The study by Wilkinson et al. adds nonhealing ulcers to the list of diabetic pathologies that are underpinned by alterations in cellular senescence and provides a novel new target for the treatment of diabetic wounds.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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Assessment of nerve endings in the skin is key for understanding and explaining dermatological pathologies, such as atopic dermatitis and psoriasis. Recently, the Journal of Investigative Dermatology published an approach to 3-dimensionally (3D) image and analyze the innervation of skin (Tan et al. 2019). This method is fundamentally different from traditional approaches that are based on 2-dimensional (2D) histology slides, as the imaging beam must pass through the superficial layers of the skin. Scattering properties of the stratum corneum and epidermis distort light beams in unpredictable ways such that the images typically become blurry. Tan et al. (2019) used extensive tissue processing that included optical clearing techniques to overcome this obstacle.

Volumetric nerve maps of skin enable the analysis of innervation architecture in normal skin and pathological situations in a way that is fundamentally different than is possible using 2D histology slides. The quantity of nerve endings, their connectivity, and their shapes can be assessed in more detail and more precisely in 3D. Typically, these architectural characteristics are quantified by single numbers, for example, the nerve fiber density or the number of branch points per volume. These quantitative metrics can be used to compare a variety of samples and identify potential biomarkers indicating pathologies. However, if this analysis is based on the manual tracing of nerve fibers, an error due to subjective decisions by raters might be introduced and affect the derived quantitative metrics. Ultimately, intravital imaging techniques are necessary to noninvasively examine cutaneous nerve endings and monitor pathologic developments.

3D versus 2D: What a third dimension has to offer
Free nerve endings are highly dispersed throughout the papillary dermis and epidermis. To quantify numbers of nerve fibers, they must be traced to their branch points and associated with their connections. In a 2D histologic slide, it is often impossible to trace single fibers and reconstruct connections of nerve fiber fragments to come to valid conclusions. Furthermore, 3D representations of nerves allow tracking of the entire fibers, enabling more precise assessment of characteristics, such as fiber shape and orientation, which is impossible using 2D histologic slides.

The advantages of 3D imaging come at the price of longer image acquisition times. Using confocal microscopy, the imaging beam is scanned point by point, forming the image pixel by pixel.

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Clinical Implications

- Optical clearing enables 3-dimensional histologic imaging of skin.
- Volumetric images provide more precise information to identify biomarkers for pathologies.
- Focus of future work: Intravital and functional imaging of nerve endings.

With an additional dimension, this procedure multiplies the number of points that must be scanned, leading to much longer acquisition times than in 2D. Moreover, for 3D scans, the imaging beam must penetrate the skin surface and pass through superficial layers of the skin that possess different optical properties (Obeidy et al., 2018). Typically, the epidermis strongly scatters and absorbs light of the visible range of the spectrum, which makes it a challenging sample for optical imaging modalities like confocal microscopy. Optical clearing, as utilized by Tan et al. (2019), lowers the scattering properties of the tissue and enables volumetric imaging of the skin. Sample preparation for 3D analysis may be more difficult than for standard 2D histology, however.

Nerve fiber segmentation: manual versus automatic

To analyze the innervation of the skin, nerves must be traced throughout the volumetric image data. Therefore, every pixel of the image needs to be assigned to either representing a nerve fiber or background. This so-called segmentation map is typically obtained by the manual assessment of experts. As pointed out by Tan et al. (2019) this rather time-consuming work can be influenced by subjective decisions that are made by raters. To compensate for rater-induced errors, it is useful to combine several manual segmentations of different raters on the same image data. With algorithms like the Simultaneous Truth and Performance Level Estimation, several manual segmentations can be combined into a single compromise solution (Warfield et al., 2004). This comes at the high cost of additional, time-consuming manual segmentation work, though. Also, the resulting error can only be estimated, and errors that are introduced will influence subsequent analyses of segmentation.

To analyze large data sets quickly and to minimize subjective errors, automated segmentation algorithms have to be developed in the field of image processing science (computer vision). To find algorithms for reliable and automatic detection of fine nerve fibers is challenging, especially in images with low contrast or low signal-to-noise ratios (SNR) as presented in the work by Tan et al. (2019).

Recently, deep learning-based image analysis emerged in the field of computer vision (Litjens et al., 2017). Using Convolutional Neural Networks in U-Net architectures, segmentation tasks are accomplished with impressive accuracy—even for low SNR data sets. However, training and testing of artificial neural networks require substantial collections of manually segmented data. In light of the consequential value of manual segmentations for the development of machine-learning algorithms for nerve fiber tracing, the collaboration of the whole field and researchers like Tan et al. (2019) to share their labeled data is required.

Intravital and functional imaging

To follow disease development and treatment responses, techniques for noninvasive intravital imaging are necessary. Not only does in vivo imaging provide immediate results, it also allows acquisition of data at several sites of subjects to minimize uncontrolled variables. However, current skin innervation imaging modalities, such as confocal microscopy, have limitations and require the application of optical clearing and contrast agents. To perform in vivo imaging, concerns related to extensive tissue processing procedures must be overcome.

Alternatively, or in addition to optical clearing, two-photon microscopy has proven to be a promising tool in dermatological research (Obeidy et al., 2018). Because it utilizes infrared light pulses, it is less prone to scattering in skin and offers increased SNR and imaging depth compared with confocal microscopy. Two-photon microscopy has already been applied to longitudinally monitor nerve fiber endings in mouse skin in vivo (Yuryev and Khiroug, 2012).

However, the point-by-point scanning approaches mentioned here are time-consuming, especially when performed in 3D. This is problematic for intravital imaging situations because motion of subjects during image acquisition leads to imaging artifacts. Swept confocally aligned planar excitation (Bouchard et al., 2015) and Talbot holographic illumination non-scanning fluorescence microscopy (Luo et al., 2014) are promising full-field acquisition approaches that greatly reduce acquisition times. Dermatological application of these approaches needs to be investigated, perhaps in combination with prior optical clearing.

Approaches to image the nerve endings in the skin aim to provide morphologic information that is prerequisite for understanding diseases. To fully characterize complex dermatological pathologies, functional information regarding nerve endings is key. Existing nerve fibers might exhibit alterations in activity and viability due to disease states. The functionality of the nerve endings has not been addressed in previous work, including that of Tan et al. (2019). Calcium signaling has proven to be a valuable tool to image intravital neural activity in neuroscience (Tian et al., 2009), and it has been shown specifically for sensory nerve fiber endings in ex vivo rodent cornea (Gover et al., 2003). However, for applications in skin, other sources of calcium signals (e.g., as shown by Tu et al., 2019) must be considered carefully as they could interfere with signals of neural activity.

Overview

Recent developments in optical imaging techniques and computer vision provide the tools to obtain deeper insights into dermatological pathophysiology. The work of Tan et al. (2019) describes the efficacy of optical clearing and the advantages of 3D imaging. However, drawbacks associated with confocal microscopy must be overcome to pave the way for in vivo.
optical imaging. Methods for automatic segmentations need to be adapted to optical nerve fiber imaging to analyze the data quickly and impartially. Furthermore, techniques for functional imaging of neural activity must be developed for dermatological applications to study pathophysiology more systematically.

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