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Biofabrication of phenotypic pulmonary fibrosis assays

Cameron Yamanishi^{1,2}, Stephen Robinson^{1,2}, Shuichi Takayama^{1,2,3}

¹Wallace H Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory School of Medicine, Atlanta, United States of America

²The Parker H Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, United States of America

Abstract

Biofabrication techniques have enabled the formation of complex models of many biological tissues. We present a framework to contextualize biofabrication techniques within a disease modeling application. Fibrosis is a progressive disease interfering with tissue structure and function, which stems from an aberrant wound healing response. Epithelial injury and clot formation lead to fibroblast invasion and activation, followed by contraction and remodeling of the extracellular matrix. These stages have healthy wound healing variants in addition to the pathogenic analogs that are seen in fibrosis. This review evaluates biofabrication of a variety of phenotypic cell-based fibrosis assays. By recapitulating different contributors to fibrosis, these assays are able to evaluate biochemical pathways and therapeutic candidates for specific stages of fibrosis pathogenesis. Biofabrication of these culture models may enable phenotypic screening for improved understanding of fibrosis biology as well as improved screening of anti-fibrotic therapeutics.

Keywords

idiopathic pulmonary fibrosis; biofabrication; collagen gel contraction; phenotypic assays; lung; tissue engineering; biomaterials

1. Introduction

The field of biofabrication, the generation of complex structures from cellular and tissue building blocks, has seen widespread growth. These techniques may be useful in a wide variety of applications, including tissue engineering and regenerative medicine [1, 2], developmental biology, disease modeling, drug/toxicity screening models, and others. Much attention has been paid to the manufacturing aspect of biofabrication [3], but we focus here on a framework to consider and evaluate the application of biofabrication to disease modeling and drug screening. The design parameters for a biofabricated structure should consider the disease pathology, cellular composition, and structure. With cellular systems, unexpected structures and functions may arise in interesting ways [4]. As these systems are explored and better understood, application to disease pathology is critical for translation to

³Author to whom any correspondence should be addressed. takayama@gatech.edu.

effective drug screening. For this review, we will focus on biofabrication techniques in the context of generating phenotypic assays of idiopathic pulmonary fibrosis (IPF).

IPF is a devastating lung disease, in which normal lung parenchyma is progressively replaced with fibrotic scar tissue. An estimated 45% of deaths in the US are attributed to disorders that are characterized by varying degrees of fibrosis, with a rising incidence [5]. Although the pathology of IPF is varied and still controversial, it generally involves dysregulation of the normal wound healing process. In a prevailing disease theory [6–9], an initial insult to the alveolar epithelial layer leads to recruitment of macrophages and other immune cells in a pro-inflammatory response (figure 1). The epithelial injury leads to clotting and fibrin formation, accompanied by fibroblast invasion to plug the wound. The fibroblasts then proliferate and activate. Activated fibroblasts, termed myofibroblasts due to the expression of alpha-smooth muscle actin (α -SMA), contract the tissue and deposit extracellular matrix (ECM) to close the wound. In a healthy response, the myofibroblasts would subsequently apoptose to be cleared by macrophages, facilitating re-epithelialization and a return to the original healthy state. In pulmonary fibrosis, however, the myofibroblasts become senescent [10] and continue to generate stiff tissue. Lung stiffening is accompanied by irreversible alveolar collapse, ultimately preventing breathing in patients [11].

Treatment options for pulmonary fibrosis are sparse, highlighting the need for effective cellular and tissue models of the disease [12]. The standard animal model of pulmonary fibrosis uses bleomycin to generate scar tissue in rodents. This model yields lungs similar in histological appearance to fibrosis, but subtle differences from the human disease have led to misuse of the model [13]. In parallel with animal studies, there has been considerable progress in development of complex *in vitro* models of pulmonary fibrosis [14]. A subset of these models provides readouts of cell and tissue level phenotypes associated with pulmonary fibrosis. Phenotypic assays are complementary to mechanistic assays, because they can account for complex, multifactorial cell behaviors producing sometimes unexpected and emergent behavior [15–18]. Phenotypic assays have proven generally useful in the discovery of first-in-class therapeutics [19], although not yet for fibrosis. Biofabricated phenotypic assays have aided in understanding of disease pathology, particularly the role of mechanotransduction and matrix stiffness in a feedback loop of fibroblast activation [20–22]. Some of these discoveries have led to the investigation of new therapeutic targets [23, 24]. In this review, we will examine and contrast a variety of biofabrication techniques to assess pulmonary fibrosis phenotypes in the *in vitro* setting. This analysis aims to summarize progress and highlight challenges and opportunities in this field. We also note that while this review focuses on pulmonary fibrosis, many of the concepts are applicable to biofabrication of a broader range of diseases that involve fibrosis and we cite relevant examples from some of these other areas.

2. Techniques for *in vitro* phenotypic analysis of pulmonary fibrosis

This review is organized by describing *in vitro* cell-based assays that aim to recreate various aspects of pulmonary fibrosis pathogenesis roughly in their order of progression *in vivo*: epithelial damage, clotting, fibroblast invasion, fibroblast activation, myofibroblast contraction, and myofibroblast persistence. These techniques are summarized in figure 1,

where they are sorted roughly by the phenotypes they cover and when those phenotypes occur in a simplified model of IPF pathogenesis. Table 1 of this review further summarizes the techniques.

2.1. Phenotypic assays incorporating epithelial damage

In the context of pulmonary fibrosis, epithelial damage could arise from a variety of sources including aging, smoking, infection, inhaled toxins or nanoparticles, and a variety of other insults [55]. A common observation among IPF patients is telomere shortening. The telomere shortening hypothesis of disease pathogenesis is consistent with the strong correlation between prevalence and patient age. Assays of epithelial damage in the context of IPF should therefore include some consideration of aging, repetitive damage, or similar types of stress. Downstream biochemical signaling has been reviewed elsewhere [56–58], and should also be considered. A plethora of techniques for culturing and assaying epithelial cells exist, but this review will cover those assays that pertain most specifically to epithelial-fibroblast interactions in pulmonary fibrosis.

Several techniques have been developed to physically induce epithelial damage, the presumed first step towards development of pulmonary fibrosis. Some of these techniques use custom-built devices to impose cyclical strain on epithelial cells, similar to that seen in healthy breathing. In one iteration, alveolar epithelial cells were seeded onto a flexible silicone membrane, anchored with a washer [26]. This membrane was cyclically stretched by pressing on the underside of the membrane with a motorized cam mechanism. The authors demonstrated that epithelial cells were damaged by strains of ~25%. Furthermore, additional culture time prior to cyclic strain had a protective effect on the epithelial cells. Another version of the technique comprised a stretching microdevice with flexible polydimethylsiloxane (PDMS) membrane and syringe pump hydraulic actuation [27]. This study found that air-liquid interface (ALI) culture exposed cells to high damage from cyclic stretching at ~35% strain. They also examined fluid mechanical stress from liquid plug movement, finding that the damage could be reduced by addition of pulmonary surfactant. These studies examined physiological strain, but they could be adapted to replicate the fibrotic lung, which is less compliant than healthy lungs. Although hyperventilation has not been causally linked to pulmonary fibrosis, mechanical ventilation has been shown to correlate with bleak outcomes for patients with pulmonary fibrosis whose hypoxemia prompts the use of mechanical ventilation [59, 60]. These cyclic stretch models provide methods to assess the epithelial contribution to disease exacerbation.

In static culture settings, membrane culture techniques, such as Transwell culture, have gained tremendous popularity. These devices comprise a porous membrane, fixed to an insert that can be submerged into a culture well, enabling physically separated co-culture between the membrane cell population and the well plate cell population [28]. Transwells have also been used extensively to generate and test ALI culture. ALI culture prompts many airway epithelial cells to differentiate into more physiologic epithelia [61]. The membrane ALI format has been adapted to allow aerosol exposure to nanoparticles in well-plate formats [29] as well as microfluidic devices [62]. In addition to cell viability and biochemical assays, membrane cultures enable measurement of epithelial barrier integrity.

Barrier function can be measured by either permeability assays using fluorescently-labeled macromolecules, such as dextran [63], or using trans-epithelial electrical resistance [64]. Transwell and membrane techniques present versatile tools for biofabrication of assays incorporating ALI epithelial components.

For analysis of epithelial injury and wound healing, the scratch wound assay is well established [25]. Briefly, a confluent monolayer of epithelial cells is denuded using a pipet tip or other blunt object to tear off the epithelial cells. The remaining edges are tracked visually as the epithelial cells proliferate and migrate to repair the wound area. A recent paper combined the scratch wound assay with Transwell co-culture [31], wherein A549 epithelial cells were grown on the top of the membrane, while primary human lung fibroblasts (healthy versus IPF) were grown on the underside of the membrane, as depicted in figure 2(A). The authors reported differing behavior in healthy and IPF patient-derived pulmonary fibroblasts. After scratching the epithelial layer, the A549 migrated over 4 d to close the wound. In co-culture with healthy fibroblasts, the fibroblasts migrated into the wound site to plug the gap and inhibited re-epithelialization. However, pulmonary fibrosis-derived fibroblasts failed to invade the wound site, allowing the epithelium to close the wound. This study prompts interesting questions about the long-term behavior of pulmonary fibroblasts in healthy and diseased states, as the healthy fibroblasts appeared more active in the short period examined than the diseased fibroblasts, contrary to what maybe expected.

Despite the progress made with biofabricated assays of epithelial damage, there remains fertile ground for research regarding long-term culture, chronic epithelial damage, and downstream signaling relevant to IPF. A particularly challenging aspect of lung research has been the inability to culture alveolar epithelial cells. However, recent work has indicated that co-culture with pulmonary fibroblasts may help solve the problem [66]. New biofabrication methods could provide additional benefits by working directly with clinicians to establish cell cultures directly from the source.

2.2. Phenotypic assays incorporating fibroblast invasion

Following epithelial damage, a coagulation cascade results in the formation of the provisional ECM. Fibrinogen from blood plasma is cross-linked into fibrin, which is supported hydrostatically by glycosaminoglycans such as hyaluronic acid [67]. Fibroblasts from surrounding tissue then invade this provisional ECM in order to initiate the remodeling process. As illustrated in figure 1, formation of the provisional ECM and invasion of fibroblasts is an early component of the wound healing response, where it can be exaggerated in fibrotic phenotypes [7]. A number of techniques have been developed to recapitulate behavior of fibroblasts in the provisional ECM, both for evaluation of the physiology of fibroblast invasion and for screening of potential therapeutics.

One such approach utilized a fibroblast-containing collagen matrix, formed on top of a dehydrated/rehydrated fibrin gel in media. Fibroblasts were then able to invade the fibrin matrix. This was used to evaluate the influence of fibronectin during fibroblast invasion into a fibrin matrix, and it was found that patterns of fibronectin provide a conduit for fibroblast transmigration from collagenous stroma into the fibrin clot provisional matrix. Fibroblast migration began as early as 24 h into the assay, but was significantly slowed by the removal

of fibronectin [68]. This approach was used to further establish the necessity of CD44 and Syndecan-4 Proteoglycan in fibroblast invasiveness [34, 69].

Miron-Mendoza *et al* used a similar assay to evaluate the differences in fibroblast migration through collagen compared to migration through fibrin. Cell-laden collagen discs were coated with fibrin for an easily imaged cell invasion assay (figure 2(B)). This was used to show that supplemental fibronectin was necessary for the invasive fibroblast phenotype, and fibroblast-fibronectin network formation was dependent on $\alpha 5 \beta 1$ integrin. Fibronectin tracks (fluorescently labelled) were formed at the leading edge of migrating cells and fibroblasts preferentially migrated through pre-existing fibronectin tracks laid down by other cells [65].

Other phenotypic assays for fibroblast invasiveness have focused on migration from 2D adherent culture into fibrin matrices. One such approach used microdroplets of fibroblast suspension patterned on a 6-well plate and covered with a fibrin matrix [70]. Through this approach, migration from the 2D culture into the fibrin gel was used to evaluate invasiveness of fibroblasts isolated from patients with acute lung injuries. This was used to evaluate the role of CD44 in mediating fibroblast invasion and subsequent tissue fibrosis. By blocking the function of CD44 with monoclonal antibodies, fibroblast invasion into a fibrin matrix was inhibited [35].

An alternative, matrix-free approach focused on fibroblast invasion into cancer spheroids. This was used for evaluation of therapeutic candidates to combat fibrotic processes in cancer, but could be applied as a phenotypic assay for invasiveness in other forms of fibrosis. The approach was optimized for 384-well microplates for high throughput evaluation of drug libraries. Tumor spheroids were formed in agarose-coated wells, as depicted in figure 2(C). A suspension of fluorescently labeled fibroblasts was then added and invasiveness was evaluated via confocal spinning disc microscopy to locate the fluorescent lung fibroblasts that had invaded the spheroids. A 480-compound library of bioactive substances was evaluated for anti-invasiveness, where several prostaglandins and ROCK inhibitors were identified as potential therapeutics [37].

Lewis *et al* used a co-culture technique involving a photodegradable polyethylene glycol (PEG)-based hydrogel in order to evaluate the impact of crosstalk between fibroblasts and cancer cells on invasiveness of the fibroblasts. An Operetta high content imaging machine was used for high-throughput migration tracking in order to show the increased invasiveness and matrix metalloproteinase (MMP) expression of fibroblasts under the influence of cross-talk with cancer cells compared to fibroblasts alone [30].

Despite the involvement of the fibroblast invasion into the provisional ECM during fibrosis, there remains a question of whether it is necessary for the pathogenesis of fibrotic disease. In the case of pulmonary fibrosis, fibrin deposition does not itself appear to be required for pathogenesis because fibrinogen-null mice are still able to develop lung fibrosis after bleomycin-induced lung injury [71, 72]. Assays based on fibroblast invasion into the provisional ECM should therefore establish physiological importance before being used to evaluate compound libraries for potential therapeutic candidates.

2.3. Phenotypic assays incorporating fibroblast contraction

A histological hallmark of IPF is the presence of fibroblastic foci, dense clusters of cells and ECM in the lung. The characteristic cell type in these clusters is the myofibroblast. These cells are a differentiated and activated form of fibroblasts, which synthesize high amounts of collagen and contract the ECM. The myofibroblast is considered the primary effector cell in driving the progression of IPF [73]. There are extensive reviews covering the various roles and behaviors of myofibroblasts [73–77]. A plethora of biofabrication techniques have been developed to measure contraction, an identifying characteristic of the myofibroblast phenotype. The bulk of these techniques are based on collagen contraction, but there are exceptions. Tuan *et al* used a fibrin gel scaffold in their model as an analog to the provisional ECM in wound repair. Gel contraction and collagen production were evaluated in order to show matrix remodeling under transforming growth factor (TGF- β 1) stimulation [36]. The use of other hydrogel alternatives to collagen is already covered in detail by other reviews [14, 78, 79].

2.3.1. Collagen gel contraction assay—Collagen gel contraction is the gold standard of phenotypic readouts for evaluation of fibrosis, with widespread use. The first reported use of a collagen gel contraction assay established the standard contraction assay format in 1979 [41]. When fibroblast-laden collagen gels are cultured in media with serum, they exhibit a high baseline level of contraction, shrinking gels to as small as 1/7th the original area within 24 h [41, 80]. The collagen gel may adhere to the walls of the dish, so most protocols physically detach the gel from the wall using a sterile pipet tip after polymerization. The need for this step limits the throughput of collagen gel contraction assays. These assays are often called floating collagen gels [81]. The contraction has been divided into three phases: slow lag phase, rapid contraction phase, and sustained slow contraction phase [82]. During the lag phase, fibroblasts recover from trypsinization and elongate along collagen fibers. As previously reviewed [74], tensile force measurements of collagen gels indicated that the lag phase correlates with fibroblast adhesion and cell movement within the collagen gel. Therefore, the lag phase has also been called the traction phase [74]. The length of the lag phase can be shortened by using more cells, more serum, or cells at higher passage number [82]. Following the lag phase, the collagen gel is rapidly contracted and remodeled, at rates sped up by high cell number or serum concentration and slowed down by higher initial collagen concentration or cytoskeletal inhibitors. Lastly, the system enters a state of slow, prolonged contraction.

Contraction by fibroblasts is regulated to a large extent by TGF- β 1 [83], which can be added exogenously to induce contraction [84] or it can be produced or activated by fibroblasts themselves [85], among other sources [85–87]. The combination of TGF- β 1 and tensile stress induces fibroblasts to differentiate to myofibroblasts, identified by their expression of α -SMA, stress fibers, and high contractility [83]. It should be noted that tensile stress is also a necessary and critical component for myofibroblast differentiation [88]. The capacity for tensile stress within collagen gels can be increased by tethering gels to the dish [41] or altering gelation parameters to form stiffer gels. Gels formed at more acidic conditions down to pH 5.5 have demonstrated higher tensile strength than gels formed at more neutral or

alkaline conditions up to pH 8.0 [89]. Collagen stiffness can also be increased by using higher concentrations of collagen [90].

Following the initial development of the collagen gel contraction assay, many adaptations to the format have been added. The conventional collagen contraction assay is low throughput and high cost, due to the large consumption of collagen and cells. More modern techniques enable higher throughput with drastically lower collagen gel volumes. In addition to the cost benefit, low volume collagen gels also reduce the diffusion distance necessary for media and relevant exogenous biomolecules [39].

One approach to improve assay consistency is to confine the collagen gel within a glass capillary tube, yielding a single, linear measurement, as in figure 3(A) [43]. Suspended human bladder-derived smooth muscle cells in an uncured collagen solution were aspirated into glass capillary tubes and polymerized. The collagen gels were then detached from the capillary by gently applying air pressure to one end. Over 48 h of culture, cell viability was consistent between the capillary culture method and the traditional whole well culture method. Due to the diffusive barrier introduced by the glass capillary, the stimulants and inhibitors were added to the gel prior to polymerization. Standard deviations ran close to 1% between replicates. The authors also suggest that the capillary technique is amenable to future addition of electrical current. Although the capillary technique is limited to short-term culture applications due to diffusive limitations, it provides a consistent, easy-to-read assay that uses low volumes of collagen.

Although fibroblasts can contract collagen gels shortly after seeding, the gel can often attach to the walls and bottom surface of the well, introducing inconsistencies when measuring and analyzing the contraction. In addition to the pipet tip manual detachment method, several groups have developed techniques to consistently seed gels that cover only a fraction of the well area, often employing non-adherent surfaces to further reduce friction [42].

Sage and coworkers published a series of papers using the collagen gel contraction assay, with an array of techniques to reduce gel adhesion. They initially used agarose-coated microwells to reduce the adhesion of the collagen gels to wells [80]. In a subsequent study, however, their group designed a water-in-oil system to contain the collagen gel to a small Teflon-coated area in the center of the well, as shown in figure 3(B) [45]. In this technique, a Teflon glass cloth was pinned to the bottom of a 12-well tissue culture well with a Teflon-coated washer. The pieces were then submerged in mineral oil. Collagen with suspended adult human dermal fibroblasts was then pipetted into the center of the Teflon washer ring and incubated to polymerize the gel. Interestingly, the authors found that as the collagen gel contracted, it expelled media, leaving a gel in water in oil 3-phase system. The oil and Teflon modified collagen gel contraction assay performed better than the conventional assay on multiple fronts. The gels maintained a more consistent circularity compared to the conventional assay, wherein gels had a tendency to adhere to the well and form elongated shapes, stretched tightly between the anchor points. In addition to altering the gel shape, the anchor points in the conventional method also prevented full contraction, introducing noise to the measurements.

Furthermore, conventional assays exhibited cupping, a result of cells sedimenting to the bottom of the collagen gel as it cured, producing vertically asymmetric contraction. In the large gels, cupping can interfere with microscopy, as cells in the center of the gel are pulled to a higher plane than the cells at the bottom of the well. The pre-warmed oil facilitated more rapid polymerization, trapping cells in a more uniformly distributed state. In studies using platelet-derived growth factor (PDGF-BB) to stimulate contraction and measuring the reduction of area, Sage and coworkers achieved standard deviations of roughly 5% between replicates. While the oil and Teflon technique provides a more uniform and consistent contraction assay, it does lose some of the mechanical stress that has previously been shown to be a driving factor for fibroblast differentiation and activation [88]. The authors did not comment on this point. Additionally, the technique is rather low throughput and still uses large volume gels. Nonetheless, their study presents a compelling case for addressing assay consistency. The widespread availability of non-adherent microplates for spheroid culture provide an accessible alternative to the Teflon-coated method.

More recently, aqueous two-phase systems (ATPSs) have been used to generate collagen microgels for contraction assays [39] (figure 3(C)). Scaling down to single μl gels facilitates diffusion of stimulatory or inhibitory proteins and molecules into the center of the gel. However, these small gels evaporate during the 30 min collagen polymerization process, often killing the embedded cells. The authors used ATPS to provide an aqueous buffer against evaporation, while confining the collagen and cells into the gel during polymerization. ATPS are phase separating solutions reminiscent of oil-water two-phase systems, but both phases contain primarily water. The distinction between phases in this particular ATPS is the presence of immiscible polymers: PEG and dextran. Conveniently, many cells and proteins partition preferentially to one phase, allowing ATPS to be used to spatially confine cells and collagen. After the collagen gel formed, the PEG and dextran were washed out and replaced with culture medium, leaving a 5 μl cell-laden collagen gel. This technique is compatible with conventional liquid handling tools and imaging, making it appropriate for high-throughput studies. A follow-up study used the technique to produce 1 μl collagen gels and showed excellent robustness of the technique to assess cytotoxicity in collagen gels [40]. However, the consistency and reproducibility of the microgel technique to assess contraction have not yet been established.

Several microfluidic approaches have facilitated formation of sub-millimeter collagen gels, as reviewed recently [91, 92]. These techniques use photolithography to generate channels 10s–100s of μm in width for precise and controlled fluid mixing. Flow-focusing devices introduce a collagen-containing aqueous phase into a stream of oil to produce microdroplets of controlled size [93]. Several groups have utilized these device geometries to generate and cure cell-laden collagen microgels 10s–100s of μm in diameter [51, 53, 94]. However, these studies have not yet examined the use of microfluidic collagen microgels in contraction assays.

2.3.2. Mechanics of collagen gel contraction—In parallel with the development of techniques for assay consistency and throughput, several groups have designed methods to measure more detailed mechanical information from collagen gel contraction assays. Liu *et al* demonstrated the importance of substrate stiffness in pulmonary fibrosis by generating a

stiffness-gradient collagen gel using photopolymerization of polyacrylamide [20]. They showed that fibroblasts are more active and responsive to TGF- β 1 on stiff substrates and less active on soft substrates. The lack of fibroblast activation was also found by inhibiting focal adhesion kinase formation with prostaglandin E2, a metabolic product produced by epithelial cells that has also been shown to suppress fibroblast activation in co-culture [95]. This technique was demonstrated as both a stiffness gradient gel and a 96-well discrete stiffness array, highlighting its versatility in probing the contractile phenotype of fibroblasts. It also provided evidence for the critical insight that fibroblasts enter a positive feedback loop, activating in response to a stiff microenvironment and contracting to exacerbate the stiffness.

A separate study used agarose to modulate collagen gel stiffness [54]. More agarose yields macroscopically stiffer gels, which can activate fibroblasts [20]. However, the agarose also reduces the effective local stiffness experienced by the cells, inhibiting the ability of the cells to cross-link and locally stiffen the matrix [54]. This study indicated that collagen concentration can increase contractility by increasing ligand availability, possibly also by reducing the ability of the collagen fibrils to slide past the agarose. The competing factors of agarose-to-collagen ratio and the collagen concentration allow fine tuning of both collagen stiffness and ligand availability.

Although the strength of fibroblast contraction can be inferred in relative terms by monitoring the shrinkage of the collagen gel, more granular mechanical information requires more advanced techniques. A variety of mechanical sensors and accompanying theoretical frameworks have been built into or applied externally to the collagen gels [96, 97]. Harley *et al* made a collagen-glycosaminoglycan scaffold by freeze-drying [98]. The process generated somewhat uniform structures with repeating lattice units, allowing for single-cell microscopic measurements to be incorporated into a whole-gel solid mechanics model. The authors generated both homogeneous, isotropic structures and heterogeneous structures for comparison. They used an Euler model of buckling stress to determine how much force a cell must exert on the lattice to buckle a strut, given the strut's thickness and the overall elastic modulus of the material. Depending on the thickness of a given strut, the dermal fibroblasts exerted contractile stress of 11–52 nN per cell. The authors examined many individual cells buckling struts of various thicknesses and found an upper bound of 450 nN, which the cell was unable to contract. The mode of contractile failure was proposed to be at the focal adhesion. While this model provides granular information about the mechanical stresses exerted by cells, the collagen glycosaminoglycan structure represents a far stiffer matrix (~5 MPa linear elastic modulus) compared to the healthy or fibrosis lung (1–20 kPa) [20]. The analysis provided in this study could be adapted in future studies examining biofabricated materials with stiffness more relevant to pulmonary fibrosis, as well as biochemical stimuli relevant to IPF.

Another treatment of collagen gel contraction mechanics derived a solid mechanics model around a cylindrical collagen disc model [49]. The model assumes an incompressible solid with a non-linear strain-dependent elastic modulus. It uses linear elastic mechanics to relate the area reduction and the elastic modulus (measured with a nano-indentation probe) to the bulk cell contraction force. These calculations yielded a contractile force of 1.41 nN

(baseline) to 4.15 nN (activated) per human aortic adventitial fibroblast cell. The differences in substrate stiffness and cell type confound any comparisons between this method and the collagen-glycosaminoglycan scaffold calculations. One drawback to this model is the incompressible solid assumption. Several studies cited in this review indicate the collagen gels lose water as they compress and decrease volume [43, 45], invalidating the incompressible assumption. Nonetheless, this model provides a general method to approximate contractile forces from area reduction measurements for cylindrical collagen gels.

2.3.3. Strain gauge techniques—Several techniques have been developed to measure contractile forces using macroscale [50] and microscale strain gauges [96]. In most of these systems, cell-laden collagen gels are formed to span between two anchors. One or both of these anchors acts as a strain gauge. Early methods used large electronic strain gauges, converting the strain (or displacement) into a change in resistance using piezoelectric materials [50]. If the geometry and elastic modulus of the anchoring material is known, the force exerted by the cells can be calculated. Modern methods apply the same principle, but use microscale anchors and detect strain by timelapse imaging. Because the force exerted by cells is relatively small, strains on the materials commonly used for cell culture (plastics, PDMS, etc) can be difficult to detect. A common workaround forms the scaffold into tall micropillars, where the long torque arm and cantilever action amplifies the distance that the micropillar moves in response to the nN-scale cell contractile forces [96].

Another technique to measure the contractile force of fibroblasts used supersoft PDMS to generate microposts inside donut-shaped wells, as shown in figure 4(A) [44]. Although hydrogels and other supersoft materials can be produced, these materials often suffer from tearing during demolding in traditional replica molding techniques. The supersoft PDMS technique addressed the problem by using ‘hard candy’ recipes to generate sacrificial replica molds [44]. This technique enabled fabrication of PDMS microwells with 200 μm diameter microposts and an elastic modulus on the order of 0.1 kPa. Fibroblastladen collagen gels were centrifuged into the microwells, where they formed a ring around the post. When the fibroblasts contracted, they squeezed the post. Solid mechanics analysis enabled the calculation of contractile force on the post by measuring the reduced post area. The authors reported the force exerted as a bulk radial stress and did not calculate a per-cell force. The authors also demonstrated the ability to detect an initial contraction, apply an inhibitor, then see the cells and the post relax back toward its original shape, demonstrating reversible elastic compression.

Chen *et al* modified the tethered collagen gel format to work at the microscale, as shown in figure 4(B) [33]. They tested epithelial cells by treating the cells with multi-walled carbon nanotubes (MWCNTs) in the culture flask prior to seeding in the microdevice. Cells were then suspended and seeded into the microdevices along with type I collagen by centrifugation. The microtissues formed and detached from the outer walls within 12–24 h, with cultures lasting for 1 week without detaching from the micropillars, due to their goblet shape. The authors found that low concentrations of MWCNTs increased contraction force, while high concentrations inhibited contraction force. The low dose conditions corresponded to a state of inflammation and activation, indicated by contraction, presence of reactive

oxygen species (ROS), and miR-21 expression. The high dose caused considerable cell death, offsetting the activation, as measured by the contraction force. This study demonstrates the need for multifactorial analysis of phenotypic screens, as counteracting influences may confound the interpretation of results. In this case, measurement of both cell activation (contraction force and ROS production) and cell viability elucidated the behavior along a dose-response curve that indicated peak bulk pathologic behavior at a medium concentration.

The same group used a similar approach to evaluate anti-fibrotic therapeutics using their *in-vitro* assay [32]. Fibroblasts were suspended by micropillars in a super thin (35–55 μm) collagen layer arranged in a leaflet shape in order to mimic the geometry of alveoli. Contractile forces in the collagen were measured by deflection of the micropillars that suspended the collagen and differentiation of fibroblasts into myofibroblasts was confirmed by staining for α -SMA. This assay was then used to evaluate the therapeutics Nintedanib and Pirfenidone through measurement of both changes in tissue compliance and differentiation of fibroblasts into myofibroblasts. They confirmed anti-fibrotic activity with both drugs. This paper also used an alternative implementation of the micropillar supported collagen membrane to evaluate traction bronchiectasis, a condition involving irreversible dilation of bronchi and bronchioles as a result of pulmonary fibrosis. By modulating the rigidity of the micropillars, it was shown that increased stiffness in lung tissue due to fibrosis would compound lung damage as seen in traction bronchiectasis.

2.3.4. Co-culture collagen gel contraction assays—The interplay between epithelial cells and fibroblasts may be a key component of pulmonary fibrosis pathology [8]. In an early study, A549 or bronchial epithelial cells were seeded directly on top of fibroblast-laden collagen gels [46]. Compared to the fibroblast monoculture, the co-culture gels showed less total contraction over 15 d. The authors fit an exponential decay curve to the area reduction, finding that the co-culture gel had the same time constant as the monoculture, despite the differences in total extent of contraction. Furthermore, the authors used two-photon microscopy to show that co-culture decreased the degree of collagen crosslinking, as inferred by two-photon fluorescence intensity.

In another co-culture study, Ishikawa *et al* prepared a similar collagen contraction gel with human bronchial epithelial cells seeded directly on top of the gel. However, this study also introduced ALI culture, as depicted in figure 5(1). These gels were not manually detached and remained in place for 21 d before stimulation. The gels detached by cell contraction when cells were stimulated with TGF- β 1. Histology slices of the microtissue demonstrated that TGF- β 1 also induced a decrease in epithelial layer thickness. Immunostaining in these histological slices also revealed changes in MMP and tissue inhibitor of metalloproteinase expression due to the crosstalk between co-cultured cells. These techniques demonstrated the synergistic interactions often seen in co-culture approaches.

The biochemical mechanisms of epithelial-fibroblast co-culture have been reviewed [56]. Growing evidence indicates that epithelial cells secrete a variety of biochemical signals to regulate fibroblast behavior. Biofabrication of phenotypic IPF assays would benefit from consideration of this cross-communication.

2.3.5. Alternative techniques to measure fibroblast contraction—A recently developed technique enables measurement of cell contraction based on the cell coverage of the culture surface. The xCELLigence platform uses electrodes patterned on the surface of a culture plate to measure the electrical impedance past the cells [48]. As the cells contract, they tighten and narrow, exposing electrode surfaces. The exposed surfaces pass current more easily, as the resistance from the cells is removed. One of the key benefits of this technique is its rapid response time. However, that may be more useful in studies of rapid contraction, as seen in muscle and cardiac tissues rather than the progressive contraction typically observed in fibrosis.

2.3.6. Discussion of contraction assays—As the key effector phenotype of IPF, considerable progress has been made to develop biofabrication techniques assessing contraction. However, one key phenotype that is missing from these cell-based contraction assays is the divergence between physiological contraction, which resolves, and pathophysiological contraction, which persists and progresses. The standard collagen gel contraction assay moves only in the direction of contraction, with little consideration or potential for reversal. The ability of epithelial cells to delay activation and contraction of fibroblasts [47] presents a promising building block for future biofabrication of assays that reflect the divergence of healthy contraction resolution and pathologic IPF persistence.

2.4. Phenotypic assays incorporating collagen production and deposition

In addition to collagen contraction, the production and deposition of new collagen by fibroblasts is a key event in disease progression. Collagen is produced by multiple cell types, but fibroblasts and myofibroblasts are responsible for the increase in collagen deposition seen in pulmonary fibrosis. The excessive accumulation of collagen seen in fibrosis is a product of both increased collagen production due to changes in transcriptional regulation and decreased degradation modulated by altered expression of various MMPs and cathepsins [99]. In order to evaluate the contributions to collagen accumulation, many approaches have paralleled the clinical approaches based on staining biopsies and evaluating serum biomarkers [100, 101]. Histologic approaches generally utilize sirius red and picrosirius red, however these methods lack specificity for collagen types [102]. Immunostaining enables identification of specific collagens, however quantification of collagen can still be challenging with these approaches. Methods based on soluble byproducts of collagen production and degradation have utilized radiolabelled amino acids and ELISAs [100, 101]. Other approaches specific to *in-vitro* models have focused on evaluation of transcription PCR or electrophoretic mobility shift assay methods [103]. An alternative approach to evaluation of collagen remodeling focuses on quantification of collagen alignment [104]. Despite the wide array of collagen production assays, few biofabrication studies have utilized them.

A study of kidney mesangial cells in 2D culture indicated that they respond to TGF- β 1 by forming clusters, or nodules, that somewhat resemble the nodules seen in human pulmonary fibrosis [38]. Immunostaining of these nodules indicated localized deposition of collagen. The nodule assay was later adapted in several different publications, one of which used the assay to screen Chinese herbal medicine extracts for inhibition of kidney fibroblast nodule

formation [105]. Another similar study found that macromolecular crowding with dextran sulfate causes much higher collagen deposition by fibroblasts [52]. Neutral macromolecular crowding with Ficoll produces less elevated, but more sustained collagen deposition with greater cross-linking. Collagen deposition was assessed and screened using immunochemical staining and optical microscopy of the cluster formation. This 2D method lacks the complex microenvironment of some of the other techniques, but it does provide disease-relevant information by assessing the collagen deposited by the fibroblasts.

In addition to collagen, fibronectin and elastin play substantial roles in pulmonary fibrosis. Fibronectin is also an important ECM component that shows increased expression in various forms of pulmonary fibrosis [106] and can be quantified by analogous methods to those used for collagen. Globular fibronectin in blood serum is integrated in the fibrin matrix during formation of the provisional ECM [107] and plays a major role in invasion of fibroblasts into the provisional matrix [68]. Fibronectin expression can be up-regulated by treatment with TGF- β 1 [108]. Fibronectin also binds a large number of growth factors that may promote myofibroblast differentiation [109]. Furthermore, a comparison between 2D and 3D models of fibroblast adhesion highlighted the importance of 3D scaffolds in interactions between fibronectin and integrins [110]. This demonstrates the importance of 3D biofabricated models in evaluation of ECM remodeling. Elastin is another ECM component of interest in pulmonary fibrosis due to its roles in absorption of mechanical loads and preventing tissue damage [111]. This role in maintaining elasticity and breaking strength in lung tissue makes elastin homeostasis vital in maintenance of lung function. However, elastin expression is up-regulated in pulmonary fibrosis, contributing to the increased stiffness of fibrotic lung tissue [112]. Due to the importance of ECM composition in lung function, future biofabricated phenotypic assays for pulmonary fibrosis may consider including evaluation of expression of various ECM components.

3. Challenges in biofabrication of pulmonary fibrosis models

Despite the considerable progress in the field of phenotypic assays, there remains opportunity to design microtissues that better capture the complex microenvironment of the lung. Specifically, there is a lack of appropriate alveolar models. Existing alveolar models typically use artificial substrates to provide the thin membrane structure [113]. Although most lung microtissue models use static air or flow-over gas exchange, recent work has incorporated more physiologic diaphragmatic breathing [113]. However, these models have not been shown to allow the cells to remodel the space, generating healthy or fibrotic tissue. Furthermore, these models generate cell monolayers, lacking the complex and interconnected structure of native alveoli, as in figure 6. This type of architecture and cell-driven assembly and construction is a ripe area for the field of biofabrication, biomaterials, and bioprinting. In this section, we discuss relevant physiological parameters and considerations for adaptation into biofabricated microtissue.

3.1. Suggested design parameters for phenotypic assays of pulmonary fibrosis

Problems associated with pulmonary fibrosis are predominantly caused by the accompanied decline in respiratory function. Excess accumulation of fibrous connective tissue interferes

with the architecture and function of the lungs, such that gas exchange is restricted [115]. This restriction is caused by a number of factors including a decrease in number of functional alveoli and an increase in the mechanical stiffness of lung tissue [116]. Figure 6 illustrates how fibrotic foci in the lung can interfere with gas exchange at the level of individual alveoli.

To extract biological information from a fibrotic microtissue, the cells should have an opportunity to remodel their own microenvironment. Compared to the phenotypic assays presented in this review, modern biofabrication techniques, such as bioprinting, hold additional potential for their ability to use bioactive and degradable bioinks, while still establishing complex structures. A major shift in microtissue phenotypic assays would examine restoration of function, rather than only measuring therapeutic interventions at the point of disease progression/exacerbation.

To recapitulate the restoration of lung function, consideration of relevant patient-level measurements may be informative. In patients, overall lung decline is typically measured in four ways: forced vital capacity (FVC—the maximum volume of air that can be inhaled and exhaled), carbon monoxide diffusion capacity (DLCO, a measure of gas transfer efficiency across the alveolar-capillary membrane), dyspnea (shortness of breath) and oxygen saturation after exertion (6-minute walk test). Each of these measurements reflect aspects of the total lung decline in pulmonary fibrosis. The FVC reflects a decline in compliance and the fraction of volume occupied by gas. Both DLCO and the 6-minute walk test assess the ability of the lungs to transfer oxygen species into the blood, while dyspnea may reflect an inability to effectively remove carbon dioxide. The reduction in gas exchange results from both a thickening of the alveolar wall and the reduction in the volume of gas exchanged in a full breath, as indicated by the reduction in FVC. Typical values for these measurements are included in table 2. Incorporation of these patient-level measurements to a biofabricated model would provide more directly relevant phenotypic readouts.

3.2. Biomaterials

As biofabricated models of pulmonary fibrosis become more sophisticated, it is important to mimic the physiological parameters of both healthy and fibrotic lung tissue, particularly with the selection of biomaterials. General guides for selecting biomaterials for biofabrication have been published [121–124]. In the lungs, the dominant ECM components are collagen IV, laminin, entactin, and proteoglycans in basement membranes; and fibrillar collagens, elastic fibers, and proteoglycans in interstitial connective tissue. Fibronectin and tenascin are also present in the provisional ECM, which is important during development and wound repair [125]. In fibrosis, there is increased expression of elastin, fibronectin and collagens I, III, and IV. This alteration in ECM composition increases the stiffness of lung tissue, consequently decreasing the lungs' ability to exchange gas [126]. In healthy tissue, alveolar compliance has been measured at 1–5 kPa, while fibrotic lung tissue is much stiffer, at 20–100 kPa [127].

Cellular composition of lung tissue also changes significantly during the pathogenesis of fibrosis. Cells found in the lungs include epithelial cells, endothelial cells, interstitial cells, macrophages, fibroblasts, and immune cells among others. These cell types are covered in

greater detail in prior reviews [128, 129]. As stated previously, following epithelial injury, inflammatory cells such as macrophages, neutrophils, and T cells migrate to damaged epithelium where they produce a variety of cytokines and chemokines that amplify the inflammatory response. This also triggers fibroblast recruitment, proliferation, and activation of myofibroblasts [130]. Small aggregates of proliferating fibroblasts, myofibroblasts, and immune cells are concentrated in areas known as fibrotic foci. These areas are the sites of excessive ECM production where scarring disrupts lung architecture and function [6]. Figure 6 demonstrates the effect that fibrotic foci can have on gas exchange function.

The micro-scale structure of alveoli has been analyzed through a variety of imaging methods, including *in vivo* fluorescence microimaging [131], cryosectioning [132], and casting [133]. These measurements converge on alveolar diameter on the order of hundreds of microns and alveolar mouth openings that are 85%–90% of the alveolar diameter (figure 6 and table 3). Furthermore, many of the gaseous alveolar compartments abut capillaries, enabling gas exchange. In healthy patients, this translates to a fractional tissue volume (ml tissue to ml of total lung) of 0.106–0.191 between inspiration and expiration [134]. However, in fibrotic lungs, the accumulation of tissue increases the fractional tissue volume to 0.220–0.345 [134]. In the diseased state, fibroblast foci generate local alveolar collapse [135], making measurements of individual alveolar geometry difficult. Accurately designed micro-alveolar tissues should approximate these ratios of gas to tissue, reflecting both the healthy and transition to disease.

The use of exogenous biomaterials, such as polymers, can augment mechanical properties [20] or structure [32, 33, 44] of biofabricated models [121]. However, these exogenous materials can disrupt local mechanical properties in often unexpected ways [54], in addition to potential questions of cytotoxicity. Nonetheless, the interplay between exogenous and native biomaterials may produce interesting discoveries and further inform biofabrication of future models.

3.3. Bioprinting techniques

The choice of biofabrication technique may pose additional constraints on the selection of biomaterials. The technique of 3D bioprinting remains poorly explored in the area of fibrosis assays, but the general field of bioprinting has been reviewed extensively. A recent review of bioinks elaborated on several approaches to print fibroblast-laden hydrogels, however none of these methods were used to evaluate fibrosis [140]. Another review covering 3D bioprinting techniques evaluated bioprinting for creation of complex 3D biomimetic architectures to provide improved *in vitro* analogs to *in vivo* tissue organization [141]. Decellularized ECM bioprinting techniques provide additional promise, as they already include native architecture [142]. These various techniques could be utilized to provide an added level of sophistication to fibrosis assays in order to evaluate a broader range of contributors to fibrosis pathogenesis.

4. Conclusions and future directions

Considerable progress has been made in the past two decades to biofabricate complex 3D lung culture environments that recreate aspects of pulmonary fibrosis pathogenesis. These

encompass epithelial damage, fibroblast invasion, contraction, and ECM deposition. Furthermore, some of these techniques have been adapted and applied as medium-throughput phenotypic screens for potential therapeutics.

However, there remains unexplored territory, particularly in the areas of alveolar architecture, provisional ECM and long-term disease progression. These areas present particularly challenging hurdles for cell-based assays. Clotting is a complex process involving many different factors from blood. Incorporation of endothelial compartments or fibrin assays has somewhat addressed the issue. Recent literature implicates aging and fibroblast senescence as key differences between fibrotic and healthy tissue in both animal models [143] and in human patients [144]. The role of senescence in fibrosis has been reviewed recently [10]. Maintaining long-term culture has proven difficult, although multicellular systems have shown some success, including the direct contact epithelial-fibroblast culture that lasted 21 d [47]. Furthermore, we have deliberately neglected the ever-present immune system in this review. The exact role of the immune system in IPF remains controversial and the challenges surrounding biofabrication of immune components are immense, but new work in this area could have tremendous benefits.

Development of future phenotypic assays should carefully consider the unique features of the lung environment: mechanical compliance, tissue geometry, and the composition of cells and matrix proteins. Furthermore, selection of appropriate readouts will be critical to answering the right questions about disease progression. If the assays are to be adapted to high-throughput screens, then extensive optimization of the reproducibility and performance of the assay should be performed. Biofabrication of culture models that enable phenotypic screening of pulmonary fibrosis presents a compelling approach to finding new therapies in pulmonary fibrosis and potentially other related aberrant wound healing diseases.

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Figure 1.

In a simplified model of pulmonary fibrosis, an epithelial injury precedes formation of the clot-like provisional extracellular matrix (ECM). Subsequently, fibroblasts invade the wound site and differentiate into myofibroblasts. The myofibroblasts accelerate wound closure by contracting and depositing extracellular matrix components, predominantly collagen. The assays covered in this review are listed and roughly sorted by the phenotypes present in each assay, labeled by the gray arrows. Additional information for each technique is in table 1. Note that some assays span multiple phenotypes that may split across the graph.

(a) Transwell Scratch and Invasion

(b) Collagen Plug Covered in Fibrin

(c) Fibroblast Invasion into Spheroids

Legend:

- Epithelial Cell

- Fibroblast

- Cancer Cell

- Myofibroblast

Figure 2.

Phenotypic assays for invasion. (a) In the Transwell scratch and invasion assay, fibroblasts are seeded on the underside of the Transwell, while epithelial cells are seeded on the top side. The epithelial layer is gently scratched with a pipet tip to remove a section of epithelial cells. After several days of culture, epithelial cells and fibroblasts may migrate into the opened area [31]. (b) The collagen plug covered in fibrin assay monitors fibroblast migration from an encapsulated collagen gel out into the surrounding fibrin gel [65]. (c) In a spheroid

fibroblast invasion assay, spheroids are seeded in agarose-coated 384-well microplates. Fibroblasts added after spheroid formation can be observed invading the spheroids [37].

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(a) Glass Capillary Confinement

(b) Teflon and Oil Lubrication

(c) ATPS Microprinting

Figure 3.

(a) Soluble, cell-laden collagen is pipeted into glass capillary tubes. After gelation, the collagen contracts linearly, enabling easy measurement of contraction [43]. (b) Teflon-coated surfaces contain the collagen gel, allowing smooth contraction without friction-induced deformations. The mineral oil layer helps confine the gel to the Teflon-coated contraction region [45]. Reprinted by permission from [45], © Society for In Vitro Biology 2002. (c) Aqueous two-phase systems (ATPS) enable formation of collagen microgels without excessive evaporation by confining soluble collagen to a dextran-rich phase until

gelation. The ATPS components are washed out and floating collagen gel assay can contract [39].

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(a) Supersoft Pillar Compression

(b) PDMS Micropillars Bending

Figure 4.

Microfabricated devices for fibroblast contraction. (a) Cell-laden collagen gels are formed inside of microfabricated, supersoft, donut-shaped wells. The cells are able to contract and deform the low elastic modulus pillars [44]. (b) Cell-laden collagen gels are seeded around micropillars. When the cells contract, the micropillars bend as predicted by cantilever solid mechanics [33]. Reproduced from [33].CC BY 4.0.

Direct Contact ALI with Contraction

Figure 5.

Epithelial cells are seeded on top of a fibroblast-laden collagen gel in a Transwell insert. After confluence is reached, culture media is removed from the top well, generating an air–liquid interface culture. The epithelial layer differentiates in response to the air–liquid interface. After stimulation, the collagen gel contracts, separating from the walls of the well [47].

Figure 6.

Healthy function of the lungs requires compliant tissue that can readily expand and contract in response to diaphragmatic breathing in order to enable gas volume exchange during respiration. The line represents the volume of gas inside the lung over time. Normal tidal breathing uses a small fraction of the total lung capacity. A full inhale, followed by a full exhale (leaving residual gas volume behind) moves a volume of gas equal to the forced vital capacity (FVC). Formation of fibrotic foci interferes with this compliance due to the heterogenous accumulation of fibrous connective tissue. In the advancement of IPF, both FVC and residual volume progressively decline [114]. Models of pulmonary fibrosis could benefit from mimicking alveolar architecture and enabling fibrotic remodeling of alveolar tissue. Furthermore, phenotypic evaluation of more clinically relevant metrics such as gas volume exchange and tissue compliance may better suit evaluation of anti-fibrotic therapeutics.

Table 1.

Biofabricated phenotypic assays for pulmonary fibrosis. Colored dots correspond to fibrosis phenotypes, as in figure 1.

Technique	Phenotypes	Cell types used	Exogenous materials	Additional equipment	Applications and findings	References
Scratch wound assay	Re-epithelialization	Primary endothelial cells and NIH3T3	None	None	Decreased migration of primary ECs upon deletion of focal adhesion kinase	[25]
Alveolar epithelial stretching damage	Cell death	Rat alveolar epithelial type II cells	Silicone membrane	Cam motor, gasket setup	Cell death reduced by limiting the amplitude of deformation	[26]
Alveolar epithelial stretching damage	Cell death and detachment from surface	Mouse alveolar epithelial cells	PDMS membrane	Syringe pump, microfabrication	Meniscus propagation in addition to cyclic stretch resulted in additional monolayer detachment	[27]
Air Transwell co-culture	Protein expression	SAEC and endothelial	Transwell	None	Exposure of small airway epithelial cells to multi-wall carbon nanotubes induced pro-inflammatory responses in co-cultured endothelial cells	[28]
Transwell with aerosol exposure	Viability, cytokine expression	A549	Transwell with 1 μ m pore	Nebulizer, gas containment chamber	ZnO nanoparticles induced pro-inflammatory and oxidative stress responses only above occupationally allowed exposure levels	[29]
Photodegradable microbeads in hydrogel co-culture	Migration	A549, human normal lung fibroblasts CCL-210.	Home-made microspheres, hydrogels	Operetta high content imaging system (Perkin Elmer)	A549 epithelial cells stimulated fibroblast migration	[30]
Transwell scratch and invasion	Migration, wound closure, re-epithelialization	A549 and fibroblast (NHLF versus IPF)	Transwell with 8 μ m pore	None	Fibroblasts from IPF patients had an impaired response to epithelial injury compared to healthy	[31]
Tethered fibrotic microtissue array	Contraction, tearing epithelial layer, activation	Primary NHLF, human primary SAECs	PDMS, collagen, matrigel, fibronectin	Micropillar PDMS device	Pirfenidone and Nintedanib both reduce contractile and fibrotic phenotypes	[32]
PDMS micropillar tethered collagen gel	Contraction	BEAS-2B normal lung epithelial cells	PDMS micropillars, collagen	Microfabrication	Short multi-wall carbon nanotubes stimulate contraction and pro-fibrotic phenotype	[33]
Fibroblast invasion from collagen into fibrin	Invasion	Human adult dermal fibroblasts	Dried fibrin gels, collagen, fibronectin	None	Dermatan sulfate is a necessary proteoglycan for fibroblast invasion into fibrin gels	[34]
Fibroblast invasion from 2D into fibrin gel	Invasion	Isolated human lung mesenchymal cells	Fibrinogen, thrombin	Inverted phase contrast microscope	Fibroblast invasion into fibrin gels is CD44 dependent	[35]
Fibrin contraction Assay	Proliferation and collagen synthesis	Human Dermal Fibroblasts	Fibrinogen, thrombin,	Inverted microscope to measure height	TGF- β induced fibrin gel contraction and collagen synthesis	[36]
Fibroblast invasion into spheroids	Invasiveness	T47D (breastcancer), MCF7 + H1299 (lung cancer), HPF (pulmonary fibroblasts)	480 compound library	Agarose for non-adherent surface for spheroid formation	Prostaglandins and ROCK inhibitors reduced fibroblast invasion into spheroids	[37]

Technique	Phenotypes	Cell types used	Exogenous materials	Additional equipment	Applications and findings	References
2D nodule formation assay	Nodule formation	Many different cells examined	None	None	Alk5 inhibitors and tranilast inhibited nodule formation	[38]
Stiffness gradient collagen gel	Activation, collagen production	Human lung fibroblasts (CCL-151)	Collagen, acrylamide, bisacrylamide, Irgacure 2959	UV light source	Fibroblasts activate in response to stiff environments through COX-2 dependent pathway	[20]
Aqueous two-phase system microprinting	Contraction	HEK 293, NIH 3T3, MC 3T3	Polyethylene glycol, dextran, collagen	Robotic pipettor	Small volume collagen gels contract in response to 1 min of TGF- β 1 stimulation	[39, 40]
Floating collagen gel contraction	Contraction	Human foreskin fibroblasts monoculture	Collagen	None	High population doubling level cells maintain contractile ability; gold standard method to assess contraction	[41]
Low-adherence plates facilitated collagen gel shrinkage	Contraction	NB1RGB normal human dermal fibroblasts	Collagen	Flexcell compression	Cyclic compression induced hyaluronic acid production in fibroblasts	[42]
Glass capillary confinement	Contraction	Primary human bladder-derived smooth muscle cells (SMC)	Glass capillary	None	U46619, a prostaglandin H ₂ analog, induces SMC contraction	[43]
Supersoft micropost lithography	Micropost area reduction	HS-5 stromal fibroblast cell line	Microfabricated device	Microfabrication	LPA reversibly induces contractile stress in fibroblasts	[44]
Teflon and oil lubrication	Contraction	Normal adult human dermal fibroblasts	Teflon-coated glass, teflon-coated washer, mineral oil	None	PDGF-BB induces fibroblast contraction	[45]
Direct contact collagen gel co-culture	Macroscopic and microscopic contraction	A549, 16HBE14o-, CCD-18 lung fibroblast	Collagen	2-photon microscope for collagen sub-analysis	Epithelial cells slow fibroblast contraction	[46]
Direct contact ALI with collagen gel contraction	Gel contraction	IMR-90 human fetal lung fibroblasts, normal human bronchial epithelial cells (HBECs)	Collagen	None	Epithelial cells contribute to fibroblast expression of MMP-9 and MMP-2	[47]
Electrical impedance	Contraction	Human primary bronchial smooth muscle cells (BSMCs), primary lung fibroblasts (HLFs)	Gold electrode arrays	xCELLigence RTCA MP instrument	Identified non-covalent agonists and antagonists of TRPA1, applications in asthma and allergies	[48]
Nano-indentation with collagen gel contraction to measure contractile forces	Contractile force	Human aortic adventitial fibroblasts	Collagen type I	Depth-sensing nanoindentation tester	Histamine-induced collagen gel contraction by fibroblasts reduced the stiffness	[49]
Collagen gel tethering	Direct force measurement via strain gauge	Normal and diseased human and calf skin fibroblasts	Strain gauge	Custom strain gauge	Fibroblasts generate tensional homeostasis	[50]
Microfluidic droplet generation	Not reported	3T3 fibroblast cells	porcine gelatin, rat tail collagen type I	Blue LED for photocrosslinking, microfabrication	Fibroblasts spreading and invasion was enhanced by increased microgel stiffness	[51]

Technique	Phenotypes	Cell types used	Exogenous materials	Additional equipment	Applications and findings	References
Optical measurement of collagen cluster formation ●	Deposition	Normal primary human lung fibroblasts (WL-38)	Macromolecules (optional)	None	Known antifibrotic compounds reduced scarring; newer compound: microRNA29c found to be antifibrotic	[52]
Collagen microsphere generation ●	None	MDA 231 cells	Collagen	Microfabrication lab	Cells were successfully encapsulated in collagen microgels and remained viable	[53]
Agarose-embedded collagen gels ●	Contraction	U373-MG human glioma cells	Collagen, agarose	None	Microscale mechanics are more important for cell behavior than macroscale mechanics	[54]

- Epithelial injury and clot formation.
- Fibroblast invasion and differentiation.
- Contraction and ECM remodeling.

Table 2.

Patient-level phenotypic measurements in IPF.

Macroscopic patient phenotypic parameter	Description	Functions measured	Numbers	Reference
Forced vital capacity (FVC)	Max volume of air that can be inhaled and exhaled in a single breath.	Volumetric gas exchange	>10% decrease indicates disease progression	[117, 118]
Dyspnea	Shortness of breath	Gas exchange, inability to respond to ventilator needs	NA	[118, 119]
6-minute walk test.	The patient is instructed to walk for 6 min, after which the blood oxygen saturation is measured.	Gas exchange measurement with stress test	6MWT followed by desaturation (<88%)	[118]
Carbon monoxide diffusion capacity (DLCO)	Measure of the conductance of gas transfer from inspired gas to red blood cells	Gas exchange at the blood level	Lower in IPF [120] Drop of >15% indicates disease progression	[117]

Table 3.

Selected physical properties of the lung.

Property	Values in healthy people	Values in IPF patients
Compliance	1–5 kPa [127]	20–100 kPa [127]
Fractional tissue volume (ml tissue per ml tissue and gas)	0.106–0.191 (inspiration–expiration) [134]	0.220–0.345 [134]
Alveolar mouth opening	278 μm [131]	291.4 μm [136]
Alveolar depth to mouth diameter	0.85–0.9 for residual capacity to total lung capacity, 0.95–1.5 below residual capacity—results in collapse [132]	NA
Alveolar half-opening angle	60 degrees [133, 137]	NA
Alveolar lining layer thickness	0.1–0.2 μm [137, 138]	NA
Alveolar diameter	225 μm [139]	NA