

Antibodies, Fluorescent Markers and Their Conjugates. Preparation and Purification of Fluorescently Labeled Antibodies

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Abstract: Immunoassays are highly selective bioanalytical methods that detect or quantify analytes using antibodies or antigens as biorecognition elements. In fluorescent immunoassays, fluorescent probes serve as sensitive and versatile detectable signals, enabling quantitative and multiplexed analysis. The performance of these assays critically depends on the properties of the antibodies, fluorescent markers, and their conjugates. This review summarizes both classical fluorescent dyes, derived from cyanine, fluorescein, rhodamine, and coumarin, which established the foundations of immunofluorescence, and modern fluorophores developed over the last decade with improved photostability, brightness, and spectral definition. Particular emphasis is placed on chemical strategies for the preparation of antibody-fluorophore conjugates, as well as on purification and validation methods that ensure conjugate stability and preservation of antigen-binding activity. Recent advances in fluorophore development and conjugation approaches relevant to diagnostic and biomedical applications are also discussed, highlighting the transition from traditional random labeling methods to more controlled and reproducible practices.

Keywords: Antibody, Fluorophore, Conjugates.

Introduction

Fluorescence immunoassay (FIA) is a simple, rapid, and sensitive technique used to measure a wide range of analytes, including drugs, hormones, proteins, and cells. Immunofluorescence analysis has historically relied on monoclonal antibodies (MAbs) modified by derivatization with fluorescent labels [6]. The coupling of fluorescent moieties to antibodies, first reported by Coons and collaborators more than six decades ago [10, 11], represents a foundational milestone in biological sciences and medicine. These early studies established the feasibility of antibody-based fluorescence detection and laid the groundwork for subsequent methodological developments.

In classical labeling strategies, a succinimidyl-ester functional group is attached to a fluorophore core, conferring reaction specificity toward primary amines. However, the presence of multiple lysine residues, including those located near or within antigen-binding regions, often leads to heterogeneous labeling and may compromise antigen-binding activity [17, 19]. Recognition of these limitations has driven the development of improved conjugation strategies.

Monoclonal antibodies represent the largest and fastest-growing class of biologics, with expanding applications in diagnostics, targeted therapy, autoimmune disorders,

and oncology, largely driven by advances in antibody engineering and production technologies [20]. In parallel, significant progress over the last decade in fluorophore chemistry has resulted in dyes with enhanced photostability, brightness, and extended spectral coverage [21, 22, 30, 35, 45, 53, 61]. These developments, combined with modern detection systems of increased sensitivity, have positioned fluorescence-based assays as a robust and increasingly preferred alternative to radioactive probes [63]. Compared with radioisotopes, fluorescent dyes offer lower cost, ease of handling and disposal, comparable sensitivity, and the ability to perform multicolor labeling – an essential requirement for multiplexed immunoassays and high-content analysis [63].

In this review, the main methods for obtaining antibody – fluorescent dye conjugates are described, together with purification and characterization strategies, with clear distinction between historically established approaches and current best practices.

Antibodies and fluorescent markers

Antibodies

The primary component of a fluorescence immunoassay for cell diagnostics is the fluorescently labeled antibody. All immunoassays rely on the highly specific recognition between an antigen (Ag) and an antibody (Ab), mediated by weak non-covalent interactions such as hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic interactions [65]. These principles represent a fundamental immunochemical framework that remains unchanged in contemporary assay design.

Antibodies are immunoglobulin (Ig) serum glycoproteins produced by the immune system against foreign materials. Although five major classes of antibodies exist (IgA, IgD, IgE, IgG, IgM), approximately 80% of total immunoglobulins in human serum are IgG, which are therefore most commonly used in immunoassays (Fig. 1).

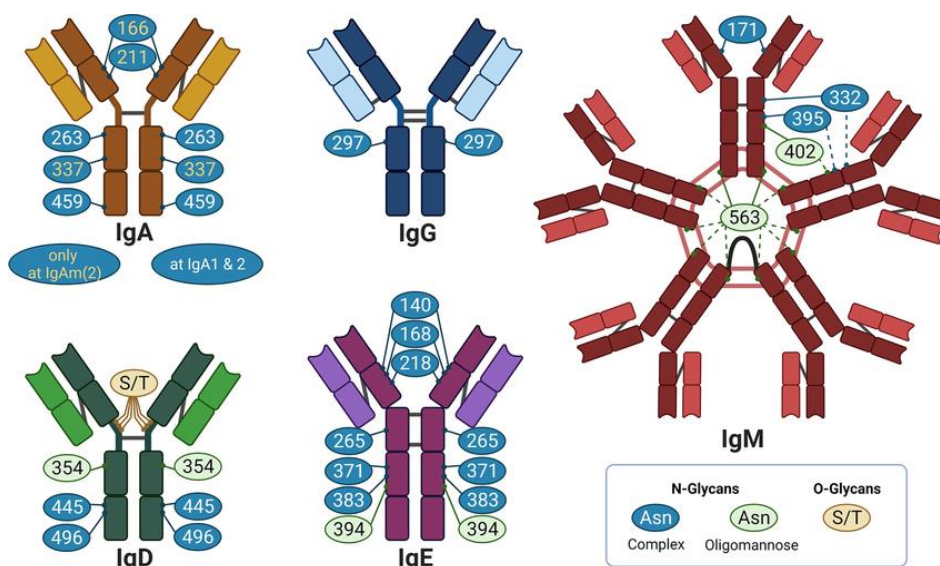


Fig. 1 Overview of different Ig: IgA, IgG, IgM, IgD, and IgE in individual colors. The bright color illustrates the light chain, the dark color the heavy chain. The grey lines indicate disulfide bridges. Created with BioRender.com [69].

Antibodies used in immunoassays can be monoclonal or polyclonal. Monoclonal antibodies (mAbs) are homogeneous immunoglobulin populations recognizing a single epitope, typically

produced from mouse or rabbit hybridomas (Fig. 2), [71]. These classical production methods remain widely used; however, recent advances increasingly favor recombinant antibody technologies that offer improved reproducibility and molecular control. Polyclonal antibodies (pAbs), while more tolerant to antigen variation, exhibit greater heterogeneity and an increased risk of cross-reactivity.

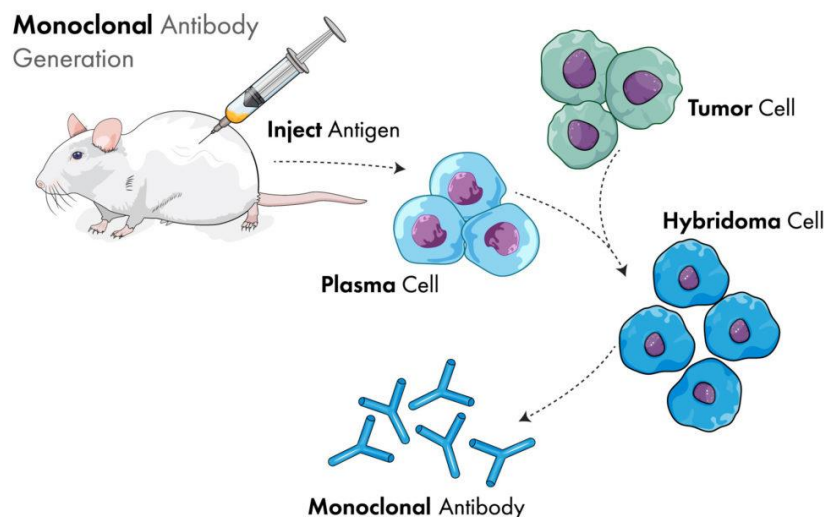


Fig. 2 Production of monoclonal antibodies.

The generation of monoclonal antibodies is shown through the fusion of B cells from an animal (e.g., a mouse) with myeloma cells, forming hybridomas that produce homogeneous antibodies against a specific epitope of the antigen [71].

Immunoglobulin G (IgG) antibodies are predominantly employed in immunological tests. Their basic structure consists of two identical heavy and two identical light chains connected by disulfide bonds (Fig. 3). The antigen-binding site is formed by the variable regions of the heavy and light chains, particularly within the complementarity-determining regions (CDRs). The Fc region, although not involved in antigen binding, mediates important effector functions.

While full-length IgG molecules remain widely used, recent studies demonstrate that recombinant antibody fragments, such as Fab and single-chain variable fragments (scFv), retain antigen-binding activity while offering advantages including reduced molecular size, improved tissue penetration, and enhanced compatibility with site-specific fluorescent labeling strategies [3]. These developments illustrate the shift from traditional antibody formats toward engineered constructs optimized for modern fluorescent assays.

Fluorescent markers

An important component of fluorescent immunoassay for cell recognition and counting is the fluorescent marker of the specific antibody. Fluorescent molecules, also called fluorochromes or fluorophores, absorb light at a precisely defined wavelength (absorption) of the visible and ultraviolet regions and subsequently emit light at a greater, lower-energy wavelength (emission). Fluorochromes are characterized by their absorption and emission spectra [4, 5]. The most intense fluorescence (emission) occurs when the molecule is irradiated with a wavelength close to the peak of the absorption spectrum. Each dye is characterized by precisely defined wavelengths for absorption and emission. The ratio between the emitted and absorbed photons is called *quantum yield*, and it generally has values in the interval 0÷1.

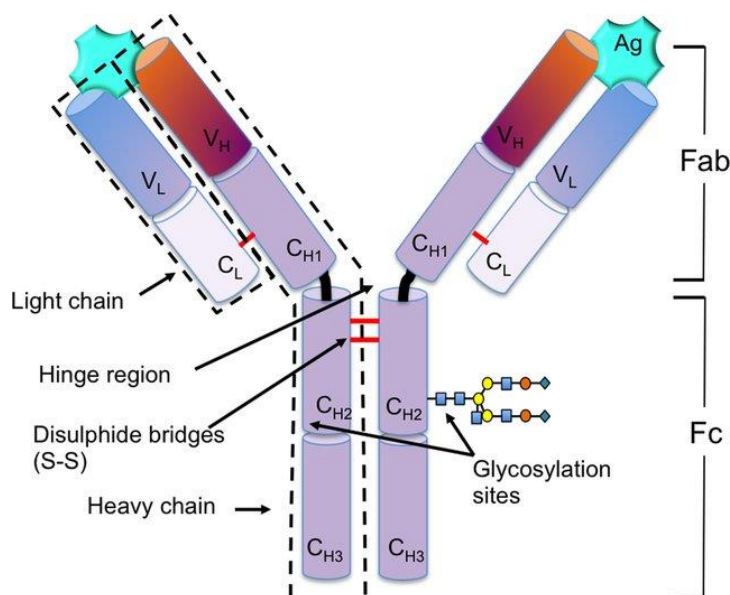


Fig. 3 Immunoglobulin G (IgG) antibody structure.

An antibody consists of two identical heavy chains and two identical light chains linked by disulfide bonds. The variable regions of the heavy and light chains form the antigen-binding site (Fab), whereas the constant regions of the heavy chains constitute the Fc fragment, which is responsible for effector functions such as complement binding [2].

The higher the value of the quantum yield, the more efficient the emission process is. To be used as a label for molecular tagging, a fluorescent dye must exhibit high photostability, minimal environmental sensitivity, high quantum yield and a large extinction coefficient, parameters that critically determine assay sensitivity and reproducibility [53]. The most commonly used fluorescent dyes are derivatives of *cyanine*, *fluorescein*, *rhodamine*, and *coumarin* (Table 1), [7, 9, 12, 14]. Fluorescein isothiocyanate (FITC) finds extensive application among the *fluorescein* derivatives. It is used for labeling antibodies [15], in histological experiments for typing the blood-brain barrier [16], for detecting proteins after electrophoretic separation [18], for microsequencing of proteins and peptides [23], in the analysis of molecules by capillary zone electrophoresis and for tracking and detection of molecules involved in various biointeractions. It is highly reactive toward amino, amide, sulfhydryl groups, and tyrosine residues [24, 25]. The most stable conjugate is obtained when the dye interacts with the primary amino groups of the antibody. The isothiocyanate group is relatively stable in an aqueous solution only for a short time, after which it degrades. Upon storage, FITC loses its activity. *Cyanine dyes* are composed of 2 aromatic rings connected by a polymethine bridge. Typically, the names of the dyes are accompanied by a number that corresponds to the number of carbon atoms in the polymethine chain (tricyanine – Cy3, pentacyanine – Cy5, etc.). The longer the chain, the higher the wavelengths of absorption and emission. For example, Cy3 absorbs and emits light at wavelengths above 500 nm, Cy5 – above 600 nm, and Cy7 – above 700 nm. To make them more soluble and prevent interaction between the individual dye molecules, sulfo groups are introduced to the aromatic ring of the cyanine dyes. Cyanine dyes are a mixture of several isomers with different properties. Since the equilibrium between the isomers depends on temperature or other environmental factors, the absorption and emission of such dyes are not precisely defined. In contrast, modern fluorophores developed over the last decade, such as ATTO dyes, exhibit high photochemical stability and well-defined spectral properties largely independent of solvent conditions [61]. ATTO dyes are derivatives of *coumarin* and

rhodamine, and contain different functional groups for conjugation: dyes with a carboxyl and an NHS-ester group conjugate with the amino group of the bioagent, dyes with a maleimide group conjugate with thiol groups, and biotin derivative dyes conjugate with avidin or streptavidin labeled compounds, etc.

Table 1 Properties and applications of some the most commonly used fluorescent dyes

Fluorescent dye	Absorption, (nm)	Emission, (nm)	Application	Reference
Propidium Iodide (PI)	535	617	Staining of dead cell nuclei	[27, 46]
Thiazole Orange (TO)	508	530	Staining of nuclei of living and dead cells	[28, 51]
TO3CN	548	604	Nucleic acid staining	[31, 47, 68]
Peridinin-Chlorophyll-Protein (PerCP)	483	676	Flow cytometry, multicolor panels	[32, 52]
Fluorescein isothiocyanate (FITC)	495	518	Protein and antibody labeling	[34]
ATTO620 NHS ester	620	642	Quantitative protein labeling	[60]
R-phycoerythrin (PE)	546	578	Protein labeling, flow cytometry	[36, 52]
dR110	491	518	Staining of dead cell nuclei	[37, 64]
7-AAD	546	647	Live/dead discrimination	[40, 46]
Alexa Fluor 647	650	668	Immunofluorescence, flow cytometry	[42, 52, 62]
Alexa Fluor 750 (NIR)	749	775	<i>in vivo</i> imaging, multiplex assays	[47, 49, 52, 62]
DyLight 650	654	673	ELISA, Western blot, IHC	[43, 52, 62]
DyLight 800 (NIR)	783	812	Quantitative Western blotting	[43, 52, 62]
IRDye 680RD (NIR)	680	694	Two-color Western blotting	[58]
IRDye 800CW (NIR)	774	789	<i>in vivo</i> imaging, protein quantification	[58]
CF647	650	668	Immunofluorescence microscopy	[48, 59]
CF750 (NIR)	755	777	<i>in vivo</i> imaging, low background	[48, 59]

* NIR - near-infrared fluorophores

Phycoerythrin (PE) has a molecular mass of 240 kDa and is a highly water-soluble fluorescent protein derived from cyanobacteria and eukaryotic algae. Its conjugates are often used in flow cytometry and immunoassays [26]. These fluorescent markers are characterized by strong absorption, a high fluorescent quantum yield, exceptional photostability, and good water solubility. In a single sample, fluorochromes can be used individually or in combination with others to create diverse, contrasting fluorescent staining with clearly differentiated components.

Near-infrared (NIR) fluorophores (650-900 nm) have gained particular importance due to reduced tissue autofluorescence and deeper light penetration, making them especially suitable for *in vivo* imaging [70], flow cytometry, and multiplexed immunoassays. Widely used contemporary fluorophores include Alexa Fluor, DyLight, IRDye, and CF dyes, which combine high brightness, narrow spectral bands, and excellent photostability (Table 1), [42, 43, 47, 49, 52, 58, 59, 62].

Alexa Fluor dyes (Thermo Fisher Scientific) cover a wide spectral range – from visible to near-infrared regions (e.g., Alexa Fluor 647, 680, 750). They are widely used for antibody labeling, immunofluorescence microscopy, flow cytometry, and super-resolution microscopy due to their high brightness and resistance to photobleaching [42, 47]. DyLight dyes are structurally optimized cyanine dyes, showing improved stability and lower pH sensitivity compared to classical Cy dyes. They are primarily applied in Western blot analysis, ELISA, immunohistochemistry, and fluorescent gel scanning [43, 52]. IRDye fluorophores (LI-COR Biosciences) are typical NIR dyes (e.g., IRDye 680 and 800), specifically designed for quantitative immunoassays, dual-channel Western blot detection, and *in vivo* optical imaging [58]. Their NIR spectra allow simultaneous detection of multiple targets with minimal signal overlap. CF dyes (Biotium) represent improved alternatives to Alexa Fluor and Cy dyes, combining high brightness, good water solubility, and chemical stability. They are used for fluorescent labeling of proteins, nucleic acids, cellular structures, and antibodies, including in multichannel and quantitative analyses [48, 59].

The adoption of these modern dyes significantly expands the analytical capabilities of immunofluorescent methods, enabling higher sensitivity, improved multiplexing, and more reliable quantitative measurements compared with classical fluorophores [66].

Methods for preparation and purification of antibody-fluorescent marker conjugates

An important prerequisite for the preparation of a conjugate is that the link between the antibody and the marker must be a strong chemical bond, as the conjugate is stored and used repeatedly over a relatively long period of time. Furthermore, they are subjected to separation and purification operations such as gel chromatography, high-performance liquid chromatography, etc., which require a strong antibody-fluorescent marker bond. Thiol and amino groups are the only two naturally occurring functional groups in antibodies that are routinely exploited for covalent modification in aqueous media; however, conventional amine-targeted approaches often result in heterogeneous labeling, prompting increasing interest in site-specific conjugation strategies [1, 3, 8]. Although the thiol group is the easiest functional group to modify with high selectivity, amino groups are the most frequent target for antibody modification. Virtually all proteins have lysine residues, and most have free terminal amino groups. The lysine ϵ -amino group is basic in nature and reacts with acylating reagents. The concentration of the free basic forms of aliphatic amines at a pH lower than 8.0 is very low. Therefore, the kinetics of the acylation reactions of amines by isothiocyanates, succinimidyl esters, and other reagents are highly dependent on pH. A pH of 8.5-9.5 is considered optimal for the modification of lysines [1].

Classical amine-reactive approaches, although robust and easy to implement, often produce heterogeneous conjugates with limited control over labeling sites. Consequently, current best practices increasingly emphasize site-specific conjugation strategies, which improve reproducibility and preserve antigen-binding activity [1, 3, 8].

Methods for preparation of antibody – fluorescent dye conjugates

The carbodiimide method is one of the most widely used classical approaches for conjugating carboxyl and amino groups. Despite its historical importance and simplicity, the method suffers from limited control over reaction specificity and labeling stoichiometry. Carbodiimide is active at low pH. For the amino group of the antibody to react, it is necessary for it to be in its non-protonated form. Consequently, the pH of the solution must be alkaline to achieve a high concentration of non-protonated amino groups. Since these facts are contradictory, it is accepted that the reaction should be carried out at pH 5-6 [13]. Furthermore, the use of acetate buffer should be avoided as it reacts with the carbodiimide. Activation of the carboxyl group of the fluorescent marker (Flu) leads to the formation of an active intermediate compound, which subsequently reacts with the amino group of the antibody. The most commonly used carbodiimides are dicyclohexyl carbodiimide and the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Fig. 4).

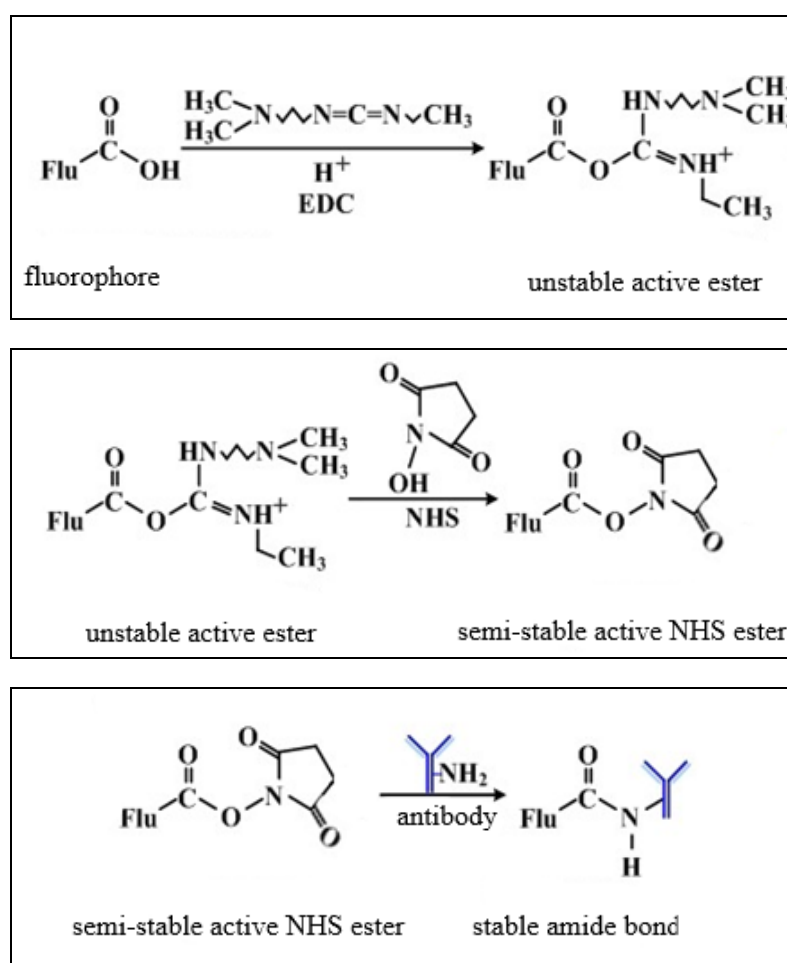


Fig. 4 Conjugation of an antibody with a fluorescent dye using the carbodiimide method (Adapted and modified from [33])

Since activation with carbodiimide replaces the negative charge of the marker's carboxyl group with a positive one, this can lead to repulsion of the antibody molecule. Furthermore, water-soluble carbodiimide esters are unstable, which can lead to hydrolysis and regeneration of the carboxyl group. Activation with a mixture of carbodiimide (EDC) and N-hydroxysuccinimide (NHS) leads to the formation of a relatively stable NHS-ester intermediate, which reacts efficiently with amino groups to form amide bonds (Fig. 4);

despite its simplicity, this method may affect antibody functionality due to random modification and limited control over fluorophore-to-protein ratio [8, 53].

Reaction with Isothiocyanates. Isocyanates (R–NCO) have not found application as reactive compounds due to their high reactivity toward water and OH-containing compounds [29]. Isothiocyanates, R–N=C=S, are moderately reactive but are more stable in water and other solvents. However, its susceptibility to hydrolysis and potential impact on antibody activity have prompted the development of alternative labeling chemistries (Fig. 5).

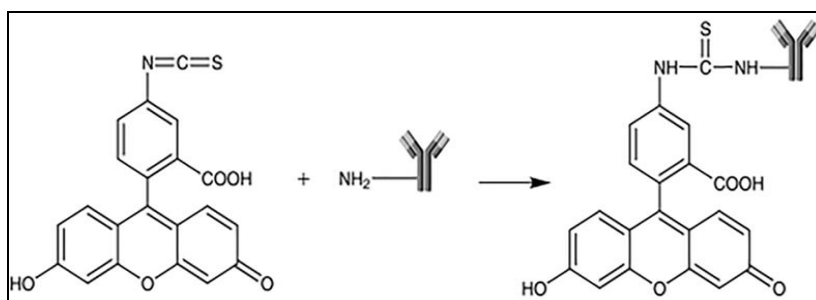


Fig. 5 Direct conjugation of FITC to an antibody
(Adapted and modified from [38])

The main drawback of the **glutaraldehyde method** is the potential formation of antibody and dye polymers, which can negatively affect assay performance and reproducibility; recent studies emphasize strict control of reaction conditions to minimize these side reactions [39]. Glutaraldehyde has two aldehyde groups, one of which reacts with the amino group of the antibody and the other with the amino group of the dye, forming Schiff bases. The reaction is conducted in an alkaline medium (Fig. 6). Since Schiff bases are unstable, the bond can be stabilized by adding a reducing agent such as NaBH₄, NaCNBH₃, and others.

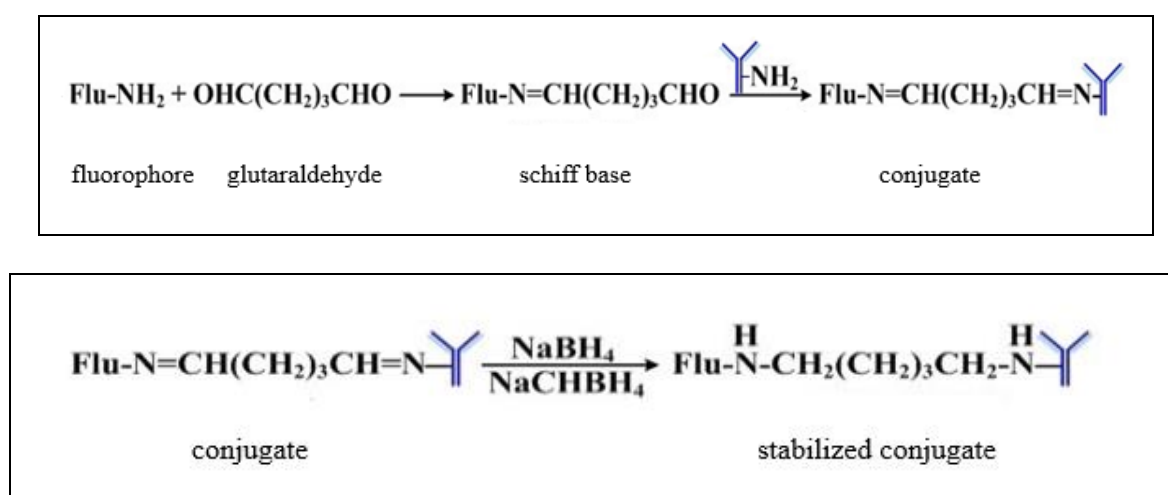


Fig. 6 Conjugation of an antibody with a fluorescent dye
using the glutaraldehyde method (Adapted and modified from [41])

The glutaraldehyde solution must be prepared fresh and must not have polymerized, which is checked by adding a few drops of water to the solution – the appearance of a precipitate or cloudiness in the solution is an indicator of polymerization. The main drawback of this method is that, besides the desired conjugate, marker polymers may also form. This can be

avoided by reducing the conjugation time, stopping the reaction by adding an excess of an amine-containing compound, and other methods. Two glutaraldehyde methods are distinguished: the **one-step method** (the reagent is added to the mixture of antibody and dye) and the **two-step method** (the antibody reacts with an excess of glutaraldehyde, the unreacted aldehyde is removed, and the fluorescent dye is added to the remaining mixture).

Glutaraldehyde-based conjugation methods are effective but prone to polymer formation and reduced reproducibility. Recent studies emphasize strict control of reaction conditions and often recommend alternative approaches for applications requiring quantitative accuracy and long-term stability [39].

Methods for purification of antibody – fluorescent dye conjugates

Following the preparation of the conjugate, purification is required, the goal of which is the removal of unreacted reagents that could interfere with the analysis, particularly the presence of chemically reactive dye. Purification of the conjugate is recommended using gel chromatography, where molecules are separated based on their size.

Gel filtration chromatography remains one of the most commonly applied methods for separating antibody-fluorophore conjugates from unreacted dyes and low-molecular-weight byproducts, although method selection should consider conjugate size distribution and labeling heterogeneity [8, 44]. The system consists of a stationary phase (gel) made of particles that have a defined pore size. A sample containing substances of different sizes (molecular masses) is introduced via a mobile phase (eluent). As they pass through the gel, each substance is retained in the column for a different amount of time. Substances with the largest molecular mass exit the chromatographic column first, while those with the smallest exit last. Smaller particles are retained longer because they enter the pores of the gel, and this delays their exit from the column. As matrices (stationary phases), Sephadex, Sephacryl, Superdex, and Superose with various particle sizes can be used.

Gel filtration chromatography remains a widely applied and reliable method for removing unreacted dyes and low-molecular-weight impurities. Classical matrices such as Sephadex and Superdex are still commonly used. However, recent advances include micropreparative gel electrophoresis and high-resolution chromatographic techniques that provide improved separation and analytical characterization of conjugates [50, 54].

Gel electrophoresis is a physicochemical process in which molecules are separated based on their size and electric charge by applying an electric current in a gel-like medium (polyacrylamide gel). HPLC is a method for separating and characterizing the composition of complex mixtures. It is based on the distribution of the components of a mixture between two phases, one of which is a liquid (mobile) phase. The other phase (the stationary phase) consists of solid particles that fill the chromatographic column. A dynamic process of adsorption–desorption occurs on the surface of the stationary phase.

Methods for characterization of antibody – fluorescent dye conjugates

One of the most important steps in the preparation of a conjugate (antibody-fluorescent dye) is its characterization (or validation), i.e., confirming the binding of the antibody to the fluorescent dye. In some cases, the conjugate may be a colored compound, which is a useful quantitative indicator of successful binding between the individual components. In other cases, it is a colorless compound, which necessitates its characterization.

UV-Vis spectrophotometry remains a fundamental tool for conjugate characterization, enabling estimation of protein concentration and fluorophore-to-protein (F/P) ratio. Contemporary analytical approaches combine classical spectroscopic methods with correction models and optimization strategies developed over the last decade [53, 55, 56]. This method can be used if the antibody has a characteristic absorption spectrum in these regions that differs from the spectrum of the dye. The degree of binding can be determined even when the antibody has a wavelength (λ_{max}) close to that of the marker. The difference in absorption between the conjugate and the starting antibody is proportional to the quantity of antibody bound.

An optimal F/P ratio, typically between 3 and 10, is critical for balancing signal intensity and preservation of antigen-binding activity [57]. Excessive labeling remains a key source of background signal and reduced assay performance.

Biotechnological processing involves the use of specific methods and often expensive consumables. This is a prerequisite for the integration of research within scientific and applied science projects, as well as joint work with similar areas [71].

Conclusion

Fluorescently labeled antibodies remain indispensable components of immunoassays, combining high selectivity with sensitive detection. Classical conjugation methods and fluorophores established the foundation of immunofluorescence but often yield heterogeneous conjugates with limited reproducibility. Modern developments over the last decade – including photostable fluorophores, near-infrared dyes, and site-specific conjugation strategies – have substantially improved assay performance.

Clear distinction between foundational methodologies and current best practices provides a framework for selecting appropriate labeling strategies for both routine diagnostics and advanced biomedical research. Future developments are expected to focus on precision conjugation, advanced fluorophore design, and integration with next-generation analytical platforms, further enhancing the reliability and applicability of fluorescent immunoassays.

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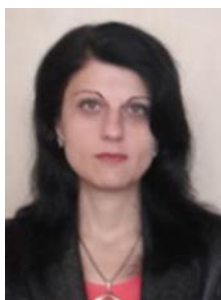
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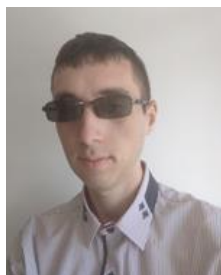
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