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REVIEW



# Disentangling the fibrous microenvironment: designer culture models for improved drug discovery

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**ABSTRACT**

**Introduction:** Standard high-throughput screening (HTS) assays rarely identify clinically viable 'hits', likely because cells do not experience physiologically realistic culture conditions. The biophysical nature of the extracellular matrix has emerged as a critical driver of cell function and response and recreating these factors could be critically important in streamlining the drug discovery pipeline.

**Areas covered:** The authors review recent design strategies to understand and manipulate biophysical features of three-dimensional fibrous tissues. The effects of architectural parameters of the extracellular matrix and their resulting mechanical behaviors are deconstructed; and their individual and combined impact on cell behavior is examined. The authors then illustrate the potential impact of these physical features on designing next-generation platforms to identify drugs effective against breast cancer.

**Expert opinion:** Progression toward increased culture complexity must be balanced against the demanding technical requirements for high-throughput screening; and strategies to identify the minimal set of microenvironmental parameters needed to recreate disease-relevant responses must be specifically tailored to the disease stage and organ system being studied. Although challenging, this can be achieved through integrative and multidisciplinary technologies that span microfabrication, cell biology, and tissue engineering.

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## 1. Introduction

Initially, promising hits in high-throughput screening (HTS) drug assays that turn out to be 'dead-ends' create costly inefficiencies in the drug development pipeline. These expenses must be absorbed by successful therapeutics. Hence, it cost an average of 648 million USD to develop a single new drug in 2017 [1], and these numbers are projected to continue increasing. Since 90% of drugs fail phase I clinical trials [2], an ability to identify dead-ends prior to expensive testing would significantly reduce the average costs of pursuing the most promising therapies.

Cells exist in extraordinarily complex environments, where they reside within a meshwork of supporting fibrous proteins. This extracellular matrix (ECM) both supports and drives cellular organization into complex structures, and relays a variety of biochemical and biophysical signals to regulate cellular response. The fibrous ECM meshwork can consist of ~100 different fibrous proteoglycans, polysaccharides, and proteins (the most abundant of which include Type I collagen and elastin), and a further ~100 ECM associated proteins such as soluble factors that bind to this mesh [3]. The biochemical complexity of the ECM plays a critical role in driving cellular response [4] and has been shown to affect therapeutic response [5]. These features have been extensively reviewed elsewhere [6–9]. More recently however, *biophysical* signals provided by the fibrous microenvironment have emerged as pivotal regulators of cell function. Changes in biophysical properties such as tissue architecture and mechanics are associated with disease progression in a wide variety of diseases including cancer, fibrotic disease, and osteoarthritis [7,10–12], but the hard, flat, plastic surfaces of conventional drug screens do not capture this complexity. Thus, it is not surprising that cells do not respond to candidate therapeutics in a realistic manner. Developing biomimetic, disease-specific, and precisely defined environments should therefore improve the translational potential of these assays. For example, simply including protein fibers in drug screening culture models significantly impacts observed drug efficacy [13–17]. Methods to recreate tissue complexity and architecture that occur at various stages of disease progression may therefore be a viable strategy in streamlining the drug development pipeline.

Although promising, this general approach presents two contrasting challenges. First, how do we decide which set of 'microenvironmental' features are both necessary and sufficient to produce translational results in HTS platforms? Second, how do we implement these features in HTS drug screening systems? While the idealistic design strategy would be to recreate the entire tissue, current technologies limit our capacity to replicate this immense complexity, particularly for HTS applications that require robustness for intensive scale-up. Furthermore, tissue characteristics are both patient- and disease-specific, making it challenging to determine the generalized features needed to recreate a target disease. Finally, the specific features within

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**Article highlights**

- Biophysical features of the extracellular matrix have emerged as critically important regulators of cellular function, but the increased complexity of these systems provides considerable challenges in designing next-generation HTS platforms.
- Identifying a minimal set of biophysical features needed to prompt realistic cellular activity is essential, but is likely to be disease- and tissue-specific.
- Tissue architecture and mechanical behaviors are intimately linked, and critically important for cell function; and their individual contributions can be identified using well defined in vitro culture technologies.
- Cellular structures, fiber microstructure and organization; and biomechanical parameters including tissue stiffness, viscoelasticity, and plasticity are more complex than originally thought, and can have disease-specific effects on cell-response.
- These culture models have strong potential utility in designing next generation breast cancer screening platforms.
- To streamline the drug development pipeline, there is a need for fundamental disease-specific knowledge about the key microenvironmental drivers of cell function to emerge in parallel with novel technologies to recreate these features in HTS-compatible formats.

This box summarizes key points contained in the article.

a fibrous tissue that drive realistic drug responses remain unclear. Architectural parameters of overall cellular structure, along with fiber length, density, and organization all integratively contribute to mechanical characteristics such as bulk and localized stiffness, viscoelasticity, and plasticity (Figure 1). Each of these features progressively changes during disease evolution in a highly disease-specific manner [7,11]. These changes can direct diseased cell phenotypes [18], and in many cases, their effects on drug efficacy remain unclear.

Here, we review emerging strategies to isolate and manipulate specific parameters in the fibrous tissue microenvironment, and ultimately identify those cues important to disease progression and drug response. We limit the scope of this review to the emerging role of biophysical tissue features in driving cell behavior, and consider the integrated effects of the highly localized tissue architecture, along with arising micro- and macro-scale mechanical tissue characteristics (Figure 1). To highlight the potential for designer culture strategies in improving drug screening, we then provide a disease-specific context for these findings, by reviewing the impact of fibrous cues on our understanding of breast cancer, a disease for which improved HTS strategies are urgently needed. We conclude with an expert opinion on how these fundamental studies can contribute to developing the next generation of HTS platforms.

## 2. Deconstructing tissue architecture

The need to include three-dimensional (3D) cultures in drug screening has been well-established [19–22]. 3D culture provides critically important microenvironmental cues, and supports analysis of functional cell behaviors such as tissue branching, tissue permeability, and cell invasion [23–26], that would not be possible in 2D. As multiple excellent reviews already support the importance of 3D cultures over 2D systems [9,15,21,27,28], here we focus on specific aspects of 3D tissue architecture, including microstructural organization of the surrounding ECM network, the importance of cellular structures within 3D tissues, and their interrelated roles in driving overall tissue function. We consider these characteristics in the context of dissecting and ultimately reducing the complexity of the environment needed for translational HTS.

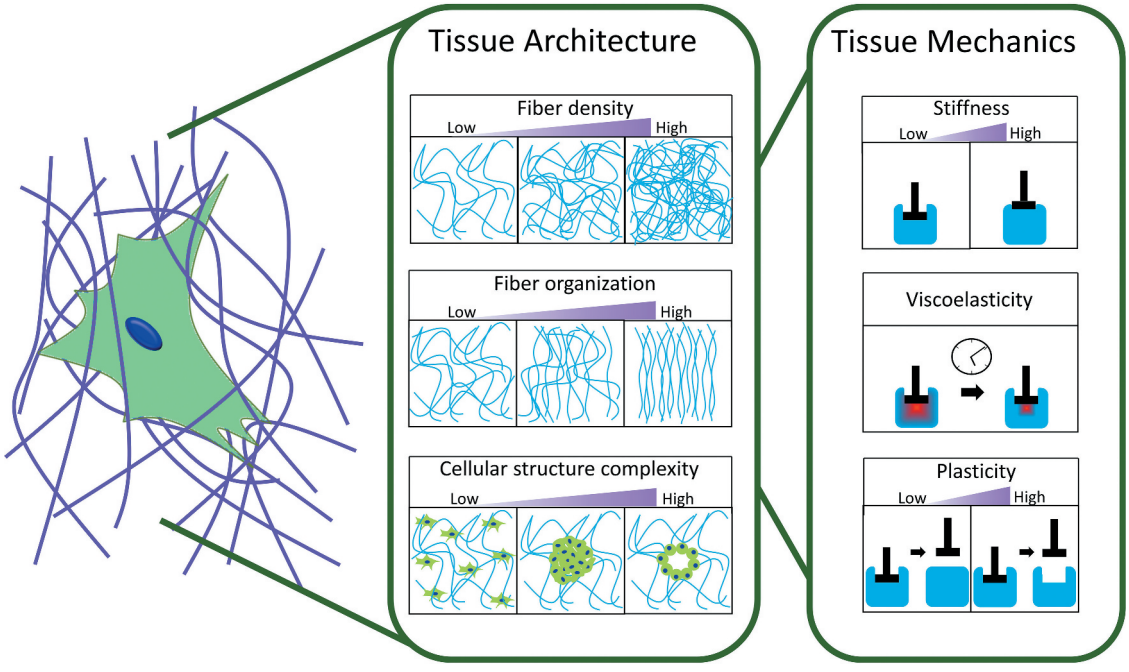


Figure 1. The cues presented by the fibrous microenvironment are widely varied. Here we consider the highly localized effects of tissue architecture on cell function, including features such as fiber density, spatial organization, and structural complexity. These features also influence local and bulk tissue properties including stiffness, viscoelasticity, and plasticity; each of which may be critically important to recreating disease-specific drug screening platforms.

### 2.1. Microstructural features of the fibrous ECM

Physical characteristics of tissue ECM at the microscale include fiber thickness, length, density, and organization, which vary substantially *in vivo*. These microstructures vary based on tissue location, with fiber diameters ranging from subcellular (<100 nm) collagen fibrils present in basement membrane [29], to 500–800 nm collagen fiber bundles [30,31] in connective tissues, or larger micrometer-wide fibrils [32,33]. Increases in ECM density, through increases in fiber thickness and/or quantity, are accompanied by a corresponding reduction in average matrix pore size. These changes have been observed in several diseases, including tumor desmoplasia and fibrotic plaque development [11,34]. Finally, distinct degrees of fiber network organization and alignment are also associated with disease progression and cell function. Each of these features has emerged as a pivotal player in tissue organization and disease progression; and identifying their precise roles is an important step in determining which parameters must be replicated for HTS assays (Figure 1).

Architecture of the fibrous matrix can be tuned through a variety of biomaterial engineering strategies. For example, simply gelling natural collagen matrices at various temperatures can tune collagen fiber bundling and increase pore size without drastically affecting collagen content or overall matrix stiffness [35]. Incorporating small molecular weight polymers that do not interact with cells, can change fiber lengths and pore sizes [36–38]. Alternatively, designing dual hydrogels with interpenetrating networks may be used to decouple fiber density from bulk mechanical properties of the matrices [39]. More precise control over these properties may be obtained by electrospinning polymers, to control fiber geometry, density, and adhesive patterns of a synthetic polymer mesh [33]. Furthermore, these defined scaffolds can then be incorporated within various hydrogels, producing a fiber-reinforced composite [33]. Overall organization of fiber mesh can be tuned by incorporating a moving collector plate in the electrospinning process to bias the alignment of fibers [40]. More advanced methods have also been developed, including incorporating magnetically activated, cell-adhesive microgel rods to align a fibrous mesh [41]; a composite hydrogel system, where swelling of one hydrogel results in the uniaxial alignment of the second [42]; or using evaporation-mediated flow [43] or external vibration [44] techniques to align fibers during matrix gellation.

These techniques focus on specific features of the fibrous environment, and the results demonstrate the important role of 3D fibrous matrix architecture on cell function. Breast cancer cells in matrices of short fibers (and correspondingly small pore sizes) appear circular with short-lived, weak protrusions [36], exhibit reduced cell contractility [38], and increased oxidative stress [36]. Larger pore sizes give rise to a spindle-shaped, mesenchymal-like phenotype [36,38]. Although increasing fiber density generally appears to increase cell spread area, cells exhibit a biphasic change in morphology, depending on the number of fibers locally available for contact guidance [33]. Cells also generally align along fibers and extend protrusions in low fiber-density environments, but adopt a pancake-like spread morphology in high fiber-densities [33]. These observations suggest that there is a fiber length and density threshold, below which cell spreading and contractility is impaired [36,38], factors which directly affect downstream cellular responses.

While each of these studies demonstrates significant architectural effects on a cellular behaviors, these approaches to modulate fiber parameters do not allow consistent and cellular-scale control of these features. Instead, cells experience a heterogeneous ensemble of stimuli, and read out an ensemble average of cellular responses. Furthermore, these approaches do not allow the precision to individually tune each fiber parameter. For example, changes in fiber size affect both adhesive ligand availability and spatial distribution, which may affect cells through distinct mechanisms. Finally, emergent larger-scale mechanical properties of the matrix (section 3) are also prone to change with many of these strategies. To better understand the specific influences of fiber features on cells, it is helpful to design experiments that consider the separate roles of three-dimensional, topographical structures and adhesive patterns on cell function, both of which may independently affect cell response.

Fiber-like topographies alone can be recreated by molding linear, nano-grooved substrates such as poly(DL-lactic acid) (PLA) [45], optical adhesives [46], or polyurethane [47], and independently drive uniaxial cell alignment, contact guided migration, and control over morphological phenotypes such as cell aspect ratio. Alternatively, the spatial distribution of adhesive cues that accompany fiber microstructure may drive cell function. To study these factors, several groups have devised methods to pattern adhesive proteins on topographically flat and otherwise non-adhesive 2D surfaces in fiber-like patterns. This can be accomplished by laser-based removal of a non-adhesive surface [48], exposing adhesive sites on a blocked surface by mechanical fracture of the non-adhesive layer [49,50], microcontact printing [51], e-beam lithography [52], and electrospinning tunable fibers over open wells [40,53]. Micropatterns have also been created along three-dimensional surfaces to study the effects of individual fiber tortuosity on cell morphology [54]. These approaches enable independent assessment of the role of fiber alignment (anisotropy), spacing (density), and contact area (fiber width) on cell behavior.

Collectively, these studies have demonstrated that subtle characteristics of the supporting fibrous ECM mesh affect virtually every aspect of cell function. Cell shape is a well-established predictor of fate, function, and proliferative state [55–57], and is hence of crucial importance. Cells preferentially align with and migrate along pathways formed in the fibrous mesh of ECM proteins [58], and adopt elongated cell morphologies that closely correspond to the orientation of the underlying matrix [48,51,54]. This characteristic is independent of culture platform dimensionality, and has been consistently observed on 1D linear arrays, on flat [48] and topographically complex surfaces [54], and on aligned, fully 3D matrices [42], given sufficient contact area and spacing between fibers is maintained [54]. Fibroblasts cultured on 1D linear fiber arrays, as compared to unaligned surfaces, exhibit uniaxial cell spreading, polarization, and migratory behaviors that strongly resemble those cultured on 3D cell-derived matrices [48,51].

This behavior is independent of the ligand type and density, suggesting that the linear directional cue provided by the cell-ECM contact plays an important role in determining uniaxial phenotype [48].

### 2.2. Cellular structures within tissues

In contrast to cells constrained on flat tissue culture plates, cells in three-dimensions can be organized into a wide variety of structures that present cells with distinct cues, driving behavior and overall tissue function. For example, healthy breast epithelial cells can be induced to grow from single cells to form hollow, polarized spheres resembling native breast tissue structures when cultured in 3D-reconstituted basement membrane (rBM) [59,60]. Indeed, these structural features also direct homeostasis of healthy tissue and tissue degradation during disease: when formed into hollow, polarized spheres, healthy breast epithelial cells are growth-arrested and show high resistance to apoptotic signals compared to growth on a 2D dish [59]. In contrast, breast tumor cells that also begin as single cells in 3D matrices form a disorganized mass of aggregated cells that may ultimately shed cells into the surrounding matrix. These architectures are also cell-type specific: single fibroblasts will remain spaced apart in 3D matrices [33]. Each of these tissue structures present distinct microenvironmental cues to their component cells, including control over cell shape [55,61] and tissue curvature, which provides stress gradients that affect invasion and migration [25,62–64].

Recreating these tissue structures presents considerable challenges and opportunities for HTS platforms. Engineered tissues are generally achieved via three main mechanisms: precision assembly, guided assembly, and self-assembly (Figure 2). In self-assembled tissues, cells themselves direct their formation through processes of growth, invasion, and matrix remodeling, as is the case with the acinar structures described above, and organoid models derived from stem-cell precursors [65]. While tissue self-assembly is relatively straightforward to implement at scale, there is no control over tissue architecture, and cells can often form undesired structures. For example, placental organoids form a fused syncytial mass within the organoid, rather than on the surface as in human placental villi [66], and this may not be desirable for the specific screening application being developed. On the other end of the spectrum, precision assembly involves precisely positioning individual cells in pre-defined locations within an encapsulating matrix [67–69]. Limitations in speed of precision-assembly strategies currently prevent scale-up toward high-throughput drug screening, and are therefore not considered in this review.

An alternative and promising strategy is to provide cells with a pre-organized template that can support the formation of more advanced and controlled structures. For example, either the cells or the surrounding matrix can be pre-molded into a specified shape. Cells packed into these shapes are then

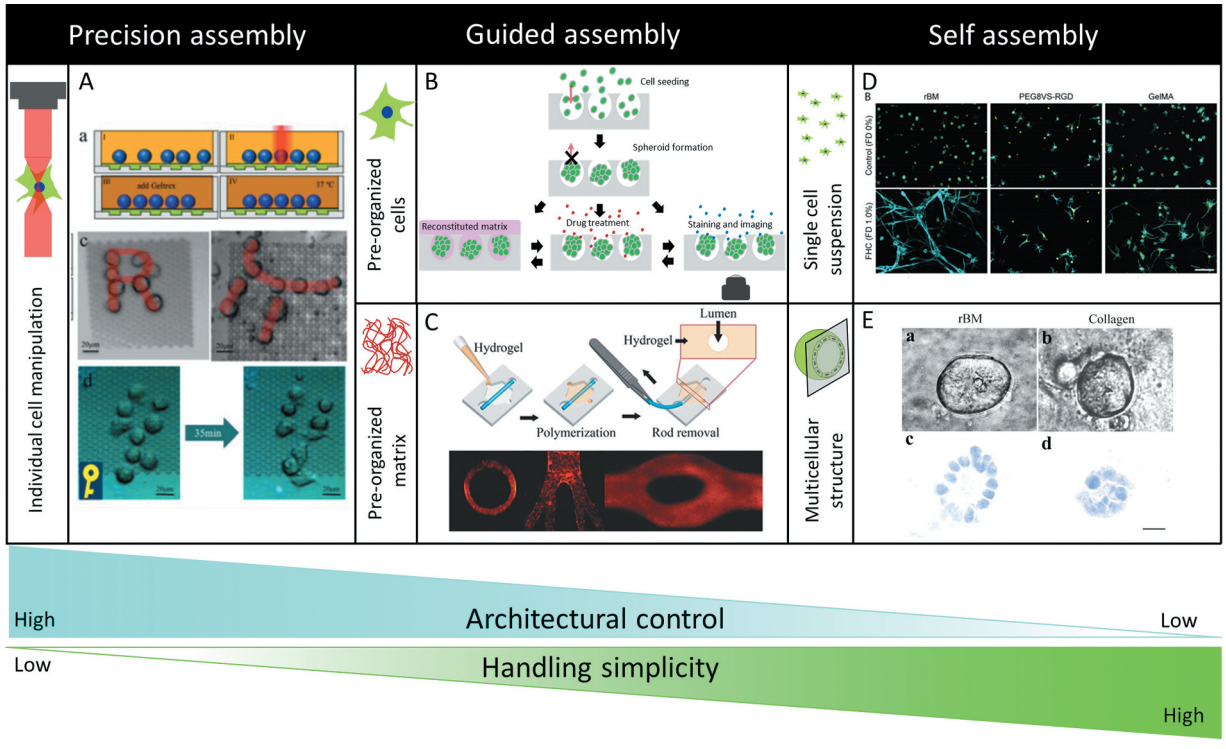


Figure 2. 3D tissue engineering approaches for specific structural outcomes. (A) Precision assembly uses tools such as optical tweezers for fine cell placement within an overall tissue structure (Adapted with permission from [69] © The Optical Society). Pre-organizing either the (B) cells into spheroids [70], or (C) matrix into ducts are both examples of tissue guided assembly (reproduced with permission from [23] © John Wiley and Sons). Tissue self-assembly into structures includes (D) single-cell suspensions of fibroblasts, with different spread morphologies depending on the ECM suspended in (scale: 100 μm; reproduced with permission from [33] © (2019) American Chemical Society), or (E) multicellular spheres containing either polarized cells with a hollow lumen or solid structures depending on the ECM suspended in (scale: 25 μm; reproduced with permission from [71] © The Company of Biologists Ltd.).

allowed to self-assemble to create tight junctions with fine control over tissue structure. Multicellular aggregates or spheroids can be considered to form via this guided assembly, as they are formed by culturing cells in close proximity to each other, using techniques such as the hanging drop method for micromass culture, aqueous two-phase system (ATPS), or micropocket devices [70,72,73]. Such approaches are often used to generate three-dimensional tumor models that may be applied to screening [72,74,75]. While these approaches do increase handling complexity (Figure 2), they provide a potentially scalable route for designer high-throughput studies [76]. For example, tissues with hollow lumens are required to understand the role of blood vessels and ducts for systemically transported drugs, and it therefore becomes important to capture the lumenized morphology of these tissues. Lumens can be engineered into three-dimensional tissues using a variety of strategies. A simple and accessible method to achieve this is to polymerize ECM around a removable rod made of various materials such as needles, polydimethylsiloxane (PDMS) [23], gelatin [24], or even a highly viscous fluid [22,77]. These materials are subsequently removed to leave a hollow tube through the matrix. Endothelial or epithelial cells can then be seeded into these tubes. Similarly, more complex templates that mimic structures such as branches can also be used to micromold appropriately shaped cavities, and many of these presented lumen-engineering strategies have been designed with increased throughput in mind [23,25,26,78,79].

In the context of HTS assays, every additional processing step introduces potential for reduced assay robustness and reproducibility. Hence, tissue engineering for HTS systems is akin to solving the 'Goldilocks' problem: how do we reduce culture complexity enough to manage HTS standards, while maintaining adequate tissue complexity to produce translational results, thereby achieving a 'just right' screening platform. Guided assembly culture models may provide a realistically attainable and sufficient solution.

## 3. Deconstructing tissue mechanics

The mechanical properties of biological tissues arise from the highly local, microstructural features described in section 2, and these mechanics, felt at both a local and larger scale, have now been implicated as powerful regulators of cell functions. Changes in local tissue mechanics occur in a wide variety of diseases [80–83], and since these changes play a fundamental role in cell response, it is likely of critical importance to include these mechanical parameters in drug screening applications. However, tissue mechanics can be quite complex, and distinct mechanical features are emerging as being differently important for various applications, making it important to identify the precise parameters needed to incorporate into a specific HTS assay.

Biological tissues exhibit elastic, viscoelastic, and plastic behaviors (Figure 3). Elastic properties of the material allow it to store energy in the form of internal stress while being deformed, and return to the original shape once an applied load is removed. In contrast, viscoelastic materials dissipate internal stress over time through internal reorganization,

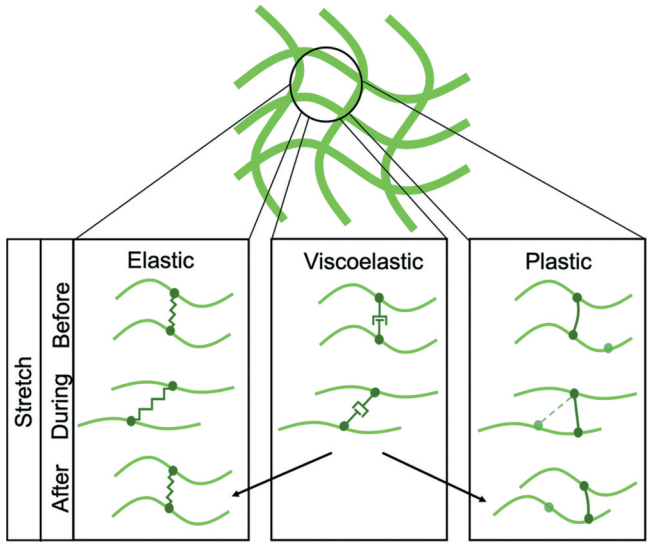


Figure 3. Elastic, viscoelastic, and plastic behaviors arise from unique mechanisms during ECM stretch. Elastic materials behave like rubber bands, where energy from an applied force is stored in the material deformation. Hence, when the force is removed, the material will rapidly return to its original shape. Viscoelastic materials display both elastic and viscous properties, and rather than the stress remaining stored in the material, a time-dependent stress decrease occurs. Viscoelastic materials may or may not return to their original shape when the force is removed, depending on elastic recovery force strength and the mechanism of viscous dissipation. Plastic materials display permanent deformation following an applied force, often due to new bond formations which are stronger than elastic recovery forces.

allowing the material to gradually flow under an applied load. Plasticity describes permanent deformation of a material, which does not return to its original shape after the load is removed. Although the impact of matrix stiffness has been established for over a decade [84,85], the importance of viscoelasticity and plasticity have only recently emerged (reviewed in [86,87]). However, manipulating these parameters individually can be quite challenging, and in this section, we review models and approaches to understand how complex tissue mechanics might influence cell function and drug response.

### 3.1. Elasticity

The mechanical elasticity (or stiffness) of tissues is a measure of how resistant the tissue is to applied deformation. Increases in tissue stiffness are often associated with disease progression in a wide variety of organ systems, including osteoarthritis [12], liver fibrosis [88,89], and breast cancer [10,90,91], amongst many others. To better understand how these disease processes arise, multiple biomaterial models with tunable stiffness have been developed, each presenting distinct advantages.

The simplest and most common strategy to tune stiffness in biomaterials is to increase crosslinking density in the hydrogel network. The earliest example of this is in polyacrylamide, a hydrogel material in which monomer and crosslinker components can be adjusted to create stiffness-tunable 2D culture surfaces [92]. Functionalization of polyacrylamide surfaces with an extracellular matrix protein or peptide allows precise definition of the adhesive molecules

presented to cells, and this material has been broadly used in a variety of culture formats including within microfluidic channels [93], at air-liquid interfaces [94], and as a substrate in pseudo-3D 'sandwich' systems [95–97]. However, the harsh crosslinking reactions required for gelation limit the use of this material as a true 3D culture system, and similar tuning strategies have been developed for synthetic polymers such as polyethylene glycol (PEG), which can be used for 3D applications [98]. These materials can also be functionalized with candidate peptides and dynamically stiffened or softened on exposure to light by incorporating photosensitive crosslinkers [99,100].

An important consideration of these synthetic hydrogel systems is that they form with pore sizes smaller than individual cells, creating a cage that prevents cell spreading in 3D [101]. Therefore, they must be engineered with cell-cleavable crosslinks [102] to allow cell spreading within the matrix. How these cleavable sites affect the local rigidity surrounding individual cells is uncertain, and processing strategies such as gas foaming, freeze-thawing, and including porogens have been developed to tune pore size in these materials [103]. Alternatively, using naturally derived polymers such as gelatin [104–106], collagen, or hyaluronic acid [107–109] resolves these issues, and can be chemically modified to stiffen by addition of crosslinkers, or interpenetrating networks of other hydrogels [110–114]. However, the use of natural biomaterials does come with a significant caveat, in that cells may remodel the matrix through enzymatic activity, ECM deposition, and fiber reorganization. Hence, when changes to fiber architecture occur, there is a corresponding change in fiber mechanics. For example, increased collagen fiber density and linear organization increase the stiffness, or stiffness anisotropy of the tissue [115,116]. Synthetic, elastic matrices on the other hand, can be engineered to present stable mechanical properties throughout the culture period [117].

The use of both synthetic and natural biomaterials do present some challenges in precisely decoupling stiffness from other variables, and cells still experience heterogeneous matrix properties such as porosity (and associated stiffness), which are not precisely controlled throughout the materials. The overall strategy to tune stiffness, requiring changes in crosslink density and material microstructure, has also generated some controversy as to which features drive cell response [118,119], and microfabricated analogs may provide unique strategies to independently manipulate adhesion and mechanical stiffness. For example, micropillar arrays have been fabricated with precise control over pillar diameter and pillar height, to independently tune these features [120,121]. To capture fiber-like phenotypes, stiffness-tunable electrospun biomaterials have also been developed to independently manipulate fiber stiffness, geometry, and network architecture [40,122].

The above studies generally demonstrate that stiffness affects a wide variety of cellular processes. On 2D hydrogel surfaces, stiffer materials induce cell spreading and proliferation [120], provided that adhesion sites are spaced closely enough to allow spreading. In contrast, increased stiffness in 3D fibrous matrices decreases cell spread, migration, and proliferation [98,99,102,105–107,109,111,112,123,124]. These responses are likely also dependent on cell type [125,126] and disease context, and must therefore be carefully considered in the specific context of the HTS assay being developed.

### 3.2. Viscoelasticity

Viscoelasticity is a measure of internal stress dissipation (Figure 3). Changes in viscoelastic parameters accompany diseases such as osteoarthritis [127–129], tissue fibrosis [130–132], and breast cancer [133–141], and designing biomaterials to independently tune and capture the effects of both viscous and elastic properties is important in identifying relevant HTS parameters.

Two general strategies exist to incorporate viscoelasticity into materials. First, for stiffness-tunable materials, adjusting the concentration of crosslinkers to monomers can put the material outside a linear-elastic regime, as demonstrated in 2D cultures on polyacrylamide [142–144], gelatin [145,146] and PDMS [147], due to the formation of defect structures in the poorly connected gel networks [142], or inherent viscoelastic characteristics in crosslinked matrices. Since crosslinking must be carefully limited to produce these hydrogels, their fabrication and gelation often require precise control over temperature and oxygen conditions. Alternatively, the viscosity of the gel's liquid phase can be modified with additives [148–150], without drastically affecting elastic properties, while the liquid phase provides the dissipative element. Although technically simple, these methods do not allow dynamic, on-demand changes in material properties, which have proven useful for many applications [146,151,152].

Customized crosslinkers can also be used to tune viscoelastic properties [153], and these approaches can be compatible with 3D culture formats. Reversible bonds including ionic [154–156], guest-host [152,157], hydrazine/aldehyde [158,159], or thioester [160] crosslinks are some examples. These crosslinkers exhibit half-lives of minutes to months, which enables well-defined control over stress-relaxation rates. However, manipulating crosslinks also effects elastic properties, making it quite challenging to fully de-couple elastic and viscous effects, without incorporating additional covalent bonds, as demonstrated in guest-host hyaluronic acid gels [152] and polyacrylamide [143,144]. Alternatively, alginate hydrogels can be modified with spacers grafted onto the polymer backbones to sterically hinder crosslinking of the alginate chains, with higher spacer densities and lengths both leading to faster stress relaxation [161]; or developed with covalent binding sites and calcium-activated ionic binding sites, to tune how much stress dissipates from the material independently of stiffness [162].

In general, increased stress relaxation prompts increased cell spreading and proliferation for fibroblast-like cells [156,160,161], and myoblasts [159], but decreased spreading in hepatic stellate cells [152] and hMSCs [151]. Once again, these responses seem dependent on cell type, culture dimensionality, and disease context, suggesting that disease-specific experiments are necessary prior to designing appropriate HTS assays.

### 3.3. Mechanical plasticity

While tuning viscoelasticity involves controlling the time-dependent properties, the degree of plastic deformation sustained by a material during these energy dissipation processes is also emerging as a key parameter to consider in designing biomaterial culture platforms. In real tissues such as excised human breast tumor masses, plasticity is observed [163], and mesenchymal cells produce stresses large enough to plastically deform these biomaterials [164–166]. Plasticity in real materials is dependent upon applied stress, stress relaxation times, and mechanical stiffness [86]. Hence, deconstructing the specific role of plasticity in tissue response requires novel biomaterial designs. Alginate polymers have previously been crosslinked with rBM in an interpenetrating network, to tune mechanical plasticity. Changing the molecular weight of the alginate, in combination with the calcium crosslinking density has been shown to independently tune plasticity [163], as does changing the fraction of covalent crosslinks present in the matrix, using enzymatic crosslinking [167]. However, the degree to which this property is distinct from viscoelastic behavior is unclear, as viscoelasticity also involves crosslink breakage and reformation. Viscoelasticity and plasticity thereby have overlapping mechanisms, and local permanent deformation of a material arises when the elastic portion of the hydrogel network is insufficient to enable the return of the material to its original position after unloading.

## 4. Contributions to breast cancer research

Although the concepts of deconstructing various elements of the fibrous tissue microenvironment may seem academic, these fundamental observations of cell–environment interactions suggest important design strategies for future HTS drug platforms. While the deconstruction techniques have been applied in a variety of disease models, here we briefly review their impact on our current understanding of breast cancer, a disease that affects one in eight women in North America, has a 15% mortality rate, and has seen no improvements in survival for women under 50 since 2007 [168]. Current HTS systems are not effective, suggesting the need for more advanced and realistic platforms.

3D culture of breast cancer cells has now been established to influence breast cancer drug screening results. Cells cultured in a fibrous matrix are much less responsive to accepted chemotherapies than in 2D systems [17,59,169–171], and culture in 3D spheroid models confers further resistance [172], perhaps due to differences in proliferation and consequently uptake, for drugs such as paclitaxel that selectively target rapidly-dividing cells [173].

During cancer progression, collagen fiber density and crosslinking increases, matrix pore size decreases, and fiber linearization increases [115,116]. Increased tissue density is generally considered a risk factor for breast cancer disease progression [83], and may also further influence drug uptake by limiting delivery of therapeutics [174] or altering cell function. Drug dosage is an important element of any drug discovery or screening study and must be considered carefully, further supporting the need to conduct testing in realistic environments. Moreover, decreased pore size confines cells to limit spreading and mobility, and when cultured within these confining 3D fibrous meshes, cells appear to compensate by upregulating cell-cell adhesion genes [38] and attaching to each other instead, forming multicellular tubular network structures [37]. Some interpret these structures as resembling lobules and ducts in normal breast tissue [38]; however, based on β1-integrin upregulation in these networks, others suggest this phenotype is more similar to vasculogenic mimicry, which is associated with poor prognosis for breast cancer patients. This suggests that confining architecture is important during metastatic disease progression [37], and should be considered in HTS assays. Fiber linearization is also influential, as directed cell migration is observed on aligned 3-D matrices which enhance the migratory behavior of metastatic cells. This enhanced migratory behavior was lost on isotropic matrices and with non-metastatic lines on pre-aligned matrices [42].

The above architectural changes must be accompanied by changes in fiber mechanics. Importantly, stiffer matrices have been demonstrated to drive an increase in proliferation and invasion amongst nonmalignant cells [110], but seem to have the opposite effect on invasive breast cancer cell lines [111]. Of interest, invasive breast cancer cells may adapt to the surrounding mechanics. When cultured within high density, stiffened collagen, invasive speed is initially slowed [37] similar to their deceleration within synthetically stiffened environments [111]. However, following a cell cycle, the speed of cells greatly increases [37]. This suggests that breast cancer drug screening timelines should last longer than a cell cycle.

Interestingly, the dynamic stiffening of 3D matrices also influences drug response, and invasive breast cancer cell lines show greater chemoresistance within these dynamic cultures than cells cultured in static high-stiffness matrices [175], but no effect was noted on less aggressively invasive cell lines. These effects are not observed in 2D settings, further suggesting the need for disease-specific and stiffness-tunable 3D screens. The fact that dynamic stiffening is required also strongly indicates that viscoelastic and plastic behaviors that contribute to these changes may be important to consider.

Finally, invasive breast cancer cell lines display higher invasive behaviors in highly plastic matrices, such as higher cell spread, motility, and protrusions; even though other matrix properties remained the same [163,167]. Plasticity may hence be an important driver of breast cancer specifically.

## 5. Expert opinion

While the capacity to dissect the microenvironment and fundamentally understand cell-matrix interactions is certainly of academic value, we ask here whether these theoretical insights might allow us to bridge the gap toward practical gains in drug discovery. We believe that while such knowledge translation is both possible and highly desirable, implementing these approaches for next-generation HTS in particular presents unique challenges and opportunities for knowledge acquisition, development of insight, and technological innovation.

First, the technologies developed to disentangle the fibrous microenvironmental parameters simultaneously highlight the importance of these factors, as well as our relatively limited knowledge of their specific impact in various organ systems. The studies conducted to date demonstrate that common rules for all cell types are a myth. Precisely defined microenvironmental cues appear to affect cells differently. For example, endothelial and glioma cells seem to be largely unaffected by the local presence of surrounding fibers [35,39], whereas fibroblasts and breast cancer cells exhibit different morphologies and phenotypes within matrices of different densities. Similarly, while the role of mechanical stiffness has now been well established in a variety of tissues, the effects of viscoelasticity and plasticity have only recently been elucidated, and their importance is tissue-specific and requires further investigation. Moreover, whether temporary viscoelastic deformations are fundamentally different from permanent plastic deformation is unclear, and further fundamental studies are needed in tissue systems specific to the disease being screened.

Second, while these microenvironmental factors have an established impact, it remains unclear what the *in vivo* microenvironmental conditions actually are, particularly during disease progression. For example, breast tumors have demonstrated both greater and lower viscous behavior than healthy tissue [134,135,141], and the factors that cause these changes remain unknown. Changes in fiber composition, density, organization, and crosslinking that lead to these viscous changes are largely speculative, although a few *in silico* and *in vitro* approaches have recently been developed to address this [165,176]. These differences may also arise from measurements made at different length-scales, and we therefore argue that it is important to characterize tissue biophysics at the length scale of individual cells within living tissues undergoing disease progression. To this end, the recent development of cell-sized sensors that can be embedded in human tissue for long-term measurements of force [177–180], stiffness [181,182], and viscoelasticity [183] may prove particularly valuable.

Third, while identifying the fundamental microenvironmental parameters underlying disease progression is a good first step, developing scalable culture systems that implement these specific features for HTS presents unique technological challenges. Running millions of assays in a typical HTS screen requires robustness, reproducibility, automation, and optimization of assay costs and time. In some cases, these fundamental studies immediately provide strategies to scale-up screening: for example, the use of 1D and 2D adhesive patterns on substrates can prompt cells to behave as if they were in 3D [29,45,48,51], and this strategy can be directly applied to conventional HTS microscopes and data analysis workflows. Where fibrous 3D cultures are required, techniques must be developed to address difficulties in forming 3D structures with sufficient throughput, handling these structures during requisite wash steps, and 3D imaging.

Integrating microfabrication technologies with tissue engineering strategies may provide valuable tools to address these challenges. For example, arrays of micro-reservoirs have been developed on-a-chip, into which nanoliter volumes of prepolymerized tissue may be loaded, cultured, and assayed [184,185]. Liquid-in-liquid patterning techniques have been developed to 'print' microscale tissue volumes in existing well-plates, using standard robotic pipetting infrastructure [186]. Similarly, 3D bioprinters now offer such capabilities [187]. Using a microfabricated hydrogel template, tissue-engineered structures such as spheroids [70] and lumenized vessels [23,25] can also be rapidly formed, stimulated, and analyzed without handling issues. These techniques have added advantages of using small volumes of cells and reagents, which reduces assay cost and analysis time, and may ultimately enable precision medicine approaches on a patient-specific level. Finally, considerably more advanced microfabricated platforms (organ-on-a-chip systems, recently reviewed in [188]) are being actively developed by multiple research groups, and may eventually be scalable for HTS applications.

Collectively, these challenges and innovations demonstrate that while umbrella solutions for HTS were initially effective in identifying low-hanging fruit, the development of next-generation therapeutics will require targeted HTS strategies customized to mimic the microenvironment of the specific target disease. The diverse tissue structure in the human body, the variability that arises during disease progression, and the specificity of a cell's interaction with the microenvironment, each suggests that disease-specific HTS approaches are needed. Therefore, we conclude that understanding the tissue microenvironment, incorporating those cues most relevant to the disease of interest, and developing high-throughput microfabricated HTS assays will be required to strip away unnecessary costs and complexities in the drug development pipeline, and focus platforms toward identifying high-value therapeutic targets.

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## Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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