WORKSHOP
Ex Vivo Explant Models: Unique Insights Offered by Studying Disease in a Dish

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In Vitro Models for Elucidating the Mechanical Regulation of Tendon Physiology and Disease

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Abstract: At first glance, tendon may seem to be a relatively simple tissue with the straightforward function of transferring muscle forces to the bony skeleton. A closer look reveals that it is actually a complex physiological system, with tightly coordinated interplay between an "intrinsic compartment" comprising the fibrous collagen core (tendon cells and the multiscale arrangement of collagen assemblies), and an "extrinsic tendon compartment" consisting of synovium-like tissues that interface with the immune, vascular, and nervous systems. The extent of intrinsic and extrinsic compartment coordination in functional repair, and discord in degenerative processes, is still poorly understood. In this workshop segment, we aim to make the case that explant models from rodent tail tendon provide a powerful platform to identify, investigate and understand aspects of the complex cellular and cell-matrix interplay in tendon tissue, and offer potential to reveal central mechanisms of connective tissue homeostasis and repair.

Background: Tendon is an under-researched tissue, a fact that that can be at least partly attributed to a shortage of physiologically and clinically relevant research models. Almost all existing data regarding basic mechanisms of tissue physiology or tendon damage and repair thus stem either from a limited range of non-primate animal models or from *in vitro* experiments on isolated animal or human tendon cells in 2D or 3D tissue culture. Although 2D and 3D culture systems are widely used due to their practicality, these experimental models are really only amenable to the study of robust cellular mechanisms that are realtively insensitive to physiological context and/or subtleties of the biochemical and biophysical cellular niche. The physiological contexts that 2D and 3D adequately capture arguably include neotendon formation after an acute injury, or the study of acute interactions between recruited tendon cells and an implanted biomaterial.

In contrast, the physiological contexts of tendon tissue homeostasis, disease, and repair of focal tissue damage involves cellular niches in which the extracellular matrix (ECM) architecture, ECM biophysics, context dependent cell-matrix interactions, and spatially regulated cell-cell contacts all play critical yet still poorly defined roles in system behavior. These aspects of the extracellular niche are extremely difficult, if not impossible, to adequately recapitulate *in vitro* using engineered 2D or 3D culture systems.

Tendon explant models, in contrast to 2D and 3D tissue culture, include key anatomical and biophysical aspects that other in vitro systems cannot currently capture. In this workshop, we present some early investigations from rodent tail tendon explants that demonstrate their potential utility in controlled parametric investigation of tendon physiology and pathology. We aim to highlight the potential value of these systems for making rapid progress in identifying central mechanisms of human tendon disease and repair. We also attempt to provide a balanced look at their limitations, and how these models may optimally complement *in vivo* animal models and ultimate verification using human cells and tissues.

The rodent tendon fascicle as an *in vitro* model of tendon - advantages and limitations: A tendon fascicle can be considered the basic "functional unit" of tendon. These units are ordered into higher structures that yield a wide range of biomechanical properties. In contrast to highly cross-linked load-bearing tendons, rodent tail tendon fascicles can be readily extracted from these positional tendons with minimal mechanical or biological damage. Given their ease of isolation and high degree of mechanical and biological reproducibility, rat and mouse tail tendons have played an important historical role in studies of tendon structure-function (Rigby et al., 1959).

There is widespread dogmatic skepticism regarding the physiological and clinical relevance of rodent tail tendon as a model of human tendon diseases and repair. The most commonly cited shortcomings include 1) the disparity in the functional role of the tail tendon as a positional tendon and suspected related deviations at the cellular (epigenetic) level 2) the lack of covalent collagen cross-links in the extracellular matrix and 3) its non-human origin (genetic disparities). However, we have argued that a tail tendon explant nonetheless represents the most reproducible and human-relevant *in vitro* model of "core tendon" physiology that is now available (Fessel and Snedeker, 2011; Snedeker and Foolen, 2017). Mechanical properties are comparable to mature human tendon, with rodent fascicles ranging in elastic modulus from

several hundred MPa to over 1 GPa, depending on the anatomical location of tail tissue harvest, as well as the age, breed, sex, and/or diet of the animal Additionally, the failure properties of isolated tail tendon fascicles reflect those of whole human tendon, with failure stresses on the order of 80 MPa and failure strains of approximately 10% However, it must be acknowledged that mechanical properties at the fascicle level depend highly on the structural organization of the collagen fibers and the degree of cross-linking within and between fibers, and that the nature of rodent tail tissue cross-linking (divalent aldimine crosslinks) diverges substantially from human load bearing tendon (combined divalent aldimine and trivalent histidine crosslinks) (Avery and Bailey 2005). In this sense, the tail tendon fascicle is likely not well suited for investigation of tendon damage from mechanical overload or fatigue. Still, within non-damaging physiological ranges of mechanical loading, the tail tendon model seems to be exceptionally well suited for investigations of biophysical cell-matrix interactions. Aside from the noted molecular differences in collagen crosslinks, the extracellular matrix biochemical composition and architectural structure (including cell-cell and cell-matrix interfaces) of rodent tendon is similar to healthy human tendon (Arnoczky et al. 2002, Gautieri et al. 2017). Similarly, the genetic regulation involved in native tissues and in response to tendon injury is remarkably close to that of human tendon – suggesting that many of the basic biological mechanisms of tendon are conserved across species and anatomical origin (Snedeker and Foolen, 2017).

Tail tendon models of tendon pathology *(credit Stefania Wunderli, University and ETH Zurich):* Our ongoing work investigates the reaction of the tendon core ("intrinsic compartment") to unloading as modulated by the extrinsic niche - specifically tissue vascularity. In these studies, explanted murine tail tendon fascicles serve as a model of an independent and underloaded unit of the intrinsic compartment. These studies investigate tendon fascicle behavior in niches of varying temperature, oxygenation and nutrient supply that are conceived to mimic pathological and vascularized states of different severity (Figure 1). Using this approach, we have compared the evolution of tissue mechanical properties, cell viability, metabolic activity, tissue morphology, transcriptome and secretome. Below we briefly summarize the methods, and initial findings from this work.

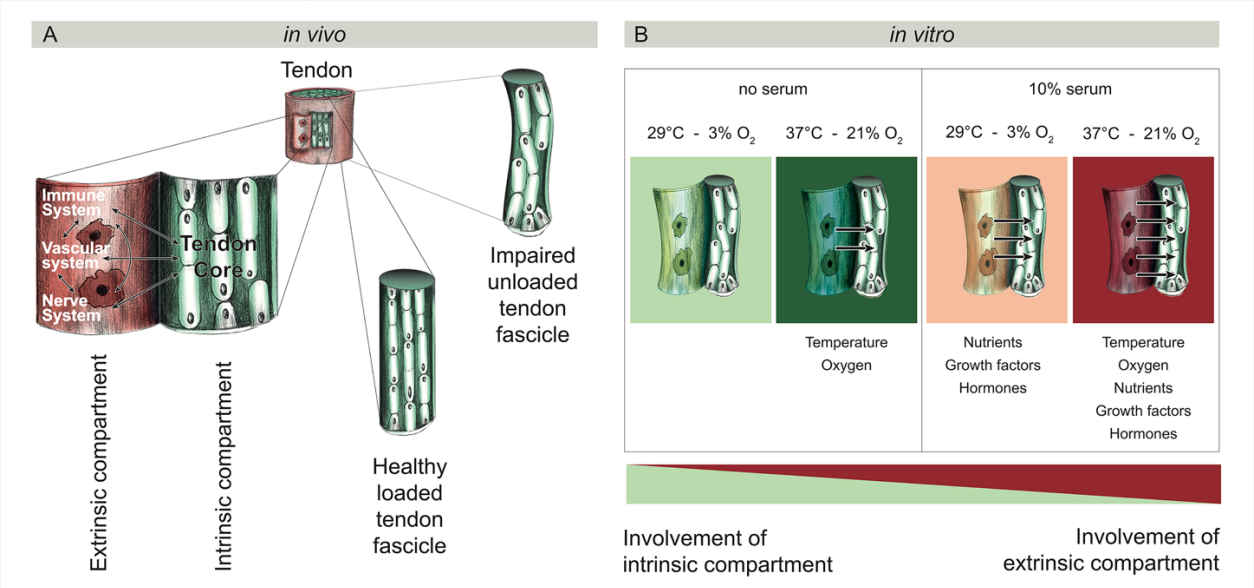
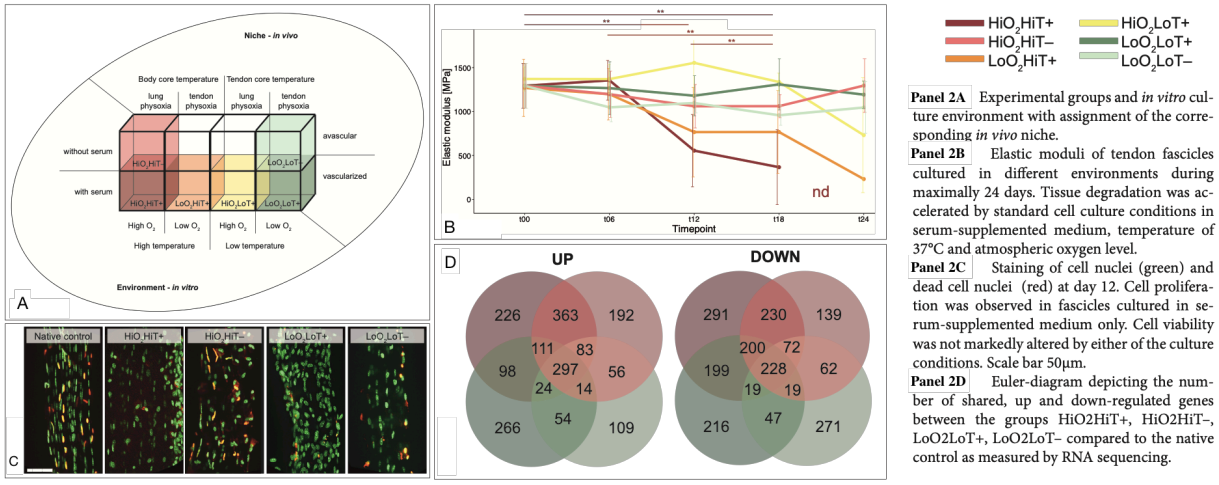


Figure 1: (A) Tendon is a hierarchically structured tissue consisting of densely packed collagen type I matrix and tendon cells (tenocytes) that are responsible for tissue maintenance. The tendon fascicle is the basic functional unit of the tendon (tendon core) and represents the intrinsic tendon compartment. Due to tendon overloading, it is thought that ruptured tendon may remain in an unloaded state. The extrinsic compartment comprises a wrapping layer with synovium-like character that interconnects the immune, vascular and nervous systems. Wrapping layers are present at several hierarchical levels and are named endotendon, epitenon and paratenon (from lowest to highest level). It is thought that intrinsic and extrinsic compartments interact with each other at all levels to ensure proper tissue maintenance. (B) In our ex vivo model the impaired tendon core is represented by an unloaded murine tail tendon fascicle. By varying temperature, oxygen and serum content of the culture conditions we mimicked the degree of involvement of the extrinsic compartment on the cell-driven healing process.

Methods: Tendon fascicles were extracted from tails of freshly euthanized 12-13 weeks old wild-type C57BL6/J mice and distributed among the treatment groups in a non-biased fashion (Fig. 2A). The treatment groups comprised different culture environment and were analyzed at fixed timepoints (6, 12, 18, 24 days). Tissues were cultivated in absence of mechanical load. Culture environments were varied by a combination of different oxygen saturations ('LoO2' = 3% O2 or 'HiO2' = 21% O2), temperatures ('LoT' = 29°C or 'HiT' = 37°C) and serum contents ('+'=with serum or '–' = without serum) (Figure 2A). To discriminate active cell-mediated extracellular matrix remodeling from passive thermodynamic or proteolytic effects of the culture medium, control tissues were devitalized by three freeze-thaw cycles before incubation. Cell viability and proliferation were assessed by fluorescence staining (Hoechst and Ethidium Homodimer-1) and microscopic image analysis by normalizing values to tissue volume (n=2). Mechanical properties were also evaluated (n=6). RNA sequencing was used to determine differentially expressed genes between cultured, "pathologic" (HiO2HiT+, HiO2HiT–, LoO2LoT+, LoO2LoT–, all at 6 days) and healthy, native fascicles (n=3). Differential expression between the HiO2HiT+ and LoO2LoT+ group and HiO2HiT+ and HiO2HiT– was used to detect possible temperature / oxygen or serum dependent processes involved in matrix degradation, respectively. Process and pathway analyses were performed with MetaCore database via GeneGo tool from Thomson Reuters (threshold: fold change = 2, p-value = 0.01). All animal experiments were ethically approved by the Cantonal Veterinary office of Zürich.

Results: Control tendons were functionally stable across all time points, with elastic moduli of devitalized tissues being unaffected by all tested culture conditions. Similarly, intact fascicles cultured in serum-free medium ("–") displayed no mechanical degradation by day 24 (Fig. 2B). In contrast, macroscopic tissue contraction was evident in fascicles cultured for 12 days in serum-containing environment, with length at preload (0.015N) increasing significantly (Ctrl: 20.46mm, sd = 0.15mm | HiO2HiT+: 22.05, sd=1.03mm | LoO2HiT+: 21.74mm, sd=0.48 | HiO2LoT+: 21.06mm, sd=0.43mm | LoO2LoT+: 21.34mm, sd=0.47mm). Culture for 12 days at high temperature in serum resulted in significantly decreased tissue elastic modulus (HiO2HiT+, LoO2HiT+). Lower temperature slowed the degradation process and in combination with low oxygen levels did not affect tissue integrity within 24 days. Cell viability was not markedly altered by either of the culture conditions, however a tendency towards increased cell density in the experimental groups cultured in serum-supplemented medium was observed, while cell number in the serum-free group remained essentially constant (Fig. 2C). The total number of differentially regulated genes was lowest in the LoO2LoT– group (Fig. 2D), suggesting this environment as closest to the healthy native condition (Fig. 2C). In contrast with the microscopic analysis, next generation RNA sequencing showed a re-regulation of genes involved in mitotic cell division processes in all the analyzed test groups, independently of the presence of serum in the medium. In the group mimicking severe tendon damage/pathology (HiO2HiT+) transcriptome analysis also revealed the activation of pathways involved extracellular matrix and structure organization. Further, pairwise comparison between the HiO2HiT+ and the LoO2LoT+ group showed that temperature and oxygen had a large impact on immune system related genes.



HiO2HiT+ HiO2LoT+
HiO2HiT– LoO2LoT+
LoO2HiT+ LoO2LoT–

Panel 2A Experimental groups and *in vitro* culture environment with assignment of the corresponding *in vivo* niche.

Panel 2B Elastic moduli of tendon fascicles cultured in different environments during maximally 24 days. Tissue degradation was accelerated by standard cell culture conditions in serum-supplemented medium, temperature of 37°C and atmospheric oxygen level.

Panel 2C Staining of cell nuclei (green) and dead cell nuclei (red) at day 12. Cell proliferation was observed in fascicles cultured in serum-supplemented medium only. Cell viability was not markedly altered by either of the culture conditions. Scale bar 50µm.

Panel 2D Euler-diagram depicting the number of shared, up and down-regulated genes between the groups HiO2HiT+, HiO2HiT–, LoO2LoT+, LoO2LoT– compared to the native control as measured by RNA sequencing.

Discussion*:* Using a tail tendon explant model, we demonstrate that cellular regulation and downstream mechanical properties in load-deprived tendons are heavily dependent on the niche mimicked by applied explant culture conditions. Culture environments that simulate increased tissue vascularity (serum, elevated temperature and oxygen levels), strongly induce cell proliferation and tissue degradation as reflected in gene expression and tissue biomechanical function. Our results suggest that loss of tissue integrity driven by the activation of catabolic enzymes in the initial phase of healing is temperature dependent and can be slowed in low oxygen conditions. However, this catabolic potential was effectively absent in quiescent cells (no serum). Summarizing, minimal culture conditions (low temperature and oxygen without serum) are favorable for maintaining tissue homeostasis ex vivo. Further studies are needed to examine whether mechanical load may protect the tendon collagen matrix from catabolic turnover within a pathologic environment. The analysis of gene regulated signaling in these conditions may identify possible key targets underlying diminished regenerative response and potentially provide a focus for the development of novel therapies.

This study shows that standard culture conditions mimic a pathological environment for tail tendon explants that results in accelerated tissue proteolysis of the tendon core. Unravelling the contribution of different extrinsic factors on tissue degradation (temperature, oxygenation and nutrient supply among others) may provide important insight into the remodelling process of tendon tissue after damage.

Conclusion: Rodent Tail tendon is a highly tractable experimental model that provides an extraordinary playground for experimental investigation. We demonstrate, for instance, how tissue culture conditions can be varied to explore sensitivity to physiological context (wound healing). The model also lends itself exceptionally well to basic study of matrix structure function, and biological investigation of tendon cell mechanotransduction and focused study on tendon cell-matrix interactions.

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Utilizing *in vitro* organ culture for mechanistic studies of the intervertebral disc

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Abstract

The intervertebral disc (IVD) is a cellularized fibrocartilaginous structure that serves to transmit and dampen loads in the spine. Within the IVD there are microstructures that contain distinct cell types as well as unique extracellular matrices. Whole organ culture of the FSU preserves the native extracellular matrix, cell phenotypes, and cellular-matrix interactions. Thus, organ culture techniques are particularly useful for investigating the complex biological mechanisms of the IVD. Like all model systems, however, it is critically important to acknowledge the limitations of this approach. Here, we discuss our implementation of whole lumbar mouse FSUs in *in vitro* organ culture, as well as practical considerations in designing experiments that allow the mechanistic investigations of the IVD. As a case study, we describe our implementation of the organ culture method in conjunction with genetically modified animals for the precise molecular monitoring of the IVD in response to injury and therapies.

Introduction

Low back pain (LBP) is the leading factor for global disability and lost productivity in the workplace, and intervertebral disc (IVD) degeneration is among the most significant risk factors for LBP[1]. Although animals model are widely accepted as preclinical models for investigating disease progression and therapies, the complex interactions of multiple circulating factors and confounding responses of the *in vivo* environment poses challenges in isolating the precise causal mechanisms and their downstream effects. Cell culture models are essential for understanding molecular pathways and the cell's response to engineered environments[2], but they do not allow observations are limited in to fully understand how the cells interact with their native extracellular environment. Organ culture systems, whereby surgically isolated IVDs or functional spine units (FSU) are kept intact and incubated under media conditions, offer a reductionist compromise between cellular and organismal models[3-7]. These systems are reduced to only the native cell population and the surrounding extracellular matrix, thus enabling the direct interpretation of the effects of external stimuli on IVD biology. A practical benefit of organ cultures is the ability to longitudinally and non-destructively monitor the expression of cytokines, proteases, and metabolites by assaying the media throughout the culture period.

Organ culture models of the IVD have been implemented for humans[8], bovine[3], rat[9,10], and mice[5-7,11,12]. As with all model systems, there are strengths and limitations to each, and several considerations are listed in Table 1. The dimensions and the size of the size for several different animals and how they compare with human IVDs have been described else where[13], and the dimensions directly impact the ability of the cells within the organ explant to receive sufficient nutrition. For larger systems such as bovine and human, bioreactors may be required to deliver sustenance to the cells furthest away from the tissue surface. It is also important to consider whether the inclusion of the adjacent vertebrae and encapsulating end-plates are necessary to impair the free swelling of individual IVDs[14]. Likewise, to control the effects of swelling, it may be important to mechanically constrain the IVD. The desired outcome measures will also influence the selection of the animal / human model. For example, if MRI is desired, then the large animal or human tissues should be utilized in order to achieve adequate spatial resolution relative to the whole IVD. The final two considerations are somewhat inter-related: availability of tissues and genetic homogeneity – rodent tissues are relatively abundant and are genetically controlled, while it may be challenging to assure such consistency in larger organisms.

Table 1: Considerations when selecting the appropriate model system for IVD organ culture

- Dimension / size of the IVDs
- Passive diffusion vs bioreactor
- Free floating vs mechanically constrained
- Outcome measures: mechanical, imaging, compositional, gene expression, cytokines, histology, etc.
- Availability of tissues
- Genetic homogeneity

Here we focus on the utilization of mouse models. Mouse models are attractive due to their relatively low cost and scalability, as well as the large number of genetically modified animals that allow for the rapid targeted screening of degenerative mechanisms and potential therapies. In particular, using our prior works as a case study, we demonstrate the use of a reporter mouse for monitoring the IVDs' homeostatic, mechanical, structural, and inflammatory patterns over time[6,7].

Methods

Animals and specimen preparation

For details on specific methods, please consult references 6 and 7. Briefly, all animal experiments were performed with approval from the Washington University Animal Studies Committee. Two strains of mice were used for this study, BALB/c and a nuclear factor kappa-B-luciferase reporter animal (NF-kb-luc) which is bred on a BALB/c background (Taconic Model #10499, BALB/c-Tg(Rela-luc)31Xen). After euthanasia, a longitudinal vertical cut was made using a no. 11 blade scalpel on the dorsal surface of the mouse to expose the body cavity. The lumbar spine from each animal was dissected out and excessive soft tissues surrounding the spinal column were removed. Spinal columns were then further dissected into functional spinal units (FSUs) (Fig. 1).

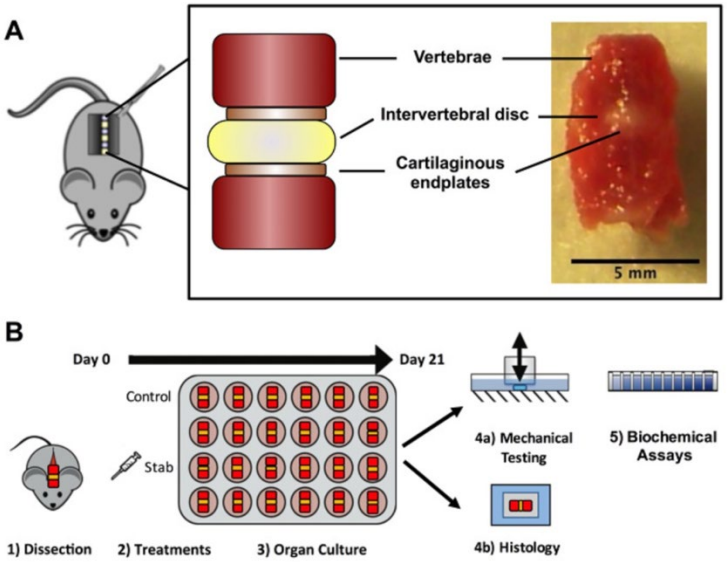

Figure 1: (A) Intervertebral disc functional spinal units (FSUs) were dissected from the lumbar segments of the mouse; the FSUs contained two intact vertebrae, the cartilaginous endplates, and the intervertebral disc. (B) Following dissection, samples were divided into treatment groups and cultured in vitro for 21 days. Afterwards, samples was used for mechanical testing, biochemical assays, and histological analysis. Adapted from (7).

Validation assays

Verification that the culture conditions has maintained the cellular function and viability is a critical first step in validating the *in vitro* organ culture. Moreover, since no assay is perfect, it is important to utilize multiple assays to verify cellular and tissue function. A number of assays are suggested in Table 2. For example, the alamaBlue metabolic assay provides a longitudinal assessment of whether the organ is metabolically active and offers the advantage of requiring just the exchanged media. However, it is important to couple this assay with the tetrazolium nitro blue assay to identify the specific cells which are active and viable, however this can only be conducted after the culture period has ended and requires histological preparation (Fig. 2). The cultured samples should also have appropriate negative and positive controls, but it should also be compared with the freshly extracted samples whenever possible. As an example of a positive control, lipopolysaccharide (LPS), an exotoxin that is commonly used for induce NFkB activity, is used to ensure that the IVD robustly expresses the reporter for the duration of the culture period (Fig. 3). In contrast, the negative control containing only the base media, shows little luciferase activity.

Table 2: Validation assays

- Diffusion (fluorescein)
- Metabolism and viability (tetrazolium nitro blue / DAPI; alamarBlue metabolic assay)
- Mechanical function (Dynamic microcompression)
- Structure (MicroCT and laser micrometer)
- Composition (DMMB and collagen assays)
- Cytokine production (ELISA and reporter activity)
- **Comparison with Fresh**
- **Comparison with Negative Control**
- **Comparison with Positive Control**

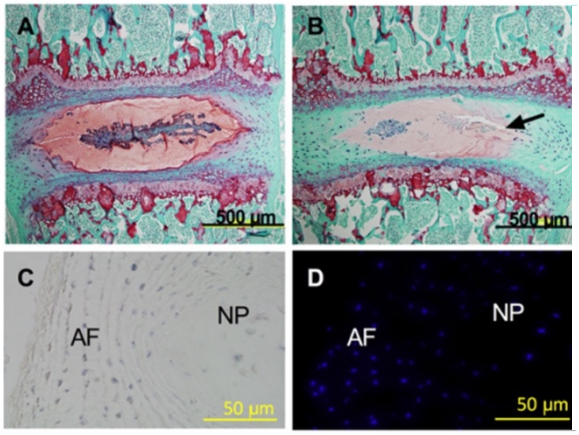

*Figure 3: (**A**) After 21 days in culture, Safranin O staining (red) shows that proteoglycan content is maintained in both the AF and NP in the Control samples. (**B**) The Stab samples (puncture site indicated by arrow) showed decreased proteoglycan content in both the AF and NP. (**C**) Tetrazolium blue staining (blue) shows co-localization. (**D**) DAPI staining shows that cells are metabolically active and viable after 21 days in organ culture. Adapted from (7).*

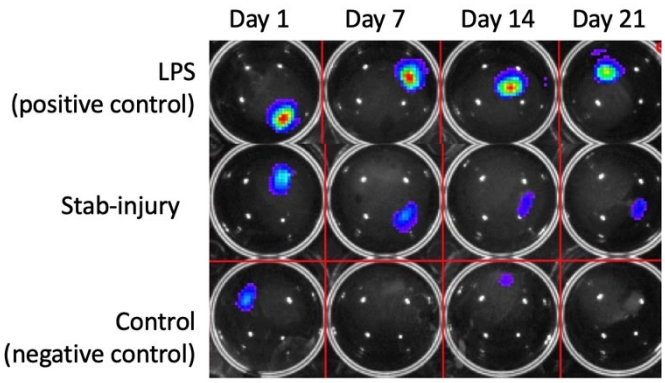

Figure 2: Positive and negative controls are used to verify the responsiveness of the NFkB-luc reporter in both the injury condition and the exposure to the exotoxin, lipopolysaccharide (LPS). Bioluminescence activity confirms that LPS induces the elevated and sustained activity of NFkB, and the negative control expresses minimal NFkB activity. These controls help to contextualize the stab-injury group as a pro-inflammatory condition.

Discussion

The organ culture approach can also be revealing of tissue-specific mechanistic effects of a treatment or therapy. For example, it is reasonable to conclude that the responses observed in the organ culture is the result of IVD cell adaptations. The ability to conduct high-throughput analyses, in combination of molecular reporters, make them an attractive choice for the first-order screening of translational and preclinical therapies. However it is critical to verify that the culture methodologies do not alter the relevant characteristics of the IVD, and careful controls are required to ensure the validity of the experimental results. Certainly as with all models, one must be mindful of the limitations of the system, and in this system, mechanical loading / constraints were not implemented. Despite this, there were no swelling or detectable differences in tissue mechanical behavior, suggesting that at least in this experimental setup, the lack of directed mechanical stimulus does not trigger an anabolic response of the explants. In our example here, we are able to concurrently maintain a high number of mouse IVD FSUs on 24-well culture plates. Moreover, we are able to conduct real-time monitoring through novel imaging methods (bioluminescence) and ELISA of the exchanged media. Taken together, the IVD organ culture is a powerful platform for revealing mechanistic insights to IVD homeostasis as well as rapid screening of drugs and therapies.

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Review

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Experimental studies of bone mechanoadaptation: bridging *in vitro* and *in vivo* studies with multiscale systems

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Despite advancements in technology and science over the last century, the mechanisms underlying Wolff's law—bone structure adaptation in response to physical stimuli—remain poorly understood, limiting the ability to effectively treat and prevent skeletal diseases. A challenge to overcome in the study of the underlying mechanisms of this principle is the multiscale nature of mechanoadaptation. While there exist *in silico* systems that are capable of studying across these scales, experimental studies are typically limited to interpretation at a single dimension or time point. For instance, studies of single-cell responses to defined physical stimuli offer only a limited prediction of the whole bone response, while overlapping pathways or compensatory mechanisms complicate the ability to isolate critical targets in a whole animal model. Thus, there exists a need to develop experimental systems capable of bridging traditional experimental approaches and informing existing multiscale theoretical models. The purpose of this article is to review the process of mechanoadaptation and inherent challenges in studying its underlying mechanisms, discuss the limitations of traditional experimental systems in capturing the many facets of this process and highlight three multiscale experimental systems which bridge traditional approaches and cover relatively understudied time and length scales in bone adaptation.

1. Introduction

The skeleton is often thought of as a static organ system whose main function is support for locomotion and protection of internal organs. Yet, the skeleton is made up of, arguably, some of the most dynamic tissue in the body. In fact, more than 100 years ago, German surgeon Julius Wolff recognized the dynamic nature of healthy and unhealthy bone and developed a theory, known today as Wolff's law, positing that changes in the physical requirements for bone to support the human body are manifested as changes in the internal architecture and external form of the bone [1,2]. A century later, much research has supported the outcome of Wolff's law, demonstrating that bone tissue is capable of adapting to altered mechanical environments to either improve bone quality when greater support is required or diminish bone structure when that support is no longer necessary. To the former point, a seminal study in 1977 compared cortical thickness in the arms of professional tennis players and found an approximate 30% difference between the serving and non-serving arms, with repetitive serving conferring an advantage [3]. Multiple studies since have reported impressive positive gains in bone mass and quality in response to exercise [3,4]. At the other end of the spectrum, the loss of bone and ensuing diminishment of bone quality has been reported in situations of unloading such as paralysis, spinal cord injury [5], bed rest [6] and microgravity [7,8]. Further, the adaptation of bone does not just occur in extraordinary mechanical environments; bone turnover is constant throughout life. Unfortunately with age, it is thought the ability of bone to adapt to its mechanical environment diminishes and that this loss of dynamic function underlies several skeletal

pathologies of great clinical concern, including osteoporosis and related fractures [9]. It is estimated that osteoporosis will affect 10% of females worldwide that reach age 60 and 40% of those that reach age 80. The disease also affects men, though to a lesser extent [10]. Thus, with an ageing population worldwide, there is considerable motivation, in both clinical and basic science, to elucidate mechanisms guiding this adaptive process. A better understanding of the skeleton's ability to adapt to physiologic mechanical loads has potential to reveal new strategies for mitigating the more extreme demands placed on bones in pathologic conditions and to inform novel, innovative and more precise treatments for patients with bone disease.

2. Mechanoadaptation: a multiscale process

While experimentation has convincingly demonstrated the general relationship between mechanical stimulation and bone adaptation, the underlying mechanisms of mechanoadaptation continue to challenge researchers. At the crux of this challenge lies the fact that mechanoadaptation involves many steps traversing varying length and time scales. That is, forces applied to the bone are transmitted from the tissue level down to cells within the bone, and then different cells release factors, initiate cascades and modify bone accordingly, resulting in changes that scale back up to the tissue level over time. A complete understanding of mechanoadaptation must therefore consider mechanisms underlying these processes at specific spatial (subcellular to tissue) and temporal (milliseconds to months) scales, as well as explore mechanisms that integrate these scales (figure 1).

At the cellular level, there are three major types of bone cells. Osteoblasts are derived from the mesenchymal lineage and are responsible for bone matrix deposition. Osteoclasts are large, multi-nucleated cells of haematopoietic origin that resorb bone. The coupled activity of osteoblasts and osteoclasts—with bone formation following resorption—is called remodelling, whereas modelling refers to formation or resorption on independent surfaces. Imbalanced activities of osteoblasts and osteoclasts can result in either excess bone formation or resorption, both typically pathologic outcomes. These two cell types make up less than 10% of the bone cell population at a given moment and, with lifetimes estimated in only weeks, represent a rather transient population. The majority of bone cells are osteocytes: mature cells embedded within the bone matrix. Osteocytes make up more than 90% of the bone cell population and can live for years [11].

In order for mechanoadaptation to initiate, certain cells must be sensitive to a changing mechanical environment. Osteocytes are considered ideal mechanosensors due to their abundance and intricate arrangement within the bone tissue [12–16]. They are stellate cells characterized by numerous cellular processes—or dendrites—emanating from the cell body and connecting osteocytes both with one another and with cells on the bone surface [17]. Osteocyte cell bodies reside in lacunae, and the dendrites extend through channels within the bone tissue called canaliculi. The extensive network created by these structures is called the lacunocanalicular system (LCS).

This intricate cellular arrangement is likely to be a critical feature for bridging the cellular- and tissue-level responses to mechanical forces in bone. Mechanotransduction refers to the conversion of a physical stimulus, such as applied load,

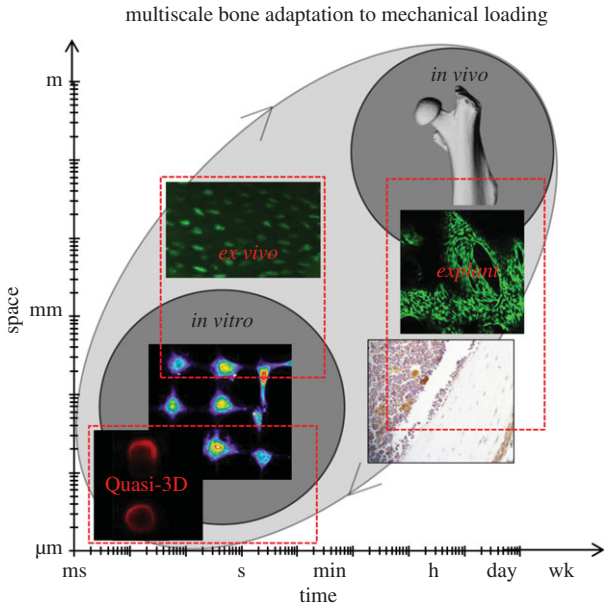

Figure 1. Mechanoadaptation (light grey oval) is a feedback process with underlying mechanisms spanning large spatial and temporal scales. Briefly, forces applied to the whole bone are transmitted from the tissue level down to cells within the bone, and then different cells release factors, initiate cascades and modify bone accordingly, resulting in changes that scale back up to the tissue level over time. Traditional experimental approaches (dark grey circles) typically focus at narrow scales. *In vivo* studies produce outputs at later time points and are often interfaced with μCT and coupled with histology. The top right image shows a sample μCT rendering of proximal mouse femur and the bottom right image shows a longitudinal section of mouse femur with osteocytes embedded in cortical bone (right) and osteoclasts in marrow (left). *In vitro* experiments typically focus on early responses of cell populations. The centre image on the left shows calcium responses of osteocytes cultured in micropatterned networks. Multiscale experimental systems (red dotted rectangles) are necessary to bridge traditional systems and cover more comprehensive time and length scales. Three such systems—*ex vivo*, explant and quasi-three-dimensional—are highlighted in this review. (Online version in colour.)

to a biochemical response [18]. In bone, mechanotransduction covers an expansive time scale. Mechanotransduction includes immediate and gradual biochemical responses of osteocytes, such as the quick release of second messengers (in seconds) or early changes in gene expression (minutes), which sometimes translate to later protein expression changes (hours). Over time, these biochemical responses act on osteoblasts, osteoclasts and other supporting cell types to modulate the tissue composition and architecture, resulting in adaptation of the whole bone (in days). In health, these processes are coordinated to maintain tissue homeostasis. In disease, disruptions at one of these scales may lead to the overall pathologies that present at the tissue level. Thus, there has been considerable motivation to reveal mechanisms of mechano-sensation and -transduction in order to more effectively understand bone adaptation in health and disease.

3. Traditional approaches to the study of mechanoadaptation

The motivation to uncover these mechanisms has generated an emphasis in the last few decades on osteocyte mechanobiology,

particularly what stimuli osteocytes are experiencing and what biochemical responses they are generating. The function of osteocytes was enigmatic for a long time, largely due to their inaccessibility. However, with the introduction of an osteocyte-like cell line [19] and new technologies to probe their functions *in vitro* and *in vivo* [20], the role of osteocytes in bone adaptation has received considerable attention over the last few decades. Numerous studies both *in vitro* and *in vivo* have now demonstrated the importance of osteocytes in the orchestration of bone turnover in response to changing mechanical demands [15,16]. Following is a brief outline of some key findings of traditional *in vitro* and *in vivo* approaches. The interested reader is encouraged to consult more exhaustive reviews of osteocytes and mechanoadaptation for thorough discussion of key developments in the field [12,13,15,16,20–24].

3.1. Mechanosensation

Physiologic loads from activities such as running and jogging can generate strains on the bone surface in the range of 2000–3000 microstrain (0.2–0.3%) [25]. However, when similar strains were used to stimulate bone cells *in vitro*, they were not sufficient to engender a biological response, suggesting that cells embedded within the bone experience a different mechanical stimulus than strain on the whole bone surface. Using poroelastic models to relate whole bone strains to cell-level mechanical signals, it was proposed as early as the 1970s that small shear stresses acting on osteocytes within the LCS are induced by the flow of interstitial fluid driven by matrix deformation [26–28]. In the 1990s, a seminal theoretical paper predicted that the relatively small deformations to the whole bone tissue could translate to fluid shear stresses similar to those in vascular tissues [26] (reviewed in [29]). Since then, numerous studies have demonstrated that osteocytes cultured *in vitro* are sensitive to similar levels of shear stress as predicted by modelling.

3.2. Mechanotransduction

Multiple *in vitro* systems have since been developed to apply dynamic shear stress profiles to monolayer cultures of cells with varying magnitudes and frequencies. Gene and protein expression changes are common endpoints, and early biochemical responses can also be observed using biochemical assays at appropriate time points. Indeed, a major advantage of *in vitro* studies is the ability to control precisely defined inputs and outputs at these early time scales (seconds, minutes or hours).

In addition, by coupling these systems with fluorescence microscopy, real-time cellular responses to mechanical forces can also be studied *in vitro*. One of the earliest responses (in seconds) of bone cells to fluid shear is a rise in cytosolic calcium (Ca^{2+}), which was first demonstrated in osteoblasts [30]. Due to the aforementioned challenges to studying osteocytes, the characterization of osteocyte Ca^{2+} responses to mechanical loading came nearly a decade later, with the first studies applying different mechanical stimuli from fluid flow. Osteocyte stimulation by microneedle displacement was shown to induce a Ca^{2+} response that could be propagated to neighbouring cells [31–33], and osteocytes were more sensitive when stimulated along a dendritic process rather than at the cell body [34], a result also confirmed using localized hydrodynamic forces [35]. A unique pattern in Ca^{2+} signalling was discovered by our group when osteocytes cultured in micropatterned networks were exposed to fluid shear. Distinct from the calcium responses of osteoblast precursors [36,37], robust, un-attenuated Ca^{2+} oscillations were observed in the osteocyte cell line MLO-Y4 exposed to steady flow [36,38]. Taken together, these studies implicate Ca^{2+} as an important biochemical signal in response to mechanical load. In addition to an immediate Ca^{2+} response, *in vitro* studies subjecting osteocytes to fluid shear have shown that loading can enhance the release of prostaglandin E_2 (PGE_2) [39,40], nitric oxide [41,42], osteopontin [43], Wnts [44] and modulate the ratio of receptor activator of nuclear factor kappa-B ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) [45,46] over the course of hours and days. The RANKL/OPG ratio is of particular interest because it modulates the differentiation of osteoclasts.

While *in vitro* experiments have certainly demonstrated that osteocytes possess necessary qualities to act as mechanosensors and mechanotransducers, there are limitations to these experiments. First, *in vitro* studies are typically limited to short time points, capturing only early biochemical changes (seconds to hours). Next, it can be difficult to determine whether these biochemical responses would be translated to an adaptive response. Furthermore, these studies are limited to osteocytes in the absence of interactions with other bone cells. In order to determine whether cells produce functional proteins that would ultimately change the activity of effector cells downstream, *in vitro* studies rely mainly on conditioned medium experiments, though some co-culture systems exist that can better approximate cellular communication in native tissue. A few studies have demonstrated that conditioned medium from osteocytes exposed to fluid flow decreases osteoclastogenesis [45,46], inhibits osteoclast resorptive activity [47] and promotes osteoblast differentiation [48]. However, conditioned media studies fail to capture any coordination of tissue adaptation at particular sites. Lastly, native geometry and connectivity of osteocytes, both between one another and other bone cells, is not often achieved, which can obscure some of the emergent properties of mechano-sensation and -transduction that would arise at the tissue scale or at longer time points. Therefore, a gap still remains regarding how biochemical signals produced by osteocytes in response to mechanical loading regulate bone turnover, and studies coupling early osteocyte responses to mechanical loading with the resultant bone formation or resorption responses could immensely inform our understanding of bone adaptation.

3.3. Tissue-level adaptation

Unlike *in vitro* studies, *in vivo* studies keep cellular sources and interactions intact, which is important for the translation of mechanical interventions to tissue responses. Though other animal models exist, the predominant animal model in the study of bone adaptation continues to be the mouse, due in large part to its robust breeding, relatively short time to skeletal maturity, and amenability to genetic manipulation [49]. For loading, murine ulnar and tibial models are used to induce bone gain, and strain values for whole bones in each of these have been characterized with strain gauges and finite-element (FE) modelling [50,51]. For unloading, tail suspension [52], limb immobilization and botox-induced muscle paralysis have all been shown to induce bone loss

(reviewed in [53]). With all of these models, studies are often interfaced with high-resolution imaging such as micro-computed tomography (μCT) in order to monitor and quantify whole bone and microarchitectural changes, or coupled with histology to relate tissue-level protein expression and dynamic histomorphometric parameters to mechanical interventions. *In vivo* μCT imaging of animal skeletons also allows assessments of dynamic bone formation and resorption, and μCT-based FE analyses allow precise correlations between local mechanical stimuli and bone remodelling dynamics [54–56]. *In vivo* studies are also starting to integrate better with cell studies as transgenic technologies advance to include more cell-specific promotors and lineage tracing [57,58]. Additionally, more precise time points can be explored using inducible expression of these transgenes [59].

In vivo systems have tremendously advanced the understanding of some underlying mechanisms of mechanoadaptation. First, in transgenic mice with ablated osteocytes, mice were resistant to unloading-induced bone loss [60]. Next, recent studies using osteocyte-specific inducible knockouts of RANKL suggested that osteocytes are the primary source of RANKL for modulating osteoclast activity [61,62]. Furthermore, whole bone mechanical loading models indicate that osteocytes can also control osteoblast activity through the Wnt/β-catenin inhibitor sclerostin, which suppresses the activity of osteoblasts. Studies of both ulnar and tibial loading in mice have been shown to decrease levels of the sclerostin protein expression, whereas unloading has been shown to increase levels of sclerostin [63]. These changes are highly correlated with sites of anabolic bone formation in the loading models [63–66]. Taken all together, these studies highlight the essential role of osteocytes in both immediate mechanosensation and the coordination of later adaptive responses.

As exciting as these findings have been in the field, *in vivo* models are not without limitations. Animal models are inherently complicated by compensatory mechanisms, off-target effects and the inability to selectively interrupt pathways in cells that share lineages. This last point is especially pertinent as osteocytes are derived from osteoblasts. As of now, most osteocyte-specific knockouts use Cre recombinase driven by dentin matrix protein-1 (DMP-1), which also affect late-stage osteoblasts and some chondrocytes [67]. These latter cells are known to produce some of the same proteins implicated in mechanotransduction as osteocytes, such as RANKL and PGE_2. The inability to truly separate osteocyte function from late-stage osteoblast function is then a major challenge to drawing inferences from *in vivo* studies.

4. Multiscale experimentation

One multiscale approach to the study of bone adaptation is to use more traditional techniques and emerging technologies to expand the time and spatial information that can be gathered. Trüssel *et al*. [68] recently outlined a mechanical systems biology approach in the study of bone adaptation. Briefly, the authors demonstrate that μCT scans taken at regular intervals of a loading treatment can be registered using advanced imaging software in order to identify local regions of bone adaptation. When combined with traditional histology, histomorphometric techniques and FE modelling, local biochemical changes can then be correlated to local tissue-level strains and sites of adaptation. In addition, new techniques of microdissection are enabling single cells to be extracted from histology samples and gene information to be recovered. Thus, by combining the more traditional single-scale techniques with new technology, which enables the extraction of data from both multiple time points and multiple length scales in the same sample, a multiscale picture of local adaptation to mechanical stimuli can be captured.

Another, and possibly complimentary approach, is to develop multiscale experimental systems that study mechanoadaptation at spatial and temporal scales not covered by *in vitro* or *in vivo* systems, serving to enhance our knowledge of this overall process while simultaneously bridging the information generated by traditional approaches (figure 1). To date, many of these approaches in studying bone are founded on computational models, freed from the inherent challenges found in experimentation with living systems (reviewed in [69]). Yet, a full understanding and validation of the mechanisms underlying mechanical adaptation will require the integration of experimentation which can explore among a variety of dimensions, simultaneously.

Currently, only a few experimental systems exist which attempt to connect traditional scales. For example, the introduction of three-dimensional cell cultures has added some complexity to *in vitro* studies and has proved immensely valuable in unveiling cell behaviours that are intricately tied to the native morphology and parameters of the microenvironment [70–74]. The opposite approach—stripping down native tissues to fewer, more controllable features—has also provided unique platforms for connecting these scales. By nature of their source, major advantages of these *ex vivo* or explanted tissues are that they maintain native tissue architecture, cellular composition and cell–cell arrangement. Indeed, the cellular network formed by osteocytes in the LCS integrates information from whole bone deformation at different locations in the organ, communicates biochemical responses to bone surfaces where effector cell populations reside and coordinates the sites of bone formation and resorption responses to adapt the bone structure. Recapitulating this network should therefore offer insights into the role of osteocyte spatial arrangement in mechanoadaptation. Furthermore, explants can also be used to experiment at vastly different time scales, adding another level of interpretation to experimental studies that may be more difficult to achieve with traditional approaches. Finally, experimental systems can be designed to push the boundaries of this multiscale process, interfacing with other disciplines, such as biophysics, to explore some of the earliest events in mechanosensation at the single-cell level.

For the purpose of illustrating the role of multiscale experimentation in the study of bone adaptation, the remaining sections of this paper highlight three systems employed by our group capable of simultaneously observing behaviours at multiple length scales spanning very early to extended time frames. The experimental systems presented herein each address an important aspect of mechanoadaptation not covered by traditional approaches (figure 1, red dotted boxes). The *ex vivo* system explores early mechano-sensation and -transduction in osteocytes in the cortex of an intact whole bone, which enables the real-time response (seconds to minutes) of osteocytes to whole bone mechanical loading to be assessed, while the osteocytes are maintained in their native network arrangement. This *ex vivo* system has been used to verify mechanisms underlying mechanosensation in bone and to validate *in vitro* studies exploring Ca^{2+} signalling

in osteocytes. The second system discussed is a trabecular bone explant model used to identify pathways critical to tissue-level responses to mechanical load coordinated by osteocytes. This system spans a large spatial scale, enabling links to be drawn first between genetic changes and cell-level responses and then between cell-level responses and architectural changes at the tissue level. In addition, the explant system can be kept viable in culture for up to four weeks, allowing for data to be collected at both short- and long-term time points within the same experimental sample. Lastly, we discuss experimentation at the single-cell level to explore subcellular mechano-sensation and -transduction (in seconds). This technique creates a potential link between cellular and subcellular responses that inform osteocyte function as well as instantaneous and short-term cellular responses to stimuli.

4.1. Mechanosensation and early mechanotransduction in osteocytes *ex vivo*

As previously mentioned, models dating back to the 1970s predicted that mechanical loads sensed by osteocytes were the result of fluid shear stresses induced by interstitial fluid flow within the LCS. However, no experimental systems existed to validate this prediction until the introduction of an *ex vivo* whole bone mechanical loading system [75]. In this system, intact tibiae harvested from mice after injection of a sodium fluorescein tracer compound were placed in a mechanical loading apparatus mounted on a confocal microscope. Cyclic compressive loads were applied to the whole bone to mimic physiological loading, and diffusive transport was measured by fluorescence resonance after photobleaching imaging of osteocyte lacunae embedded within the bone. Fluorescence recovery was faster in lacunae from loaded samples, indicating load-induced convection, and coupling these results to a fluid transport model of canalicular flow demonstrated an enhanced fluid flow in response to whole bone deformation.

While traditional *in vitro* studies allow for the short-term response of loading to be assessed and *in vivo* studies typically require the evaluation of set time points after a loading event, this *ex vivo* system has also created opportunities to explore the immediate response of osteocytes within the actual native environment while the whole bone is loaded. In other words, this system is unique in that it directly couples real-time early responses (milliseconds to minutes) to much higher length scales than are typically studied, pushing the boundaries of traditional mechanotransduction experiments. In particular, early events typically explored only *in vitro* in cells plated in two dimensions are now probed at the cellular network level and coupled to tissue-level stimulation.

One way that this system has been used, in fact, has been in validating that immediate phenomena observed through *in vitro* studies on osteocytes can also be observed in an actual living bone. Though *in vitro* studies subjecting osteocytes to fluid shear strongly support a role for Ca^{2+} signalling to encode information important for mechanotransduction in osteocytes, limited systems were available to show that Ca^{2+} signals in osteocytes embedded within the bone could be induced by load. In explanted fragments of chicken embryonic calvariae, bone cells were demonstrated to exhibit autonomous Ca^{2+} responses [76], and embedded osteocytes were found to respond with elevated Ca^{2+} to bone matrix deformation [77] and shear stress applied over the explant surfaces [78]. We recently modified the *ex vivo* system described earlier to observe Ca^{2+} responses in live osteocytes in a mouse long bone subjected to dynamic, deformational loading (figure 2*a*). Osteocytes exhibited robust oscillations in Ca^{2+} in response to load in a load-dependent manner (figure 2*b*) [79]. This pattern was more pronounced than the autonomous Ca^{2+} responses of osteocytes, which were undetectable, as well as the loading-induced and autonomous responses of cells on the bone surface (figure 2*b*) [76,79]. A representative confocal image and corresponding osteocyte Ca^{2+} time courses are shown in figure 2*c*. Furthermore, in a previous study, we had explored signal propagation between osteocytes and the dependence of that propagation on distance between cells, which enables us to not only look at the level of the individual cell, but also the importance of parameters like cell density and spacing at the near-tissue level [37], which could be incorporated into this *ex vivo* model. This work is also amenable to extending to more complex *in vivo* models which capitalize on the genetic manipulability of the mouse. For instance, future studies could use strain gauges to match strains engendered on the surface of tibia from mice with genetic modifications targeting specific pathways considered important in osteocyte mechanotransduction, such as sclerostin knockout mice (figure 2*d*). Additionally, the generation of FE models from μCT images of these mice could be used to more thoroughly explore or extrapolate these findings to regions within the bone experiencing different strain levels (figure 2*e*). Finally, this model could be used to study the effects of long-term processes like aging on real-time mechanosensation.

Taken together, these *ex vivo* systems have provided novel platforms for studying early biochemical responses in bones under mechanical loading. Furthermore, they have validated mechanosensation in living bone by connecting theoretical models to the abundant *in vitro* experiments showing cellular responses to flow, allowing these studies to progress and add valuable information regarding the underlying cellular mechanisms of mechanotransduction. Finally, current and emerging imaging and modelling techniques can be coupled with these systems to relate immediate cellular responses within the intact bone to the tissue responses measured at the whole bone level.

Of course, there are inherent limitations to the *ex vivo* system. Primarily, the specimen cannot be kept alive indefinitely, limiting the time scale for observation to hours, far short of the time needed to capture tissue-level adaptation. Furthermore, the effect of other organ systems known to influence bone, such as muscle and the nervous system, are absent from these studies. However, isolating the whole bone for observation during these initial time points allows this system to traverse a larger spatial scale in early osteocyte mechanotransduction.

4.2. A three-dimensional trabecular bone explant model of bone adaptation

The previous studies highlight the ability of explant systems to probe fundamental questions in mechanotransduction at early time scales, but they can also be used for the opposite. Mechanical adaptation results in tissue-level changes over a much longer time scale (weeks). During physiologic bone remodelling, for instance, bone formation is coupled to sites

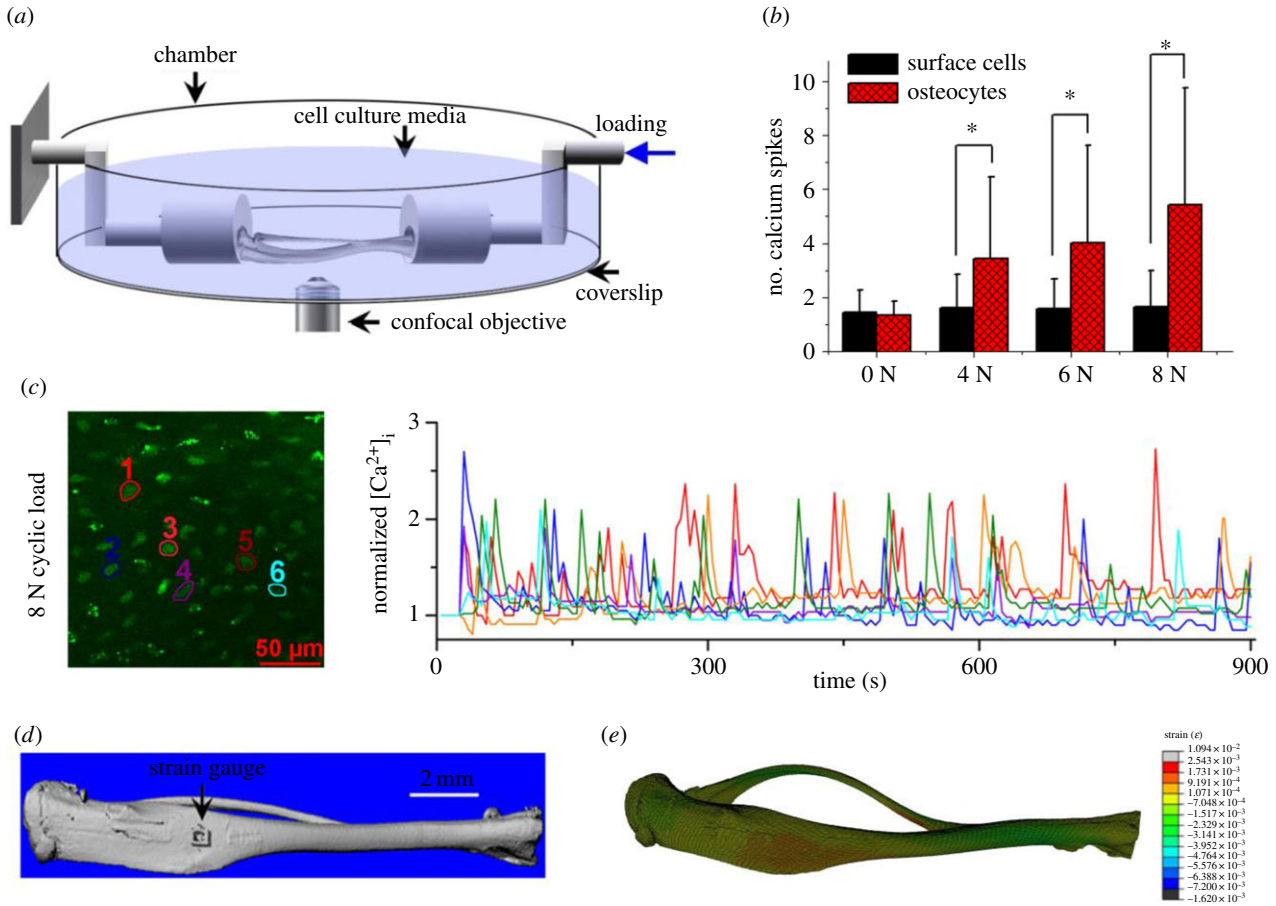

Figure 2. An *ex vivo* murine tibia model to study real-time mechanotransduction in *in situ* osteocytes. (*a*) Schematic of the mechanical loading system used for confocal imaging experiments. Axial, cyclic compressive loads are applied to the distal end of the murine tibia. (*b*) Average number of Ca^{2+} responses (excluding non-responsive cells) over a 15 min experimental period. $*p < 0.05$. (*c*) Representative imaging field and Ca^{2+} traces of Fluo-8-stained osteocytes under 8 N cyclic load. (*d*) Strain gauges were placed on the anteromedial surface to quantify load-induced strains. This represents the region used for Ca^{2+} imaging studies. (*e*) Specimen-specific linear FE models can be generated from µCT images by imposing prescribed displacements. Note that the anteromedial surface experiences the highest strains. (Online version in colour.)

of bone resorption, where osteoclasts may degrade the bone for days before apposition by osteoblasts begins. It is thought that osteocytes coordinate this process by signalling to these cells, but the exact mechanisms remain unknown. Therefore, there exists a need for studies coupling early osteocyte responses to mechanical loading (days) to later tissue-level changes (weeks).

Few bone explant cultures have been established to investigate the adaptive response of bone tissue to mechanical cues, largely due to the requirement to sustain viability of the explanted tissue in culture over long enough periods. The introduction of the ZetOS bioreactor overcame these limitations by providing a system to simultaneously perfuse nutrients through trabecular bone explants and apply dynamic loading [80,81]. Bovine bone cores complete with surface cells and bone marrow were sustained for three weeks in culture, with osteocyte viability remaining above 60% and osteoblast/osteoclast populations responding to mechanical and chemical stimuli [82]. The application of simulated jumping strains resulted in increased bone formation parameters in some samples, and most importantly, architectural changes in the trabecular bone tissue [82]. Furthermore, application of load resulted in changes in the apparent stiffness of the bone samples [83]. Similar results were reported in a rabbit trabecular bone explant model cultured in a perfusion/loading system, where mechanical loading resulted in new bone deposition demonstrated by osteoid formation and the presence of double fluorochrome labelled surfaces [84]. Comparable systems have been developed to sustain viability in whole bone organ cultures [85]. These improvements in culture conditions lay a strong foundation for using trabecular bone explants to study the mechanisms underlying mechanical adaptation.

A major advantage of transferring tissues to culture environments is the ability to adapt the tissue to remove confounding variables and isolate critical interactions. We developed a trabecular bone explant model of osteocyte–osteoblast interactions to evaluate histological and mechanical property changes in response to loading that had been built on previous explants used for short-term loading experiments [86–90]. In this system, bovine trabecular bone cores were thoroughly cleaned to remove bone marrow, disrupted nerves and vasculature, as experimental studies have demonstrated an influence of these systems in bone. The explants were also treated to remove any surface cells, which have been shown to populate the bone surface after a few days in culture in an uncontrolled manner. A controlled number of primary osteoblasts could then be seeded back onto the surface. The resulting explant provides a system in which osteocytes in their native environment can send signals to osteoblasts, and bone deposition and changes in mechanical properties can be measured in response to load.

In our first study, hydrostatic pressure loading enhanced osteoid production in these explants, but the effect was

limited to only 8 days in culture [91], which led us to also develop a loadable perfusion bioreactor to maintain cell viability (figure 3*a*,*b*). Low-level perfusion was used to maintain osteocyte viability without inducing a cellular response to fluid flow, so that changes in mechanical load were only a result of applied dynamic deformation. In this system, osteocyte viability was maintained for up to four weeks, even with abundant osteoblast population of the bone surface, as evidenced by confocal images showing live osteocytes within the bone (figure 3*c*) and histological assessment (figure 3*d*). Mechanical loading resulted in early biochemical responses, such as the release of PGE_2 which remained elevated from within hours to a couple days of culture (figure 3*e*), as well as the deposition of osteoid, and changes in apparent elastic modulus of the explant after four weeks (figure 3*f*). Blocking the PGE_2 response abrogated the anabolic effects of loading (figure 3*e*,*f*) [92]. As both osteocytes and osteoblasts are capable of releasing PGE_2 in response to mechanical stimulation, it is unclear from this study if this response was mediated primarily through osteocyte mechanosensing. A critical advantage of this explant system, however, is the ability to selectively manipulate the osteoblast population to disrupt PGE_2 signalling in osteoblasts only, a condition that cannot be created by *in vivo* studies, which once again highlights the critical role of explant systems for delineating mechanisms underlying bone responses to loading.

While powerful as a model system, limitations do exist. The added challenge of keeping osteocytes viable addressed above makes decoupling the long-term effects of loading versus transport difficult. Additionally, while native arrangement of the osteocytes and connection with surface cells is achieved with the explant, the bone core size and shape do not capture the whole bone response to mechanical loading as faithfully as the *ex vivo* model. Further, the removal of all cell types except osteocytes and the seeded cell type of interest may obscure the essential interactions of other native cells in the adaptive process. Despite these limitations, explanted trabecular bone cultures can be used to probe outcomes of mechanical loading at very different time points with a simplified cell composition in a still intricate arrangement.

4.3. Single-cell studies to probe subcellular mechanosensation in osteocytes

The previously described explanted bone tissue models cover considerable ground between the time and length scales encompassed by traditional experimental approaches. Bridging these studies will be immensely important for interpreting the results from these approaches and enhancing our understanding of bone mechanoadaptation. In the spirit of embracing a multiscale approach, one can also extend these experimental studies to additional time and length scales not typically considered in the study of adaptation, such as subcellular mechanosensation.

In addition to probing downstream osteocyte responses, many *in vitro* studies have explored the role of subcellular organelles in contributing to these biochemical responses. For instance, both the primary cilium [93] and the endoplasmic reticulum (ER) have been demonstrated to contribute to Ca^{2+} signalling in osteocytes, and mechanosensitive channels within the cell membrane have also been shown to contribute to these responses [40]. Furthermore, subcellular structures influence the transmission of mechanical forces to cells. For instance, the cell cytoskeleton and its associated molecules relay this mechanical information to locations within the cell containing mechanosensitive proteins, such as the nucleus or the ER [94]. Thus, the cytoskeleton of osteocytes likely plays a critical role in flow-induced mechanosensing [95–97].

The actin cytoskeleton is very pronounced in osteocytes, with dense perinuclear actin networks and actin filaments extending along the entire length of osteocytes processes [98]. Actin filaments maintain cell shape, dendritic morphology and support membrane tension in osteocytes as evidenced by studies using latrunculin B to depolymerize actin [71,98]. Indeed, the dramatic differences in cytoskeletal components between osteocytes and osteoblasts, particularly in dendrites, suggest that actin may play a critical role in osteocyte mechanosensory function [71]. Furthermore, differences in osteocyte morphology in long bones compared with those that experience relatively mild mechanical environments suggest the importance of the cell cytoskeleton and conferred cell shape in bone adaptation. Since most *in vitro* studies observe flat, spread cells that form actin stress fibres, capturing this native morphology *in vitro* is another important bridge between traditional approaches.

Our laboratory developed a technique to observe the deformation of cytoskeletal elements in rounded osteocytes under fluid flow at high temporal resolution (less than 1 s) in both bottom- and side-view [99] (figure 4*a*). Using this technique, we can reconstruct a quasi-three-dimensional image of the cell under fluid flow (figure 4*b*). Traditional bottom-view imaging of osteocytes transfected with a fluorescent protein tagged to the actin cytoskeleton showed time-dependent deformation of the cytoskeleton under loading, with moderate creep and recovery of the actin cytoskeleton in the normal strain directions (figure 4*c*). The addition of side-view imaging revealed more pronounced cytoskeletal deformations and the observation of a shear strain (figure 4*d*). In a later study, the cortical actin network in osteocytes was found to be more responsive to oscillatory flow than microtubule networks when the temporal resolution was sufficient to probe subcellular responses within a single oscillation period (figure 4*e*) [100]. In these studies, high temporal resolution enabled the characterization of the instantaneous mechanical behaviours of cytoskeletal elements within osteocytes, with more than 10 images collected in a single oscillatory flow waveform at physiologic frequency of 1 Hz. Future studies coupling similar experiments with additional fluorescent probes to look at signal activation at subcellular locations could enable more precise characterizations of mechanotransduction in osteocytes. For example, it is possible to observe how deformation of the cytoskeleton within milliseconds of mechanical stimulation may influence Ca^{2+} signals, which typically initiate within 10–20 s after flow onset.

The temporal resolution opens up many exciting avenues of experimentation; however, there are some key limitations in this system. In order to keep the cell body more physiologically rounded, it is plated in a way that prevents the formation of dendrites. However, this could be improved by incorporating microcontact printing to form controlled osteocyte shape with dendrites [101]. As we have previously discussed, the morphology and interconnection of osteocytes is thought to play a major role in their function and response. Furthermore, the high-frequency image acquisition limits the time frame to only a couple of minutes due to inherent photobleaching effects. However, this quasi-three-dimensional system is exploring the x, y and z spatial dimensions at a

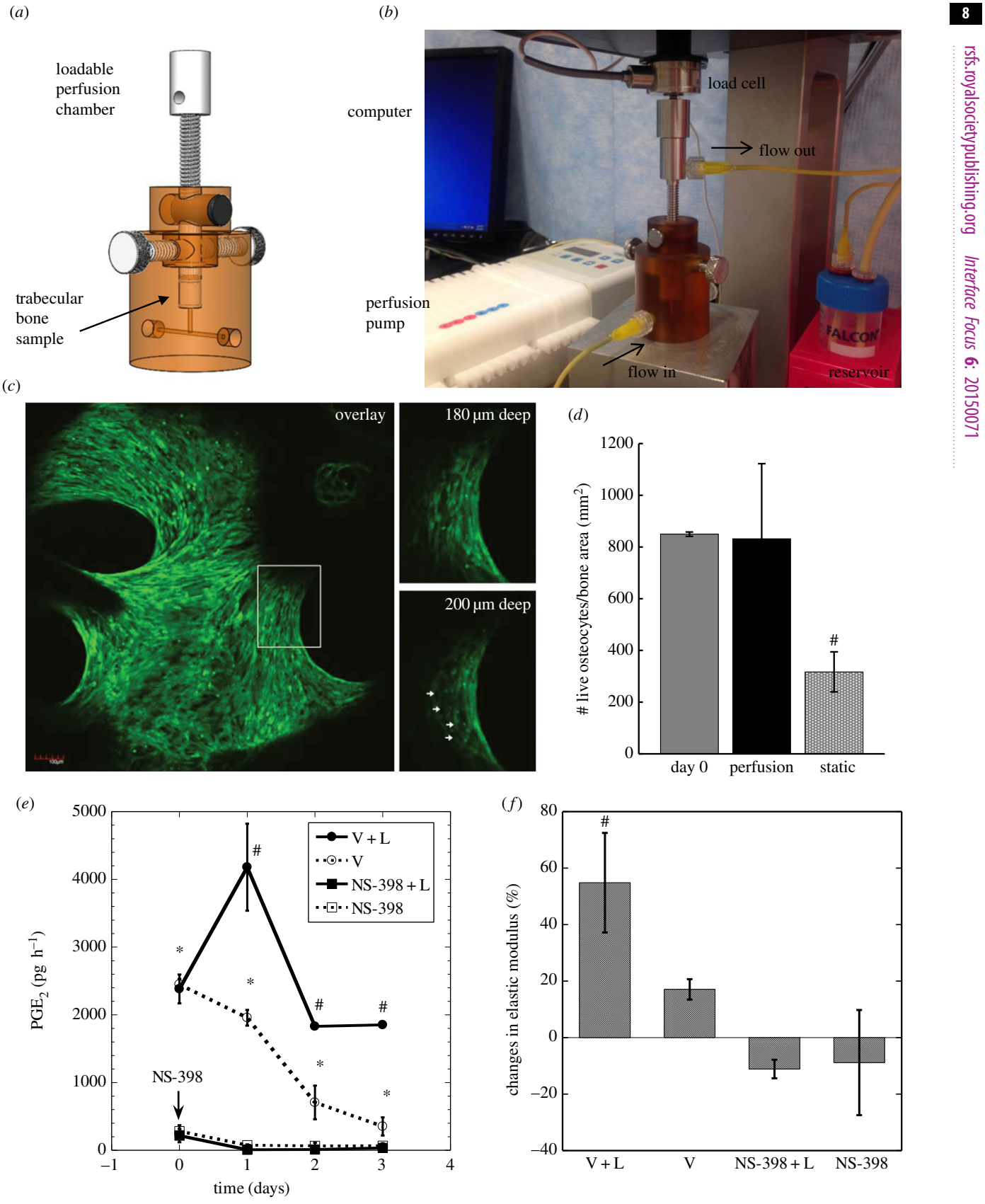

Figure 3. A trabecular bone explant system to study tissue-level adaptation to applied mechanical loads. (*a*) Loadable perfusion chamber to sustain growth of trabecular bone samples. (*b*) Set-up of the chamber connected to a perfusion pump and mounted in a loading device. (*c*) Overlaid confocal images of an explant stained with a Live/Dead viability stain. Osteocytes remain viable when perfused at 0.05 ml min^{-1}, compared to static culture. Insets demonstrate osteocytes (white arrows) residing in the bone 20 µm below osteoblasts on the surface. Scale bar, 100 µm. (*d*) Histological assessment of osteocyte viability in perfusion or static groups compared to initial values. #$p < 0.05$ versus Day 0. (*e*) PGE_2 release during three-day dynamic deformational loading (loading at first, second and third day). Groups include vehicle-treated controls (V), vehicle-treated samples subjected to dynamic loading (V + L) and corresponding non-loaded (NS-398) and loaded (NS-398 + L) groups treated with the PGE_2 antagonist NS-398. *$p < 0.05$ versus NS-398 groups, #$p < 0.05$ versus all other groups. (*f*) Changes in apparent elastic modulus of bone explants after four weeks in culture (#$p < 0.05$ versus all other groups). Error bars are standard deviations. (Online version in colour.)

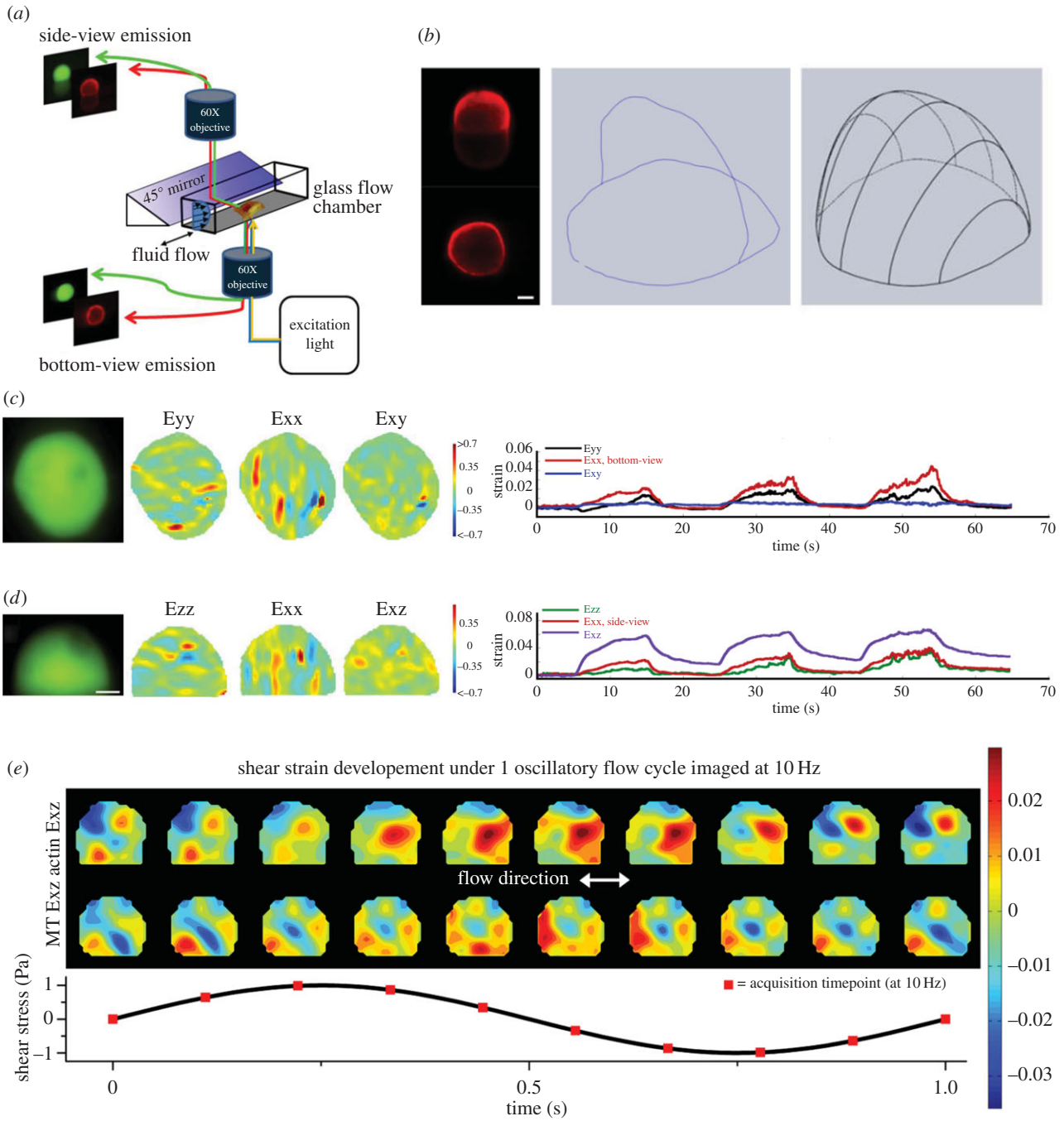

Figure 4. Single-cell imaging of osteocytes under fluid flow in quasi-three dimensions. (*a*) Schematic of the quasi-three-dimensional microscopy system. (*b*) Conversion of cell plasma membrane boundaries from side- and bottom-view images to obtain a whole-cell volume. Scale bar is 5 µm. Average actin network strains and contour plots for a sample osteocyte in bottom- (*c*) and side- (*d*) views. From the bottom-view image, plots of the average normal strains Eyy, Exx and shear strain Exy are obtained. From the side-view images, plots of the average normal strains of Ezz, Exx and shear strain Exz are obtained. Scale bar is 5 µm. (*e*) Actin and microtubule strain contour plots of a cycle of oscillatory flow (10 images were taken per cycle). The areas of oscillating strains in the two networks are distinct, with actin networks responding to flow first. (Online version in colour.)

much smaller overall length scale of the single cell and may add critical information to our understanding of osteocyte mechanosensation. Furthermore, identification of new subcellular pathways in osteocyte mechanobiology could lead to new avenues of exploration in *in vitro* and *in vivo* studies.

5. Conclusion

It is increasingly evident that advancement in understanding complex physiologic and pathologic processes, such as mechanoadaptation, will require a multiscale approach and an integration of computational modelling and experimentation. In addition to interfacing traditional experimental platforms with emerging computational models and advanced imaging techniques, we suggest that the addition of multiscale experimental platforms to such approaches can further strengthen the link between these various scales and allow for even deeper understanding of the complex relationship between mechanical stimulation and tissue adaptation. The three examples highlighted in this review also demonstrate key advantages of multiscale experimental systems in general. First, they form a bridge between *in vitro* and *in vivo* studies, filling in gaps left by the limitations of traditional approaches and isolating the most critical pathways conserved among experimental scales. Second, these systems explore events at the

cellular and tissue level simultaneously, and can therefore provide invaluable experimental validation and refinement in existing computational models or can be used to test hypotheses generated from these models in order to select promising lines of experimentation. Finally, a multiscale approach is a way to uncover more fundamental mechanisms that underlie our health and guide the development of more targeted and precise modes of treatment. With approaches such as these, the field moves forward.

As a final thought, Wolff may have first hypothesized adaptation of bone to mechanical loading, but the last decades have demonstrated increasing evidence that this process underlies the homeostasis and disease of multiple tissues of the body. Cervical tissue, vasculature, pulmonary tissue and muscle tissue, to name a few, have all been shown experimentally to remodel in response to changing mechanical load. It is possible that multiscale experimental approaches in these systems may prove equally as fruitful in understanding their inherent physiology and pathologies. This could also mean that discoveries of the deeper mechanisms, which conserve across scales in one system, such as bone, also translate to other organ systems: an exciting motivation to promote multiscale experimentation.

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