TO THE EDITOR
While it has been known for more than 5 decades that nerve endings are found in the epidermis (Arthur and Shelley, 1959), it remains unclear how the cutaneous nervous system is altered in pruritic skin, as existing studies have yielded conflicting results. Although pruritic skin is commonly thought to be hyperinnervated (Taneda et al., 2011; Tominaga et al., 2009), several studies reported demonstrable reductions in nerve densities in itchy skin (Johansson et al., 1991; Maddison et al., 2008). One plausible explanation for this could be the disparate types of readouts used for nerve density analysis, for instance, counting the number of nerves per unit length (Kim et al., 2014) as opposed to measuring fiber length per epidermal unit area (Maddison et al., 2011) (Supplementary Table S1 online).

Currently, cutaneous nerve density is determined by blinded manual counting of intra-epidermal nerve fibers (IENFs) in 50-μm sections, and this is routinely used to diagnose small fiber neuropathy (Hoeijmakers et al., 2012). According to existing guidelines, linear IENF density is calculated by counting the number of nerve filaments crossing the basement membrane per unit length of skin (Lauria et al., 2005). Nerve fragments generated from sectioning are not counted; this method of quantification also cannot fully account for the actual extent of nerve innervation in the epidermis, as a short nerve fiber or a filament with extensive secondary branching traversing the basement membrane are both counted as one filament. In order to capture this information and reduce the generation of nerve fragments, we developed a workflow to perform 3-dimensional (3D) volumetric analysis of immunostained and optically cleared whole skin samples (Supplementary Figures S1 and S2 online). Optical clearing reduces light scattering, rendering it

**See related commentary on pg 999**

**3-Dimensional Optical Clearing and Imaging of Pruritic Atopic Dermatitis and Psoriasis Skin Reveals Downregulation of Epidermal Innervation**

**REFERENCES**


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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.11.006.

**ABBREVIATIONS**

AD, atopic dermatitis; 3D, 3-dimensional; IENF, intra-epidermal nerve fiber.

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possible to perform 3D imaging of whole samples without sectioning (Richardson and Lichtman, 2015).

Institutional approval and written consent was obtained prior to recruitment of atopic dermatitis (AD), psoriasis patients and healthy individuals (Supplementary Table S2 online). Skin biopsies of lesional and non-lesional skin obtained from patients and healthy volunteers were immunostained, optically cleared (Figure 1a, Supplementary Methods online), and analyzed. Importantly, optical clearing did not cause any significant volume change in the skin (Figure 1b, 1c). While a 2-dimensional view can provide a cross-sectional view of epidermal and dermal nerves typically seen in literature, the 3D view in our study comparatively reveals a dense cutaneous nerve network innervating the epidermis, where secondary branching of epidermal nerves occurs in all three dimensions (Figure 1d, Supplementary Movie S1 online). To further validate the reliability of our method, we carried out nerve tracing using the healthy skin data set to determine the inter-rater (0.915) and intra-rater reliability rates (0.936) and found them to be good (Supplementary Table S3 online).

From our analyses, we observed a general trend of atrophy in nerves in lesional skin of pruritic patients in comparison to healthy individuals (Supplementary Figure S5 online). Fiber length per unit volume was substantially reduced in lesional skin of AD (59.56% reduction) and psoriasis (73.98% reduction) patients as opposed to healthy skin (Figure 2a, Supplementary Table S4 online), which correlated with a lower dendrite volume (Figure 2b). Non-lesional skin of pruritic patients tended to have less innervation in comparison to healthy individuals, but the difference was only statistically significant for nerves in non-lesional skin of AD patients. The lower fiber length per unit volume is likely due to the significant reduction in the number of dendrites in lesional skin of AD and psoriasis patients compared to healthy subjects (Figure 2c). This corresponded with a decrease in the amount of nerve branching in lesional skin of pruritic patients (Figure 2d).

We believe the lack of consensus regarding nerve innervation in pruritic skin is caused by differing methodology for analyzing IENF density (Supplementary Table S1). Here, we demonstrate a possible standardized method for calculating IENF density by tracing the nerves in whole skin biopsy in a 3D manner to minimize

Figure 1. 3D analysis of cutaneous nerves via optical clearing and imaging. (a) Photomicrograph of uncleared and optically cleared human skin. (b) Outlines of the same human skin section before (blue) and after clearing (red). (c) Normalized linear expansion of 300-µm-thick human skin sections before and after optical clearing (mean ± standard deviation; n = 4). Uncleared skin sections have a value of 1 after normalization. (d) 2D slice view and 3D image of immunostained healthy skin. 2D, 2-dimensional; 3D, 3-dimensional. Epidermal nerves, green; dermal nerves, blue. Dotted region in the 2D slice view demarcates epidermal nerves. Scale bar of 2D slice view = 100 µm; scale bar of 3D overview image = 200 µm; scale bar of zoomed in image = 20 µm.

Y Tan et al.
3D Optical Clearing and Imaging of Pruritic Skin
nerve fragments from 2-dimensional sectioning, and to account for nerve branching and total epidermal volume in our IENF density calculations. Our observed lower IENF density in pruritic skin compared to data reported by other methods is probably because we have accounted for the higher epidermal volume in pruritic patients (Supplementary Figure S6 online).

Notably, 2-dimensional studies using methods to calculate nerve density by measuring nerve fiber length per unit area of epidermis (or a similar method calculating percentage of nerve stained pixels out of total number of epidermal pixels) reveal a similar trend—a decrease in IENF of lesional AD skin (Tsutsumi et al., 2016), keloids (Tey et al., 2012), and lichen amyloidosis (Maddison et al., 2008) in comparison with healthy skin. While it may seem counterintuitive that epidermal innervation is reduced in pruritic skin, ongoing itch could possibly be driven by hypersensitization of epidermal nerves from an upregulation of itch receptors, such as IL-31 receptor (Nattkemper et al., 2018).

In summary, our study has demonstrated a 3D visualization of the skin biopsy using optical clearing, and we found a statistically significant reduction in epidermal nerve innervation in pruritic lesional skin of AD and psoriasis patients compared to healthy individuals using this approach. Future development of accurate automated segmentation and tracing methods could make 3D IENF density determination faster and easier. This volumetric visualization approach also has the potential to provide important spatial information in other types of skin histologic analysis, such as studying the location of known itch-mediating small molecules or cells in relation to the cutaneous nervous system.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

LAN, GY, LGN, and HLT obtained funding; YT, LGN, and HLT conceptualized and designed the study; YT, WJN, and SZXL acquired the data; BTKL helped with data analysis; YT, LGN, HLT performed data analysis and interpretation, and wrote the manuscript. All authors reviewed the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

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**REFERENCES**


Kim TW, Shim WH, Kim JM, Mun JH, Song M, Kim HS, et al. Clinical characteristics of pruritus...


