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What lies beyond—Insights into elastic microscaffolds with metamaterial properties for cell studies

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Recent advances in additive manufacturing have opened up new possibilities to print almost arbitrary structures with submicrometer resolution. An intriguing application is the fabrication of metamaterial-based scaffolds with unprecedented precision and with defined effective elastic properties for mechanobiological research. This field of study has already led to promising results but remains wide open. The vast possibilities, together with the high interdisciplinary character and current lack of established protocols or literature on the subject, are intriguing on the one hand but might discourage researchers who are new to this field. In this review, we aim to provide insights into the work with such microstructured biometamaterials, mainly based on our own experience with 2D systems, hoping to encourage further mechanobiological studies. Finally, we present some considerations for expanding to the third dimension to more closely resemble the *in vivo* situation.

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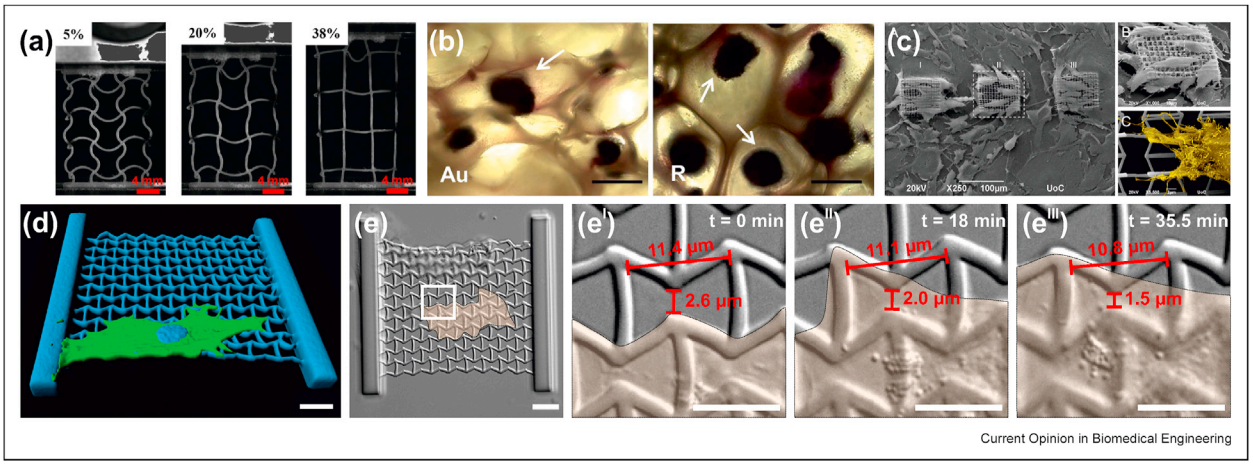
Introduction

Cellular behavior is influenced not only by biochemical cues but also by the physical properties of their microenvironment. In a process termed mechanotransduction, cells translate mechanical signals of their surroundings into biochemical signals, which allows them to adapt to their environment. Despite major progress in controlling the mechanoregulation of cells using hydrogel substrates with different degrees of crosslinking thus changing stiffness [1–4], the potential of nonconventional mechanical materials has often been neglected. With the introduction of a new material class, the so-called metamaterials, previously inaccessible or rare mechanical properties can now be achieved by rational design and appropriate microstructuring. These properties include (but are not limited to) viscoelastic behavior, as exhibited by cells and tissues [5–7], the resistance to shear forces or material characteristics, such as the Poisson's ratio (PR), representing the ratio of the material's transversal deformation to longitudinal deformation. While all of these properties are present *in vivo*, their impact on cell behavior is yet poorly understood. In the recent past, negative Poisson's ratio, as observed for tendons, the tibia bone, or even the skin [8–11], gained increasing attention. For these so-called auxetic materials, a contraction perpendicular to the axis of compression is observed. Because of this unique behavior, their potential medical applications have been discussed in detail in a review by Lvov *et al.* [12]. Only recently, Dong *et al.* explored auxetic composite patches for myocardial damage repair, thus creating an adjustable basis for advancing biomimetics for heart tissue repair [13]. While these materials mostly reside within the macroscopic scale, first efforts in applying auxetic materials for cell studies revealed an impact on cell behavior and stem cell differentiation [14,15]. Furthermore, mesenchymal stem cells as well as fibroblasts were shown to invade into and deform 3D auxetic metamaterials, adapting to their geometries in doing so [16,17]. Exemplary images of these approaches for biological application of auxetic materials on different size scales are given in Figure 1 (a)–(c). Although all of these pose great opportunities for exploring auxetic materials in the biological context, they all possess drawbacks for mechanobiological studies

Figure 1



Examples of auxetic materials in biological applications. (**a**) Cardiac patch with auxetic properties made from polycaprolactone under different strains [13]. (**b**) Aggregates of human-induced pluripotent stem cells in polyurethane foams. Auxetic scaffolds (Au) are obtained by auxetic conversion of regular foams (R). Scale bar: 200 µm [15]. (**c**) NIH3T3 cells cultured on 3D-printed bowtie metamaterials [16]. (**d**) 3D reconstruction of a rat embryonic fibroblast (REF) cell on a bio-metamaterial scaffold. Cell area derived from actin staining is depicted in green, IP-PDMS scaffold and nucleus are depicted in blue Scale bar: 20 µm (e) Differential interference contrast (DIC)-image of a REF cell on a bio-metamaterial scaffold. Scale bar: 20 µm. (e^{I}-e^{III}) Magnified images of the area marked in C at different timepoints during live cell imaging. Deformations of the metamaterial are indicated. Scale bars: 10 µm.

due to either stiff compound materials or pore sizes larger than biological cells.

In 2021, a novel polydimethylsiloxane (PDMS)-based commercial resist termed IP-PDMS opened up new possibilities to generate soft and deformable metamaterials by 3D laser microprinting. Using IP-PDMS-based scaffolds, we fabricated metamaterial nets for single-cell studies. We were able to develop an unprecedented workflow for fabrication of metamaterials and analysis of forces exerted by the cells upon culturing on metamaterial nets [18]. Figure 1 (e)−(e^{III}) shows an exemplary fibroblast cell adhering to and deforming the underlying metamaterial net.

In the following, we would like to share some insights into the work with 2D bio-metamaterials to provide a comprehensive beginners' guide for researchers new to this emerging field of study. Starting from initial metamaterial design, we will address the individual steps involved, including the analysis of the experimental data. As an overview, the key points discussed in the following are summarized in Table 1.

Design

Metamaterials are rationally designed materials with unusual properties that are derived from their microstructure and go beyond those of the constituent material [20]. Some examples are metamaterials with negative refractive indices, ones that expand upon uniform compression, or metamaterials that exhibit mechanical properties of solids and fluids [21−23]. Here, we focus on mechanical metamaterials for biological application as a new tool to mechanically regulate the cell fate.

In order to study the cellular mechanoresponse, it is crucial to precisely control the mechanical environment. Mechanical metamaterials enable such control, without the need to screen various base materials exhibiting the desired properties. The underlying concept of such studies is visualized in Figure 2. In the literature, one can find different materials which, in principle, could be called mechanical "metamaterials", ranging from elastic pillar arrays with defined stiffnesses based on column height [24] to purely geometry-based scaffolds without consideration of effective properties [16,17]. We here refer to metamaterials as microstructured continuous materials (not arrays of individual geometries) characterized by their effective properties. In theory, arbitrary asymmetric geometries could be fabricated [25]. It is apparent that with the free choice of the design of the inner structure of a material, one could therefore, in principle, realize almost any desired properties (obeying conservation of energy, causality, and stability). In practice, however, due to metamaterials typically being highly symmetric with periodic unit cell arrangement, this number can be narrowed down to three parameters per spatial direction: the aforementioned PR, the Young's modulus (YM), which characterizes the material's resistance to axial compression or extension, and the shear modulus, which describes the materials

Table 1

Key considerations for metamaterial-based studies.

	Key considerations	Examples
Design	• Isotropic or anisotropic features • Aimed at effective properties • Unit cell structure based on available designs or design from scratch • Unit cell size	• Auxetic geometries [19] • Pore sizes larger [15] and smaller [16] than biological cell size
Fabrication	• Sufficient resolution • Fabrication time • Limitations in material choice	• Sub-millimeter: extrusion printing [33] • Sub-micron: photolithography [35], 3D laser microprinting [36] • Nanometer: EBID [34]
Material	• Mechanical properties of bulk material • Swelling in aqueous environment • Cytotoxicity • Protein adsorption • Autofluorescence and optical properties	For 3D laser microprinting: • Soft: PEGDA [14], IP-PDMS [18] • Intermediate: Oil ink [49] • Stiff: SZ2080 [16], PETA [18]
Analysis	• Reliable and comprehensive computational approaches • Appropriate control experiments to allow interpretation of results	• Finite element method [17,18] • Nematic actin order parameter [18,55] • YAP distribution [18,56,57]

EBID, electron-beam-induced deposition; PEGDA, poly(ethylene glycol) diacrylate; PETA, pentaerythritol triacrylate; YAP, yes-associated protein.

response to shear forces. For anisotropic materials, the effective parameters are highly anisotropic too. For the ease of understanding, a simplified visualization of these descriptors of material properties is given in the box in Figure 2.

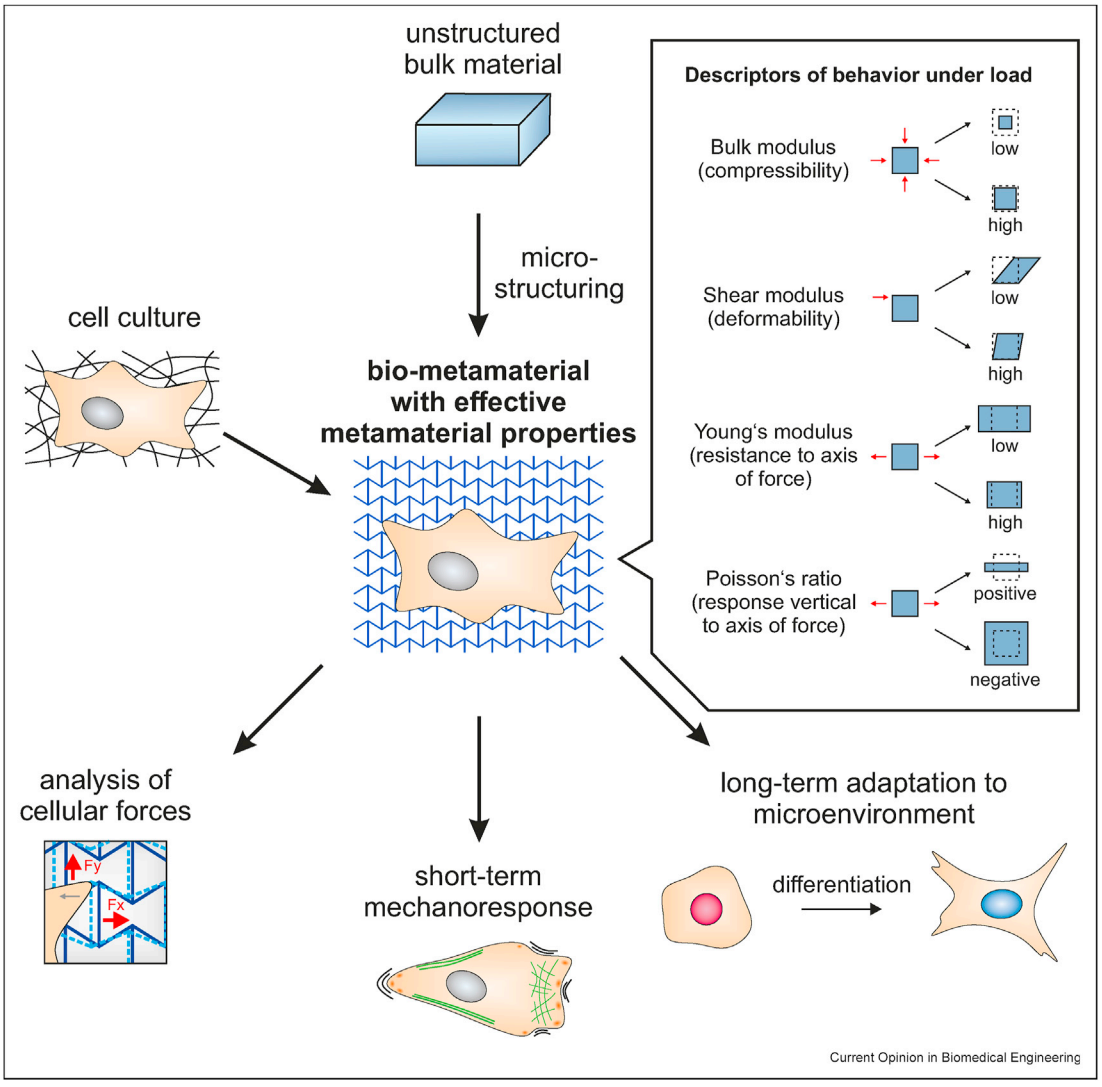
Despite the theoretical possibility for a 2D metamaterial to tune these six parameters independently, it is geometrically not trivial to design arbitrary combinations of them. Usually, the PR and YM influence the shear modulus, rendering it dependent on the other two parameters. To design a metamaterial with desired mechanical parameters in the vast space of possible geometries is challenging. Generally, this is an inverse problem because there can be multiple geometries leading to the same effective parameters. This nonunique mapping means that solving the inverse problem often requires exploring many possible solutions, rather than finding a single, straightforward answer. Typically, metamaterials are designed by modifying existing designs, by physical intuition, and with the help of simulations [26]. Topology optimization has been adopted as a method to solve the inverse problem [27]. However, the method is computationally demanding due to its iterative optimization nature. This makes topology optimization less attractive for biomaterial applications in which one aims at solving the inverse problem many times for a certain class of metamaterials and properties. Machine learning is a new direction for the inverse problem [28]. Here, a large computational effort is needed once to set up a metamaterial database and to train a neural network for the inverse problem. However, once the neural network is well-trained, metamaterials with targeted effective mechanical properties can be rapidly solved in under a second. One should be aware of several aspects: first, a correct architecture for the inverse neural network is critical for its efficiency. Suitable choices include the Generative Adversarial Networks (GANs) [29] or the conditional Variational Auto-Encoder (cVAE) [30]. Second, the designed metamaterials from neural networks cannot be 100% faithful. It is necessary to double-check the effective properties by using a faithful numerical homogenization method. Lastly, machine learning can be very efficient in interpolating between entries of an underlying database, but it typically does not efficiently extrapolate. Therefore, it is crucial to start from a good database covering the relevant target property space.

Another point to consider is the unit cell size. Interesting mechanobiological effects can be observed when biological cells do not feel the underlying microstructure but experience the resulting effective mechanical properties. This requires the unit cells of the metamaterial to be sufficiently smaller than the biological cell itself. We found that roughly 3–5 times smaller unit cells are sufficient to induce a mechanoresponse in mesenchymal stem cells [18]. However, this might differ for other cell types and metamaterials. Additionally, the filling fraction, referring to the proportion of the material's volume consisting of constituent material, must be kept constant between different designs for an unbiased comparison. Previous micropatterning approaches revealed a dependency of cell adhesion on adhesive area, thus posing another influencing factor [31,32].

Fabrication

Once the metamaterial design is decided on, the question of fabrication arises. The optimal fabrication method depends on the desired spatial resolution and

Figure 2



Underlying concept of bio-metamaterials for mechanobiological cell studies. The mechanical properties of a bulk material can be altered by appropriate microstructuring. The resulting effective properties can include unusual or previously inaccessible mechanical behavior, rendering such bio-metamaterials an interesting tool for the use in mechanobiological studies. Perspectives for analysis include forces exerted onto the metamaterial or the response to said effective mechanical properties on shorter time scales. For longer culture periods, the adaption to the microenvironment such as the selective differentiation of stem cells can be a potential field of study.

Table 2

Key characteristics of fabrication methods for 2.5D/3D scaffolds.

	Extrusion printing	3D laser microprinting	Photolithography	EBID
Resolution	~50 μm	~500 nm	Few nm	~1 nm
Speed	Depends on aimed at resolution	Up to ~500 nm/s	Parallel illumination, seconds to minutes	~10 nm/s
Materials	Polymers, cells	Mostly polymers	Mostly polymers	Mostly metals
Advantages	Material versatility, low cost	Truly 3D	Fast due to parallelization	Very high resolution
Disadvantages	Comparably low resolution	Requires powerful lasers	Only 2D/2.5D	Requires SEM or STEM, biocompatibility of metals
References	[42,43]	[41]	[47,48]	[44–46]

EBID, electron-beam-induced deposition; SEM, scanning electron microscopy; STEM, scanning transmission electron microscopy.

structure size of the metamaterial, oftentimes accompanied by certain restrictions in ingredient material choices.

Large metamaterials with large unit cells can be fabricated by, e.g. extrusion printing with a resolution in the submillimeter range [33]. At the other end of the spectrum, electron-beam-induced deposition (EBID) allows for nanometer resolution [34]. However, this technique is mostly applicable to metals and has a low deposition rate. Large arrays of 2D or 2.5D structures with a submicron resolution can be fabricated by photolithography [35]. Here, masks are used to expose defined regions of resists to ultraviolet light to create the desired structure. However, this technique cannot produce arbitrary 3D structures and requires new masks for each new design.

With a resolution well below the size of biological cells and the possibility to rapidly print polymers, 3D laser microprinting is a reasonable choice for fabricating metamaterials for mechanobiological experiments. In 3D laser microprinting, a laser beam is scanned through a liquid photoresist, which polymerizes upon energy deposition of the laser. The remaining liquid resist is then washed away during a developing step in a solvent. It is apparent that, apart from hollow structures, in theory, any shape can be realized, including overhanging or interlocking parts [36]. The sequential laser scanning with high resolution can lead to a fabrication time in the region of minutes to hours for millimeter-sized samples. 3D laser microprinting has already been used to print structures in a biological environment and for biological research [18,37–39]. The resolution and printing speed of 3D laser microprinting depends on the photoresist, wavelength, and optical setup. However, the wavelength-dependent limitation of the printing resolution has already been overcome by the use of stimulated emission depletion (STED). [40]. The resolution of 3D laser microprinting is a few hundred nanometers, allowing for unit cell and feature sizes smaller than the typical size of cells. A comparison of key characteristics of the mentioned fabrication methods is given in Table 2.

Material requirements

To narrow down the choice of materials, we focus on polymers in the context of 3D laser microprinting. Although some of the discussed points may be specific for this application, others might apply universally.

Mechanical properties of base materials

The question on what polymer to choose depends on the aimed-at application. For measuring cellular forces, the photoresist should be sufficiently soft to allow deformation. By varying the degree of crosslinking during printing by altering laser power and scan speed, the stiffness of the printed structure can be further reduced [50]. Especially for less crosslinked materials, further softening might occur due to incorporation of the aqueous medium, which is required for maintenance of cells [51]. This can lead to swelling, potentially distorting the initial metamaterial design. Depending on the degree of swelling, this might interfere with the aimed-at effective properties.

Cell culture compatibility

Apart from structure quality and stability, a few more considerations arise from the cell biology perspective. In the case of 3D laser microprinting, most resists consist of a monomer to which a suitable photoinitiator is added. Especially the latter oftentimes induces cytotoxic effects [52]. To avoid this, thorough removal of unpolymerized resist and nonreacted initiator or the use of biocompatible initiator systems is recommended [53]. To perform cell studies on any printed material, the cells need to be able to attach to the structure. *In vivo*, cells attach to a meshwork of proteins *via* the so-called focal adhesions. Here, integrin receptors bind to motifs of extracellular matrix proteins [54]. Hence, to facilitate adhesion of cells to 3D structures, the easiest way to achieve this is by coating the structure with an extracellular matrix protein such as fibronectin *via* physisorption from aqueous solution to the polymer.

Compatibility with microscopic observation

In order to properly image cells and scaffolds together, the printed structures should be optically clear and not exhibit extensive diffraction. This holds especially true for microscopy techniques such as phase contrast or differential interference contrast microscopy. Depending on the microscopy setup and desired magnification, height restrictions might occur due to the working distance of the objective and increased optical aberrations. In cell biology, samples are often fixed and embedded onto a microscopy slide. To bypass height limitations, samples can be embedded between two thin coverslips rather than on a thick microscopy slide to allow imaging from either side.

A common method to visualize cellular markers is fluorescent labeling of proteins. While many fluorophores are commercially available, the choice is often restricted by the excitation wavelengths of the available microscope. This choice can be further limited, if the printed scaffold exhibits autofluorescence over a broad wavelength region. To avoid this, selecting a resist with low autofluorescence or using postprint bleaching may be helpful.

Analysis and interpretation

While bio-metamaterials provide new possibilities for cell studies, this field is equally unexplored. On the one

hand, this may give rise to many new insights; on the other hand, there are basically no established guidelines or protocols on how to analyze or handle the obtained data. Compared to other, more straightforward approaches, the complexity of metamaterial architecture can make it difficult to translate observed effects into biological explanations. Previous endeavors mainly focused on alignment of cells with the structure geometry and the degree of deflection/deformation of the printed scaffold. In our proof-of-concept publication [18], we added another dimension to this analysis by deriving force fields from structure deformations *via* numerical calculations. In the following, we want to highlight a few of the challenges that we faced when analyzing the effects of metamaterials on cells.

Numerical force calculation

In the first step, the experimentally observed displacements were translated into deformation vectors based on image-cross-correlation analysis. Because of the periodic nature of the metamaterial and the dynamic change in deformation, the correct tracking of markers can be difficult. This aspect can be improved by choosing unique and recognizable areas in the lattice, e.g. crossings, but requires careful control of the obtained displacement vectors. Especially in case of very large or very small deformations, a faulty tracking can occur.

For the calculation of the applied force field, ideally, the effective material properties can be measured and serve as a baseline for the numerical analysis. However, due to the relatively low filling fraction, the high elasticity of the resist, and the aqueous environment, this proved difficult in our case. To nonetheless reflect the correct effective material properties, the theoretical metamaterial properties, the bulk material characteristics, and the boundary restrictions need to be considered. While the YM of the bulk material can be measured by, e.g. atomic force microscopy (AFM) nanoindentation, boundary restrictions and metamaterial characteristics are typically harder to access. In our case, the beams for suspension of the metamaterial induced a stiff boundary in one axis, which introduces a high anisotropy in the structure, and hence needed to be included in the numerical calculations. Additionally, in the case of anisotropic metamaterial designs, one might need to spend some thought on which axis should be analyzed to connect metamaterial characteristics and experimental deformations.

Biological observations

From a biological viewpoint, a few more challenges arise in addition to the force-field analysis. It is well known, that many cells show increased spreading on and migrate toward stiffer substrates [4], which might lead to an unfavorable localization of the cells in multimaterial scaffolds, thus reducing experimental output. This can be reduced by using a protein-repellent resist where applicable. Furthermore, while the cellular response to different YMs is addressed in many publications [24,58–61], the influence of physical characteristics such as PR remains mostly unknown, making it difficult to interpret experimental observations or even spot the caused effects. Furthermore, cells are susceptible to changes in their adhesion geometry [62], which poses another challenge interpreting the obtained results. Combined, these effects require a well-designed strategy and proper control experiments to entangle the influences of different parameters such as anisotropy, geometry, or filling fraction.

Toward 3D

While 2D structures serve as helpful tools to gain an initial understanding of the cellular response to metamaterials, 3D environments certainly allow for more accurate simulation of the *in vivo* situation [63]. A recent study was successful in using a 3D metamaterial environment to influence osteogenic differentiation and matrix mineralization of preosteoblast cells [64]. When transitioning to 3D, the third dimension introduces yet more challenges in addition to common considerations for 3D scaffolds and 3D cell culture, which are briefly discussed in the following.

Design

When turning toward truly 3D designs, structure stability can pose an additional restraint. Especially in case of column-like designs, the ratio of the height to the area connected to the substrate is crucial to prevent tumbling over or detaching of the structure. For the cells to experience the 3D environment, the unit cells need to be sufficiently large for the cells to penetrate the scaffold but small enough to prevent "falling through" to the bottom of the scaffold. This is mainly restricted by the size of the cell nucleus, which is only deformable to some degree. Furthermore, as stated for the 2D case, unit cells should be smaller than the biological cell for it to experience metamaterial properties.

Additionally, even more possible designs and effective parameters are realizable than in 2D. However, choosing highly anisotropic designs might make it more difficult to extract the influence of each parameter.

Fabrication

With 3D laser microprinting, 3D structures with submicrometer resolution can be realized. With soft photoresist and a porous structure, however, the printed 3D object might buckle. Fabricating microstructures with increased height is possible, but it imposes some constraints. To use the full range in the Z-direction, structures need to be printed in dip-in mode, where the photoresist is put directly on the objective lens. The refractive index of the lens and that of the resist have to match to not induce optical distortions.

Imaging and analysis

To properly image deformations of 3D metamaterials, advanced microscopy techniques are required. Depending on the experimental conditions, confocal optical microscopy might suffice, whereas in other cases, techniques such as light-sheet optical microscopy might be necessary to achieve reasonable imaging speed. While endpoint analysis might still be easier to achieve, the extraction of dynamic displacements or force fields of 3D data sets, as shown for the 2D case, comes with a huge increase in computational cost and might be limited. Especially when it comes to interpretation of the results, one might want to consider the relatively limited knowledge of 3D mechanobiology. While this appears intriguing on the one hand, it might be harder to make sense of the obtained observations.

Conclusion

In conclusion, bio-metamaterials offer an interesting and mostly unexplored playground for cell-biological experiments. Because of the sheer endless possibilities in geometries, they allow the realization of a huge space of effective properties — even for using only a single fixed ingredient or constituent material. Thus, metamaterials allow for rapid testing of a multitude of properties without the need to search for new materials and repeated adaptation of fabrication strategies. However, as a relatively new field of study with a highly interdisciplinary character, interpretation of results or starting points for analysis might be difficult to find. As there are little references or protocols available, this field is as scientifically intriguing as challenging to enter. We hope this review might motivate more researchers to nonetheless use these promising materials in their own research, so that with a growing pool of insights, a few of these challenges might subside and allow accessing the full potential of these bio-metamaterials.

Declaration of competing interest

The authors report no declarations of interest.

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Data availability

Data will be made available on request.

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