

Machine learning-assisted SHG morphometry reveals distinct collagen microarchitectures of trabecular bone and fibrosis in bone marrow biopsies

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Abstract

Collagen microarchitecture in bone marrow biopsies represents a largely underexplored source of quantitative biomarkers for histopathological diagnostics and analysis of tissue remodeling. While second harmonic generation (SHG) microscopy has been increasingly applied to fibrosis assessment, the collagen organization of trabecular bone in bone marrow trephine biopsies remains poorly characterized. Here, we combined high-resolution SHG microscopy with shallow machine learning–assisted morphometry to compare collagen architecture in structured trabecular bone, unstructured trabecular bone, and fibrosis in bone marrow biopsies from patients with primary myelofibrosis. SHG image segmentation was performed using the LabKit plugin in Fiji. Several annotation strategies were evaluated to identify classifier configurations that preserved fibrillar structures. Quantitative morphometric analysis revealed marked differences in collagen organization between tissue types. Fibrosis exhibited significantly thinner collagen fibers and reduced branching complexity compared with structured trabecular bone. In contrast, unstructured trabecular bone showed extensive network branching accompanied by shorter skeleton branch length, consistent with remodeling-associated alterations of trabecular collagen architecture. Our results further demonstrate that annotation strategy substantially influences segmentation outcome and downstream morphometric measurements in SHG-based collagen analysis. Overall, this study establishes a reproducible workflow for machine learning-assisted SHG morphometry and highlights its potential for quantitative assessment of fibrosis, bone remodeling, and extracellular matrix organization in bone marrow pathology.

1. Introduction

Second harmonic generation (SHG) microscopy has emerged as a powerful label-free imaging modality for quantitative characterization of collagen organization in biological tissues [1–3]. Biomedical applications of SHG collagen imaging span major histopathological domains including skin [4, 5], liver fibrosis and steatohepatitis [6–8], bone [2, 9], as well as brain tumors and meninges [10–12]. Among its important biomedical applications are structural studies of fibrosis and pathology-related bone remodeling [13]. This rapidly developing field has important potential for the development of novel quantitative diagnostic approaches and for studies of pathological mechanisms in bone marrow diseases. Despite a handful of highly important pioneering studies [3, 14], SHG-based analysis of collagen microarchitecture in bone marrow biopsies remains severely underexplored. Because SHG signal generation is highly sensitive to fibrillar collagen organization, and packing geometry, the method provides unique opportunities for quantitative analysis of extracellular matrix microarchitecture in pathological tissue remodeling [15].

The bone marrow microenvironment plays a central role in the development and progression of myeloproliferative neoplasms and other hematological malignancies [16–18]. Increasing evidence suggests that tumor-associated vascular, endosteal, and fibrotic niches critically influence tumor progression, stem cell maintenance, inflammatory signaling, and therapy response [16, 19]. In particular, fibrosis-associated extracellular matrix remodeling and pathology-associated alterations of trabecular

bone architecture represent important but still poorly understood components of the bone marrow tumor niche microenvironment [20–22].

In recent years, SHG imaging has been increasingly applied to fibrosis assessment in bone marrow biopsies, including automated grading approaches for myelofibrosis [3]. However, most studies have primarily focused on fibrotic collagen accumulation, while the collagen architecture of trabecular bone within trephine biopsy samples remains largely unexplored. This limitation is notable because myeloproliferative neoplasms are frequently associated not only with fibrosis, but also with profound alterations in bone remodeling and osteosclerotic microarchitecture [20, 21, 23]. Given the high sensitivity of SHG microscopy to fibrillar collagen organization [1, 2], the method may provide unique opportunities for quantitative analysis of both fibrosis and bone structure within the same biopsy specimen.

Recent advances in machine learning–assisted image analysis further expand the diagnostic and analytical potential of SHG microscopy for collagen-rich tissues. In particular, shallow learning approaches integrated into user-friendly image analysis environments provide important advantages for exploratory histopathological studies because they require minimal training datasets while allowing rapid generation of reproducible segmentation workflows. We recently demonstrated the applicability of the LabKit shallow learning framework for SHG image analysis of collagen fiber network microarchitecture [12]. Another line of evidence suggests that Labkit shallow learning annotation strategies critically influence segmentation outcomes and downstream morphometric parameters in biomedical image analysis [24].

A growing body of literature has further emphasized that the amount, quality, and selection strategy of annotated training data are key determinants of segmentation performance in biomedical image analysis. Accordingly, considerable efforts are currently directed toward annotation-efficient learning strategies aimed at reducing expert annotation burden while preserving segmentation accuracy [25, 26].

Here, we applied high-resolution SHG microscopy combined with machine learning–assisted morphometry to quantitatively analyze collagen microstructure in bone marrow trephine biopsies from patients with primary myelofibrosis. We demonstrate that structured trabecular bone, unstructured trabecular bone, and fibrosis exhibit distinct collagen microarchitectures based on fiber local thickness, branching, and branch length measurements. We further address the efficiency of different annotation strategies for robust segmentation of the collagen SHG images. Our findings suggest a novel and affordable framework for quantitative tumor diagnostics and studies of the bone marrow tumor microenvironment and, more broadly, for quantitative analysis of bone- and fibrosis-associated collagen microstructure across a wide range of human pathologies.

2. Results

2.1. High-resolution SHG imaging of trephine biopsy samples.

We first aimed to obtain high-resolution SHG images of bone and fibrosis in trephine biopsy samples from human patients diagnosed with primary myelofibrosis (Fig. 1A-E). While SHG imaging is generally considered to be comparable to confocal microscopy in terms of spatial resolution limit [27, 28], we encountered a major limitation in image resolution due to sample photodamage when using high-NA objectives (20×, 63×). The highest spatial resolution we could obtain was at a pixel size of 200 nm using a 10×/0.4 objective (Fig. 1). We collected 4096 × 4096 pixel images containing bone and myeloid tissue (Fig. 1E). We observed high-resolution, high-contrast images of collagen fibers both within the bone and in fibrotic areas (Fig. 1E). The bone images contained both highly structured areas of parallelly oriented fibers that we term here as structured bone (Fig. 1F, Fig. 7A) and relatively poorly structured areas (unstructured bone) (Fig. 1G, Fig. 7B). The extent of fibrosis varied from mild cases with sparse fibers (Fig. 1E, H) to extensive collagen networks spanning large areas of the sections (Fig. 1E, I).

2.2 Creation of LabKit classifiers.

We then asked whether the recently developed shallow learning-based image segmentation algorithm LabKit [29] can be used for the segmentation of bone and fibrosis SHG images from trephine sections. We created several LabKit classifiers to assess how robustly the algorithm performs on fibers of various sizes, shapes, and SHG intensity levels (Figs. 2, 3). One crucial advantage of the LabKit shallow learning method is that it allows a skilled researcher to create a single classifier in less than one minute, which can then be applied to multiple images.

We tried a broad repertoire of various annotation strategies to elaborate efficient shallow learning-based collagen SHG image segmentation (Fig. 2). The foreground sampling varied in terms of SHG intensity, fiber straightness/tortuosity, fiber number. For the creation of classifier 1, two fibers were selected: one exhibiting medium SHG signal intensity and the other showing SHG signal intensity close to the maximum observed values. For classifier 2, nine representative fibers distributed across the low-to-medium SHG intensity range were selected. Background regions were simultaneously annotated alongside the fibers. For classifier 3, a single fiber exhibiting the highest SHG signal intensity was selected, and a corresponding background region was annotated in parallel. For classifier 4, a low-intensity fiber segment was selected. The foreground annotation line was curved and discontinuous. A single straight line was used to annotate the background parallel to the foreground annotation. For classifier 5, five fiber segments were selected, three of which belonged to the same fiber. The selected segments exhibited medium SHG signal intensity. Background regions were annotated on both sides of the selected segments using straight annotation lines. For classifier 6, two low-intensity segments of the same fiber were selected, and background regions were annotated on both sides in parallel.

2.3 Comparison of classifiers.

Comparison of the resulting segmentation masks demonstrated that classifiers 1 and 2 segmented structures exhibiting medium SHG signal intensity with comparable performance (Fig. 3). However, classifier 2 produced a more detailed segmentation pattern; nevertheless, in regions of high SHG signal intensity, adjacent structures frequently merged into larger connected objects.

Classifier 3 segmented primarily structures exhibiting high SHG signal intensity, whereas structures with low SHG signal intensity were largely excluded from the segmentation results. In addition, high-intensity structures were frequently segmented as single merged objects.

Classifier 4 segmented structures across the full range of SHG signal intensities with consistently high performance, while minimizing the formation of merged objects in high-intensity regions.

Classifiers 5 and 6 showed similar performance when segmenting structures exhibiting low-to-medium SHG signal intensity. However, classifier 5 produced a more detailed segmentation due to its superior detection of low-intensity fibers (Fig. 3).

Based on the comparison of segmentation results obtained for structured bone, unstructured bone, and fibrotic tissue, classifier 4 demonstrated the best overall performance. It provided the most accurate segmentation across all SHG signal intensity ranges, avoided artificial merging of bright structures, and preserved low-intensity fibers that were frequently missed by other classifiers, thereby generating the most detailed morphological representation (Fig. 3).

2.4 Training Rules for Successful Classifiers.

Systematic comparison of the foreground and background annotation strategies (red and blue annotation lines in Fig. 2) enabled us to formulate a set of practical guidelines for training efficient Labkit classifiers for bone marrow SHG image segmentation. The proposed rules are as follows: 1) Foreground and background annotation lines should be placed in close proximity to one another; 2) Background annotations should preferably be drawn on both sides of the foreground annotation. If only one background line is used, it should be positioned in a region exhibiting the greatest possible intensity contrast relative to the foreground. 3) Foreground annotations should follow continuous collagen fibrils rather than interrupted signal fragments and should include both high- and low-intensity pixels whenever possible. 4) The foreground annotation should span the full width of the collagen fibril rather than only its central axis.

To evaluate these guidelines experimentally, we generated an independent set of six additional classifiers: three trained according to the proposed annotation rules and three intentionally trained in violation of these rules (Fig. S1). Segmentation accuracy was assessed by calculating two overlap metrics, the Dice coefficient and Intersection over Union (IoU), between manually generated expert masks and the corresponding Labkit-derived segmentation masks. Across a dataset comprising nine ROIs of structured trabecular bone and nine ROIs of fibrosis obtained from three independent patients, classifiers trained according to the proposed annotation guidelines consistently achieved higher Dice and IoU values than classifiers deliberately trained in violation of these guidelines (Fig. 4).

2.5 Fiji Pipelines with Embedded Machine Learning.

The Labkit plugin was originally developed to facilitate integration into reproducible Fiji workflows and high-throughput image analysis pipelines [29]. Taking advantage of these capabilities, we developed two Fiji-based workflows for semi-automated and automated analysis of bone marrow SHG images.

Pipeline 1 operates on 4096×4096 pixel images. The workflow begins with SHG image segmentation using a pre-trained Labkit classifier, which generates a binary mask (Fig. 5). The center of each region of interest (ROI) is then selected manually using the PointPicker tool. Each ROI measures 400×400 pixels, and multiple ROIs may be selected during a single pipeline run.

The resulting binary masks are subsequently subjected to two complementary morphometric analyses using Fiji plugins: (1) Local Thickness analysis, which generates a fiber width histogram and a pseudocolor thickness map; and (2) Skeleton analysis, which extracts quantitative parameters describing collagen network branching geometry.

All morphometric measurements are automatically exported to output files for subsequent statistical analysis. Additional image-processing operations and morphometric measurements can be incorporated into the workflow with minimal modifications. Compared with manual image processing, this workflow increases analysis speed by approximately 8.5-fold while substantially reducing operator intervention throughout the analysis procedure. Pipeline 2 processes a predefined set of ROIs and performs Labkit-based segmentation followed by the same morphometric analysis steps as in Pipeline 1.

2.6 Quantitative analysis of the bone and fibrosis collagen microstructure.

After we selected the classifier 4 as an optimally working one, we used it for the quantitative analysis of the bone and fibrosis collagen microstructures. Binarized masks of 820×820 mm SHG images were obtained using the classifier 4 (Fig. 5A). We then selected ROIs of 80×80 mm size for fibrosis, structured bone and unstructured bone areas of the trephine sections (Fig. 5B). Ten ROIs of each tissue type per patient were selected from the 3 patients. The Local thickness and Skeleton plugins were applied in Fiji to retrieve quantitative parameters of the collagen microstructure.

2.6.1 Collagen fiber width.

We observed remarkable differences in the fiber width distribution between the structured trabecular bone, unstructured trabecular bone and fibrosis (Fig. 5). Most significantly, the fibers were clearly thinner in fibrosis as compared to the trabecular bone (Fig. 6H, J). Next, the fiber width distribution is clearly shifted to the left for the unstructured bone as compared to the structured bone for all three patients (Fig. 5H, I). The structured trabecular bone demonstrates consistent profile of the fiber thickness distribution for all 3 patients with the common maximum at about 4 mm (Fig. 5E-H). By contrast, both

fibrosis and unstructured bone exhibit remarkable variability in fiber thickness, suggesting different states of the pathologic process.

2.6.2 The collagen network geometry.

- **Gross analysis.**

By using the Skeleton plugin in Fiji we retrieve several quantitative parameters of the trabecular bone and fibrosis collagen network, such as the number of branches, the number of joint points, the mean and maximum branch length, etc. (Figs. 5, 6). The plugin detects both small unbranched objects and networks of various size and complexity (Fig. 8A-C). We first compared average values for the geometry parameters of all objects between structured bone, unstructured bone and fibrosis (Fig. 7). The structured bone network was more branched than fibrosis with no clear difference in the branch length (Fig. 7A-D). In turn, the unstructured bone was more branched than the structured bone and exhibited systematically shorter branches (Fig. 7E-H).

- **Branching-based analysis.**

The Skeleton plugin detects objects of various size and geometry in SHG images of trephine sections: point single-pixel objects (constituting the majority of objects by number), short unbranched sticks, small, medium size and very large, highly branched networks of fibers. Averaging structural parameters over that highly diverse ensemble of various architectures gives a very gross estimation of real geometry variants with limited ability to detect possible differences between collagen networks in bone versus fibrosis. We therefore divided the detected skeletons into four subgroups based on the branch number: 1) objects of 0–1 branches; 2) 2–10; 3) 11–100 and 4) > 100 branches (Fig. 7A-C, Fig.S). We then compared values for the geometry parameters between structured bone, unstructured bone and fibrosis in those subgroups.

0–1 branches. Highly consistent difference between structured and unstructured bone was observed for all patients in terms of branching and branch length for objects of 0–1 branches. No clear difference was observed between structured bone and fibrosis in this object group (confident intervals included 0).

2–10 branches. Strong difference was observed between structured bone and fibrosis for maximum branch length (mean difference of 5 mm) for objects of 2–10 branches (Fig.S). Within the same object group (2–10 branches) strong difference was observed also between structured and unstructured bone in terms of the mean and maximal branch length and the number of slab voxels .

11–100 branches. Very strong and highly consistent difference was observed 1) between structured bone and fibrosis; 2) between structured and unstructured bone in terms of branching for objects of 11–100 branches. The effect was accompanied by strong difference between structured and unstructured bone in terms of branch length (Fig. 7).

Over 100 branches. Those large collagen networks demonstrated the most consistent difference 1) between structured bone and fibrosis; 2) between structured and unstructured bone. For those paired comparison analyses strong difference was observed both in terms of branching and branch length (Fig. 7).

2.6.3 Collagen orientation.

Because SHG signal intensity depends on the orientation of non-centrosymmetric collagen fibrils relative to the optical axis and polarization of the imaging system (Ref), we next examined collagen orientation in the three tissue types using the Fiji Directionality plugin (Fourier components method). Structured trabecular bone exhibited pronounced preferred fiber orientations in all three patients, as demonstrated by distinct peaks in the pooled orientation histograms (Fig. S2A). In contrast, orientation distributions in unstructured trabecular bone and fibrosis were considerably broader and generally lacked pronounced dominant directions (Fig. S2B,C). Interestingly, although the three patients displayed distinct orientation profiles (Fig. S2A), the local thickness histograms showed highly similar peak positions across patients (Fig. 6E–G), indicating that the observed collagen thickness measurements are largely independent of the overall fiber orientation. To quantify the observed differences in orientation distributions, we compared the angular dispersion values obtained from the Directionality plugin (Fig. 10). Structured trabecular bone consistently exhibited lower angular dispersion and stronger directional alignment than either unstructured trabecular bone or fibrosis (Fig. 10A–I). By contrast, both unstructured trabecular bone and fibrosis showed substantially broader orientation distributions, reflecting a more isotropic collagen organization.

Together, these findings indicate that angular dispersion provides an additional quantitative descriptor of collagen microarchitecture, complementing local thickness and skeleton-based morphometric parameters for SHG analysis of bone marrow collagen.

3. Discussion

The extracellular matrix is increasingly recognized as an active component of the bone marrow niche rather than a passive scaffold. Collagen organization influences tissue mechanics, cell adhesion, migration, and signaling, thereby contributing to the regulation of hematopoietic and neoplastic cell behavior. The present results demonstrate that structured trabecular bone, unstructured trabecular bone, and fibrosis represent distinct collagen microarchitectural states of the bone marrow microenvironment. The approach described here suggests that collagen microarchitecture may provide a quantitative structural descriptor of bone marrow niches, thereby adding a new dimension to diagnostics and mechanistic studies of bone marrow pathologies. Indeed, in tumors, collagen alignment, bundling, and fiber organization influence cancer cell migration, invasion, mechanotransduction, immune cell infiltration, and therapy response [30–33]. Likewise, alterations of collagen organization in bone are associated with changes in tissue mechanical properties and are increasingly recognized as important contributors to osteoporosis and pathology-associated bone remodeling [9, 34, 35]. From this perspective, SHG-based collagen morphometry may complement existing cellular and molecular

approaches by providing quantitative structural descriptors of the extracellular matrix component of the bone marrow niche microenvironment.

From a diagnostic pathology perspective, the present approach may provide information that is complementary to conventional fibrosis grading. Rather than quantifying collagen abundance alone, SHG morphometry enables quantitative assessment of collagen organization, branching architecture, and structural heterogeneity, thereby introducing additional descriptors of extracellular matrix remodeling in bone marrow disease [3, 13, 14, 16, 36].

Although the present study was not designed to correlate SHG-derived parameters with established fibrosis grades, the observed differences in collagen microarchitecture suggest that future studies may evaluate whether quantitative morphometric descriptors provide information complementary to current WHO and European consensus fibrosis grading systems [3, 19].

While histopathological assessment of bone marrow fibrosis has traditionally relied on the extent of collagen deposition and fibrosis grading, the present results suggest that SHG imaging may provide several additional quantitative descriptors of collagen organization, including fiber thickness, branching complexity, and network geometry. These parameters may represent an additional dimension of bone marrow pathology and could potentially expand the repertoire of quantitative tools available for differential diagnostics [13, 15]. Future studies may determine whether SHG-derived collagen microarchitectural descriptors differ between primary myelofibrosis, post-polycythemic myelofibrosis, post-essential thrombocythemia myelofibrosis, reactive fibrosis, and other bone marrow pathologies [16, 17].

An important methodological aspect of the present study is the use of shallow machine learning–based segmentation with the LabKit platform. While deep learning approaches dominate current biomedical image analysis, shallow learning methods provide substantial practical advantages for exploratory histopathological applications, including rapid classifier generation, low annotation burden, and direct integration into Fiji workflows. Our analysis further demonstrates that annotation strategy critically influences segmentation outcome and derived morphometric parameters. This observation is particularly important for fibrillar structures, where preservation of low-intensity collagen fibers substantially affects skeleton topology and quantitative branching analysis. More broadly, it aligns with a growing body of literature emphasizing that annotation strategy itself represents a major determinant of machine learning performance in biomedical image analysis. Recent annotation-efficient approaches aim to reduce expert workload by optimizing the selection and representativeness of training annotations while maintaining segmentation quality [25, 26].

To our knowledge, shallow learning approaches have not previously been applied to SHG microscopy image segmentation (except for a recent proof-of-concept study on mouse brain meninges in Paveliev et al., *BiophysRev* 2025 [12]). Therefore, the approach described here opens a new instrumental perspective that may be valuable for the broad and rapidly growing field of SHG biomedical imaging. Moreover, thorough literature search suggests that bone collagen structural analysis and fibrosis histological

analysis have not previously been addressed with shallow learning either, highlighting the above-mentioned methodological advantages of shallow learning for these important fields of medical research and diagnostics.

In contrast to end-to-end deep learning approaches, the present workflow is based on shallow machine learning–assisted segmentation followed by extraction of biologically interpretable morphometric parameters. As a result, the final diagnostic descriptors remain directly linked to observable collagen features such as fiber thickness, branching complexity, branch length, and orientation dispersion. This transparency facilitates biological interpretation, validation by pathologists, and identification of potential segmentation artifacts. Such explainable morphometric workflows may offer advantages for clinical implementation because the diagnostic decision can be traced back to specific structural features rather than inferred from latent representations of a neural network [37, 38].

Interestingly, a conceptually similar trend towards explainable machine learning can be observed in recent SHG studies of myelofibrosis. In their pioneering work, Yu et al. [3] first extracted biologically interpretable collagen descriptors including fiber width, length, straightness, orientation and fiber count, and only subsequently applied a support vector machine classifier for fibrosis grading. Thus, even when deep learning is used for diagnostic classification, the decision remains grounded in explicit morphometric features rather than directly inferred from raw image data. This approach provides important perspectives for the development of novel diagnostic strategies based on label-free multiphoton microscopy and quantitative tissue assessment. Such quantitative imaging approaches may be highly beneficial for a broad range of diagnostic and biomedical applications, including but not limited to gastrointestinal stromal tumors, breast cancer, and pancreatic ductal adenocarcinoma [8, 39, 40].

A substantial part of the present study was devoted to creation, selection and testing of classifiers that perform efficiently both on the bone and fibrosis images (Figs. 2–3). As a result, we created the classifier 4 fulfilling that condition and successfully working on images from independent samples and repetitive experiments. Importantly, this result allows using a unified procedure at all steps of the study from tissue imaging to image segmentation and subsequent analysis with local thickness and skeletonisation algorithms. To further explore the procedure of efficient classifier creation we asked whether we could formulate the “rules of thumb” for the bone marrow SHG image annotation within the Labkit classifier training. We previously addressed the same question for a relatively simple object – confocal images of GFAP-immunostained astrocytes in mouse brain sections in the experimental model of neuroimplantation-induced glial scarring [24]. In the present study we extended the approach to formulate and test certain rules for training efficient Labkit classifiers on the bone marrow SHG images. By using two different metrics (the Dice index and IoU) we demonstrate that rule-compliant classifiers are more efficient as compared to rule-violating classifiers both on the structured bone and fibrosis images (Fig. 4). This result suggests that annotation design of efficient shallow learning classifiers is affordable for a range of biomedical applications and could be further studied for expanding applicability and increasing efficiency of shallow learning tools.

Our results demonstrate that SHG microscopy combined with machine learning–assisted image analysis enables quantitative characterization of collagen microarchitecture in bone marrow trephine biopsies. Previous SHG studies of bone have described both highly ordered lamellar collagen organization and more disorganized fibrillar arrangements associated with remodeling, repair, or pathological bone formation [1, 2]. In agreement with these observations, we detected both structured trabecular regions composed of parallel collagen bundles and less organized fibrillar patterns characterized by extensive branching and shorter skeleton segments.

Importantly, most previous SHG studies of bone collagen organization were performed in developmental, fracture-healing, osteoporosis, or experimental remodeling models, whereas trabecular collagen architecture within bone marrow trephine biopsies has received substantially less attention. This limitation is particularly relevant in myeloproliferative neoplasms, where fibrosis is frequently accompanied by profound alterations in bone remodeling and osteosclerotic microarchitecture [20, 21, 23]. Our findings suggest that SHG-based morphometric analysis may provide a quantitative framework for assessing not only fibrosis, but also pathology-associated remodeling of trabecular bone collagen organization within the bone marrow microenvironment.

The highly reproducible local thickness maximum observed at approximately 4 μm in structured trabecular bone is remarkably consistent with the known hierarchical organization of bone collagen. Trabecular bone lamellae were previously reported to be approximately 6 μm thick [34], whereas mineralized collagen fibrils assemble into bundles of approximately 2–3 μm that form lamellar bone architecture [9]. Therefore, the SHG-derived local thickness maximum observed here may reflect a characteristic structural scale of collagen bundle organization within trabecular lamellae.

Altogether, we used four classes of metrics to characterize collagen geometry: fiber thickness, branching, branch length, and orientation dispersion, all of which demonstrated substantial differences between structured trabecular bone and the other two tissue types (unstructured trabecular bone and fibrosis). Future studies may combine multiple SHG-derived morphometric parameters into multidimensional feature spaces enabling automated classification of collagen microarchitectures.

The “unstructured” SHG pattern observed in some trabecular regions likely reflects a combination of biological and geometrical factors. On one hand, increased branching and fragmentation of the skeletonized collagen network may correspond to remodeling-associated disorganization and osteodestructive alterations of trabecular collagen architecture. On the other hand, SHG signal morphology is strongly influenced by fibril orientation relative to the imaging plane and excitation geometry. Consequently, regions containing collagen bundles oriented outside the section plane may also contribute to fragmented or poorly aligned SHG patterns. Therefore, the observed morphometric differences likely reflect both true pathological remodeling and orientation-dependent projection effects associated with complex three-dimensional collagen organization.

Dissecting the relative contributions of the biological and geometrical factors to the resulting SHG images would be a highly relevant issue to address in future studies. In that regard, measuring the

collagen network parameters on the morphometry data from 3 patients and averaging over 10 ROIs per patient allows us to partially compensate for SHG dependence on fiber orientation, when comparing microarchitecture of structured bone versus fibrosis and structured vs unstructured bone, as the resulting data represent a broad range of fiber orientation angles relative to the optical axis (Fig. S2). The robustness of the proposed morphometric pipeline with respect to collagen anisotropy is supported by the very good agreement of the peak positions of fiber thickness distributions in structured bone across the three patients (Fig. 6E–G), despite markedly different fiber orientation profiles in the pooled histograms (Fig. S2A).

Although the present study focused on bone marrow trephine biopsies from patients with myelofibrosis, the methodological framework developed here is not limited to hematological pathology. SHG microscopy is increasingly used for investigation of fibrosis and extracellular matrix remodeling in a broad range of tissues, including liver, skin, lung, kidney, and tumor stroma [1, 6, 7, 41]. Likewise, quantitative characterization of collagen organization is becoming increasingly relevant in studies of osteoporosis, fracture healing, osteosclerosis, and pathological bone remodeling [2, 9, 34]. The ability of the present workflow to quantify fiber thickness distributions, branching architecture, and collagen network topology using an affordable and reproducible image analysis pipeline suggests potential applicability to a wide spectrum of fibrosis- and bone-related pathologies.

Overall, the present findings demonstrate that SHG microscopy combined with reproducible shallow machine learning–assisted morphometry enables quantitative analysis of both fibrosis and trabecular bone microarchitecture in bone marrow pathology. Beyond conventional assessment of collagen deposition, the proposed approach provides quantitative descriptors of collagen organization, branching architecture, and structural heterogeneity, thereby expanding the analytical toolbox available for studies of bone marrow remodeling. These results support the concept that collagen microarchitecture represents an informative and largely underexplored component of the bone marrow niche microenvironment. More broadly, the workflow presented here may provide an affordable and scalable platform for quantitative investigation of fibrosis, bone remodeling, and extracellular matrix organization across a wide range of human pathologies.

4. Materials and Methods

4.1. Tissue samples.

Bone marrow trephine biopsy specimens were obtained from patients diagnosed with primary myelofibrosis during routine diagnostic procedures approved by the local Ethics Committee of the Russian Scientific Center of Surgery named after Academician B.V. Petrovsky (approval No. 9/27.10.2023). All samples were anonymized before image acquisition and quantitative analysis. Histopathological diagnosis was established according to the WHO Classification of Haematolymphoid Tumours (5th edition, 2022) using conventional clinical, histopathological, and laboratory criteria.

Trephine biopsy samples were fixed in 10% neutral buffered formalin (NBF), decalcified in EDTA, embedded in paraffin and sectioned for microscopy. Decalcification was performed in a saturated EDTA (disodium ethylenediaminetetraacetate) solution prepared as follows: 150 g of disodium EDTA was dissolved in 850 mL of distilled water preheated to 50–60°C until a slight insoluble excess remained, followed by the addition of 150 mL of 40% formaldehyde solution. The pH was adjusted to 7.0–7.5 with 40% aqueous NaOH. Decalcification was carried out at 37°C for 4–5 days. After decalcification, the specimens were rinsed in running water for 30–60 min prior to dehydration. Sections used for second harmonic generation (SHG) imaging were cut at 2 µm thickness and mounted in Leica mounting medium.

Three independent primary myelofibrosis cases were included in the present exploratory morphometric study. Representative regions containing structured trabecular bone, unstructured trabecular bone and fibrosis were selected for SHG imaging and quantitative analysis.

4.2. Microscopy.

Biopsy samples were sectioned at 2mm thickness and mounted in Leica mounting medium. Backward SHG imaging was conducted on a Leica TCS SP5MP confocal microscope (Leica, Germany) with 800nm Mai Tai Ti:Sapphire tunable laser excitation and 390-410nm bandpass acquisition. Imaging was performed with an oil immersion objective (HC X PL APO CS 10×/0.40 IMM UV; Leica Microsystems, Wetzlar, Germany) at a pixel size of 200 nm.

4.3. Image analysis.

For local thickness and skeleton geometry measurements 10 ROIs 80x80 mm size were selected for each type of SHG images – for structured bone, unstructured bone and fibrosis. Data were averaged over those 10 ROIs.

Collagen orientation was quantified using the Fiji Directionality plugin (Fourier components method). The plugin estimates the dominant fiber orientation and the angular dispersion around the dominant direction by fitting the orientation histogram.

4.4. Statistical analysis.

Data were analyzed using estimation statistics, reporting mean paired differences with bootstrap-derived confidence intervals, rather than relying exclusively on null-hypothesis significance testing [42, 43]. Standardized paired effect sizes (Cohen's *dz*) were calculated to quantify within animal differences [44, 45]. For the analyses presented in Figs. 5, 7–9, statistical comparisons were based on averages per patient (3 patients; *N* = 3 independent experiments). For each patient measurements were averaged for 10 regions of interest per tissue type (structured trabecular bone, unstructured trabecular bone, and fibrosis) per patient.

Declarations

Author Contributions: Conceptualization, M.P., Z.P.A., A.V.A., and A.A.E.; methodology, M.P., D.V.S., D.A.P., Z.P.A., A.A.M.; software, A.A.E., M.P., A.A.R., and I.I.V.; validation, M.P., Z.P.A., L.F.N., D.A.P., S.M.S., A.A.M., and D.S.V.; formal analysis, M.P., A.A.E., D.A.P., and D.S.V.; investigation, M.P., Z.P.A., L.F.N., D.A.P., A.A.M., S.M.S., and D.S.V.; resources, D.V.S., A.A.E., L.F.N., A.V.A., D.E.C., Yu.A.K.,; data curation, Z.P.A., D.A.P., S.M.S., M.E.F., and D.S.V.; writing—original draft preparation, M.P.; writing—review and editing, Z.P.A., A.V.A., A.A.E., D.E.C., Yu.A.K., A.A.M., M.P.; visualization, M.P., D.A.P., A.A.M.; supervision, M.P., A.A.E., A.V.A., Z.P.A.; project administration, A.A.E., A.V.A., D.V.S., and M.P.; funding acquisition, A.A.E., M.P., A.V.A., D.V.S., L.F.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the Russian Scientific Center of Surgery named after Academician B.V. Petrovsky (approval protocol number 9, date of approval 27.10.2023) for studies involving humans.

Data Availability Statement: Data are available upon request.

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Figures

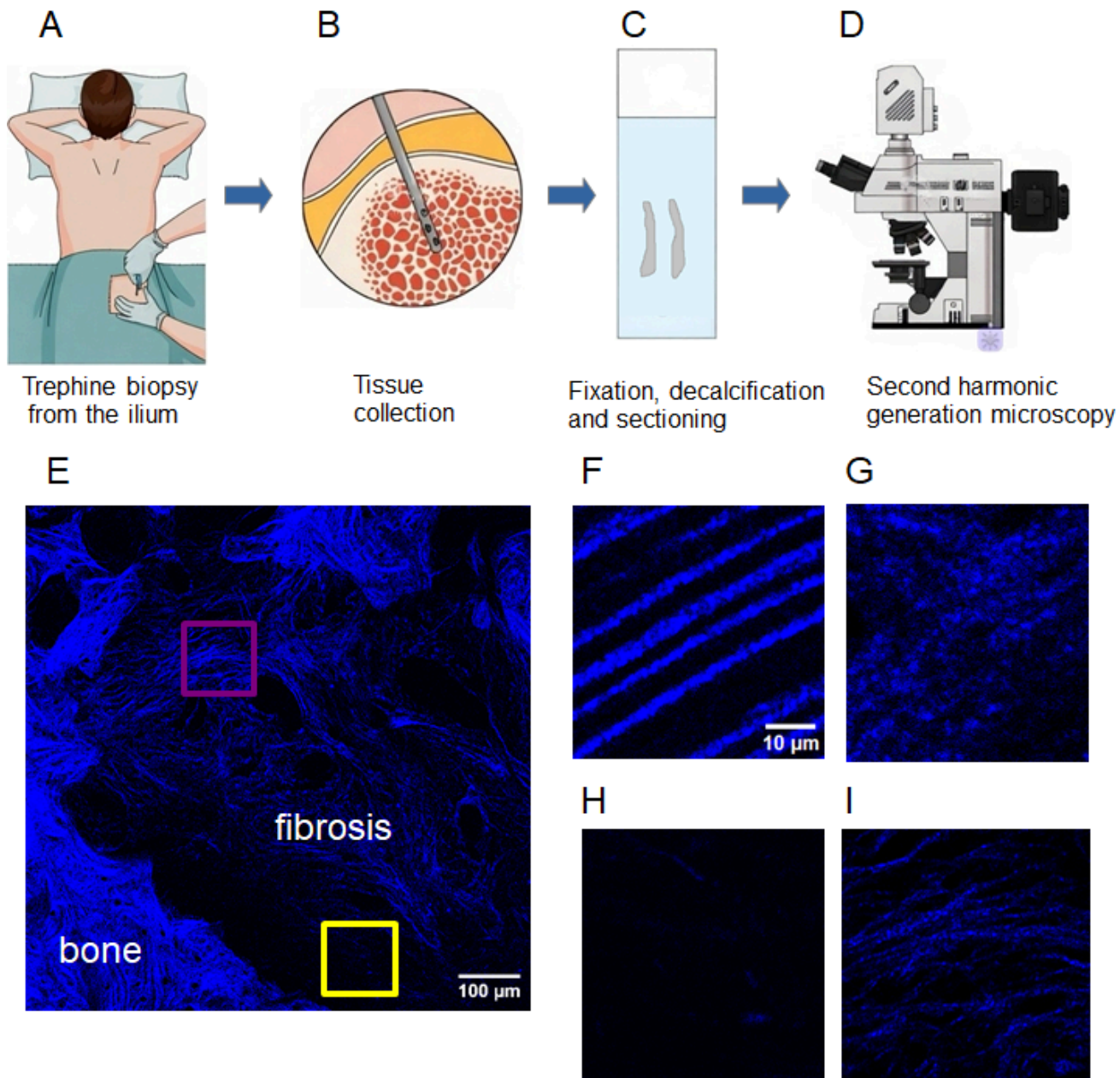
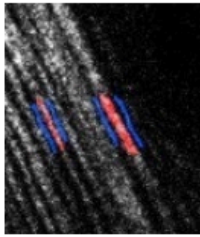


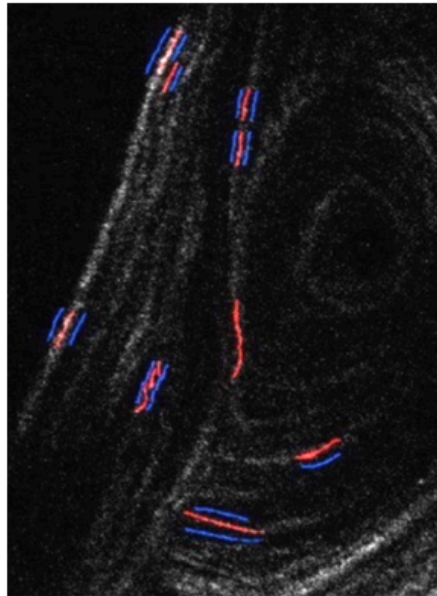
Figure 1

Second harmonic generation microscopy of bone marrow. Trephine biopsy of ilium (**a**) was performed to collect bone marrow samples (**b**), subjected to decalcification, sectioning (**c**) and SHG microscopy with 800nm excitation (**d**). Images of 4096x4096 pixel size were collected (**e**) at pixel size 200 nm. Structured bone (**f**), unstructured bone (**c**), sparse fibrosis (**h**, yellow squared area in **e**) and intense fibrosis (**i**, purple squared area in **e**) were observed.

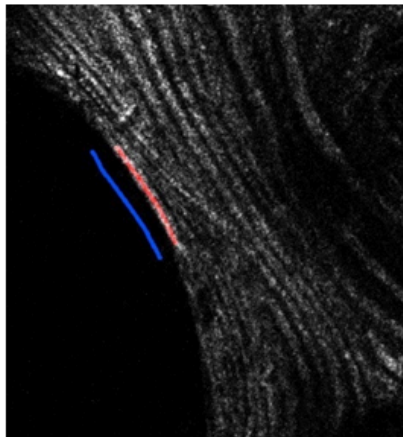
A Classifier 1



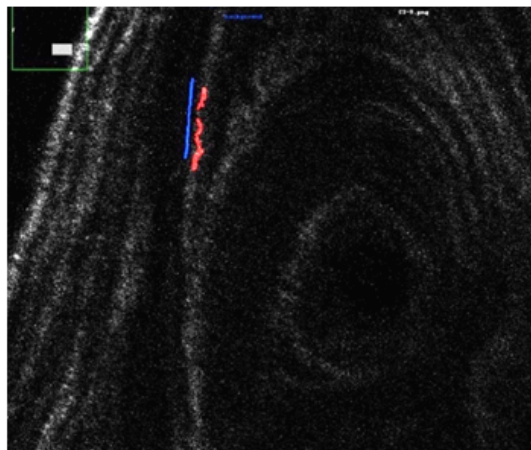
B Classifier 2



C Classifier 3



D Classifier 4



E Classifier 4

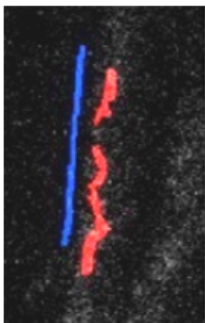


Figure 2

Labkit shallow learning classifier creation. Several classifiers were produced implementing various annotation strategies in terms of SHG intensity, fiber straightness/tortuosity, fiber number, and the number of background lines. Foreground (red) and background (blue) annotation is shown for classifiers 1-4 (**a-e**).

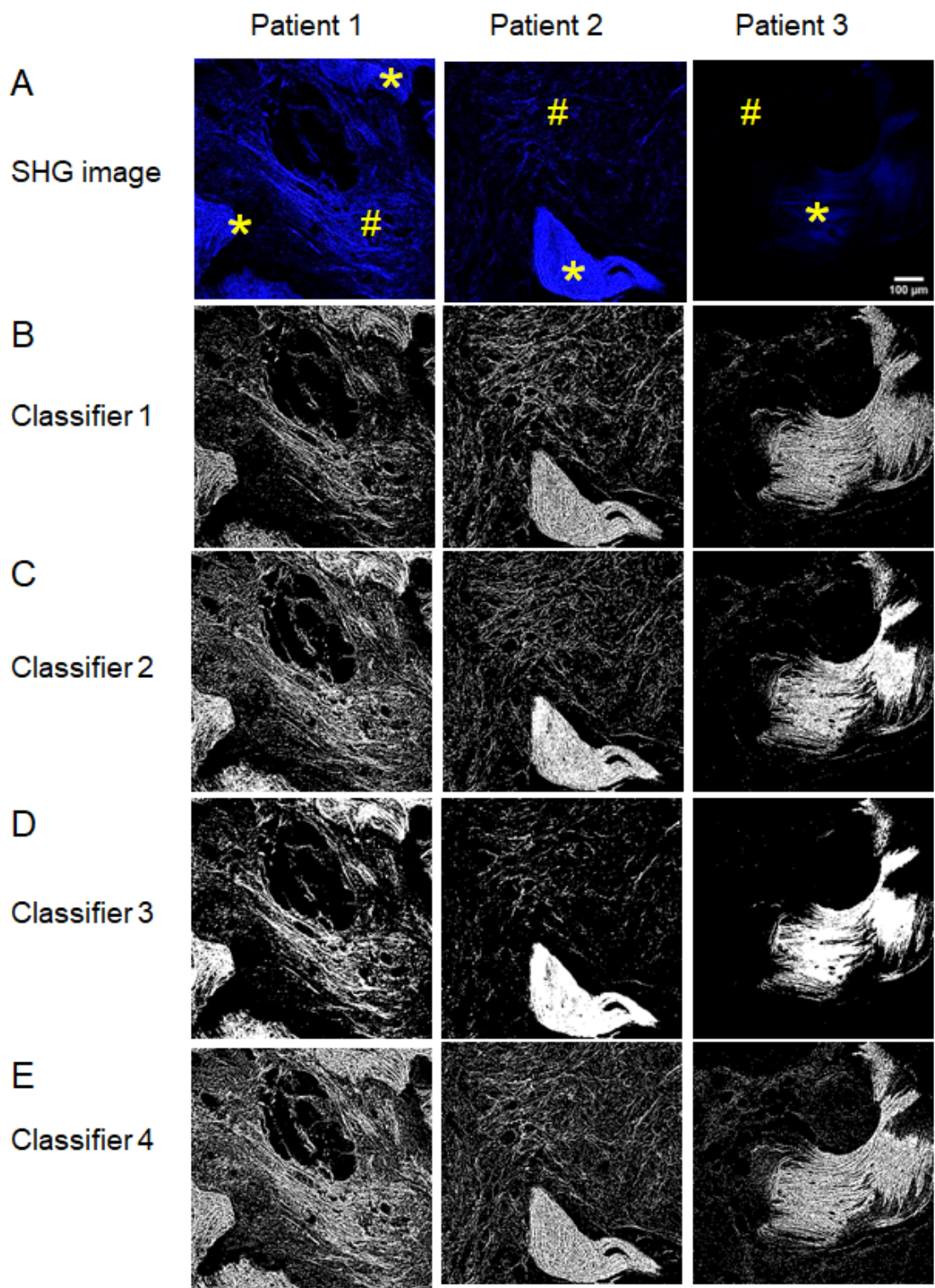


Figure 3

Labkit shallow learning classifier testing. Representative raw SHG images containing bone (marked with asterisk) and fibrosis (hashtag) are shown for three patients (**a**). Labkit segmentation results are shown for classifiers 1-4 (**b-e**).

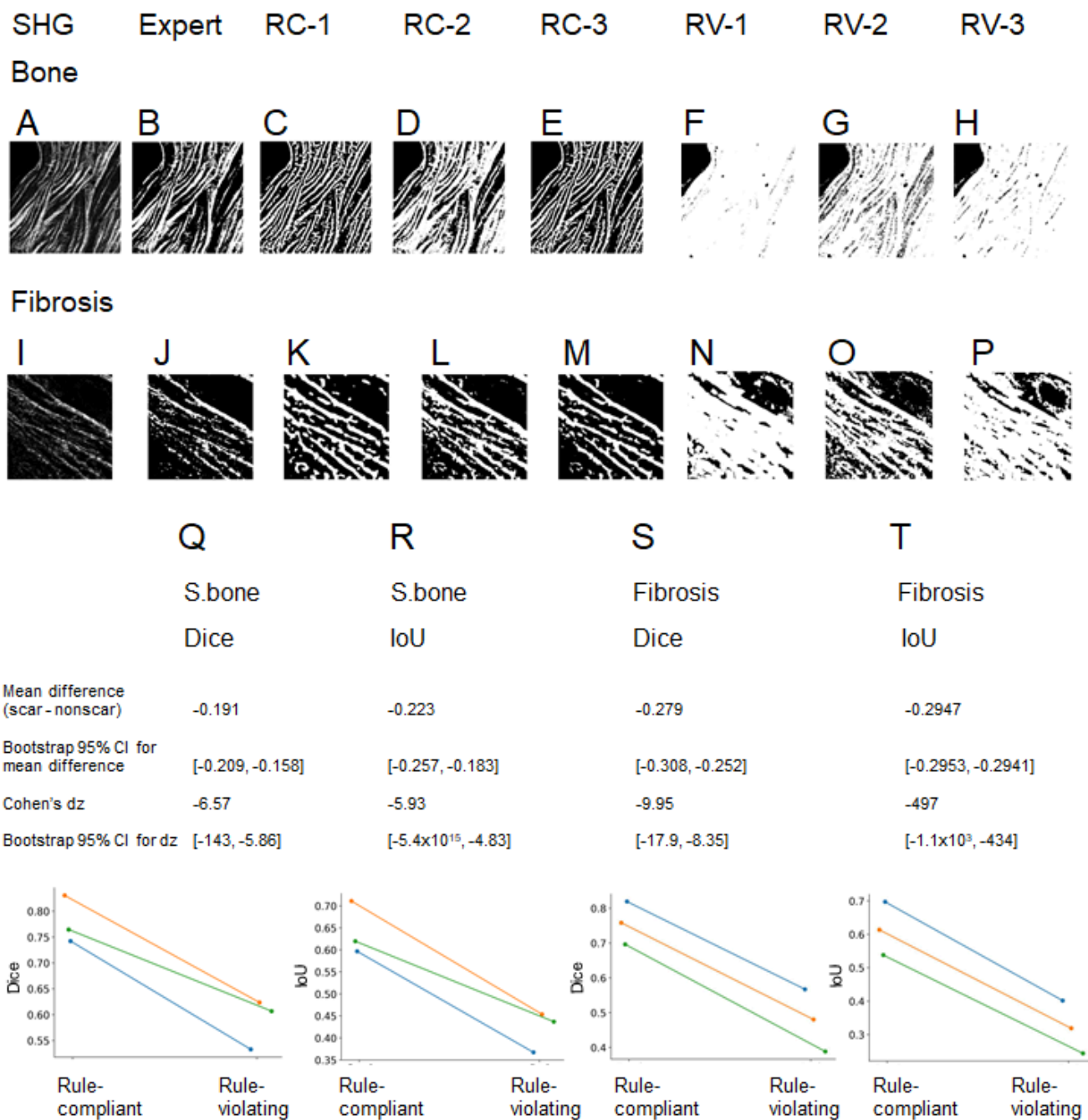
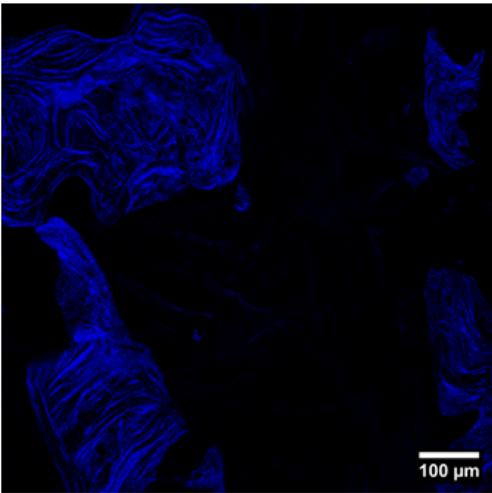


Figure 4

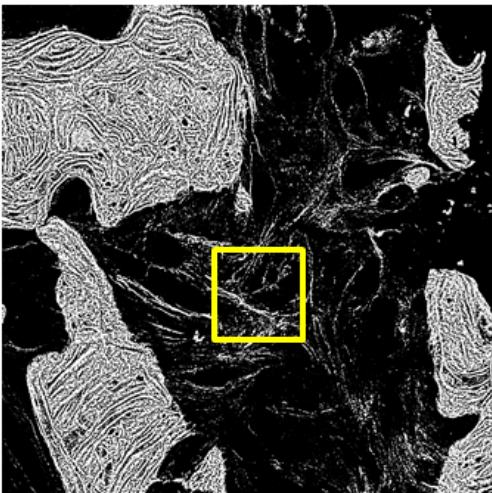
Segmentation performance is affected by training rules for shallow learning. **(a,i)** – SHG images of structured bone and fibrosis. **(b,j)** – Manual thresholding-based binarized masks for A, I, threshold set by a human expert. **(c-e)** – bone segmentation results for rule-compliant classifiers 1-3, correspondingly. **(f-h)** - bone segmentation results for rule-violating classifiers 1-3, correspondingly. **(k-m)** - fibrosis segmentation results for rule-compliant classifiers 1-3, correspondingly. **(n-p)** – fibrosis segmentation

results for rule-violating classifiers 1-3, correspondingly. Two different match indices—the Dice coefficient and Intersection over Union (IoU)—were calculated between the expert- and Labkit-derived masks, and the values were averaged across the three rule-compliant and three rule-violating classifiers for structured trabecular bone (**q, r**) and fibrosis (**s, t**). Data are presented as averages per patient for 3 patients (N =3). The dataset included 3 ROIs from each patient, for a total of 9 ROIs for s.bone and 9 ROIs for fibrosis.

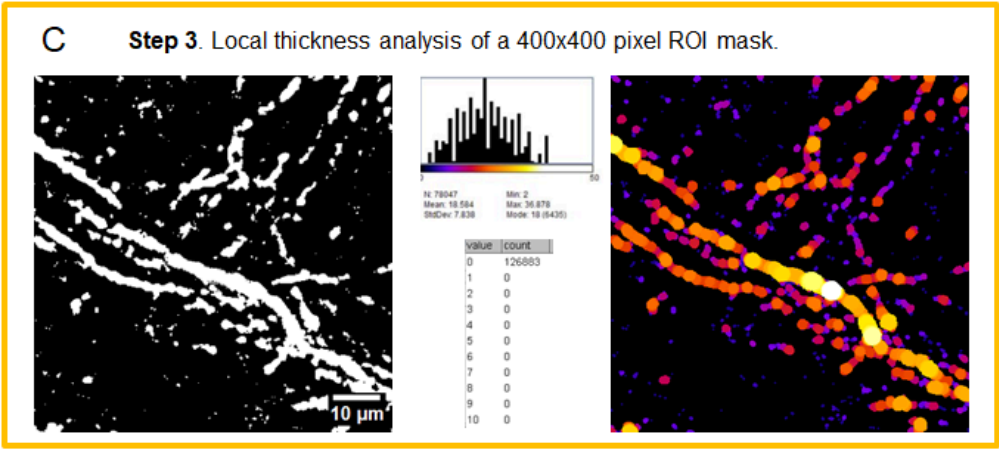
A Step 1. Labkit segmentation of a raw 4096x4096 pixel SHG image.



B Step 2. Selection of 400x400 ROI(s) within the binarized mask.



C Step 3. Local thickness analysis of a 400x400 pixel ROI mask.



D Step 4. Skeletonization analysis of a 400x400 pixel ROI mask.

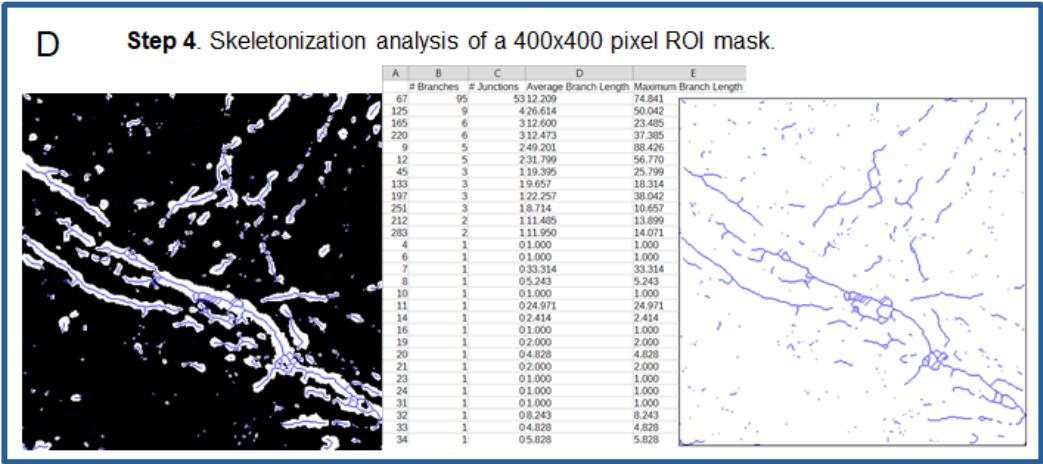


Figure 5

Schematic representation of the pipeline 1. Labkit segmentation (**a**) is followed by ROI selection (**b**), local thickness (**c**) and skeleton (**d**) analysis.

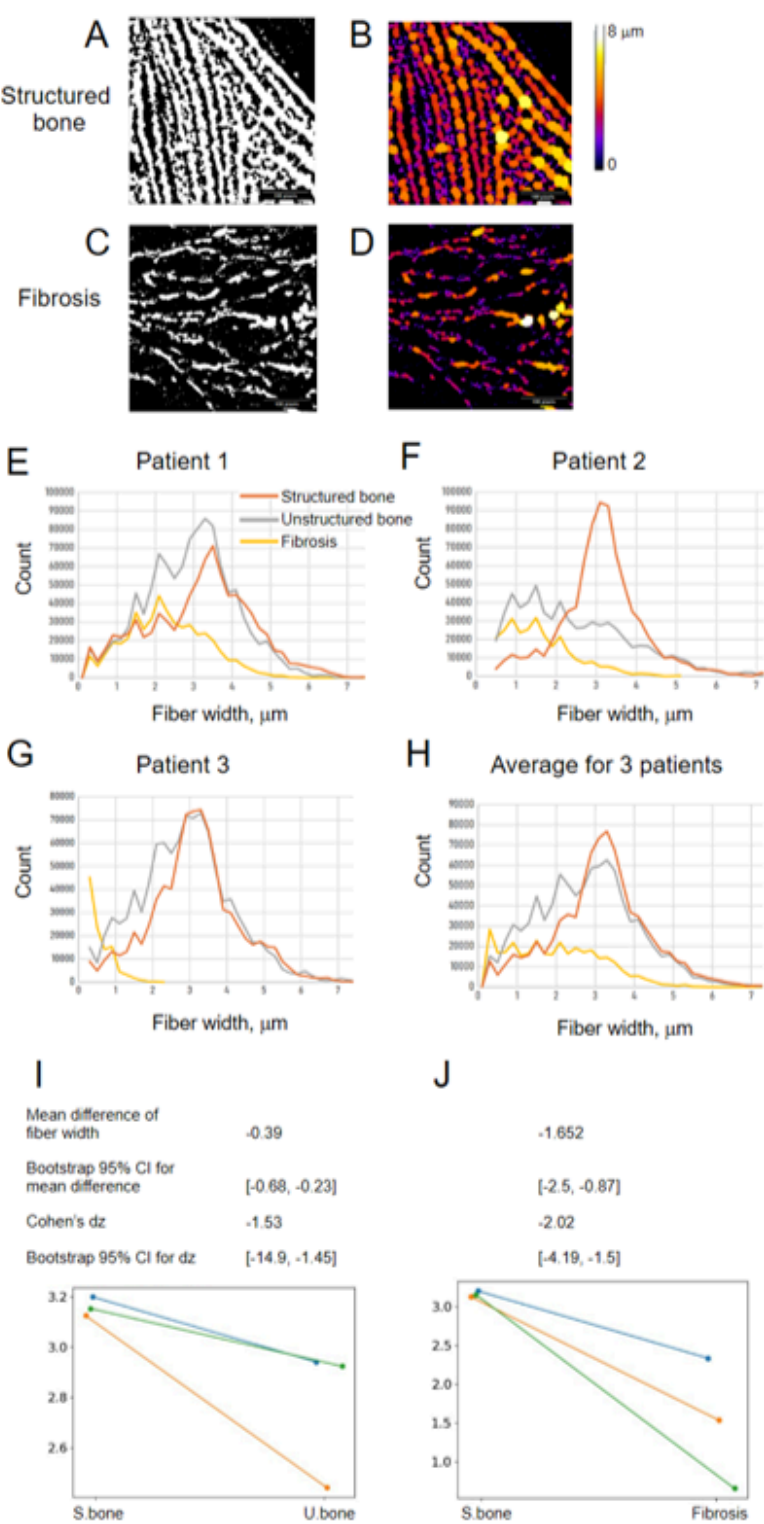


Figure 6

Collagen fiber width quantification in bone marrow SHG images. Following Labkit segmentation of raw SHG images with classifier 4 binarized masks of bone (**a**) and fibrosis (**c**) were processed with the Local thickness algorithm in Fiji (**b**, **d**, respectively). Histograms of the fiber local thickness (mm) measurements are shown for structured bone, unstructured bone and fibrosis (averaged over 10 ROIs in each case) for patients 1-3 (**e-g**, respectively) and averaged for 3 patients (**h**). Bootstrap analysis shows very strong difference in mean fiber thickness between structured bone and fibrosis (**i**) as well as between structure and unstructured bone (**j**). The local thickness pseudocolor code in (**b**) is valid for (**b**, **d**).

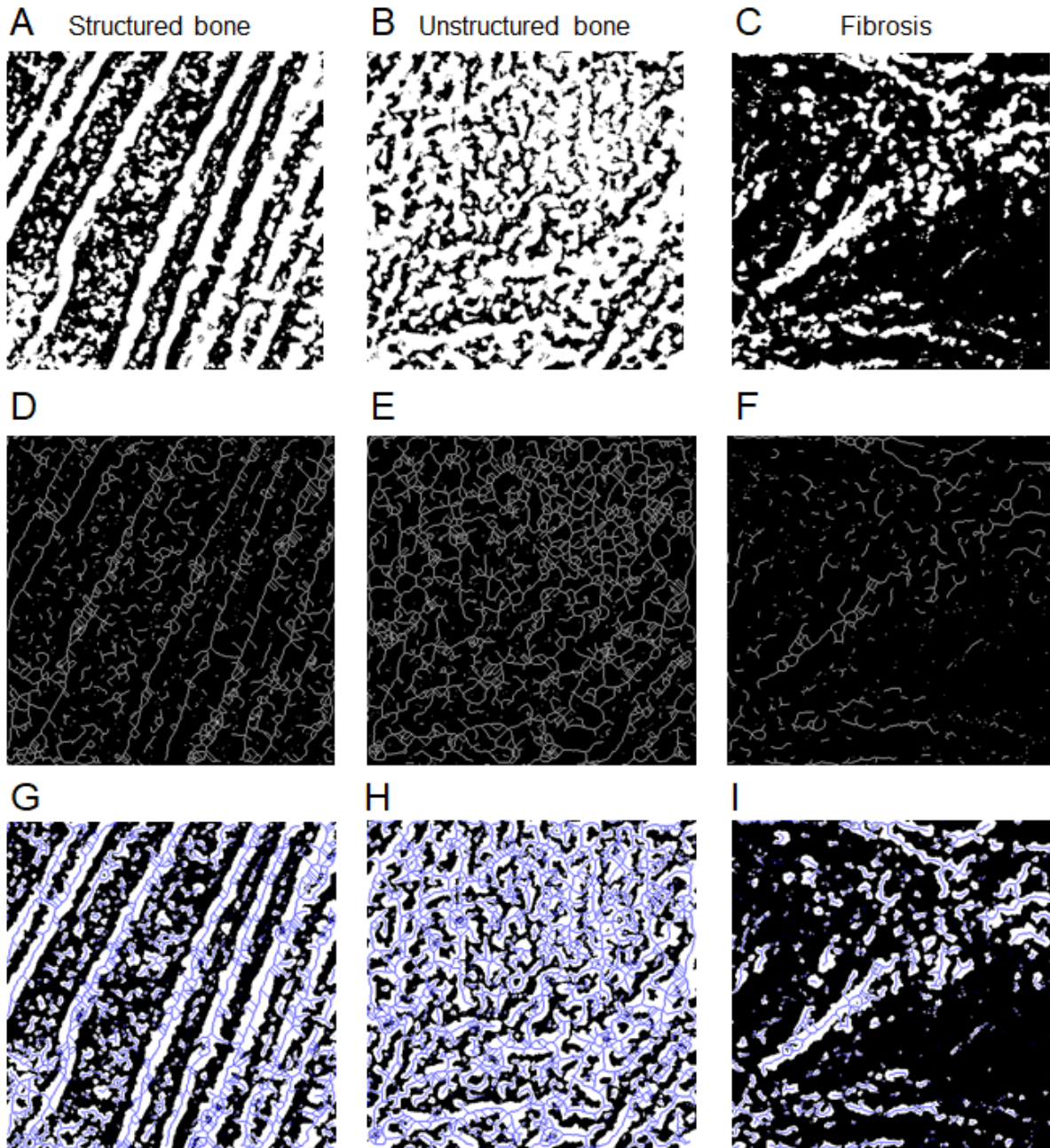


Figure 7

Skeletonization of Labkit-segmented SHG bone marrow images. Binarized masks of structured bone (a), unstructured bone (b) and fibrosis (c) were processed with the Skeleton plugin in Fiji. The resulting skeleton network architecture (d-f) exhibits clear difference between the three types of SHG images. (g-i)The skeleton overlay on SHG masks.

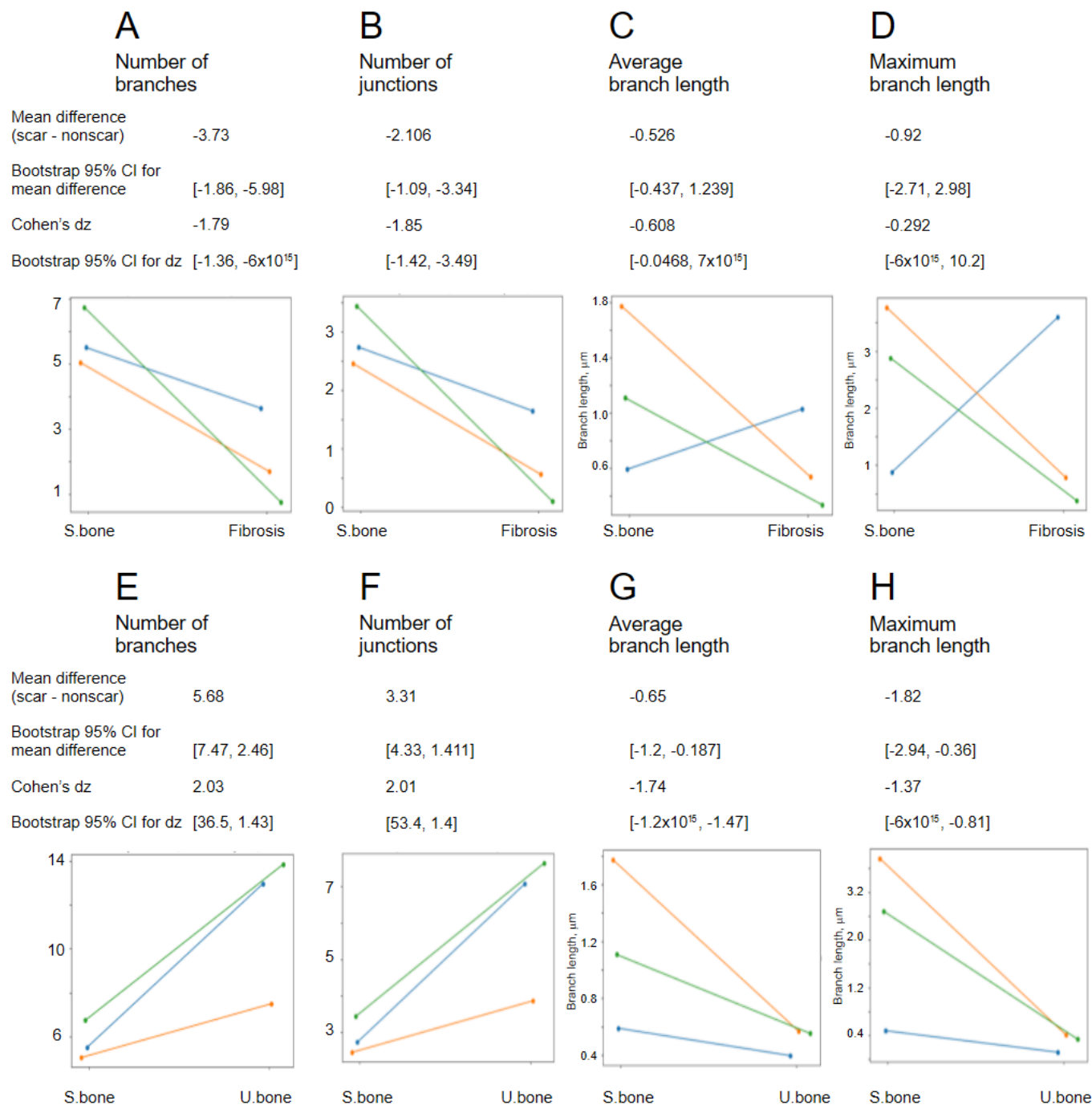


Figure 8

Collagen fiber network skeletonization-derived parameters reveal the network geometry difference between structured bone, unstructured bone and fibrosis. Branching is consistently higher for the structured bone as compared to fibrosis (**a, b**) and consistently higher for unstructured bone as compared to the structured bone (**e, f**). Average and maximal branch length does not exhibit consistent difference between structured bone and fibrosis (**c, d**), but those parameters show consistently higher

values for structured bone as compared to unstructured bone. Data are presented as an average over objects of all sizes and branching complexity levels.

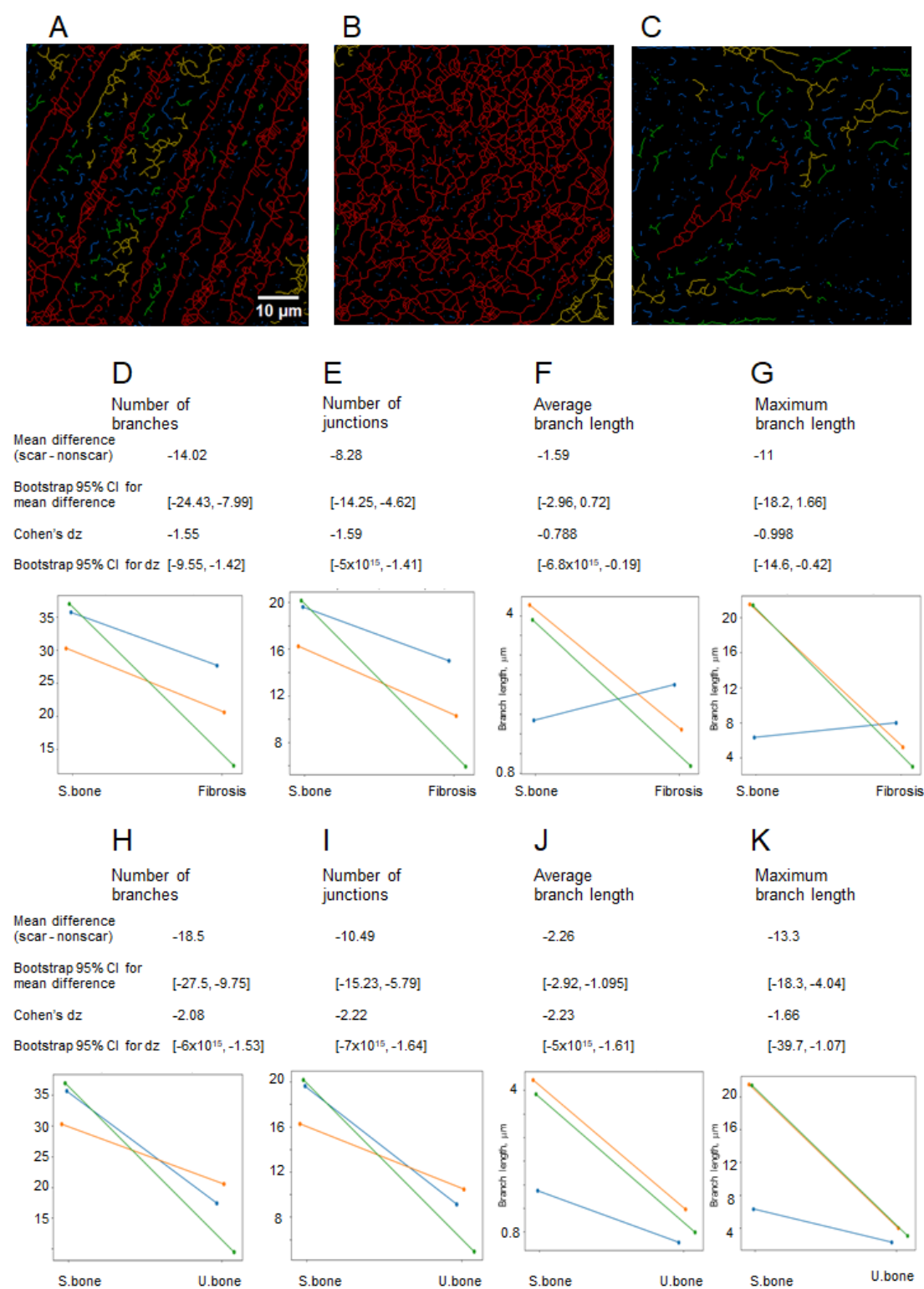


Figure 9

Differential analysis of the collagen fiber network skeletonization-derived parameters based on the network branching complexity. **(a-c)** The skeletonization-derived objects were divided into four bins

based on the number of branches: 0-1 branches (colorcoded in blue), 2-10 (green), 11-100 (yellow), over 100 branches (red). The same representative masks for structured bone (a), unstructured bone (b) and fibrosis (c) are shown as in Fig.5. (d-k). Bootstrap analysis for the bin of 11-100 branches. Branching is consistently higher for the structured bone as compared both to fibrosis (d-e) and to the unstructured bone (h, i). Average and maximal branch length does not exhibit consistent difference between structured bone and fibrosis (c, d), but those parameters show consistently higher values for structured bone as compared to unstructured bone.

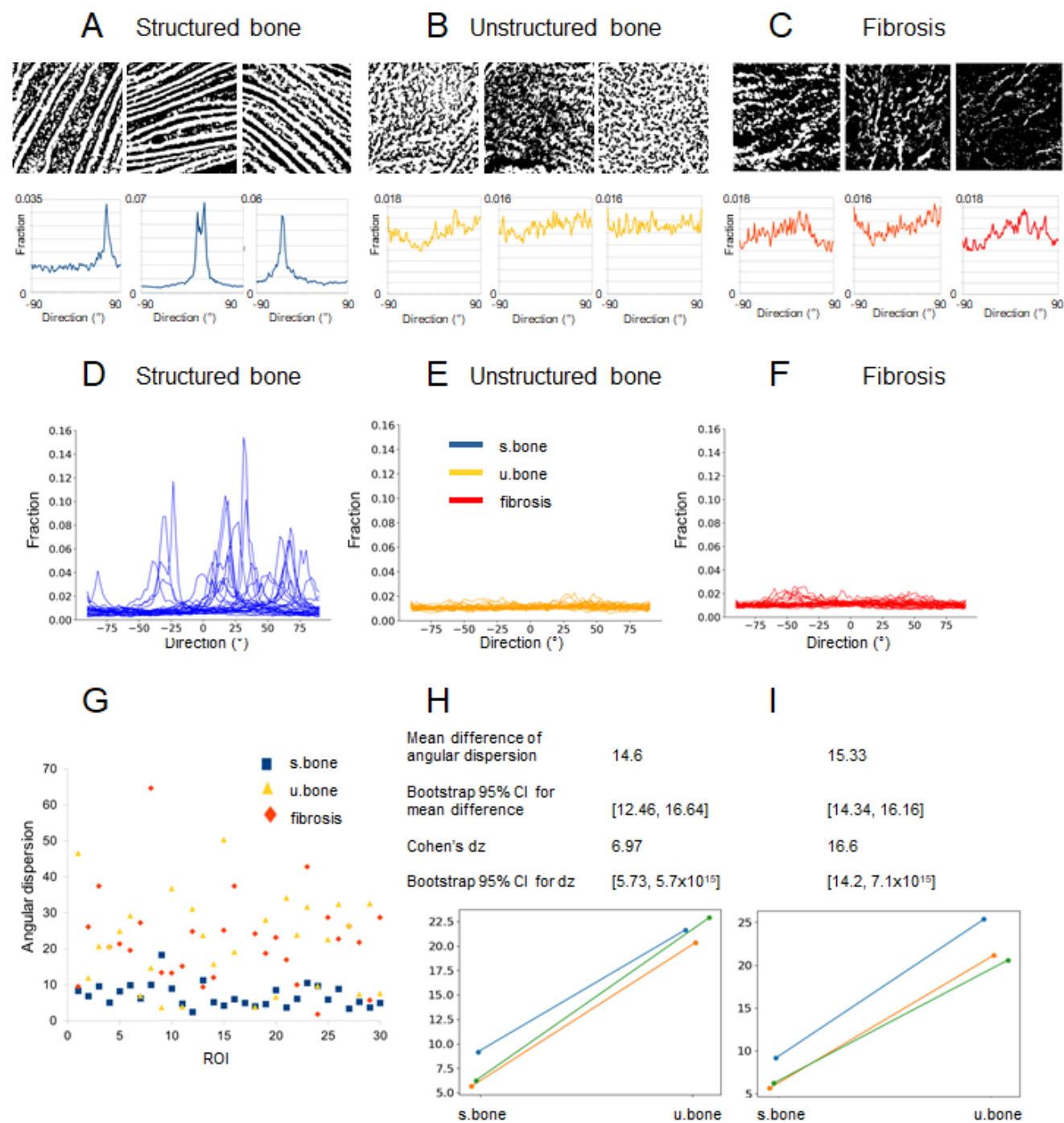


Figure 10

Angular dispersion of collagen orientation reveals quantitative differences between structured trabecular bone, unstructured trabecular bone, and fibrosis in SHG images. **(a-c)** Representative images from a single patient are shown for structured trabecular bone **(a)**, unstructured trabecular bone **(b)**, and fibrosis **(c)**. Corresponding orientation histograms are shown below each image. Note the different y-axis scales across panels. Orientation distributions were obtained using the Fiji Directionality plugin (Fourier components method). **(d-f)** Orientation histograms for individual ROIs of structured trabecular bone **(d)**, unstructured trabecular bone **(e)**, and fibrosis **(f)**. Data are shown for 30 ROIs per tissue type (10 ROIs per patient per tissue type across 3 patients). **(g)** Single-ROI angular dispersion scatter plot for the same dataset as in **(d-f)**. **(h,i)** Structured trabecular bone exhibits systematically lower angular dispersion compared to unstructured trabecular bone **(h)** and fibrosis **(i)**.