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Serration pattern analysis for differentiating epidermolysis bullosa acquisita from other pemphigoid diseases

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Supplemental: table S1, figures & step-by-step protocol; video cryomicrotomy 4 µm sections

ABSTRACT

Background: Direct immunofluorescence microscopy (DIF) of a skin biopsy specimen is the reference standard for the diagnosis of pemphigoid diseases (PD). Serration pattern analysis enables differentiation of epidermolysis bullosa acquisita (EBA) from other PD using DIF microscopy alone. However, practice gaps need to be addressed for implication of this technique in daily routine diagnostics.

Objective: to determine and optimize the technical requirements for serration pattern analysis of DIF microscopy and determine inter-rater conformity of serration pattern analysis.

Methods: we compared serration pattern analysis of routine DIF from Groningen and Lübeck laboratories with four blinded observers. Skin biopsies from 20 patients with EBA and other PD were exchanged and analysed. Various factors of section thickness, transport medium and biopsy processing were evaluated.

Results: inter-rater conformity of four observers was 95.7%. Recognition of serration patterns was comparable in samples transported in saline and in Michel's medium and with section thicknesses of 4, 6 and 8 µm.

Limitations: sample size and availability of 20 samples retrospectively compared.

Conclusion: DIF serration pattern analysis is not restricted by variation in laboratory procedures, transport medium, or experience of observers. This learnable technique can be implemented as routine diagnostic method in extension to DIF microscopy for subtyping PD.

Key words: basement membrane – dermatopathology - autoimmune bullous disease – pemphigoid – epidermolysis bullosa acquisita – direct immunofluorescence microscopy – diagnostic technique

List of abbreviations:

bSLE: bullous systemic lupus erythematosus

DIF: direct immunofluorescence microscopy

EBA: epidermolysis bullosa acquisita

EBMZ: epidermal basement membrane zone

PD: pemphigoid diseases

INTRODUCTION

In patients with pemphigoid diseases (PD), direct immunofluorescence (DIF) microscopy on a skin biopsy is considered as the reference standard for diagnosis.[1] PD are autoimmune bullous diseases of the skin and mucosa characterized by circulating and tissue-bound autoantibodies against structural proteins in the epidermal basement membrane zone (EBMZ). PD includes the subtype epidermolysis bullosa acquisita (EBA) characterized by autoantibodies against type VII collagen.[1,2] Differentiating EBA from other PD is relevant for both the treatment and prognosis of patients. Diagnosis of PD can be challenging because of the heterogeneous clinical presentation and histopathology.[1,3] For diagnosis of EBA in vivo-bound linear immunodepositions can be detected along the EBMZ in a skin biopsy by DIF microscopy and using serration pattern analysis[4], or detected by direct immuno-electron microscopy.[5] [6,7] Circulating autoantibodies against type VII collagen can be detected in serum by indirect immunofluorescence (IIF) microscopy on salt-split skin (SSS), immunoblot, or ELISA.[3,6,8,9] However, labeling of autoantibodies to the dermal side (floor) of the artifical split in IIF SSS is also seen in patients with anti-laminin-332 mucous membrane pemphigoid and anti-p200/laminin γ1 pemphigoid.[10-12] Additionally, the technique of DIF microscopy using salt-split patient skin may destruct the tissue sample and cannot differentiate EBA from other PD.[6] Furthermore, serological diagnosis of EBA is hampered by the low sensitivity of these immunoserological tests, with no detectable circulating autoantibodies and possible misdiagnosis in approximately 50% of patients with EBA.[7,13,14] Although the concept of DIF serration pattern analysis was described in 2004 and incorporated into standard textbooks, its use in routine DIF was limited, perhaps by perceived hurdles of high technical requirements.[4,15,16] Consequently, EBA is likely underdiagnosed, especially in seronegative patients.

By DIF, two serrated patterns of linear immunodeposits (IgG, IgA, C3) along the EBMZ can be identified in PD: the u-serrated pattern in EBA and in the more rare bullous systemic lupus erythematosus (bSLE) and the n-serrated pattern in all other PD

including bullous pemphigoid and mucous membrane pemphigoid. The u-serrated pattern is recognizable by finger-like structures or spikes (figure 1 a,b) and is pathognomonic for EBA/bSLE, where immunodeposits are located below the lamina densa associated with type VII collagen as part of anchoring fibrils. The linear n-serrated pattern (figure 1 c,d) can be seen in all other PD where the linear deposits are located on or above the lamina densa.[4,17] By comparing various technical factors of routine DIF procedures of two laboratories, including the use of saline versus Michel's transport medium, the thickness of cryosections, and conformity among observers with different levels of expertise, this study sought to optimize the DIF serration pattern analysis for the implementation in routine diagnostics.

MATERIALS AND METHODS

A total of 20 perilesional skin biopsy specimens of patients with suspected PD and positive findings of linear IgG immunodepositions along the EBMZ by DIF were analysed. Archived biopsies were included of patients diagnosed with EBA (n=10) based on a linear u-serrated IgG immunodeposition pattern (100%). These EBA patients were either seropositive with circulating autoantibodies against type VII collagen confirmed by ELISA and/or immunoblotting (60%) or seronegative (40%). Biopsy specimen of patients diagnosed with other PD (n=10) were included based on a linear (n-serrated) IgG immunodeposition pattern along the EBMZ. For comparative purposes biopsies transported in saline (Groningen) and Michel's transport medium (Lübeck) were exchanged between the two laboratories and processed according to routine diagnostic procedures for DIF microscopy of each laboratory (see below). This type of study with a retrospective reanalysis of left-over patient materials for diagnostic purposes is exempted from the review by the medical ethical committees in the Netherlands and Germany.

Observers and study procedure

Four observers with different grades of experience independently examined routine 6µm sections for serration pattern analysis, including a dermato-pathologist (GD) and dermatologist (MJ) from Groningen, and a dermatologist (ES) and resident in dermatology (AV) from Lübeck. Sections were presented at random (JM and IA) to the observers and the observed immunodeposition patterns were classified as linear n-serrated, linear u-serrated, or undetermined. The intensity of fluorescence of the IgG immunodepositions was graded as + (weak), ++ (moderate) or +++ (bright). To evaluate the influence of section thickness on serration pattern analysis, from all biopsy specimens 4, 6 or, 8 µm thick sections were cut in the Groningen laboratory and randomly examined by two observers (GD and MJ).

DIF laboratory procedures

In the Groningen routine DIF laboratory procedure, perilesional biopsies were placed in saline in polypropylene vials, stored overnight in saline (12-16 hours) and processed the next day. Biopsies were then placed in aluminium vials, snap-frozen in liquid nitrogen and stored at -80℃ until processing. Temp erature of cryostate (Leica CM3050S, Leica, Wetzlar, Germany) and microtome knife was regulated between as low as -20 and -35℃ to prevent compression and def ormation of the sections. For biopsy fixation, first a small wetted piece of filter paper was applied to the fixation table, a circle of Tissue-Tek O.C.T. Compound (Sakura, Alphen aan den Rijn, The Netherlands) was added to the filter paper, frozen and filled up with another drip of O.C.T. Compound for fixation of the biopsy specimen (figure S1, S2). The biopsy specimen was only partially embedded with the base in O.C.T. Compound and the top of the specimen protruding for cutting (figure S2, S3). Cryosections were cut with tungsten carbide conventional microtome knives (Type C 160mm, Spikker Specials, Zevenaar, The Netherlands). The microtome knives were standard wedge shaped with a bevel angle of 20° and resharpenable (figure S3). Cryosections were mounted on polysine® glass slides sideways with no pressure applied (supplemental video). The sections were stained for 30 minutes in a moist chamber at room temperature using

fluorescein FITC-labeled Fc-specific goat $F(ab')_2$ antibody against human IgG (Protos 311, Protos immunoresearch, Burlingame, CA, USA). SlowFade™ Antifade Reagent (ThermoFisher Scientific Inc., Waltham, MA, USA) was added to inhibit photobleaching. Sections were examined with a Leica DM2000 microscope and Leica HCX PL Fluotar 40x/0.75 dry objective and 10x ocular (total magnification x400) (Leica, Wetzlar, Germany).

The Lübeck routine DIF laboratory procedure only showed minor differences compared to the Groningen procedure. Biopsies were either kept in Michel's medium or snap frozen at -20℃ until processing. Biopsy speci mens were fixated by completely embedding the material in Tissue-Tek O.C.T. Compound and cryosections were cut using disposable microtome blades S35 (Feather, Osaka, Japan). Sections were stained using a monoclonal mouse anti-human IgG-FITC antibody (Bio-Rad, Hercules, CA, USA) and examined with a Olympus BX40 microscope and 40x/0.75 dry objective (UPlan FI, Olympus, Tokio, Japan). Photos, video and extended methods are available in supplemental material.

For additional automated serration pattern recognition, all slides were photographed with total magnification x400 with standardized settings and processed with Leica Application Suite imaging software. The automated technique by Shi *et al.* (under review) was developed based a trainable inhibition-augmented COSFIRE filter with detection of the distinctive spikes (ridge-endings) found in u-serrated patterns, similar to recognition of fingerprints.[18]

Statistical analysis

Data were analyzed using commercially available software (SPSS version 23, IBM, Armonk, NY, USA). Qualitative data were expressed as frequencies and percentages. Statistical analysis of recognition rates of serration pattern analysis was done by the McNemar test. For comparison of recognition rates between 4µm, 6µm and 8µm sections the Cochran's Q test was used. Conformity among four observers (inter- and intra-observer variability) was assessed of routine serration pattern analysis of 6 µm

sections and of fluorescence intensity of IgG staining and was calculated with two-way mixed intraclass correlation coefficient (ICC 3,4). All tests were two-tailed, a p-value of less than 0.05 was considered significant.

RESULTS

The main outcome for diagnostic purposes in daily practice was the recognition rate of serration patterns of routinely cut 6 µm sections (n=20) from both laboratories (figure 1). The mean recognition rate by four observers was 97.5% and the intraclass correlation coefficient ICC(3,4) for the conformity among four observers (inter- and intra-observer variability) was 0.957 (95% CI 0.916-0.981). Moreover, no erroneous serration patterns were classified in the complete experimental setup, differences only occurred in classification of 'undetermined' immunodepositions (Supplemental material, table S1). Sections were classified as n-serrated in 48.8%, u-serrated in 43.8% and undetermined in 7.5%. Of interest was one specific case classified by all observers as 'undetermined' with a homogeneous immunodeposition of IgG instead of a linear immunodeposition (supplemental material, table S1, case GR5). In retrospect this biopsy proved to be of a patient with porphyria cutanea tarda who was initially misdiagnosed as mechano-bullous EBA (seronegative) and is subject of current research.

Biopsies transported in Michels's transport medium or frozen at -20℃ showed a higher background fluorescence in the dermis compared to biopsies transported in saline (figure 1). However, no significant difference was seen in the recognition of serration patterns comparing 6µm sections of samples transported in Michel's transport medium from the Lübeck laboratory to samples transported in saline from the Groningen laboratory (p=0.49). For interpretation of these data, fluorescence intensity of IgG staining of the random selected biopsies was classified as 1+ (11.3%), 2+ (48.7%) and 3+ (40.0%). The classification of fluorescence intensity of IgG staining

among the four observers showed a high conformity (ICC(3,4) 0.836). The overall serration pattern recognition rate of sequentially cut 4, 6 and 8 µm sections (n=60) was 95.8%. No statistical significant differences in recognition rates were seen between the groups with 4, 6 and 8 µm sections (p=0.368, supplemental figure S4), or between the two observers (p=0.063).

DISCUSSION

The implementation of DIF serration pattern analysis in daily routine diagnostics is possibly hampered by presumed technical hurdles, such as the inability to use Michel's medium for transport, the need of cutting very thin <4 µm sections, the requirement of high-magnification ocular-objectives (x600), and the need of a trained pathologist. We found in two independent laboratories that not a single technical factor really limits the performance in routine diagnostics. Using DIF serration pattern analysis and recognizing the pathognomonic u-serrated pattern may increase the number of diagnosed cases of EBA substantially and is the most informative and cost-effective diagnostic test for PD.[13,14] This learnable technique can be trained online with DIF photos of serrated patterns at our website: nversusu.umcg.nl.

In more detail, a higher background fluorescence was observed in biopsy specimens transported in Michel's medium compared to saline (figure 1).[19] A decreased signal to noise ratio was mainly observed in 8 µm cryosections, which may lead to false-negative findings in cases with low staining intensities. The additionally performed automated pattern recognition based on the DIF photos of all slides showed a recognition rate of 90% of u-serrated patterns, confirming that the spikes or ridge-endings of the u-serrated patterns are distinctive recognizable features (Shi *et al.* under review).[18,20] The recognition rate decreased with thicker 8 µm sections of biopsies transported in Michel's medium, because of the loss of details of spikes with the higher background fluorescence.

Exchange of experiences in two laboratories

Although the various technical factors did not critically impair the DIF serration pattern recognition, the exchange of experiences of observers and laboratory technicians taught us the following on optimizing the DIF procedure. The non-disposable wedge-shaped microtome blade with a bevel angle is optimal, however, cutting cryosections of 4 µm was also possible with disposable microtome blades. Only partially embedding the biopsy tissue in O.C.T. compound improved the cutting characteristics, resulting in more stretched sections that are better microphotographed within the depth of field (figure S2, S3 and supplemental video). In contrast, details can be lost in overexposed areas and micrometering is needed to focus in 8 µm cryosections. Use of saline transport medium and cutting thin sections decreases background staining, which is necessary to distinguish serrated patterns in specimens with low amounts of IgG deposition. The limiting factor for saline transport is the required transport time to be less than 48 hours from physician to (reference) laboratory.[19] Alternatively, Michel's medium allows transport times longer than 48 hours. We investigated in both laboratories pre-incubation of biopsy specimens in saline prior to storage in Michel's medium with various time intervals, and also washing of biopsy specimens with saline and various buffers after storage in Michel's medium. However, these alternative detours did not decrease background fluorescence.

In conclusion, serration pattern analysis in routine DIF microscopy can differentiate EBA from other PD and is essential for a definitive diagnosis of EBA in patients with no detectable circulating autoantibodies in serum. The very high recognition rate and conformity among various observers in this study show that serration pattern analysis is not critically restricted by variations in technical laboratory procedures, transport mediums, and the experience of the observers. This easily learned technique can be implemented as routine diagnostic method in extension to DIF microscopy for the diagnosis and subtyping of PD.

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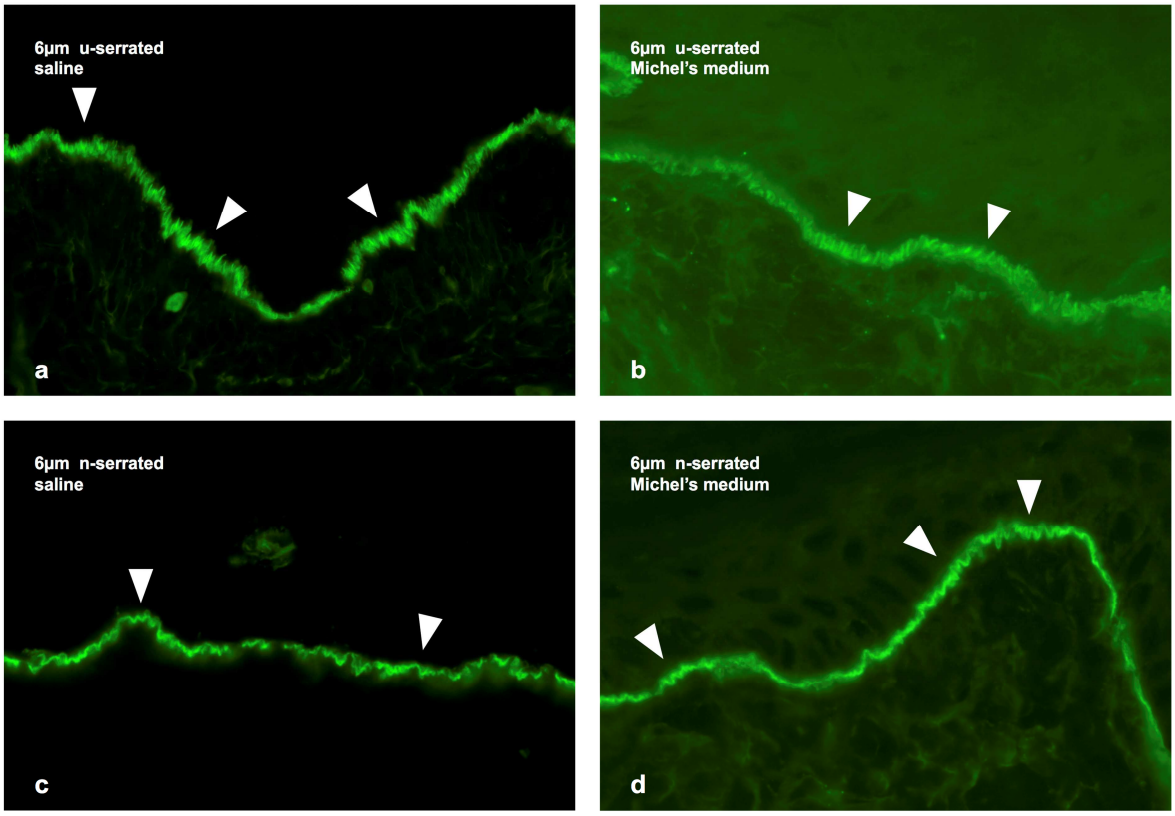
LEGENDS TO FIGURES

Figure 1

Direct immunofluorescence microscopy serration pattern analysis: u-serrated and n-serrated patterns in pemphigoid diseases. Linear u-serrated IgG deposition patterns along the EBMZ in patients with EBA with biopsies transported in saline (a) and Michel's medium (b). Note distinct spikes or 'growing grass' in u-serrated patterns (arrowheads, a and b). Linear n-serrated IgG deposition patterns along the EBMZ in patients with other pemphigoid diseases with biopsies transported in saline (c) and Michel's medium (d). Note a continuous curved linear deposition with closed arches at the tops (arrowheads, c and d). All 6µm cryosections were cut and processed in the Lübeck laboratory. Original magnification x400.


6µm u-serrated
saline
a
6µm u-serrated
Michel's medium
b
6µm n-serrated
saline
c
6µm n-serrated
Michel's medium
d

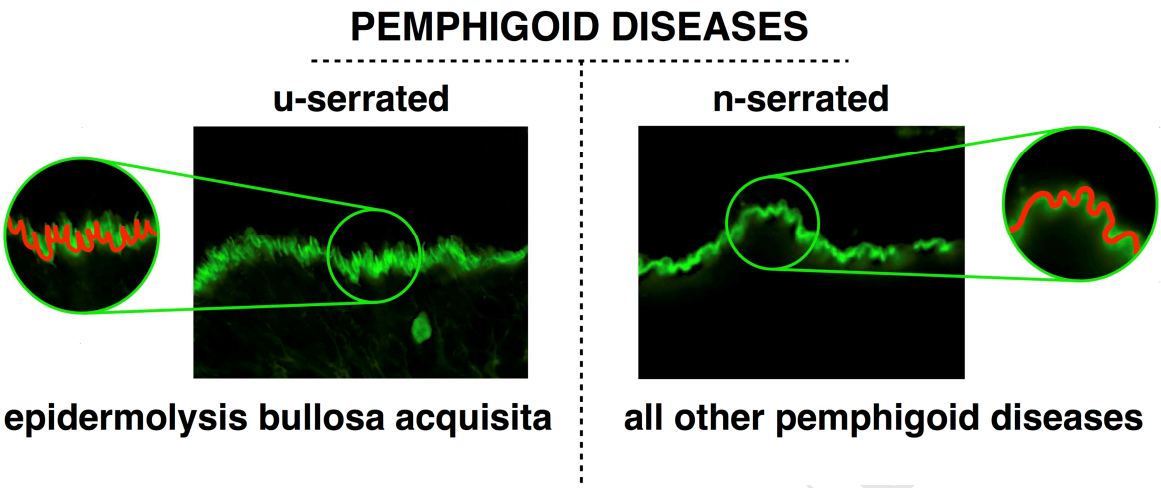


Figure S1 - Biopsy fixation. A circle of Tissue-Tek O.C.T. Compound (Sakura, Alphen aan den Rijn, The Netherlands) is applied to the wetted filter paper and frozen with freezer spray.

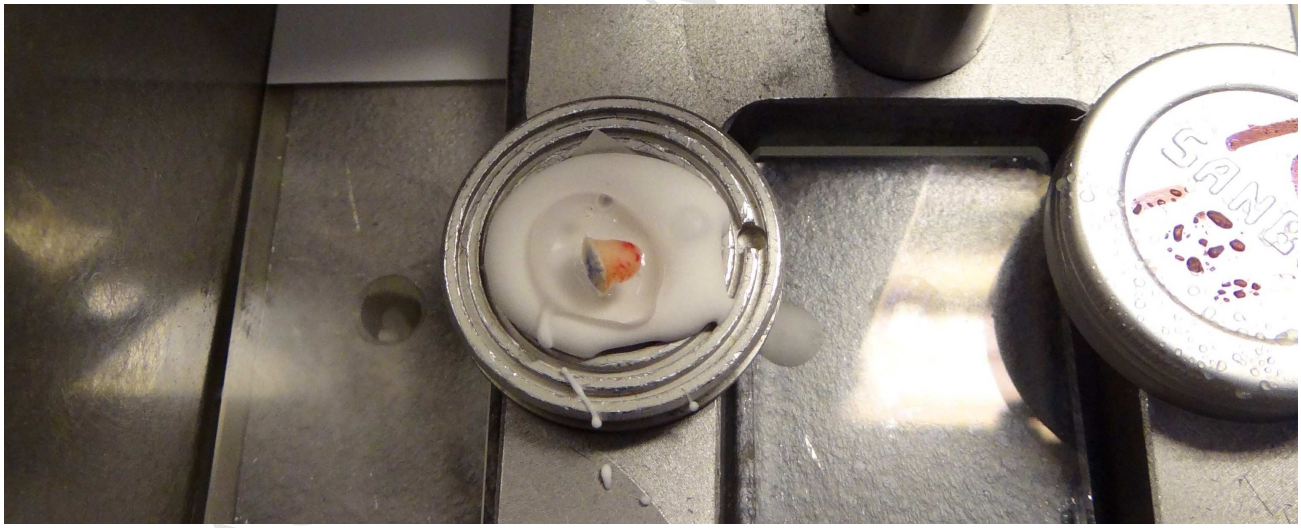


Figure S2 - Biopsy fixation. The circle is filled up with another drip of OCT Compound for fixation of the biopsy specimen. The biopsy specimen is not completely, but only partially embedded in OCT Compound and protrudes for cutting only the biopsy tissue.

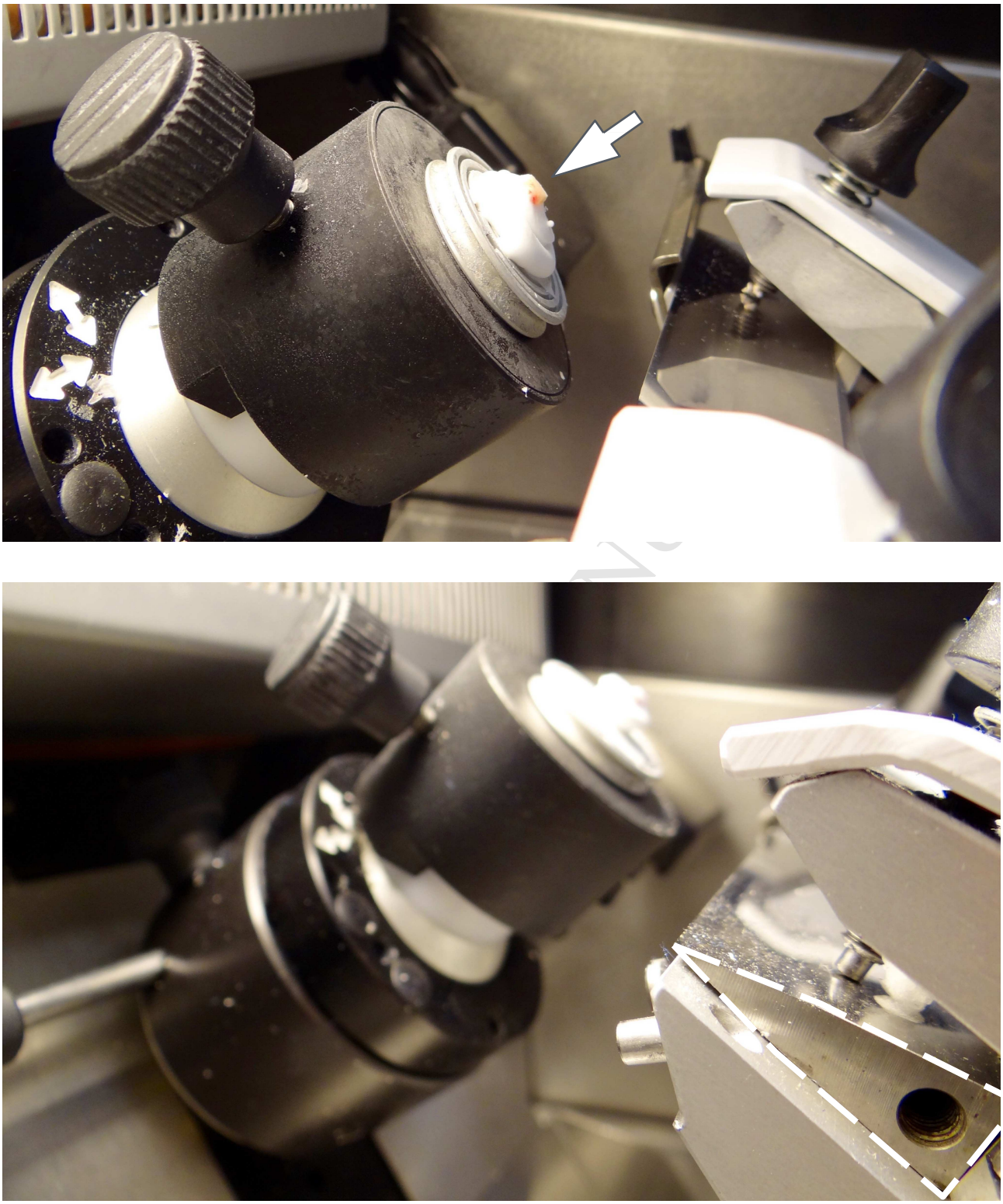


Figure S3 - Biopsy fixation and microtome blade. The biopsy specimen is not completely, but only partially embedded in OCT Compound and protrudes for cutting only the biopsy tissue. Cryosections of are cut with resharpenable wedge shaped tungsten carbide microtome blades with a bevel angle (Type C 160mm, Spikker Specials, Zevenaar, The Netherlands).

Supplemental video - Cutting and processing cryosections. Cryosections are aligned on the microtome blade, mounted on polysine® glass slides sideways with no pressure applied and quality checked for artefacts with light microscopy.

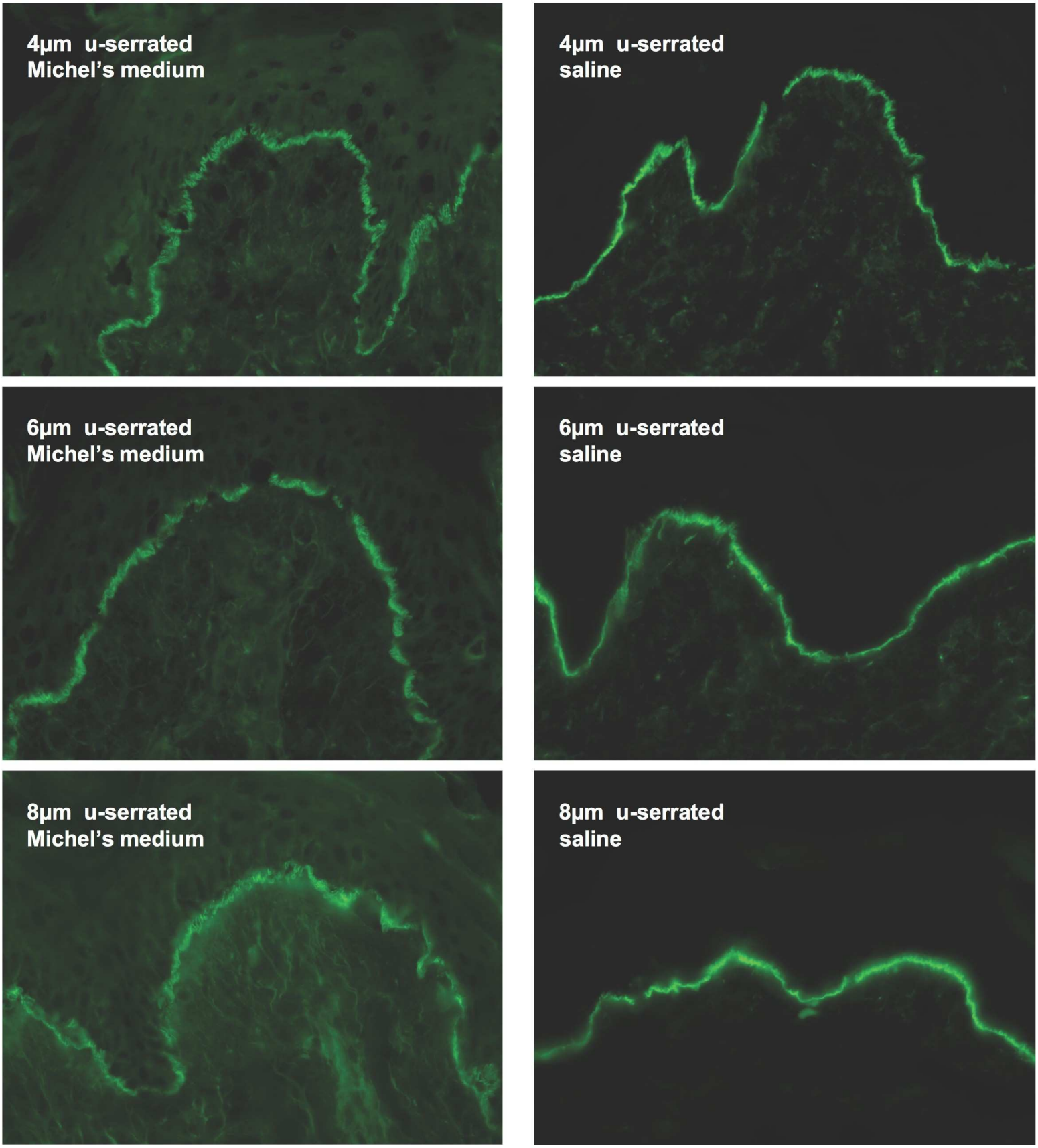


Figure 54 - Direct immunofluorescence of epidermolysis bullosa acquisita: effect of section thickness and transport medium. Comparison of 4f.Jm, 6f.Jm and 8f.Jm thick cryosections on linear u-serrated lgG immunodepositions by DIF. Biopsy specimens transported in Michel's medium (Lubeck, left panel) and saline (Groningen, right panel). Cryosections were cut and processed in the Groningen laboratory. Original magnification x400.

Extended methods DIF laboratory procedures

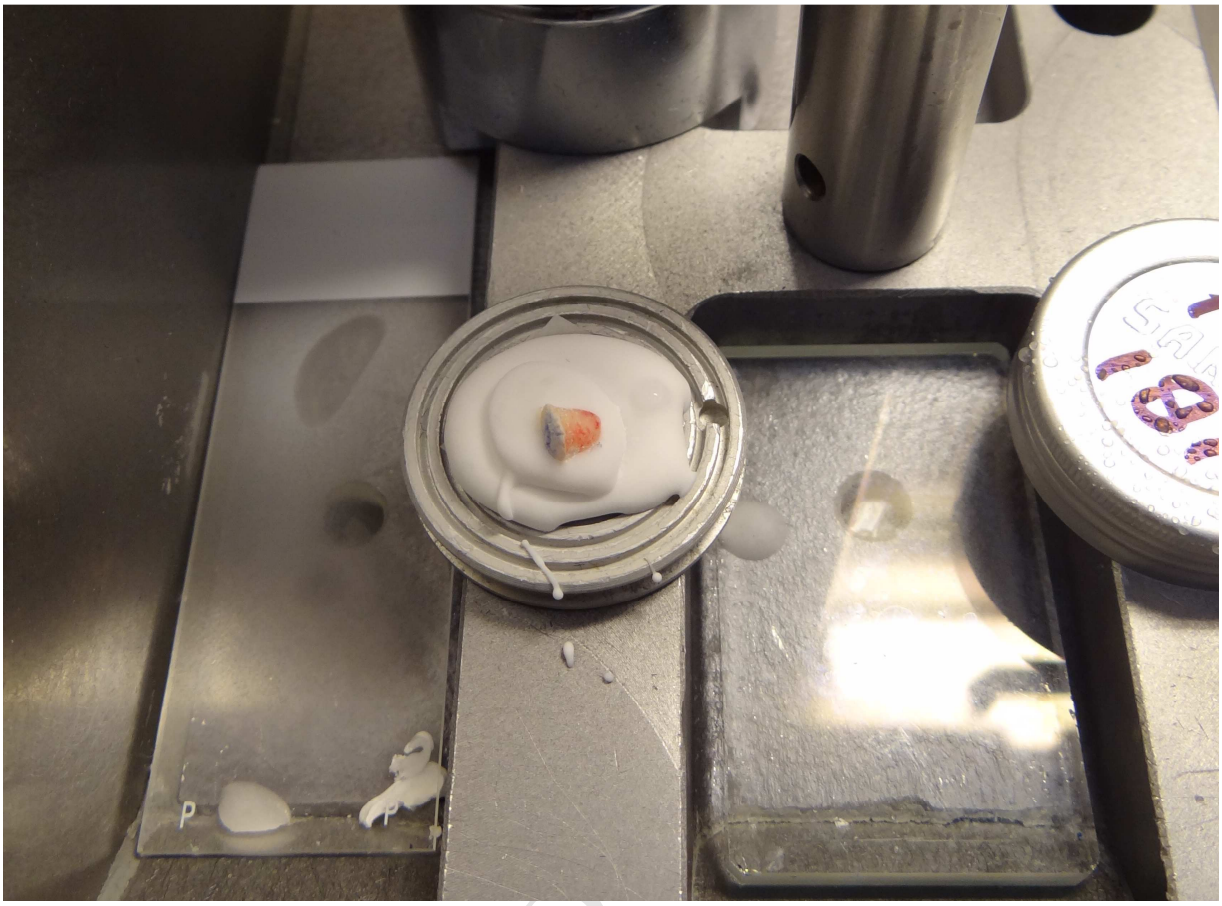
In the Groningen routine DIF laboratory procedure, perilesional biopsies were placed in saline in polypropylene vials, stored overnight in saline (12-16 hours) and processed the next day. Biopsies were then placed in aluminium vials, snap-frozen in liquid nitrogen and stored at -80℃ until processing. Temperature of cryostate (Leica CM3050S, Leica, Wetzlar, Germany) and microtome knife was regulated between as low as -20 and -35℃ to prevent compression and deformation of the sections. A small piece of filter paper was applied to the fixation table inside the cryostate with one drip of water and frozen. A circle of Tissue-Tek O.C.T. Compound (Sakura, Alphen aan den Rijn, The Netherlands) was added to the filter paper, frozen with freezer spray and filled up with another drip of Tissue-Tek for fixation of the biopsy specimen (figure S1). The biopsy specimen was orientated with epidermal side left and not completely, but only partially embedded in Tissue-Tek with the base of the specimen for cutting only biopsy tissue (figure S2, S3). After freezing, the fixation table was placed in the specimen block and cryosections of 4µm, 6µm and 8µm thickness were cut with tungsten carbide conventional microtome knives (Type C 160mm, Spikker Specials, Zevenaar, The Netherlands). The microtome knives were standard wedge shaped with a bevel angle of 20° and resharpenable (figure S3). The clearance angle between the lower facet of the knife and specimen block was positioned parallel to the specimen and slightly raised. Cryosections were aligned on the microtome knife, mounted on polysine® glass slides sideways with no pressure applied (supplemental video) and quality checked for artefacts with light microscopy. Then, air-dried for 15 minutes in front of a fan, rinsed with PBS and encircled with a hydrophobic emulsion (DAKO, Glostrup, Denmark). The sections were then stained for 30 minutes in a moist chamber at room temperature using fluorescein FITC-labeled Fc-specific goat F(ab')2 antibody against human IgG (Protos 311, Protos immunoresearch, Burlingame, CA, USA), rinsed and washed with PBS and SlowFade™ Antifade Reagent (ThermoFisher Scientific Inc., Waltham, MA, USA) was added to inhibit photobleaching. After washing in PBS for 30 minutes, sections were coversliped and slide preparations stored at 4-8℃. Sections were examined with a Leica DM2000 microscope and Leica HCX PL Fluotar 40x/0.75 dry objective and 10x ocular (total magnification 400x) (Leica, Wetzlar, Germany).

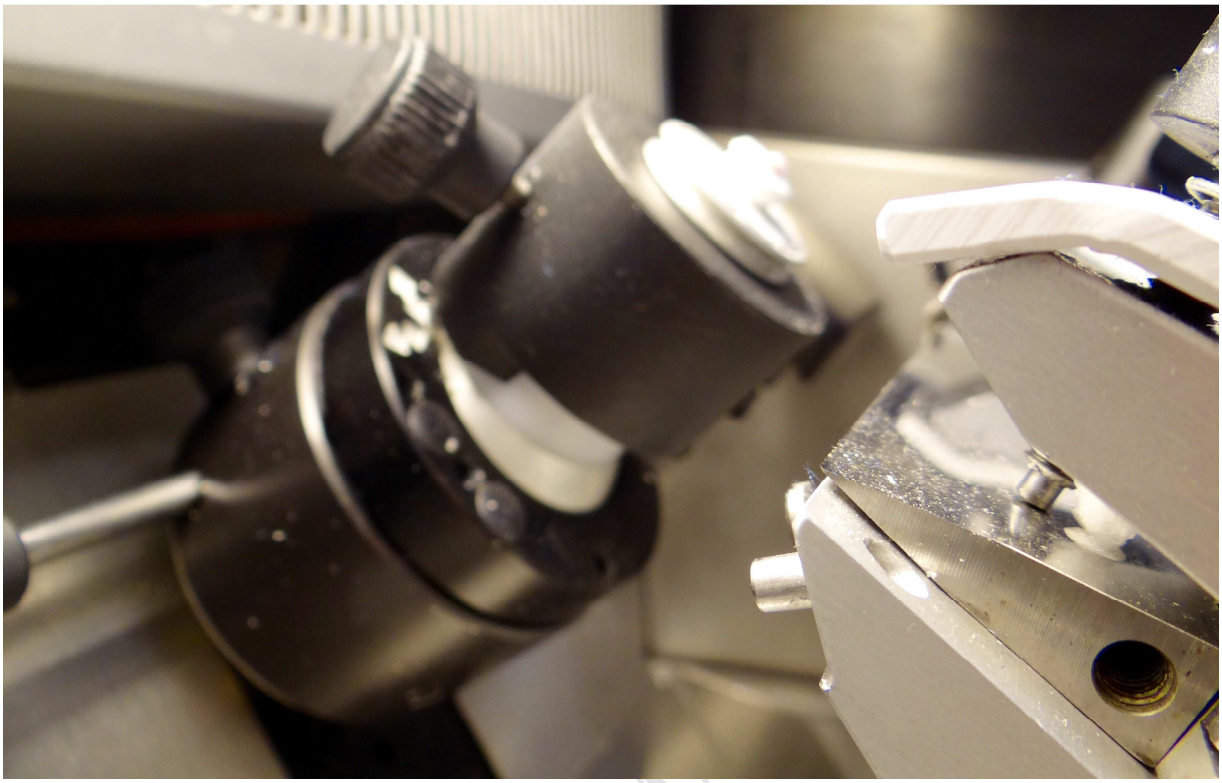
The Lübeck routine DIF laboratory procedure only showed minor differences compared to the Groningen procedure. Perilesional biopsies were either kept in Michel's medium or frozen at -20℃ until processing. Biopsy specimens were fi xated by completely embedding the material in Tissue-Tek O.C.T. Compound and 6µm cryosections were cut using disposable microtome blades S35 (Feather, Osaka, Japan) and the same cryostate. Sections were stained using a monoclonal mouse anti-human IgG-FITC antibody (Bio-Rad, Hercules, CA, USA) and examined with a Olympus BX40 microscope and 40x/0.75 dry objective (UPlan FI, Olympus, Tokio, Japan).

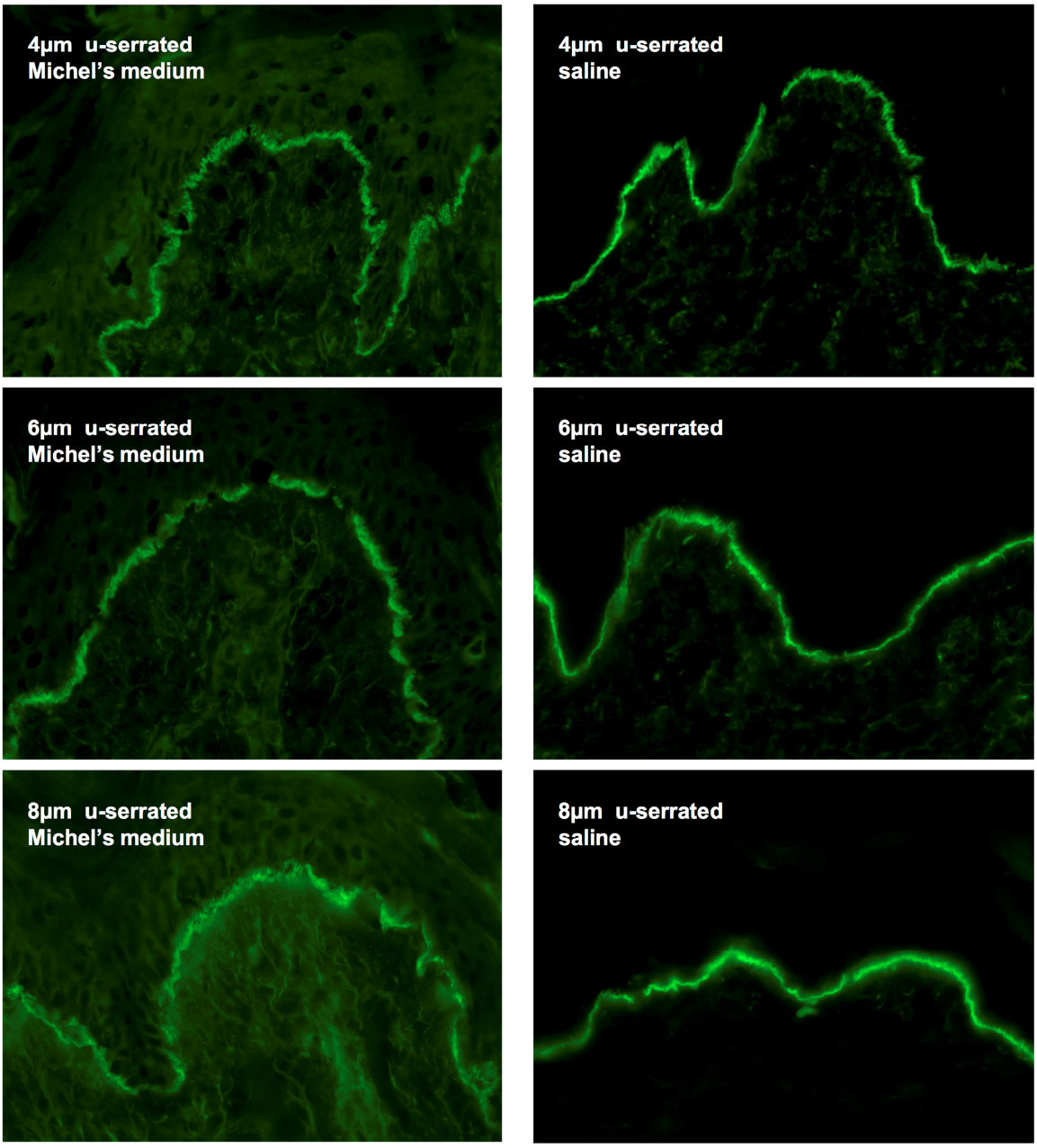
Supplemental table S1. Classifica3on of serra3on pa7erns of random DIF sec3ons by four observers.

Laboratory & sample no.	Transport medium	Diagnosis	Observer 1	Observer 2	Observer 3	Observer 4
LU1	Michel's	EBA	u---serrated	u---serrated	u---serrated	u---serrated
LU2	Michel's	EBA	u---serrated	u---serrated	u---serrated	u---serrated
LU3	Michel's	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
LU4	Michel's	EBA	u---serrated	*undetermined*	u---serrated	u---serrated
LU5	Michel's	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
LU6	Michel's	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
LU7	Michel's	EBA	u---serrated	u---serrated	u---serrated	u---serrated
LU8	Michel's	Pemphigoid	n---serrated	*undetermined*	n---serrated	n---serrated
LU9	Michel's	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
LU10	Michel's	EBA	u---serrated	u---serrated	u---serrated	u---serrated
GR1	Saline	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
GR2	Saline	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
GR3	Saline	EBA	u---serrated	u---serrated	u---serrated	u---serrated
GR4	Saline	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
GR5	Saline	Porfyria	*undetermined*	*undetermined*	*undetermined*	*undetermined*
GR6	Saline	EBA	u---serrated	u---serrated	u---serrated	u---serrated
GR7	Saline	EBA	u---serrated	u---serrated	u---serrated	u---serrated
GR8	Saline	Pemphigoid	n---serrated	n---serrated	n---serrated	n---serrated
GR9	Saline	EBA	u---serrated	u---serrated	u---serrated	u---serrated
GR10	Saline	EBA	u---serrated	u---serrated	u---serrated	u---serrated

LU; Lübeck laboratory. GR; Groningen laboratory. EBA; epidermolysis bullosa acquisita.






4µm u-serrated
Michel's medium
4µm u-serrated
saline
6µm u-serrated
Michel's medium
6µm u-serrated
saline
8µm u-serrated
Michel's medium
8µm u-serrated
saline

CAPSULE SUMMARY

- Epidermolysis bullosa acquisita can be differentiated from other pemphigoid diseases by direct immunofluorescence serration pattern analysis of a skin biopsy specimen.

- In the current study high inter-rater conformity was shown independent of laboratory procedures, transport medium, or experience.

- Serration pattern analysis can be implemented as a routine diagnostic technique in extension of direct immunofluorescence microscopy.