Fluorescence Overlay Antigen Mapping of the Epidermal Basement Membrane Zone: III. Topographic Staining and Effective Resolution

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Received for publication September 6, 1994 and in revised form December 2, 1994 and February 1, 1995; accepted February 16, 1995 (4A3483).

In this third study on the fluorescence overlay antigen mapping (FOAM) technique, we have addressed the question of which differences of antigen distributions close to the resolving power of the light microscope can be distinguished. An answer to this question should provide clues to future applications of the technique aiming at the topographic differentiation of IgG deposits displayed at the epidermal basement membrane zone (EBMZ) in certain bullous skin disorders. For the present purpose we have developed a topographic staining model in human skin, using structural EBMZ antigens as topographic reference markers. The distribution of these markers relative to one another is visualized in FOAM images obtained by selective double immunofluorescence tracing and videomicroscopic overlay imaging. The theoretical resolution limit of the technique is discussed and suggests an effective lower limit of some 60–65 nm. Although this limit is not reached under present conditions, our results show that it is possible to distinguish topographic differences of antigen distributions with an upper resolution limit of 200 ± 50 nm. Furthermore, our findings indicate that collagen Type VII and β4 integrin are the most suitable molecules to serve as topographic reference markers in future applications of the technique aiming at the differentiation of bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA). Preliminary results on this topic are most promising indeed. (J Histochem Cytochem 43:649–656, 1995)

KEY WORDS: Antigen mapping; Epidermal basement membrane zone; Extracellular matrix proteins; Multicolor immunofluorescence; Topographic staining; Overlay imaging; Videomicroscopy.

Introduction

Fluorescence overlay antigen mapping (FOAM) is based on two techniques: (a) multiple antigen detection by selective double- or triple-wavelength band immunofluorescence and (b) overlay of the fluorescence images by photomicrographic or videomicroscopic procedures. We are still refining the technique to identify relative antigen distributions close to the resolving power of the light microscope, particularly in tissue sections (2,3). One of our ultimate goals is to identify topographic differences of immune deposits across the epidermal basement membrane zone (EBMZ) as found in certain autoimmune bullous skin disorders (1,12). Of special interest are autoimmune IgG deposits at the EBMZ in bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA). Immunoelectron microscopic studies have shown that these deposits are displayed at different locations on one side or the other of the EBMZ (13,18,20,22). It seems far beyond reach to detect such differences by routine immunofluorescence microscopy. In fact, it has been stated earlier that the resolution of the light microscope is insufficient "to determine whether such deposits are within the lamina lucida or below the lamina densa" (14). More recent studies indicate that the topographic difference of the IgG deposits ranges from about 200 nm to some 700 nm (15,27). It is rather challenging to find out whether the FOAM technique is able to show such differences. Therefore, in the present study we have addressed the question of which differences of antigen distributions close to the resolving power of the light microscope can be distinguished by visual inspection of FOAM images. For this purpose we have developed a topographic staining model in human skin, according to the principle of performance testing. In this model, pairs of structural EBMZ antigens are used as topographic reference markers (TRMs). These TRMs are visualized in FOAM images obtained by double immunofluorescence tracing and computer-aided overlay imaging. The TRMs studied have fairly well-determined relative distributions (Figure 1), and include antigen distributions simulating those of the above-mentioned skin-bound IgG deposits in BP and EBA. This should provide an answer to the key question of which TRMs are

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the most suitable to use in future diagnostic applications of the technique. Furthermore, to ensure the geometric fidelity of FOAM images, we have refined the staining procedure by using a double fluorescent geometric verification marker (GVM). This permits accurate correction of image shifts that may routinely occur in the overlay procedure (3).

This study shows that visual inspection of FOAM images offers the possibility to distinguish between antigens displayed on one side or the other of the EBMZ. Thus, the topographic staining efficacy and effective resolution appear to be adequate to distinguish antigenic sites on the hemidesmosome complex from those on the anchoring fibril structures below the lamina densa. Because these antigen distributions are comparable to those of skin-bound IgG deposits in BP and EBA, this shows enough promise to embark on the diagnostic application of the FOAM technique. Furthermore, the staining and overlay imaging of topographic and geometric markers can be adapted to studies other than the analysis of EBMZ antigens, to which it has been first applied.

Materials and Methods

Terminology.

FOAM (fluorescence overlay antigen mapping): an immunofluorescence double (or triple) staining technique whereby the unknown distribution of antigens that are in close proximity can be located relative to a suitable TRM (see below). Assessment of antigen distributions relative to one another (topographic mapping) is done by visual inspection of an overlay image showing the distinctly labeled TRM and antigen(s) of interest.

TRM (topographic reference marker): a distinctly labeled marker with known tissue distribution used to locate unknown antigenic sites that are in close proximity.

GVM (geometric verification marker): a double- or triple-labeled in situ marker used to correct and verify image shifts that may routinely occur during overlay of fluorescence images (see FOAM).

Skin Specimens. We used clinically normal skin specimens obtained by punch biopsy (4 mm) under local anesthesia with 2% lidocaine. We studied three skin samples obtained from three white subjects: one sample from the extensor surface of the forearm, one from the shoulder, and one from the buttock. These samples were selected from a larger pool on the basis of overt expression of the structural EBMZ antigens studied (see below). The samples were snap-frozen in liquid nitrogen and stored at -80°C. An additional skin sample from the groin was used for transmission electron microscopy (Figure 1). Routine immunofluorescence microscopy did not show any abnormalities in these skin samples.

Antibodies. Monoclonal antibodies (MAbs) used to stain the epidermal GVMs included rat IgG1 anti-HSP73, dilution 1:100–1:300 (Stress-Gen Biotechnology; Victoria, BC, Canada), previously described as MoAb 1B5 (8), and mouse IgG1 anti-CK10,11, known as CK8.60 (10), dilution 1:4000 (Sigma; St Louis, MO).

Antibodies used to stain TRMs are summarized in Table 1. The MAbs HD121, dilution 1:100, and ID1, dilution 1:40, were obtained through the courtesy of Dr. K. Owaribe. MAb GoH3, dilution 1:20, was provided by
Table 1. Primary antibodies directed against structural EBMZ molecules used to assess the topographic staining efficacy of the FOAM technique

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen specificity</th>
<th>Ultradevelopment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD121</td>
<td>HD1 (500 KD)</td>
<td>Intracellular HD</td>
<td>9</td>
</tr>
<tr>
<td>SI-BP230</td>
<td>BP230 (BPAG1)</td>
<td>Intracellular HD</td>
<td>11</td>
</tr>
<tr>
<td>J1D1</td>
<td>BP180 (BPAG2)</td>
<td>Extracellular HD</td>
<td>19</td>
</tr>
<tr>
<td>GoH3</td>
<td>66 integrin</td>
<td>Extracellular HD</td>
<td>24,25</td>
</tr>
<tr>
<td>439-9B</td>
<td>B4 integrin</td>
<td>Extracellular HD</td>
<td>16,24</td>
</tr>
<tr>
<td>Lam1</td>
<td>Laminin 1</td>
<td>Lamina lucida</td>
<td>7</td>
</tr>
<tr>
<td>GB3</td>
<td>Laminin 5 (niccin)</td>
<td>Interface laminin /lamina densa</td>
<td>28</td>
</tr>
<tr>
<td>LH7:2</td>
<td>Collagen Type VII</td>
<td>Lamina densa and sublamina densa</td>
<td>17,23</td>
</tr>
<tr>
<td>II-32</td>
<td>Collagen Type VII</td>
<td>Lamina densa and sublamina densa</td>
<td>15,21</td>
</tr>
</tbody>
</table>

All antibodies are monoclonals except for SI-BP230 and Lam 1 (see Materials and Methods). According to recent nomenclature, the original EHS tumor-derived laminin is referred to as laminin-1 and niccin as laminin-5 (5).

HD, hemidesmosome complex (see also Figure 1). The data referring to the distribution of HD1 and BP180 antigen are based on studies in bovine cornea (9,19). The other data apply to human skin.

Dr. A. Sonnenberg, and MAb 439-9B, dilution 1:50, by Dr. S.J. Kennel. MAb GB3, dilution 1:200, was obtained by Dr. J.P. Ortonne, and MAb LH7:2, dilution 1:20–1:50, was generously provided in bulk by Dr. I.M. Leigh. Polyclonal rabbit anti-laminin 1, dilution 1:80, and MAb II-32, dilution 1:5000–1:10,000, were purchased from Telios Pharmaceuticals (San Diego, CA). The antibody St-BP230, dilution 1:30–1:20, was obtained as IgG fraction from the serum of a patient with BP. Analysis by Western immunoblotting, using the soluble fraction of cultured human epidermal cells as antigenic substrate, showed that SI-BP230 was reactive with the 230 KD (BPAG1) but not with the 180 KD (BPAG2) pemphigoid antigen.

Secondary polyclonal antibodies included (a) biotinylated γ-chain-specific goat Ig anti-mouse IgG1, dilution 1:100–1:200 (Southern Biotechnology; Birmingham, AL), (b) fluorescein isothiocyanate (FITC)-conjugated γ-chain-specific goat Ig anti-rat IgG, dilution 1:50 (Southern Biotechnology), (c) dichlorotriazine-fluorescein (DTAF)-conjugated donkey Ig anti-rat IgG, dilution 1:200 (Jackson Immunoresearch; Avondale, PA), (d) lissamine rhodamine B sulfonyl chloride (LRSC)-conjugated donkey Ig anti-rat IgG, dilution 1:100–1:200 (Jackson Immunoresearch), (e) LRSC-conjugated donkey Ig anti-rabbit IgG, dilution 1:100–1:300 (Jackson Immunoresearch), (f) LRSC-conjugated donkey Ig anti-mouse IgG1, dilution 1:200 (Jackson Immunoresearch), (g) FITC-conjugated mouse IgG2a anti-human IgG, dilution 1:20 (Zymed Laboratories; San Francisco, CA), and (h) FITC-conjugated γ-chain-specific goat Ig anti-mouse IgG1, dilution 1:100 (Southern Biotechnology). Binding of the biotinylated antibody was visualized by incubation with DTAF-conjugated streptavidin, dilution 1:200, and/or LRSC-conjugated streptavidin, dilution 1:200–1:600 (Jackson Immunoresearch). All antibody dilutions were made in PBS (0.01 M, pH 7.3) enriched with 5 x crystallized ovalbumin, 4% w/v (Serva; Heidelberg, Germany). Before use the diluted antibodies were centrifuged at 13,400 × g for 3 min.

Slide Preparation. Cryosections (4 µm) were mounted on chrome gelatin-coated glass slides, air-dried before a fan for 30 min, and pre-washed for 5 min in PBS (0.01 M, pH 7.3). The sections were pre-treated for 15 min with Triton X-100 (0.125% w/v in PBS), except in the case of staining integrin antigens, and were encircled with a hydrophobic emulsion (PAP pen; Dako, Glostrup, Denmark). Staining steps of 30 min each were alternated by thorough washing for 15 min in PBS. All incubations (see below) were done in a moist chamber at ambient temperature. The sections were mounted in PBS–glycerol (50% w/v, pH 7.3) containing 1.5 mg/ml of the anti-fading reagent p-phenylenediamine (Sigma). Before mounting, the hydrophobic PAP ring was removed to minimize the distance between sections and coverslip.

Topographic Immunostaining. The staining efficacy and effective resolution of the FOAM technique were studied by performance testing. For this purpose we identified the co-localization of different EBMZ antigens with fairly well-established ultrastructural location (Figure 1), using video microscopy and computer-aided overlaying (see below). In all, we studied the relative distribution of seven pairs of red- and green-labeled EBMZ antigens, referred to as red TRM and green TRM (see Table 3). The co-localization of these TRMs was studied in nine skin sections cut from three biopsy samples. The immunostaining procedure used to locate the TRM pair, BP230 and collagen Type VII (Figures 2a–2c) is detailed in Table 2. The same staining principle was employed to locate the other TRM pairs (Table 3), using the primary antibodies listed in Table 1. In addition to the TRMs stained, the topographic staining model includes simultaneous red and green labeling of a geometric verification marker (GVM), allowing accurate control of the geometric fidelity of the overlay images (3). For this purpose we used either the epidermal suprabasal antigen HSP73 (heat-shock protein 73) or CK10,11 (cytokeratin 10,11). The specificity of the staining protocols was thoroughly checked, using well-established principles of replacement of the relevant antibodies and conjugates by irrelevant ones and/or by reagents yielding purposely undesired specific staining (26). In an additional control we exchanged the fluorochromes used to label the TRM pair, u6 integrin and collagen Type VII. This showed the same relative antigen distribution with reversed position of their colors. Last but not

Table 2. Example of a five-step immunostaining procedure used to assess whether the topographic difference of the BP230 antigen and collagen Type VII antigen can be distinguished by visual inspection of FOAM images

<table>
<thead>
<tr>
<th>Step</th>
<th>Immunostaining reagents</th>
<th>Dilution</th>
<th>Antigen marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a.</td>
<td>RTlgG anti-HSP73</td>
<td>1:200</td>
<td>Red + green GVM (HSP73)</td>
</tr>
<tr>
<td>2a.</td>
<td>HuIgG anti-BP230 (SI-BP230)</td>
<td>1:10</td>
<td>Green TRM (BP230)</td>
</tr>
<tr>
<td>2b.</td>
<td>DnlqG anti-RTlgG/DTAF</td>
<td>1:300</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>MolqG2 anti-HuIgG/FTTC</td>
<td>1:20</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>MolqG1 anti-collagen VII</td>
<td>1:30</td>
<td>Red TRM (Collagen VII)</td>
</tr>
<tr>
<td>5.</td>
<td>Streptavidin/LRSC</td>
<td>1:200</td>
<td></td>
</tr>
</tbody>
</table>

* The reagents of Steps 1a,b and Steps 2a,b,c were used as a cocktail. Rt, rat; Dn, donkey; Hu, human; Mo, mouse; Go, goat. DTAF and FITC are green fluorescent tags and LRSC is a red fluorescent tag (see also Table 4).

* Geometric verification marker and topographic reference markers as illustrated in Figures 2–2c.
Figure 2-4. Three panels of video microscopic images illustrating the topographic staining efficacy of the FOAM technique applied to frozen sections of normal arm skin. Each panel shows the "linear" fluorescence pattern of the EBMZ, marked by immunostaining of two different EBMZ antigens in red (a) and green (b) respectively. In addition, each figure panel shows the cytoplasmic fluorescence pattern of the suprabasal epidermal layer, visualized by a simultaneously red and green fluorescent geometric verification marker used to correct for image shifts. Furthermore, each panel shows an overlay (c) of the red and green image, illustrating the distribution relative to one another of the EBMZ antigens (TRM pair). Bar = 10 μm.

Figure 2. (a) The red staining pattern of the EBMZ antigen, collagen Type VII, and of the cytoplasmic marker, HSP73. (b) The green staining pattern of the EBMZ antigen BP230, and of the same HSP73 marker. Note that (c) the shift-corrected overlay shows complete overlap of the red and green HSP73 marker, in contrast to the red collagen Type VII and green BP230 antigen. The reagents and procedure used to stain these antigens are detailed in Table 2. Comparable staining procedures were used to identify the other TRM pairs illustrated, using the primary antibodies indicated in Table 1.

Figure 3. (a) The red staining pattern of the EBMZ antigen, collagen Type VII, and of the cytoplasmic marker, CK10,11, using MAb CK8.60 as the primary antibody. (b) The green staining pattern of the EBMZ antigen, α6 integrin, and of the same CK10,11 marker. (c) The overlay shows still distinctive red collagen Type VII beneath the yellow-orange interface of the EMBZ antigens.

Figure 4. (a) The red staining pattern of the EBMZ antigen, laminin-1, and of the cytoplasmic marker HSP73. (b) The green staining pattern of the EBMZ antigen BP180 and of the same HSP73 marker. (c) The overlay shows an indistinct EBMZ pattern consisting of mixed colors of the red laminin-1 and of the green BP180 antigen. Note the green intercellular α6 staining in the epidermal basal layer (Figures 3b and 3c), and the red laminin-1 staining of a blood vessel wall (Figures 4a and 4c), indicating the specificity of the immunostaining procedures.

At least, it is essential that additional requirements of color fidelity (Table 4) are fulfilled as well (12).

Fluorescence Microscopy. The sections were examined with a Leitz Orthoplan microscope equipped with a xenon short arc lamp (XBO 75W/2), a Mercury short arc lamp (HBO 50W/AC/L2), and a stepless turnable mechanical stage (110° rotation). Wavelength selection was made by a 4λ-Ploemopak unit for incident light excitation (Table 4). For selective emission of FITC and DTAF fluorescence, the original 12/3 filter set (Leitz/Leica; Wetzlar, Germany) was modified by simply sliding a shortpass interference filter SP560 (Omega Optical; Brattleboro, VT) in the emission path.

Video Microscopy and Image Processing. Pairs of red and green fluorescent microscopic images were acquired as separate 8-bit gray-scale TIFF files using a low-cost image processing system, based on an 80486 IBM-PC/AT-compatible computer. The system is equipped with a MATROX MVP-AT processor (MATROX Electronic Systems; Dorval, Quebec, Canada), a black-and-white Fairchild CCD 5000/1 camera (Fairchild Weston Systems; Sunnyvale, CA), and an expansion board for exposure time control (29). The software for image acquisition was developed with the aid of the
Table 3. Results of topographic antigen mapping using defined pairs of separate red and green fluorescent topographic reference markers and a concomitant red and green fluorescent geometric verification marker (compare Figure 1)

<table>
<thead>
<tr>
<th>Green TRM*</th>
<th>Red TRM*</th>
<th>nm</th>
<th>EBMZ visual appearance*</th>
<th>Distinct</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP230 (BPAG1)</td>
<td>Collagen VII</td>
<td>560</td>
<td>Green, yellow-orange, red</td>
<td>Yes</td>
</tr>
<tr>
<td>α6 integrin</td>
<td>Collagen VII</td>
<td>460</td>
<td>Green, yellow-orange, red</td>
<td>Yes</td>
</tr>
<tr>
<td>β4 integrin</td>
<td>Collagen VII</td>
<td>460</td>
<td>Green, yellow-orange, red</td>
<td>Yes</td>
</tr>
<tr>
<td>Laminin 1</td>
<td>Collagen VII</td>
<td>370</td>
<td>Orange-yellow, green</td>
<td>Yes</td>
</tr>
<tr>
<td>BP180 (BPAG2)</td>
<td>Laminin-1</td>
<td>60</td>
<td>Yellow-orange</td>
<td>No</td>
</tr>
<tr>
<td>β4 integrin</td>
<td>Laminin-5</td>
<td>30</td>
<td>Yellow-orange</td>
<td>No</td>
</tr>
</tbody>
</table>

* For marker antibodies and ultrastructural localization, see Table 1.

** Estimated maximal distance (nm) between antigen distributions of each TRM pair, based on mean values of the maximal width of the EBMZ layer in which the respective antigens are supposed to reside (11, 15, 27).

The fluorescence colors of light perceived are arranged from the epidermal to the dermal compartment of the EBMZ. Distinctive colors are separated by a comma.

Table 4. Filter sets, wavelength band characteristics, and light sources used to ensure color fidelity, using LRSC, FITC, and DTAF fluoroprobes

<table>
<thead>
<tr>
<th>Filter set*</th>
<th>LRSC (red)</th>
<th>FITC</th>
<th>DTAF (green)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter set</td>
<td>TXR (XF 44)</td>
<td>BP540-580</td>
<td>BP540-690</td>
</tr>
<tr>
<td>Excitation filter</td>
<td>BP490</td>
<td>&gt;SP510</td>
<td>SP560</td>
</tr>
<tr>
<td>Emission filter</td>
<td>BP605-665</td>
<td>LP510</td>
<td>SP560</td>
</tr>
<tr>
<td>Light source</td>
<td>Mercury arc (HBO)</td>
<td>Xenon arc (XBO)</td>
<td></td>
</tr>
</tbody>
</table>

* Filter sets are indicated by trademark names. The TF 12/3m is a modified 12/3 filter set (2). DRLP, dichroic reflection longpass filter (dichroic mirror); BP, bandpass filter; LP, longpass filter; SP, shortpass filter. The wavelength band of these filters is expressed in nanometers. A 4-mm BG38 red suppression filter was always inserted in the lamp housing.
the yellow-orange interface. As the green fluorescence merges into the overlap color yellow, these spots are hard to reproduce in the overlay. The staining patterns of the TRM pair, a6 integrin and collagen Type VII, are illustrated in Figures 3a and 3b. Although still distinctive, the overlay pattern (Figure 3e) showed a smaller red fluorescence layer merging into the broader yellow-orange interface (compare Figure 2c). This pattern was also found with the TRM pair, β4 integrin and collagen Type VII (not shown). Surprisingly, overlay of the TRM pair, β4 integrin and HDL (red), yielded a small but distinctive green layer beneath an orange-yellow layer (not illustrated). This suggests that the average distance between these molecules is somewhat larger than indicated in Table 3. By contrast, the TRM pair, laminin-1 and collagen Type VII (Figures 4a and 4b) yielded an indistinct yellow-orange EBMZ pattern in the overlay, as illustrated in Figure 4c. Comparable indistinct overlay patterns were obtained with the TRM pair, BP180 and laminin-1, and with the TRM pair, β4 integrin and laminin-5 (not shown). To this can be added that close inspection of the separate red and green images of these latter TRM pairs did reveal subtle morphological differences lengthwise along the EMBZ. Comparison of Figures 4a and 4b may clarify this point. Future studies involving elaborate digital pattern recognition should establish whether such morphological differences have any discriminating value.

Unfortunately, we were unable to determine the relative distribution of the TRM pair, BP180 and collagen Type VII, because of crossreactive specific staining yielded by their IgG1 subclass antibodies. However, the findings obtained with the TRM pair, β4 integrin and collagen Type VII, may serve instead, as the location of the β4 antigen roughly compares with that of the BP180 antigen. The two different MAbs (LH7:2 and I1-32) used to identify collagen Type VII could be exchanged without essentially modifying the results presented in Table 3. The only difference observed was the more conspicuous staining by MAb II-32. This allowed better crossreactive specific staining yielded by their IgG1 subclass antibodies. However, the findings obtained with the TRM pair, β4 integrin and collagen Type VII (not shown). To this can be added that close inspection of the separate red and green images of these latter TRM pairs did reveal subtle morphological differences lengthwise along the EMBZ. Comparison of Figures 4a and 4b may clarify this point. Future studies involving elaborate digital pattern recognition should establish whether such morphological differences have any discriminating value.

Discussion

The present study has evolved from the underlying question of whether the FOAM technique potentially enables identification of topographic differences of autoimmune deposits at one side or the other of the EBMZ, such as are found in the disease variants BP and EBA. Before any such elaborate work is undertaken, the soundness of the underlying techniques must be assessed (2.3). We do not claim that the topographic staining model used in this study offers the ultimate possibility to establish the lower resolution limit of the technique (see below). We do claim that this model is the best one can get to formulate an answer to the underlying question mentioned above. Our results show that it is indeed possible to distinguish antigenic sites on the hemidesmosome complex from those on the anchoring fibril structures below the lamina densa. This seems at variance with an earlier statement that the resolution of the fluorescence microscope is incapable of determining such topographic differences (14). In view of the dimensions of the EBMZ layers (4, 27), direct microscopic identification of such differences appears very difficult, if not impossible. Even the use of multiband filter sets for concomitant observation of the red and green fluorescence signals would make little difference, because shades of red, green, and yellow-orange are very difficult to define under the conditions specified (personal observation, SB and MCMdE). Our present technique appears to be more promising, provided that due consideration is given to the geometric fidelity (3) and the color fidelity (2). The strict requirements of staining specificity may give rise to hopeless problems in devising the desired staining protocol. Most of these problems arise from the mere fact that the bulk of MAbs available are of the mouse IgG1 subclass. One of the underlying problems is the necessity to have strict control over the correct geometric position of the red and the green TRM. For this purpose we included a simultaneously red and green fluorescent GVM in the FOAM procedure. Ideally, a GVM should fulfill the following requirements: (a) suitability to be labeled reproducibly by at least two distinct fluorescent tags which do not interfere with TRM staining; (b) morphologically discrete presence at tissue sites where the TRMs of interest reside; and (c) no spatial interference with the TRM distribution pattern(s). Including such an in situ marker allows accurate alignment of images without having to worry that the slightest touch of the microscope can easily disturb the calibration settings of the system, as needed with external markers. Ideally, the fluorescence intensities of both the GVM and TRMs should match completely, yielding red and green digitized images of comparable saturation and brightness. In practice this is almost impossible. However, our technique offers the possibility to improve poorly balanced fluorescence intensities by selective adjustment of exposure times (29) and a simple digital image enhancement procedure. This enabled us to correct geometric errors (image shifts) with an accuracy of 200 ± 50 nm. It should be realized that this value applies to tissue sections of 4 μm thickness, a numerical aperture of only 1.00, and wavelength bands of 510–560 nm and 605–665 nm. These data give an upper limit to the effective resolution of the FOAM technique. Further studies using digital image analysis could well show that finer detail can be detected reproducibly. In theory, one might expect that the resolution limit of the microscope is the lower limit (~250 nm). This limit holds only for objects emitting light of the same wavelength. In the present case, the objects to be distinguished emit light of separate wavelengths, which leads to a rather different situation. If the image is sampled at the Nyquist frequency (125 nm/pixel), it should be possible to determine the center of gravity of each line to within about 30 nm (0.25 pixel). The difference between these centers can then be determined with an accuracy of about 45 nm. To compensate for residual errors in the correction of geometric errors, the same procedure should be used on the GVM, which would estimate this error to within the same accuracy. This suggests an effective lower resolution limit of some 60–65 nm. We are presently...
working on digital image analysis techniques to see whether this limit can be reached at all. In addition, further exploration of the combined use of FOAM and confocal microscopy could well show that even finer detail can be detected, particularly in tissue sections (6).

The results presented on the topographic staining efficacy show that the procedures specified enabled visual distinction of some, but not all, TRM pairs studied. In view of the upper limit of the effective resolution, it is apparent from Table 3 that some discrepancies were observed. A sound interpretation of these findings should take into account the following facts. First, the numerical dimensions provided are derived from electron microscopic studies and do not immediately apply to the findings presented here. Simple calculation will show that, e.g., the width of the EBMZ layer stained by anti-collagen Type VII (Figure 2a) approximates 3.75 μm. This value is about 11 times the dimension found in electron microscopy (15). Second, the dimensions do not represent volumetric data, as required by strict morphometric principles. Third, the antibodies (Table 1) react with specific epitopes that do not necessarily represent the volumetric dimension of the antigens, let alone the ultrastructural dimension of the EBMZ layer in which the antigens reside. With these reservations in mind, the results presented in Table 3 show enough promise to use collagen Type VII as the prime TRM for differentiating between BP and EBA. It can be expected that the staining pattern of this TRM will show substantial similarity and will overlap with that of skin-bound IgG deposits in EBA (18,23). By contrast, the staining pattern of IgG deposits in BP (20,22) is likely to be distinctive from that of collagen Type VII. The reverse probably holds for the distribution patterns of α6 integrin, β4 integrin, and BP180 and BP230 antigens, which are likely to show gross overlap with the distribution of IgG deposits in BP but not with that in EBA. Given some restrictions (see below), this implies that both collagen Type VII and β4 integrin are the most promising TRMs for the purpose mentioned. Unfortunately, the use of α6 integrin seems less attractive because of its variable presence in human skin. The use of BP180 and BP230 may present some difficulty, because antibodies to these antigens are not yet commercially available.

In conclusion, we have shown that the currently developed procedures offer the possibility to distinguish topographic differences in antigen distributions with an upper resolution limit of 200 ± 50 nm. Furthermore, our findings indicate that collagen Type VII and β4 integrin are the prime molecules to serve as TRM in future applications of the technique aiming at the diagnostic differentiation of BP and EBA. Preliminary results on this topic are most promising indeed.

Acknowledgments
We gratefully acknowledge Mr Jacob Pleiter for his kind help with the Lasermark film recorder and Mr Peter van der Syde and Mr Dick Huizinga for skillful assistance in the processing of color reproductions. We wish to thank Mr A Pasma for giving us access to the dual-band filter set XF53 (Omega Optical).

Literature Cited
1. Bhogal BS, Black MM: Diagnosis, diagnostic and research techniques.

11. Ishiko A, Shimizu H, Kikuchi A, Ebihara T, Hashimoto T, Nishikawa T: Human autointibodies against the 230-kD bullous pemphigoid antigen (BPAG1) bind only to the intracellular domain of the hemidesmosome, whereas those against the 180-kD bullous pemphigoid antigen (BPAG2) bind along the plasma membrane of the hemidesmosome in normal human and wrine skin. J Clin Invest 91:1608, 1993
17. Leigh IM, Purks PE, Bruckner-Tuderman L: LH7.2 monoclonal antibody detects type VII collagen in the sublamina densa zone of ectodermally-derived epithelia, including skin. Epithelium 1:17, 1987


