Research Techniques Made Simple: Immunofluorescence Antigen Mapping in Epidermolysis Bullosa

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Inherited epidermolysis bullosa is a group of genetic blistering diseases with a broad spectrum of clinical severity and molecular defects. Epidermolysis bullosa results from mutations in genes encoding proteins involved in cell-cell and cell-matrix adhesion in the epidermis. Immunofluorescence antigen mapping makes use of monoclonal antibodies against proteins of the dermal-epidermal junction zone to determine the layer of skin where cleavage occurs and the relative protein abundance. It allows the diagnosis of the type and subtype of inherited epidermolysis bullosa and sheds light on molecular mechanisms underlying the disease. Immunofluorescence mapping steps include obtaining a skin biopsy sample, processing the biopsy material, antigen-antibody interaction on tissue, washing, incubation with fluorescently conjugated secondary antibodies, mounting, observation under a fluorescence microscope, and interpretation. A minimal antibody panel allows discrimination of the main epidermolysis bullosa subtypes. Extended panels can be used depending on the diagnostic or scientific question to be addressed. Immunofluorescence mapping contributed to significant progress in understanding epidermolysis bullosa, including identification of new underlying genetic mutations, mutation mechanisms, and the presence of revertant mosaicism. It is also an important tool in the assessment of the efficacy of experimental therapeutic approaches.

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INTRODUCTION

Inherited epidermolysis bullosa (EB) is a group of genetic diseases that is defined by fragility of the skin and mucous membranes. EB is characterized by a broad spectrum of molecular defects and clinical severity, with more than 30 subtypes described so far (Fine et al., 2014). EB is the consequence of mutations in genes coding for proteins involved in adhesion of epidermal keratinocytes to each other or to the underlying dermis (Figure 1). Development of monoclonal antibodies enabled identification of dysfunctional proteins by immunofluorescence antigen mapping (IFM). Sequencing of gene panels or whole-exome sequencing may allow direct discovery of the underlying genetic defect. Importantly, IFM provides complementary information to mutation analysis, allowing clinicians and researchers to understand the consequences of the genetic defect on a protein and tissue level.

HISTORY

IFM was first described as a method to determine changes in antigens within the dermal-epidermal junction in mechanobullous diseases in 1981 (Hintner et al., 1981). The method further developed with the generation of improved, domain-specific antibodies to detect adhesion proteins and with advances in understanding the molecular mechanisms of EB (Pohla-Gubo et al., 2010). Currently, IFM is available in

BENEFITS OF IFM

- Indicates the layer of skin where split formation occurs and identifies the nonfunctioning protein in severe types of EB.
- Is a valuable, rapid tool for uncovering effects of known gene mutations on protein expression and for establishing diagnosis of skin fragility disorders.
- Allows characterization of changes in expression of key skin structural proteins if patients have unclassified genetic variants of unknown pathogenic relevance.
- Is a rapid and powerful tool for the diagnosis of EB in neonates.
- Has prognostic value. The fluorescence intensity of an antigen shows protein abundance and correlates with the type of mutation and severity of the phenotype.

LIMITATIONS

- Artificial skin splits may occur during sampling, shipment, or storage.
- In cases of mild skin fragility, no skin cleavage may be present in the sample and no changes in the immunoreactivity of the markers may be observed.
- Changes in multiple markers may occur.
- Interpretation requires basic knowledge of the molecular architecture of cell-cell and cell-matrix adhesions in the epidermis and depends on the experience of the investigator.
- The antibody costs for an extended IFM panel are high.
many countries worldwide, even in those where sophisticated genetic techniques are not affordable.

**MOLECULAR BASIS OF EPIDERMAL AND DERMAL-EPIDERMAL ADHESION**

The main adhesive structures in the skin examined with IFM are supramolecular protein complexes including desmosomes, hemidesmosomes, the basement membrane, and anchoring fibrils (Figure 1). Desmosomes are major intercellular junctions that provide a high degree of resistance to mechanical forces through stable molecular interactions between desmosomal plaques composed of desmoplakin, plakoglobin, plakophilins, and keratin intermediate filaments. The desmosomal cadherins, desmogleins (1–3), and desmocollins (1–3) insert with one end into the desmosomal plaque and accomplish intercellular connections through interactions between their extracellular domains. In basal keratinocytes facing the basement membrane, keratin intermediate filaments consisting of keratin-5 and -14 heterodimers insert into the inner plaques of the hemidesmosomes containing bullous pemphigoid antigen 1 (BPAG1e, BP230) and plectin, which then interact with the transmembrane proteins, integrin-β4 and collagen XVII (BPAG2, BP180). On the extracellular side, integrin-α6β4 and collagen XVII bind to laminin-332, which is inserted into the lamina densa, a tight molecular network of collagen IV and proteoglycans. Here, collagen VII is attached from the dermal side, in the form of anchoring fibrils, which ensure stable adhesion between the basement membrane and the underlying dermis.

**HOW IS IFM PERFORMED?**

**Biopsy technique and tissue processing**

The choice of the biopsy site is critical for maximizing the quality of IFM results. The skin sample should be taken from skin around a recent blister (less than 12 hours). In older blisters, inflammation and tissue regeneration may induce artifacts. If no fresh blister is present, the skin should be rubbed with an eraser to induce new blister formation, and

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**Figure 1.** Morphologic and ultrastructural features of human skin and level of skin cleavage in EB. Transmission electron microscopy pictures depicting desmosomes (upper panel) and the basement membrane zone (lower panel) are shown. The disease-relevant molecular components of desmosomes (upper panel) and the basement membrane zone (lower panel) are depicted schematically. (Transmission electron microscopy courtesy of Dr. Ingrid Hausser, Heidelberg, Germany). Classically, EB was divided into three main types according to where skin cleavage occurs: in the basal epidermal layer, in the lamina lucida, or below the lamina densa of the basement membrane. Subsequently, new genes were discovered, and currently the EB spectrum includes Kindler syndrome and disorders exhibiting epidermal fragility (modified from Has et al., 2013, with permission from Elsevier and Dr. Ingrid Hausser). BPAG1e, bullous pemphigoid antigen-1e; DEB, dystrophic epidermolysis bullosa; EBS, epidermolysis simplex bullosa; JEB, junctional epidermolysis bullosa.
the biopsy sample should be taken several minutes later. A 3–4-mm punch biopsy should be performed at the rubbed area; if more detailed RNA studies and extended IFM panels are desired, at least a 4-mm punch is recommended. To preserve the proteins and epitopes, the biopsy sample should either be immersed in Michel’s transport medium or normal saline or be directly snap frozen. Recently, ex vivo blister induction was proposed as more sensitive than in vivo blister induction (Mozafari et al., 2014). To avoid artificial cleavage of the skin and degradation of proteins, shipment of samples to the laboratory to perform IFM should not exceed 1–3 days.

Antigen-antibody interaction and visualisation
Monospecific antibodies at experimentally determined dilutions are directly applied to a 5-μm tissue section on a glass slide and incubated for 2 hours or overnight (Figure 2). Usually no blocking or permeabilization step is necessary. The choice of the primary antibodies depends on the available clinical data and on the complexity of the question. A minimal panel of antibodies against collagen types IV, VII, XVII and laminin-β3 chain can be used. For detailed analyses, extended panels can be applied, including antibodies against any protein involved in rare EB subtypes (see Supplementary Materials online). Four washing steps of 5 minutes each are carried out to remove excess primary antibody. For dilution of antibodies and washing, Tris- or phosphate-buffered saline can be used. A secondary antibody against the IgG of the host species of the primary antibody, conjugated with a fluorescent compound, is then applied to the skin section for 1 hour.

![Figure 2. Schematic diagram of the immunofluorescence antigen mapping technique.](image)

**Table 1. Interpretation of the results of IFM**

<table>
<thead>
<tr>
<th>EB Type</th>
<th>Target Genes/Proteins</th>
<th>Characteristic Immunofluorescence Features</th>
<th>Limits/Challenges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial EB simplex</td>
<td>TGM5/transglutaminase-5</td>
<td>Subcorneal cleavage Reduced transglutaminase-5 abundance or activity</td>
<td>May be interpreted as an artifact The transglutaminase activity assay is challenging and requires a fresh frozen skin sample</td>
</tr>
<tr>
<td>DSP/desmoplakin</td>
<td>Acantholysis throughout the basal and spinous layers Desmoplakin or desmoplakin tail absent</td>
<td>Domain-specific antibodies</td>
<td></td>
</tr>
<tr>
<td>JUP/plakoglobin</td>
<td>Acantholysis throughout the basal and spinous layers Plakoglobin staining negative</td>
<td>Several desmosomal markers may be attenuated</td>
<td></td>
</tr>
<tr>
<td>PKP1/plakophilin-1</td>
<td>Acantholysis throughout the basal and spinous layers Plakophilin-1 staining negative</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Basal EB simplex</td>
<td>KRT5, KRT14/keratin-5, -14</td>
<td>Cleavage in the basal epidermal layer Keratin-14 negative in autosomal recessive forms No marker changed</td>
<td>In dominant EB simplex no split may occur, and immunoreactivity of antigens may be not changed; in severe generalized EB simplex, acantholysis in the spinous layer may occur</td>
</tr>
<tr>
<td>PLEC/plectin</td>
<td>Cleavage low in the basal epidermal keratinocytes Plectin negative or attenuated</td>
<td>Results are dependent on the epitope recognized by the primary antibody</td>
<td></td>
</tr>
<tr>
<td>DST/BPAG1</td>
<td>Cleavage low in the basal epidermal keratinocytes BPAG1 negative</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>EXPH5/exophilin-5</td>
<td>Cytolysis in the basal epidermal layer Exophilin-5 negative</td>
<td>Available antibodies of low quality</td>
<td></td>
</tr>
<tr>
<td>Junctional EB</td>
<td>COL17A1/collagen XVII</td>
<td>Collagen XVII negative or attenuated</td>
<td>May be attenuated in other types of junctional EB</td>
</tr>
<tr>
<td>LAMA3, LAMB3, LAMC2/laminin-332</td>
<td>Laminin-332 negative or attenuated</td>
<td>May be attenuated in other types of junctional EB</td>
<td></td>
</tr>
<tr>
<td>ITGA6, ITGB4/integrin-α6-β4</td>
<td>Integrin-α6-β4 negative or attenuated</td>
<td>Integrin-β4 is often attenuated in other subtypes of junctional EB</td>
<td></td>
</tr>
</tbody>
</table>
The most commonly used fluorescent compounds are fluorescein isothiocyanate (excitation and emission at 495 and 519 nm, respectively) and Alexa Fluor (e.g., 488 cyan-green, excitation and emission at 495 and 519 nm, respectively; Invitrogen, Karlsruhe, Germany), the latter having greater photostability and higher fluorescence intensity. After washing, the stained specimen is mounted with a coverslip using a fluorescence mounting medium (e.g., Dako, Hamburg, Germany). Because fluorochromes are prone to photobleaching, rapid but careful observation with fluorescence microscopy is required.

**Controls**

As a positive control against which to compare a patient’s samples, a normal skin sample should be stained in parallel using the same reagents. As a negative control, secondary antibodies without primary antibodies should be applied.

**INTERPRETATION OF THE RESULTS**

The interpretation of the results is described in detail in Table 1. In brief, the position of the cleavage plane relative to collagen IV, a marker of the basement membrane that is not affected in EB, indicates a junctional (collagen IV at blister floor) or a dermal (collagen IV at blister roof) blister. In EB simplex, the cleavage mainly occurs within the basal epidermal layer; the fluorescent signals for keratin, plectin, bullous pemphigoid antigen-1 (BPAG1), collagen XVII, or integrin-α6β4 appear at the floor of the blister. In Kindler syndrome, the layer of skin cleavage is variable: intraepidermal, junctional, or dermal. In addition, the intensity of

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</thead>
<tbody>
<tr>
<td>Dystrophic EB</td>
<td>ITGAV3/Integrin-α3 subunit</td>
<td>Integrin-α3 negative</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>COL7A1/collagen VII</td>
<td>Collagen type IV at the roof of the blister</td>
<td>In dominant dystrophic EB no split may occur, and immunoreactivity for collagen type VII may be unchanged; in such cases GDA-J/F3 (assim et al., 1991) may be reduced and indicative</td>
</tr>
<tr>
<td>Kindler syndrome</td>
<td>FERMT1/kindlin-1</td>
<td>Mixed layers where skin cleavage occurs: intraepidermal, junctional, or dermal</td>
<td>Kindlin-1 staining in normal skin is faint, probably because of the discrete distribution of β1 integrin adhesions in the skin</td>
</tr>
</tbody>
</table>

Table 1. Continued

**Abbreviations:** BPAG1, bullous pemphigoid antigen-1; DEJ, dermal-epidermal junction; EB, epidermolysis bullosa.

![Figure 3](https://example.com/figure3.png)

Figure 3. Immunofluorescence antigen mapping performed on skin sections from a healthy control (Co) and a patient with interstitial lung disease, nephrotic syndrome, and EB with an intronic unclassified variant. Confocal microscopy was used for visualization. Integrin-α3, integrin-α6, laminin-α3, and collagen VII appear in green. The positions of the blisters are depicted by a cross, and nuclei appear in blue. Scale bars = 100μm (reprinted from He et al., 2016, with permission from Elsevier).
the immunoreactivity as compared with normal skin reflects the relative protein expression in the skin of the patient and has prognostic value. Assessment of the immunofluorescence staining intensity can be done by the observer using a subjective scoring method or by using appropriate software (e.g., ImageJ, available from the National Institutes of Health at http://imagej.nih.gov/ij/).

**COMPARISON BETWEEN IFM AND OTHER METHODS**

A comparative study between transmission electron microscopy and IFM indicated the superioriity of IFM in the diagnosis of EB (Yiasemides et al., 2006). Nevertheless, in particular situations transmission electron microscopy may deliver important morphologic details (e.g., the discovery of a particular subtype of EB simplex because of exophilin-5 gene mutations) (McGrath et al., 2012). IFM

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**Figure 4.** Immunofluorescence antigen mapping performed on skin sections from a healthy control (Co) and three patients with junctional epidermolysis bullosa and COL17A1 mutations (confocal microscopy). Immunofluorescence staining was performed with the following domain-specific antibodies against collagen XVII: Endo2, NC16A, and Hk139. Patients 1 (P1) and 2 (P2) had the mutation p.R1303Q, and patient 3 (P3) had loss-of-function mutations leading to absence of collagen XVII. Note apicolateral staining in the basal keratinocytes with the antibody Endo2 in P1 and P2, similar to the control skin. Immunostaining with HK139 and NC16A, which recognize the ectodomain of collagen XVII, showed a broad, irregular distribution below the level of the basement membrane in intact skin of P1 and P2 and presence of the signal at both roof and base of a blister in P3. Immunofluorescence staining with an antibody against the laminin-γ2 chain (clone GB3) shows broad, irregular distribution below the level of the basement membrane in the intact skin of P1. Laminin-γ2 immunostaining is present at both roof and floor of the blister in P2, and only at the blister floor in P3. Crosses indicate blisters, and nuclei are stained with DAPI. Scale bars = 50 μm (modified from Has et al., 2014, with permission from Elsevier).
and genetic mutation detection are complementary approaches. An example for this is shown in Figure 3: the pathogenic role of the unclassified intronic sequence variant, c.1383-11T>A, found in a patient with severe interstitial lung disease and nephrotic syndrome, was uncertain. IFM showed lack of integrin-α3 in the skin of the patient, demonstrating the disease-causing role of the intronic variant. Detailed analysis of RNA extracted from the skin sample further showed the molecular mechanisms underlying this unusual mutation (He et al., 2016).
ACKNOWLEDGMENTS
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SUPPLEMENTARY MATERIAL
Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

REFERENCES


