



## Application of Immunofluorescence in the Diagnosis of Oral Diseases

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### Abstract

Immunofluorescence (IF) is a technique widely used in medical research and diagnostics due to its simplicity, accuracy, and rapidity. In this article, we present the applications of IF in the diagnosis of seven oral diseases; namely, pemphigus vulgaris, mucous membrane pemphigoid, linear IgA disease, oral lichen planus, oral mucosal lupus erythematosus, epidermolysis bullosa acquisita, and chronic ulcerative stomatitis. A brief outline of each disease, in terms of its clinical features and underlying pathophysiology (based on current understanding), is also provided so that the relevance of the IF findings may be better understood and appreciated. This review aims to serve as a ready reference for both clinicians and students alike, as well as to demonstrate how this 76-year-old technique remains relevant in the field of oral medicine today.

### Introduction

Immunofluorescence (IF) is a fluorescent staining method which uses antibodies conjugated to a fluorescent labeller (fluorochromes, enzymes, radioactive compounds) to visualize specific proteins/antigens in cell or tissue sections [1]. This technique was first described by Albert Coons and colleagues back in 1941, where they successfully produced fluorescein-conjugated anti-pneumococcal-3 antibodies to detect type 3 *Streptococcus pneumoniae* [2,3]. Since then, IF has made its way into a wide variety of scientific fields as both a research and diagnostic tool due to its simplicity, accuracy, and rapidity. The principle of IF takes advantage of the fundamental structure of all atoms, where electrons are arranged in discrete energy levels around the atomic nucleus. When an electron absorbs the energy of a passing photon, it jumps to a higher, less stable energy level. The 'excited' electron does not remain in this state for long (half-life of approximately 10 seconds) and quickly drops back down to its ground (non-excited) state. During this process, a small amount of energy is lost as heat while the remainder is released in the form of an emitted photon. Thus, the emitted photon has lower energy (and hence longer wavelength) compared to the initially absorbed photon.

There are two main types of IF: direct and indirect. In direct IF (DIF), tissue samples are exposed to a primary antibody that is directly conjugated to a fluorescent dye. This contrasts with indirect IF (IIF), where tissue samples are first exposed to an unlabelled primary antibody and then a secondary antibody conjugated to a fluorescent dye is used to bind to that primary antibody to achieve fluorescence. Both types of IF have their advantages and disadvantages, which means they each excel in specific applications. In general, direct IF is simpler and quicker (because it involves fewer steps and reagents) but also costlier (because labelled primary antibodies are more expensive than unlabelled versions) compared to indirect IF; whereas the use of secondary antibodies in indirect IF allows it to be more cost effective and provides a means for signal amplification. When interpreting IF samples, the five main features that allow identification of a diagnosis are the (a) type of immunoreactant, (b) location of the immune deposits, (c) extent of the staining, (d) intensity of the staining, and (e) pattern of the immune complex [4]. In the field of oral medicine, IF is most commonly used to aid in the diagnosis and prognosis of immunobullous disorders, as many oral lesions tend to be difficult to diagnose clinically alone. Hence, in this process, IF findings are correlated with clinical and histomorphological information to either strengthen the diagnosis or modify it.

This article focuses primarily on the IF aspects of the diagnostic process, summarizing its applications in the diagnosis of oral diseases. It is hoped that the information contained in this article may serve as a ready reference for both clinicians and students alike, as well as to demonstrate

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**Table 1:** Example direct IF findings in pemphigus vulgaris.

	IgG	IgA	IgM	C3
Laskaris [24]	98% (57/58)	14% (8/58)	7% (4/58)	41% (24/58)
Daniels and Quadra-White [23]	100% (10/10)	10% (1/10)	10% (1/10)	60% (6/10)
Judd and Lever [25]	100% (47/47)	51% (24/47)	40% (19/47)	49% (23/47)
Rogers and Johnson [26]	73% (11/15)	60% (9/15)	53% (8/15)	100% (15/15)

how this 76-year-old technique remains relevant in the field of oral medicine today.

## Pemphigus Vulgaris

Pemphigus is a group of blistering autoimmune diseases that affects the skin and mucous membranes of the oral cavity. Worldwide, this condition affects fewer than 5 in 1,000,000 people every year but if left untreated, pemphigus can be life-threatening due to the increased risk of skin infection, sepsis, and dehydration [5]. Pemphigus occurs due to the presence and circulation of autoantibodies against desmoglein, a cadherin-type cell adhesion molecule that forms desmosomes and binds keratinocytes together. Thus, keratinocytes separate from each other (acantholysis) in the suprabasal layer of the stratified squamous epithelium and this presents clinically as superficial skin blisters which easily rupture and heal poorly. There are two main types of pemphigus: pemphigus vulgaris (PV) and pemphigus foliaceus (PF). In PV, the autoantibody present is predominantly anti-Dsg3 (anti-desmoglein-3) while in PF, the predominant autoantibody is anti-Dsg1. Precisely how these intercellular antibodies cause acantholysis is unclear but several mechanisms have been proposed in the literature, such as blocking of adhesion sites on desmoglein or interference with the structure, function, or assembly of desmosomes [5-8]. Exacerbation of acantholysis and cellular damage occurs due to complement activation [9-15], while proteolysis occurs due to increased production of plasminogen activator [16-18].

PV is the type that usually affects the mouth because the oral mucosa mainly expresses Dsg3, whereas Dsg1 is poorly expressed [19]. Cases involving only anti-Dsg3 result in mucosal-dominant PV (affecting oral mucosa only), but mucocutaneous PV can also occur (affecting oral mucosa and skin) when both anti-Dsg3 and anti-Dsg1 are expressed. Direct IF is useful for assessing the presence of immunoglobulins in the intercellular space of the tissue samples in question. When viewed under a fluorescence microscope, direct IF tissue samples that are positive for pemphigus vulgaris have a characteristic uniform, reticular intercellular space fluorescence pattern, indicating the presence of autoantibodies binding to desmosomes on the keratinocyte surface. This feature is mandatory for the diagnosis of pemphigus vulgaris, although it alone does not immediately rule out other differential diagnoses. Direct IF analyses have revealed the deposition of IgG, IgA, IgM, as well as C3 (complement component 3) in the intercellular spaces of lesional and perilesional tissues (Table 1) [20,21]. IgG is found in nearly all PV cases whereas IgA, IgM, and C3 occur with greater variability. Interestingly, it has also been found that C3 has the tendency to be confined to acantholytic areas [22,23].

Indirect IF may also be used to detect the presence of circulating anti-Dsg (IgG isotype) in the serum of patients, where samples positive for PV will also present with a characteristic reticular intercellular space (ICS) fluorescence pattern [24]. Commonly used epidermal substrates include normal human oral mucosa or animal mucosae (such as rabbit, monkey, or guinea pig esophagus).

**Table 2:** Example indirect IF findings in pemphigus vulgaris.

Study	Presence of Circulating Anti-ICS Antibodies (No. of cases)
Zagarodniuk et al. [31]	81% (26/32)
Laskaris [24]	86% (50/58)
Meurer et al. [32]	80% (8/10)
Tuffanelli [28]	0% (0/4)
Trotter [33]	50% (1/2)
Eversole et al. [34]	100% (1/1)
Hasler [35]	100% (1/1)
Bean et al. [36]	0% (0/1)
Shklar & Caltaldo [37]	0% (0/1)
Chorzelski & Beutner [27]	100% (1/1)
Peck et al. [38]	100 (1/1)

Circulating pemphigus antibodies are occasionally difficult to detect with indirect IF when the disease affects only the oral mucosa, is minimal or inactive, or if the patient is on immunosuppressive therapy [24]. Possible reasons for these ‘false-negative’ results are (a) antibody species or organ specificity, (b) the prozone phenomenon, (c) interference by other antibodies in the sera of patients, and (d) technical errors [27,28]. However, Laskaris (1981) noticed that circulating anti-ICS antibodies were more detectable when normal human mucosa was used as the epidermal substrate (86.2%) compared to when animal epithelial tissue was used (48.3%), which could also explain the data variation among the different studies (Table 2) [24]. In PV, serum antibody titres are typically low, ranging from 1:10 to 1:160. Although there is a correlation between antibody titre and disease activity [25,29,30], using it as a parameter to guide or monitor therapy is not recommended due to insufficient reliability [20,24].

## Mucous Membrane Pemphigoid

Pemphigoid is a group of autoimmune skin diseases characterised by subepithelial blistering. Types of pemphigoid include bullous pemphigoid, mucous membrane pemphigoid (previously known as cicatricial pemphigoid), and pemphigoid gestationis. Of these, mucous membrane pemphigoid (MMP) is the main type that affects the oral mucosa [39,40]. MMP occurs due to the presence and circulation of autoantibodies against hemidesmosomal components in the basement membrane zone (BMZ), such as the  $\beta 4$  subunit of  $\alpha 6\beta 4$  integrin, laminin-5, laminin-6, type VII collagen, and bullous pemphigoid antigens 1 and 2 (BP230 and BP180 respectively) [40,41]. The exact pathogenesis is unclear but it is suggested that binding of pemphigoid autoantibodies to the autoantigens triggers the host immune system, resulting in the release of inflammatory mediators and subsequent migration of leukocytes (neutrophils, eosinophils, lymphocytes, and mast cells) to the BMZ. Damage at the epithelial-BMZ junction then ensues either through direct cytotoxic action or proteolytic enzyme action, resulting in epithelial detachment from the underlying tissues [40-43].

In addition to the clinical presentation of subepithelial blistering in the oral mucosa, immunopathologic evidence is also needed for a diagnosis of MMP to be established, per the International Consensus on MMP [44]. Direct IF of perilesional tissue in MMP-positive cases typically reveals a linear, homogenous, ribbon-like deposition of IgG and/or C3 (and occasionally IgA) along the BMZ. For example, Ahmed & Hombal (1986) collated data from nine studies and reported

that direct IF detected immunoglobulin deposition at the BMZ in 84% (90/107 cases) of oral mucosal specimens [45]. Circulating anti-BMZ antibodies (IgG isotype) can also be occasionally detected by indirect IF, usually only when mucosal substrates are used [46]. This corresponds with the clinical presentation of subepithelial blistering in MMP, where it mainly affects mucous membranes and rarely the skin. However, these circulating autoantibodies are generally difficult to detect, as is evident from the study by Ahmed & Hombal (1986) which found positive indirect IF findings in only 19% (28/144) of cases [45]. Suggested reasons for this are that (a) MMP is a localized disease, hence only small quantities of anti-BMZ antibodies are produced with most of them being bound, and (b) routine indirect IF techniques are not sensitive enough to detect very low titres [47-49]. Where detectable, the serum antibody titres in MMP are also low, ranging from 1:10 to 1:40 [46,49,50], and there are conflicting opinions in the literature regarding the correlation between antibody titre and disease activity [51,52].

### Linear IgA Disease

Linear IgA disease (LAD) is an autoimmune skin disease characterized by subepithelial blistering of skin and mucous membranes which are similar in appearance to other blistering diseases, such as pemphigoid and dermatitis herpetiformis. LAD is extremely rare with an estimated incidence of 5 in 10,000,000 people in Western Europe, affecting both children and adults [53]. It usually initiates spontaneously but can be triggered by certain drugs or medications, such as vancomycin [54-57]. LAD occurs due to the presence and circulation of IgA autoantibodies against antigens in the BMZ, such as BP180, BP230, and LAD285 [58]. Deposition of these IgA autoantibodies leads to complement activation and neutrophil migration to the site, resulting in damage at the epithelial-BMZ junction [53,59]. Hence, LAD has similarities with MMP not only in terms of clinical presentation but also in disease immunology. IF techniques are therefore paramount for distinguishing LAD from MMP and other similarly-presenting diseases.

The characteristic feature of LAD-positive samples by direct IF analysis is the linear deposition of IgA along the BMZ [60]. When this feature is present exclusively (i.e. without the involvement of other immunoglobulins), a diagnosis of LAD can be made confidently. However, there may be additional involvement of other immunoreactants, such as C3, IgG, and very rarely IgM, in a small number of cases [53,60,61]. Circulating anti-BMZ IgA antibodies may occasionally be detected by indirect IF in low titres, ranging from 1:2 to 1:64 [61].

### Oral Lichen Planus

Oral lichen planus (OLP) is a chronic inflammatory autoimmune disease that affects the mucous membranes of the oral cavity, presenting clinically as white lacy lesions, papules, or plaques; sometimes resembling keratotic diseases (e.g. leukoplakia) [62]. Although most oral lesions are asymptomatic, the atrophic and erosive forms of the lesion can be considerably painful. The causes or triggering factors of OLP are largely unclear, but one key early event in the pathophysiology of OLP is the increased production of Th1 cytokines, which leads to the activation and migration of T cells to the oral epithelium [62,63]. There, the T cells bind to keratinocytes and IFN- $\gamma$ , followed by the upregulation of metalloproteinase-1 (MMP1), MMP3, and p53 [64-66]. Thus, apoptosis is induced and destruction of epithelial basal cells ensues. In chronic OLP, the white lesions

may occur due to keratinocyte hyperproliferation, which is caused by inhibition of the TGF- $\beta$ /Smad signalling pathway and activation of the nuclear factor NF- $\kappa$ B pathway [67,68]. In OLP, there is also a small risk (approximately 1.09%) of malignant transformation [69].

The diagnosis of OLP is usually made by correlating clinical and histomorphological information. However, interpretation of the latter can often be subjective [70], hence the poor correlation between the two in approximately 50% of cases [71]. Thus, in such cases, direct IF may be used to add certainty to the diagnosis or suggest another. In OLP-positive cases, direct IF typically reveals linear fibrinogen deposition along the BMZ, extending into the papillary lamina propria in a 'shaggy' pattern [72]. Additionally, deposition of IgM, IgA, IgG, and C3 on cytoid bodies at the BMZ or papillary lamina propria may be detected [72,73]. The presence of both these features are usually required for diagnosis of OLP by direct IF [23,73]. Ig deposits along the BMZ are rarely seen in OLP, occurring only in 3-30% of cases [74]. Because there is no known antibody-mediated component in the pathogenesis of OLP, indirect IF is generally not indicated. It may, however, be useful in diagnosing lichenoid drug reactions [75,76].

### Oral Mucosal Lupus Erythematosus

Lupus erythematosus (LE) is a term given to a group of chronic autoimmune diseases that can affect many different organs in the body. The exact pathogenesis of LE remains unclear but the potential complex mechanisms involved are described in the literature [77,78]. Oral mucosal manifestations can occur either with systemic LE (SLE), discoid LE (DLE), or alone. These oral lesions have a wide range of clinical presentations, from white keratotic striae resembling OLP to erythematous patches and ulcerations [79]. Despite these non-specific presentations, Schiødt (1984) and Karjalainen & Tomich (1989) have elucidated several histomorphological features characteristic of oral lupus that allow for a diagnosis of oral lupus to be made at the light microscopy level [79,80]. However, the authors acknowledged that other diagnostic methods, including IF, can offer valuable information in cases of uncertainty.

When oral mucosal LE samples are analysed by direct IF, a coarse granular band along the BMZ formed by C3 and IgM deposition is most commonly seen [23,74]. The presence of IgM, particularly, favours the diagnosis of LE as opposed to OLP or leukoplakia [81]. Deposition of IgG and IgA may be detected in less than one third of cases [74]. When present, the fibrinogen and cytoid body deposition patterns resemble those seen in OLP. Patients with DLE more commonly exhibit fibrinogen deposition (89% of cases) compared to patients with SLE (67%), but cytoid bodies are more common in SLE cases (43%) than in DLE (27%) [74]. Hence, the main feature distinguishing oral mucosal LE from OLP is the presence of immunoglobulin deposits, since this feature is rarely present in OLP.

### Epidermolysis Bullosa Acquisita

Epidermolysis bullosa acquisita (EBA) is a rare, acquired autoimmune disease characterized by subepithelial blistering. The clinical presentation of EBA is similar to that of the dystrophic forms of hereditary epidermolysis bullosa (EB), which is why EBA was historically considered part of the EB group of diseases [82]. Instead, EBA is distinguished from EB by its later onset (most commonly in the fourth and fifth decades of life) with no obvious inheritance pattern [82,83]. EBA occurs due to the presence and circulation of autoantibodies against collagen VII (C7), a major component of

anchoring fibrils (AF) that functions to link the BMZ lamina densa to the papillary lamina propria.

Direct IF analysis conducted on lesional and perilesional samples typically reveals an intense, linear deposition of IgG along the BMZ, occasionally with the involvement of C3, IgA, and IgM [84]. This deposition pattern closely resembles those seen in pemphigoid and it is extremely difficult to differentiate the two by direct IF alone. However, one subtle difference is that the IgG band in EBA tends to be more intense and broader compared to that of pemphigoid [84]. Circulating IgG anti-BMZ autoantibodies may also be found by indirect IF and the frequency of detection varies depending on the stage of the disease; i.e. higher in the early and inflammatory phases, whilst lower in the mechanobullous phase [84,85].

### Chronic Ulcerative Stomatitis

Chronic ulcerative stomatitis (CUS) is a recently described disease that presents clinically as chronic and recurrent oral mucosal erosions and ulcerations [86,87]. The most commonly affected sites are the tongue, buccal mucosa, and gingiva (in order of decreasing frequency), but occasionally the skin may also be involved [88]. CUS predominantly affects Caucasian women in their fifth decade of life who have had a long history of undiagnosed oral pain [86-88]. Currently, the pathogenesis of CUS is still unknown due to the small number of reported cases in the literature, but the target antigen in CUS is reported to be  $\Delta$ Np63 $\alpha$ , a protein normally present in basal and parabasal cells of stratified squamous epithelia [87]. Both clinical and histomorphological features of CUS are non-specific; hence direct IF is the current gold standard test for diagnosing CUS [89]. An accurate diagnosis is critical because medications conventionally used to treat immune-mediated diseases (e.g. glucocorticoids) are not effective in CUS, which requires treatment with hydroxychloroquine instead [87,90].

Direct IF analysis of CUS samples reveals the presence of IgG autoantibodies on the nuclei of keratinocytes, predominantly at the basal and parabasal layers, in a speckled deposition pattern [91,92]. The detection of these unique stratified epithelium-specific antinuclear antibodies (SES-ANA) is characteristic of CUS. Fibrinogen deposition in a pattern similar to that of OLP (i.e. along the BMZ in a 'shaggy' pattern) may be observed in some cases, but this finding is not commonly reported as it is not thought to be a diagnostic criterion for CUS [87,93]. Circulating autoantibodies detected by indirect IF also exhibit the SES-ANA deposition pattern, but only when stratified squamous epithelial substrates (e.g. guinea pig, monkey, or human esophagus) are used [87].

### Conclusion

When IF was first described by Albert Coons and colleagues in 1941, it was used to detect one species of bacteria – type 3 *Streptococcus pneumoniae* – as a proof of concept. Today, 76 years later, its use has exploded into an immensely wide range of fields, including oral medicine. IF has proven to be a valuable tool in the diagnosis of oral diseases, allowing conditions that have extremely similar clinical or histomorphological appearances to be differentiated with greater accuracy. This, in turn, has enabled treatment and management to be more targeted and efficient, resulting in improved patient outcomes. As technologies develop, it is expected that the new will replace the old. However, just as IF has stood the test of time today, it would not be surprising if it remains to be relevant for many more years to come, proving itself to be one of the most resilient innovations of

mankind.

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