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Review of state-of-the-art micro and macro-bioreactors for the intervertebral disc

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ABSTRACT

Lower back pain continues to be a global epidemic, limiting quality of life and ability to work, due in large part to symptomatic disc degeneration. Development of more effective and less invasive biological strategies are needed to treat disc degeneration. In vitro models such as macro- or micro-bioreactors or mechanically active organ-chips hold great promise in reducing the need for animal studies that may have limited clinical translatability, due to harsher and more complex mechanical loading environments in human discs than in most animal models. This review highlights the complex loading conditions of the disc in situ, evaluates state-of-the-art designs for applying such complex loads across multiple length scales, from macro-bioreactors that load whole discs to organ-chips that aim to replicate cellular or engineered tissue loading. Emphasis was placed on the rapidly evolving more customizable organ-chips, given their greater potential for studying the progression and treatment of symptomatic disc degeneration. Lastly, this review identifies new trends and challenges for using organ-chips to assess therapeutic strategies.

1 Introduction

Lower back pain (LBP) is a leading cause of physical disability worldwide. In the United States, annual costs associated with LBP are greater than $100 billion, as more than 70% of Americans experience debilitating back pain at least once in their lifespan, limiting quality of life and ability to work. [Katz, 2006, Rubin, 2007, Vos et al., 2012, Wu et al., 2020] Causes for LBP are multifactorial and include risk factors such as smoking tobacco, obesity, diabetes, occupation, fat infiltration of muscles, facet degeneration, and most commonly, disc degeneration. [Zhou et al., 2023, Frymoyer et al., 1983, Kalichman et al., 2010, Lewinnek and Warfield, 1986, Teichtahl et al., 2015, Zhang et al., 2018, Alpantaki et al., 2019, Battié and Videman, 2006, Miller et al., 1988, Powell et al., 1986] Disc degeneration has been diagnosed in nearly 40% of LBP patients with higher prevalence in elderly patients (70-75%). [Peh, 2005, Schwarzer et al., 1995, Teraguchi et al., 2014] Obesity, occupation, and recreational activity are all linked to excessive loads on the spine. For example, competitive rowers are five times more likely to have lumbar disc degeneration than the general public due to repetitive high loads on the disc in a flexed position. [Cross et al., 2014, Hosea and Hannafin, 2012, Reid and Mcnair, 2000]

The intervertebral disc includes the annulus fibrosus (AF), a highly organized multi-layered collagenous tissue made up of fibroblast-like cells that surrounds and supports a gelatinous nucleus pulposus (NP). [White and Panjabi, 1978, Zeldin et al., 2020] The healthy disc provides flexibility and structural support during complex loading conditions experienced

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with daily activities. As the disc is compressed through muscle forces, the AF acts like a thick-walled pressurized vessel with fibers being engaged through hoop tensile stresses, creating multiaxial or complex stresses at the cellular level (see Fig. 1). [Chan et al., 2011] As the disc undergoes degenerative remodeling, NP pressure decreases resulting in the AF absorbing greater stresses during daily activities, which can lead to AF buckling or bulging, narrowing of the spinal canal, and nerve impingement. [Kalichman et al., 2010, Luoma et al., 2000, O'Connell et al., 2007, O'Connell et al., 2015, Will et al., 2018, Stefanakis et al., 2014, White and Panjabi, 1978]

Current treatment strategies for debilitating LBP are limited and include non-surgical options such as Non-Steroidal Anti-Inflammatory Drugs (NSAIDS), physical therapy, or steroid blockers. [Padayachee et al., 2018] For advanced symptomatic degeneration, the gold standard for treatment is removal of the disc, followed by fusion of the spinal section, reducing flexibility. There has been growing interest in developing minimally invasive biological strategies to treat symptomatic disc degeneration, including molecular and cell-based therapies, or injectable biomaterials; [Ju et al., 2020,Loibl et al., 2019,Sampara et al., 2018, Schmitz et al., 2020, Vadalà et al., 2019, Yamada et al., 2022] however, clinical uses of these approaches have been limited.

Preclinical models are important for assessing new therapeutic options. Commonly, animal models are used to study new therapeutics by first inducing disc degeneration. These studies can be costly, under-powered for strong statistical analysis, and the method used to create disc degeneration may not accurately represent degeneration in humans. [Poletto et al., 2023] Moreover, species-dependent differences (e.g., presence of regenerative notochordal cells) can result in limited translational value from *in vivo* models to clinical observations. [Loibl et al., 2019, Maltman and Przyborski, 2010] Species-related differences are partially due to the larger size of human discs, resulting in a harsher mechanical and chemical environment with worse nutrient transport that may limit the lifespan of any injected cells. [Peng and Li, 2022] As the FDA (Food and Drug Administration) moves away from requiring animal studies prior to clinical trials, there is a greater need for *in vitro* model systems that accurately replicate the human disc microenvironment. [Wadman, 2023] In the past couple of decades, bioreactors have been used to simulate the harsh microenvironment of the degenerated human disc, and these models are becoming increasingly important for understanding disease progression, drug screening, and tissue regeneration. [Baumgartner et al., 2021, Sanapati et al., 2018, Peroglio et al., 2018, Tang et al., 2022]

While each aspect of the microenvironment is important for cell health and the degenerative process, this review focuses on bioreactor platform design for replicating the complex mechanical microenvironment. Non-mechanical or chemical cues (e.g., osmolarity oxygenation, nutrients, inflammatory compounds, pH) were not included since they can be changed with culture media or incubator conditions. [O'Connell et al., 2014, Wuertz et al., 2007, Bush et al., 2005, Griffith and Swartz, 2006, Neidlinger-Wilke et al., 2012]

Bioreactor designs were reviewed and categorized as either a micro- or macro-bioreactor, providing a multiscale perspective. Macro-bioreactors or *ex vivo* organ-culture models refer to whole disc or tissue culture, whereas organ-chips refer to mechanically active organ-chips. This review highlights the importance of complex loading in the disc, evaluates state-of-the-art designs for applying such complex loads across multiple length scales, and identifies new trends and challenges for using mechanically active devices to assess therapeutic strategies.

2 Considerations for Designing a Disc Bioreactor of Any Scale

Complex loading conditions in the disc have been investigated by tracking intradiscal tissue movement during six-degree-of-freedom loading. [Ahmed et al., 2019, Amin et al., 2019] Finite element models coupled with noninvasive imaging (e.g., Magnetic Resonance Imaging (MRI)) have also been used to elucidate intradiscal loading. [O'Connell et al., 2007,Zhou et al., 2021a] Such studies help establish the range of multiaxial strains experienced during physiological loading and define the range of strains that should be replicated in a bioreactor system. As disc degeneration progresses, AF and NP cells are exposed to more hypoxic conditions, hyperosmotic stresses, greater strains, and deficiencies in nutrient delivery and waste removal. [Loibl et al., 2019]

Loading type and magnitude determines whether a cell will have a catabolic or anabolic response. For example, dynamic loading to moderate physiological strains (e.g. 5% strain) creates an anabolic response, while high strains (e.g. 20% strain) or prolonged static loading ($>$ 24 hours) leads to a catabolic response. [Vadalà et al., 2019, Vergroesen et al., 2015, Gawri et al., 2014] However, accurately accounting for the strains applied to cells in macro-bioreactors or organ-chips is not straightforward, as anywhere from 50 to 80% of applied strain is transferred from the 2D substrate to the cells (i.e., 50-80% Strain Transfer Ratio (STR)). The STR of *in vitro* systems does not replicate *in situ* conditions as seen with chondrocytes, where the extracellular matrix provides protection from cellular overloading through strain transfer attenuation (30-65% STR). [Gilchrist et al., 2007, Han et al., 2013, Lee et al., 2018] The effect of STR can be accounted for by reducing the applied strain or by choosing alternative cellular models that would allow for 3D culture.

Cellular orientation can also be effected by loading, as demonstrated by Abbott et al. who showed AF cell actin and focal adhesion redistribution within 24 hours of shifting the cyclic load path. [Abbott et al., 2012] Work by Chen et al. showed how cell orientation differs in 2D and 3D environments for cells responding to the same load. Rat cardiac fibroblasts loaded uniaxially on an elastic 2D substrate (Figure 3A) aligned perpendicularly to the loading direction, while cells in a 3D

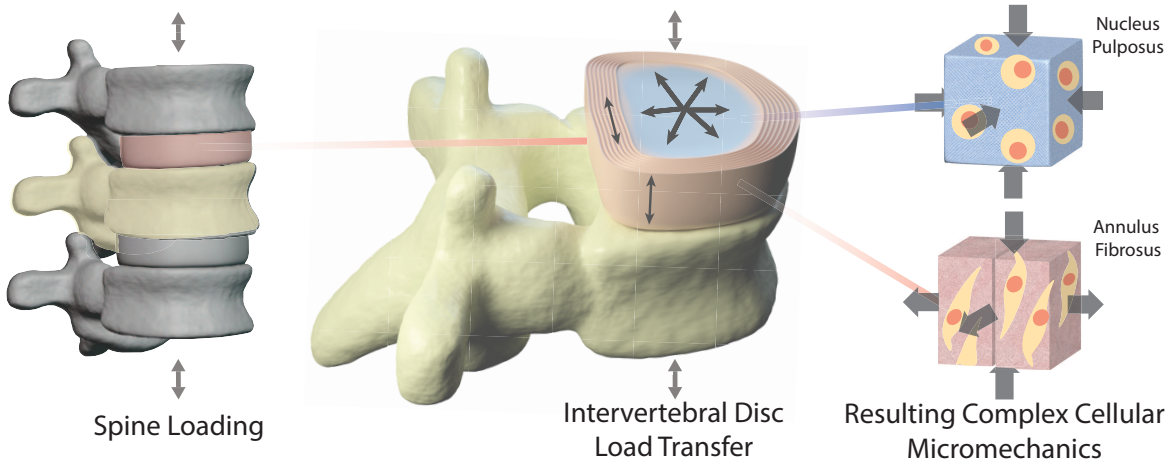

Fig. 1. Multiscale loading in the disc. Compressive forces applied at the organ-level results in compressive, tensile, and shear stresses throughout the NP and AF, which are experienced by embedded cells. In the healthy disc, the AF experiences both direct axial compression and tensile stresses due to internal pressure from the NP. Furthermore, off-axis loading is created from supporting trunk muscles during cyclic bending or axial rotation during diurnal loading (represented with double-sided arrows).

collagen gel aligned parallel to the loading direction as expected from native tissues. [Chen et al., 2018]

More than just alignment, previous studies show a relationship between cell phenotype and applied mechanical loading. [Lee et al., 2015, Ujihara et al., 2015] NP cells are embedded within a tissue without strong collagen alignment and therefore have a rounder morphology that fluctuates with diurnal loading due to changes in hydrostatic pressure. [Wilke et al., 1999] Meanwhile, work by Bonnevie and coworkers showed that rabbit AF cells lose the healthy spindle-like morphology and exhibit signs of degeneration such as spread morphology and apoptosis, after AF fiber strain and alignment is altered due to damage. [Bonnevie et al., 2019]

Additionally, cell response depends on the stiffness of the surrounding material. 2D monolayer culture on stiff tissue culture plastic causes cells to put more energy towards cell proliferation than matrix production, and results in cell spreading, dedifferentiation, localized cell alignment and elongation at confluence. [Lindberg et al., 2023] Gruber et al. showed that human AF cells in 3D gels in static culture led to greater collagen I and II production than human AF cells in 2D monolayer static culture. [Gruber and Hanley, 2000]

Collagen fiber orientation and fiber engagement play an important role on cell morphology and behavior. AF cells align parallel to the highly aligned collagen fibers. [Torre et al., 2019] For this reason, collagen fibers or electrospun fibers have been added to engineered tissues to encourage cell contraction and alignment in 3D culture, including disc implants. [Mauck et al., 2009] When large diameter fibers are prearranged to act like cell substrates, fibroblast orientation and expression of collagen-I are altered which can dictate complex load paths. [Eichinger et al., 2021, Mascharak et al., 2017, Zhou et al., 2021b] Additionally, Bowles and coworkers showed that in some cases cells can align fibers instead of the other way around. When ovine AF cells were embedded in a ring-shaped collagen gel around a porous rigid or soft core during a collagen gel contraction assay, the gel contracted (80% reduction of area), and both cells and collagen fibrils aligned along the circumferential direction having started in a random pattern. [Bowles et al., 2010, Zhang et al., 2019]

3 Macro-Bioreactors for Disc Culture

Macro-bioreactors or organ-culture for the disc simulate loads experienced by the human spine during daily activity using cell-based engineered scaffolds or whole discs (Fig. 2 – bottom half). [Ahmed et al., 2019] Macro-bioreactors load the entire excised disc from a cadaver, along with part of the superior and inferior vertebral bodies. These devices can culture cadaveric human discs for an extended period of time (i.e., weeks or months) to study progression of disc degeneration. Such systems are ideal for isolating disc response to an injectable treatment or an inflammatory culture condition with physiological loading. [Du et al., 2020, Navone et al., 2018] Despite differences in cellular response between human discs and other species, researchers have cultured rat, ovine, caprine, and bovine along with human discs, in addition to engineered disc-like constructs with human cells. [Jünger et al., 2009, Ahmed et al., 2019, Gantenbein et al., 2015, Daly et al., 2016, O'Connell et al., 2007] Many macro-bioreactors ensure nutrient diffusion and nonuniform tissue strain by application of dynamic uniaxial compression. [Costi et al., 2011, Wall et al., 2007] In these examples, uniaxial loading is applied (e.g.,

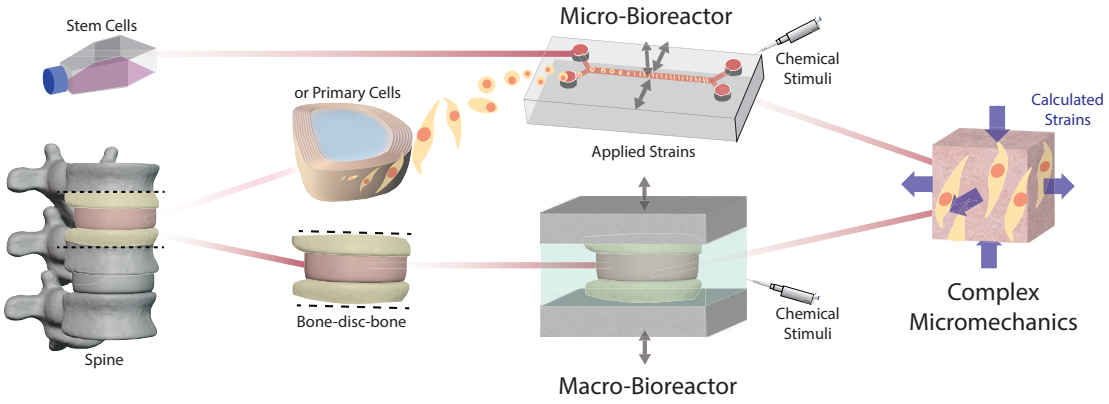

Fig. 2. Mechanically active devices for the disc are categorized as either macro-bioreactors for whole discs or micro-bioreactors for disc cells, otherwise known as mechanically active organ-chips. Grey arrows indicate either applied loads, displacements, or strains of known magnitude, direction, and frequency. Blue arrows indicate unknown strains that need to be measured or calculated.

axial compression alone) while strain magnitude and frequency are varied while comparing to a control group under static loading conditions.

Few studies have evaluated differences between single and multi-loading modalities (e.g., combinations of tension, compression, and/or shear) on cell response and tissue production. Studies that decouple complex mechanical stimuli into its simple components help identify benefits of complex loading with respect to tissue health and integrity, such as cell viability and tissue remodeling. A whole disc macro-bioreactor was designed to apply bilateral bending with compression on human discs. When bovine discs were used for initial testing, better cell viability was achieved after 14 days than static culture at the same time interval. [Beatty et al., 2016] However, more work is needed to better match *in vivo* loading conditions (magnitude and frequency) with *ex vivo* conditions set in a macro-bioreactor.

In a separate whole disc complex loading counter example, Chen and coworkers applied uniaxial compression (0.2 MPa static or 0.6 $\pm$ 0.2 MPa at 0.2 Hz dynamic) plus axial rotation or torsion ($\pm 2^o$, 0.2Hz) which lead to a decrease in cell viability in bovine discs compared to compression or torsion alone. [Chan et al., 2013] At 2-weeks in culture, cell viability in the AF was approximately 70% for all loading groups and was not statistically different from fresh tissue. However, the most complex loading regime (cyclic compression combined with cyclic torsion) lead to $>$ 80% decrease in NP cell viability and a change in NP cell morphology from rounded chondrocyte-like cells to spindle shaped cells. Upregulation of genes related to matrix remodeling (collagen, biglycan, MMP13, and ADAMTS4) in the AF under dynamic compression with torsion suggests that such a system may be useful for studying the impact of excessive spinal loading (e.g., higher loads or more dynamic loads of construction or factory workers).

A whole disc macro-bioreactor has also been used to model trauma to human discs by subjecting them to acute high speed and high magnitude impacts (30% compression in one second). Significant cell death, loss of GAG, damage to aggrecan, neurite sprouting with increased nerve growth factor and cartilage endplate cracking resulted. [Alkhatib et al., 2014]

3.1 Challenges of Disc Macro-bioreactors

Culturing the entire disc with attached endplates presents significant challenges in sample preparation, including maintaining sterility, preventing damage to the endplate, providing adequate nutrient diffusion throughout the disc during culture, and replicating physiological loading conditions. [Chan and Gantenbein-Ritter, 2012] To achieve proper fixation between the mechanical loading device and the sample which is necessary for replicating physiological loading conditions, endplates are carved and potted in bone cement to form an artificial endplate. [Secerovic et al., 2022] As a result, the healthy nutrient flow through the endplate to the NP or inner AF is disrupted, replicating a culture condition that is similar to discs with calcified endplates. [Jackson et al., 2011] Without access to capillary beds in the endplates of adjacent vertebral bodies for nutrient and metabolite exchange by way of diffusion (small molecules) and mechanically driven convection (large molecules) the disc is starved of nutrients. [Martin et al., 2022, Urban et al., 2004] While this can mimic endplate degeneration such as calcification, the lifespan of disc cells are limited. [Haglund et al., 2011] To mimic fluid exchange seen *in vivo*, platens from the testing equipment can be made porous for partial nutrient diffusion or entirely different methods such as rotating bioreactors can be considered to extend cell life. [Stannard et al., 2012]

Macro-bioreactors, however, need to be static to apply loading. These static reservoirs of media can lead to free swelling of the disc which can alter load transfer from the disc to the cells and can change biochemical response including GAG

production. [Ishihara et al., 1997] This free swelling can be addressed with precise control of annulus fibrosus osmolarity for accurate tissue mechanics and composition. [Werbner et al., 2022]

While the whole disc organ-culture models provide the most physiologically relevant modeling of tissue structure and function, the study of cell response is limited to post experiment analysis, such as histology and gene expression. Moreover, macro-bioreactor tests tend to run much longer than conventional cell culture (e.g., weeks rather than days), which increases the risk of contamination and decreases throughput. Lastly, with an already complex system, whole disc models rarely incorporate additional cells types that may be crucial for studying disease and pain progression (e.g., immune cells). [Gantenbein et al., 2015] To eliminate the need for whole disc culture, researchers have been harvesting various types of cells to include in a single chip that aims to represent the mechanical and cellular environment of a specific organ.

4 Current Micro-bioreactors or Mechanically Active Organ-chips

Micro-bioreactors, commonly known as mechanically active organ-chips, offer control over nutrient flow, drug delivery and applied strains (Fig. 2 - top half). [Figallo et al., 2007] Organ-chips are often designed with deformable microporous polydimethylsiloxane (PDMS) membranes as cell substrates to apply strains using pneumatics that mimic physiological loading. [Huh et al., 2012, Huh, 2015, Thompson et al., 2020] Such organ-chips have multiple use cases including drug discovery, drug dosing/toxicology, and studying biological mechanisms of disease progression with human cells, which helps to improve translatability when compared to rodent models.

Compared to organ-culture systems, organ-chips only include the most relevant tissue and functional responses. [Leung et al., 2022] Devices include micron-sized channels, where 10-1000+ cells can be cultured and capillary-like system to deliver specific fluids can be created. [Griffith et al., 2020, Young and Beebe, 2010] In addition, mechanical loading can be applied with real-time monitoring of cell behavior (e.g., migration or change in morphology/strain). By using a similar manufacturing process used for computer chips, an array of dozens of devices within a relatively small footprint (size of postage stamp) can be created. As a result, researchers can greatly increase the number of replicates for higher statistical power. The multitude of devices also enables sensitivity studies across given variables, such as strain magnitude. [Lee et al., 2018]

Organ-chips have been used to study several other mechanically active tissues such as blood vessels and alveolar sacs under fluid shear and tensile strain respectively; however, there has been limited research for studying the disc. [Thompson et al., 2020, Mainardi et al., 2021] To recapitulate disc degeneration, organ-chips need to include the inflammatory response and tissue remodeling in the presence of dynamic loading. Compared to whole disc organ-culture models, at first glance organ-chips can be more accessible as human disc cell donations are more frequent than whole discs given the greater prevalence of procedures that excise disc tissue (e.g., discectomies and spinal fusion) compared to cadaveric harvesting. [Wuertz et al., 2007] However, the disease state of the tissue at harvest continues to be a consideration as it may alter cell response to treatments that may be ideal for early-stage degeneration but ineffective for later-stage disease.

Organ-chips have yet to be designed for the intervertebral disc, however, the more common musculoskeletal organ-chips could be adapted given similarities in applied loads, cell type, structure, and functionality. [Skommer and Wlodkowic, 2015, Mainardi et al., 2021]

4.1 General Advancements in Mechanically Active Organ-chips

Since the NP is constrained by stiffer cartilage endplates and the deformable AF, confined compression loading may be more physiologically relevant for NP cells embedded within a hydrogel. Full confinement of a high aspect ratio micro tissue on three sides with a rigid yet deformable fourth side enabled the cartilage-on-a-chip to apply uniform strain (upwards of 30% compression) along the length of a gel to study pathogenesis (Fig. 3 - C). [Occhetta et al., 2019] However, a confined chamber may limit nutrient transport. Therefore, undersized gaps between the cell chamber walls and scaffold act to mimic capillaries in the body. When hyperphysiological compression is applied, cells exhibit signs of catabolism and inflammation similar to the clinical outcomes of osteoarthritis. With respect to tuning the device for use in the disc, similar compressive loads (both static and dynamic) could be applied to NP and inner AF cells. The ability to apply hyperphysiological loads (20% strain) drives cytokine and inflammatory responses important for studying disc degeneration. [Gawri et al., 2014]

In 2019, an organ-chip was created to apply complex loads on hydrogels. Lee et al. built a microfluidic platform that applied varying magnitudes of cyclic load (compressive strain up to 34%) across an array of alginate scaffolds embedded with chondrocytes (5×5 separate gels; Figure 3B). [Lee et al., 2019] The spacing of the array allowed sufficient fluid flow around each specimen to remove waste, distribute nutrients or administer drugs. The resulting unconfined compression loading is a scaled down yet comparable version of the well-researched macro-bioreactors used to improve *de novo* tissue production (Fig. 3 - B). [Ahmed et al., 2019] Furthermore, strain attenuation from the device was comparable to *in vivo* observations, where 34% compression from the device resulted in 16% strain on the cell (50% STR). This kind of device would enable the quick replication of testing samples in the disc.

Under unconfined tension or compression, the transverse direction experiences compression or tension, respectively.

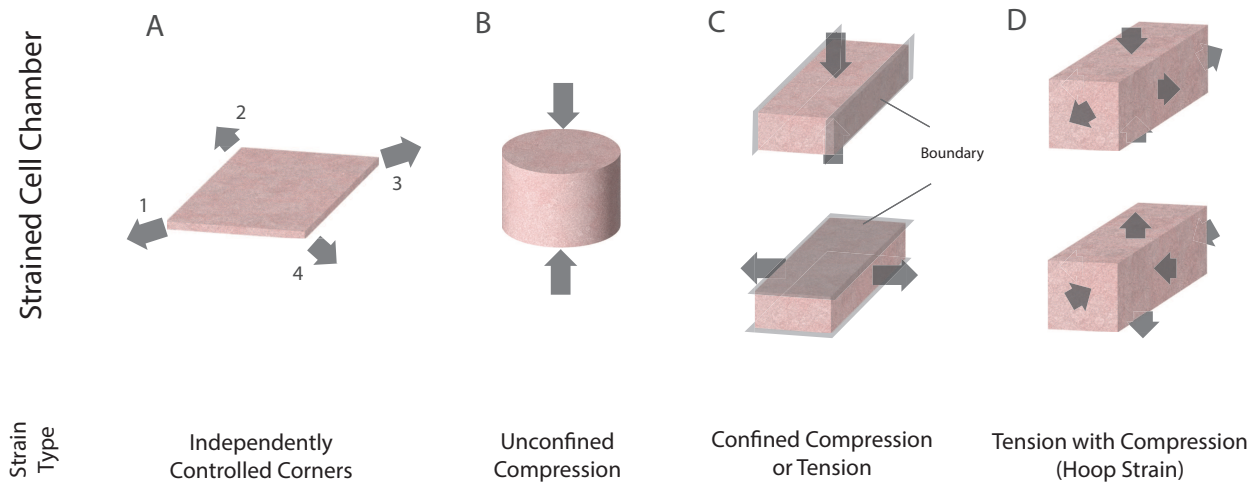

Fig. 3. Overview of the loading environment created by micro-bioreactors or mechanically active organ-chips. Except for the (A), dimensions for cell chambers are on the order of tens or hundreds of microns. (A) While still 2D, a PDMS membrane with independently actuated corners represents a departure from conventional uniaxial or biaxial stretch culture that could be programmed to achieve more disc-like strains. [Gizzi et al., 2017] (B) Cylindrical scaffolds are loaded in unconfined compression, and are commonly used to increase matrix production in engineered cartilage. [Lee et al., 2018, Mauck et al., 2003] (C) Devices have also been developed to provide confined compression or tension loading, creating more complex strains relevant tissue interfaces (e.g., articular cartilage-bone interface). [Occhetta et al., 2019, Marsano et al., 2016] (D) Hoop strain, a combination of tension and compression indicated with the arrows, is applied to cells when embedded within a bulk PDMS form that is bent. Stain can be reversed to achieve tension instead of compression and vice versa when the PDMS is bent in the opposite direction. Complex strain in the form of hoop strain is relevant for annular structures such as the annulus fibrosus, cardiac vessels or the cervix. [McKinley et al., 2022]

However, studies that assessed intradiscal strains showed that regions of the disc may experience biaxial tensile strains. [O'Connell et al., 2011, Costi et al., 2007] In 2022, we developed the flexing Annulus-On-a-Chip (AoC) as a proof-of-concept design for mimicking complex AF strains on a cellular level when the whole disc is under axial compression (Fig. 4 - A and B). Strains are applied uniformly across a large cell chamber (16mm in length) and designed to accommodate a wide range of strain magnitudes in both tension and compression (19% compression to 12% tension). Instead of the use of pneumatic actuation, complex strains were achieved through bending the deformable PDMS chip. The location of the cell chamber was moved within the PDMS chip with respect to the bending axis to achieve a target strain. The device was able to create bi-axial strain ratios (tension with compression) that encapsulated the entire 3D complex micromechanical engineered environment reported in the literature (Fig. 3 - D). [O'Connell et al., 2007, Amin et al., 2019] The device satisfies the gap in applied mechanical activity by applying both tensile and compressive strains, as seen in the hoop stresses in the AF, however, the device is yet to be tested with human cells in 3D.

A different form of multiaxial strain was achieved by using vacuum-driven actuators on four sides of a 2D membrane. [Tremblay et al., 2014] Gizzi et al. accomplished programmable multi-axial loading on the membrane by independently controlling the four vacuum-driven actuators, which could create injury related strain concentrations (Fig. 3 - A). [Gizzi et al., 2017] Added control enabled testing ranging from simple loading all the way to irregular injury-like loading. While the device is two-dimensional, three-dimensional hydrogels bound by fibers to the device membrane could translate loads on embedded cells into three dimensions in an effort to increase translatability for modeling injuries to the AF.

Bulk shear, a common load type connected to endplate junction failure and disc herniation, was replicated for an articular model using a series of pneumatic chambers. [Jacobs et al., 2011, Amin et al., 2019, Zhou et al., 2022] Three pneumatic chambers were created along the length of a micro tissue made up of chondrocytes embedded in a gel (Fig. 4 - C). [Paggi et al., 2020] Each chamber was independently programmed to either be pressurized or to be held under vacuum to achieve equally distributed confined compressive strains (5-12%), bulk shear (9.8 +/- 2.9 milliradians), or a gradient of compressive strains on the tissue. This device would only need to be tuned for use with the new AF cell type.

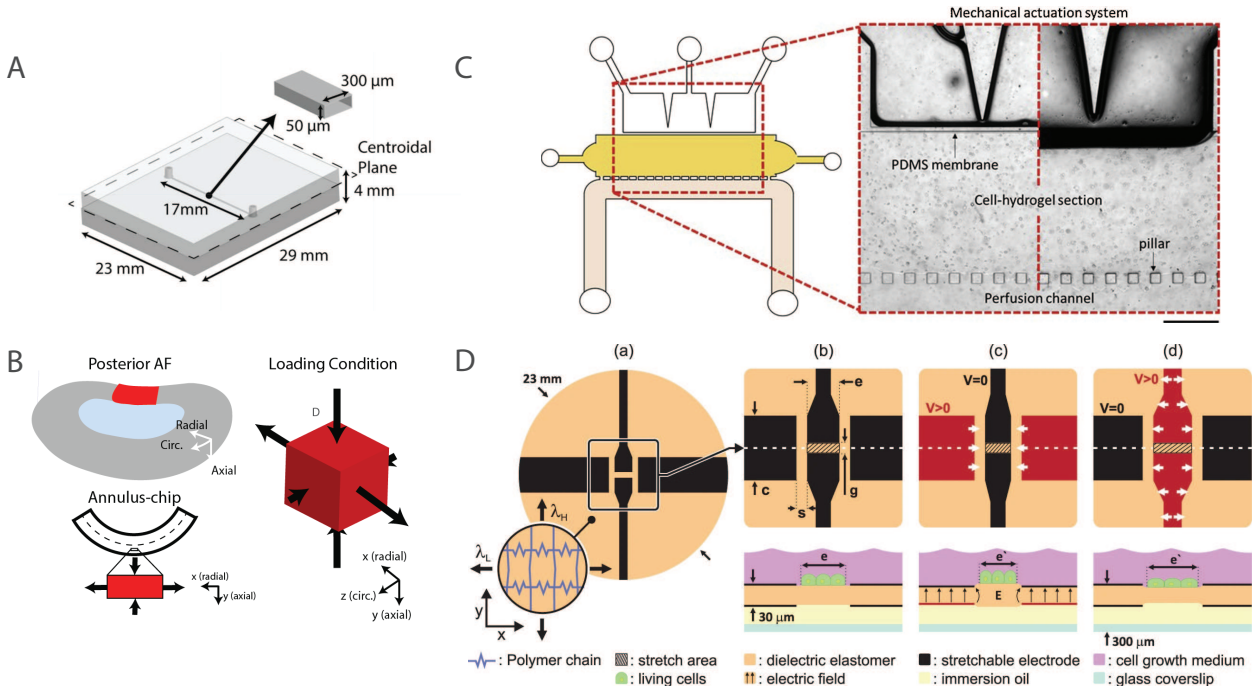

Fig. 4. (A) Schematic and dimensions of the Annulus-on-a-Chip (AoC) including the embedded channel. The figure and text is reproduced from the reference. [McKinley et al., 2022] (B) When the posterior AF is under combined flexion, compression, and axial rotation, it assumes a state of strain where the axial and radial strains are inversely proportional and the circumferential strains are minimal and variable in comparison. This strain condition was replicated in the AoC channel when the device is flexed. The figure and text is reproduced from the reference. [McKinley et al., 2022] (C) Design of the microfluidic platform to mimic articular cartilage that has use in disc research. Left: Top view of the device comprising (from top to bottom): a mechanical actuation section composed of 3 connected actuation chambers separated from the rest of the system by a thin vertical PDMS membrane; a 3D cell culture chamber; an array of pillars; a medium perfusion channel. Right: Microscopic picture showing a section of the system (red dashed square on the left picture) containing a chondrocyte-laden agarose matrix. Left, static condition; Right, homogeneous compression (1000 mbar). Scale bar: 500 microns. The figure and text is reproduced from the reference. [Paggi et al., 2020] (D) Design of DEA device capable of tension and compression within the same cycle at high speeds if needed. a. The actuator consists of an elastomer membrane with four stretchable electrodes patterned on each side. The membrane is non-equibiaxially prestretched to suppress the electromechanical instability in the DEA and to set the preferred actuation direction. The elastomer is much stiffer in the y-axis than the x-axis due to the hyperelastic behavior of the silicone elastomer. b. Cells are cultured at the surface of the device and immersed in growth medium. The bottom side is covered by a thin oil film and sealed with a glass coverslip. Upon actuation, precise strain can be generated on cells located at the center of the device (hatched rectangle). c. When up to 4 kV are applied between the top and bottom horizontal electrodes (in red), the electrodes expand horizontally, hence compressing the cells in the center of the membrane. d. When up to 4 kV are applied between the top and bottom vertical electrodes (in red) they expand horizontally, hence elongating cells located at the center. The figure and text is reproduced from the reference. [Poulin et al., 2018]

5 Future of Micro-bioreactors or Mechanically Active Organ-chips

5.1 Replacing External Actuators with Active Driven Membranes

Nearly all organ-chips operate with passive driven membranes in that the membrane deformations within the device are driven by external stimuli by way of hydrostatic pressure, pneumatic pressure, or a linkage from a motor. However, there is a new class of materials that use active driven membranes with the use of electromechanically active polymers, piezoelectric or smart polymers. [Koike et al., 2020] Controlled by numerous types of inputs (temperature, magnetism, light, humidity, electricity, chemistry, bio-activation, or mechanical force), these smart polymers can deform the cell scaffold itself or make dielectric elastomer actuators (DEAs) for a microfluidic device to apply stimulus to the scaffold as seen in muscle tissue. [Dong et al., 2021] DEAs controlled by cyclic voltage can apply relatively high stress (100 kPa) and large strains (10-50%) which can be effective for disease models. [Hajiesmaili and Clarke, 2021] The use of smart polymers to stimulate cells has long been studied for simple loading applications, however, the technology is largely unexplored for use in organ-chips that apply complex strain. [Clark et al., 2000, Costa et al., 2020]

One promising DEA based design for use in the disc is capable of applying tensile (upwards of 38%) strains followed

by compressive strains (12%) within the same cycle at ultra-high frequencies if needed (Fig. 4 - D). [Poulin et al., 2018] Alternating compression and tension enables the modeling of disc tissues that experience both forms of mechanical stimulation during flexion and extension of the spine. [Amin et al., 2019] Meanwhile, high speed actuation capabilities enables the modeling of traumatic injuries as well as degeneration from vibration, an occupational hazard for helicopter pilots and truck drivers. [Byeon et al., 2013, De Oliveira and Nadal, 2005, Lan et al., 2016, Poulin et al., 2018]

5.2 Combining Macro-bioreactor and Organ-chip Design Features for the Best of Both Worlds

Between the *in vitro* organ-chips and the *ex vivo* macro-bioreactors lies an exception to the framework outlined in this manuscript; devices that utilize aspects of both device classes, such as the combination of microfluidics with whole *ex vivo* discs. Several examples have been included below.

In one example, whole discs from mice were placed in perfusion chambers where media for nutrients were precisely controlled with the use of microfluidic channels. [Dai et al., 2019] Compared to the loose lamella and dead cells in the static group, precise perfusion of nutrients in the disc-on-a-chip maintained a disc structure of dense collagen fibers and cell viability for much longer (three weeks instead of less than a week). The inclusion of complex cyclic loading may extend the health of these discs, as indicated in the torsional macro-bioreactor study above. In total, perfusion by way of microfluidics or mesofluidics could be used to eliminate contamination, continuously elute metabolites, and deliver drugs in either micro- or macro-bioreactors.

In a second example, our proof-of-concept tests indicate that microfluidics could be inserted into existing mechanical testing equipment used for compressing whole discs. In this scenario, the whole disc would be replaced by a manufactured PDMS disc with similar size, shape, and material properties. Microfluidic channels embedded in a bulk PDMS form could then be actuated if a mechanical tester compressed the entire form. Disc-like material properties (Young's modulus and Poisson's ratio), for both the NP and the AF, could be achieved by altering the PDMS component ratios of cross linker and matrix. [Wang et al., 2014] The softer PDMS mix for the NP could be poured in the middle of the AF PDMS mix to achieve a disc shape with a soft interior, a more rigid exterior, and a gradient of material properties in between. Complex strains like those measured in MRI studies of whole loaded disc could be applied to cells if microfluidic channels were placed in one of three locations: 1) within the bulk PDMS disc, representative of the native AF position, 2) the NP, or 3) the boundary in between. [O'Connell et al., 2007] Degeneration could be modeled by adding cuts in the PDMS exterior as if internal disc disruption was present. While adjacent perfusion channels would also experience the actuation, the design of these channels could still allow for fluid flow, drug administration, and gene expression sampling.

5.3 Modeling Multiple Cell Types

Healthy discs are isolated from immune and nerve cells until injury or the degenerative cascade disrupts the outer AF. [Sun et al., 2020] Therefore, a multiple cell-type environment is needed, where neurons, immunocytes, and vascular endothelial cells, are included to replicate the microenvironment of the injured disc which leads to pain, inflammation, and matrix remodeling as most often studied in mice. [Shi et al., 2018] Due to its higher complexity, a multiple cell type model needed to bridge the gap with mouse models is rare.

Hwang et al. developed one such model using a microfluidic device for co-culturing disc cells, neuronal cells, and vascular cells or for culturing disc cells while subjecting them to the conditioned media of another cell type. [Hwang et al., 2017] Multiple cell types on one device such as immune cells with musculoskeletal cells are rare due to added complexity, however, the use of conditioned media is more common. In this example, conditioned media from immune cells that contained pro-inflammatory cytokines (interleukin IL-1β and tumor necrosis factor-alpha (TNF-α)) were added to disc cells to initiate a degenerative cascade. After enough time (72 hours), disc cells showed signs of degeneration, which helped establish a degenerative cellular model. Ma et al. used a similar conditioned media approach to demonstrate multiple cell type communication for chronic back pain by first inflaming rat and bovine dorsal root ganglia before using the resulting media to treat spinal cord cells. [Ma et al., 2021]

Navone et al. meanwhile demonstrated that macro-bioreactors could be used to create conditioned media for use in organ-chips or other cell cultures. A combination of a macro-bioreactor and an *in vitro* cell culture was used to establish the effects of disc degeneration on neighboring nueroinflammatory microglia cells, which play a role in the disc degeneration cascade in general and in back pain more specifically. Using a macro-bioreactor, Navone et al. first mechanically-induced degenerated intervertebral whole discs with high frequency cycle compression and limited nutrition. [Navone et al., 2018] Conditioned media containing interleukins (IL-8, IL-17), nerve growth factor, and interferon from the degenerating disc was then collected, analyzed, and applied for 48 hours to bovine microglial cells harvested from the spinal cord. Telltale signs of nueroinflammatory response were observed given stimulated microglia proliferation and activation. In general, these highly observable and measurable organ-chip platforms allow for the analysis of phenotypic and genotypic changes in a variety of cell types important to the disc, while macro-bioreactors are well suited for creating the initial conditioned media from the disc.

5.4 Challenges of Designing Organ-chips for the Disc

The benefits of organ-chips include greater sample sizes and higher quality modeling for clinical translation, however, compared to macro-bioreactors, there are numerous additional challenges in designing and manufacturing organ-chips to study disc mechanobiology. [Parrish et al., 2019]

Specifically, designs that aim to replicate the entire disc, rather than regions of the disc, need to include a homogeneous hydrogel for the NP and a more fibrous structure for the AF which is yet to be accomplished on a microscale. Rather than self-assembled monolayer tissues that have cells in close contact, disc organ-chips require an engineered scaffold to provide 3D structure and to limit cell-cell connections. [Berrier and Yamada, 2007]

Without vasculature, nutrient supply through the disc tissue is critical for maintaining disc health and therefore critical to consider for culturing disc cells *in vitro*. [Grunhagen et al., 2006] Organ-chip design can influence diffusion of nutrients, gas, and cell waste throughout the device by altering channel size and spacing for increased flow of media. [Rosser et al., 2019] However, the scaffold dictates permeability and therefore movement of these molecules closest to the cell. [Bott et al., 2010, Castro and Lacroix, 2018, Tolabi et al., 2023, Liu et al., 2019] There are several permeable hydrogels that balance mechanical properties for desired disc tissue strength and cellular response (low proliferation rates yet high GAG secretion), however, only some are workable on-chip. [Banh et al., 2022, Cha et al., 2013, Picollet-D'Hahan et al., 2016]

The transfer of strain from scaffold to cell is an important consideration that depends on viscoelasticity, focal adhesion site density, and fiber inclusion among other factors. [Bott et al., 2010, Castro and Lacroix, 2018] Strain on cells embedded in the scaffolds can be measured using digital image correlation. [Lee et al., 2019] Due to the opacity of PDMS, which is typically used to make organ-chips, cells can be visualized for cell kinetics or morphology with immunofluorescence staining, prior to measuring gene expression (e.g., qPCR or RNA sequencing) or protein production. [Hwang et al., 2017] Signal from fluorescent labels provides 'texture' for measuring cell or scaffold strain depending on the image resolution. Alternatively, embedded microbeads have been used to track scaffold deformations. [Paggi et al., 2020, O'Connell et al., 2011] However, as cells deposit extracellular matrix, imaging and analysis becomes more challenging. [Wall et al., 2007]

Even though organ-chips typically have a much higher cell density than native AF or NP tissues, low cell counts still make gene expression analysis more complicated. [Alonso and Hart, 2014] Gene expression analysis is limited to either PCR-ELISA (Polymerase Chain Reaction - Enzyme-Linked Immunoassay) or conventional ELISA with the pooling of several devices to reach sufficient cell counts. Advances in single cell RNA sequencing and the dropping costs to run such analyses may eliminate this challenge.

Lastly, as with all organ-chip designs, a clean room is required to manufacture such devices, as the small scale increases the risk of clogging the cell or nutrient channel. [Tajeddin and Mustafaoglu, 2021] The reliance on such facilities increases the challenges to designing and manufacturing complex systems for the disc. However, once a reusable mold is made using photolithography, devices can be manufactured inexpensively in parallel.

6 Review Limitations

This review is limited to experimental devices and does not include computational modeling, which more readily enables the study of both the healthy and the degenerate conditions. Most applied strain considered in this review corresponds with healthy tissues; however, tissue strains are more than 2X greater in degenerated or injured tissue. [Heuer et al., 2008] Moreover, most existing bioreactors can not switch between tension and compression and have a limitation on maximum applied strain, which limits the ability of such designs to replicate the wide range of physiological to hyperphysiological strains. [White and Panjabi, 1978] Ultimately, computational modeling could be used in tandem with bioreactors to fill in gaps of knowledge in experimental work. One such example is the study of collagen fiber and tissue matrix interactions with or without injury. [Nerurkar et al., 2010, Zhou et al., 2022] Fibers are an important element of load bearing soft tissue that are challenging to incorporate in scaffolds for organ-chips, but can be easily incorporated in finite element models. [Filippi et al., 2022] Lastly, this review did not cover single cell loading since there is a review on microfluidics for single-cell manipulation. [Ito and Kaneko, 2020] Cellular response to complex loading is just as important as the complex loading itself, therefore additional study of cellular mechanosensitive markers are needed. [Fearing et al., 2018]

7 Conclusion

Between computational, *in vitro*, and animal models, there are numerous options to study minimally invasive therapies to address the global impact from intervertebral disc degeneration. However, of these options, only some micro and macro-bioreactors can replicate the complex and harsh 3D strain environment in human intervertebral discs. A literature review was conducted with three aims: determining the importance of these complex loads, determining the state-of-the-art of complex loading disc models across multiple scales, and identifying new trends for future mechanically active device design.

Complex load bearing disc-like tissues in musculoskeletal research proved useful when completing these aims. Bioreactors capable of comparing complex and simple loading identified enhanced tissue response under complex loads in both disc and disc-like tissues. Within existing bioreactors, even the most simply loaded macro-bioreactors on a whole disc or large

tissue sample scale applied complex loading at the cellular scale. However, micro-bioreactors or organ-chips proved capable of applying these complex loads with more control and with greater variety. Ultimately, from a study design standpoint, the control of the microenvironment down to small cell populations in combination with a highly measurable and customizable platform made organ-chips the most promising for studying drugs and treatments to fight disc degeneration. Capabilities of future organ-chips include multicellular or conditioned cell modeling, active driven membrane actuation, and high speed actuation which would greatly extend cellular modeling capabilities for both the nucleus pulposus and the annulus fibrosus in the disc. Both micro and macro-bioreactors come with their unique benefits and challenges. Ultimately, a combination of bioreactors in addition to small or large animal *in vivo* models is best to understand disc degeneration and related non-invasive treatments prior to clinical studies in the future.

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