Structural Assessment of Scars Using Optical Techniques

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19.1 Introduction

The skin, the largest organ of the body, consists of an epidermal layer and a dermal layer underneath. The thin epidermal layer functions as a barrier to the outer world. The dermis is much thicker, and its structural organization is responsible for the functional qualities of the human skin. It consists for almost 80% of collagen, elastin, and different types of cells, which render the skin strong and at the same time remarkably pliable. In scar tissue, visual aspects (discoloration and texture [1]), functional aspects (stiffness [1] and moisture retention [2]), and sensory functions [3] of the skin differ significantly from those of healthy skin.

In clinical practice, objective assessment of cutaneous scars remains a challenging task. The use of scar assessment tools has increased significantly in the last decade. The clinical follow-up of scar treatment necessitates precise measurement of thickness, surface area, and relief. Further, a better visualization of the precise 3D microstructural organization is crucial in understanding the etiology of pathological scarring. As most aspects of clinical scar assessment scales are based on the visual appearance, the potential for optical techniques as an objective assessment tool is high. With a so-called optical biopsy, it is possible to assess the microstructure below the surface of the skin with optical methods, negating invasive physical biopsies. Furthermore, a range of extensions for these techniques allow simultaneous structural, mechanical, and even chemical characterization.

This chapter aims to describe current progress in objective imaging techniques used in the clinical follow-up of scar progression on the one hand and some of the most powerful techniques for basic structural research of scar microstructures on the other hand. For the sake of brevity, we limit our discussion to techniques and devices that have been shown to be valid and reliable. For a complete overview of available devices, we refer the reader to an extensive review by Lee et al. [4].

19.1.1 Volumetric Analyses

The number of devices that measure scar volume has seen a rapid growth in the last decade, partly advanced by increased attention to wound and burn research.

Volumetric aspects of scars discussed here are surface area, thickness, and surface topology. Although there exists significant overlap between these aspects and some devices measure multiple aspects (surface roughness can be considered as fluctuations in scar height/thickness; newer analysis methods of surface area are often intrinsically three dimensional and therefore include information on surface topology), we dis-

cuss them separately as the reliability (the consistency of the measurements) and validity (whether the device measures what it intends to measure) of individual aspects of a device aren't necessarily similar.

Volumetric assessment has gone from manual paper tracing to high-tech 3D cameras.

Although manual tracing of scar surface area is reliable in the case of linear postoperative scars [5], for scars with irregular boundaries, the reliability decreases with scar size [6]. The LifeViz 3D camera (Quantificare SA, Valbonne, France) has been extensively tested in both the surface area and volumetric analysis mode by Stekelenburg et al., who reported it to be a reliable and valid tool [7, 8]. The LifeViz 3D works by comparing two photos from different angles, from a predefined distance. The Vectra 3D is a speckle pattern-based system that utilizes six cameras to construct a 3D image. Its interrater reliability was found to be excellent in a trial on raised scars [9]. A device with a similar working mechanism, MAVIS III (Photometrix, Pontypridd, United Kingdom), was assessed in a study on burn scars. The authors note that poorly delineated scars on curved surfaces pose a challenge in the boundary selection and subsequent analysis [10].

Rashaan et al. tested another stereophotogrammetry device, the Artec MHT (Artec 3D, San Diego, CA, USA), and found it to be highly reliable and valid in burn scars [11].

Other strategies that include laser scanning cameras and structured light scanners need additional clinical evaluation.

19.1.2 Surface Topology

Roughness, or surface irregularity, has a significant impact on a patient's perception of scars [12]. Furthermore, the predictive validity of long-term scar quality was found to be significantly influenced by the "relief" item on the POSAS observer scale [13] (see ► Chap. 16 for more on scar assessment scales). Most scar assessment scales include such a texture item, and even though subjective analysis isn't necessarily inferior to objective analysis, quantification of these aspect remains challenging.

Early attempts at quantitative evaluation of roughness entailed the casting of a positive or negative mold of the scar surface and its subsequent optical or mechanical analysis [14]. This process, although accurate, is overly cumbersome and time-consuming. Current contactless techniques use a combination of illumination and analysis of disturbance patterns. The most frequently used device, the Phaseshift Rapid In Vivo Measurement of the Skin (PRIMOS, Canfield Scientific, Inc., Parsippany, USA), has been shown to reliably mea-

sure scar topology [15, 16]. PRIMOS projects patterns of light onto the skin surface. Fluctuations in the surface profile cause disruptions in these patterns that are subsequently analyzed.

Antera 3D (Miravex, Dublin, Ireland) is based on photometric stereo, in which light from different angles produces shade based on surface topology. The reflected light is analyzed to form a surface reconstruction. Similarly, LifeViz Micro (Quantificare SA, Valbonne, France) is one of the devices in the LifeViz series mentioned earlier. Although they have been described in the context of clinical scar assessment [17, 18], their clinimetric properties have not been analyzed for the surface roughness aspect.

The Visioscan VC 98 uses software analysis of grayscale values in UVA-illuminated photographs (Visioscan VC 98). Reliability in healthy skin relies heavily on the chosen outcome measure of the “surface evaluation of living skin” method [16].

The analysis of relief can be considered a structural analysis of scar volume. Indeed, most devices offer some volumetric analysis. Furthermore, the Antera 3D offers additional colorimetric analysis. The abovementioned devices show great heterogeneity in measured parameters, resolution, and field of view, and thus, it is difficult to make a qualitative comparison. In the future, further cross-pollination between techniques can be expected. Askaruly et al. showed the potential of optical coherence tomography (OCT) to measure relief [19]. Although their study was not on scar tissue, the possibility of combining 3D surface analysis with structural analysis looks promising.

19.1.3 Thickness

Follow-up of scar thickness is especially important in both hypertrophic and keloid scars, where the aberrant deposition of ECM proteins leads to significant expansion. Therapies are mostly focused on reducing the scar's prominence over the surrounding tissue, and correctly analyzing the thickness of cutaneous scar is paramount.

Although not an optical technique, we discuss high-frequency ultrasound here as it's by far the most commonly used technique. Its inferior resolution compared to optical equivalents is mitigated by its superior penetration depth allowing thickness analysis, even in severe scar thickening. The working mechanism is based on reflection of sound waves of structures with different acoustic impedances and the analysis of the reflection time to determine the depth of the structure. The penetration depth ranges from the upper dermal layers to full-thickness skin and subcutaneous structures, depending on the employed frequency.

Numerous ultrasound systems exist that are marketed for dermatological applications; we succinctly

limit our discussion to systems that have been applied to scars. The frequency used by these systems ranges from 5 to 50 MHz and forms the most important limitation in scars, as this limits the penetration depth. For example, the most frequently described device, the Dermascan C (Cortex Technology ApS), utilizes a frequency of 20 MHz which has a penetration depth described as inadequate in up to a third of burn scars in a study by Gankande et al. [20] (using the DermaLab Combo).

Reliability of these instruments has been tested in several studies and shows excellent inter-observer [20–23] and intra-observer reliability [20, 21, 23–25].

From a validity standpoint, it is of interest to note that a golden standard does not currently exist. Comparison with scar assessment scales has yielded inconsistent results, possibly due to heterogeneity in scar scales and ultrasound frequency [23, 26]. Histology has several drawbacks that include deformation during the biopsy process dependent on resting skin tension, manipulation during fixation, and slicing [27]. Agabalyan et al. found poor correlation between 20MHz ultrasound analysis and histological analysis of skin graft scars in a small group of patients [26]. Andrew et al. found biopsies to be significantly thicker after excision, advising the use of pinning during formaldehyde fixation to mitigate this effect [27]. Li et al. compared ultrasound measurements with the use of a micrometer [24], theoretically bypassing the negative effects of sample preparation for histology. Although they achieve a high correlation, sonographic analysis was performed on porcine excised tissue in which it is conceivably easier to recognize the skin's boundaries.

Scar height can be measured using volumetric analyses like the 3D camera LifeViz 3D (Quantificare SA, Valbonne, France), although studies comparing this device with ultrasound measurements have favored the latter [23, 28]. It seems that the reliability is suboptimal due to the device having difficulty scanning over curved surfaces.

19.2 Experimental Techniques

Scarring involves the aberrant deposition of matrix molecules. The collagen content of scars, especially, differs from healthy skin in terms of amount, type, and, importantly, organization. In the past decades, quantification of the extracellular matrix (ECM) organization was mostly limited to two-dimensional images from fixated and stained tissue. Depending on the orientation of histological slices relative to the skin surface, analysis of orientation will yield different results. For example, Verhaegen et al. found significantly different supramolecular organization depending on which cross section was imaged (e.g., perpendicular to the surface or in the

parallel plain) [29]. Gaining a better three-dimensional perspective of ECM organization is paramount in elucidating the etiology of pathological scars.

As the goal is not only to assess scar tissue based on texture, volume, and color but also to perform microstructural analysis, more advanced microscopic tools are required. An ideal optical scar assessment technique would have a resolution with which microstructural changes can be clearly identified, even in the deeper parts of the dermis. However, in optics, there exists a common trade-off between spatial resolution and penetration depth, and the marked thickening of hypertrophic and keloids scar complicates this trade-off. In this sense, a “perfect technique” does not exist. A technique’s suitability depends merely on whether the application matches its strength. In the following, we will discuss several microscopic techniques and their applications in scar assessment and research.

19.2.1 Optical Coherence Tomography

Colloquially known as “Ultrasound based on light,” OCT is a promising technique based on low-coherence interferometry. In short, light with a broad bandwidth is split into a sample arm and a reference arm. The interference pattern that arises from the combination of the reference arm and the sample arm, containing reflected light from different depth layers of the sample, creates a depth profile of the sample. Combining a lateral series of these profiles creates a cross-sectional image (B scan) or a three-dimensional reconstruction when a stack of B scans is combined. Scanning approaches with full-field illumination allow the acquisition of stacks of “en face” scans. Typically, penetration depths of 1–2 mm can be reached, at a resolution of 4–10 μm [30].

The capability of OCT to create high-resolution tomography first piqued the interest of the ophthalmological community, where it is now a staple technique in retinal assessment. Naturally, attributes like fast acquisition time, high-resolution images, noninvasiveness, and multifaceted structural assessment make OCT an interesting technique for dermatology. Moreover, OCT devices are often manageable handheld scanners, increasing their usability in the clinic.

Polarization-sensitive OCT (PS-OCT) has been described as a quantification tool for collagen density and alignment by means of tissue birefringence [31]. Jaspers et al. compared burn scars and healthy skin using a volume-based birefringence method, which showed strong correlation to histologically determined collagen content [32]. By applying speckle decorrelation, Liew et al. visualized the microvasculature of burn scars in three dimensions [33]. A similar strategy was used in the clinical follow-up of burn scars undergoing laser

ablation [34], allowing the authors to distinguish mature and immature scars [35]. Comparable strategies have been applied to lymph vessels [36]. Combined vascularity, lymph vessels, and birefringence analyses were used by Park et al. to longitudinally follow wound healing in a mouse model, showing angiogenetic changes and realignment of birefringent signals [37].

Simultaneous mechanical testing allows the correlation of local structural changes to variations in stiffness. Es’haghian et al. showed that combining a handheld OCT probe with a mechanical loading device permits concurrent imaging of the *in vivo* scars and stiffness mapping [38, 39]. Vibrational perturbation of the skin and the analysis of resonance frequencies while imaging the layers of the skin have also received some attention recently for bulk measurements of mechanical properties [40, 41].

OCT has been described as a tool for measuring thickness of individual skin layers. Because the limited penetration depth makes full thickness imaging impossible, its application is limited to the epidermal thickness [42, 43].

19.2.2 Confocal Microscopy

Higher-resolution images to assess structures with submicron resolution in 3D can be obtained using various microscopic techniques. In confocal fluorescence microscopy, the addition of a set of pinholes to a traditional fluorescence microscope allows for the selection and detection of a small focal volume. Selective labeling with a fluorophore allows for the imaging of a particular substance of interest by excitation of the fluorophore by a lamp or laser and detection of the fluorescence emission signals. Images are obtained by scanning the laser or the sample, yielding images with a resolution of $0.4 \lambda/\text{NA}$ where λ is the wavelength of the emitted light and NA is the numerical aperture of the objective, which is an improvement of ~30% compared to traditional fluorescence microscopy. Another advantage over widefield microscopy is that the labeling with fluorophores can be sparser, allowing for a fairer comparison of relative quantities of structures.

Confocal microscopy has been used to determine the ratios of collagen of different scar types [44], cell-signaling molecules [45] or innervation [46] of different scar types and as an *ex vivo* tool in evaluation of micro-needling penetration depth [47]. Furthermore, the superior resolution allows for quantitative collagen orientation analysis [29, 48]. Devices like the VivaScope (MAVIG, Munich, Germany) [49] are based on reflectance confocal microscopy (RCM), in which backscattered light from unlabeled structures is analyzed, based on changes in refractive index. The *in vivo* applications of RCM lie predominantly in the diagnosis of various

skin cancers [50], as its penetration depth is limited to the epidermis and upper part of the dermis. However, attempts to analyze collagen production after micro-needling of acne scars have been described *in vivo* with a combination of dermoscopy and RCM [51]. The limited penetration depth seems a significant hurdle in scar research; however, combining the device with an OCT arm allowed Iftimia et al. to analyze both structural and birefringence properties in burn scars [52].

19.2.3 Nonlinear Optical Microscopy

19.2.3.1 Introduction

As discussed above, confocal microscopy allows for three-dimensional imaging; however in this technique, samples require specific staining and sometimes also fixation and slicing, which may affect ECM structure and organization. Nonlinear optical (NLO) microscopy involves label-free imaging of unfixed tissue in three dimensions and has strong potential in the visualization of the scar microstructure. Here, we discuss several types of NLO microscopy, namely, higher harmonic generation microscopy (HHG) and multiphoton excited autofluorescence (MEA), and explain their strengths in label-free imaging of scars.

Nonlinear microscopy can be described as the conversion of multiple photons into a single photon, based on the specific nonlinear behavior of certain molecules or structures. This process occurs only when the intensity of the incident light is sufficiently high. To keep the average laser power on the sample sufficiently low, short-pulsed lasers are used (generally femtosecond lasers) [53].

MEA involves the absorption of two or more photons by an intrinsic fluorophore and the recording of the subsequent fluorescence emission, which is then at a wavelength shorter than of the excitation light. Alternatively, external fluorophores can be applied to the sample to label specific structures. In contrast to single-photon fluorescence, excitation wavelengths typically lie in the infrared region, allowing deeper tissue penetration of up to 1.6 mm [54].

Comparably, in the HHG processes, two or three photons are converted into a single photon. No energy transfer takes place however, and the emitted photons have exactly half (second harmonic generation, SHG) or a third (third harmonic generation, THG) of the wavelength of the incident photons and exactly double or triple the energy, respectively. This allows the detection of the specific signal using narrow-banded filters, improving contrast between structures. Simultaneous registration of these signals using one system allows for label-free visualization of the most important structural proteins of the ECM.

19.2.3.2 Two-Photon Excited Autofluorescence (2PEF)

MEP involves two or three photons being absorbed by a fluorophore and the subsequent emission of a single photon of a shorter wavelength. Two-photon excited autofluorescence (2PEF) has been described extensively in skin research.

2PEF is applicable to fluorophores used in single-photon fluorescence. As this is a fundamentally different process, excitation wavelengths will not equate to exactly double the wavelength of the single-photon excitation. The emission spectrum however will be identical. Fluorophores naturally present in biological tissue have well-described excitation and emission spectra, and extrapolation to the two-photon excitation wavelength is usually warranted. Although penetration depths more than one millimeter have been described in the context of brain tissue [54], the density of the skin and the relevant excitation wavelengths do not permit such deep penetration. Imaging systems are conventionally limited to several hundred micrometers in depth.

Both collagen and elastin, the most important structural proteins of the extracellular matrix (ECM), have convenient 2PEF profiles [55]. On the cellular level, the abundantly present cytoplasmic metabolites NADH and FAD are prolific emitters in their reduced and oxidized states, respectively. An example of 2PEF imaging of dermal fibroblasts can be seen in ■ Fig. 19.1.

■ **Fig. 19.1** Combined two-photon excited fluorescence image (2PEF, green) and second harmonic generation image (SHG, red) of cultured human dermal cells and their derived collagen matrix, respectively. Collagen fibers induce a bright SHG signal, whereas intrinsic fluorophores in the cytoplasm of the dermal cells outline the cells. *In vitro* images were acquired using a previously described setup [87]. Scale bar: 500 μm

Simultaneous recording allows a redox ratio to be calculated. The feasibility of this strategy was shown in an *in vivo* mouse model by Quinn et al. [56], visualizing metabolic changes during wound healing. Another *in vivo* study used the same concept to investigate the depth-dependent metabolism of keratinocytes [57]. Melanin content has been used as an *in vivo* marker for several dermatological disorders [58].

19.2.3.3 Second Harmonic Generation (SHG) Microscopy

The introduction of SHG microscopy has sparked a slew of research in dermatology. Also known as frequency doubling, it involves the interaction of two photons of the same wavelength with a nonlinear material and the subsequent creation of a single photon with double the frequency and energy. The ability of a material for second harmonic generation, described by the nonlinear susceptibility coefficient $\chi(2)$, depends on the non-centrosymmetric structure. Biologically significant harmonophores include collagen [59], myosin [60], and microtubules [61].

In the case of fibrillar collagen, the repeating dipoles of peptide bonds and the structural alignment on all levels of the molecular and supramolecular structure (parallel peptide bonds are assembled into parallel fibrils; fibrils are aligned into fibers) result in coherent amplification of the emitted second harmonic signal refs. This also means that the structural alignment of collagen fibers influences the SHG signal intensity [62]. As this is not the case for non-fibrillar collagen types, SHG microscopy is specific for type I and II collagens and forms a highly specific tool for 3D imaging of unprocessed skin samples as showed in ■ Fig. 19.2 Abnormal ratios of collagen I:III have been described in multiple fibrotic processes [63]. Although collagen type III, often deposited alongside type I, is a fibrillar collagen, it produces only weak SHG signals [64]. As the SHG signal is dependent on phase-matching conditions, most of the signal forwardly propagated. The thin scattered fibers of collagen type III and their associated reduction in phase matching appear to be distinguishable from type I by comparing forward- and backward-scattered SHG images [65, 66]. The opacity of collagenous tissues however makes the detection of this signal impractical, rendering this technique possible only in thin specimens.

Multiple strategies to distinguish scar types using SHG images have been described. Most of these focus on software-based analysis of collagen fiber orientation and density [67–69]. Matrix organization and orientation have been described as abnormal in scars [29]. Non-software-based approaches rely on the dependence of

the nonlinear susceptibility of collagen on the polarization of the incident light. Polarization-sensitive SHG (PS-SHG) probes the organization of collagen fibers by sequentially rotating the polarization relative to the optical axis and probing the SHG response. By analyzing the SHG intensity at different angles of polarization, orientation vectors can be reconstructed [70, 71]. Note that because of the strong dependence of SHG on polarization, software-based analyses should be performed on images from circularly polarized light sources.

Using unstained tissue allows simultaneous biomechanical testing during imaging. For example, Rosin et al. assessed the scarring capacity of healing split-thickness skin grafts by microstructural SHG imaging of collagen and biaxial stretch testing [72]. The authors correlate the loss of waviness patterns to increased stiffness.

The reduced phototoxicity (as no external fluorophores are used) from these optical processes allows *in vitro* and even *in vivo* imaging, for example, skin equivalents [73], lab-grown wound models [74], and antifibrotic treatments [75].

Several research groups have made attempts to include SHG in clinical decision-making. Predominately applied to neoplastic lesions, consecutive follow-up of

■ **Fig. 19.2** Combined 2PEF (green) and SHG (red) image of elastin and collagen fibers, respectively. Unprocessed *ex vivo* human skin, specifically the reticular dermis, was imaged using the setup described in [87]. A stack of 35 images was acquired in the Z-axis. Image processing in ImageJ resulted in the 3D projection seen here [88]. Scale bar: 500 μm

scars and topical therapies has also been described. Commercial translation has already resulted in multiple devices marketed for in vivo dermatological applications. The Dermaspect (JenLab GmbH, Berlin, Germany) device entails a multimodal (2PEF, CARS, SHG) microscope with an adjustable scan head for in vivo skin imaging [76]. Innovations in laser technology allow for increasingly smaller and cheaper devices [77, 78].

19.2.3.4 Third Harmonic Generation (THG) Microscopy

The propensity of a material for third harmonic generation depends not only on its nonlinear susceptibility coefficient $\chi(3)$ but also on whether partial phase matching can be achieved by a small inhomogeneity at the focus, making THG an interface-sensitive technique [79]. THG is less specific than SHG, increasing the number of possible applications. By setting the focal volume of the incident laser beam to several times the size of a typical tissue structure, a geometry can be created where the phase-matching conditions enable efficient THG. As the THG contrast comes from inhomogeneities and tissue interfaces with a high $\chi(3)$, cell membranes and cell nuclei are clearly displayed with THG microscopy, since they contain lipids which are known to have a high $\chi(3)$ [80]. Not surprisingly, the multilayered structure of the epidermis provides excellent contrast. ■ Figure 19.3 shows a THG cross section of the epidermis in a drug penetration experiment. To illustrate the potential in dermatology, THG was shown to be a viable imaging technique for label-free tracking of melanoma cells [81]. The THG contrast from melanin and structural features allowed differentiation of skin cancers [82].

In the context of scar tissue, the surface of elastin fibers is an adequate THG emitter [83]; thus, combining SHG and THG potentially allows for three-dimensional imaging of unstained, unfixed, and unsliced tissue.

Although an analysis of scar tissue has not been described yet, THG has strong potential as a label-free imaging technique because of reduced phototoxicity. Indeed, several articles describe the in vivo “optical biopsy” technique in the skin [78, 84].

19.2.3.5 Coherent Anti-Stokes Raman Spectroscopy (CARS)

The structural information provided by SHG and THG can be supplemented by chemical information using another nonlinear process, Coherent anti-Stokes Raman Spectroscopy (CARS). Here, two lasers with different frequencies are focused on the sample, to probe the vibrational fingerprint of the molecules in the focal point. The CH₂ content of lipids provides excellent contrast when imaging phospholipid-rich cell membranes and adipocytes. The OH signal from water molecules makes this technique especially applicable for research into transepidermal water loss [76, 85].

19.3 Future Perspectives

The enormous increase in available devices for volumetric analysis is indicative for the increased attention for quantification tools in scar research. Technological advances and the need for point of care solutions have already resulted in smaller and manageable devices suitable for bedside use. This trend can be expected to continue with smartphone-based solutions already being investigated in wound evaluation [86]. Both researcher and clinicians should however be mindful of the fact that most devices are not adequately tested on their clinimetric properties.

This trend is also applicable to experimental techniques. As image acquisition times decrease and devices get smaller, we can expect integration of different imaging techniques, each bringing their own strengths in elucidating the multifaceted issue of pathological scarring.

■ **Fig. 19.3** Nonlinear microscopy image of a cross section of ex vivo skin. The THG signal (green) depicts the layers of the epidermis; collagen fibers in the papillary dermis give rise to the SHG sig-

nal in red. In this experiment, fluorescently labeled (magenta) hyaluronic acid (250 kDa) was applied to the skin's surface to quantify epidermal skin penetration. Scale bar: 500 μm

Take Home Messages

- Technological advances will increase the number of devices for scar assessment, while reducing their size.
- The integration of multiple techniques has the potential to improve scar assessment.
- For most devices, the clinimetric properties are insufficiently determined.
- SHG microscopy is a valuable tool for experimental research into scar pathophysiology and potential therapeutics.

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