

A Starter Guide to Immunofluorescence Testing in Dermatology

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RESIDENT PEARL

- Direct immunofluorescence, indirect immunofluorescence, and enzyme-linked immunosorbent assay are important tests for residents to have in their diagnostic tool box, especially when evaluating patients with blistering diseases.

Immunodermatology laboratory testing, including direct immunofluorescence (DIF), indirect immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA) are powerful tools that can aide dermatologists when diagnosing autoimmune blistering diseases. Understanding these tests is important to ensure appropriate use and optimum results. This article is intended to serve as a helpful primer for immunofluorescence testing in dermatology, with an overview of the tests available as well as pragmatic tips for optimal biopsy sites and specimen transport.

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Direct immunofluorescence (DIF) is the go-to diagnostic test when evaluating vesiculobullous eruptions, connective tissue disease, and vasculitis. This specialized test allows visualization of autoantibodies and their reaction products in the epidermis and dermis (skin) and epithelium and subepithelium (mucosa). Indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA) are additional tests that can help in the diagnosis of autoimmune blistering disease. In the blistering autoimmune diseases, the autoantibodies target components in skin and mucous membranes that

are essential for cell-cell and cell-matrix adhesion causing separation within or beneath the epidermis, depending on where the target components are located. This article is intended to serve as a helpful primer for immunofluorescence testing in dermatology, with an overview of the tests available as well as pragmatic tips for optimal biopsy sites and specimen transport.

Direct Immunofluorescence

Immunofluorescence techniques date back to 1941 when Albert Coons, an American physician, pathologist, and immunologist, fluorescently labelled antibodies to visualize pneumococcal antigens in infected tissues.¹⁻³ In dermatology, similar methodology was used to visualize the deposition of immunoglobulins and complement in the skin of patients with systemic lupus erythematosus in 1963.⁴ Basement membrane zone antibodies were first visualized via DIF in bullous pemphigoid in 1967.⁵ This elegant test utilizes specific antibodies labeled with fluorophores that are then incubated with the patient's tissue, ultimately forming antibody-antigen conjugates that can be visualized with a fluorescent microscope. Antibodies usually include IgG, IgM, IgA, fibrinogen, and C3. Some institutions also evaluate for IgG4.

Transport medium is critical for proper evaluation of tissues using DIF. Inappropriate storage of tissue can degrade the antigen and confuse the interpretation of specimens. An acceptable medium for DIF includes Michel transport medium, which allows tissue to be stored for days while being transported at ambient temperature without loss of signal.^{6,7} Zeus medium also can be used and is more readily available. Alternatively, biopsy tissue can be snap frozen using liquid nitrogen.

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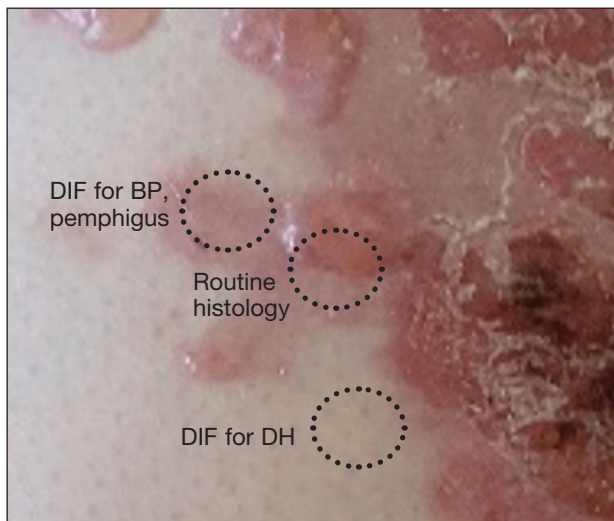
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Specimens also may be stored on saline gauze but should be analyzed within 24 to 48 hours.⁸ Most importantly, do not place the specimen in formalin; even a brief soak in formalin can greatly alter results, especially when trying to diagnose pemphigus.⁹ Proper transport conditions are critical to prevent autolysis, mitigate putrefaction, and preserve morphology while maintaining antigenicity.¹⁰

Indirect Immunofluorescence

Indirect immunofluorescence can be helpful for detecting antibodies circulating in patient serum. Indirect immunofluorescence can be used to help diagnose pemphigoid, pemphigus, epidermolysis bullosa acquisita, bullous lupus erythematosus, and dermatitis herpetiformis. Serum testing also can be a helpful alternative when obtaining tissue is difficult, such as in children.

Indirect immunofluorescence is a 2-part technique that takes a bit longer to assay than DIF.¹¹ The first step involves incubating prepared tissue substrates with patient serum. Unlabeled antibodies in the patient serum are allowed to bind to antigens in the substrate tissue for about 30 minutes. Doubling dilutions of patient serum can be performed to titer antibody levels. The second step uses fluorescein-labeled antihuman antibodies to recognize the antigen-antibody conjugates. Normal whole tissues (eg, monkey esophagus for pemphigus vulgaris, rat bladder for paraneoplastic pemphigus, salt-split normal human skin substrate for pemphigoid and epidermolysis bullosa) are the usual substrates for testing.^{11,12} Again, this test requires serum and should be collected in a red-top tube or serum-separator tube. Usually, a minimum of 0.5 mL is required for testing, but check with your preferred immunodermatology send-out laboratory before collecting.¹³



Preferred sites for biopsy specimens for direct immunofluorescence (DIF) in autoimmune bullous disorders. BP indicates bullous pemphigoid; DH, dermatitis herpetiformis.

Indirect immunofluorescence usually involves an initial screening panel using 1 or 2 tissue substrates followed by individual antigen-specific assays that correspond to the clinical suspicion and IIF screening results.¹¹ Salt-split skin is used to localize basement membrane zone autoantibodies to either the epidermal (roof) or dermal (floor) side. Although many dermatopathology laboratories offer DIF testing, IIF is more specialized and may be a send-out test at your institution.

Enzyme-linked Immunosorbent Assays

Another tool in the immunodermatology armamentarium is ELISA. Commercial ELISA systems are available for the detection of autoantibodies against bullous pemphigoid (BP) antigen 180, BP230, type VII collagen, desmoglein (Dsg) 1, Dsg3, and envoplakin.¹¹ This test allows semiquantitative measurement of antibody levels and thus can be used to monitor response to treatment or identify relapse and treatment failure.¹¹ For example, in BP, significantly increased baseline anti-BP180 IgG levels correlate with 1-year mortality rates ($P=.001$) and relapse rates ($P=.041$).^{14,15} Numerous additional studies support the observation that monitoring anti-BP180 as a potential marker of disease relapse can be helpful.^{16,17} In pemphigus, the presence or increase of autoantibodies at remission, either anti-Dsg3 or anti-Dsg1, may be a useful tool in predicting disease relapse.¹⁸ It is important for physicians to be aware of this to be able to offer guidance on prognosis.

Where Should I Biopsy?

Knowing where to biopsy can be confusing when beginning residency. But the short answer is, it depends. Let your clinical suspicion guide your specimen site. The Figure provides a quick reference for which location will give you the highest yield for a specific diagnosis.

A few cardinal rules should guide which site is biopsied. Avoid obtaining specimens from the lower extremities as much as possible, as this site has been linked with false-negative results, especially in bullous pemphigoid.^{19,20} As a dependent area prone to stasis, this site gets a lot of abuse and inflammatory changes secondary to everyday insults that can theoretically alter DIF findings, especially fibrinogen deposition.

Although tissue sent for hematoxylin and eosin staining should be lesional, biopsy for DIF ideally should not contain a new or active blister, ulcer, erosion, or bulla. Immunoreactants are more likely to be degraded in these areas, and DIF may be falsely negative.²¹

It is worthwhile to briefly discuss the definitions of the terms *perilesional* and *nonlesional*. Perilesional skin most frequently refers to skin adjacent to a bulla or vesicle. This skin can be erythematous/inflamed or appear normal. When obtaining tissue for a diagnosis of blistering disease, the general recommendation is to obtain the biopsy from lesional nonbullous skin or perilesional uninvolved skin within 1 cm of the bulla.²²⁻²⁴ The only exception to

this is dermatitis herpetiformis, which is best diagnosed on tissue obtained from normal-appearing perilesional skin within 1 cm of an active lesion.²⁵ Additionally, if your patient has oral disease, the recommendation is to obtain the biopsy from nonlesional buccal mucosa, especially if there is desquamative gingivitis.^{26,27}

The ideal biopsy size is 4 or 5 mm. If considering both DIF and histopathology, it is best to procure 2 separate specimens. One larger biopsy can be carefully bisected in 2 but often is subject to more handling artifacts, which can affect findings. In the case of 1 biopsy bisected into 2 specimens, the punch should be at least 6 mm. Shave biopsies also can be performed as long as they extend into the reticular dermis.²³

For vasculitis, biopsies for DIF should be taken from lesions that are less than 24 hours old for highest yield, as the level of tissue immunoreactants tends to decline over time.²⁸ This guideline does differ from hematoxylin and eosin specimens sent for evaluation of vasculitis, which ideally should be lesional tissue over 72 hours old. When evaluating for lupus (including subacute cutaneous lupus, discoid lupus, and systemic lupus), DIF is more likely to be positive in well-established, active lesions.

Which Test Should I Order?

The answer to this question depends, but the use of all 3 tests has a specificity close to 100% when evaluating for autoantibody-associated diseases.²³ For autoimmune blistering disease, DIF is considered the diagnostic standard. The sensitivity of DIF for diagnosing BP is in the range of 82% to 90.5%, while specificity is 98%.²⁹⁻³¹ Other autoimmune blistering diseases, such as pemphigus or dermatitis herpetiformis, have even higher sensitivities and specificities. Direct immunofluorescence often is used as a screening test, but false negatives do occur.^{32,33} Although rare, false positives also can occur, especially in cases of infection, and should be suspected when there is a lack of clinicopathologic correlation.³⁴ If DIF is negative but clinical suspicion remains high, IIF should be ordered to directly evaluate a patient's serum for autoantibodies.

In acute cutaneous lupus, subacute cutaneous lupus, and discoid lupus, DIF of active lesions may be helpful if histopathologic examination of a cutaneous lupus erythematosus lesion is nondiagnostic. However, histopathologic examination of formalin-fixed tissue remains the standard for these diagnoses. In vasculitis, while DIF is not used for diagnosis, it is useful to evaluate for IgA deposition. This is important in adults, as IgA deposition has been associated with a greater risk for developing end-stage renal disease.³⁵

Final Thoughts

This is an overview of the tests available for diagnosing autoimmune blistering diseases. Residents should keep in mind that these tests are just one part of the puzzle when it comes to diagnosing these diseases. Results of DIF, IIF,

and ELISA testing should be considered in conjunction with patient history and physical examination as well as histopathologic examination of lesional tissue when evaluating for dermatologic diseases with autoantibodies.

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