Tips and Tricks for Cell-Based Imaging Assays: Part 2, Immunocytochemistry

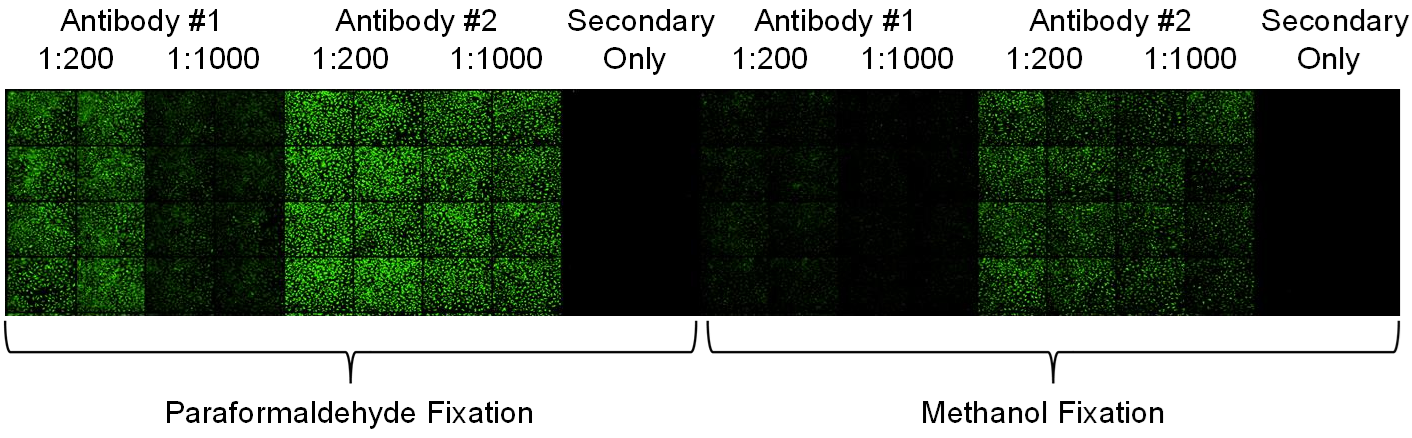
We are delighted to present the fifth installment in a series of white papers published by Scintillant Bioscience, related to the services we offer. This paper constitutes the second publication in our series dedicated to providing practical tips and tricks for the development and execution of cell-based assays. Within this paper, we will explore the practical considerations surrounding the technique known as immunocytochemistry (ICC). ICC is a powerful method employed to visualize specific proteins or antigens within cells. It harnesses the binding specificity of antibodies in conjunction with immunofluorescence microscopy, enabling the examination of subcellular structures. The ICC process involves a series of critical steps, namely fixation, permeabilization, blocking, antibody binding, and washing, all of which are essential for attaining accurate and reliable results. In this paper, we will limit the discussion to ICC, and not address the similar method known as immunohistochemistry (IHC), which is used with fixed tissue sections, rather than 2D cultures of dissociated cells.

While numerous tutorials and detailed ICC protocols can be found online, we have chosen not to provide a redundant replication of such resources. Instead, this white paper assumes a basic understanding of ICC on the reader's part and aims to serve as a practical guide for optimizing ICC assay development. It will discuss various tips and tricks for enhancing the efficiency of fixation, permeabilization, blocking, antibody binding, and washing, while also addressing other practical considerations.

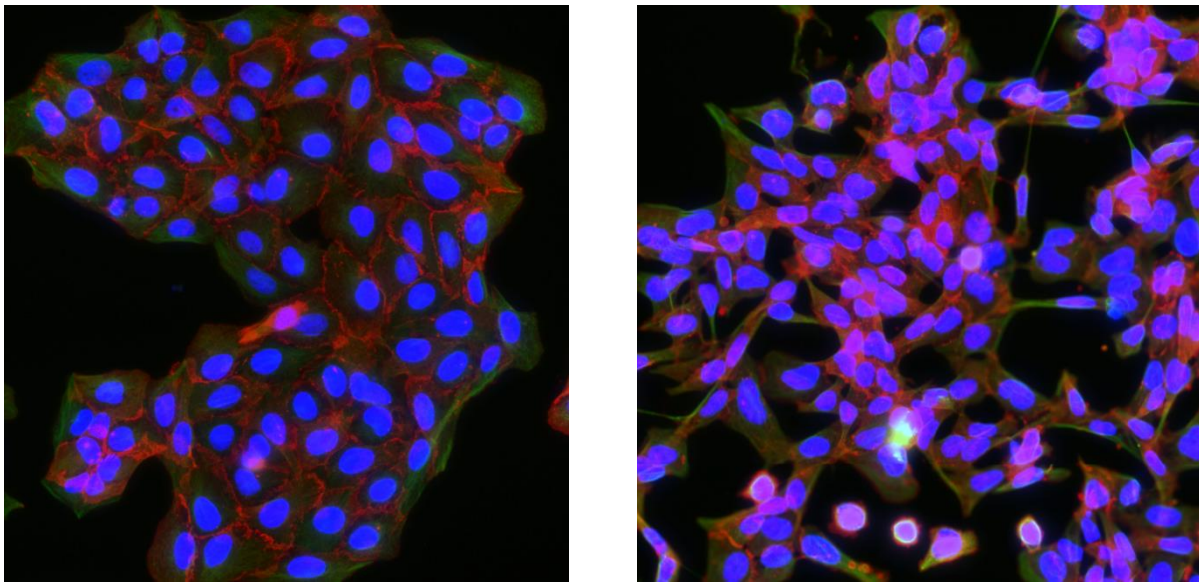
Significantly, we will emphasize the execution of ICC in 96-well and 384-well plates. Some researchers informally refer to this general approach as an "In-Cell Western," although "In-Cell Western™" is a trademark of LI-COR Biosciences. This approach combines antibody detection and quantification of protein expression levels, akin to Western blotting, but in a high-throughput format.

Before diving into the detailed tips and tricks for improving ICC experimental parameters, we present a general yet simplified depiction of the ICC assay development process in 384-well plates, as shown in the figure below. Using 384-well plates, researchers can array different experimental conditions across columns and rows, greatly facilitating assay development and optimization.

The figure below showcases thumbnail images from a subset of wells in a 384-well plate, showing four technical-replicate wells for each condition. This experiment focused on investigating the effects of different fixation conditions, primary antibodies, and antibody concentrations across the plate's columns. We examined two primary antibodies targeting a protein of interest, each at two dilutions: 1:200 and 1:1000. The results revealed that Antibody #1 was incompatible with methanol fixation, while paraformaldehyde produced superior outcomes for both antibodies. Additionally, it was found that the 1:1000 dilution of Antibody #1 was inadequate. Wells labeled as "Secondary Only" served as controls, receiving only the secondary antibody without prior incubation of the primary antibody. This control should display a dark background to ensure that the observed antibody staining pattern and intensity are not due to non-specific binding of the secondary antibody. Within the 384-well format, assay parameters beyond fixation conditions and antibody dilutions can be varied, including methods of permeabilization after fixation and compositions of blocking and binding buffers, as discussed further below.

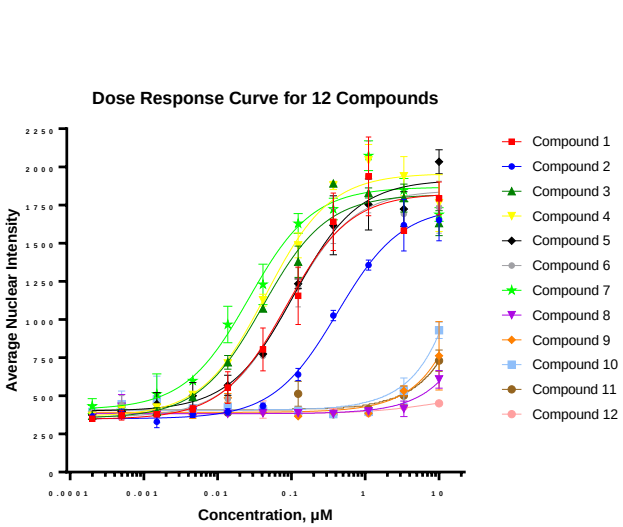


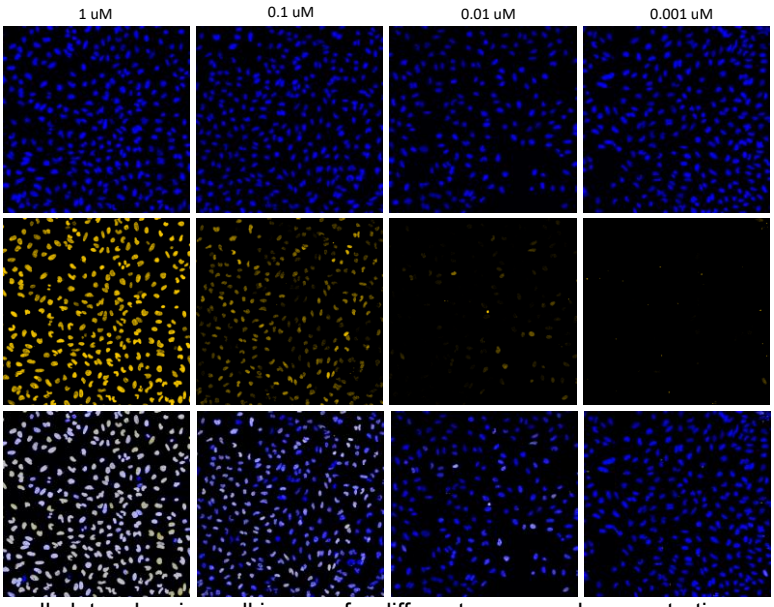
Now let's explore how ICC can be utilized in the development of a drug-discovery assay. Drugs have the potential to influence the expression levels, distribution, movement, or morphology of proteins and organelles. Following drug treatment, cells can be fixed at a specific time point for ICC analysis. By staining the target(s) of interest using immunofluorescence, compound-treated cells can be compared to untreated cells. In the figure below, the drug treatment caused a change in the localization of beta-catenin, which can be quantified through automated image analysis. The quantified impact on protein translocation can be represented as a dose-response curve.



Left panel: Beta-catenin (red immunofluorescence) localized at cell-cell adherens junctions.
Right panel: Translocation of beta-catenin to the nucleus in response to drug treatment.

Using another example shown in the figure below, a drug may alter the abundance of a protein, in this case the expression level of a protein in the nucleus involved in DNA repair. We generated 10-point dose response curves by testing 10 different concentrations of a set of compounds in 384-well format, followed by ICC analysis of the nuclear protein target. We analyzed the average fluorescence intensity per cell within the nuclei (yellow staining in images below) and plotted the average fluorescence intensity data as dose-response curves.





Right Panel: Example images of ICC data obtained from a 384-well plate, showing cell images for different compound concentrations.
Left Panel: Dose-response curves generated from ICC data. Fluorescence intensity was quantified by creating a mask of Hoechst-stained nuclei (blue) and measuring the average intensity per nucleus in the second fluorescence channel (yellow). Four regions per well were captured, and four technical-replicate wells were used for each compound concentration to generate dose-response curves.

The previous page's figures demonstrate the application of ICC as a readout in drug-discovery assays. The reliability and precision of the data heavily rely on the optimization procedures employed during assay development. To assist you in efficiently developing, optimizing, and validating drug-discovery assays using ICC techniques, we provide the following tips and tricks. These recommendations are applicable to ICC experiments conducted in various formats, including high-throughput formats, like 96-well and 384-well formats.

Fixation

Fixation is a critical step in ICC, as it preserves cellular morphology, immobilizes target antigens, and facilitates antibody binding. The choice of fixative significantly impacts the success and quality of ICC experiments. Here we provide an overview of different fixatives commonly used in ICC, with a discussion of their applications, and the reasons why one fixative may be preferred over another.

Paraformaldehyde (PFA), a formaldehyde polymer, is a widely used fixative in ICC. PFA preserves cellular structures, retains antigenicity, and is compatible with a variety of antibody-based techniques. It is effective for capturing both cytoplasmic and membrane-associated antigens. PFA fixation is usually performed at room temperature or slightly higher. In ICC experiments, 4% PFA is typically applied for 10 – 20 minutes. PFA is particularly suitable for preserving delicate cellular structures, such as neuronal processes, and maintaining antigen integrity. Additionally, PFA-fixed samples can be stored for extended periods, allowing flexibility in experimental timelines.

Methanol is another commonly used fixative in ICC, especially for intracellular antigens. It rapidly permeates cells, making it effective for preserving intracellular epitopes. Methanol fixation is typically performed at -20°C or lower, and fixation times are relatively short, usually ranging from a few minutes to tens of minutes. Methanol fixation is particularly advantageous for detecting nuclear antigens, phosphoproteins, and some cytoskeletal proteins. However, methanol fixation can cause cell shrinkage and disruption of certain cellular structures, limiting its utility for preserving fine morphological details.

Ethanol is a fixative that shares similarities with methanol but is less commonly used in ICC. Like methanol, ethanol rapidly penetrates cells and denatures proteins. Ethanol fixation is often performed at -20°C or lower and can range from a few minutes to tens of minutes. Ethanol fixation is suitable for preserving intracellular antigens, and it is particularly effective for detecting lipid-rich structures. However, similar to methanol, ethanol fixation can cause cellular shrinkage and structural alterations, potentially affecting antigen localization and morphological integrity.

Acetone is an organic solvent fixative sometimes used for ICC, but more commonly used for IHC of frozen sections. It effectively permeabilizes cells and preserves antigenicity. Acetone fixation is typically performed at -20°C or lower, and fixation times are relatively short, ranging from a few minutes to tens of minutes. Acetone fixation is particularly useful for preserving delicate cellular structures and preserving antigen integrity. It is commonly employed in ICC for viral antigens, complement components, and some cytoskeletal proteins. However, acetone fixation can cause cellular dehydration, leading to shrinkage and loss of soluble cytoplasmic antigens.

The choice of fixative depends on several factors, including the nature of the antigen, the desired cellular morphology, and the experimental requirements. PFA is often preferred when preserving cellular morphology is crucial, especially for tissues with intricate structures. Methanol and ethanol are often preferred for detecting intracellular antigens and phosphoproteins due to their ability to rapidly permeabilize cells and preserve antigenicity. Acetone is commonly used for frozen sections and is effective in preserving delicate structures and maintaining antigen integrity. When fixing cells with organic solvents, it is advisable to wash the cells once or twice with phosphate-buffered saline (PBS) before applying the fixative. This eliminates traces of cell-culture media as organic solvents precipitate serum proteins in the media.

It is important to note that fixative preference can also be influenced by the specific experimental setup and the compatibility of the fixative with subsequent ICC steps, such as permeabilization and antibody binding.

Optimization of fixation conditions, including concentration, temperature, and duration, is necessary to ensure proper antigen preservation and minimize artifacts.

Following fixation, cells are usually washed with PBS three times for 5-minutes per wash. If permeabilizing with PFA, this is done with ice-cold PBS to quench the PFA fixation, which is a poor fixative at cold temperatures.

Permeabilization

Following fixation, the crucial next step in the ICC workflow is cell permeabilization, which allows antibodies to access their target antigens within the cell. Various approaches to cell permeabilization exist, each with its advantages and limitations. This section of the paper aims to explore different methods of cell permeabilization after fixation in ICC and discuss why one approach may be better suited than another.

Triton X-100 and NP-40 are non-ionic detergents commonly employed for cell permeabilization. They effectively disrupt the plasma membrane by solubilizing lipids and creating transient pores. These detergents are efficient in permeabilizing most cell types, enabling antibodies to penetrate the cell and bind to target antigens. They also partially dissolve the nuclear membrane, making them suitable for nuclear antigen staining. Typically, they are used at concentrations ranging from 0.1% to 0.2%, and in some cases up to 0.5%, for a duration of 10 to 30 minutes. However, it is important to consider that Triton X-100 and NP-40 are relatively harsh detergents. When used at higher concentrations or for prolonged incubation times, they may lead to the loss of soluble cytoplasmic and membrane proteins, perturbation of cellular structure, and potential alterations in antigen distribution and cellular integrity.

Saponin and digitonin are milder membrane solubilizers compared to Triton X-100 and NP-40. They can be used at concentrations of 0.2% to 0.5% for up to 30 minutes. These detergents create pores of sufficient size for antibody penetration without dissolving the plasma membrane. Saponin and digitonin bind to cholesterol in the plasma membrane, forming pores that serve as gateways for antibody entry. This approach is particularly advantageous when preserving delicate cytoplasmic structures and maintaining the integrity of intracellular membranes is critical for accurate analysis. However, it is worth noting that these detergents may be less effective in permeabilizing certain cell types compared to other permeabilization agents. One significant advantage of saponin and digitonin is their selective permeabilization of the plasma membrane while leaving intracellular membranes intact. It is important to emphasize that saponin and digitonin are not recommended for permeabilizing the nuclear membrane.

Tween-20 is a mild detergent that is occasionally used for permeabilizing fixed cells but is more commonly employed in blocking and binding buffers to help prevent non-specific antibody binding.

Fixation with organic solvents such as methanol or acetone can also achieve cell permeabilization in ICC. These fixatives extract lipids from the plasma membrane, creating openings for antibody penetration. Methanol fixation is often preferred for intracellular antigens, while acetone fixation is effective for both surface and intracellular antigens. However, this approach can result in protein denaturation and precipitation, leading to reduced antigenicity and potential loss of fine cellular structures. Although fixation with organic solvents permeabilizes cells, it may be necessary to further permeabilize organelles with detergents, such as Triton X-100. Conversely, fixation with PFA, followed by permeabilization with an organic solvent, may be optimal for some antigens.

For robust permeabilization of most cell types, Triton X-100 is often a reliable choice. It provides broad permeabilization and is suitable for general immunolabeling. However, if the preservation of delicate cellular structures or intracellular membranes is a priority, saponin permeabilization may be preferred. Organic solvent-based permeabilization methods like methanol or acetone fixation offer simplicity and compatibility with a wide range of antibodies but should be used cautiously to avoid protein denaturation.

The choice of cell-permeabilization method in ICC depends on multiple factors, including the nature of the target antigen, primary antibody, cell type, desired preservation of cellular structures, and experimental objectives. While no single method is universally superior, the considerations described above can guide the selection

process. The various detergent-based and organic solvent-based permeabilization methods all have their advantages and limitations. We recommend testing these various approaches empirically, ensuring optimal visualization of target antigens and reliable data acquisition.

Blocking

Blocking buffers are formulated to saturate non-specific binding sites, ensuring that the primary antibody exclusively targets the desired antigen, thereby minimizing background staining. These buffers typically contain serum proteins and non-ionic detergents dissolved in a balanced salt solution. Commonly used salt solutions for blocking include phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline (DPBS), or Tris-buffered saline (TBS). The pH and ionic strength of the binding buffer play essential roles in maintaining the stability of antibody-antigen interactions. For most immunocytochemistry experiments, it is generally recommended to use a slightly alkaline pH (around 7.4) and physiological ionic strength, such as PBS, to optimize antibody binding specificity.

To prevent non-specific binding, bovine serum albumin (BSA) is commonly utilized. However, a more effective approach involves utilizing serum from the same species in which the secondary antibody was raised. This takes advantage of endogenous immunoglobulins present in the serum, which can occupy non-specific binding sites on the cells. Notably, you should not use serum from the same species in which the primary antibody was raised because your secondary antibody will bind both the intended primary antibody and antibodies in the blocking serum, creating very high background. In addition to serum proteins, low concentrations of mild detergents like Tween-20 can further reduce non-specific binding.

The duration of the blocking step may vary but typically ranges from 20 minutes to one hour before applying antibodies to cells in a binding buffer. It is crucial to ensure effective blocking to minimize background noise. However, it is important to avoid excessive blocking, as it can interfere with specific antibody binding, leading to a reduction in signal strength.

Antibody Binding

Antibody binding is a crucial step in immunocytochemistry (ICC) assays. In the conventional approach, primary antibodies specifically recognize the target antigen, while secondary antibodies conjugated with fluorophores enable detection. It is important to choose validated primary antibodies for ICC and determine the optimal antibody concentration for the target of interest. It is recommended to assess primary antibody specificity using appropriate controls.

One essential control is to bind the fluorophore-conjugated secondary antibody without the primary antibody. This control confirms that observed staining is not due to non-specific binding of the secondary antibody. More sophisticated controls can involve binding the primary antibody in a cell line known to express the target protein abundantly and a cell line lacking the target protein (a knock-out cell line). Such controls provide valuable insights into binding specificity. Some antibody vendors demonstrate specificity by showing lack of binding in a knock-out cell line. Additional controls involving pharmacological or other perturbations can also be useful for assessing antibody binding specificity and validating assay readouts.

An alternative and simplified approach to ICC involves using a primary antibody conjugated directly to a fluorophore, eliminating the need for a secondary antibody. However, it is important to note that many fluorophore-conjugated primary antibodies may not have been validated for maintaining optimal binding potency to their antigen targets, and the choice of available fluorophores is typically limited.

Binding buffers are designed to facilitate specific interactions between the primary antibody and the target antigen. These buffers optimize antibody-antigen binding affinity, signal strength, and signal-to-noise ratio. Binding buffers for ICC often share components with blocking buffers, including serum from the same species as the secondary antibody and a low concentration of a mild detergent like Tween-20 dissolved in a balanced

salt solution at pH 7.4. Typically, no washing is performed between blocking and antibody binding steps, and the blocking buffer is replaced with the binding buffer containing primary antibodies.

During the primary antibody incubation, it is recommended to use an orbital shaker. Incubation times may vary but typically range from one to two hours at room temperature or overnight at 4 °C. This parameter should be optimized during assay development.

Appropriate dilution of the primary antibody is critical to ensure optimal binding specificity. Too high a concentration can result in non-specific binding, while too low a concentration may yield weak or undetectable signals. Optimization experiments should be conducted to determine the optimal antibody dilution for each specific antigen and experimental setup. Secondary antibody dilutions may also require optimization depending on the abundance of the target.

At Scintillant Bioscience, we commonly use goat-derived fluorophore-conjugated secondary antibodies to bind mouse and rabbit primary antibodies. Our blocking and binding buffers typically contain 5-10% goat serum and 0.1% Tween-20, which effectively prevent non-specific binding for most antibodies and antigens.

To remove unbound primary antibodies, several wash steps are necessary between primary and secondary antibody applications. A typical protocol involves washing three times with phosphate-buffered saline (PBS) containing 0.1% Tween-20 for 5 minutes per wash. Following these wash steps, secondary antibodies are typically applied for one hour at room temperature on an orbital shaker, followed by additional wash steps before imaging, as described below.

Washing

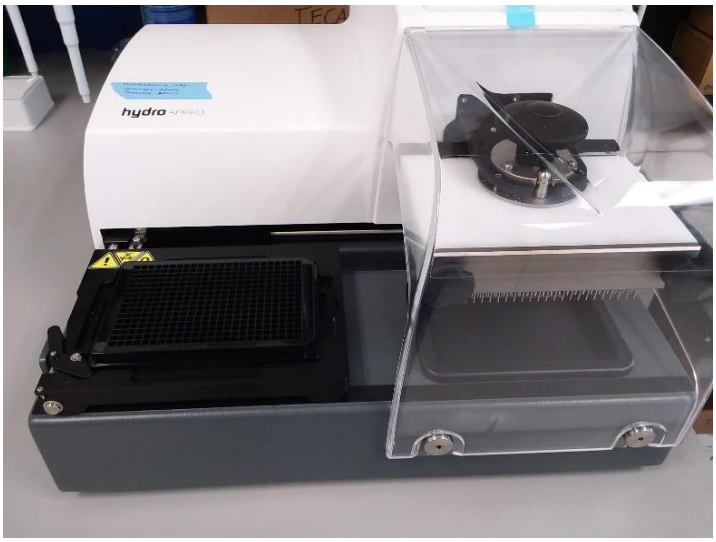
Wash buffers are used to wash away unbound primary and secondary antibodies. This step ensures that only specific antibody-antigen complexes remain bound to the cells, reducing background noise and non-specific binding. Wash buffers contribute to minimizing background staining by removing any non-specifically bound proteins or antibodies that may interfere with the specific antigen detection. Proper washing steps help enhance the signal-to-noise ratio, improving the sensitivity and specificity of the immunocytochemical staining.

Wash buffers are formulated with specific components to effectively remove antibodies and minimize non-specific binding. To maintain a stable pH during the washing process, buffering agents like Tris or phosphate-based buffers are crucial. This pH control ensures optimal interactions between antibodies and antigens while preventing non-specific binding and protein denaturation. Typically, a physiological salt solution like PBS is used as the wash buffer, often supplemented with detergents like Tween-20 or Triton X-100. These detergents aid in removing non-specifically bound proteins or antibodies by disrupting weak interactions, while still preserving the integrity of the antigen-antibody complex. The wash buffer conditions can be optimized based on experimental requirements, cell or tissue type, and the antibody-antigen affinity. Adjustments in pH, salt concentration, and detergent concentration can enhance the specificity and sensitivity of immunocytochemical staining. After removing excess secondary antibody using a detergent-containing wash buffer, it is advisable to perform two or three final washes without detergent to minimize any fluorescence background caused by the detergent in the wash buffer.

The number of washes performed is crucial for effective removal of unbound antibodies and reducing background staining. Typically, multiple washes (e.g., 3-5 times) are performed to ensure thorough removal while minimizing the loss of specific antibody-antigen complexes. The duration and intensity of washing are important considerations. Gentle agitation or shaking on an orbital shaker during the wash steps helps dislodge unbound antibodies efficiently. The duration of washing should be optimized to achieve optimal removal without excessive exposure that may lead to the loss of specific antibody-antigen complexes.

Although manual washing can be done effectively in 96-well plates, it is general not feasible to pipette solutions out of the wells in 384-well plates. Manual washing of 384-well plates is typically accomplished by dumping

solution from the wells in the plate and replacing it with wash buffer. This works but tends to dislodge cells from the plate bottom and becomes tedious and impractical when processing many plates at once.



Automated plate washers, such as the one depicted on the left, offer significant benefits in performing ICC wash steps for the 384-well format. In this format, the use of an automated plate washer is particularly advantageous. To optimize washing efficiency, it is recommended to incorporate a detergent, such as 0.1% Tween-20, in the wash buffer. In addition to the role of a detergent in helping to remove non-specifically bound antibodies, the detergent aids in reducing the surface tension of the aqueous solution. Without detergent, a wash buffer lacking surfactants can exhibit sufficient surface tension, leading to the formation of air pockets at the bottom of the well, which hinders proper washing. For automated plate washing, detergents play a role in disrupting this surface tension, allowing for more effective and thorough washing.

Conclusion

In conclusion, this white paper offers practical tips and tricks for optimizing immunocytochemistry (ICC) assays, specifically focusing on fixation, permeabilization, blocking, binding, and washing steps. It also demonstrates the application of ICC in drug-discovery assays, highlighting its effectiveness in studying protein localization and abundance in response to drug treatment.

The choice of fixative, such as paraformaldehyde or methanol, depends on the target antigen and desired cellular preservation. Various cell permeabilization methods, including Triton X-100, saponin, and organic solvents, offer different levels of permeability and cellular structure preservation. Blocking and binding buffers, containing various concentrations of serum proteins and detergents, play a crucial role in minimizing non-specific antibody binding. Finally, critical washing steps ensure optimal signal over background.

To enhance the accuracy and reliability of ICC assays, we recommend carefully testing and optimizing the experimental parameters mentioned above. We recommended arraying different experimental conditions across rows and columns of 96-well or 384-well plates, allowing for empirical identification of optimal assay conditions. With experience, this optimization process can be done efficiently in 384-well plates within two or three iterations, resulting in a high-quality ICC assay for drug discovery.

In summary, this white paper serves as a practical guide for researchers aiming to improve their ICC experiments. By understanding the significance of optimizing fixation, permeabilization, blocking, binding, and washing parameters and implementing the recommended tips and tricks, scientists can significantly enhance the success rate and quality of their ICC experiments.

Please feel free to reach out to us with questions or comments at any time.

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