Texas Red®	595/613	Orange	Yellow-Green (561 nm)	Very bright fluorescence; use a tunable dye laser to avoid leaking when multiplexed with PE
Alexa Fluor® 594	590/617	Orange	Yellow-Green (561 nm)	Better photostability than Texas Red
DyLight™ 650	654/673	Red	Red (633 nm)	
APC	650/660	Red	Red (633 nm)	Bright fluorescent protein; do not use with DyLight™ 650 due to overlapping emission spectra
Alexa Fluor® 647	650/665	Red	Red (633 nm)	Extremely photostable, good replacement for Cy5 or APC
Cy5™	647/665	Red	Red (633 nm)	Some fluorescence quenching when conjugated

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Introduction

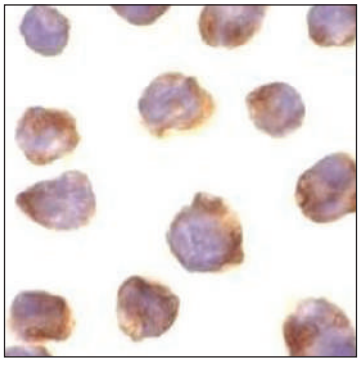
Immunocytochemistry (ICC) refers to immunostaining of cultured cell lines or primary cells including smears, swabs, and aspirates. ICC offers a semi-quantitative means of analyzing the relative abundance, conformation, and subcellular localization of target antigens. Traditional ICC techniques use chromogenic detection in which enzyme conjugated antibodies convert chromogen substrates to a colored precipitate at the reaction site. However chromogenic detection has lost favor with the advent of immunofluorescent labels. In immunocytochemistry/immunofluorescence (ICC/IF) assays, the cellular antigens are visualized using either fluorochrome-conjugated primary antibodies (direct detection) or a two-step method (indirect detection) involving an unlabeled primary antibody followed by a fluorochrome-conjugated secondary antibody. By combining different fluorochrome-labeled antibodies, multiplex ICC/IF can detect several antigens in the same sample.

While ICC is often used interchangeably with ICC/IF (immunocytochemistry/ immunofluorescence) and another related term, IF, significant differences exist between them in reference to the method of detection and starting sample types involved. Some of the key differences between ICC, ICC/IF and IF are illustrated in the table and images below:

Assay Terminology	Sample	Detection
ICC (traditional)	Cells	Enzyme-labeled antibodies and corresponding chromogen substrates (chromogenic detection)
ICC/IF	Cells	Fluorochrome-labeled antibodies (immunofluorescent detection)
IF	Tissues or Cells	Immunofluorescent detection, can be applicable to ICC and/or IHC

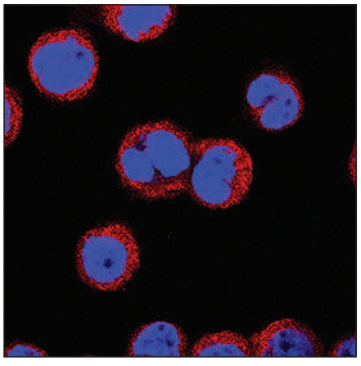
Figure 1. Examples of staining using traditional ICC, ICC/IF, and IHC with IF based detection

Traditional ICC



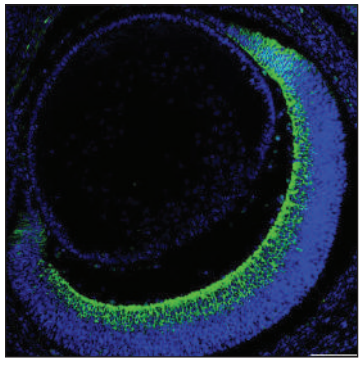
IRAK4 was detected in K562 cells using 10 µg/ml of IRAK4 antibody [NBP1-77231] and HRP conjugated secondary antibody. Cells were stained with DAB (brown) and nuclei were counterstained with hematoxylin (blue).

ICC/IF



IRAK4 was detected in THP 1 cells using 15 µg/ml IRAK4 antibody [AF3919]. The signal was developed using NorthernLights™ 557-conjugated secondary antibody (red) and nuclei were counterstained with DAPI (blue).

Example of IF in IHC

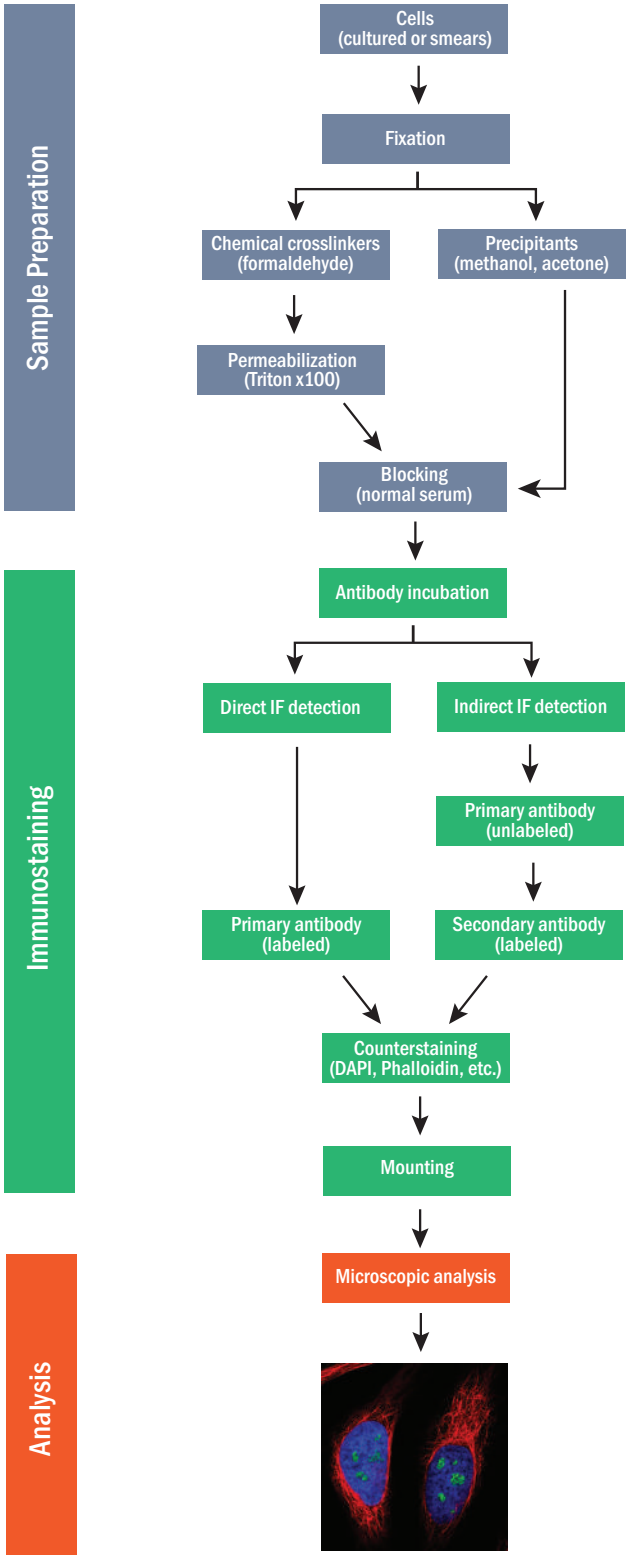


GAP43 was detected in a formalin fixed paraffin embedded section of E15.5 mouse embryo using 1ug/ml of GAP-43 antibody [NB300-143] followed by Alexa Fluor® 488 labeled secondary. DAPI was used as a nuclear counterstain.

Basic ICC/IF Protocol

A standard ICC/IF protocol involves fixation, permeabilization, blocking, immunolabeling, counterstaining, and microscopic imaging of stained cells (see the flow chart in Figure 2). Each step of the ICC/IF protocol requires optimization as experimental variables in each step can significantly impact staining outcome.

Figure 2. General protocol steps in an ICC/IF assay



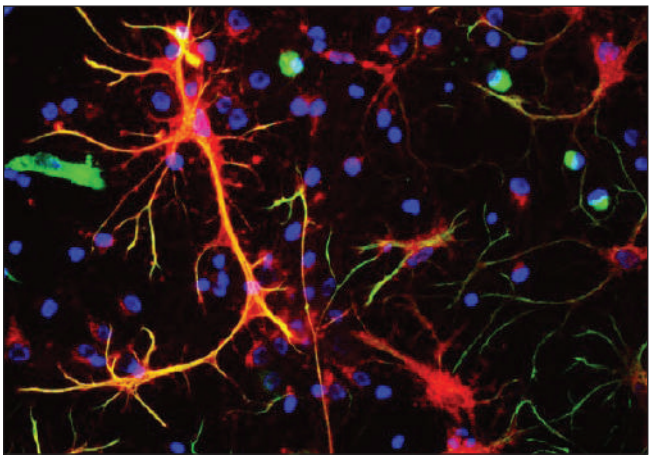
Multicolor ICC/IF

Why use IF based detection?

The benefit of fluorescence detection is the ability to simultaneously detect multiple structural and functional molecules (multiplex analysis) with high contrast and visibility. Due to the narrow spectral properties of most fluorochromes, the expression of multiple proteins can be detected at the same time and better distinguished relative to chromogen based detection methods, which demonstrate broad spectral properties. Often fluorescent imaging is used to compare the subcellular localization or distribution of two or more fluorescently labeled molecules. These comparisons can then be used to understand protein function, including intracellular transport, response to biological stimuli or disease, and target protein co-localization with a marker or organelle. Unlike fluorescence detection, the broad emission spectra of chromogens make it difficult to distinguish individual colors that coincide on the same structure/organelle (see Figure 3). Thus, the relative simplicity of multiplex analysis and ability to clearly detect co-localized antigens make fluorescence detection more desirable than chromogen based detection methods in ICC.

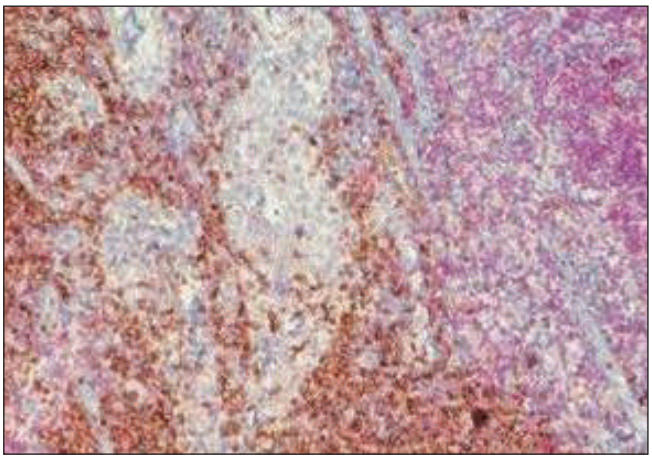
Figure 3. Comparison of staining with IF and chromogenic detection

IF Detection



Neuron-glia cell mixed cultures were stained with ALDH1L1 antibody (2E7) [NBP2-50033] (red) and Vimentin antibody [NB300-223] (green) with DAPI as a counterstain for the nuclei. ALDH1L1 protein is present in the astrocytic cell bodies and processes. The fibroblastic cells contain only Vimentin and so are green, while astrocytes contain either Vimentin and ALDH1L1, so appearing golden, or predominantly ALDH1L1, in which case they appear red.

Chromogenic Detection



Immunostaining of a human lymph node tissue section with Lightning-Link HRP [701-0000] labeled CD20 antibody (brown) and an alkaline phosphatase labeled CD3 antibody. CD20 positive cells are visualized with DAB (brown) and CD3 with ALP's substrate Fast Red (red). The nuclei of the cells were counter-stained with hematoxylin.

Multicolor ICC/IF - limitations?

ICC/IF offers significant advantages over the traditional ICC but it also has certain limitations. Although fluorescence detection makes the design of multiplex ICC experiments easier, fluorochrome combinations must be carefully considered to limit emission spectra overlap. Poorly optimized fluorochrome combinations may obscure detection of individual proteins, especially the detection of co-localized proteins. Autofluorescence, the natural emission of light by biological structures, contributes to background staining and can produce false-positive results. Non-specific staining can also be the result of cross-reactivity or nonspecific binding of primary and/or secondary antibodies.

Considerations Before You Start

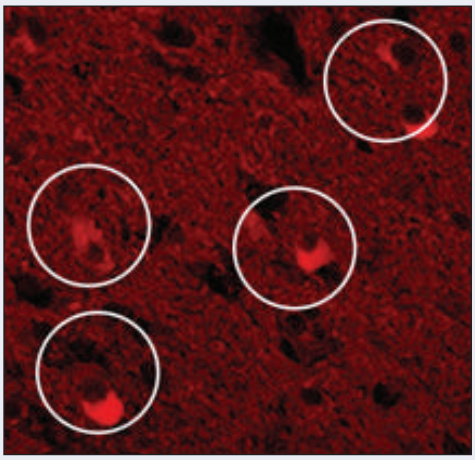
Autofluorescence

Autofluorescence can contribute to background signal and result in false-positive staining. It is important to observe cells under a microscope before immunostaining to determine if autofluorescence is present in your samples. Based on its origin, autofluorescence is classified into two major types: (i) biological/inherent, and (ii) fixative-induced autofluorescence as discussed below:

Biological/inherent autofluorescence (the color within)

Cells or tissues often contain biological components which emit fluorescence signal when excited by a suitable wavelength. Biological autofluorescence originates from mitochondria, lysosomes, and aromatic amino acid containing components including flavin coenzymes (FAD, FMN) and pyridine nucleotides (NADH). Refer to Figure 4A for an example of autofluorescence emitted by Lipofuscin, a pigment that accumulates with age in many tissue types. Lipofuscin has autofluorescence properties that overlap with the emission spectra of commonly used fluorochromes.

Figure 4A.
Biological autofluorescence

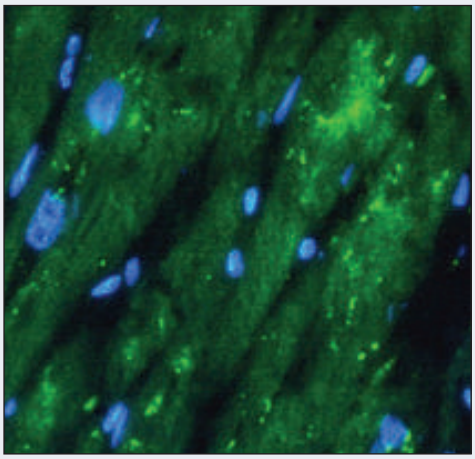


Circled in the micrograph above are Lipofuscin-containing neurons that may appear labeled using fluorescence microscopy in the red spectrum.

Fixation induced autofluorescence (the artifactual color)

In samples fixed with aldehydes, such as formaldehyde, the aldehyde group can react with amine groups of proteins, resulting in end products which exhibit fluorescent properties (see Figure 4B). Aldehyde fixative- induced autofluorescence can be blocked by reducing the aldehyde group to a hydroxyl group, or by treating fixed cells with sodium borohydride. It is pertinent to note here that quencher treatment time should be optimized in order to quench autofluorescence while minimizing the impact on specific immunostaining.

Figure 4B.
Fixative-induced autofluorescence



Formalin-fixed sample showing strong autofluorescence (green) in human cardiomyocytes. The blue color represents nuclear staining with DAPI.

Fluorochrome Selection

Rules for selection of fluorochromes

Designing a successful ICC/IF experiment requires the selection of suitable fluorochromes. Beginners often find it challenging to choose optimal fluorochrome combinations. Some tips are provided below to simplify the selection process and to minimize potential errors.

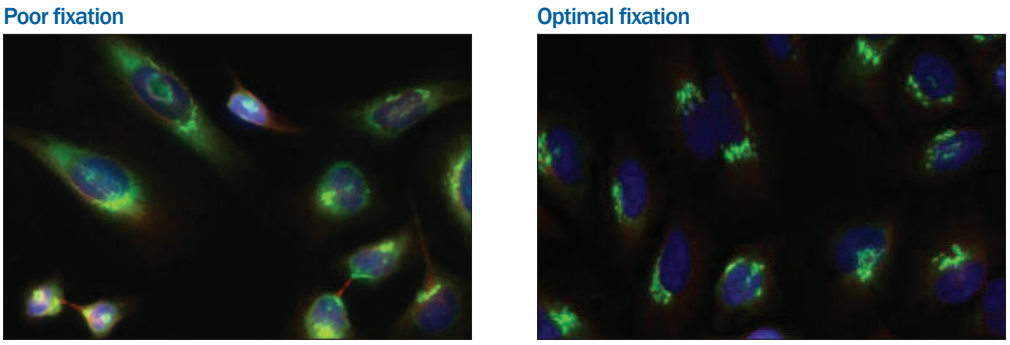
Fluorochrome and microscope compatibility	Become familiar with the lasers and filter sets of your microscope to ensure you are selecting fluorochromes that are optimally excited and detected by them. Spectra viewers and fluorochrome reference charts are useful tools to determine the maximum excitation and emission wavelengths of the fluorochromes under consideration. Some filters such as FITC/TRITC indicate the fluorochrome to use in the filter name. In addition, a rough guide for laser/filter selection is provided by the number listed after some dyes, such as 488 in Alexa Fluor® 488 or DyLight™ 488.
Extinction coefficients (ε) and brightness of fluorochrome	The brightness of fluorochromes is directly proportional to the extinction coefficient, a measurement of the fluorophore's probability of absorbing a photon of light. For example, a dim fluorochrome, such as DyLight™ 350, has ε value = 15,000, whereas a brighter member of this family, DyLight™ 650, has an ε value = 250,000. In multi-color ICC/IF, it is advisable to associate antigen abundance with the brightness of a fluorochrome. In general, we recommend using the brightest fluorochrome to detect the least abundant antigen and to select the dimmest fluorochrome to detect the most abundant antigen.
Quantum yields (Φ) of fluorochrome	Fluorescence quantum yield is a measurement of emission efficiency and is calculated by the formula: ΦF = number of emitted photons/number of absorbed photons. The maximum quantum yield possible (ΦF = 1) signifies a 100% efficient fluorescence process. An example of a fluorochrome with high quantum yield is Alexa Fluor® 488 which has ΦF= 0.92.
Photobleaching & pH compatibility	Photobleaching refers to a catabolic photochemical reaction that results in a reduction in fluorescence intensity. Conventional fluorochromes such as FITC and PE are sensitive to photobleaching whereas fluorochromes including Alexa Fluor®, DyLight™ or HiLyte™ dye families are more photostable. If using FITC or PE in an experiment, lower the intensity or exposure time of the excitation light or use an anti-fade reagent containing mounting medium to reduce potential issues. The fluorescence intensity of some conventional fluorochromes, including FITC is highly sensitive to pH and alcohols, which limits their usefulness in protocols using acidic buffers and alcohol-containing reagents. In contrast, improved dyes such as Alexa Fluor®, Dylight™ etc. are more tolerant to acidic buffers and alcohols.
Spectral overlap, the bleed-through effect	Bleed-through or spill-over effect is the detection of fluorochromes in adjacent filter sets. This can make it difficult to distinguish interfering fluorescence from the fluorochrome of interest, which is particularly problematic for co-localization studies. For example, the most commonly used nuclear counterstain DAPI cannot be used in combination with blue emitting fluorochromes such as Alexa Fluor® 405, DyLight™ 405 or HiLyte 405. Therefore, it is critical to select fluorochromes with minimal spectral overlap for multi-color ICC/IF.

Sample Preparation

How important is it?

Sample preparation is a critical step for achieving accurate expression and localization data in multicolor ICC/IF. Before starting an experiment, review the scientific literature for information about the expression and localization of your protein of interest. If the target protein is not expressed under basal conditions, you may need to apply a stimulus (e.g. protein or chemical) to your cells to induce protein expression. Another critical factor to consider during the cell culture phase of your ICC/IF experiment is cell confluency. A high cell density can negatively impact cell architecture, while low cell density may require imaging at a lower magnification or capturing multiple images to ensure a sufficient number of cells are imaged. Optimal cell density is also critical to maintain cell-to-cell contacts, and is of particular importance when studying proteins, such as cell junction markers, including claudin-1 or ZO-1. Typically, a cell density of 60-80% is optimal for ICC/IF assays. Getting the right working conditions help achieve quality data while failing at the sample preparation or the following steps, especially the fixation and permeabilization, can result in false or misleading staining. See figure 5 for an example of poorly and optimally prepared samples.

Figure 5. ICC/IF staining of GM130/Golga2 in underfixed and adequately fixed HeLa cells



ICC/IF staining of GM130/Golga2, a golgi marker, using 5µg/ml of GM130 antibody [NBP2-53420] showing different staining in two sets of HeLa cells. The signal was developed using DyLight™ 488 conjugated secondary antibody and the counterstaining was performed using phalloidin for cytoskeleton (red) and DAPI for the nuclei (blue). First set shows the cells which were not fixed properly and the antigen seems to have dispersed into the nucleus and the cytoplasm. The second image shows a properly fixed sample with Golga2 protein localized into the Golgi apparatus.

Coverslip preparation and coating

Cell culture is performed under aseptic conditions and coverslips must be sterilized using an autoclave or UV enabled laminar flow culture hood. Following sterilization, coverslips should be washed with ethanol in the culture hood and once dried, placed in culture wells using tweezers. Apply a coating matrix to glass coverslips if cells do not properly adhere to the surface. The type of coating matrix is dependent on the cell type, but poly-L-lysine works well for most cell lines. Other matrix proteins and their compatibility with various cell types are listed below:

Cell Types	Suggested Coating Material
Embryonic stem cells	Laminin
Primary keratinocytes/hepatocytes	Collagen
Microvascular endothelial cells	Gelatin
Neural stem cells	Fibronectin

Fixation

Why fix the cells?

Fixatives prevent proteolytic enzyme induced autolysis of cells and the process of putrefaction or cellular decay. It is critical to fix cells immediately following removal from cell culture to limit these effects. Fixatives also enhance the rigidity and mechanical strength of cells. This preserves cellular morphology and structure, which is critical to withstanding the various, sometimes rigorous, steps of the immunostaining procedure.

Two major types of fixative

Cross-linking fixatives

Aldehydes, such as formalin or formaldehyde, are the preferred fixative for preserving cell morphology and are well suited for immunostaining of membrane proteins. These fixatives crosslink proteins via free amine groups, forming intermolecular bridges and a network of linked antigenic proteins. Crosslinking masks antigens and reduces target antigenicity which may be perpetuated by a long fixation time. To fix with an aldehyde, cells are typically incubated with 4% formaldehyde or 10% formalin solution for 10-20 minutes at room temperature.

Precipitating fixatives

Some organic solvents, such as methanol, acetone, and picric acid, act as strong dehydrants and cause the precipitation of cellular proteins. While these fixatives are effective at preserving cellular architecture, they can remove small soluble molecules and lipids. In addition, precipitating fixatives are not recommended for use with overexpressed fluorescent proteins (e.g. GFP) because they can denature these proteins. In a standard fixation protocol, ice-cold methanol (solution kept at -20˚C) is added to cells for 10-20 minutes at 4˚C and the cells are then rinsed with buffer. Other protocols may involve acetone, a milder fixative compared to methanol or a mixture with an equal ratio of chilled methanol and acetone (1:1). Cells fixed with either methanol or acetone may not require a permeabilization step.

Fixative	Major advantages	*Major disadvantages
**Formaldehyde	Universal fixative, recommended for testing novel antibodies or antigens. Ideal for preserving morphology and staining membrane proteins.	Some antibodies may not detect formaldehyde fixed antigens. Excessive crosslinking of antigens may reduce the signal.
Methanol	Good choice for aldehyde sensitive epitopes. Additional permeabilization is not required.	Some antibodies may not detect methanol fixed antigens. Highly volatile and flammable. Not good for over-expressed fluorescent probes.
Acetone	Good choice for aldehyde or methanol sensitive epitopes. Additional permeabilization is not required. Less harsh than methanol.	Some antibodies may not detect acetone fixed antigens. Highly volatile and flammable. Not good for over-expressed fluorescent probes.

** Always check the application notes and the customized protocols, if available, on the datasheet of antibodies for these limitations.*

***Note: Paraformaldehyde (PFA) is a powder form of polymerized formaldehyde that needs to be dissolved in PBS before its use as a fixative. Formalin is a commercially available, saturated formaldehyde solution (37% w/v) that also contains methanol as a stabilizer to prevent the polymerization of formaldehyde. A 3.7% PFA solution is equivalent to a 10% formalin solution.*

Permeabilization

Under normal conditions, antibody molecules are too large and ionic to pass through the cellular membrane. Permeabilizing the cells through methanol or acetone fixation, or with the use of a detergent, allows antibodies to pass through the cellular membrane and enter the cell. This step is necessary when staining intracellular proteins. The most common detergent used for cell permeabilization is Triton X-100, which is non-ionic in nature. Other permeabilizing agents include digitonin or related saponin compounds which are considered milder and are useful when cells are sensitive to Triton X-100.

Permeabilization Agent	Working Concentration and Incubation Time	Major Advantages	Major Disadvantages
Triton X-100	0.1% -0.4% in PBS, 10-15 minutes	Most common, permeabilize all lipid bilayers including the nuclear membrane.	High concentrations or longer incubation may lyse cells. Non-selective.
Saponins/Digitonin	0.1 % in PBS, 5-7 minutes	Reversible. Maintains the integrity of protein surface antigens. Great alternative for cells sensitive to Triton X-100.	Doesn't permeabilize the nuclear membrane.
Methanol	100%, 10 minutes	Dual agent with fixation and permeablization properties. Great for phosphorylated and nuclear antigens.	Can damage the cell membrane, the microtubules and other organelles.

Blocking

The significance of blocking?

In ICC/IF, antigen detection is dependent on specific binding of the antibody to its epitope. This binding in turn is governed by hydrophobic interactions, ionic interactions, hydrogen bonding, and other intermolecular forces. Besides facilitating specific antigen-antibody binding, the same attractive forces/bonds can also contribute to non-specific binding. The blocking step minimizes non-specific interactions that result in background and false-positive staining (artifacts). It is important to use reagents and blocking conditions that reduce nonspecific binding while having a negligible impact on specific antigen-antibody binding.

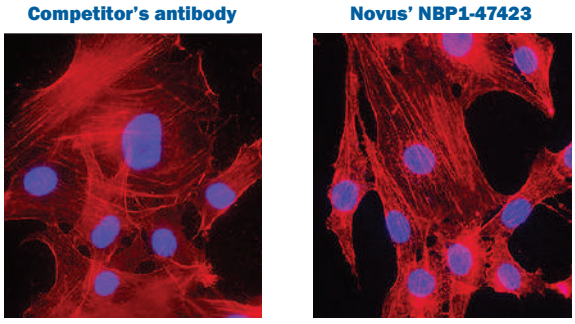
Blocking buffers

Before the antibody incubation step, the cell should be blocked with a blocking buffer containing heat-inactivated normal serum, from the same species as the host of secondary antibody. Other, less preferred, blocking agents include fetal calf serum (FCS), bovine serum albumin (BSA), casein protein, non-fat dry milk, and gelatin. To facilitate the entry of blocking buffer into the cells and to minimize the effects of non-specific hydrophobic interactions, it is essential to add a non-ionic detergent (Triton X-100 or Tween 20) to the blocking buffer as well as to the antibody diluent and wash buffers which can help reduce non-specific hydrophobic interactions. To reduce non-specificity from ionic interactions, it is suggested to increase the ionic strength of the fixative and/or the antibody diluent buffer. However, increasing the ionic strength of buffers is not recommended for monoclonal antibodies. Because of their single epitope specificity, monoclonal antibodies are more vulnerable than polyclonals to impairment of performance with increasing ionic strength.

Antibodies

It is critical to select antibodies that are specific to the antigen of interest. Selecting the optimal antibody should include a review of available validation data, peer reviewed product citations, and end user feedback/ reviews. A low-affinity antibody may compromise staining, while a cross-reactive antibody may result in non-specific background or a false positive result. The figure on the right shows a side-by-side comparison of Novus' beta-actin antibody (NBP1-47423) with a leading competitor. The diffuse signal shown by the competitor's antibody is non-specific background.

Figure 6. Example of non-specific background staining



ICC/IF analysis of beta-actin in NIH-3T3 mouse embryonic cells using Novus Biologicals' NBP1-47423 and a leading competitor's antibody. The competitor's antibody shows a high level of diffuse background/nonspecific staining.

When choosing an antibody, it is important to consider the following factors:

Reactivity

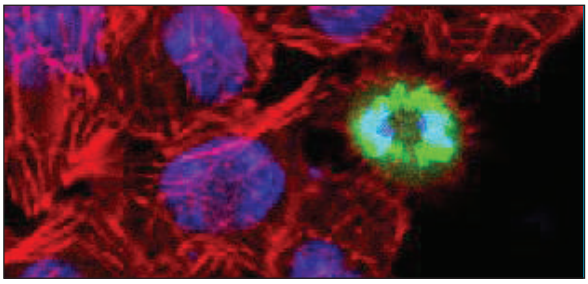
Reactivity refers to the species of the antigen detected by a given antibody. When working in a human cell line, it is important to determine if the antibody has been tested against the human antigen. In some cases, a commercially available antibody reactive against the desired species may not be available. In this case, comparing the epitope sequence of the verified species to the sequence of the unverified species can predict antibody reactivity. An antibody may work in an unverified species if the epitope recognized by the antibody shares a high level of sequence similarity with the non-validated species. The datasheets of Novus' antibodies list the guaranteed and predicted species with homology details (100% backed by our Risk Free Testing Program).

Specificity

Specificity* of an antibody is its capability of binding specifically to the unique epitope on the target antigen. Antibody specificity is critical when detecting various post-translational modifications, sub-cellular domains or specific isoforms of a given protein. For example, detection of phospho-Ser10 Histone H3, a mitosis marker, requires an antibody that specifically recognizes Histone H3 protein when it is phosphorylated at Serine 10 (See Figure 5A).

**Note: Specificity of an antibody is often better confirmed with immunoassays other than ICC, such as Western blot and ELISA.*

Figure 7. ICC/IF detection of phospho-Histone H3



ICC/IF analysis of HeLa cells using Histone H3 (p Ser10] antibody [NB21-1091] with Alexa Fluor® 488-conjugated goat anti-rabbit IgG secondary antibody (green). Actin filaments were labeled with Alexa Fluor® 568 labeled phalloidin (red) and DAPI was used to stain the cell nuclei (blue).

Antibodies

Clonality

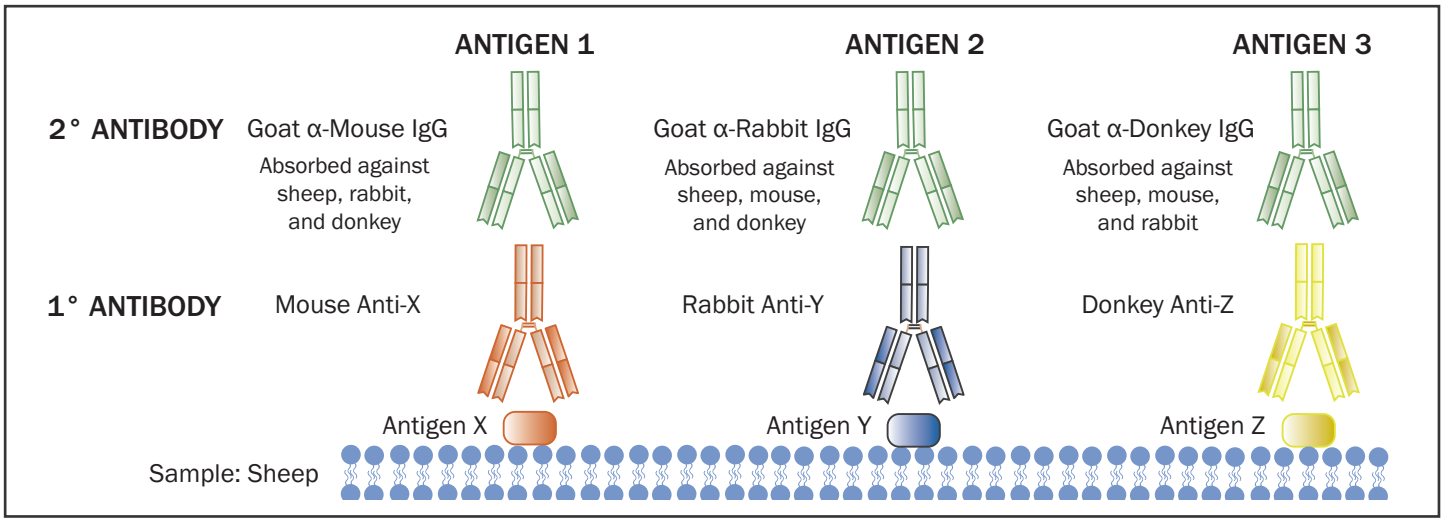
Antibodies are categorized as monoclonal and polyclonal based on the method of production and the number of epitopes detected. Key differences between monoclonal and polyclonal antibodies are listed below:

Clonality	Monoclonal	Polyclonal
Production	Produced from a single B cell clone.	Produced from mixed or multiple B cell clones.
Number of Epitopes Detected	Always detects a single epitope.	Multiple when immunogen sequence is long or the full length protein. Single in the case of small peptides or modification related antigens.
Cross-reactivity	Highly specific, less likely to be cross-reactive unless the epitope is conserved among various species.	More likely to be cross-reactive, especially antibodies generated against full length protein immunogens.
Working concentration	1-15ug/ml	5-25ug/ml

Host

The host of an antibody refers to the species of the animal used to raise that antibody. In indirect detection, the host species of the primary antibody should be carefully considered. This is because each secondary antibody is directed against the species of the corresponding primary antibody (see figure below). For example, the mouse primary antibody 1 requires an anti-mouse secondary antibody 1. Since more than one primary antibody is used in multicolor ICC, selecting primary antibodies raised in different species will minimize secondary antibody cross-reactivity and improve specificity. For instance, the anti-mouse secondary antibody 1 specifically binds to the mouse primary antibody 1, whereas the anti-rabbit secondary antibody 2 specifically recognizes and binds to the rabbit primary antibody 2. If the primary antibodies were both raised in the same species (mouse), then the corresponding secondary antibodies may bind to the primary antibodies indiscriminately. When the options of primary antibody species are limited, multiplexed immunoassays may use subclass specific primary and secondary antibodies (such as IgG1, IgG2b, IgG3, etc.).

Figure 8. Multiple staining with primary antibodies raised in different species



See our *Secondary Antibody Handbook* at novusbio.com/support/SecondaryAntibodyHandbook for more information on staining with primary antibodies raised in the same species.

Antibodies

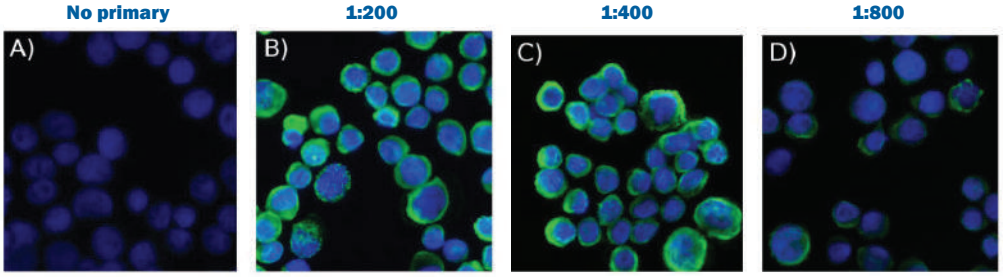
Application Compatibility

When choosing an antibody, confirm that the antibody under consideration is recommended for use in ICC/IF assay. If options are limited or if the intention is to optimize an antibody which has not been validated in ICC/IF yet, try a few different conditions/ variables to see what works the best for generating an accurate expression and localization data. To our experience, the antibodies with the ability to detect protein in its native conformation are more likely to work in ICC/IF.

Concentration and Incubation Time

Some factors such as the working concentration of the antibody, diluent of the antibody, and incubation conditions (time and temperature) can affect antigen-antibody and nonspecific binding. A high antibody concentration and a longer incubation time can result in overstaining of the cells. To determine the ideal antibody concentration and incubation time, review the recommendations provided in the antibody datasheet, as well as methods provided in peer-reviewed publications. When testing new antigens or non-validated antibodies, test a range of antibody concentrations, while incubating samples at room temperature for 1 hour. Be sure to include the appropriate controls to validate the results.

Figure 9. Effect of different dilutions of the primary antibody on staining outcome



ICC/IF staining of cytokeratin in MCF7 cells using biotin conjugated pan Cytokeratin antibody (clone AE1 + AE3) [NBP2-33200B] followed by SAv-A488. The data shown here is for different dilutions of the primary antibody: no primary control (A), 1:200 dilution (B), 1:400 dilution (C), and 1:800 dilution of NBP2-33200B (D). This data suggests 1:400 as an optimal dilution of primary under the tested conditions.

Antibody Washes

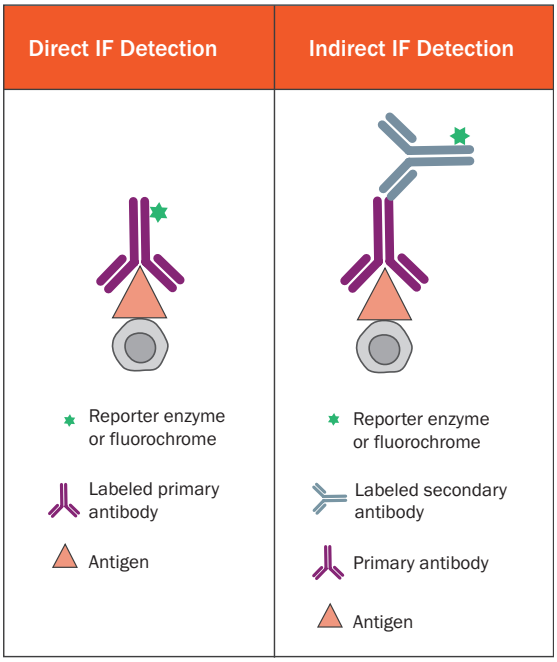
Antibody washes refer to the addition, incubation and discarding of a wash buffer to the cells after primary or secondary antibody incubations. This step is necessary to remove the residual as well as the nonspecifically bound antibody fractions from the samples. An inadequate washing may lead to non-specific background and over-staining of the cells. However, it is important to note that time between wash steps should be limited to prevent cells from drying out, which can result in staining artifacts. Moreover, the samples incubated for extended time in wash buffer can undergo cell lysis, despite the low concentration of the detergent in most wash buffers. Care should be taken to avoid excessive incubation of cells in wash buffer. We recommend 5 minute wash steps in PBS with a final concentration of 0.1% Triton X-100 or 0.05% Tween 20.

Detection Methods

Direct and indirect detection

Direct detection in multicolor ICC/IF uses primary antibodies that are conjugated to a fluorochrome. In indirect detection, the primary antibody targeting the molecule of interest is unlabeled and a labeled secondary antibody is directed against the constant region of the primary antibody.

Figure 10. Diagrammatic representation of direct and indirect detection



Which detection method is better?

The choice of detection method varies from experiment to experiment, and is often scientifically contextual. The factors and considerations listed in the table below can help in deciding which method would suit the best for a given experimental scenario.

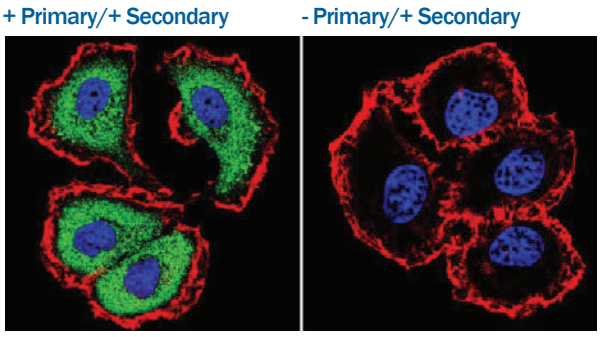
	Direct detection method	Indirect detection method
Sensitivity	The direct method of detection is less sensitive because the amplification of signal afforded by the secondary antibody is lost.	The indirect method of detection is highly sensitive as more than one secondary antibody can bind to a single primary antibody molecule.
Assay Time	The direct method protocol is shorter due to elimination of the secondary antibody incubation steps.	The indirect method protocol is longer due to secondary antibody incubation and the related wash steps.
Multiplexing	Multiplexing is easier with direct detection because multiple primary antibodies from the same host species can be used together.	Indirect method multiplexing is more complicated due to secondary antibody cross-reactivity.
Background	Non-specific background signal is lower in the direct method as secondary antibody mediated cross-reactivity is eliminated.	Non-specific background staining is generally higher in the indirect method due to secondary antibody cross-reactivity.
Flexibility	The direct method is less flexible since the selection of directly conjugated antibodies can be limited.	The indirect method is more flexible because it is possible to use different conjugated secondary antibodies depending on your experiment.

Controls

There are several variables in ICC/IF which can contribute to background staining and false-positive results. To identify potential sources of non-specific staining, the appropriate controls should be included in each experiment.

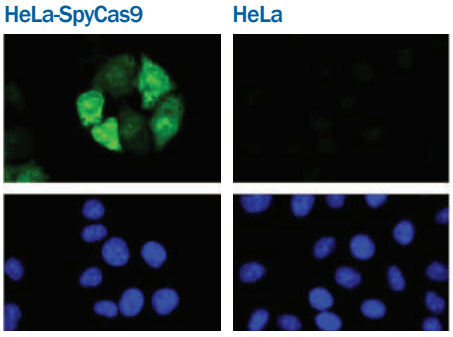
Type of Control	User Notes
Auto-fluorescence control	Unstained samples (fixed, blocked, permeabilized, but not immunostained).
No primary control	Sample fixed, blocked, permeabilized and incubated with secondary antibody only with no primary antibody (Figure 11).
Peptide block (aka absorption control)	To confirm the specific binding of the primary antibody with the antigen of interest, the antibody may be blocked with a specific blocking peptide or protein which is often the immunogen used for antibody production. Either no or reduced staining in blocked antibody sample will signify the inhibition of the antibody activity by the blocking peptide.
Isotype control	Non-immune serum or immunoglobulin molecules of the same isotype (e.g., IgG, IgG1, IgG2a, IgM etc.) used at the same working concentration as the primary antibody.
Expression controls	Positive control- cells with known or transfection induced expression of the target protein e.g. CRISPR-Cas9 transfected HeLa cells as a positive control for CRISPR-Cas9 (Figure 12), and Chloroquine treated HeLa cells as positive control for LC3B. Negative control- Cells with known siRNA/shRNA gene-mediated silencing, or cells derived from knockout models that lack or have reduced expression of the target protein. Examples include HIF1 alpha knock-out MEFs as a negative control for HIF1 alpha, and MMP7 null cell line SW480 as a negative control for MMP7.

Figure 11. No primary, secondary only control



ICC/IF analysis of NFATc1 in MCF7 cells using NFATc1 antibody (7A6) [NB300-620] with DyLight™ 488 conjugated secondary antibody (green). Phalloidin (red) and DAPI (blue) were used for counterstaining the cytoskeleton and nuclei, respectively. The image on the right shows a control with all conditions being the same but without addition of primary antibody.

Figure 12. Overexpression positive control



CRISPR-Cas9 was detected in HeLa cells expressing Flag-tagged SpyCas9 using CRISPR-Cas9 antibody (clone 6G12) [NBP2-52398] with Alexa Fluor® 488 conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342.

Counterstaining and Mounting

Counterstaining

In addition to immunofluorescence, cells are typically counterstained with DNA or cytoskeleton-binding dyes to identify a particular cellular organelle or structure, and to easily identify individual cells. Dyes such as Hoechst and DAPI are able to penetrate the nucleus without permeabilization and intercalate into the DNA helix. Other examples include staining the cytoskeletal marker actin with fluorescently-labeled phalloidin, or the plasma membrane using fluorescently-tagged wheat germ agglutinin (WGA). When designing an ICC experiment, it is critical to ensure that the counterstain and the selected fluorochrome display limited spectral overlap so they can be sufficiently distinguished from one another. For example, DAPI should not be used in combination with other blue emitting fluorochromes, such as Alexa Fluor® 405, DyLight™ 405, or HiLyght™ 405.

Listed below are the dyes available from Novus which are useful for counterstaining of the nuclei and other organelles.

Dye	Excitation/Emission Max (nm)	Usefulness
Acridine Orange hydrochloride [NB5092]	460/650 (RNA), 500/526 (DNA)	Acridine Orange is a membrane permeable nucleic acid binding dye which emits green fluorescence when bound to double-stranded DNA and red fluorescence when bound to RNA or single-stranded DNA. It is useful in cell cycle and apoptosis studies and as a lysosomal dye.
Calcein AM [NB5119]	495/515	Calcein AM is a cell permeable, non-fluorescent chemical compound that is hydrolyzed inside living cells by esterases to become fluorescent calcein. It is useful for monitoring cell viability, chemotaxis, cell adhesion, and multidrug resistance on live (unfixed) cells.
DAPI [NBP2-31156]	358/461	DAPI is a common nuclear counterstaing dye which intercalates into double-stranded nucleic acids and fluoresces brightly around 461nm. It is taken up by dead/dying or permeabilized cells.
Hoechst 33258 [NB5824]	352/461	Hoechst 33258 is a fluorescent dye which binds the minor groove of DNA at A and T rich regions. It can be used as an indicator of apoptosis.
Hoechst 33342 [NB5117]	350/461	Hoechst 33342 is a cell permeable fluorescent DNA stain which binds minor groove of AT-rich regions. It is useful for quantification of DNA in viable cells.

Mounting

After staining, cells on coverslips must be mounted onto microscope slides prior to imaging. The use of mounting media protects stained cells from dehydration and makes the sample suitable for microscopy. It is important to match the refractive index of your mounting media with microscope objectives to generate high-quality images. Most mounting media contains anti-fading agents to protect samples from photobleaching. Some mounting media also includes DNA stains, such as DAPI, eliminating the need for a separate counterstaining step. While mounted slides can be stored for months in the dark at -20°C, the fluorescence intensity will slowly decay over time.

PROTOCOL: Multicolor ICC/IF

Multicolor ICC/IF can be performed either simultaneously using an antibody cocktail or sequentially by probing one antigen after another. In both simultaneous and sequential multicolor staining protocols, the choice of directly labeled - primary antibodies or secondary antibodies is critical. Simultaneous and sequential staining follow the same basic protocol, but considerable variations exist in the blocking and antibody incubation steps.

ADHERENT CELLS (CULTURE ON COVERSLIPS)

01 Coat the coverslips with poly-L-lysine, dry and sterilize them.

Tip: Coverslips can be sterilized by dipping in ethanol and flaming them or by placing them into tissue culture dishes and exposing them to UV radiation (available in most tissue culture hoods).

02 Seed the cells on sterile glass coverslips (poly-L-lysine coated). Some cell types may require further coverslip treatment for optimal adhesion and growth. Grow the cells to semi-confluency.

Tip: Multiple coverslips can be seeded in a single dish which helps reduce the number of dishes stored in the incubator. Keep all mechanical manipulations to a minimum to avoid compromising the quality of the final cell images. Always treat the cells and coverslips gently. Never let them dry out and avoid directly pipetting solutions onto the cells.

03 After desired treatments schedules, if any, cells on the coverslips may be processed for the next step (fixation).

NON-ADHERENT CELLS (SMEARS)

01 Fix the non-adherent cells with 4% PFA for 20 minutes at room temperature. The fixative should be added directly to the culture media in a ratio of 1mL fixative for every 1 x 10^6 cells.

02 Once the cells are fixed, add 1 mL of the cell solution to a 1.5 mL microfuge tube. After 30 seconds spin down of cells in a microcentrifuge, discard the supernatant and resuspend the cell pellet in 1 mL of deionized water. Spin for 30 seconds, discard the supernatant and resuspend the pellet in 1mL deionized water. Spin again for 30 seconds, discard the supernatant and resuspend the pellet in 200µL of deionized water. Use 5 µL of the cell suspension for making spears on a gelatin-coated slide with a pipette tip. After drying the slide on a hot plate with low heat setting, the sample may be processed for the next step (blocking).

FIXATION

01 Aspirate the culture medium from the dish or remove the coverslips from culture with tweezers, and gently wash them with PBS at room temperature.

02 Incubate the coverslips in freshly prepared 4% paraformaldehyde/PBS (neutral pH) at room temperature for 10 minutes. Alternatively, the cells can be fixed for 10 minutes in chilled methanol (pre-equilibrated at -20°C) on ice.

Tip: Alternative fixation methods may be tested and compared to determine which is best at preserving the structure and epitope of the protein of interest.

03 Wash the coverslips of fixative in PBS for 2 minutes.

PROTOCOL: Multicolor ICC/IF

PERMEABILIZATION

01 Incubate the coverslips in 0.5% Triton X-100 in PBS (PBST) at room temperature for 5 minutes. Test different detergents (ex. digitonin, Tween-20) in a range of concentrations to find the optimal condition that best preserves cell structure and the target protein.

Tip: A permeabilization step is not required with methanol fixation because methanol acts as a dual fixing and permeabilizing agent.

02 Wash the coverslips of the permeabilization buffer by incubating in PBS for 5 minutes.

BLOCKING

01 Block the coverslips in 1-5% normal serum prepared in PBST for 1 hour at room temperature.

Tip: Normal serum for blocking should be from the same host species as the secondary antibody. For example, if you are using a goat anti-rabbit secondary antibody, then block with 5% normal goat serum. Also, it is critical to use normal serum different from the host animal source of the primary antibodies to avoid cross-reactivity with secondary antibodies. Alternative blocking agents are 1% gelatin or 1-5% BSA in PBST.

IMMUNOSTAINING

Multicolor labeling experiments are best carried out by sequentially incubating cells with primary and secondary antibodies, however it may be performed by employing one of the following three options:

Option 1: Sequential incubations with unlabeled antibodies

This method is most useful when using primary antibodies from different host species (e.g. mouse monoclonal against antigen-X, rabbit polyclonal against antigen Y, and sheep polyclonal against antigen Z), and the antibodies display aggregates formation in the simultaneous incubation method.

01 Blocking step #1: incubate cells with blocking buffer solution #1 (5% serum from the same species as the host of secondary antibody #1) for 30 minutes at room temperature in the dark.

02 Primary antibody incubation #1: incubate cells with primary antibody #1 in 1% BSA or 1% serum in a humidified chamber for 1 hour at room temperature or overnight at 4°C.

03 Decant the antibody solution and wash the cells 3 times in PBS-T (5 minutes for each wash).

04 Secondary antibody incubation #1: Incubate cells with secondary antibody #1 in 1% BSA for 1 hour at room temperature in the dark.

05 Decant the antibody solution and wash the cells 3 times in PBS-T (5 minutes for each wash).

Tip: Process the cells for next step immediately and do not let the cells dry out at any stage.

06 Blocking step #2: incubate the cells with blocking buffer #2 (5% serum from the same species as the host of secondary antibody #2) for 30 minutes at room temperature in the dark.

07 Primary antibody incubation #2: Incubate cells with primary antibody #2 in 1% BSA or 1% serum in a humidified chamber in the dark for 1 hour at room temperature or overnight at 4°C.

08 Decant the antibody solution and wash the cells 3 times in PBS-T (5 minutes for each wash).

09 Secondary antibody incubation #2: Incubate cells with secondary antibody #2 in 1% BSA for one hour at room temperature in the dark.

10 Decant the secondary antibody solution and wash 3 times with PBS for 5 minutes each in the dark.

Tip: Repeat steps 1-5 for additional primary antibodies. During various wash steps, handle the cells carefully to minimize potential artifacts.

Option 2: Simultaneous incubation with directly labeled primary antibodies

This method is useful when the primary antibodies are from the same host. For example, a mouse monoclonal against antigen-X and a mouse monoclonal against antigen-Y.

01 Create an antibody cocktail by adding the recommended amount of each directly conjugated antibody to an appropriate volume of blocking buffer.

02 After the blocking step, incubate the cells with the antibody cocktail of directly labeled primary antibodies (in the blocking buffer) in a humidified chamber for one hour at room temperature or overnight at 4°C in the dark.

Tip: The use of fluorescently labeled phalloidin is a good alternative to immunostaining actin as a reference marker in ICC.

03 Decant the antibody solution and wash the cells 3 times in PBS-T (5 minutes for each wash).

Tip: Additional optimization may be required to determine the working number and time of washes.

Option 3: Simultaneous incubation with unlabeled primary antibodies

This method is useful when the primary antibodies are from different hosts. For example, a mouse monoclonal antibody against antigen-X and rabbit polyclonal antibody against antigen Y.

01 After the blocking step, create an antibody cocktail by adding the recommended amount of each unconjugated antibody to an appropriate volume of blocking buffer. Incubate the cells with a mixture of both unlabeled primary antibodies cocktail in the blocking buffer in a humidified chamber for one hour at room temperature or overnight at 4°C.

Tip: The blocking buffer can be prepared by mixing the serum from each host of the secondary antibodies. Alternatively, 5% BSA in PBS-T can be used as a blocking buffer.

02 Decant the antibody solution and wash the cells 3 times in PBS-T (5 minutes for each wash).

03 Create a secondary antibody cocktail by adding the recommended amount of each directly conjugated secondary antibody to an appropriate volume of blocking buffer. Incubate the cells with a mixture of both secondary antibodies in 1% BSA for one hour at room temperature in the dark.

Tip: The secondary antibodies often come with a broad range of working dilutions. It is recommended to choose the dilutions very carefully and to employ additional optimization to see which dilution combination gives the best possible staining.

04 Decant the secondary antibody solution and wash 3 times with PBS for 5 minutes each in the dark.

Tip: Additional washes may be required for a cleaner staining.

Counterstaining, Mounting, and Imaging

01 When all the necessary washing steps have been completed, cell nuclei can be counterstained with DAPI or Hoechst (1-10 µg/mL).

02 Invert the coverslip, placing it cell side down on a glass slip and add a drop of mounting media containing the anti-fade agent.

Tip: If a coverslip of cells is accidently dropped and “cell side up” orientation is lost, you can still salvage the experiment by carefully picking up the coverslip with tweezers. Additionally, gently scraping one surface of the coverslip with a pipette tip is helpful to see if any cells are visibly removed.

03 Carefully remove the excess mounting media, if necessary, and seal the edges of the coverslip with nail polish if needed.

Tip: Some mounting media solutions have DAPI already added and will harden after exposure to air, eliminating the need to seal the edges of the coverslip.

04 Examine the cells under a fluorescence microscope and collect images if needed.

Tip: Avoid long exposure to light and the laser to prevent photo-bleaching. The slides mounted with glycerol-based mounting media should be stored at -20°C.

Pros and cons of multi-staining methods

	Sequential incubations with unlabeled primary antibodies (indirect)	Simultaneous incubation with unlabeled primary antibodies (indirect)	Simultaneous incubation with directly labeled primary antibodies (direct)
Sensitivity	This method is more sensitive since multiple secondary antibodies can bind a single primary antibody.	This method is sensitive but cross-reactivity of secondary antibodies may increase background signal.	This method is less sensitive because signal amplification afforded by the secondary antibody is lost.
Assay Time	This method is time consuming as a result of multiple antibody incubations and wash steps.	This method is relatively less time consuming as the primary antibodies and secondary antibodies are incubated at the same time.	This method is the least time consuming as it eliminates the secondary antibody related incubation and wash steps.
Multiplexing	Multiplexing using this method is more complex than direct detection due to potential cross reaction of secondary antibodies.	Multiplexing using this method is more complex than direct detection due to the potential cross reaction of secondary antibodies.	Multiplexing using this method is less complex than indirect detection methods as multiple primary antibodies from the same species can be incubated together.
Background	This method gives the cleanest staining. In comparison to simultaneous incubation methods, additional wash steps (related to sequential incubations) reduce non-specific back-ground.	Staining using simultaneous incubation with unlabelled primary antibodies generally increases non-specific background because secondary antibodies can cross-react with multiple primary antibodies.	In this method, the non-specific background is generally low because secondary antibody cross-reactivity is eliminated.
Flexibility	Staining using sequential incubations with unlabelled antibodies offers the most flexibility.	This method is flexible due to the diversity of commercially available fluorochrome-conjugated secondary antibodies.	This method is the least flexible due to a limited selection of commercially available directly conjugated antibodies.

Troubleshooting Tips

NO SIGNAL

Potential Issue	Reccomendations
Antibodies	Increase the concentration or incubation time of the primary or secondary antibody.
Permeabilization Buffer	Use the proper permeabilization reagent for the target protein's localization. Triton detergent is necessary for mitochondrial or nuclear proteins but will dissolve the outer membrane and disrupt proper membrane localization. Increase incubation duration or detergent concentration.
Cell Fixation	Over fixation can cause epitope masking. Decrease the fixation incubation time or concentration of the fixative.
Antibody Compatibility	Confirm that your primary and secondary antibodies are compatible by checking the species reactivity. The secondary antibody should be raised against the host species of the primary antibody. For example, a mouse monoclonal primary antibody requires an anti-mouse secondary antibody. Confirm that the antibody can be used for assays in which the protein is in its native conformation. Ensure that the fluorochrome conjugated to the secondary antibody is compatible with your microscope's filter sets.
Target Availability	Use an overexpression assay or positive control cell line known to express the protein of interest. Cell Viability - Confirm cell viability before starting the staining procedure (for example by using Trypan Blue).
Microscope Adjustments	Increase the manual exposure time of your camera or use the auto-exposure option that will adjust the gain automatically to compensate for low-intensity fluorescence signal.

HIGH BACKGROUND

Potential Issue	Reccomendations
Antibody Concentration	Decrease the concentration of the primary/secondary antibody. Blocking- Increase the incubation time or concentration of serum in the blocking buffer. Use blocking buffer for primary and secondary antibody incubations.
Antibody Application	It is recommended to incubate primary antibodies overnight at 4°C. Room temperature incubation may increase non-specific binding and cause higher background. Confirm that the secondary antibody is not contributing to background signal by cross-reacting with the cells. To do this, perform the assay without the primary antibody. -
Contamination	Ensure slides are clean and free of dust. Buffers should be made fresh to prevent microbial contamination.
Washing	Increase the number of washes. Add very gentle agitation to the plates with cells. Increase the concentration of Tween-20 in the PBS-T.
Spectral Overlap	If double or triple labeling the cells, confirm that there is no overlap in emission spectra of different secondary antibodies.

Common Organelle Markers for ICC/IF

AUTOPHAGOSOMES

- LC3B/MAP1LC3B
- LC3/MAP1LC3A

CELLULAR JUNCTIONS

Adherens Junctions

- E-Cadherin
- N-Cadherin
- P-Cadherin
- VE-Cadherin

Tight Junctions

- ZO1/TJP1

Desmosomes

- Desmoglein-2

Gap Junctions

- Connexin 43

CENTROSOME

- Pericentrin
- gamma-Tubulin

CYTOSKELETON ACTIN FILAMENTS

Actin Filaments

- Beta-actin

Intermediate Filaments

- Cytokeratin
- Vimentin

Microtubules

- alpha Tubulin
- beta Tubulin

ENDOPLASMIC RETICULUM

- Calreticulin
- Pdi1p
- Protein disulfide-isomerase (PDI)
- KDEL

ENDOSOMES

Early Endosome

- EEA1
- Rab5a

Recycling Endosome

- TfR (Transferrin R)
- Rab11A

Late Endosome

- CD63
- IGF-II R
- Rab7a

Caveolae & Endosomes

- Caveolin-1

GOLGI APPARATUS

Golgi

- 58K Golgi Protein
- GOLM1
- GM130/Golga2

Trans-Golgi Network (TGN)

- TGN38
- TGN46
- Furin

LYSOSOMES

- Cathepsin D
- LIMPII/lpg85
- PMP70
- LAMP-2/CD107b
- LAMP-1/CD107a

MITOCHONDRIA

- Cytochrome c
- SDHB
- Mitofilin
- HSP60

NUCLEUS

Nucleus

- Histone H2BS
- Histone H3
- Histone H4
- LSD1
- MeCP2
- ASH2L

Nuclear Membrane

- Lamin B1
- Lamin A + C

Nucleolus

- DDX21
- Fibrillarin

Centromere

- CENPA

Nuclear Speckle

- SC35

Heterochromatin Marker

- HP1 alpha

PEROXISOMES

- Catalase
- ACAA1
- PMP70

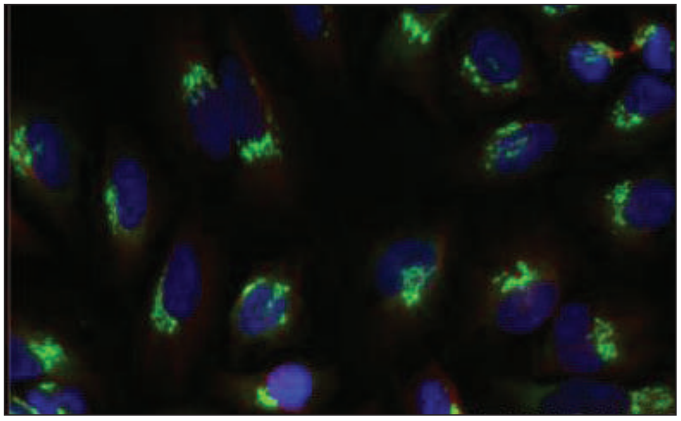
RIBOSOMES

- RPL7A
- Ribosomal Protein S6/ RPS6
- RPS3

Common Organelle Markers for ICC/IF

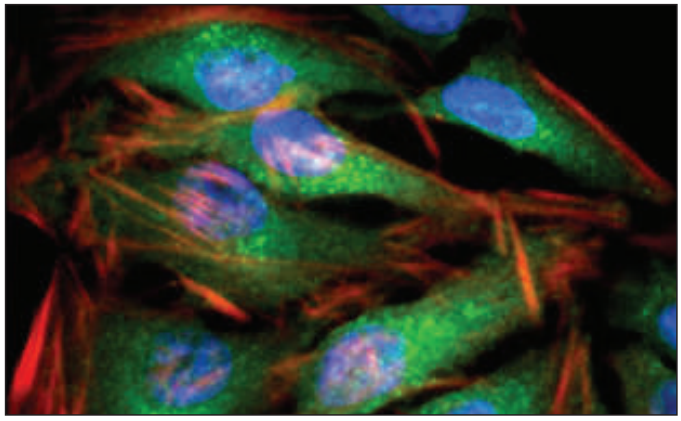
Figure 13. ICC/IF detection of select organelle markers

GM13/Golga 2, *Golgi Marker*



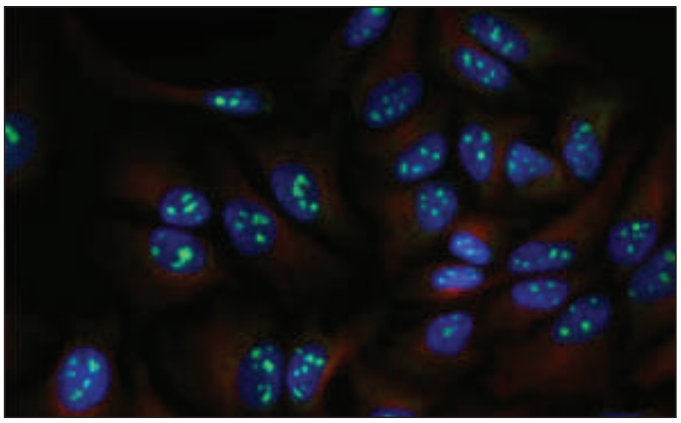
ICC/IF detection of Golga2 in HeLa cells using GM130/GOLGA2 antibody [NBP2-53420]. The cells were fixed for 10 minutes using 10% formalin and then permeabilized for 5 minutes using 1X TBS + 0.5% Triton X-100. The cells were incubated with anti-GOLGA2 at a 1:200 dilution overnight at 4˚C and detected with an anti-rabbit Dylight™ 488 secondary antibody (green) at a 1:500 dilution. Alpha tubulin (DM1A) [NB100-690] was used as a co-stain at a 1:1000 dilution and detected with an anti-mouse Dylight™ 550 (red) at a 1:500 dilution. Nuclei were counterstained with DAPI (blue).

LAMP2/CD107b, *Lysosome Marker*



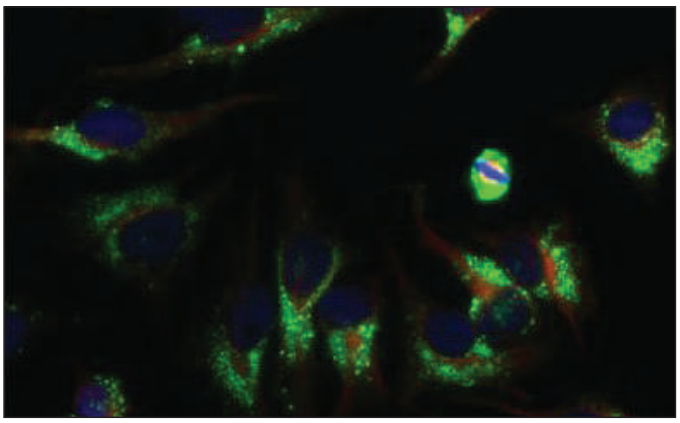
ICC/IF detection of LAMP2 in formalin fixed HeLa cells using LAMP-2/CD107b antibody (H4B4) [NBP2-22217]. After 10 minutes of fixation, the cells were permeabilized for 5 minutes using 1X TBS + 0.5% Triton X-100. Primary antibody was used at 1:200 dilution overnight at 4˚C and detected with an anti-rabbit Dylight™ 488 secondary antibody (green) at a 1:200 dilution. Alpha tubulin (DM1A) [NB100-690] was used as a co-stain at a 1:1000 dilution and detected with an anti-mouse Dylight™ 550 (red) at a 1:500 dilution. Nuclei were counterstained with DAPI (blue).

DDX21, *Nucleolus Marker*



ICC/IF detection of DDX21 in HeLa cells which were fixed for 10 minutes using 10% formalin and then permeabilized for 5 minutes using 1X TBS + 0.5% Triton X-100. The cells were incubated with anti-DDX21 [NB100-1718] at a 1:200 dilution overnight at 4˚C and detected with an anti-rabbit Dylight™ 488 (green) at a 1:500 dilution. Alpha tubulin (DM1A) [NB100-690] was used as a co-stain at a 1:1000 dilution and detected with an anti-mouse Dylight™ 550 (red) at a 1:500 dilution. Nuclei were counterstained with DAPI (blue).

LC3B, *Autophagosome Marker*



ICC/IF detection of LC3B in HeLa cells which were treated overnight with 50uM CQ, then fixed for 10 minutes using 10% formalin and then permeabilized for 5 minutes using 1X TBS 0.5% Triton X-100. The cells were incubated with 0.1 µg/ml LC3B antibody [NB600-1384] for overnight at 4C and detected with an anti-rabbit Dylight™ 488 (green) at a 1:500 dilution. Alpha tubulin (DM1A) [NB100-690] was used as a co-stain at a 1:1000 dilution and detected with an anti-mouse Dylight™ 550 (red) at a 1:500 dilution. Nuclei were counterstained with DAPI (blue).

Reagent Recipes

WASH BUFFERS

> Tween-20: PBS with a final concentration of 0.05 % Tween-20.

> Triton X-100: PBS with a final concentration of 0.1 % Triton X-100.

FIXATION SOLUTIONS

> Formaldehyde: For 1 L of 4% Formaldehyde, add 800 mL of 1X PBS to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60 °C. Take care that the solution does not boil. Add 40 g of paraformaldehyde powder to the heated PBS solution. The powder will not immediately dissolve into solution. Slowly raise the pH by adding 1 N NaOH drop-wise from a pipette until the solution clears. Once the paraformaldehyde is dissolved, the solution should be cooled and filtered. Adjust the volume of the solution to 1 L with 1X PBS. Recheck the pH, and adjust it with small amounts of dilute HCl to approximately 6.9. This solution retains its properties for about 2 weeks when stored at 4° - 8°C.

> Methanol: 100 % Methanol (-20 °C).

> Methanol/Acetone: 50 % Methanol (-20 °C) and 50 % Acetone (-20 °C).

PERMEABILIZATION BUFFERS

> Triton X-100: PBS with a final concentration of 0.1-0.4% Triton X-100.

> Saponin: PBS with a final concentration of 0.1 % Saponin.

BLOCKING BUFFERS

> BSA (bovine serum albumin): PBS with a final concentration of 1-5 % BSA.

> Normal serum: PBS with a final concentration of 5-10 % normal serum.

> Milk powder: PBS with a final concentration of 1 % milk powder.

ANTIBODY DILUENT

> PBS with normal serum or BSA to a final concentration of 0.5-1%.

> PBS only may also be used as a diluent from case to case basis.

Conjugated Antibodies

Conjugated antibodies

Can't find a labeled antibody? Try searching novusbio.com for your target and the conjugate of interest. Novus Biologicals provides over 55,000 unique antibody-dye combinations.

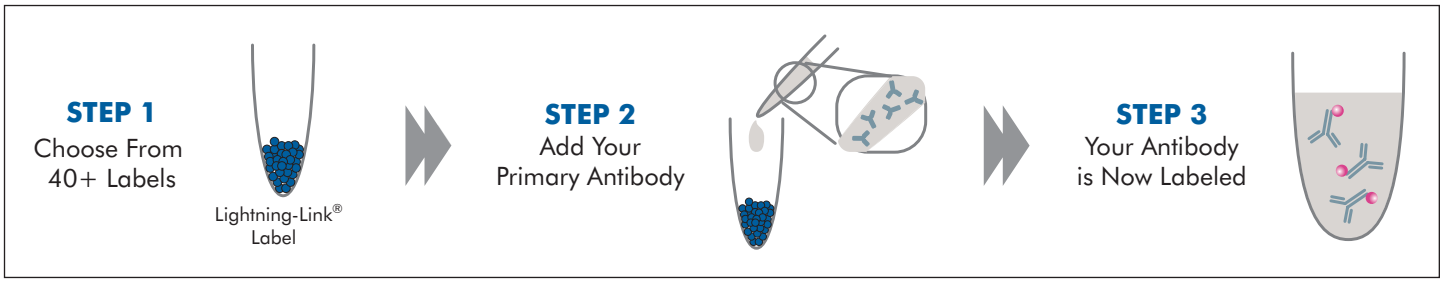
If you do not see a conjugated antibody listed on our website, ask for custom conjugations. In our in-house conjugation facility, Novus' Lab Team custom labels many of our unconjugated primary antibodies on special request. Some of the available options of fluorochromes for custom conjugations are listed to the right.

Learn more at novusbio.com/conjugatedantibodies

Figure 14. Options of fluorochromes for custom conjugations

Antibody Labeling Kits

Want to label an antibody yourself? Try Novus antibody labeling kits which enables you to conjugate an antibody in as little as 30 seconds! See below the labeling protocol flowchart and the fluorochrome options for Novus kits:



Fluorescent Dyes

AMCA	FluorProbes® 594
Atto 390, 488, 565, 594, 633, 680, 700	FITC
Cyanine Dye Cy3, Cy5, Cy5.5	Rhodamine
DyLight™ 350, 405, 488, 550, 594, 633, 650, 680, 755, 800	Texas Red®

Fluorescent Proteins

Allophycocyanin (APC)	PerCP
B-Phycoerythrin (BPE)	R-Phycocyanin (RPC)

Tandem Dyes

Allophycocyanin/Cy5.5	PE/Cy5
Allophycocyanin/Cy7	PE/Cy5.5
PerCP/Cy5.5	PE/Cy7

For a complete product list of labeling kits, visit novusbio.com/labeling-kits

R&D Systems develops and manufactures high-quality proteins and serves as a world leader in immunoassays. R&D Systems also produces quality antibodies, antibody arrays, stem cell and cell culture products, and cell selection and detection products, serving the life science and diagnostics industry. rndsystems.com

Novus Biologicals licenses, manufactures, and markets antibodies to over 20,000 unique targets to support a wide array of research areas. Novus is built on honesty, collaboration and strong relationships and continues to provide quality tools that accelerate research. Every product is backed by our 100% guarantee. novusbio.com

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