Chapter 1: Techniques for Studying Mechanobiology

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### List of abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
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<td>3D</td>
<td>Three-dimensional</td>
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<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>Ca2+</td>
<td>Calcium ion</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CFD</td>
<td>Computational fluid dynamics</td>
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<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<td>DIC</td>
<td>Digital image correlation</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>FE</td>
<td>Finite element</td>
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<td>FP</td>
<td>Fluorescent protein</td>
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<td>FRAP</td>
<td>Fluorescent recovery after photobleaching</td>
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<td>FRET</td>
<td>Fluorescent resonance energy transfer</td>
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<td>FSI</td>
<td>Fluid-solid interaction</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>ICH</td>
<td>Immunohistochemistry</td>
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<td>mPAD</td>
<td>Microfabricated post array detectors</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>PIV</td>
<td>Particle image velocimetry</td>
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<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TFM</td>
<td>Traction force microscopy</td>
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<td>UHVEM</td>
<td>Ultra-high voltage electron microscopy</td>
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ABSTRACT

Lying at the intersection between engineering and biology, mechanobiology is a nascent field of study that investigates adaptation of the structure and behaviour of tissues in response to mechanical loading. While mechanobiology has been implicated in a range of diseases and evidence of its effects is strewn across multiple scales, it is ultimately a cell-driven process arising in response to changes in local mechanical stimulation. Therefore the field presents unique challenges to researchers, necessitating techniques to shed light on complex biophysical interactions. While bioreactors, tissue engineering and animal models are utilised at organ and tissue scales, advanced microscopy and protein analysis techniques are required to capture localised responses to mechanical stimuli at the cellular and molecular scale. Additionally, computational techniques enable calculation of these localised mechanical stimuli, and linking of these stimuli across multiple scales. This chapter describes in detail a range of techniques relevant to the study of mechanobiology.

1. Introduction to Mechanobiology

Mechanobiology is a field at the forefront of biomedical investigation, situated at the interface between the fields engineering and biology. While new examples of the human body adapting or responding to mechanical loading are regularly being discovered, this phenomenon has been long been observed in multiple tissue types and across numerous anatomical locations. Examples of adaptation in response to changes in loading include bone, cartilage, tendon, vessels, heart, lung, and skin (1-7). Each of these cases involves cell-driven responses by tissues and organs to loading, requiring translation of loading that occurs at the whole-organ scale down to mechanical stimulation of individual cells. The resulting changes in cell activity are then manifested back up through the scales, causing adaption at the tissue or organ level (8).

While intricately related to what could be termed “classical” biomechanics, mechanobiology can be thought of as its mirror opposite. Biomechanics largely concerns the study of the physical effects and interactions induced by biological activity (e.g. the forces imparted onto the ground during running), whereas mechanobiology describes the biological response to an applied mechanical stimulus (e.g. the loss of bone in low-gravity environments). Therefore, while mechanobiological effects can be observed at the scale of an organ or organism, at its core they are the result of changes wrought by cells in response to mechanical stimuli (9). In fact, it has been shown that most eukaryotic cells themselves exert force on their surrounding tissues, even in the absence of any external mechanical stimulus (10, 11). Furthermore, it has been proposed that all cells are mechanosensitive (12), as forces are essential for basic cellular functions like mitosis and migration (13, 14). Thus, mechanobiology is fundamentally a multi-scale phenomenon, spanning the length scales from the very smallest molecules to whole organs, and presenting unique challenges to researchers attempting to
further our understanding. This complex relationship across multiple scales is illustrated in Figure 1.

The objective of this chapter is to introduce researchers from various backgrounds to the wide range of experimental and computational techniques being applied to advance the study of mechanobiology. The first section examines investigative methods at the organ and tissue level, including animal models and tissue engineering techniques. The second section moves towards the cell and molecular levels, introducing imaging techniques, biochemical assays and molecular analysis methods to determine the biological responses to mechanical stimuli. The final section describes computational methods, which have been applied at multiple scales to analyse imaging data, quantify loading experienced by biological tissues, and predict structural responses to mechanical stimuli.

**INSERT FIGURE 1 HERE**

![Figure 1: Schematic illustrating the multi-scale nature of mechanobiology, and some techniques applied to investigate observed adaptation in response to mechanical stimulation. Mechanical stimulation is transferred down from organ to molecular scales, with various animal models and culture techniques used to replicate this experimentally. These stimuli are then transduced into biochemical and structural responses, with a range of assays and imaging techniques applied to measure these. Computational techniques can operate multiple levels, acting as a bridge across the length scales to model in vivo mechanobiology.](image-url)
2. Animal and Tissue Engineering to study Mechanobiology

2.1. Analysis of a Single Cell

Single cell investigations are advantageous for understanding cell behaviour in response to specific stimuli (mechanotransduction). The results of single cell investigations can be used to guide the development of mechanical environments that elicit favourable cell responses and inform tissue engineering approaches (15, 16). Single cell investigations are also used to investigate cell material properties, vital information which is required for computational investigations (17). Force-application techniques are used to investigate single cell mechanics, whereby the cell is deformed in some way by a known force or stress and its mechanical and/or biochemical response is measured. Usually, the surface of the cell is indented or extended (17, 18). There are a number of force application techniques available, as discussed in detail by Rodriguez et al. (17). Optical tweezers, atomic force microscopy and micro-pipette aspiration are commonly used tools for single cell investigation, shown in Figure 2 and discussed below.

2.1.1. Force Application Techniques to Analyse a Single Cell

2.1.1.1. Optical Tweezers

Optical tweezers (often referred to as optical trap) is one method often used to apply a known force to a cell. This technique was developed by Arthur Ashkin in 1970 (19) and was originally used to trap individual atoms, viruses and bacteria (20). In this method nano- to micron-sized beads are attached to the cell membrane. Displacement of the cell membrane is controlled by directing infrared lasers at the transparent beads. When photons pass through the beads, there is a change in their direction. The change in direction causes a change in momentum, resulting in a force on the bead. This change is dependent on the refractive index of the beads. Optical tweezers can exert forces in excess of 100pN on particles ranging in size from nanometers to microns while simultaneously measuring the 3D displacement of the trapped particle with sub-nanometer accuracy and sub-millisecond time resolution (21).

2.1.1.2. Atomic Force Microscopy

Atomic Force microscopy (AFM) was first developed to probe nanoscale features of solid materials using its high sensitivity to intermolecular forces (~pN) and spatial resolution (~nm). More recently, AFM has been used throughout the literature to measure the apparent elasticity of living cells. An AFM system generally consists of a probing tip attached to a flexible cantilever which is lowered onto the cell and the deflection of the cantilever is monitored. The local Young’s Modulus (E) of a living cell can be measured by recording the force acting on the AFM tip while it is indented into a cell, which results in a force-displacement curve. This force-displacement curve can be used to calculate the force-
indentation curve by fitting it with the Hertz model (contact mechanics) allowing the estimation of the local $E$, a detailed description is provided in (22, 23). The following two conditions must be met for an accurate measurement: a) the indentation depth is not more than ~10% of the sample thickness (24, 25) and b) the indentation depth is $>200\text{nm}$ (26). Additionally, the variable shape of a typical AFM probe will determine the nature of the force-deformation curve (18). AFM indentation is typically performed on highly localised regions of the cell, probing individual structures and determining the heterogeneity of cell.

### 2.1.1.3. Micro-pipette Aspiration

A micropipette is a small glass capillary with an internal diameter smaller than that of a cell. In this technique, the micro-pipette is extended to the surface of a cell and a small negative pressure is applied to create a tight seal between the cell and the tip of the micro-pipette. Once this seal is formed, a known negative pressure is applied inducing cell deformation or “aspiration”. Micro-pipette aspiration is used to study whole-cell mechanics by investigating how much cellular material is pulled into a glass pipette in response to the known negative pressure applied. Video microscopy is used to monitor the volume of cell material outside the pipette by tracking the change in radius of material and the height of cellular material inside the micro-pipette (16, 17). The Young’s modulus of the cell can be calculated from the applied vacuum pressure, the length of the cell inside the pipette, and the inner radius of the pipette, if the cell is assumed to be a solid homogenous material (18). If the cell is assumed to behave as a viscous solid, the cell viscosity can be calculated from these aforementioned values, the radius of the spherical portion of the cell outside the pipette, and the lengthening rate of the cellular material within the pipette (17, 18). The device can measure piconewton-level forces (18). This technique has been used to determine the elastic modulus and viscoelastic properties of various cell types throughout the years (17, 27, 28). It has been extensively used to measure cells in suspension (29-34), but more recently has been used to measure cells adhered to a substrate (35, 36). This techniques has also been used to investigate the stiffness of nuclear mechanics by gently isolating the nucleus from the cell cytoplasm (32, 37).

### 2.2. Cellular Interactions with their Local Environment

In addition to the investigation of cells themselves, forces generated by the cell in their local environment are key in the study of mechanobiology and tissue engineering. Forces are produced by cells during development, contraction, migration and other common cell processes (17). Contractile cellular forces (cellular tractions) are transmitted to other cells via cell-cell interaction and to the local extracellular matrix through cell-matrix interactions. These forces generated by cells drive the bending, stretching, alignment, and repositioning required for tissue development and homeostasis, and they also regulate cell functions ranging from receptor signalling and transcription to differentiation and proliferation. Cell tractions are in the range of pico to nanonewtons and occur across small length scales (nano to micrometers), making direct measurement a particularly challenge task. Methods for measuring cellular forces include collagen contraction, tissue pillars, 2D and 3D traction force microscopy and micropillar arrays. For a review of these methods, refer to Polacheck and Chen (38), traction force microscopy and micropillar arrays are discussed briefly below.
2.2.1. Techniques to Analyse Cellular Tractions

2.2.1.1. Traction Force Microscopy

Cellular traction force microscopy (TFM) developed by Dembo and Wang (39) remains the most widely used method to measure cell forces. Traction forces generated by cells can be decomposed into a component acting parallel to the substrate surface and a normal component, which acts perpendicular to the substrate surface. The forces that act parallel to the substrate surface generate deformations in the optical viewing plane and can be visualised using wide-field microscopy. Traction Force Microscopy (TFM) involves tracking synthetic elastic polymer substrates as they move in response to cellular forces (38). Briefly, standard 2D TFM involves mixing small fluorescent beads (< 1 um) into a substrate and seeding cells on/in the substrate. The substrate used for this application must be a flat, deformable material that has well characterised mechanical properties. The material must behave as an isotropic linear elastic material under deformations likely to occur. In addition to this, the substrate must be resistant to degradation in order to decouple force measurements from changes in mechanical properties of the substrate. The fluorescent beads are optically imaged in a stresses state and then the cell traction forces are released by cell lysis, detachment, or myosin inhibition and the beads are tracked in space and time to determine their position in an unstressed state. Computational algorithms are then used to determine the displacement of the beads from the images and the force required to cause such displacements. This technique allows cellular forces to be mapped at a subcellular resolution as the size of the beads are much smaller than the size of the cells. However, complicated computation calculation are required to determine bead displacements and forces (38). Various computational techniques are discussed in detail in the following publications (40-42).

Tracking substrate deformation in a 2D plane (as described above) is not representative of a 3D environment as contractile forces generated by cells are distributed throughout the 3D space. For this reason, TFM techniques have been modified to track bead displacement in 3D with confocal microscopy. However, computing traction forces in 3D requires considerable computational resources. Measuring tractions of cells in 3D is difficult for two main reasons a) experimental/computational complexities and b) the mechanical properties of biologically relevant 3D culture substrates is much more complicated than those of well characterised non-degradable synthetic materials used for 2D TFM (38).

TFM and related techniques have enabled characterisation of the force dynamics involved in a variety of cell biological processes such as adhesion maturation (43, 44), migration (39, 45-47), differentiation (48), and malignant transformation (49). Although great progress has been made in this field over the past number of years, it still remains unclear how forces measured in vitro on mechanically simplified materials relate to forces in living tissues. Current methods measure the forces between a cell and a single material, but in vivo, cells are connected to a host of materials and other cells, all of which contribute to the generation and propagation of cellular forces (38). However, the ever growing community of engineers, mathematicians and scientists are working on the continual development of solutions to overcome the shortcomings of current methods.

2.2.1.2. Micropillar Arrays

Micropillar arrays are another method to measure cellular traction forces. In this technique, single cells are seeded onto an array of micro-sized evenly distributed pillars/cantilevers. The tops of the cantilevers serve as the cell substrate, which results in a high density of force
sensors beneath a single cell. Cellular- or subcellular-scale pillars are typically 0.5–10 μm. The displacements of each cantilever in an array can be tracked, and the observed displacements can be used to calculate the tissue traction forces using beam theory (16, 38). Furthermore, these posts can be fabricated in a cost effective manner, as described by Rodriguez et al (17). Micropillars are also known as micropost arrays or microfabricated post array detectors (mPADs) (17, 38). Micropillar arrays have been used to investigate cell spreading (50, 51), migration (52-54), contractility (51, 55, 56), focal adhesion strength (57) and cadherin junction tractions (58, 59).

2.3. Bioreactors mimic the in vivo environment

In the body, the forces experienced by tissues and cells vary both in type and magnitude depending on the physiological location. As a result, each type of tissue construct (skin, bone, cartilage, tendon, blood vessel etc.) has different requirements, making bioreactor design a complex task. For this reason tissue-specific bioreactors have been developed based on a thorough understanding of biological and engineering aspects, to generate loading conditions in vitro similar to those experienced by cells in their native niche (60). A tissue engineering bioreactor can be defined as a device that uses mechanical means to influence biological processes (61). Bioreactors are generally designed to perform at least one of the following functions, (a) provide a spatially uniform cell distribution, (b) maintain the desired concentration of gases and nutrients in culture medium, (c) facilitate mass transport to the tissue, (d) expose the construct to physical stimuli and/or (e) provide information about the formation of 3D tissue (62-64). Numerous studies have demonstrated that the application of mechanical cues assists in the differentiation and growth of stem cells and the production of functional extra cellular matrix, such as aligned tendon (65-68), cartilage (69-72) and mineralised bone (43, 73-75). Bioreactor studies are often combined with computation/mathematical modelling to advance the understanding of the dynamic environment.

2.3.1. Types of Bioreactors

Bioreactors range from advanced commercial systems to custom-built systems developed and built by researchers. Bioreactors have been specifically developed to apply mechanical stimulation via compressive loading, tensile strain, hydrostatic pressure, shearing fluid flow, or indeed a combination of these elements. These types of bioreactors are shown in Figure 3, and discussed briefly below. For a more thorough review refer to Pörtner et al. (60).

INSERT FIGURE 3 HERE

Figure 3: Perfusion, compression, tensile and hydrostatic forces applied to cells/scaffolds using bioreactors.

Flow perfusion bioreactors are most commonly used as they replicate a dynamic environment by allowing 3D structures to obtain nutrients and eliminate waste. Flow perfusion bioreactors generally consist of a pump which forces the media through a scaffold (located in a scaffold chamber) at a quantifiable flow-rate. Media can be easily changed in this experimental set-up,
however results may largely depend on the flow-rate. Microfluidics systems typically consist of cells grown on a porous scaffold or flat surface and fluid is pumped across the cell layer, whereas in microcarrier systems cells are seeded on a scaffold which is placed in an agitated solution (60, 62).

Compressive forces in vivo generate shear stress and strain as fluid is forced from the compressed area to the interstitial spaces. Both static and dynamic forces occur in vivo. Compression bioreactors are particularly important in the musculoskeletal system, specifically osteocytes and chondrocytes are particularly sensitive to compressive forces. Generally, compression bioreactors consist of a motor that provides a linear motion and a controlling mechanism providing displacements regimes. The compressive force is transferred to the construct by flat platens that distribute the load evenly. Mass transfer is usually improved in dynamic compression bioreactors as compression causes fluid flow through the scaffold (62, 76).

Tensile forces are commonly experienced in tendons, ligaments and muscles. In order to grow these tissues in vitro it is necessary to align the cell growth along the appropriate axis. Once the cells are aligned, the intracellular cytoskeleton and extracellular matrix deposition will also be aligned parallel to the strain axis. Many tensile strain bioreactors have very similar design to compression bioreactors, differing only in the direction in which the load is applied. In this case, the scaffold is clamped in position using non-slip grips and tensile strain is applied (60, 62).

Hydrostatic pressure bioreactors can be used to apply mechanical stimulus to cell-loaded constructs and are commonly used in cartilage tissue engineering. Hydrostatic pressure bioreactors generally consist of scaffold chamber which can withstand the pressure applied and a means to apply the pressure, such as an actuator controlled piston. In this case, the piston must apply the pressure via an impermeable membrane so as not to sacrifice sterility of the experimental set-up (62).

The four basic steps of bioreactor design are: (a) identifying the needs and technical requirements, (b) defining and evaluating the related concepts, (c) designing and drawing the device, and (d) building and validating the device. Furthermore, the design has to be adapted to the specific purpose of the research and how the tissues will be used (77). A description of bioreactor design requirements is provided by Partap et al. (62).

2.3.2. Future of Bioreactors
Static culture conditions do not accurately represent the dynamic in vivo environment and are being gradually replaced by bioreactor culture systems. A better understanding of the mechanobiological environment of cells in 3D is required for the successful fabrication to functional engineered tissue. Bioreactors are a vital cog in the transition to the next generation of cell research, whereby readily-available, easy-to-use systems will allow researchers to apply appropriate mechanical loading to their experiments and hence mimic the native cell environment (78). However, currently most bioreactors are specialised devices with a low volume output. Many exhibit operator dependent variability and their assemble is time-consuming and labour-intensive (62). Firstly bioreactors are required to enable us to study this complex 3D environment and following-on from this scaled-up automated bioreactors are required to produce this engineered tissue.
2.4. Animal loading Models

Animal loading models are often required to elucidate the mechanobiology of living tissue under normal and altered mechanical conditions. These models are commonly used to study bone mechanobiology, as loading is particularly important for bone development, remodelling and regeneration. Bone is constantly remodelled by the coordinated action of bone-resorbing osteoclasts and bone-forming osteoblasts. During physical activity, mechanical forces are exerted on bones through ground reaction forces and by the contractile activity of muscles (79, 80). These physical forces result in a maintenance or gain of bone mass and adaptive bone remodelling. Lack of physical activity/mechanical loading result in resorption of bone (81). Numerous animal loading models have been developed throughout the years to test specific hypothesis about bone modelling and remodelling. Animal loading models are used to apply forces at the organ scale in order to generate responses at the cellular level in an effort to determine what mechanical signals elicit specific cellular responses.

In a controlled experimental environment the force required to generate these mechanical signals can come from intrinsic sources, such as voluntary muscle contraction during a vigorous exercise session (non-invasive), or from normal activity following the surgical removal of a nearby bone that formerly shared the load (invasive). Alternatively, the load can originate from extrinsic sources, such pressure applied to skin adjacent to bone (non-invasive) or loads applied to surgically implanted pins (invasive) (82). For a review of some of the most widely used animal loading models for bone, refer to Robling et al (82).

2.4.1. Non-invasive extrinsic skeletal loading models

Early models enabling extrinsic control of load levels provided a significant insight into bone remodelling, however they typically employed invasive surgical procedures, which can present complications (e.g., infection and inflammation) in experiments and interpretation of results. This led to the development of non-invasive animal loading models that are capable of applying a well-defined mechanical signal to bone without the potential complications of surgery. Non-invasive models are technically simpler, less expensive, and do not rely on healing processes, as compared to the surgical models (82). The two most commonly used non-invasive animal loading models are the tibial four-point bending approach developed by Turner and co-workers (83) and the ulnar compression model of Lanyon and co-workers (84).

2.4.1.1. Tibial Four-Point Bend Model

The tibial four-point bend model was first described by Turner (83), whereby the rat tibia is subjected to 4-point bending in the mediolateral direction. The right hind limb of an anesthetised animal is placed between pairs of upper and lower padded load points. A downward force is applied to the upper points, and the load is transmitted to the tibia through the skin, fascia, muscle, and periosteum, resulting in the production of a bending moment in the region between the two upper points. The bending moment imposes a compressive strain on the lateral tibial surface and tensile strain on the medial surface, as shown in Figure 4A (top). On the contralateral leg of the animal, a sham configuration is implemented, whereby the upper and lower points directly oppose each other, as shown in Figure 4A (bottom). In this configuration the sham leg is squeezed but the bone does not deform (85). This model has since been scaled down for a mouse model (86).
2.4.1.2. Ulnar Compression Model

In the ulnar compression model (84), the forearm of an anesthetised rat is secured between two small metal cups which are mounted on the platens of a materials testing machine or other actuator. The elbow is secured with one cup and the dorsal surface of the volarflexed wrist is secured in the second cup. Compressive forces applied to the platens are transmitted to the ulnar diaphysis through the skin, fascia, articular cartilage (at the distal end), and ulnar metaphyseal bone, as shown in Figure 4B. The natural curvature of the ulnar diaphysis translates ~90% of the axial compression into a mediolateral bending moment (82). The ulnar loading model was also initially developed for the rat and has been modified for the mouse (87) and rabbit (88).

Figure 4: Skeletal Animal Loading Models. A: Tibial four-point bend model and B: Ulnar compression model.

Figure 3: A: Tibial four-point bend model adapted and reproduced from Robling et al. (89), B: Ulnar compression model adapted and reproduced with permission from Warden et al. (90).

In both the tibial four-point bend model and the ulnar compression model, dynamic loads are applied and the load magnitude, rate, number of cycles and duration are well controlled. These non-invasive models, combined with increased computing power, higher resolution imaging and new molecular techniques will enable systematic evaluation of loading
parameters to understand the nature of the osteogenic stimuli and pathways (91). Additionally, the explosive growth of transgenic animal technology, will undoubtedly lead to a more comprehensive understanding of the process of mechanically induced bone formation.

2.4.1. Embryonic Animal Models with an altered mechanical environment

Evidence from animal models has been key to help our understanding of the importance of movement as a regulatory tool in sculpting skeletal development. In animal models the mechanical environment can be altered in a number of ways including \textit{in vivo} immobilisation of the musculature (\textit{in ovo} immobilisation) or the use of mutant mouse embryos in which the skeletal rudiments develop with reduced, absent or non-contractile muscle (reviewed in 92). Both the chick and mouse are valuable vertebrate models used to investigate the effect of mechanical stimulation on embryonic skeletal development, due to their similarities with human musculoskeletal development.

2.4.1.1. \textit{In ovo} Immobilisation

An advantage of the chick embryonic model is that it can be physically manipulated in ways that are impossible in the mammalian embryo. The chick shares many features of embryonic development with mammals and has a huge advantage of development external to the mother, \textit{in ovo} (in the egg); which allows procedures and alterations to the embryos and resulting effects to be examined (reviewed in 93)). During chick development, innervations of chick myotomes occurs at approximately embryonic day 3 (E3) (94), and it has been reported that spontaneous limb movements occur from E3.5 to hatching (95). Immobilisation can be achieved in the developing chick embryo either surgically, by extirpation of the spinal nerves, or by application of pharmaceutical agents which block neuromuscular signals (for example 96, 97, 98). Immobilisation studies on the chick have shown that biophysical stimuli are required for correct initiation of ossification (96), several aspects of joint morphogenesis (97, 99-102) and correct spine development (98).

2.4.1.2. Mammalian Models

Essential information about regulatory genes and the role of environmental stimuli for skeletal development has emerged using the developing chick model; however for appropriate comparison with human development the mammalian murine model has been utilised. Another benefit of the murine model is the substantial knowledge of the genome, and the similarity in gene regulation mechanisms with the human. Muscle contractions begin relatively early in development, at approximately the same time as the cartilage template is taking shape, after approximately E12.5 in the mouse (103). Genetic manipulation has produced mouse models that can be used to study the effect of mechanical stimulation from movement on skeletal development; they include mice with reduced (\textit{Myf5}nlacZ/+:\textit{MyoD}-/- (104)) or immobile (\textit{Mdg}-/- (105)) or absent (\textit{Splotch} (106, 107), \textit{Splotch-delayed} (108, 109), \textit{Myf5}nlacZ/nlacZ:\textit{MyoD}-/- (110, 111), \textit{Six1}-/-:\textit{Six4}-/- (112)) skeletal muscle. These mouse models that lack normal muscle contraction show similar skeletal phenotypes to those observed in the chick immobilisation studies including; joint fusions and alterations in the ossification pattern (113).
2.4.1.3. Zebrafish models

The recent emergence of the zebrafish as a model for mechanoregulation of the skeletal system builds on work of the previously described chick and mouse. The zebrafish is a system however, in which many transgenic lines are available, specifically those that mark the various cell types of the musculoskeletal system (114). This system has aided the understanding of cellular behaviour following the manipulation of the mechanical environment (115). Paralysis of the zebrafish exhibit a reduction in the size of all pharyngeal cartilage; establishing muscle loading in this model as a regulator of chondrocyte intercalation (116). Similarly, zebrafish mutants that lack neuromuscular nicotinic receptors (nic b107) and are therefore immobile, display jaw morphology abnormalities, such as smaller and wider elements (116). Both flaccid and rigid paralysis of the zebrafish has been shown to show similar changes to the morphology and function of the jaw joint (117). It has recently been demonstrated using live zebrafish joint imaging that cell behaviours such as proliferation, migration, intercalation and cell morphology changes required to shape the jaw joint are altered under reduced biomechanical conditions (118). The malleable nature of this model, could potentially hold promise for joint malformation recovery studies following periods of immobilisation, as may occur in utero.

2.5. Fluorescent Proteins (FPs) and Imaging Techniques

The discovery of green fluorescent protein (GFP) in 1962 (119) has led to the development of a number of FPs with various hues. Fluorescent proteins are members of structurally similar class of proteins that share the unique property of emitting fluorescence at a specific wavelength when excited by a specific wavelength. FPs can be fused to virtually any protein of interest and genetically encoded into cells to analyse protein geography, movement, and chemistry in living cells (120). Fluorescent proteins have been widely used for live-cell imaging over the past 20 years and have advanced our understanding of many important molecular and cellular functions in live cells. For a thorough review on the various FPs refer to Wang et al (121). As a result of the innovation in FPs, new imaging technologies which utilise FPs have also been developed. Techniques utilising novel FPs and imaging technology have been making a substantial impact on mechanobiology research over the past number of years.

2.5.1. Fluorescent Proteins as Markers in Mechanobiology

Mechanical forces can activate a number of signalling molecules located in the cell membrane and other subcellular compartments. As FPs are genetically encoded they are well suited for the imaging of the spatiotemporal localisation and activation of signalling molecules and structures in live cells in response to mechanical stimuli. A large number of signalling molecules have been labelled with FPs and as such the position and movement of these molecules can be visualised with high spatiotemporal resolution techniques (121, 122). FPs and live-cell imaging can be used to visualise organelles, cytoskeleton, signalling molecules and gene expression in mechanobiology, as discussed in detail by Wang et al. (121).

Briefly, at the organelle level, FPs can be fused to signalling molecules that localise to subcellular organelles to monitor where the organelle resides. FPs can highlight organelles to serve as a reference points for the determination of the global mechanical properties (123-
125). FPs can also be fused to cytoskeleton molecules such as actin, microtubules and intermediate filaments making the cytoskeleton fluorescent whereby morphology and deformations of the cytoskeleton can be monitored in a dynamic fashion (126, 127). Labelling and monitoring the dynamics and intercompartmental traffic of signalling molecules has been the most successful use of FPs in mechanobiology to date. FPs have been used to observe the molecular dynamics in terms of intracellular mechanical tension/stress (128-130), extracellular mechanical environment (131, 132), external mechanical loading (133-136) and the mechanical impact the cells exert on the extracellular environment (43, 44). FPs have also been used to investigate the translocation of specific target molecules among different subcellular organelles (137-140). In gene expression FPs are fused to the promoter region of the gene of interest, when cells are exposed to various types of mechanical stimulation the up/down regulation of the gene can be monitored by the levels of expressed FPs (121).

2.5.2. Imaging Technologies using FPs

2.5.2.1. Live Cell Imaging

Time-lapse imaging is used to observe and capture cellular dynamics by imaging live cells at regular time intervals using fluorescent or indeed light microscopy. In this technique, a camera captures sequences of images which are later viewed at faster speed to track cellular responses over time. The two main experimental challenges in collecting robust live cell imaging data are to minimize photodamage while retaining a useful signal-to-noise ratio (specifically for fluorescent imaging techniques), and to provide a suitable environment for cells or tissues to replicate physiological cell dynamics. Living cells will only behave normally in a physiological environment and control of factors (temperature, cell culture medium) using an environmental chamber is therefore critically important. The single most important factor to successful live cell imaging and meaningful data is to limit excitation light as photobleaching is inevitable with this technique, as discussed by Ettinger et al. (141).

2.5.2.2. Fluorescent Resonance Energy Transfer (FRET)

Fluorescent Resonance Energy Transfer (FRET) is a phenomenon of quantum mechanics that involves the nonradiative transfer of energy from a donor to an acceptor fluorophore (molecule that fluoresces) (121). A fluorophore can serve as a FRET donor if its emission spectrum overlaps the excitation spectrum of another fluorophore, the acceptor fluorophore. When the donor and the acceptor are in close proximity to one another (<10 nm) at the correct orientation, the excitation of the donor can elicit an energy transfer, inducing emission of the acceptor. FRET efficiency is defined as the proportion of the donor molecules that have transferred excitation state energy to the acceptor molecules and is dependent on the distance and orientations between the fluorophores. FRET is a reversible reaction and occurs instantaneously (121). Genetically encoded FRET biosensors can be easily introduced into cells making this technique well suited to molecular live cell imaging to monitor mechanotransduction with high spatiotemporal resolutions. FRET-based techniques have been employed to visualise signal transduction in response to mechanical stimulation, as discussed by Wang et al. (121). Briefly, Chachisvilis et al. (142) fused ECFP and EYFP (fluorescent proteins) to human B₂ bradykinin receptor, a G protein–coupled receptor (GPCR), to detect the activation of GPCR. Using FRET they showed that shear stress activated B₂ bradykinin in bovine aorta endothelial cells, and this effect can be inhibited by B₂-selective antagonist. These results suggest that the membrane B₂ bradykinin GPCRs are
involved in mediating primary mechanochemical signal transduction in endothelial cells (142). More recently FRET has been reported in 3D where Zhao et al. demonstrate that Ca\textsuperscript{2+} and cAMP levels of live embryos expressing dual FRET sensors can be monitored simultaneously at microscopic resolution (143).

2.5.2.3. **Fluorescent recovery after photobleaching (FRAP)**

Fluorescent recovery after photobleaching (FRAP) is a technique where fluorescent signals are selectively photobleached within a subcellular region and the recovery of the fluorescence is monitored in that region over time. The fluorescent intensity of the bleached area will recover at different rates, depending on the levels of diffusion and active transportation of fluorescent molecules (121). FRAP has been widely used to investigate molecular dynamics in mechanobiology. Using FRAP, Vereecke et al. (144, 145) assessed the speed of intracellular Ca\textsuperscript{2+} wave propagation during mechanical stimulation in rat retinal pigment epithelial cells. FRAP has also been used to show that mechanical stress controls the focal adhesion assembly by modulating the kinetics of zyxin in bovine adrenal capillary endothelial cells (128).

2.5.2.4. **Confocal and Two-Photon Microscopy**

Cells in their native 3D environment behave very differently to in vitro 2D cultures (146-148). Investigation into cellular responses requires 3D images at the cellular, sub-cellular and ultrastructural levels. Imaging structures in 3D is an inherently difficult task as the contribution of a signal from above and below the focal plane produces background fluorescence, affecting the quality of the image. Depths and scattering effects (148) requires new imaging techniques to achieve high resolution images of cells and indeed FPs in 3D environments.

In confocal laser scanning microscopy a point source laser light excites a fluorophore in the sample which is either autofluorescent or has been stained with specific fluorescent dyes. The sample is imaged at sequential focal planes and a pinhole detector excludes out-of-focus background fluorescence from detection. A stack of 2D optical sections is acquired, which enables production of 3D representations of internal structures (149). However, in this technique as the excitation light generates fluorescence it also produces photobleaching and phototoxicity throughout the specimen (even though the signal is only collected from the plane of focus). The penetration depth is also limited by absorption of excitation energy throughout the beam path, and by specimen scattering of the photons (149-151).

Two-photon excitation microscopy has been developed as an alternative to conventional single-photon confocal microscopy. In two-photon excitation microscopy a fluorophore is excited by the simultaneous absorption of two long wavelength (low energy) photons. In this case, their combined energy induces excitation of a fluorophore, which normally requires the absorption of a high energy to become excited. This can only occur at a very focused area with limited volume (femtolitre scale) (121), and as such noise originating from the areas outside the focal region is eliminated. As a result of the enhanced signal-to-noise ratio, the penetration depth of imaging is improved (several hundred micrometres) without significant photobleaching (121, 152, 153). As this technique enables increased depth penetration and can be less phototoxic to live specimens it has been widely at the molecular, cellular, tissue and animal levels (154-157).
3. Molecular and Genetic techniques to study Mechanobiology

1. Analysis of mRNA expression

To understand how a biological system works, researchers seek to understand the functioning of the systems component parts. As all cells in a given organism possess an identical genetic make-up, it is the unique phenotypes, or observable characteristics, directed by differential gene expression that guide the systems complexity. The first step to understanding this complexity in assorted cell types is to discover which genes are expressed by the cells of interest, thus guiding cellular differentiation into a specific tissue types and then into functioning systems. Great progress has been made over recent years towards understanding the role that mechanical stimulation has on the development and maintenance of tissues and the impact it has in guiding cell differentiation (158-160). Much of this understanding of the integration of mechanical forces and cellular responses has been possible through the analysis of messenger RNA (mRNA) profiles and changes in gene expression following alterations in the mechanical environment. The central dogma of molecular genetics is that DNA codes for protein not directly but indirectly through processes called transcription and translation. This indirect route of information transfer involves an intermediate ribonucleic acid (RNA) molecule that relays the message. This so called messenger RNA (mRNA) carries the genetic information transcribed from DNA and is used to translate a template for protein synthesis. Through analysis of mRNA through different means, as discussed below, it is possible to understand the types of proteins that are being guided to be produced by this molecular information transfer.

The objective of this chapter is to provide researchers from traditional engineering backgrounds with the theoretical principles and practical techniques of experimental molecular biology, to utilise in the field of mechanobiology.

The first step in selecting a method of mRNA expression analysis is to assess whether a hypothesis can be tested using known specific genes that may be responsive to experimental mechanical manipulation. In these cases, methods of detection of specific individual/single genes would be appropriate. Techniques for individual/ single gene expression include in situ hybridisation (spatial expression) and quantitative real time polymerase chain reaction (qRT-PCR). In cases when specific gene changes would be unknown, a more high-throughput screening approach using techniques such as microarray or RNA-sequencing that focus on genome-wide patterns of gene expression would be more appropriate. Utilising high-throughput methods of mRNA detection offers the benefit of simultaneously capturing changes in interacting groups of genes, with the potential of illustrating novel mechanisms of mechanotransduction. This section will present the basic principles underlying the molecular and genetic techniques of high-throughput and individual level detection of mRNA expression that have been utilised for mechanobiology studies.

3.1. Microarray Analysis

DNA microarray or chip based detection was the first of its type to take a large scale screening approach to collect large data sets to allow data mining and reveal intricate functions. The methodological approach was originally used for sequence analysis, but then became widely adopted to quantitatively measure changes in gene expression (reviewed in
Microarray technology works on the principle of nucleic acid site specific sequence binding or hybridization on to synthetic sequences present on a chip. A microarray (or chip) is a flat surface in which 10,000-100,000 distinct oligonucleotide (short number of nucleotides) probes are present. These probes represent unique sequences for individual genes that will allow for complementary binding of mRNA from cell/ tissue samples. For both in vitro and in vivo experiments involving alterations of the mechanical environment, separate control and experimental groups are formed. The method includes total RNA extraction, reverse transcription of the RNA using oligo-dT primers and the inclusion of a promoter sequence. In vitro transcription is then performed to form complimentary DNA (cDNA) incorporating a fluorescent label. The fluorescently labelled cDNA is hybridised with the microarray (chip), to allow the complementary sequences specific binding of the sample cDNA with the oligonucleotide probe sets on the chip. Following rinsing and digital scanning of the chip the abundance of RNA (bound labelled cDNA) is by measuring fluorescent density (Figure 4A). Data are then normalised amongst replicates for control and experimental groups and the statistical analysis performed. Differential gene expression is generally indicated with a fold change of ≥2.0 or ≤2.0.

The molecular response following alterations in the mechanical environment have been reported in various in vitro and in vivo studies in an attempt to understand in more detail the means by which mechanical stimuli modulate the cellular response during cellular differentiation. Profiling of genome wide changes under altered mechanical environments have been carried out using in vitro culture systems in conjunction with microarray technology, including osteoblast cell-lines subjected to weightlessness or microgravity conditions (162) and chondrocytes subjected to anabolic loading (163), dynamic compression (164) or hydrostatic pressure (165). Analysis of genetic responses to altered mechanical environment during in vivo conditions have also been performed, including expression changes to an absence of movement during embryonic limb development (166).

A limiting factor of the hybridization methodology is its high background, because it is unable to distinguish RNA molecules sharing high sequence similarity. Microarrays also rely on hybridization with a labelled probe which sequence is known, while RNA sequencing technology doesn’t depend on the genomic sequences being known which allows the potential to identify novel gene sequences.

### 3.1.2. Transcriptomics: Total RNA and mRNA sequencing

A transcriptome is the whole set of RNAs transcribed by the genome from a specific tissue or cell type at a particular developmental stage and/or under a certain physiological condition. Following the sequencing of the genome, transcriptome analysis allowed researchers to understand further information on gene structure and regulation of gene expression. This technique has been utilised in multiple aspects of biology to reveal the regulation network of biological processes and guidance on aspects of diseases and drug discovery (167-169). Transcriptome sequencing is a major advance in the study of gene expression because it allows a snapshot of the whole transcriptome rather than a predetermined subset of genes. Direct comparisons between RNA-sequencing-based approaches and microarray technologies to reveal alterations in gene expression between tissues report that RNA-seq identifies a great
number of differentially expressed genes (166, 170-172) and is more sensitive in reproducibly detecting alterations in gene expression at lower quantitative levels (166).

The steps for RNA sequencing begin in the same way as for a microarray, total RNA is extracted. This RNA is then converted into a library of cDNA fragments. Sequences adaptors are added to each cDNA fragment and a short sequence is obtained from each fragment from one end (single-end sequencing) or both ends (paired) using high throughput sequencing technology ((reviewed in173)). The resulting sequence reads are aligned with the reference genome or transcriptome, or in the case where there is a limited reference genome, they can be assembled to produce a genome-scale transcription map that consisted of level of expression for particular genes (Figure 4 B).

This technique has advanced greatly over the last 10 years and is overcoming challenges with respect to cDNA library construction, bioinformatics and sequence coverage versus cost ((reviewed in173)). A factor to consider when utilising this technique for expression analysis, is the quantity of high quality RNA available for analysis. Recent work has optimised a protocol to extract high quality RNA from human articular cartilage, and performed RNA seq, this advancement could be valuable to understand more about expression changes in osteoarthritic patients (174). More recent advancements in the next generation sequencing field, has seen the emergence of single-cell RNA-sequencing technology (scRNA-Seq) which is designed to overcome population-averaged RNA-seq which may mask rare sub-populations of cells (such as stem cells). Single-cell RNA-seq attempts to investigate expression profiles at the cell level, and comparisons between tube and microfluidic based extraction methods are being explored (175, 176). The advancement of single-cell genomics had the advantage of exploring cellular process with a more accurate resolution and thus may be of benefit to understanding mechanotransduction events in multiple contexts.

3.1.3. Quantitative Real Time PCR

Quantitative real time polymerase chain reaction (qRT-PCR) is a technique that is comprehensively used to analyse the expression levels of individual gene transcripts in a particular tissue or cell population following environmental manipulation. PCR was first devised in 1985 and it has had a major impact on biological research and genetic engineering. Through which, it is now possible to analyse 40,000-year-old DNA, DNA from fingerprints, blood and tissue found at crime scenes, analyse single embryonic cells for prenatal diagnosis of genetic disorders and virally infected cells. It is no doubt that this technique has been invaluable to our understanding of mechanobiology, and the analysis of cellular changes following a change in the mechanical environment. qRT-PCR begins by converting sample mRNA into complementary DNA (cDNA) with corresponding sequences (using reverse transcriptase and DNA polymerase). PCR amplification is then performed encompassing a denaturation step (to separate DNA strands), an annealing step (to allow known sequence specific primers for a particular gene to bind to the ends of the target sequence) and an extension phase (when DNA polymerase adds free nucleotides to the end of each primer). These steps are then repeated up to 40 cycles, which results in an exponential growing population of identical DNA molecules. Inclusion of a fluorescently bound dye during the annealing phase, that fluoresces only when bound to a double stranded PCR product, is read computationally and the levels of expression, or C_t (cycle threshold) value for a particular gene can be quantified. The cycle threshold method (177) is an example of the relative
quantification approach, that compares a gene of interest between experimental and control samples, following normalization to an endogenous control gene (Figure 4C).

Changes in or identification of mechanosensitive genes following the application or removal of mechanical stimulation commonly utilises qRT-PCR to confirm high throughput data output (162, 164, 166). The use of qRT-PCR is also valuable in assessing changes in the cellular phenotype following the manipulation of the mechanical environment. Work on mechanisms of chondrocyte differentiation shows that the application of hydrostatic pressure on embryonic cells and adult derived progenitor cells results in a ‘stable’ cartilage phenotype (178-180). Work on wound healing identified that mechanical strain results in the up-regulation of matrix remodelling genes, and the production of more matrix (181). This technique has also been valuable in revealing changes in gene expression due to changes in the mechanical microenvironment in glaucomatous cells (182). The value of this approach supports the quantification of the molecular changes in a tissue or cell population, however it does not show the exact location in which these changes are taking place, this can be addressed with the technique called in situ hybridization.

3.1.4. In situ Hybridisation

In situ hybridization is a powerful tool for detecting DNA or RNA sequences in intact cells, tissues of whole organisms. Mary Lou Pardue and Joseph Gal pioneered the technique of in situ hybridisation by using a radioactive test DNA to label stationary DNA of a cytological preparation (183) (Figure 4D). This approach allowed for the first time the spatial localisation of genetic information. This technique has continued to advance and the method of complementary binding of a nucleotide probe to a specific target sequence is still applicable, with probes being labelled radioactively, colourometrically or fluorescently (184). In situ hybridization is extensively used in research, as well as clinical applications, especially for diagnostic purposes. Use of this technique has aided interpretation of phenotypic changes following the manipulation of the mechanical environment during skeletal development (Figure 5A) (96, 102, 166, 185, 186) and to elucidate the expression profile of genes in mechanosensitive regions (187).

INSERT FIGURE 5 HERE

Gene expression profiling at an individual/single gene level, that have altered the mechanical environment in vivo have investigated candidate genes for altered expression, in order to assess the molecular response to alterations in biophysical stimuli. Both qualitative and quantitative approaches, as described above, to investigate changes in these genes have been performed (96, 166, 185, 186, 188, 189). Application of various forms of mechanical stimulation during in vitro culture regimes is a major goal of bioengineering techniques in order to create tissue suitable for regeneration applications. Through the methods described, it has been possible to reveal molecular responses to mechanical stimulation and identify tissue compositions due to the known molecular identify profiles.
Figure 4: Molecular techniques using mRNA expression to analyse mechanobiology. Basic flow through of each technique as described in the text for both high-throughput identification of changes in gene expression using Microarray analysis (A) and transcriptomic mRNA sequencing (B), and more individual or single based gene expression changes using Quantitative RT-PCR (C) and in situ hybridisation (D).

Figure 5: (A) In situ Hybridization shown by Kahn et al. (185) in the developing mouse humeroradial joint at 14.5 days of embryonic development. Sections of control (m,p) and Spd muscleless mutant (m’, p’) show no Gdf5 expression in Spd (black arrow in [m’]) in contrast
to the control joint region (m). Red arrows indicate joint loss in the mutant as visualized by Sox9 (p) gene expression. (ul: ulna, h: humerus) (B) Immunofluorescent detection of nuclear or cytoplasmic YAP, shown by (190). This study investigates the effect of age and substrate stiffness on MSCs on nuclear-to-cytoplasmic location of YAP as a measure of osteogenic mechanotransductive signalling. (A and B) YAP is located in the nucleus of children-derived MSCs (C-MSCs), while (C and D) it is located in the cytoplasm of adult-derived MSCs (A-MSCs) when culture in control conditions.

3.2. Analysis at the Protein Level
How cells perceive and relay dynamic mechanical signals to illicit an intracellular response and an alteration at the mRNA transcript level still remains unclear. Other avenues to aid understanding of these changes is at the protein level. It is credible that biomechanics impacts on proteins that guide cell-matrix, cell-cell adhesion, cytoskeletal and ultimately nuclear interactions (191). This perspective in analysing mechanobiology has been driven by the recent developments in functional proteomics, and the ongoing advances in mass spectrometry quantitation (reviewed in 191)). More traditional approaches to understanding or observing changes at the protein level include the use of antibodies through spatial localisation (Immunohisto/cytochemistry) or enzyme-linked immunosorbent assay (ELISA) or identification of proteins based on molecular weight (western blotting (192)). The basis of these three techniques rely on a particular antigen-antibody complex binding.

3.2.1. Immunohistochemistry
To identify specific proteins in a tissue or cell type of interest, antibody molecules for specific target molecules are exposed to the sample. The binding of these molecules is detected by incubating the sample with a secondary antibody specific for immunoglobulin molecules and conjugated to a fluorophore (for fluorescent detection). This provides both a visible signal and amplification of the signal that can be visualised using a fluorescent microscope. Immunohistochemistry (ICH) provides information about the spatial localisation of protein expression and qualitative evaluation of expression levels. The general steps for the procedure involve fixation, embedding, and sectioning (for tissue samples), detergent permeabilisation of cell membranes, antigen retrieval (commonly used for paraffin-embedding sections to increase specificity of binding), blocking and incubation with appropriate primary and secondary antibodies. Double or triple labelling of antigens can be performed in a single sample, as long as each primary antibody is either a different isotype or raised in a different species so that each can be recognised by distinct secondary antibodies with different labels. Appropriate negative controls are required during the procedure to confirm specificity of staining. A common approach is to use “no primary antibody controls”, in which the primary antibody is omitted but the secondary is placed on the sample, this will give insight into non-specific binding.

The use of ICH in mechanobiology research has facilitated investigation into phenotypic changes in cell populations following the application of mechanical stimulation in vitro (180, 193, 194). This technique also provides value for the investigation of mechanisms of mechanotransduction, for example through the evaluation of primary cilia following the application of mechanical stimulation (195-197). The nuclear localisation of specific signalling pathway components similarly utilise this technique, in an attempt to understand how cells sense and adapt to external forces and physical constraints. An example of this is the analysis of the role of the YAP/TAZ (Hippo pathway components) as nuclear relays of
mechanical signals exerted by extra-cellular matrix (ECM) rigidity and cell shape (Figure 5B) (190, 198).

### 3.2.2. Western Blotting

Western blotting (or Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) is a technique that identifies specific proteins based on separation by molecular weight through gel electrophoresis. The theory of the procedure is as follows: sample preparation (most commonly cell lysates), gel electrophoresis (two types of agarose gels: stacking and separating), blotting (electrical transfer from gel to a membrane), washing, blocking and antibody incubation (192). The signal is then detected from the bound antibody, usually with an enzyme, this then corresponds to the target protein. Normalisation among samples and experimental groups is based on the loading of each sample lane with an equal amount of total protein. For further validation, it is common practice to re-probe the membrane for a putatively constitutively expressed protein, such as beta-actin.

### 3.2.1. ELISA

The most sensitive and quantitative technique for protein analysis, ELISA (enzyme-linked immunosorbent assay) allows high specificity, even in complex solutions such as blood. The technique uses a biochemical assay to detect the presence of an antigen in a liquid sample. Since its first description by Engvall et al in the 1970s (199), ELISA has experienced rapid adoption as a diagnostic tool in medicine, a valuable investigative method in scientific research and a quality control check in various biotech industries. While several different variations of the technique have been developed, the “sandwich” ELISA is the most pertinent and useful for analysing soluble proteins in scientific research and will therefore be discussed in detail here. A sandwich ELISA operates by using two separate antibodies that recognise different epitopes, which can be either two different monoclonal antibodies or a polyclonal antibody solution. This method allows the measurement of growth factors and/or cytokine levels in cultures or biological liquids, providing convenient assessment of the biological responses to stimuli such as mechanical loading. ELISA kits are commercially available (typically as a 96-well plate), though they can be expensive. It is recommended that for high throughput experiments for repeated analysis of particular antigens, a custom kit be developed in-house. However, custom sandwich ELISAs used for research purposes should be validated due to the risk of false positive results (200).

The initial step in a sandwich ELISA involves coating the surface of the wells such that a known quantity of the capture antibody adsorbs onto its surface. Following blocking of any non-specific binding sites, the wells should be incubated with serially diluted standards of known concentration and experimental samples. A “blank” group, one without samples or standards, should be included to allow measurement of the background signal in the assay. Next, an enzyme-conjugated detection antibody should be added, followed by a substrate that forms a soluble colorimetric, fluorescent, electrochemical or chemiluminescent product when cleaved. Between each step, extensive washing should be performed, adding a “stop” buffer at the end of the assay to terminate the enzyme reaction. A plate reader is then employed to collect the raw data, and a standard curve is generated upon removal of the background signal. Given appropriate conditions, ELISA can accurately measure sample concentrations in the low (<10) picogram/millimetre range, ELISA data is usually normalised to total protein or DNA (e.g. picogram antigen/nanogram DNA) in order to account for potential variability in cell number among samples and experimental groups (201). ELISA kits have
been applied to study mechanobiology in varied tissues and organs, for example, exploring
the effect of loading on intervertebral disc degeneration (202), examining correlation between
pressure and cell stiffness in heart valve cells (203), and incorporated into microfluidics
devices to characterise mechanotransduction in vitro (204).

3.3. Techniques for Editing Gene Function and altering the mechanical
environment

3.3.1. In vitro mutagenesis – mice
A technique called *in vitro mutagenesis* encompasses specific mutations being introduced
into a cloned gene, and the mutated gene is returned to a cell in such a way that it disables or
“knocks out” the normal cellular copies of the same gene. If the introduced mutations alter or
destroy the function of the gene product, the phenotype of the mutant cell may help reveal the
function of the missing normal protein. Using molecular and genetic techniques worked out
in the 1980’s, researchers can generate mice with any given gene disabled in order to study
the role of that gene in development and in the adult. Multiple mouse models have been
utilised to examine and investigate the role of the mechanical environment on cellular
function, some of which are detailed below.

3.3.2. CRISPR
The novel molecular technique that has become increasingly popular over the past 5 years
based on the identification of the functions of CRISPR (Clustered Regularly Interspaced
Short Palindromic Repeats) and CRISPR associated (Cas) genes, and the manipulation of
these for genome editing. The functions of CRISPR and Cas genes are essential for adaptive
immunity, enabling organisms to eliminate invading genetic material (205, 206). The relative
simplicity of the CRISPR nuclease system makes it amenable to adaptations for genome
editing, which was realised in 2012 (207). Manipulation of this system as a tool in molecular
biology allows for either gene silencing or activation. To date this tool has been used in
multiple systems, including human, bacteria, zebrafish, mice ((reviewed in 205)). This tool
has recently been utilised to eliminate gene function in a model of tendon biology in rats, to
understand the cellular responses to mechanical stress (208). This tool has the potential to
expand our knowledge of the molecular mechanisms that are involved in mechanobiology,
and the potential of its use will unquestionably be demonstrated over the next 5-10 years.

3.3.3. In ovo/ Ex ovo manipulation – chick
A powerful tool for unravelling the molecular mechanisms involved in developmental
processes is to ectopically express a gene or signalling pathway of interest and examine the
effect. The avian embryo offers many advantages for developmental studies over mammalian
embryos, due to the ease of access for *in ovo* (in the egg) manipulations. Different types of
manipulations can be performed *in ovo* including surgical and chemically induced
immobilisation (discussed below). Retroviral transmission has been used very successfully to
deliver genes into tissue locations in chick embryos ((reviewed in 209)). Retroviral
independent gene transfer can be achieved in chick embryos using *in ovo* electroporation, a
more successful technique for targeting specific embryonic tissues/cells compared to
microparticle bombardment and lipofection, offering a positive alternative to broad retroviral
infection (210, 211). The basis of the electroporation technique relies on the transient
generation of pores in the plasma membrane, to allow macromolecules to penetrate the
cytoplasm and DNA to enter due to its negative charge (212). Multiple electroporation
systems have been described with respect to targeting different tissues in the developing
chick, for example the neural tube (213), the somites (214), and the eye (215). In general, *in ovo* electroporation has been most commonly applied to chick embryos at early stages of
development, younger than Hamburger and Hamilton stage 20 (HH20/ ~E3.5). Therefore an
alternative to carrying out the DNA transfer *in ovo* for older embryos is to use shell-less
culture techniques (216-220). Such *ex ovo* methods have described using petri dishes (217),
plastic cups (220) and drinking glasses (219). These *ex ovo* methods provide additional
accessibility that may be required in order to target a specific tissue at older stages of
development. The use of this targeted technique may prove advantageous for investigating
the molecular mechanisms involved in mechanoregulation, as it may be possible to combine
this technique with that of an altered mechanical environment (described below).

4. Computational Techniques in Mechanobiology

Computational techniques for probing research questions in the field of mechanobiology have
developed alongside experimental investigations, as these techniques can both inform
experimental design and glean new information from experimental observations. As
computational power increases exponentially, these methods have become ever more
sophisticated and enlightening. Indeed, many advances in mechanobiology have been spurred
by computational investigation, shedding new light on problems ranging from the mechanical
response to loading of individual cells to predicting tissue differentiation in response to
loading.

While many experimental techniques exist to study mechanobiology, as outlined in the
previous sections, almost all involve some sort of destructive interference with the tissue or
cellular mechanical environment. Therefore, most mechanobiological problems represent
excellent examples of Heisenberg’s Uncertainty Principle, wherein it is effectively
impossible to observe an intact in vivo mechanobiological environment without interfering
with its native behaviour. Examples of this include osteocytes in bone (221), skeletal
development in utero (222, 223), cell migration in the intestinal epithelium (224), and the
growth of aortic aneurysms (225).

This section will outline a number of computational methods that have proven invaluable to
the study of mechanobiology, and will give a perspective on its future development as a field.

4.1. Computational Modelling

Computational, or in silico, modelling comprises interdisciplinary methods that apply
mathematics, physics and computer science to replicate and analyse the behaviour of
complex systems through the use of computer simulation. By characterising a system using
numerous variables, the simulation can adjust these variables and predict the resulting effects
on the system. In silico modelling of physical behaviours has developed from theoretical
origins in the early 20th Century into a powerful engineering tool to assess the mechanical
behaviour of physical structures, mechanical systems and, more recently, biological processes. Rapid advances in computational power over the past two decades have brought computational modelling to the fore as a key tool to test prevailing theories or develop entirely new ones. The primary methods by which this is achieved are finite element (FE) method and finite volume method, whereby the system is broken down into a mesh of smaller, simpler regions, allowing modelling of solid or fluid behaviours, respectively. While finite element modelling involves treating these elements like simple structures obeying physical laws, finite volume modelling calculates the change in flow of a fluid through the simple volume and into the next discrete volume. The standard physical equations solved in the elements or volumes are then assembled into a larger system of equations, allowing modelling and analysis of the entire problem (8). The use of these techniques both complements and enhances development of new and existing theoretical models in the field of mechanobiology. These techniques have been applied to a range of different tissues and diseases, a selection of which will be described in this following sections.

4.1.1. Computational Fluid Dynamics
Computational fluid dynamics (CFD) as a technique is readily applicable to the cardiovascular system, given its key role as a fluid transport system for the body. It has been applied for some time to investigate a wide range of vascular diseases, in disparate locations in the body. Since the early application of CFD methods to aneurysms in 1992 (226), they have developed rapidly to gain the confidence of clinicians as a strong diagnostic tool for predicting risk of rupture (227, 228). Similarly, the first application of CFD to coronary artery disease was published in 2000 (229), and has since been combined with significant advancements in medical imaging to develop realistic patient-specific models in 3D (230, 231). A recent, and intriguing development, is the study of mechanobiology of blood cells themselves using CFD (232), with multiple research groups simulating the interactions of crowded blood cell clusters in 3D (233-235). Similar use of CFD in mechanobiology allows modelling the vitreous humour of the eye, predicting concentrations of shear stress on the chamber wall (236).

The other major mechanobiological application of CFD has been to predict flow of marrow or interstitial fluid within bone. Early computational models were developed to characterise loading-induced fluid flow across whole bones (237). Similar techniques were used to analyse an idealised lacunar-canicular system, predicting abrupt changes in the drag forces within the canaliculi arising from changes in geometry or proximity to bone micro-porosity and the Haversian canals (238). CFD techniques facilitated analysis of models of bone cells, in particular osteocytes, with idealised models predicting high shear stresses within the canaliculi (239). More recently, CFD studies have demonstrated the importance of local geometry on fluid flow in the pericellular space, with geometries obtained from transmission electron microscopy (TEM) and ultra-high voltage electron microscopes (UHVEM) images suggested (240, 241). Additionally, numerical models have explored the effect of the pericellular matrix on flow through the canaliculus, investigating the permeability (242-244), fluid movement (245, 246), and electro-chemo-mechanical effects (246, 247). On a larger scale, shear stress within bone marrow under macroscopic loading has been characterised using CFD (248, 249), predicting important mechanical stimuli for tissue engineering of bone (250).
4.1.2. Finite Element Analysis

Given that bone is a stiff, mechanically active, adaptive tissue, FE models have been employed for decades to investigate the biomechanics of bone. Application of FE to orthopaedic tissues began in 1972 (251), and initially was largely focused either the design of prostheses or fundamental research into structural biomechanics (252). More recently, they have been used to determine adaptation of tissue structure in response to loading, as well as mechanical stimuli at the tissue and cell levels under macroscale mechanical loading. Adaption is largely modelled either through tissue growth or tissue differentiation algorithms (reviewed in (8)), with these approaches being used to successfully model fracture healing (253), skeletal morphogenesis (222, 254) and regeneration (255). A range of mechanical stimuli at the tissue or cell level can be computed from models, predicting stress and strain (256, 257), marrow shear stress (258-260) and even thermal stimuli (261) at the tissue and cellular scales. At the cellular level, first complete 3D idealised FE model of the bone cell environment predicted that strains in the lacunar walls are amplified by the local matrix geometry (262). These findings were corroborated by recent finite element studies applying accurate 3D geometries of osteocytes using scans from confocal laser scanning microscopy and X-ray nano-tomography, predicting that geometry alone can amplify strain transfer to the osteocyte in vivo (263, 264). FE models have also been applied to investigate mechanosensation of bone cells in vitro, allowing exploration of the stimulatory effects of cell morphology, focal adhesion density (265), and substrate material properties (266), as well as the translation of mechanical stimulation to the nucleus via the cytoskeleton (267).

The complex process of modelling of heart valve mechanics at the organ scale began as structural models, applying blood pressure as static loads (268-272). Dynamic loading developed later and incorporated realistic geometries (273-275), as well as anisotropic (276) and non-linear (277, 278) material properties. At the tissue scale, research has concentrated on developing constitutive models to capture the mechanical behaviour of heart valve tissue, with finite element modelling recruited to implement these models (review in detail elsewhere (279)). Modelling at the cell-scale has developed recently and advanced rapidly, applying finite element methods to either model the cell itself as a continuum (280, 281), or to characterise the structural behaviour of the cytoskeleton (280, 281).

Similar to bone, cartilage is a mechanically responsive tissue for which finite element analysis has provided many insights, including articular cartilage thickness distributions, skeletal morphology and endochondral ossification patterns (reviewed in (282)).Finite element models have also been applied to investigate the expansion and growth of skin tissue, allowing the development of algorithms to predict mechanically-controlled skin growth in health and disease (283-286). Promising research into mechanics and mechanobiology in the vocal folds (287) and the vocal ligament (288) is also being carried out using finite element analysis.
4.1.3. Multi-scale & Multi-physics Modelling

While the various models outlined above focus on research questions confined to individual loading cases of a specific tissue structure or cell type, mechanobiology in vivo occurs across multiple scales, with translation of loading to cell and molecular levels followed by transduction into responses expressed at tissue and organ scales. Therefore researchers across varying fields of study have recently applied multi-scale modelling techniques to investigate this phenomenon.

In bone tissue, multi-scale modelling techniques have been applied alongside periodic boundary conditions to determine that the strain experienced by osteocytes under the same macroscopic loading varies significantly, and strongly depends on their location relative to microstructural porosities (289). Furthermore, it was found that orientation of tissue structures such as lamellae can have a significant effect on strain experienced at level of individual bone cells (289). A similar multi-scale FE approach has been applied to cells suspended in bone marrow, demonstrating the importance of cell-cell attachments for mechanosensation within the bone marrow under macroscopic bone loading (290).

Multiscale modelling has also been applied in more disparate cases, such as modelling fluid flow and matrix deformation in the liver, allowing optimisation of perfusion conditions for tissue engineering (291). In the study of morphogenesis, multiscale models are used both to investigate tissue level effects in response to cellular- and molecular-scale events, and to study cell arrangement in developing tissue (292).

As has been discussed, cells are exposed to various types of inter-related physical stimuli and therefore reside in a multi-physics environment. Multi-physics modelling represents novel and developing array of methods which couple the effects of several physical phenomena in a single simulation or system of coupled simulations (see examples in Figure 6). The type of multi-physics modelling most applicable to the study of mechanobiology is fluid-structure interaction (FSI) techniques, which couple classic CFD and FE modelling by relaying results between solvers in an iterative manner until a solution to both is converged upon. These new methods have been applied to models of in vitro systems, allowing determination of the mechanical stimulation applied to cells by experimental settings (293), as well as the stimulation experienced by individual cells at different locations in a tissue engineering scaffold (294, 295).

INSERT FIGURE 6 HERE
In bone, FSI models have elucidated the function of the primary cilium as a mechanosensor on bone cells, determining the importance of cilia length (296). Furthermore, FSI has been applied to the complex multi-physics environments within bone, recently predicting that stimulatory magnitudes of shear stress result from macroscopic loading-induced fluid flow in accurate 3D models of osteocytes (221, 297). In an attempt to definitively compare these various mechanosensors, a comprehensive study of bone cell mechanosensation both in vitro and in vivo used FSI to predict that both integrin attachments and primary cilia are highly stimulated in vitro, but that the primary cilia is less stimulated in vivo unless embedded in the surrounding matrix (298).

Multi-scale techniques have been deployed to investigate the complex problem of in-stent restenosis alongside agent-based and cellular automata-based finite element models of cell behaviour (299-304), multi-physics modelling incorporating blood flow shear stress stimuli (305), and most recently mechanical/damage stimuli to individual cells (302), significantly advancing our understanding of this complex problem. Early advances were made in tying together mechanobiological stimuli across multiple scales and capturing multi-physics behaviour in the aortic heart valve, with different forms of FSI simulations in this area developing over a decade of research (reviewed in detail elsewhere (306)). The use of these methods has shed new light on a range of different allowing analysis of transient, three-dimensional behaviour over a range of length scales.

These multi-scale and multi-physics models demonstrate the value of computational mechanobiology models for providing information on biophysical parameters that cannot be measured experimentally, as well as the localised effects of multiple types of mechanosensors and complex patterns of physiological loading.

### 4.2. Image Analysis

One of the key problems in the study of mechanobiology is quantifying physical effects caused, or experienced, by cells, which is particularly challenging without directly interfering with them. While computational modelling attempts to recreate these effects, image analysis
allows researchers to calculate the mechanics of mechanobiological behaviour from experimental observations. These techniques can be developed in different manners and for various cells or tissues, with methods particularly useful in the field of mechanobiology discussed here.

4.2.1. Digital Image Correlation
Digital image correlation (DIC) is an optical technique that combines image registration and tracking methods for accurate 2D measurements of changes in images. Correlation theories for the measurement of alterations in data were first applied to digital images in 1975 (307). These theories have been optimised in recent years to apply to numerous applications (308), including confocal microscopy (309). DIC is based upon the calculation of a correlation coefficient that is determined from pixel intensity array subsets on multiple corresponding images and extracting the deformation mapping function that relates the images. In this manner, the displacements of individual regions in an image are tracked over a series of images, with the resulting strain calculated.

DIC can thus be applied in mechanobiology to characterise strain at the tissue level or within individual cells under loading. The various tissues for which the technique has provided detailed strain maps and material properties include skin (310, 311), the gallbladder (312), the vasculature, such as the aorta (313-315), the tympanic membrane of the ear (316), as well as individual trabecular struts within bone (317). DIC techniques have been recruited to diagnose cancer, as demonstrated by the detection of a basal cell carcinoma via strain mapping (318). DIC can also be applied at the scale of cells, and can quantify the displacement field in a substrate under cell contraction (319) (Figure 7A), or the velocity of cell migration (320). A recent study applied this technique to osteocytes and osteoblasts in vivo, allowing cellular strains to be observed for the first time in bone tissue and providing direct evidence that loading of whole bones is amplified at the cell level (321) (Figure 7B). DIC was also recently applied to analyse the beating of individual human cardiomyocytes, measuring both beating time and phases (322).

INSERT FIGURE 7 HERE
Figure 7: Contour plots developed using DIC, allowing mapping of substrate deformation due to cell contractility, and strain within ex vivo osteocytes

4.2.2. Particle Image Velocimetry
Developed over the past three decades, particle image velocimetry (PIV) has become a standard too in experimental fluid mechanics. Given its ability to measure the instantaneous velocity field simultaneously at many points, it is possible to compute fluid vorticity and strain in rapidly evolving flows (323). The technique has evolved from theory (324), with significant increases in computing power facilitating the development of digital PIV (325), while the advent and proliferation of standard digital cameras provided inputs perfectly suited to PIV (323).

PIV has been used extensively to investigate vascular biomechanics and mechanobiology, shedding light on the complex flow around heart valves and heart valve replacement devices (326-329). Further development has facilitated the use of PIV to investigate flow inside bioreactors and scaffolds used for tissue engineering (330-332), including for the study heart valve tissue mechanobiology (333). Higher resolution capabilities have facilitated the investigation of cell level flow, including the dynamics of individual red blood cells (334). Indeed, micro-PIV techniques are capable of capturing the flow around individual red blood cells (335, 336). In a fascinating application of the technology, it has recently been used at the cellular scale to calculate the shear stress affecting cell cytoskeletons (337) and to detect deformation of the cytoskeleton itself (338), as shown in Figure 8. Finally, PIV has also been applied to capture the guidance of collective cell migration by substrate geometry (339), as
well as the mechanical waves generated during expansion of tissue, as in both development (340) and cancer (341).

**INSERT FIGURE 8 HERE**

**Figure 4:** Use of PIV allows imaging of (A) movement of individual red blood cells under flow and (B) movement of the cytoskeleton within a cell

5. **Future perspectives**

Mechanobiology is a nascent field which, as is evident from the methods discussed, has benefitted from rapid advancements in molecular analysis, imaging and computational techniques. Our understanding of mechanisms of mechanosensing and mechanotransduction is deepening, alongside growing recognition in many overlapping fields of the importance of mechanical effects in cell behaviour, tissue development and various diseases. In particular, the potential of mechanobiological tools to augment tissue engineering by replicating in vivo mechanical environments provides an important avenue of study. The precision through which experimental manipulation of mechanical stimuli has advanced significantly, while improvements in measurement of cell mechanics provide opportunities to investigate mechanotransduction in ever greater detail. Furthermore, the power and sophistication of computational tools has improved significantly, and will likely spur further discoveries in the future. As engineering techniques become more intertwined with cellular and molecular analysis techniques, novel insights into the fundamental mechanisms by which cells appraise their mechanical environment will be gleaned. This will likely shed new light on the pathways by which cells transduce these stimuli into mechanical signals, presenting new therapeutic targets.
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