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Engineering Approaches to Study Fibrosis in 3-D In Vitro Systems

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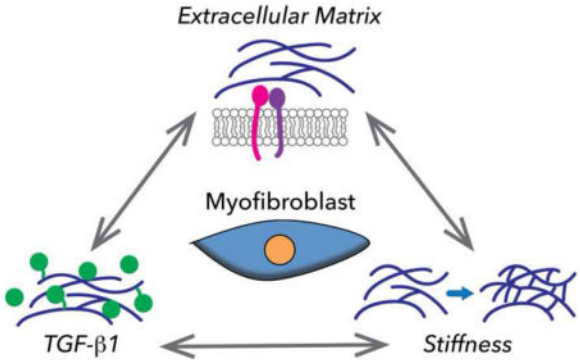
Abstract

Fibrotic diseases occur in virtually every tissue of the body and are a major cause of mortality, yet they remain largely untreatable and poorly understood on a mechanistic level. The development of anti-fibrotic agents has been hampered, in part, by the insufficient fibrosis biomimicry provided by traditional *in vitro* platforms. This review focuses on recent advancements toward creating 3-D platforms that mimic key features of fibrosis, as well as the application of novel imaging and sensor techniques to analyze dynamic extracellular matrix remodeling. Several opportunities are highlighted to apply new tools from the fields of biomaterials, imaging, and systems biology to yield pathophysiologically-relevant *in vitro* platforms that improve our understanding of fibrosis and may enable identification of potential treatment targets.

Graphical abstract



Fibrosis: When a scar goes too far

Fibrotic disease can occur in virtually any tissue in the body, spanning both systemic diseases (*e.g.*, systemic sclerosis, multifocal fibrosclerosis) and organ-specific disorders (*e.g.*, pulmonary fibrosis, liver cirrhosis, cardiac fibrosis) [1]. Combined, fibrotic diseases

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contribute to an estimated 45% of deaths in the industrialized world [2]. The common feature that ties together these diseases is the deposition of disordered and excessive extracellular matrix (ECM), particularly collagen types I and III [3]. The primary cellular mediators of this process are myofibroblasts, also known as 'activated fibroblasts', which are commonly identified by their expression of alpha-smooth muscle actin (αSMA) and contractile behavior [4]. The ECM accumulation and crosslinking promoted by myofibroblasts leads to tissue stiffening, which then acts in a positive feedback loop to sustain pathological levels of myofibroblast activity [5]. Although ECM upregulation and scar formation can be normal components of tissue repair, these events are dysregulated in fibrosis, where the heavily crosslinked, collagen-rich matrix progressively supplants the original functional tissue, eventually leading to organ dysfunction and failure.

Despite the prevalence of fibrotic diseases and the wide spectrum of tissues that they affect, there are few, if any, treatment options available [6••]. The poor biomimcry of traditional cell culture platforms is one reason for the slow progress toward identifying anti-fibrotic treatments [7]. However, recent years have seen the development of numerous approaches to create 3-D environments that possess improved pathophysiological relevance and enable precise and dynamic control over microenvironmental variables. The importance of specifically using 3-D conditions was illustrated by recent work demonstrating large differences in myofibroblast gene expression across 2-D vs. 3-D platforms possessing identical stiffness and composition [8•]. Of particular interest in designing these scaffold systems are three features that comprise the etiological backbone of fibrosis: 1) ECM composition, 2) TGF-β1 presence, and 3) mechanical stiffness (Figure 1). The use of biomaterials to study fibrosis-related behaviors has been broadly reviewed elsewhere [9•,10], while herein we focus on recent biological discoveries related to the three aforementioned features in fibrosis, advancements in the design of engineered 3-D platforms for studying these phenomena, and how this work may be combined with other emerging engineering-based approaches to further our understanding and treatment of fibrosis.

Engineering the Microenvironment

ECM Cues

Myofibroblasts interact with the ECM via integrins and other non-integrin receptors, and these specific receptor-ligand interactions are responsible for transmitting information to the cell about the composition, structure, and mechanics of their extracellular environment, all of which are altered in fibrosis. Specific integrin-ligand binding events have been found to govern fibrogenic behaviors; for instance, binding via the $\alpha_2\beta_1$ integrin exerts a protective effect in cardiac and valve fibrosis, while other β1-containing integrins (*e.g.*, $\alpha_1\beta_1$, $\alpha_5\beta_1$) increase fibrotic events in these same tissues [5]. Thus, the type of ECM ligand presented is a critically important consideration in the construction of engineered constructs, as it may influence the disease state of the system.

As reviewed elsewhere [9•–12], various native materials have been employed as 3-D constructs in the study of fibrosis, with the use of collagen-based hydrogels being the most common [7]. Other innovations in naturally-derived 3-D fibrosis culture platforms have included the development of scaffold-free 3-D strategies, such as layer-by-layer seeding [13]

or the stacking of intact cell sheets [14]. However, while these systems offer environments rich in complex biological cues, they are severely limited in their ability to be mechanically tailored or to withstand extended culture times, which is particularly problematic in the context of fibrosis, a gradual disease that is highly dependent upon matrix mechanics. These systems also do not permit controlled variation of ECM identity.

One approach to merge the need for ECM ligands, physical tailorability, and long-term culture has been the chemical modification of natural materials, such as methacrylation of gelatin (Gel-MA) or hyaluronic acid (HA-MA) [15,16]. Culture of encapsulated valvular interstitial cells (VICs, a myofibroblast precursor) was supported for several weeks in Gel-MA and HA-MA scaffolds. Moreover, the differentiation of VICs into myofibroblasts was dependent upon ECM identity, with combinations of HA-MA and Gel-MA yielding a more quiescent VIC phenotype than either component individually [15,16]. More controlled investigations of the role of ECM ligand identity in fibrosis can be achieved by modification of “blank slate” materials, such as polyethylene glycol (PEG), with adhesive peptides. While such hydrogels have been used extensively in the fabrication of engineered tissues [17], they have only recently been applied to explicitly examine fibrosis. The strong ligand-dependence of myofibroblast differentiation was illustrated by a recent study of VICs cultured within PEG-peptide hydrogels [18]; intriguingly, the $\alpha_2\beta_1$-binding peptide used in this work was associated with low αSMA expression, which is consistent with *in vivo* observations, but contrary to previous 2-D work using similar materials [19]. Together, these studies highlight not only the powerful influence that ECM identity exerts on fibrotic disease phenomena, but also the importance of controlling these cues in the *in vitro* context.

Delivery of TGF-β1

Transforming growth factor-beta1 (TGF-β1) is a molecular cornerstone in the pathogenesis of fibrosis. In simplified terms, TGF-β1 promotes fibrogenesis by increasing ECM production and decreasing its degradation [20]. Thus, delivery of this molecule to *in vitro* fibrosis platforms is a critical element in studying fibrotic mechanisms.

The delivery of TGF-β1 *in vitro* is generally performed by simple addition of soluble TGF-β1 to the culture platform. However, *in vivo*, TGF-β1 is secreted as part of a latent complex that binds to the ECM, making its presentation in a tethered form highly relevant to fibrosis. Several approaches have been described to covalently tether growth factors to biomaterial scaffolds [21], and significant increases in the production of collagen and overall ECM have been reported when smooth muscle cells or chondrocytes were cultured in scaffolds containing tethered TGF-β1 [22,23]. Recent advancements have yielded additional ways to better mimic the *in vivo* sequestration and release of TGF-β1 from the ECM. For example, latent TGF-β1 complexes were tethered to polymer scaffolds for release by encapsulated cells, resulting in increased protein and ECM synthesis [24]. Photodegradable sequences have also been used to attach TGF-β1 to polymer scaffolds, allowing temporally controlled release of tethered TGF-β1 [25,26]. TGF-β1-binding peptide sequences may also be used to achieve reversible, non-covalent sequestration [27]. To date, these systems have not been used in the study of fibrosis. However, given the importance of TGF-β1 sequestration in modulating interactions with both integrins and mechanosensing mechanisms, systems that

better mimic its *in vivo* bioavailability may greatly advance the physiological relevance of *in vitro* culture platforms.

Tailoring Matrix Mechanics

The poor success rate of anti-fibrotic drugs has also been attributed to their failure to address the mechanical events that drive fibrosis progression [5]. Fibroblasts are highly sensitive to tissue stiffness [28•–30], and there is a close connection between matrix mechanics and TGF-β1 activation, where matrix stiffening increases the release of TGF-β1 from the ECM [20], a finding with profound implications for fibrosis.

Following Engler's seminal publication describing control of cell fate by substrate stiffness [31], there has been a rapid acceleration in the use of mechanically tunable substrates to study fibrotic behaviors. A recent development in this area is the synthesis of biomaterial systems that enable dynamic, *in situ* scaffold stiffening and/or softening [28•,29,32–34]. Importantly, these systems allow cell morphology, viability, and phenotype to be consistent across all conditions at the experiment start, yielding the ability to more accurately correlate changes in cell behavior with induced changes in matrix mechanics. However, most investigations of matrix mechanics in fibrosis have been limited to 2-D, presenting a significant limitation for the study of this disease, where 3-D ECM structure and cell contractility play critical roles. A particularly interesting development is that recent 3-D studies have shown increased αSMA expression within softer materials [16,35•], a finding which is contrary to numerous 2-D studies [28–30].

Merging Cues from the ECM, TGF-β1, and Matrix Mechanics

The cues provided by ECM identity, TGF-β1, and matrix mechanics are closely intertwined (Figure 1); the latent TGF-β1 complex can directly bind to certain integrins, while integrins can sense matrix stiffness, and matrix stiffening can induce the release of TGF-β1 from the ECM [36]. Additionally, all three of these variables can directly influence myofibroblast differentiation. Combined variation of adhesive peptide sequence and stiffness has suggested that ligand identity is more influential than stiffness in regulating valvular myofibroblast differentiation [19], while another study has reported that sensitivity of pulmonary fibroblasts to exogenous TGF-β1 is increased with increasing stiffness [37]; these combinatorial approaches are needed to decipher the 'decision-making' process of cells during fibrosis. Although no system has yet combined the delivery of specific ECM cues with tethered TGF-β1 and modulation of scaffold stiffness, this is theoretically feasible with the merger of existing technologies. This approach could yield valuable information on the hierarchy and relationship of fibrotic behaviors, thus informing the development of anti-fibrotic treatments.

New Directions for the Evaluation of *in vitro* Fibrosis Platforms

In vitro fibrogenesis is typically characterized via evaluation of cell phenotype and ECM synthesis. Myofibroblasts may be identified by their expression of αSMA, as well as their contractility and activation of related signaling pathways [4] via standard biological techniques. Meanwhile, ECM synthesis is primarily monitored via the quantification of

collagen I, and, less frequently, collagen III. However, these analysis approaches do not yield information about ECM organization, a critical feature in fibrosis. The recent development of molecular imaging tools also offers an opportunity to gain insight into fibrogenic processes through the non-invasive visualization of ECM dynamics in living cultures.

Second Harmonic Generation Microscopy

In second harmonic generation (SHG) microscopy, the supramolecular assembly of collagen fibers in 3-D is imaged in the absence of exogenous molecular markers or dyes [38]. SHG yields a rich data set that quantitatively describes collagen organization; a greatly truncated set of such measurements can be seen in Figure 2. The acquisition of these data may also be automated to diagnose fibrotic conditions. For instance, multiple studies have combined SHG imaging of collagen alterations with machine learning algorithms to not only characterize the extent of fibrosis in clinical samples, but also provide automated, high-throughput diagnoses of fibrotic disease [38–40].

Other imaging advancements that may benefit the analysis of *in vitro* fibrosis platforms include a novel pairing of SHG with fluorescence lifetime imaging microscopy (FLIM), which was able to distinguish between collagen type I and type III, the two main ECM markers of fibrosis [41••]. Meanwhile, the combination of SHG with spectral lifetime imaging microscopy (SLIM) provides the ability to image real-time changes in cellular metabolites in the context of ECM alterations [42,43]. The application of these techniques to study fibrotic microenvironments has the potential to yield similar insight into the interactions between the ECM, inflammatory signals, and cellular behavior.

Molecular Probes

Multiple types of molecular probes have been developed to perform non-invasive, real-time imaging of both ECM dynamics and mechanotransduction events. For instance, collagen-binding fluorescent probes [44,45•] can enable high-resolution imaging of collagen assembly and remodeling in living tissues, producing similar quality of information as SHG [46], but without the need for specialized imaging instrumentation. Molecular sensors have also been designed to non-invasively image the real-time activity of enzymes involved in fibrotic ECM remodeling, such as matrix metalloproteinases (MMPs) [47], as well as lysyl oxidase (LOX) [48], which crosslinks fibers of collagen type I and type III, as well as elastin [49]. Also relevant to fibrosis is the recent development of molecular tension sensors [50•] which enable dynamic visualization of cell-generated mechanical forces. In combination with other imaging modalities, probes that provide such real-time measurement of ECM dynamics in living tissues may be used to track the same specimen over different stages of fibrosis and ultimately enable a more holistic characterization and understanding of fibrogenesis. Molecules that participate in ECM crosslinking and organization may also serve as targets for anti-fibrotic treatments [51].

Conclusions and Future Directions

Fibrotic diseases affect a wide range of tissues and are a significant cause of mortality, but they remain poorly understood on a mechanistic level, with few treatment options available. The development of *in vitro* platforms to recapitulate fibrotic events opens the door to not only improving our understanding of these diseases but also identifying and testing novel therapeutic approaches. Recent years have seen the generation of complex 3-D culture systems that may be used to independently vary microenvironmental properties and elucidate disease etiologies, thereby advancing our understanding of how myofibroblasts interact with their microenvironment. However, there remain numerous existing scaffold design and evaluation strategies that have yet to be applied to the study of fibrosis. As noted above, reversible tethering of latent TGF-β1 to scaffolds may permit a physiologically relevant presentation of this important pro-fibrotic molecule. Additionally, with few exceptions [52], current 3-D *in vitro* fibrosis platforms have not incorporated co-culture, despite numerous 2-D studies and advancements toward ‘organ-on-a-chip’ systems emphasizing the importance of other cell types in the progression of this disease [53,54].

With respect to fibrosis evaluation, new imaging techniques have been developed to improve the clinical diagnosis of fibrosis [38–40,55], but most of these efforts have yet to crossover to the execution of *in vitro* fibrosis studies. The application of techniques that enable non-invasive, real-time imaging of collagen assembly and remodeling in engineered fibrosis platforms has the potential to greatly advance the quality of information gained from these studies and their relevance to *in vivo* conditions. Furthermore, there are continued efforts to identify improved cellular markers for fibrosis beyond the traditional, yet non-specific, αSMA. Following the hypothesis that αSMA-positive myofibroblasts arise from the differentiation of local epithelial progenitors via epithelial-to-mesenchymal transition (EMT), transcription factors related to EMT have been quantified to monitor fibrosis [56]; however, some studies have cast doubt on this EMT-myofibroblast relationship [57]. Specific microRNAs (*e.g.*, miR-29) are also emerging as key biological regulators in fibrosis [58••], with strong potential as therapeutic targets.

Finally, despite the interconnected nature of fibrotic events and need to improve the identification and efficacy of potential treatments, computational modeling tools have rarely been applied to study fibrosis. An agent-based model was recently generated to describe cell-cell interactions in liver fibrosis and predict response to treatment [59], but most other applications of computational methods to study fibrosis have involved analysis of large datasets for gene expression or signaling, rather than prediction of cellular- and tissue-level behaviors. In addition to helping decipher the cellular decision-making processes that regulate fibrogenesis, systems biology-based approaches have the capacity to generate models that identify potential targets [60] or assess and predict drug sensitivity and resistance [61], which could yield significant advancements in the treatment of this complex disease.

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Highlights

- Development of anti-fibrotic treatments is hampered by culture system limitations
- Fibrosis involves ECM disarray, TGF-β1 signaling, and matrix stiffening
- Tunable 3-D engineered structures can recapitulate the aforementioned features
- Novel imaging and sensor tools can allow visualization of matrix dynamics *in vitro*

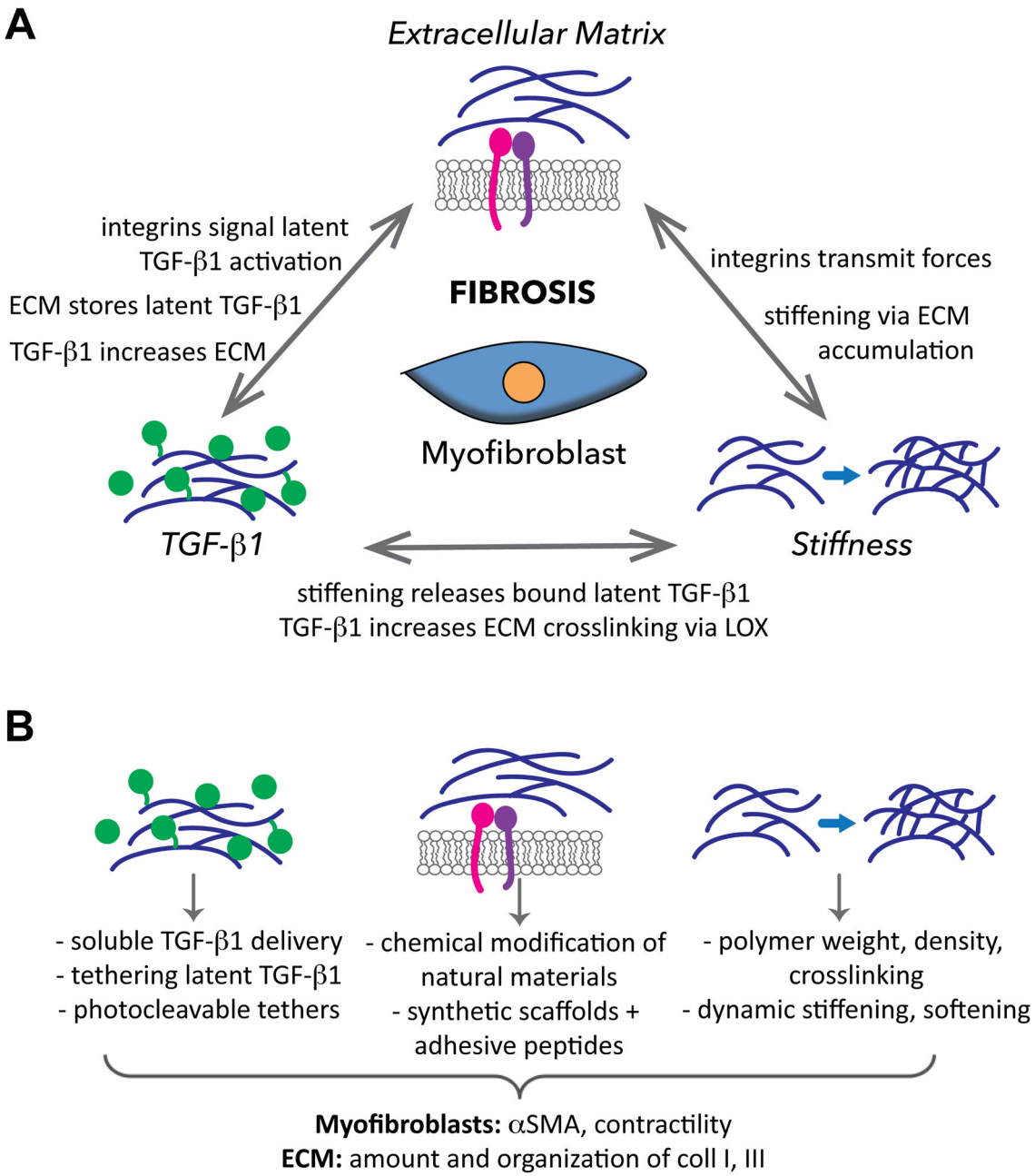
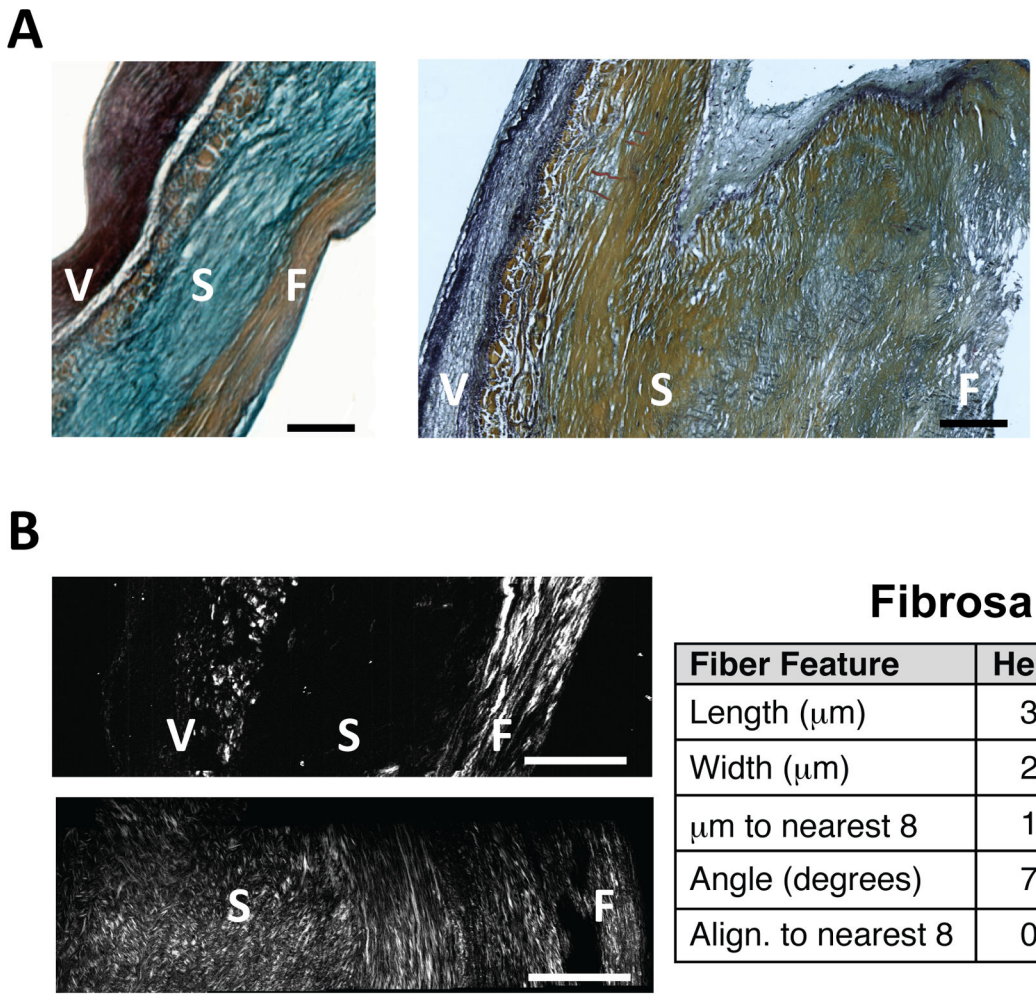


Figure 1.

A) Fibrosis is strongly influenced by extracellular matrix cues, presence of soluble and ECM-bound TGF-β1, and matrix stiffness. All three of these features are regulated by myofibroblastic activity, in addition to being able to modulate one another. **B)** Examples of 3-D *in vitro* approaches used to mimic the three aforementioned features, and outcomes that signify fibrosis.



Fibrosa (F)

Fiber Feature	Healthy	Diseased
Length (μm)	32.8	30.6*
Width (μm)	2.25	2.24
μm to nearest 8	18.0	17.6*
Angle (degrees)	78.9	71.3*
Align. to nearest 8	0.79	0.72*

Figure 2.

A) Histological sections of a healthy (left) and diseased/fibrotic (right) human aortic heart valve stained with Movat's pentachrome. Healthy leaflets possess a trilayered ECM structure, consisting of the fibrosa (F), spongiosa (S), and ventricularis (V). Yellow staining indicates collagen. **B)** Second harmonic generation (SHG) images of healthy (left) and fibrotic (right) leaflets and brief example of fiber characteristics that can be quantified by automated analysis of SHG images, illustrating the advanced ECM characterization that can be performed with alternative imaging methods. *$p<0.0001$; Scale bar = 50 μm.