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Immunological Methods for Nursing Research: From Cells to Systems

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Abstract

Scientists and clinicians frequently use immunological methods (IMs) to investigate complex biological phenomena. Commonly used IMs include immunocytochemistry (IC), enzyme-linked immunosorbent assays (ELISA) and flow cytometry. Each of these methodologies exploits a common principle in IMs—the binding of an antibody to its antigen. Scientists continue to develop new methodologies, such as high-throughput immunohistochemistry (IHC) and in vivo imaging techniques, which exploit antibody–antigen binding, to more accurately answer complex research questions involving single cells up to whole organ systems. The purpose of this paper is to discuss established and evolving IMs and to illustrate the application of these methods to nursing research.

Keywords

immunology; methods; immunocytochemistry; ELISA; flow cytometry

Researchers commonly use immunological methods (IMs) to assist them in understanding complex biological phenomena in a variety of model systems. The foundational principle of IM is antibody–antigen binding, a highly specific and intense chemical attraction that results in the affinity of an antibody for its antigen. Since its discovery, scientists have exploited antibody–antigen binding to develop numerous IMs such as immunohistochemistry (IHC), flow cytometry, and enzyme-linked immunosorbent assays (ELISA). However, IMs are only effective when the investigators making use of them understand the basic principles behind them, as these principles illuminate the assumptions that underlie each method. Our purpose in this article is to review the fundamental concepts of the antibody–antigen reaction, highlight issues investigators need to consider when designing research that makes use of IMs, and discuss the current and potential future application of IMs to nursing research. This review spans a wide range of scientific investigation, from cellular processes to system physiology.

Immunology Meets Methodology

Edward Jenner developed the first smallpox vaccine in 1796 and is credited with launching the discipline of immunology. Since Jenner's time, considerable advancements have been made in understanding the structural and functional properties of the immune system. By

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exploiting these properties, scientists have been able to apply immunological principles to develop methodologies suited to investigate complex biological phenomena.

The adaptive immune system is responsible for antibody production. An invading bacteria or nonmicrobial foreign substance, also known as an antigen, triggers the adaptive immune system to launch a specific and specialized response (Merrill, 1998; Rosner, Grassman, & Haas, 1991; Silverstein, 2004). This response occurs when lymphocytes, exposed to an antigen, release immunoglobulin (synonymous with antibodies) capable of binding specifically to the detected antigen. Researchers exploited this process in animal models to produce antibodies for a specific antigen with high antigen–antibody binding affinity (Kohler & Milstein, 1975). This seminal discovery led to the mass production of antibodies for use as manufactured reagents in commonly used IMs (ELISA, IHC, flow cytometry, etc.; Pirruccello & Aoun, 2001). Today, there are numerous antibodies available for use as immunoreagents.

Antibodies, or immunoglobulins, have a unique structure that allows them to perform their function—to recognize and bind to antigens. An immunoglobulin, traditionally depicted in a Y formation, comprises two variable arm domains (Fab) and a relatively constant (Fc) leg domain. Both antibody domains are important in IMs. The Fab domain structure is vital for the affinity and avidity of the antigen–antibody reaction, central concepts to all IMs. The amino acid composition of Fab domains is specific for the epitope—the amino acid sequence of an antigen—with which it forms an intense chemical bond. An antibody must have at least one corresponding antigen epitope to form the antibody–antigen complex necessary for most IMs; the strength of that antibody–antigen chemical bond is termed *affinity*. Varying antibody structures allow multiple binding sites, creating multiple antibody–antigen bonds. The combined and synergistic strength of these bonds is termed *avidity*. Because the number of binding sites (structure) varies across immunoglobulin subclasses (IgG, IgM, IgA, etc.), immunoglobulin avidity for an antigen of interest varies among subclasses, while affinity remains constant (Arnold, Dwek, Rudd, & Sim, 2006). An antibody's avidity and affinity for an antigen of interest are important considerations in an experimental design when selecting from the plethora of possible manufactured antibodies.

While the variable Fab region lends the antibody its specificity to the antigen of interest, the Fc region renders the antibody useful for subsequent quantification via conjugation (the joining of two elements) to chemical groups, enzymes, or fluorophores (a molecular element that is fluorescent; Woof & Burton, 2004). This conjugation is necessary for direct quantification of the antigen of interest, or instead, is recognized by secondary antibodies in indirect quantification methods. The Fc region also binds to cell receptors to amplify immune signaling and to immunomodulatory proteins, such as complement peptides, to transport antibodies across cell membranes and within body fluids (Raghavan & Bjorkman, 1996).

Commercially available antibodies may be either polyclonal or monoclonal. Polyclonal antibodies are prepared by harvesting the sera of immunized animals (Rosner et al., 1991). Contained within the sera are one or more antibodies that may react to the antigen with the desired affinity. However, polyclonal preparations also contain other nonspecific antibodies that, ultimately, affect specificity. The preparation of monoclonal antibodies is complex, yet it yields a more specific and uniform immunoreagent (Schroder, 1980). Consequently, monoclonal antibodies are likely to be more expensive compared to polyclonal antibodies. Study design does not always require a premium-priced monoclonal antibody. The researcher should consider the following when choosing an antibody for an immunoassay: (a) the type of immunoassay that will be employed, (b) the number of epitopes that are known for the antigen of interest, (c) whether cross-reactivity for similar antigens has been

reported in the literature, and (d) whether the preferred type of analysis is qualitative or quantitative. Thorough consideration of these issues may indicate the level of specificity and sensitivity a particular investigation requires and therefore the type of antibody most suited for reproducible immunoassay performance (Rosner et al., 1991). For example, one might choose to use a monoclonal antibody when quantifying a cell characteristic, such as a specific cell surface protein, epitope, or altered configuration (due to the pathophysiologic condition of interest). However, in a case where the potential for cross-reactivity or nonspecific binding is minimal, as when one is evaluating for the presence of a general class of proteins—such as the presence of all sodium channels within a cell preparation—a polyclonal antibody might be sufficient.

IMs—Past and Present

Scientists first began to exploit the antibody–antigen interaction by conjugating small chemical structures, or haptens, to antigens and antibodies, thus allowing them to characterize simple pathophysiologic conditions by describing antigen distribution and antibody diffusion (Silverstein, 2004). From this practice, scientists developed immunocytochemistry (IC) protocols, which were one of the first IMs to have a broad application in investigating biological processes. As scientists' understanding of immunology, chemistry, and biology increased in parallel with developing technologies, the early protocols of IC developed into more complex methodologies.

Advances in IM came as a direct result of advances in antibody conjugates. Coons's seminal work to develop fluorescence-conjugated antibodies (the joining of antibodies and fluorescent markers) was the cornerstone of future fluorescence-based IMs, including qualitative and quantitative fluorescence, IHC, and flow cytometry (Coons, 1961; Coons & Kaplan, 1950). Nakane and Pierce (1966) developed the enzyme-linked reaction, and Avrameas (1969) first applied it in the development of ELISA (Avrameas, 1969; Nakane & Pierce, 1966).

IC

IC is the application of antibodies to cells or tissues to identify and quantify an antigen of interest. IHC refers specifically to applications involving tissues. The earliest agents conjugated to antibodies were haptens and radioactive groups (still used today). Conjugates used more recently include fluorescent groups and enzymes, both of which significantly improve IC sensitivity. IC methods are relatively simple to actuate and require minimal specialized equipment (depending on the conjugate). In general, the sample—either plated cells or tissue section—is exposed to a conjugated antibody and allowed to incubate to maximize contact and binding to the antigen of interest. If the investigator is using a direct IC protocol, she or he then quantifies the antigen–antibody complex using the appropriate instrumentation (fluorescence microscope for fluorophore conjugates or beta/gamma counters for radioactive conjugates). If the investigator is using an indirect IC protocol, then he or she incubates a secondary antibody and/or other reagents with the sample prior to antigen quantification.

Though IC is a simple process, data collection often requires the capturing of digital images—the data. To collect meaningful data—and publishable images—it requires optimizing the experimental protocol to minimize background staining while maximizing antigen visualization. The process of optimizing a protocol begins with careful sample collection and preparation. In addition, optimization requires knowledge of all available antibody reagents (monoclonal, polyclonal, immunoglobulin type, etc.) and of the characteristics of the antigen of interest (epitopes, density, distribution, etc.). Investigators should also pay careful attention to antibody dilutions and incubation times. Though both of these variables

can often be obtained from the literature, it is usually necessary to conduct preliminary studies to validate previously published antibody dilutions, as antibody dilutions vary from laboratory to laboratory. Differences in equipment used to quantify the antigen may account for differences observed between laboratories. In addition, incubation with proteins such as albumin or animal sera is often required to reduce nonspecific binding and background fluorescence. Thus, IC can be time- and sample-consuming and may ultimately yield qualitative but not quantitative data.

IC has many applications, but it is ideally suited for identifying a particular cell type *in vitro*. Investigators may use IC to detect the presence or absence of a particular protein or to quantify a variety of phenomena in cell culture models. For example, a monoclonal antibody conjugated with a fluorophore can be used to determine the presence of VE-cadherin (qualitative), to quantify the number of cells expressing the protein, or to evaluate changes in VE-cadherin levels under experimental conditions (see Figure 1A).

IHC is a cornerstone methodology investigators use, either alone or combined with other data-collection methodologies, to generate quantitative or qualitative evidence that a phenomenon exists *in vivo*. IHC methods are applied to either frozen or paraffin tissue samples from human and animal models. Figure 1B is an example of the application of IHC to a research question aimed at determining the presence of complement peptide deposits, an indication of increased inflammatory response, in brain tissue. From the image, one can appreciate the deposition of complement peptide 3 (white) in brain tissue following ischemia and reperfusion.

ELISA

In the late 1960s, researchers developed the basic principles of the ELISA as an alternative to fluorescence IC. The ELISA became a favored alternative to fluorescence-based methodologies because it did not require dark-room processing and fluorescence microscopy (Nakane & Pierce, 1966). The ELISA, as it was first conceived and thus named, employed an enzyme-conjugated antibody specific to the antigen of interest. Figure 2 summarizes two commonly employed ELISA protocols: the direct and sandwich ELISAs. While the ELISA and IC are similar in principal—IC methods also employ enzyme-conjugated antibodies—they are distinguished from each other in their application. Whereas investigators use IC to localize and quantify antigens on plated cells or within tissue or whole organisms, they more often use the ELISA to detect and quantify antigens within fluids.

The ELISA has been improved over the years. Today, a spectrophotometer is used to quantify the colorimetric reaction (Figure 2B) and biotinylated secondary antibodies are used to amplify and speed reactions. These improvements increase specificity while decreasing experimental duration (Diacos et al., 1985; Guesdon, Ternynck, & Avrameas, 1979; Jordan, 2005). Multiple iterations of the ELISA (direct, indirect, competitive, and sandwich) can be applied to quantify antigens in fluids depending upon the antigen of interest and number of antibodies available to create the assay. For example, the sandwich ELISA greatly improves antigen specificity when two epitopes are known for the antigen of interest (Figure 2C).

The ELISA has numerous research laboratory applications. ELISA kits are readily available to quantify a multitude of cell products from cell culture supernatant and systemic antigens circulating in plasma. For example, ELISA can be used to determine cytokine production from cell cultures exposed to toxic agents. Data generated from such experiments may be useful in determining an inflammatory pathway that can be targeted for future preventative or treatment strategies.

The ELISA ensures a sensitive, specific, and repeatable assay that requires only basic lab equipment and minimal training. Thus it is both an effective and a cost-effective way to quantify an antigen of interest. A disadvantage of ELISA is that *ex vivo* sample processing may minimize nonspecific colorimetric reactions and alter the intrinsic nature of the biological sample. In addition, optimization of the sample conditions may be needed to produce meaningful data. For example, the antigen of interest may be produced in picogram quantities requiring the samples to be blocked with animal sera or albumin to reduce nonspecific binding; blocking may produce more accurate and meaningful data. Finally, the epitope for the antigen must be known and the biologic sample must be fluid.

Flow Cytometry

Flow cytometry quantifies cellular characteristics using light-scattering principles. As cells flow past a laser and series of detectors, heterogeneous cell populations are sorted, at the most basic function, by size and intracellular complexity (Figure 3). Investigators also use flow cytometry to identify and quantify cell surface markers (cluster domains [CDs]) and assess cell function (Robinson, Wayne, & Narayanan, 1997). Thus, with flow cytometry, investigators can distinguish cell populations by both general (size and intracellular complexity) and specific (CD expression) characteristics within a single sample and can compare changes in cell populations and the presence or absence of surface markers (CDs) on cells between samples.

Flow cytometry is unique among IMs because it is not limited by an assumption common to most of these methods—that a sample must consist of a single homogenous population. Thus, it is a powerful tool for measuring cellular phenomenon in a more natural context (Robinson et al., 1997). Sample processing for traditional assays (such as ELISA) may require a separation of the whole into its parts, that is, the separation of whole blood into plasma and cell products and further processing of cell products to obtain the cell type of interest (neutrophils, leukocytes, etc.). Using flow cytometry methods, however, investigators can quantify the heterogeneity of a population with minimal sample processing. Limited sample processing, with sensitivity and specificity maintained, is advantageous because it decreases the number of confounding variables entered into the study, thereby increasing experimental control while maintaining the context of the phenomenon under study.

The first flow cytometer was commercially available in 1970 and was nearly the size of a room (Shapiro, 2003). With the advent of benchtop cytometers, commercially available immunoreagents and fluidic solutions, investigators have increasingly applied flow cytology methodologies to ever more complex research questions. Flow cytometry protocols now encapsulate both cell-sorting and cell-function assays. Investigators use flow cytometry to assess how dynamically changing cell populations function after acute injury and disease and how a subset of cells function within a larger population. For example, investigators often use this method to quantify circulating neutrophil function, describe alterations in systemic neutrophil populations, or determine the functionality of changing neutrophil populations (Robinson et al., 1997). To assess neutrophil function, investigators may use flow cytometry to quantify oxidative burst, secretion of granular products (lactoferrin), and pro-inflammatory cytokines. Researchers accomplish systemic assessment of neutrophil activation by measuring surface expression of CD11b on circulating neutrophils (Maes, Davidson, McDonagh, & Ritter, 2007; Morrison, McKee, & Ritter, 2010; Robinson et al., 1997) and quantify relative changes over time in the neutrophil population in response to disease and injury using a repeated measure design. Flow cytometry can also be used to describe the occurrence of circulating cell–cell interactions (e.g., neutrophil–platelet; Ritter, Stempel, Coull, & McDonagh, 2005), which can be useful diagnostic indicators of acute and harmful inflammatory responses after injury or disease.

Correct application of flow cytometry requires sophisticated instrumentation and thorough training and is therefore a less accessible methodology as compared to IC and ELISA. An investigator must fully understand the assumptions and limitations underlying this methodology (reviewed well in Chapter 7 of Shapiro, 2003) to prevent improper data interpretation. Because acquisition and analysis of flow cytometry data are dependent on protocol and cytometer, it is necessary to consider data within the context of the reported methods and is not wise to directly compare findings across dissimilar laboratories, as protocols may differ. Investigators must also be thoroughly familiar with the function and maintenance of a cytometer. Unlike with plate-based assays, malfunctions in cytometers, and resulting invalid data, can be insidious.

The Future of IMs

Even though IMs are constantly evolving within laboratories or as a result of IM reagents' manufacturer protocol development to address increasingly complex research questions in a variety of model systems, they continue to be limited by target specificity and sensitivity and the ex vivo setting. Improvements in these elements would significantly improve diagnostic and laboratory research endeavors (Silverstein, 2004). Recent advancements in imaging, computer technology, and bioengineering have improved immunological assays. For example, the recently developed high-throughput tissue IHC microarrays and antibody-based contrast agents are assisting researchers to investigate biological phenomena without some of the limitations or disadvantages of traditional IC and IHC methods.

High-throughput tissue microarray (TMA) increases the power of IHC quantitative analysis by decreasing assay protocol variables (i.e., reagent lot, technician error, and day-to-day changes) inherent in traditional IC and IHC methods (Watanabe, Cornelison, & Hostetter, 2005). The power of TMA is derived from its single-slide platform on which the investigator places hundreds of identically oriented and sequential samples in parallel for analysis of multiple molecular markers. The single slide containing a multitude of samples allows the investigator to standardize and optimize the IHC method, thus decreasing variability relative to multi-slide techniques. In addition, data analysis for TMA staining is automated, driven by integrated imaging and computer technology, which also affects variability. Investigators are currently using TMA to validate genomic array studies, test for novel biomarkers, and assess therapeutic responses to pharmaceutical agents (Mobasheri, Airley, Foster, Schulze-Tanzil, & Shakibaei, 2004; Watanabe et al., 2005).

Alterations in the cell environment may occur when cells are isolated from a tissue preparation. This isolation may result in a change in cellular processes that would not have occurred in vivo. Advances in in vivo imaging are under development to allow investigators to more accurately determine cell and systemic response to disease and injury. Future contrast agents will combine immunoglobulins and nanoparticles to increase antigen specificity and access for in vivo identification and tracking of biological markers and disease processes within whole systems or organisms (Minchin & Martin, 2010; Reynolds et al., 2006). A promising research development is the coupling of superparamagnetic iron oxide (SPIO) with monoclonal antibodies. Using the SPIO–antibody contrast, Reynolds and colleagues (2006) demonstrated that the expression of endothelial selectin (E-selectin), an inflammatory biomarker, can be selectively imaged using noninvasive magnetic resonance imaging (MRI) in vivo. The SPIO–antibody conjugate greatly improved sensitivity and specificity over their radio-labeled antibody conjugate predecessors while also minimizing potentially harmful side effects. Nanoparticles, because of their small size, also increase the access of imaging agents to tissues and across controlled membranes (Minchin & Martin, 2010; Reynolds et al., 2006). Nanotechnology may thus allow for the determination of cell responses within an entire organism, a future development that has implications for the

translational process of research into human disease, as animal and in vitro models of disease may, at some point, no longer be needed.

IMs in Nursing Research

A thorough understanding of health and disease (pathophysiology) is imperative for the development of novel interventions to ameliorate a disease and its sequelae. IMs are important tools that nurse scientists use to investigate cellular and whole-system responses to disease and injury. Nurse scientists have used IMs as an investigative tool in a variety of research designs based on a broad spectrum of philosophical perspectives. While some scientists utilize a purely positivist paradigm, others make broader inquiries into health phenomena by using a mixed-methods research design in which they employ IMs in combination with psychometric testing or qualitative methods to explore health phenomena from multiple perspectives.

Nurse scientists may use IC to assist in the development of cell-culture model systems that can later be used to investigate disease processes (Merkle et al., 2005). They have also employed IHC to assess cell responses in tissues harvested from animal models of human disease processes (Briones, Suh, Hattar, & Wadowska, 2005). Using ELISA techniques, nurse scientists have quantified biomarkers in biologic fluids and then correlated these quantities with subject responses to questionnaire-type instruments that assess pathophysiologic human conditions (e.g., depression, stress, and postpartum fatigue; Groër et al., 2005; Kuo, Yang, Wang, Chan, & Chou, 2010; Thompson et al., 2004).

Flow cytometry has provided nursing science with biological measures to quantify cellular and tissue responses to injury, disease, and healing processes as well as to complement behavioral research—for example, to quantify immune responses while better understanding emotional responses in an Alzheimer's disease spousal caregiver population (Thompson et al., 2004), to describe cell responses during wound healing while employing wound healing products (Tsuji, Whitney, Tolentino, Perrin, & Swanson, 2010), and to describe cell responses after ischemic stroke and reperfusion (Morrison et al., 2010; Ritter et al., 2005).

The development of new IMs such as antibody-conjugated nanoparticles will move investigative research into biological phenomena forward by expanding the capabilities of in vivo and human research and by increasing sensitivity and specificity. As new methods become available more nurse scientists will apply these new IMs to complex research questions.

For nurse scientists wanting to learn more about incorporating IMs into research design or for novice researchers with an interest in IMs, there are many resources available. First, these scientists may network with other nurse scientists focused on biological measures or consider an interdisciplinary approach to network within departments of physiology and colleges of medicine in order to explore potential collaborative projects or to gain direct experience in performing IM techniques. Second, they should explore the plethora of methodology texts, tutorials, and webinars available within university libraries, university core services, and IM vendor websites. Third, the National Institute of Nursing Research (NINR) at the National Institutes of Health (NIH) offers training opportunities related to specific NINR initiatives that may provide hands-on training in IMs. Finally, they should consider collaborating with a federally funded clinical translational science center, as these centers comprise consortiums of scientists with the goal of translating bench science into clinical research.

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Figure 1.

Immunocytochemistry in prepared cell and tissue samples. A. Endothelial cells labeled with a primary antibody for VE-cadherin; a fluorescein (FITC)-conjugated secondary antibody is then coupled to the primary VE-cadherin antibody to produce a fluorescent image depicting VE-cadherin localization (arrows) in endothelial cells. Bar = 10 microns. B. Using a direct immunohistochemistry staining protocol, an FITC-conjugated antibody specific to the C3 epitope was used to produce a fluorescent image of C3 deposits (arrows) in brain tissue. Images can be used to answer qualitative or quantitative questions. Bar = 10 microns.

Figure 2.

The enzyme-linked immunosorbent assay (ELISA) method. A. Direct ELISA: Samples are added to wells of a microtiter plate where antigens bind to the surface of the plate. Sample wells are rinsed to remove excess and an antibody–enzyme complex is added to bind to the antigen of interest. Following incubation, excess antibody–antigen complex is removed with washing, and a colorimetric substrate is added. The addition of the colorimetric substrate results in a color change. B. Samples are loaded into the spectrophotometer, which measures the amount of color (optical density [OD]) of each sample. OD is directly related to the quantity of antigen present in the biologic sample (Jordan, 2005; Nakane & Pierce, 1966). Actual concentrations can be deduced through the use of a standard curve in which known concentrations are linearly related to the reported OD. Unknown sample quantities are then calculated based on the standard curve using a simple calculation (Jordan, 2005). C. Sandwich ELISA: Two antibodies are employed when two epitopes are known for the antigen of interest, increasing specificity for the targeted antigen. An antibody is added to sample wells and allowed to adhere. Excess antibody is removed and the antigen of interest (from a sample) is added and allowed to incubate. An antibody–biotin-conjugated enzyme

complex, with a different epitope for the antigen of interest, is added and allowed to incubate. Excess antibody is removed with washing, and a colorimetric substrate is added. The biotin-conjugated second antibody, which can be applied to any ELISA protocol, then interacts with the enzyme and colorimetric substrate to produce the colorimetric reaction. The biotin amplifies the colorimetric reaction due to its multiple high affinity interactions with the enzyme, thus greatly improving ELISA sensitivity at low antigen concentrations. The OD can then be assessed using a spectrophotometer as in B (Jordan, 2005). Ag = antigen present in biologic fluid; B = biotin; E = enzyme; OD = optical density.

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Figure 3.

Using laser scatter and antibody conjugates to characterize cell populations. A. As cells pass through the fluidics system of the flow cytometer, they are directed into a single cell stream and pass through a laser. The laser light that passes over the cell and scatters forward is used to assess relative cell size and is termed forward scatter (FSC). Light that scatters off the intracellular organelles in all directions is termed side scatter (SSC). Data related to the size and intracellular complexity of the cells can be recorded and displayed as a scatter plot (C) and used to distinguish different cell populations. B. To measure the presence of extracellular markers, called cluster domains (CDs), cells are first incubated with a specific fluorophore-antibody conjugate during which they bind to their respective CDs. As the cell and attached antibody pass through the laser, the fluorophore is excited and energy is released at a specific wavelength. The resulting light emission (fluorescence intensity) is quantified and recorded for each cell and can be reported as a histogram (C). C. Flow cytometry data output: cell scatter plot and fluorescence histogram.