

Imperial College London

Department of Mechanical Engineering

**Three dimensional correlative imaging for measurement of
strain in cartilage and cartilage replacement materials**

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DECLARATIONS

Declaration of originality

I hereby certify that the work presented in this thesis is the result of my own investigations, which were carried out at Imperial College London from October 2016 to February 2020, except where otherwise stated. Collaborations and contributions by other researchers have been truly and properly acknowledged.

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ABSTRACT

Articular cartilage injuries are debilitating and often lead to osteoarthritis: a foremost cause of disability worldwide. Currently no interventions provide satisfactory long-term outcomes. Successful regeneration of articular cartilage through early stage intervention would be life-changing for millions of individuals. Providing suitable mechanical stimulation to promote articular cartilage regrowth is a crucial element to achieve success. Articular cartilage is complex with in-homogenous mechanical properties which have not been well characterised: a clear barrier to designing suitable orthopaedic implants for this application. Furthermore, it is necessary to interrogate intended implants to ensure they are providing appropriate loading to cells throughout the entire volumetric device.

Digital volume correlation (DVC) offers the potential for non-destructive three-dimensional visualisation of deformation. The technique relies upon tracking the displacement of features within a sample across three-dimensional scans with successive *in-situ* loading applied. Analysis provides quantification of displacement and strain components within the three-dimensional volume.

A method was developed to utilise the cells within articular cartilage as natural features for DVC and was validated against established 2D histological analysis. DVC of human articular cartilage provided 3D strain measurements with sufficient spatial resolution to inform implant design, with measurements compared against manually-calculated values ($R^2 = 0.93$). Subsequently DVC of novel hybrid materials implants enabled strain to be visualised within individual struts of the scaffolds ($R^2 = 0.98$): providing the necessary intra-device resolution for successful matching of mechanical properties. Finally the complete tissue-biomaterial system was assessed using DVC with an explant analysed after six weeks of implantation within an ovine model, enabling measurement of implant micromotion and strain fields across the system components.

In conclusion, DVC was successfully utilised to characterise strain within articular cartilage, novel biomaterials and tissue-biomaterial systems. Incorporating the technique as part of the device development pathway could be an effective tool to regenerate articular cartilage.

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LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AM	Additive manufacturing
CT	Computed Tomography
DC	Direct Correlation (DVC approach)
DIC	Digital Image Correlation
DVC	Digital Volume Correlation
FFT	Fast Fourier Transform (DVC approach)
FFT+DC	A combined FFT and DC approach to DVC
FOV	Field of View
GAGs	Glycosaminoglycans
Micro-CT	Micro-computed tomography
MRI	Magnetic Resonance Imaging
OA	Osteoarthritis
ODD	Object detector distance
PBS	Phosphate buffered saline solution
PC	Phase contrast
PPC	Propagation phase contrast
PSF	Point spread function
PTA	Phosphotungstic acid
ROI	Region of interest

1 INTRODUCTION

1.1 MOTIVATION

Articular cartilage provides a low-friction bearing surface and acts as a shock absorber for the underlying bone in our articulating joints over millions of cycles and decades of use. Yet unlike bone, articular cartilage does not repair itself well once damaged. Surgical interventions exist which attempt to regenerate the damaged tissue at an early stage whilst it remains a localised issue yet none of these provide good long term outcomes. Eventually the damage leads to osteoarthritis, one of the most debilitating conditions affecting society and costing the UK over £3 billion a year in loss of economic production alone [1].

Tissues in the body respond to external forces and many implantable technologies have failed, in part at least, because of a mismatch in mechanical properties to that of the natural biological tissue. Engineering implantable devices with locally-matched strain may provide the answer. Limitations in suitable measuring techniques have thus far hindered evaluation of localised mechanical properties: both of the natural tissue to provide target values, and of engineered solutions which aim to solve the regeneration problem.

Digital volume correlation (DVC) was first proposed 20 years ago for measuring strain in bone [2]. When paired with *in-situ* mechanical testing and three-dimensional imaging, DVC provides the potential for full-field evaluation of strain. The technique has been applied to a variety of biological tissues and engineered materials, yet no research has focussed on dealing with the problem of articular cartilage degeneration.

The motivation of this thesis was to apply DVC to articular cartilage and novel biomaterials, with the hope that this could be implemented in device design for successful regeneration of articular cartilage.

1.2 AIMS

The main aim of this thesis was to reveal 3D strain maps in articular cartilage and implants to allow strain-matching and therefore increase the potential for regeneration of damaged cartilage. The aim of this thesis has been formalised as four specific objectives:

1. To develop a protocol to image the cells within articular cartilage in three-dimensions across a distance of several millimetres, hence providing a pattern for DVC.
2. To evaluate 3D strain maps within human articular cartilage by applying *in-situ* mechanical testing and DVC.

3. To evaluate 3D strain maps within novel biomaterials to inform device development.
4. To assess mechanics across the tissue-biomaterial interface.

With research into these aims, a framework could be developed for DVC to be utilised as part of the process for development of articular cartilage devices with the eventual aim of regenerating articular cartilage. The work of this thesis fits in to a larger portfolio of work encompassed within an EPSRC grant (EP/N025059/1). Several other researchers remain working on this project focussing on the hybrid device manufacture, tribological and biological properties.

1.3 STRUCTURE

Chapter 2 is a literature review to introduce articular cartilage, current techniques to visualise strain and the need for more research to assist with matching cartilage's mechanical properties.

Chapter 3 describes the development of a method to non-destructively visualise cells within articular cartilage in three dimensions. This includes validation against established histological analysis. This chapter is heavily based upon a paper published by the author in *Osteoarthritis and Cartilage*.

Chapter 4 illustrates the application of the imaging technique developed in Chapter 3 to human articular cartilage in combination with *in-situ* loading. DVC analysis was carried out on the image stacks to enable visualisation of strain.

Chapter 5 focusses on visualisation of strain in novel hybrid materials developed by colleagues in the Department of Materials and aiming to replace and/or regenerate articular cartilage.

Chapter 6 describes the evaluation of strain within the cartilage-biomaterial system after six weeks of implantation in an ovine model.

All four results chapters provide an introduction to the study, materials and methods, results and discussions of the main findings.

Chapter 7 summarises the main findings of each study, discusses the links and differences between chapters, considers the limitations across all studies and outlines suggestions for future work.

2 LITERATURE REVIEW

This chapter reviews the current academic literature on the topics relevant to the goals of this project. It is divided in to three main areas:

The first section describes the biological tissue of concern: articular cartilage including the issues with current treatments and the importance of developing new surgical interventions.

The second part reviews methods to evaluate mechanically deformation of local features non-destructively within samples. This is of importance both for retrieving the necessary intra-tissue strain measurements to act as a target for the design of new interventions, and then for evaluating performance of novel biomaterial devices.

The focus of the final section is the three-dimensional imaging technique of choice: micro-CT, including available techniques to increase success in visualising features within soft tissues such as articular cartilage.

2.1 ARTICULAR CARTILAGE

2.1.1 Function

Articular cartilage is a soft-tissue situated at the end of bone in articulating joints such as the knees, hips and shoulders. The tissue is critical for several key functions: it provides a low-friction articular surface for smooth motion of joints; and it is a shock absorber, minimising peak stresses on the underlying bone, allowing pain-free movement. Under normal circumstances cartilage is remarkably wear-resistant, withstanding millions of cycles performing daily activities without deforming permanently. However, unlike bone, cartilage does not naturally repair itself well and so as soon as an injury occurs, the damage tends to increase and finally results in debilitating osteoarthritis. This affects millions of people in the UK and costs the economy billions of pounds [1].

2.1.2 Structure

Cartilage as a tissue is composed of specialised chondrocyte cells and a matrix principally composed of water, collagen and proteoglycans. The components of the matrix help to retain water, which is critical in maintaining its unique mechanical properties [3]. The matrix protects chondrocytes from mechanical loading; determines nutrient diffusion; acts as a signal transducer for the cells and stores some cytokines and growth factors [4]. Central to this project, mechanical deformation of the matrix produces electrical and chemical signals which influence the functions of the chondrocytes [5].

Depending on the composition of the matrix, in addition to articular cartilage other forms of cartilage exist: elastic, fibrous and fibro-elastic [5]. These other forms of cartilage have different mechanical properties to articular cartilage and do not exhibit its excellent low friction and wear properties. The structure of cartilage is complex and composed of different zones through its height, with varying matrix composition and associated mechanical properties.

Chondrocytes are the highly specialised cells that inhabit the cartilage matrix and are responsible for both synthesis of all matrix components and maintenance of the matrix infrastructure including metabolism [6]. Chondrocytes compose only 1-5% of the articular cartilage volume and unlike other tissues, cartilage has difficulty self-healing owing to the fact that it is avascular [4]. Nutrients are supplied to chondrocytes via diffusion which is a considerably more slow method of transport [5]. Chondrocytes sit within cavities termed *lacunae* and from which they maintains a surrounding region of matrix defined as the territorial matrix. Beyond each territorial region is the interterritorial matrix through which nutrients are passed via diffusion. Water contributes between 65-80% of the wet weight of cartilage and in addition to nutrition, it provides a medium for lubrication and load-dependent deformation of the cartilage [5].

Collagen forms 10 to 20% of the wet weight of articular cartilage. Type II collagen composes 90-95% of this and provides tensile strength to the cartilage. Smaller quantities of other collagen types are also present, all providing specialised functions. Proteoglycans are protein polysaccharide molecules which compose 10-20% of the wet weight and provide compressive strength [5]. They are produced by the chondrocytes and secreted in to the matrix. Subunits of proteoglycans are called glycosaminoglycans (GAGs) and these maintain fluid and electrolyte balance within the tissue [4].

Human articular cartilage is typically 2 to 4 mm thick [3] and composed of four distinct zones categorised by depth from the surface:

1. Superficial tangential zone
2. Middle zone
3. Deep zone
4. Calcified cartilage zone

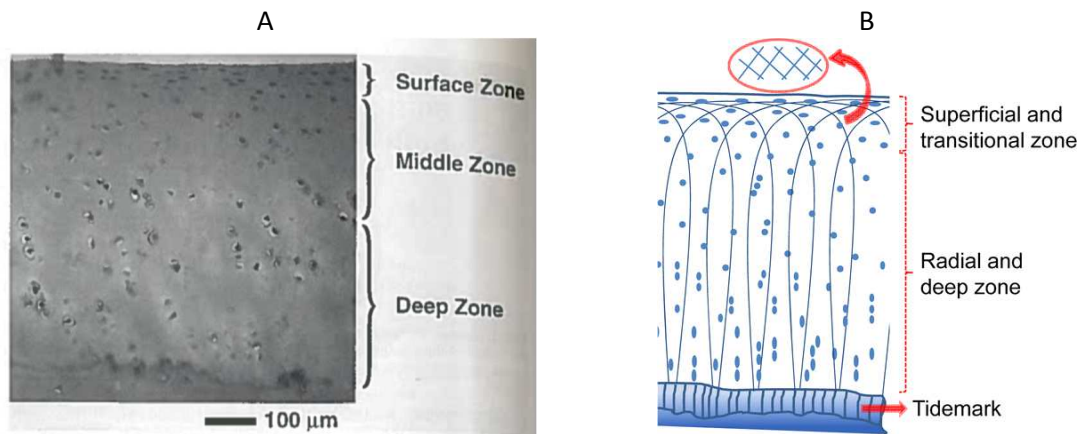


Figure 2.1. The structure of articular cartilage. Histological section showing the three non-calcified cartilage zones (A). Reprinted with permission from [7]. Schematic diagram of chondrocyte organisation in the three main zones of the uncalcified cartilage to the tidemark below which is the subchondral bone with a sagittal cross-sectional diagram of collagen fibre architecture shows the three salient zones of articular cartilage (B). Reprinted with permission from [8].

The superficial zone is the thinnest of all layers, contributing 10 to 20% of the total cartilage thickness [3]. As shown in Figure 2.1, flattened ellipsoid chondrocytes lying parallel to the joint surface and are covered by a thin film of synovial fluid, which provides the low-friction properties of articular cartilage. High production of collagen leads this zone to have the highest water content. The collagen fibres are also arranged parallel to the joint surface and hence provide the greatest shear strength and contributes to its low coefficient of friction (0.005-0.02) [9].

The middle (transitional) zone provides an anatomic bridge between the superficial and deep zones. It has lower cell density than the superficial zone, with spherical chondrocytes and randomly arranged

collagen fibres. The middle zone represents approximately 40% of the total cartilage volume and is the first line of resistance to compressive forces [3, 10, 11].

In the deep zone, cell density is lowest and the collagen fibres are at their largest diameter. Both are arranged perpendicular to the surface, providing the greatest resistance to compressive forces. Typically the deep zone represents approximately 40% of the tissue's volume.

Finally, the calcified zone represents a mineralised transition between the cartilage and underlying subchondral bone. There is a visible border between the deep and calcified zones, termed the "tidemark". Containing only a small number of cells embedded within a calcified matrix there is very little metabolic activity [5]. An important function of the cells in this zone are that they synthesise Type X collagen which is important for shock absorbance and structural integrity in relation the subchondral bone. The underlying subchondral bone itself provides a connection to the wider bone architecture as part of our musculoskeletal system and may eventually become exposed if the cartilage experiences excessive damage.

2.1.3 Cartilage Damage

Under normal circumstances cartilage is remarkably wear-resistant, withstanding millions of cycles performing various activities throughout daily life [4]. However, studies have reported that over one third of people over 45 years of age in the United States complain of joint issues from occasional joint stiffness and intermittent aching to permanent loss of motion and constant pain [12-14].

Focal cartilage lesions, involving damage specific to one or more specific locations of cartilage, are mostly commonly caused by trauma, with sporting injuries estimated to be the cause half of all injuries [15]. Articular cartilage is avascular, meaning that it has no blood vessels, and hence nutrients can only be provided via the slow process of diffusion resulting in a very limited ability to heal. The damage is initially contained but if the lesion expands the body is unable to repair the tissue. In this instance further trauma exacerbates the issue, increasing the region(s) of damage beyond repair. In addition to superficial damage to the cartilage (chondral defects), in some instances the underlying subchondral bone is also damaged, which are classified as osteochondral defects. If left untreated, eventually this leads to osteoarthritis (OA). OA is a progressive disease hence early diagnosis and intervention is critical for giving the best probability for preventing further tissue degeneration [16]. Age is one of the main predictors of OA and has been estimated to affect one third of Americans over 65 year olds [17]. Limited data is available on the costs of OA in the UK, with costs varying considerably between trusts and no studies were identified. Current estimates suggest the UK prevalence of OA alone to be 2.8 million patients. The estimated direct NHS costs of hip and knee replacements for 2010 was £852 million, an increase of 66% over the previous 10 years [1]. Indirect costs include loss of

economic production, totalling over £3.2 billion, and incapacity benefit claims for OA and related conditions of £2.41 billion in 2001 [1]. Estimates suggest that the cost of OA in the United States is annually \$185.5 billion [18]. Efforts to alleviate the burden this poses on society would have a significant positive impact on both wellbeing and the economy.

2.1.4 Regeneration & Current interventions

2.1.4.1 *Current Treatment*

In the case of early-stage focal lesions, a number of surgical techniques may be employed to attempt to treat the injury. The gold standard remains microfracture, which is a technique involving using an awl to release stem cell-containing bone marrow from the underlying bone. The intention is for the stem cells to differentiate into chondrocytes, laying down the required matrix and repairing the cartilage tissue. In reality unless the injury is very small, rather than articular cartilage, fibro cartilage is formed. It is suspected that the cause for this is a lack of load reaching the cells in the defect not providing the required mechanobiological stimuli [19]. The mechanical and tribological properties of fibro cartilage are very much different to articular cartilage and it is less wear-resistant, leading to further damage within several years [20]. Other treatments currently in use include two-step procedures: ACI (Autologous chondrocyte implantation) involves in-vitro growing of chondrocytes for several weeks extracted from a cartilage sample in the patient [21] and MACI (Matrix-induced autologous chondrocyte implantation) involves seeding of cells on to a collagen matrix [22]. Both ACI and MACI are typically used in defects up to an area of 5 cm². Although these techniques can slow the progression of OA, their use is limited in defect size and long-term efficacy is suboptimal for many patients. A one-step approach is offered by AMIC (Autologous matrix-induced chondrogenesis) which combines microfracture with a collagen matrix scaffold has shown promise though long-term outcomes have not been conclusive [23]. Widely acceptable early-intervention techniques simply do not currently exist, inevitably leaving medical professionals typically limited to treating patients symptomatically by which time significant irreparable cartilage damage has taken place [19]. New interventions are constantly in development to solve the problem [24-26] yet none have so far achieved mainstream success.

If the damage is not caught early and instead spreads to a wide area, total joint replacement remains the only reasonable option. The operation requires removal of the articular surface in addition to typically large regions of underlying bone [15]. Despite the best efforts of surgeons, engineers and materials scientists, these engineering solutions only have a finite life and often do not result in a satisfactory improvement of quality of life. Most joint replacement options only currently typically last for 20 years, thereby many patients outlive the interventions [19].

2.1.4.2 Mechanobiology

Tissues dynamically adapt to imposed loads which can dictate both the quantity and type of tissue in a given region [27]. Cell pathway differentiation depends upon mechanobiological stimulation [28, 29]. Within orthopaedics a historic issue was stress shielding, whereby after implantation of a high stiffness implant, limited load and strain is transmitted to the tissue surrounding it laterally. Biological systems are run efficiently and limited loading of the bone leads to resorption [30] (Figure 2.2). There has been a transition towards stiffness-matched implants so that load can be better shared with the surrounding tissue [31]. For non-degradable implants this can improve long term patient outcomes because the remaining tissue maintains its density and strength. The need for stiffness-matched implants is even higher for degradable implants whereby tissue regeneration is the main aim. Solving issues of degenerated tissue may be overcome by leveraging the body's own regenerative capabilities in combination with suitable designed biomaterials [32].

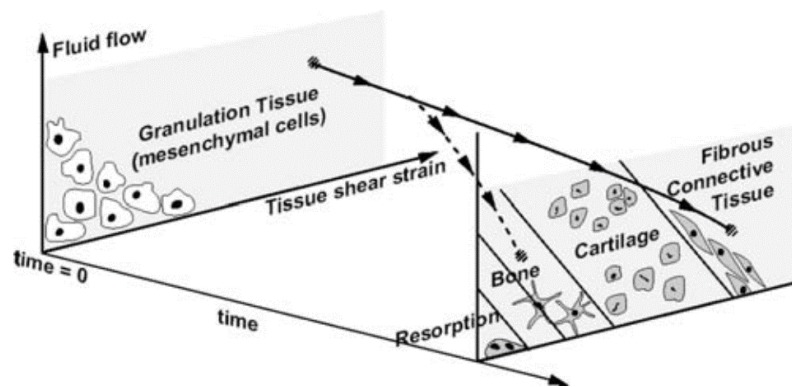


Figure 2.2. Mechanobiology of tissue differentiation. Tissue lineage is regulated by interaction with the imposed load. Reprinted with permission from [33].

One of the reasons that current cartilage treatments may have not achieved success is that the load experienced by cells has not driven differentiation to articular cartilage tissue [34]. The problem stems from data not being available for the tissue mechanical properties itself. As with other biological materials, cartilage's complicated structure means that mechanical properties vary topographically (Figure 2.3). For example, studies have shown that the compressive modulus of cartilage varies with depth [35] and stiffness varies by region [36]. Hence benchmark data is not limited to a single target value as with typical engineering materials. Once the material is considered as part of a wider biomechanical system even more variables become important, such that consideration for that the specific intended location of the implant becomes important [37]. The bulk mechanical properties of articular cartilage have been quantified though the tissue exhibits a non-linear strain dependency so care should be taken and Young's modulus cannot be reported.

Human articular cartilage can withstand compressive strain of up to 24-28% and compressive strength cited to be 22-37 MPa [38, 39]. Owing to its complicated structure, these values may be useful to gauge properties in comparison to other materials, yet to fully characterise the tissue further work is required to examine cartilage in 3D.

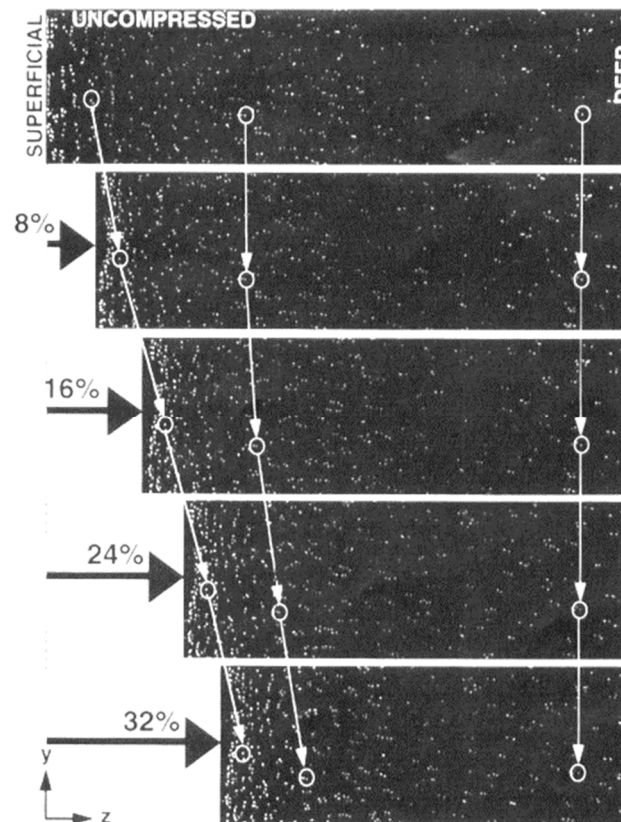


Figure 2.3. Cartilage properties vary with depth. Observed here across an exposed two-dimensional surface of bovine knee cartilage under confined compression. The white features are chondrocyte nuclei. Reprinted with permission from [35].

2.1.5 Novel Hybrid Materials

A wide variety of novel implants are being developed with the intention of cartilage regeneration including collagen composites [25] and polymeric scaffolds [40]. Materials deemed suitable must be proven with acceptable biological; chemical; mechanical and tribological properties. The interaction between the cell and the surface of the biomaterial is an important consideration when developing such scaffolds where a high degree of cell interaction and cooperation is required to achieve the intended tissue regeneration [41]. At the single-cell level, cellular attachment affects cell morphology, function and signalling mechanisms [42].

Materials such as bioactive glass have long been coveted for the positive biological response they elicit [43] but their use has remained in powder form to form putties for filling bone defects [44]. It has not been possible to produce monolithic glass components that have the required mechanical properties for cartilage applications. A recent development of combining silica glass (as in Bioglass) with polymers has produced hybrid materials which can be 3D printed [45]. Hybrids follow the same concept as composites yet are bonded at a much finer scale and are indistinguishable down to the nano-scale. The components behave as one phase yet combine the positive biological response of bioglass with the improved mechanical properties of polymers. This project focusses on the use of covalently bonded co-networks of bioactive silica and degradable polycaprolactone to form SiO₂-PCL hybrids, prepared at room temperature. The ratio of glass to polymer dictates how stiff the resulting material is and the mechanical properties can be tailored to match that of the local tissues and the material degrades congruently. The proposed implant combines an additively manufactured scaffold, with an optimised pore architecture to promote cell regeneration, and a monolithic low-friction articular surface to maintain joint mobility and enclose the cells in the scaffold (Figure 2.4). In previous lab studies, hybrid scaffolds were shown to promote collagen type II, indicating articular cartilage growth [41].

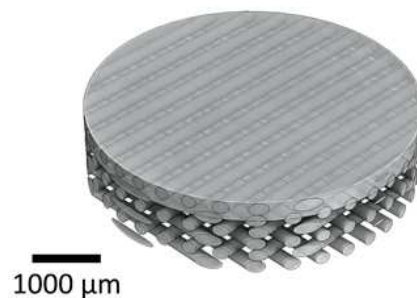


Figure 2.4. The intended hybrid device design. Layers of degradable 3D printed scaffold allow tissue in-growth and have been engineered by collaborators to have similar mechanical properties to cartilage whilst promoting cell adhesion and proliferation of type-II collagen. The scaffold is capped with a solid region of the same material, engineered to have excellent tribological properties, including very low friction, to ensure no damage is caused to the surrounding cartilage.

The biomechanical properties of the proposed implant must be considered, for two main purposes:

1. Micromotion

Several studies have shown that implant micromotion may be tolerated up to 150μm [46, 47], with 100μm or less being an acceptable threshold [48]. This is to allow cell in-growth and tissue regeneration; reduce the risk of implant rejection and encapsulation; and to maintain joint stability and limit the opportunity for damage to either the surrounding tissue or the cartilage counterface.

2. Strain-matching

It is important for the implant to share load with the surrounding the tissue, such that the cells in the scaffold are exposed to suitable mechanobiological stimuli to induce appropriate cell-signalling. For a given stress, a material's strain response is dependent on its stiffness. The ability to match the strain of the implant to that of the surrounding tissue would be advantageous, such that an appropriate strain gradient is experienced by the cells in the scaffold. This will involve accounting for the stiffness, and strain, gradient through the depth of an osteochondral defect.

Micromotion will be reduced both as a result of the strain-matching, reducing the relative movement between the implant and surrounding tissue, and by design of suitable fixation methods. Fixation may be addressed by geometric design of the implant or by use of a surgical adhesive, or both.

2.2 QUANTIFYING DISPLACEMENT AND STRAIN

Measuring local deformations in cartilage, biomaterials and tissue-biomaterial systems is a requirement of this project. To understand the function both of normal tissue and complex engineered materials it is necessary to characterise local mechanical behaviour in 3D. Complex 3D architecture, such as in articular cartilage, cannot be accurately be simplified to 2D representations [49]. Studies of cartilage mechanics using 2D techniques do exist (Figure 2.3) and these are discussed in detail in the Chapter 4. Frequently used methods for measurements on engineering materials such as linear variable differential transformers (LVDTs) are limited to surface point-readings. *In-silico* finite element models can be very useful but first require both collection of accurate and representative morphological models; associated mechanical properties which are current lacking for cartilage and finally rigorous validation [50]. Digital image correlation (DIC) is a widely used technique in biomechanical research [51] to deduce motion and strain from successively loaded photographs across a 2D plane. Stereo DIC exists, involving the use of two cameras to capture a curved surface yet the technique is still limited to surface strain measurements. Since the technique looks at many points across a plane it gives a richer dataset than LVDTs allow but with the limitation of requiring direct view of the region of interest. An obvious development would be to utilise the DIC technique with non-invasive scanning technologies such as X-ray computed tomography (CT) or magnetic resonance imaging (MRI) to allow internal features to be observed. This is provided by Digital Volume Correlation (DVC) [49].

2.2.1 Digital Volume Correlation (DVC)

2.2.1.1 Overview

DVC is a non-destructive technique which allows displacement and strain measurements within a three-dimensional volume. The technique was first employed twenty years ago by *Bay et al.* to evaluate internal displacement of bone (Figure 2.5, [2]). DVC has now been expanded to bone-biomaterial interfaces [52], implant micromotion [53] and soft tissues such as intervertebral disks [54] and lungs [55]. Volume images for DVC can be obtained from 3D imaging sources such as [56], synchrotron tomography [57] and confocal microscopy [58]. For this project micro-CT imaging (micro-CT) has been chosen for the source images due to resolution suitability and access.

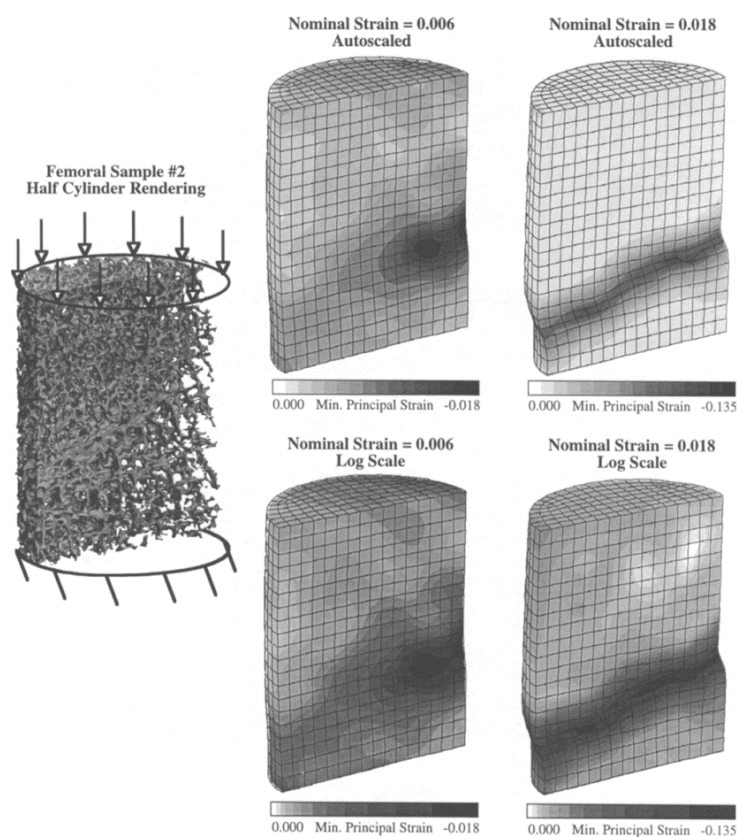


Figure 2.5. Results from the first implementation of DVC by Bay *et al.* in 1999. The internal deformation of bone under compression was observed at high resolution in three-dimensions for the first time. Reproduced with permission from [2].

2.2.1.2 Operating Principals

DVC works by correlating movement of features within the sample between the two (or more) scans based on their intensity level. A minimum of two 3D scans are required: one of the unloaded state as a reference scan, and a second with loading applied to the deformed sample. Correlation can be calculated using either global or local approaches depending on the software package [50, 59, 60]. In

either scenario, successful tracking requires there to be an internal contrast pattern within the sample (Figure 2.6). For some materials there exists a natural pattern and this includes tissues such as bone for which internal pores can be tracked [61]. The software aims to identify the pattern in both scan volumes and determine the associated displacement vector. Practically this is carried out by moving the pattern in the initial reference scan until the closest match to the deformed pattern is found (Figure 2.7). Both global and local DVC approaches exist. Global approaches compute strain through the entire 3D volume. A local approach instead divides the volume in to smaller “subvolumes” which are then computed individually. The edglength, N , of each subvolume is a critical parameter, sufficient inclusion of sample features is necessary to ensure accurate correlation and therefore tracking of deformation. A local approach, using commercially available software, was utilised throughout this project.

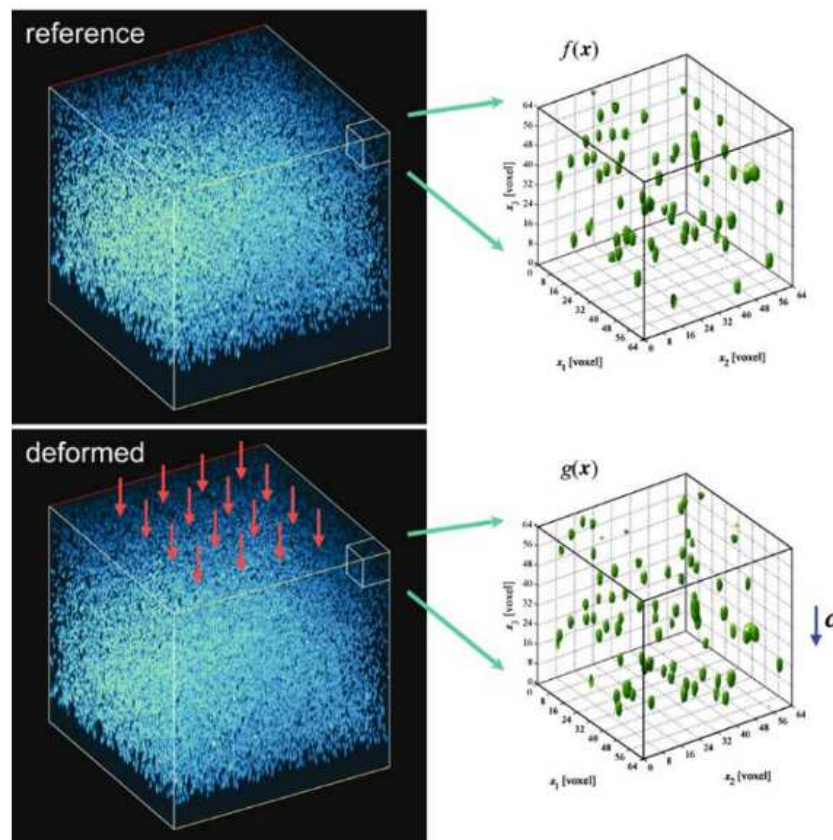


Figure 2.6. DVC requires the tracking of a pattern between 3D scans between reference ($f(x)$) and deformed conditions ($g(x)$). Often this pattern is given by internal features naturally occurring in the sample, shown here in an agarose gel. Reproduced with permission from [58].

Matching of the features is quantified with the correlation value. The commercial DVC software package for this project (DaVis 8.4, LaVision GmbH, Goettingen, Germany) utilises a normalised cross-

correlation algorithm to account for differences in intensity levels between the scans (eq 1). The value is equal to 1 when both subvolumes match perfectly.

$$C_{norm}(i) = \frac{\sum_{i=x_0}^{x_0+N-1} (A(i) - \sum_i \frac{A(i)}{N}) (B(i+dx) - \sum_i \frac{B(i+dx)}{N})}{\sqrt{\sum_{i=x_0}^{x_0+N-1} (A(i) - \sum_i \frac{A(i)}{N})^2} \sqrt{\sum_{i=x_0}^{x_0+N-1} (B(i+dx) - \sum_i \frac{B(i+dx)}{N})^2}} \quad (1)$$

where A is the reference image, B is the deformed image, x_0 denotes the initial position, N is the subvolume length and dx is the current shift position [62].

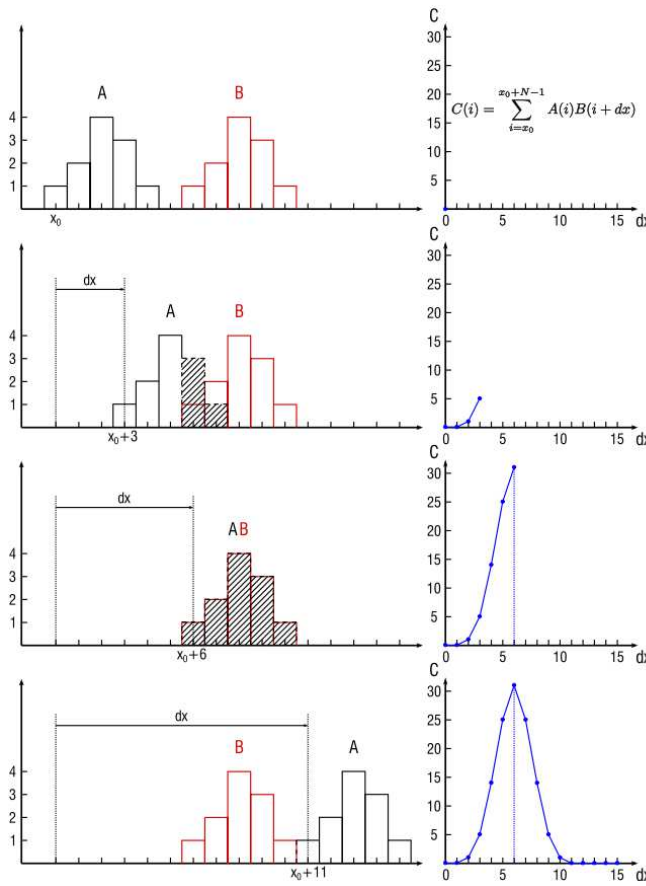


Figure 2.7. A one-dimensional example of the cross-correlation technique employed. The undeformed reference pattern (A) is shifted in increments of dx up to a maximum distance specified by the peak search radius. For each of these shifts, the product of the overlapping grayscale intensity at each point is calculated and the sum provides the corresponding correlation value. The correlation value is at its peak when the patterns have the best match. Reproduced with permission from [62].

According to documentation from LaVision, in DaVis a linear shape function is used to map between the unloaded and loaded images with 12 degrees of freedom at each point encompassing translations, rotations, normal strains and shear strains in x, y and z [62]. Once the displacement vector field has

been obtained, strain can be calculated using a centered finite differences (CFD) scheme for each subvolume [59].

The DaVis DVC software employs a local DVC approach, which involves dividing the scan volume into smaller subvolumes, which are then tracked between the scans. The subvolume size must be carefully selected: too small and each subvolume will feature insufficient trackable features for successful correlation causing erroneous vectors; too large and the spatial resolution may be insufficient to reveal localised strain concentrations.

2.2.1.3 *Parameters*

Subvolume size: The length, in voxels, of the cubic boxes which the entire volume is subdivided into for correlation with the local DVC approach.

Peak search radius: The maximum distance, in voxels, over which the subvolumes are displaced to track deformation between the scans. If the peak search radius is set insufficiently low, the true motion of subvolumes will not be detected.

Overlap: Decreasing the subvolume size can result in large errors. An alternative approach to increase spatial resolution without decreasing the size of the subvolumes is to overlap the subvolumes. For the DVC package utilised in this study offers three options for overlap: 0%, 50% and 75%.

Binning: To reduce computational burden, adjacent voxels can be averaged. For example binning of 2 reduces the number of voxels by a factor of 8.

Passes: An iterative approach can be utilised whereby the same approach is run multiple times, with each step using a predictor corrector scheme to find displacements gradually closer to the true value.

Multi-step approach: An option to iteratively refine the subvolume size, beginning with a large and coarse approach to predict large movements which are then refined with smaller subvolume sizes over a small area.

Masking: It is possible to exclude regions of the volume from the DVC calculation using either geometric or algorithmic masking. Geometric masking has been utilised in parts of this project so that inclusion of the displacement platen did not negatively affect tracking of the sample.

Required valid voxel: In the scenario where a mask is applied, this metric defines the percentage of voxels within each subvolume required for that subvolume to be included in the calculation. The default setting is 50% and was utilised throughout this study.

Correlation mode for multiple loading steps: For instances where more than two loading conditions are considered, it is possible to decide between which of the volumes the software should seek

correlation. For example *relative to first* means that each volume is correlated back to the initial undeformed state independently of each other. This provides the best accuracy but is not ideal for tracking large deformations. For this project whereby large deformations were imposed with few loading steps, the *sum of differential* mode was applied in the instance where multiple loading steps were present. This mode sums the vector fields between consecutive scans but at the expense of increased errors.

Rigid body correction: Prior to carrying out the correlation calculation there is an option to correct for rigid body movements. This motion, which encompasses both translations and rotations, can occur due to imperfect rotation or translation during the scanning procedure or misalignment if the loading rig is removed between scans. If not corrected it would otherwise lead to misalignment between successive scans.

Three different calculation methods are available with DaVis:

1. Direct Correlation (DC)

DC calculations which involve sequentially directly calculating correlation values for all subvolumes over the specified search radius as previously described. If the imposed deformation is small the DC approach can be very fast, yet if the search radius is large due to a large imposed displacement it can take prohibitively long [63]. For the DC approach there is very little cost to increasing the overlap because the software keeps track of computed correlations which are reused for overlapping regions.

2. Fast-Fourier Transform (FFT)

Instead of calculating the cross-correlation coefficient of each subvolume over a given distance individually, a Fast-Fourier Transform (FFT) operation may be used in which the analysis is instead performed on the frequency domain equivalent [63]. This has two main benefits: firstly it can decrease calculation time. Secondly, it ensures that all possible shifts within the volume are considered, providing a full correlation map. Therefore FFT is useful for large deformations which would otherwise be impractical for the DC approach. However caution should be exercised because although generally faster than DC for large deformations, FFT can be highly sensitive to increasing the overlap between subvolumes which severely increases calculation time for this approach. It has been noted that the use of 75% overlap is approximately eight times more computationally demanding than an overlap of 50% [62]. FFT may often induce higher errors than DC and is more slow for small displacements [62].

3. Combined approach: DC+FFT

Unique to the DaVis software package is the ability to combine both of the previously discussed approaches. According to documentation and conversation with the company, this harnesses the

potential of FFT to track large deformations quickly, using a coarse initial step with a large subvolume size, which then provides initial predictor of the displacement field for the DC approach [62].

2.2.1.3.1 Error Quantification

The systematic error (MAER) and random error (SDER) of DVC measurements are affected by the micro-CT equipment: beam settings, age and stability [64]; the sample type: size, morphology, mechanical fixation and density-distribution [65]; and the resulting images: spatial resolution, artefacts and contrast to noise ratio [66]. It is prudent to quantify the sum of this error and DVC studies commonly follow a protocol to quantify error in the system [67]. This is carried out by collecting two consecutive scans of the sample with no change in load between the scans. Since no load was applied between the repeat scans, no strain should be observed. Any deviation from constant-strain reveals the quantity of error in the system. Error can be quantified by calculating the mean absolute error (MAER, equation 2), representing the systematic error, and the standard deviation of error (SDER, equation 3), representing the random error inherent within the system. The random error is also calculated for the displacement components. The systematic error of displacement is not calculated because the real displacement is unknown.

$$MAER = \frac{1}{N} \sum_{k=1}^N \left(\frac{1}{6} \sum_{c=1}^6 |\epsilon_{c,k}| \right) \quad (2)$$

$$SDER = \sqrt{\frac{1}{N} \sum_{k=1}^N \left(\frac{1}{6} \sum_{c=1}^6 |\epsilon_{c,k}| - MAER \right)^2} \quad (3)$$

where “ ϵ ” represents the strain; “ c ” represents each of the six independent strain components; “ k ” represents the measurement point at each subvolume and N is the number of subvolumes measured.

These metrics are collected across a range of subvolume sizes, so that the relationship between subvolume size and error can be plotted. Typically this will follow a power law relationship whereby increasing the subvolume size yields lower errors but at the cost of poorer spatial resolution [68]. To decide upon which subvolume size to select for a study, a compromise must be sought between spatial resolution and the maximum acceptable error. Previous DVC studies have set a maximum acceptable error as 10% of the nominal strain [69]. For the samples tested in this thesis, whereby the applied strain was approximately 10%, the maximum acceptable error therefore could be calculated to be 1% strain: 10,000 $\mu\epsilon$. Once the optimum subvolume size has been selected, the compression study data

is then processed. Of note, error averaged across all six strain components is known to underestimate the largest strain error by approximately 50% [63].

A limitation of the current error quantification approach is that error is only quantified with simplified conditions [50]. Although this is useful to quantify error caused by physical components of the scanning equipment, it does not necessarily capture the impact that pattern quality within the sample will eventually have under compression conditions. It is not particularly challenging for software to correlate features between two undeformed images yet this offers no reassurance that it will work under loading for which the task of correlation is significantly more difficult. To combat this an extension of the constant-strain study has been developed whereby one of the repeated scans is virtually translated or deformed [70], creating a constant-strain and virtually deformed analysis. This allows better capture of the true error involved with multiple scans and loss of correlation due to deformation under loading. The study concluded that errors were similar at the centre of images between constant-strain and virtually deformed tests. Further approaches are being developed to quantify error in more realistic scenarios exist including the addition of artificial noise [60].

2.2.1.4 *Previous DVC studies*

Biological Tissue

Bone remains the most widely type of tissue analysed with DVC [71]. A review of all strain measurement techniques in bone focusses includes DVC on different bone types [72]. Micromotion and strain have been quantified [53, 73] across different length scales in murine [74], bovine [75] and human samples [76, 77]. Using human femoral heads both crack formation [78] and failure have been predicted [79]. A thorough study on DVC parameters was conducted on porcine bone [66]. The study made several useful contributions including concluding that a sufficient warm-up time is required for future micro-CT studies to ensure thermal stability therefore reducing strain errors. Additionally the study found that a correlation value of 0.9 or above was required for reliable tracking in bone.

The effects of synchrotron radiation on bone whereby microdamage was observed due to excessive X-ray exposure has been investigated [80]. Studies in bone have suggested that strain errors using micro-CT can be expected to be 100 $\mu\epsilon$ or below and below displacement random errors of 0.1 voxels or below [50]. Micro-CT is by far the most common imaging technique utilised, though in a move to translate findings to clinical settings studies do exist using MRI scanners [81, 82]. Commonly DVC has been paired with FE as a validation tool. In the first such comparison study, good agreement ($R^2 = 0.97$) between 3D deformation results for DVC and FE models on human bone was found in certain directions yet DVC errors were high and the authors noted that FE was not accurate in all directions for strain ($R^2 = 0.77$) [83]. Since this study DVC performance has improved and comparison between

DVC and FE results has evolved. Performance of several FE models in predicting local displacements in bone has been quantified against DVC [84]. It was suggested that errors in FE predictions could be minimised using boundary conditions derived by DVC. DVC has recently been applied to regenerated bone tissue after six-weeks of biomaterial implantation in an *in-vivo* sheep model [85], and on lab-grown extracellular collagen matrix constructs [86].

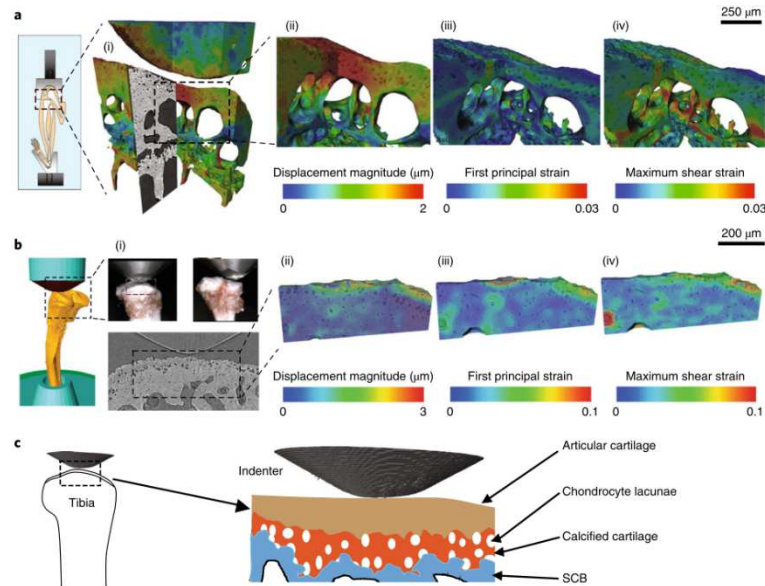


Figure 2.8. *In-situ* loading and synchrotron scanning enabled DVC on whole mouse joints. It should be noted that non-calcified articular cartilage was not included in the analysis. Reproduced with permission from [87].

Only a limited number of studies exist on soft tissues. The studies have usually taken place at synchrotron facilities [88] or using other imaging modalities depending on the tissue of interest. The vertebra-intervertebral disc interface has been studied [89], in addition to lung [55] and optic nerve tissue [90]. The internal fibrous structure allowed successful DVC study of intervertebral discs at a synchrotron [54]. The importance of sufficient time provided for stress relaxation in soft tissues prior to imaging was noted. Additional studies on the intervertebral disc (IVD) have taken place using MRI [91, 92]. DVC has even been applied to individual fluorescently-labelled fibroblast cells during cell division [93].

Cartilage DVC has previously been attempted on human participants using MRI [94]. The study was able to detect deformation but lacked resolution to provide convincing intra-tissue strain measurements. Recently a study was carried out on whole mouse joints (Figure 2.8, [87]). The study made progress in visualising deformation across the bone-cartilage interface yet crucially excluded non-calcified cartilage from their analysis.

2.2.1.4.1 Biomaterials

DVC has been used to measure deformation in manufactured biomaterials and implants for device development. Studies have been performed on micromotion of polymeric implants [53] and the stability of polymeric pegs in artificial bone [95]. Additionally studies exist on dental implants [96], scaffolds [97] and foams [98]. A constant-strain study from 2016 on artificial bone and bone-cement interface [99] was conducted and additionally performed micro-FE analysis. Their results suggest that although displacements compared well, strain results differed significantly, concluding that further work in both methods is required. Recently a study was performed to optimise image preparation on bone and bone-biomaterial systems using a constant-strain study [100]. The study compared raw images with those processed with median and non-local means filters, and also considered masking. Results from the study suggested that the non-local means filter decreased strain uncertainties and improved the average correlation coefficient in both bone and bone-biomaterial samples. Chapter 5 of the thesis details work carried out of DVC on scaffold and solid dual-composition samples produced from a hybrid biomaterial.

2.2.1.4.2 Tissue-Biomaterial Systems

The interaction of tissue and implanted biomaterial is an obvious route of analysis using DVC since no other techniques exist which can non-destructively determine internal relative deformations. Studies have been conducted on human cadaveric femurs and bone implants for validation of FE models [101]; bone-metal implant interface [102]; the bone-cement interface [103, 104] and on damage in bone caused by press-fit implants at the femoral condyles [69]. Additionally DVC has been used to estimate local strains [105] and for locating failure initiation in augmented vertebrae for which a multi-step DVC processing approach was recommended [106, 107]. The majority of studies were conducted on ex-vivo tissue though strains were successfully measured across the bone-biomaterial interface after six weeks of implantation in an ovine model (Figure 2.9, [52]). Chapter 6 of this thesis will focus on DVC of a sample from a pilot study of the hybrid scaffold implanted within an ovine osteochondral defect.

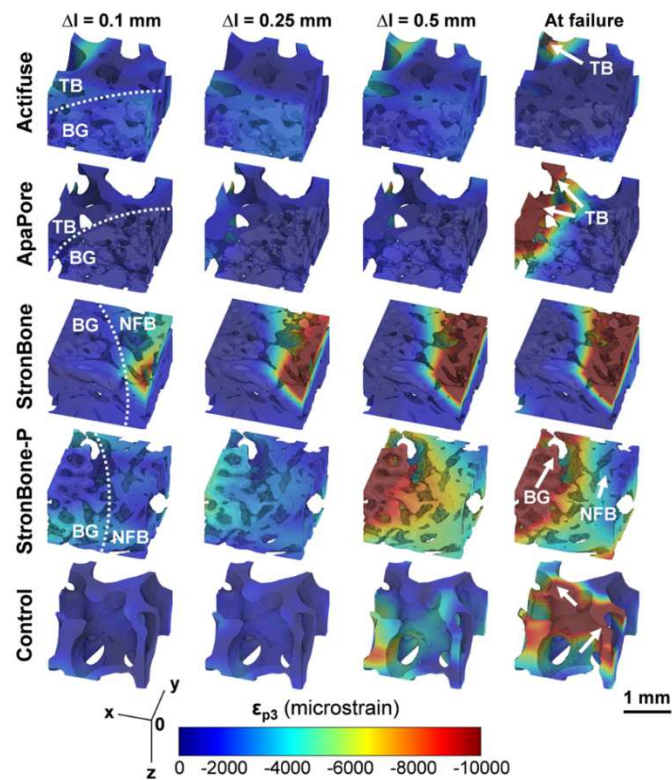


Figure 2.9. DVC has enabled strain visualisation across the bone-biomaterial interface with implants after six weeks within an ovine model. Reproduced with permission from [52].

2.3 THREE-DIMENSIONAL IMAGING TECHNIQUES

One of the critical aspects of the project is development of a robust imaging method of articular cartilage. The underlying principal of DVC relies upon successful imaging of patterns in the images that can be tracked between the successive scans. Certain materials are naturally more suited to producing suitable patterns than others. Bone is a good candidate material because the porosity provides good absorbance contrast between the bone and void components, hence micro-CT is a standard technique for quantifying trabecular bone microstructural changes [108]. The low x-ray absorption of non-mineralised soft-tissues, has meant that micro-CT has not been as immediately successful for imaging cartilage, hence research in to staining methods of cartilage has been carried out.

Several techniques are widely available to image beyond the external surfaces available with typical optical microscopy techniques. Clinically, two main techniques are used for imaging the 3D internal structure of tissue: Magnetic Resonance Imaging (MRI) and Computed Tomography (CT). These clinical instruments usually provide a maximum spatial resolution in the order of 0.1 mm: useful for visualising tissue over a large ($> 5 \text{ cm}$) field of view yet not particularly efficient for fine intra-tissue details. Techniques such as scanning confocal laser microscopy do exist which can provide sub-micron

resolution and allows imaging of internal structures [7]. Yet due to optical limitations of diffusion and scattering it is inherently lacking in penetration depth to only approximately 100 microns from the sample surface so has limited applicability for full-field 3D mechanical testing [35]. The most common approach providing the necessary resolution is micro computed tomography (micro-CT): a high resolution instrument using the same principles as clinical CT. Generally speaking, MRI is advantageous for imaging of soft tissue and avoids concerns over irradiation damage caused by exposure to X-rays during CT. Since a large part of the focus of this project was soft-tissue, micro-MRI could have been an ideal instrument to use yet they are not widely available and may not have been well suited to imaging the intended biomaterial. For these reasons X-ray micro-computed tomography (micro-CT) was chosen for this study.

2.3.1 Micro-CT

2.3.1.1 Introduction

The principal of micro-CT is similar to clinically-implemented CT but is applied with higher resolution to a reduced field of view. The technique permits visualisation of samples up to approximately 10 cm with voxel (3D pixel) resolution of 1 – 50 μm and potential for sub-micron resolution. Nano-CT is possible, looking at even smaller length scales for smaller fields of view. Images are acquired as two-dimensional projections and are subsequently reconstructed to form an image stack creating a 3D volume (Figure 2.10). This can be extended to four dimensions with sequential scanning to capture temporal changes. Micro-CT can be implemented either at a synchrotron or with laboratory facilities. Laboratory micro-CT was the focus of this project and therefore will be the focus of this thesis.

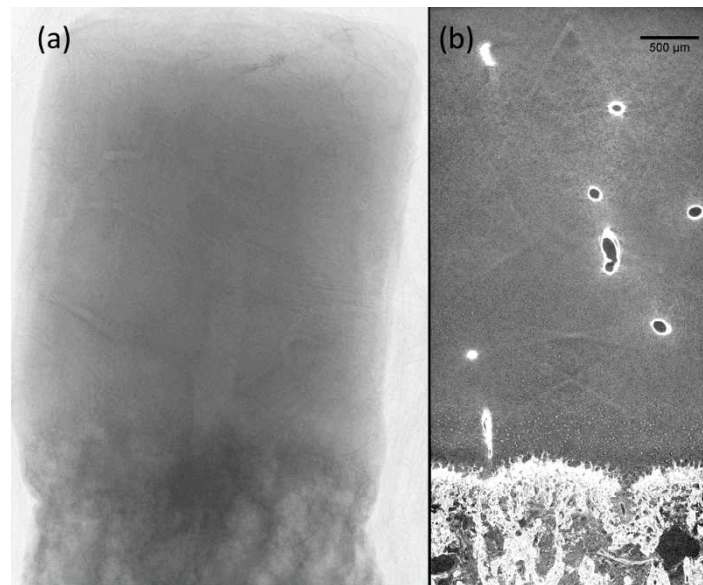


Figure 2.10. a) A single projection of a bovine osteochondral plug. b) A reconstructed slice from the final 3D volume. Notice how each individual projection shows very limited detail and image contrast.

2.3.1.2 Image Acquisition

Micro-CT first involves generation of X-rays at the source: composed of a filament which when provided with a high voltage electric input will emit X-rays. X-rays form a high-energy part of the electromagnetic spectrum, with shorter wavelengths shorter than visible light. Filaments of different material compositions are available, with tungsten being frequently used [109]. The X-rays are then projected by the source towards the sample (Figure 2.11). With a laboratory system this is typically in a cone shape whereas a parallel beam can be achieved at a synchrotron. Depending on the density and thickness of the sample, a certain proportion of the X-rays will interact with the sample and be absorbed by the sample whilst others are transmitted to the detector (Figure 2.10). Beyond the sample is the detector: the array of 2D sensors which receive the remaining X-ray signal. A scintillator converts the X-ray signal in to that of visible light, allowing use of an imaging sensor similar to that as seen in conventional digital cameras. The specifics of the setup vary by manufacturer. Some micro-CT systems include optical lenses between the sample and the detector, allowing optical magnification and therefore increased resolving power. The majority of the work described in this thesis was carried out on a Zeiss system (Versa 520, Germany) which uses an aforementioned lens system.

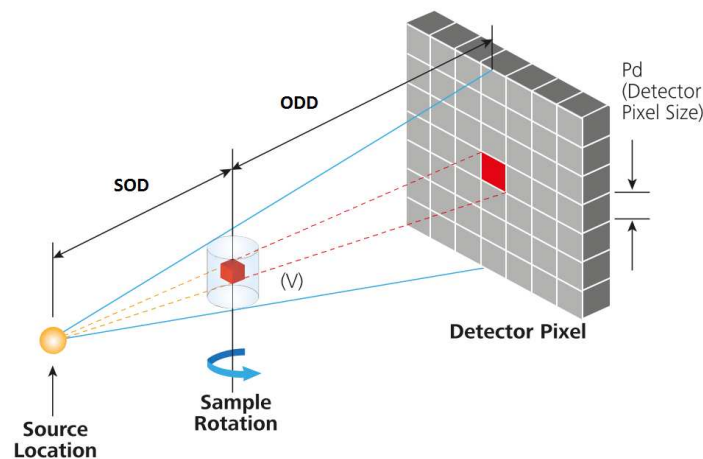


Figure 2.11. Principles of micro-CT image acquisition. SOD = Source to Object (Sample) Distance, ODD = Object (Sample to Detector Distance). Reproduced with permission from [110].

A single projection (radiograph) image is captured (Figure 2.10a), the sample is rotated on a stage by a certain amount and another projection is taken. This is progressively carried out until the sample has been rotated 360°, typically resulting in hundreds or thousands of projections in total. 180° scans are also possible yet were not utilised in this study. After scanning, the projections are reconstructed, typically using a filtered back projection algorithm, and a 3D volume is constructed composed of a stack of images (Figure 2.10b) which can be exported for analysis. Each voxel (3D pixel) has a certain greyscale value related to the x-ray transmission profile of the sample.

2.3.1.3 Imaging Modes

2.3.1.3.1 Absorption Contrast

Absorption contrast is the typical imaging mode used in micro-CT systems and is recommended for most typical sample types. Successful imaging requires both partial and differential absorption requirements are fulfilled. Partial absorption means that a portion of the X-rays emitted by the source are absorbed by the material whilst others are transmitted to the detector: if all X-rays were absorbed by the material, no signal would reach the detector and conversely if all X-rays reached the detector, unaffected by interaction with the sample, no information could be gained from the material. Differential absorption means that different materials have different absorption characteristics, thereby creating contrast within the sample: without this the sample would appear as a single intensity value. Absorption contrast can be defined as equation 4 [111]. Applying Lambert's law for image attenuation [112], equations 5 and 6 can be obtained:

$$C_{att} = \frac{I_1 - I_2}{I_1} \quad (4)$$

$$I_1 = I_0 e^{(-\mu_1 t_1)} \quad (5)$$

$$I_2 = I_0 e^{[-\mu_1(t_1-t_2)]} e^{(-\mu_2 t_2)} \quad (6)$$

where I_0 is the X-ray intensity approaching the object from the source, I_1 is the intensity transmitted towards the detector outside of the object and I_2 is the X-ray intensity transmitted to the detector having passed through the object, μ_1 and μ_2 are the attenuation coefficient of the background medium and sample detail respectively and t_1 and t_2 are the thickness, or length, of the medium and sample detail respectively. The more thick, t_2 , and dense, μ_2 the sample, the more X-rays it will absorb and therefore attenuation reduces at the detector. Graphically this is represented in Figure 2.12.

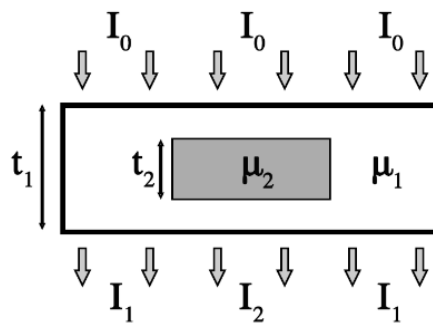


Figure 2.12. Differential attenuation due to absorption of X-rays, creating contrast. Figure created by the author based off material by [111]

Inserting equations 5 and 6 into equation 4, one obtains equation 7 which highlights a main limitation with absorption contrast: if there is a low difference in attenuation (μ_1 and μ_2) or if the detail to be visualised is small (t_2), there will be low contrast in the image.

$$C_{att} = 1 - e^{[(\mu_1 - \mu_2)t_2]} \quad (7)$$

The attenuation coefficient of the material, μ_2 , is defined by the density of the material: influenced by the atomic number (Z) of the elements composing the material. High-Z elements naturally provide good contrast with the surrounding background. Intra-sample differential contrast requires a difference in contrast, and therefore density of elements, within the sample. Pores within samples naturally provide a perfect example of this. Low contrast is typically seen in cartilage and other soft tissues in their native state. The chondrocytes (cells) of articular cartilage are of similar density and attenuation coefficient compared to the surrounding matrix. Techniques exist to increase this contrast, some of which can be highly destructive. For example for observational studies it may be appropriate to fully dehydrate and dry the sample [10, 113] yet such techniques were not considered for this study since the aim was to develop a method to allow biomechanical testing which requires somewhat physiologically intact tissue.

Two techniques which may be compatible and increase contrast will now be discussed in detail: phase contrast and contrast agents.

2.3.1.3.2 Phase Contrast

In addition to the absorption signal, it is possible to also collect what is known as the phase contrast (PC) signal. A modification can be made to Figure 2.12 to consider the X-rays as wave fronts rather than ray optics, as shown in Figure 2.13. The distortion to the wave front caused by the detail, δ_2 , creates detectable changes to intensity at the detector.

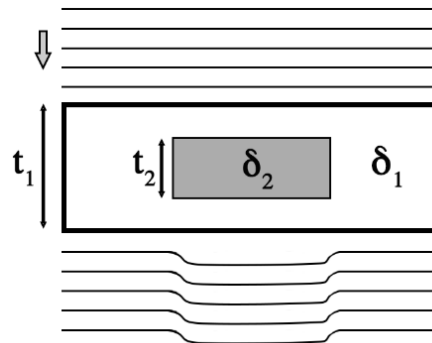


Figure 2.13. The wave effects of the X-rays interacting with the details within a sample may be considered as an alternative to ray optics method. The arrow indicated the direction of approaching wave fronts. Figure created by the author based off material by [111]

There are several, distinctly different, means to generate and detect an X-ray PC signal. There are broadly two main principles applied: firstly that of detecting interference patterns or secondly the exploitation of X-ray refraction [111]. Success with X-ray phase contrast imaging first came with interferometry in 1965 when the principles of visible light phase contrast imaging were applied to X-rays [114]. This technology uses an interferometer to recombine the distorted and undistorted patterns to detect interference of the sample detail. A simplification of the same principle came with the analyser-based imaging (ABI) which uses a single crystal [115]. The technique has been in frequent use, largely at synchrotron facilities, for the past 25 years [116]. The second technique uses a different working principle of X-ray refraction and for which the wave must be given sufficient physical distance for propagation to result in a detectable intensity change. The technique is known by many names including Propagation Phase Contrast (PPC) [117-119].

First proposed at a similar time to X-ray interferometry, again PPC was initially intended for use with synchrotron radiation [117]. The benefit of this technique is that no modification of equipment is required, as long as the working distance between the sample and detector can be sufficiently increased. Given sufficient distance, the distorted wave front will itself generate an interference pattern. Other techniques of phase contrast imaging exist [56] yet PPC is the focus of this study because it requires no modification of equipment. Two approaches may be considered to visualise the PPC effect: refraction and diffraction. Firstly, the phenomenon is commonly thought of as X-ray refraction (Figure 2.14a) linking the angle of the X-ray to the gradient of the refractive index of the sample. This gradient is at a maximum at the boundary of the sample detail, leading to refraction of the X-rays and a local maxima and minima of X-ray intensity which is therefore detected. An alternative approach is to consider again wave optics and the phase-shift created by sample detail compared to the surrounding medium (Figure 2.14b). The distorted wave front leads to Fresnel diffraction fringes in the resulting image, enhancing contrast at the boundary of the detail. Both diffraction (ray optics) and refraction (wave optics) effects yield indistinguishable results [120] when applied to systems with limited coherence: that is to say imperfect wave frequency reproducibility.

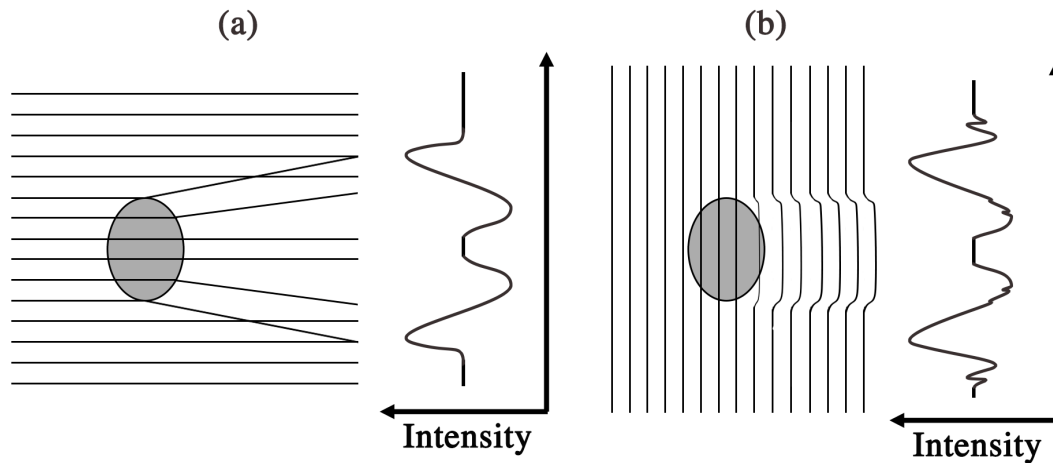


Figure 2.14. Two approaches to consider the propagation phase contrast (PPC) effect: (a) The simplified ray tracing interpretation, refraction causes an intensity gradient (b) The more complex, wave front interpretation whereby diffraction creates the intensity fluctuations. Figure created by the author based off material by [111].

PPC is most easily achieved at a large-scale synchrotron facility where the sources are often monochromatic with high spatial coherence. Experiments are possible though with laboratory systems if the effective spot size is small and the detector resolution is high [121]. Figure 2.14 shows a greatly exaggerated effect for illustrative purposes. In reality, these effects are not typically witnessed because PPC is implemented by significantly increasing the object to detector distance (ODD). By increasing the ODD, the disturbed X-rays propagate enough to be detected to produce images with enhanced contrast at the edges of features: at the boundary between physical phases. It is worth noting that due to divergence of the X-ray beam, laboratory sources require an increased ODD to display the same PPC effects as a synchrotron source [121]. Typically, to reduce scanning time, the ODD would be minimised therefore the potential PPC signal is lost.

Previous studies have found that the optimum ODD for contrast in their system decreased with higher resolution, for example at 0.3 m and 1.5 m for voxel sizes of 0.65 μm and 6.5 μm respectively for soft tissues in mice [121]. It is worth noting that the X-ray signal becomes weaker with increased ODD (and the source to object distance, SOD) therefore the distance for optimum contrast is not optimum for other aspects of the experiment given the required increase in exposure time and / or source power increasing the time increase for the experiment and thereby increased risk of X-ray damage, sample denaturing or undergoing micromotion. A compromise is sought to provide sufficient contrast whilst not making the experiment prohibitively long and resource expensive. To the author's knowledge no previous studies have sought to optimise the ODD for articular cartilage. The maximum possible ODD for our system is approximately 0.3 m. A by-product is increasing the ODD oftentimes requires adjustment of the source to object distance (SOD, Figure 2.11) to result in the desired field of view.

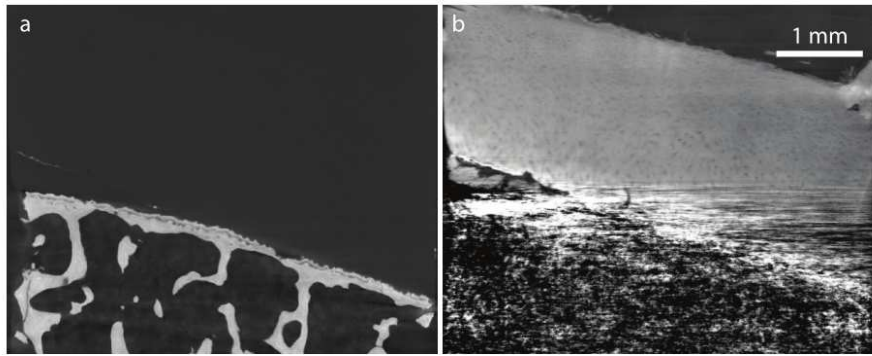


Figure 2.15. Unstained human osteochondral samples imaged with laboratory micro-CT and absorption contrast (a) and X-ray grating interferometry phase contrast at the ESRF synchrotron (b). Reproduced with permission from [122].

Phase contrast is particularly useful for low-contrast biological tissue and has been employed to enable micro-CT of wide ranging soft tissue [121, 123-126]. Attempts have been made to image articular cartilage with phase contrast, both with laboratory micro-CT [127, 128] and highly specialised synchrotron sources [122, 129]. Data indicates PPC in the synchrotron is able to detect the structure of cartilage [130] (Figure 2.15b), but lab micro-CT has not yet generated comparable images [122, 131] (Figure 2.15a).

2.3.1.4 Staining with Contrast Agents

A complimentary means to phase contrast to increase contrast in the image is by staining the sample with contrast agents. Typically composed of high density (high-Z) heavy-metals, the stains increase the density and therefore absorption of X-rays within the sample, or certain components of it. The dye is dissolved within a solvent at a specific concentration, which the sample is then exposed to for a certain length of time.

It is not particularly useful to alter the attenuation of X-rays for the entire sample (μ_1 and μ_2) since this does not increase contrast of certain features. At the organ level, often times staining will be used to alter attenuation of certain type of tissue: for example to increase contrast with soft tissues in comparison to bone. Traditionally this has been the main use of contrast agents, yet it is possible to go a step further and provide differential absorption to structures within tissue and it is this form of staining which is of interest for this research for provision of a “pattern” which can be tracked for strain measurements.

A myriad of stains exist, with different chemical bonding mechanisms and imaging effects. Among the factors contributing to their choice, contrast agents are selected based on: the tissue of interest; the type of contrast sought (i.e. intra-tissue differential contrast vs inter-tissue contrast); tissue preparation processing requirements and finally time and cost limitations.

The electrical charge of the agent affects which of the constituents of the tissue it will bind to [132, 133]. Approximately 40% of the dry weight of articular cartilage are proteoglycans, which carry a negative charge due to the high density of sulphated glycosaminoglycans (GAGs) [108]. The interstitial fluid carries a correspondingly positive charge and the type II collagen carries a close-to neutral net charge, resulting in overall electroneutrality [134]. Certain dyes exploit the negative GAGs charge, with many studies having explored use for diagnosis of osteoarthritis [135-140]. Anionic (negatively charged) and cationic (positively charged) products exist. Iodine-based stains have been popular for their use in soft tissue components. For example a common anionic iodine-based contrast agent is ioxaglate (otherwise known as Hexabrix). The negatively charged agent permeates cartilage reversely proportional to the concentration of GAGs. Ioxaglate has been used to infer the composition of the articular cartilage matrix in non-human [108, 134, 141] and human [137, 138, 140, 142] samples. An interesting development by *Saukko et al* was to mix ioxaglate with bismuth oxide nanoparticles (BINPs) to provide additional contrast [143]. Siebelt et al applied ioxaglate diluted in saline via intra-articular injection on human cadaveric for localised staining with both CT and μ CT techniques [140].

Cationic iodine stains have been applied to non-human [144-148] and human cartilage [136], menisci [149] and metacarpal [150] samples to consider GAGs and human menisci composition. A concern with iodine stains is that they are known to leach from the sample [148]. Therefore they would not be ideal for this project for which several scans of each sample are required including loading, with correlation between the scans. The effects of static mechanical compression on transport of contrast agents has been studied [151]. The results suggested that compression decreases diffusion of the certain stains tested but it should be noted that this was carried out without staining.

Pauwels et al conducted an investigation of 28 potential contrast agents [152]. The study found that three stains were the most promising for soft tissue applications, and importantly did not significantly leach from the intended tissue components. These stains were Mercury chloride (HgCl_2), phosphotungstic acid (PTA) and phosphomolybdic acid (PMA). PTA has been used for staining articular cartilage of non-human [132, 152-154] and human samples [155-157]. Studies suggest that PTA preferentially binds to collagenous structures [133] but the binding mechanisms are not fully understood [133]. Typically a concentration of 1% (w/v) is recommended, providing a pH of approximately 2.7 [132]. At low pH collagen has a positive net charge and therefore binds to the negatively charged PTA. Unlike ionic contrast agents, evidence suggests that PTA uptake in articular cartilage is not affected by concentrations of GAGs. This can be seen as a weakness because GAGs loss is an early sign of OA onset but also a positive because it allows successful staining and cartilage imaging at all stages of OA [153]. PTA has been compared against PMA as potential markers to reveal the spatial distribution of collagen [155]. PTA showed the most prominent features and strongest

association with collagen distribution. Other stains exist but have not been widely applied [158-160]. The most promising images for adding recognisable texture to cartilage were shown by Nieminen et al, who used a 1% (w/v) solution of PTA in 70% ethanol [155] (Figure 2.16).

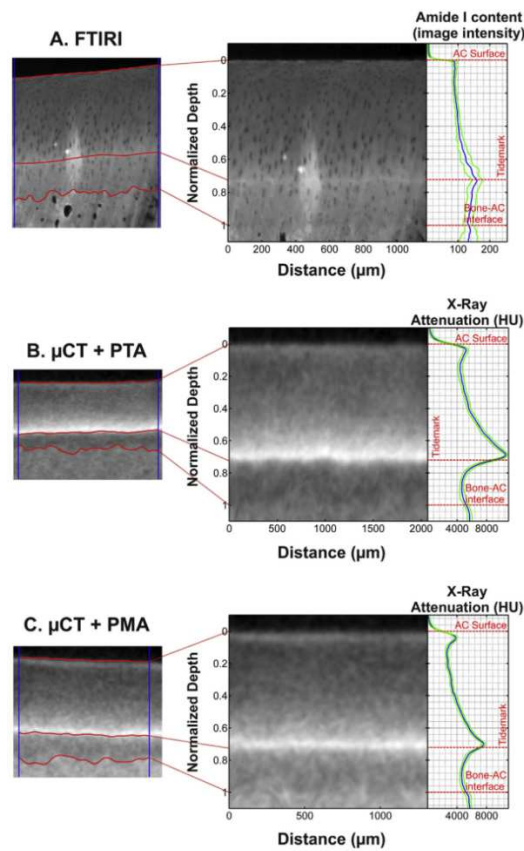


Figure 2.16. Staining of horse articular cartilage with PTA (B) and PMA (C) imaged with 3D laboratory micro-CT compared against an unstained section using 2D FTIRI (A). Cellular features visible are well resolved with FTIRI. The stained micro-CT samples show promise (B and C) but the nature of the greyscale pattern is not defined. Reproduced with permission from [155].

Intra-tissue differential contrast is required in this project to provide a pattern which can be tracked between micro-CT scans with and without mechanical loading applied. Although many of the aforementioned studies have provided contrast within the cartilage, this has only indirectly inferred the location of components within articular cartilage: namely collagen and GAGs. None of the studies have attempted to directly resolve features, instead attempting to use relative differences in bulk attenuation as a sign of differences in concentration. This would not provide sufficient markers for this project's intended aims. Of articular cartilage's constituents, the chondrocytes are the only component of a length scale appropriate for full-field visualisation through the whole cartilage thickness. Studies have had success imaging features but no studies have aimed to visualise chondrocytes with appropriate validation: this will be the focus of chapter 3, with a view to carrying out volumetric strain measurements in chapter 4.

The concentration of contrast agent solution is an important consideration when staining. Studies have found that high concentrations of ioxaglate provided excellent visualisation of exterior cartilage surfaces but hindered identification of the interface between the cartilage and underlying bone [108, 146]. Dilution decreased attenuation but provided differential contrast between the tissue types. Caution should be observed with the acidic stains such as PMA and PTA since at high concentrations they are highly acidic and may interfere with the samples including dissolving of bone [152]. Care must be taken to appropriately select both stain and concentration for the given application. Additionally, staining may affect the tissue's mechanical properties. PTA staining and dehydration with ethanol likely affect cartilage's properties [161, 162].

2.3.1.5 *Imaging Artefacts*

It is important for researchers to bear in mind limitations which can be imposed by artefacts [60]. Two of the most common ones encountered with X-ray micro-CT, in particular with laboratory systems will now be briefly discussed:

2.3.1.5.1 Beam Hardening

If a polychromatic beam is utilised: that is to say an X-ray source with a range of energies, then the lowest energy X-rays will be readily absorbed at first contact with the sample and therefore the X-rays travelling through the remainder of the sample will have a higher average energy profile. This is known as beam hardening. The resulting image can exhibit what appears like a halo close to the perimeter of the sample, and streaks or shadows around the high density materials. The effect is seen most commonly for dense materials such as metals. It is most prevalent if it is scanned in tandem with a lower density material creating significant streaks and other related artefacts (Figure 2.17). Beam hardening is less of an issue when the material is of low density, such as cartilage, yet staining changed the density and therefore it is worth being aware of.

To overcome beam hardening it is possible to physically filter the X-rays between the source and the sample. The filter absorbs the low energy X-rays therefore eliminating them before they reach the sample. This creates a more consistent final image and is often done for imaging of dense materials where a high source voltage is used.

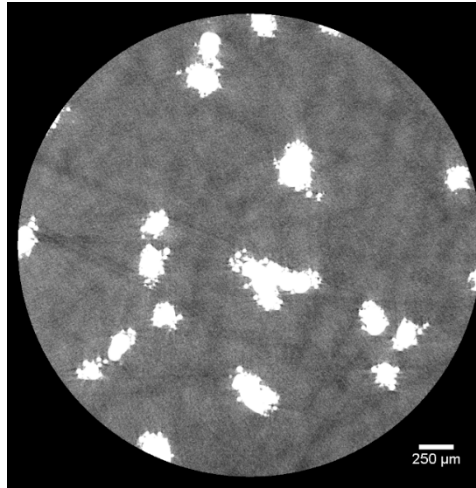


Figure 2.17. Beam hardening, observed in this instance as streaks during a scan of a high density steel scaffold

2.3.1.5.2 Ring Artefacts

The detector is composed of an array of millions of sensor elements. During the scan it is possible for these elements to over or underestimate attenuation values due to temperature fluctuations or X-ray damage. At the most extreme example the element may be irreversibly damaged, creating a “dead pixel” in the final image. As the sample rotates, these imperfect recordings may create a ring which can be visible in the final image (Figure 2.18).

To reduce ring artefacts, the sample is intentionally jittered during the scan, aiming to offset pixels and eliminate the risk of one single pixel create a continuous artefact. During reconstruction this jittering is compensated for during projection alignment. Additionally, algorithms are often used to further reduce the effects though they may not always be successfully eliminated.

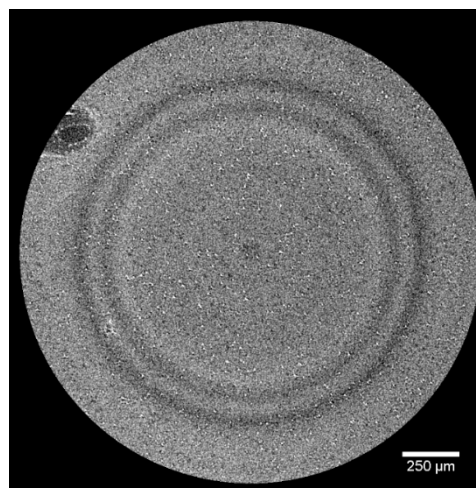


Figure 2.18. Ring artefacts are clearly visible in this micro-CT scan of bovine cartilage. Contrast has been enhanced to exaggerate the effect.

3 THREE DIMENSIONAL IMAGING OF CELLS WITHIN ARTICULAR CARTILAGE

This chapter describes the development of a protocol for 3D imaging of chondrocytes within articular cartilage across samples of several millimetres diameter. Successful imaging would allow visualisation of a pattern required for successful DVC of cartilage.

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Propagation phase-contrast micro-computed tomography allows laboratory-based three dimensional imaging of articular cartilage down to the cellular level, Clark, J.N., Garbout, A., Ferreira, S. A., Javaheri, B., Pitsillides, A. A., Rankin, S. M., Jeffers, J. R. T., Hansen, U. *Osteoarthritis and Cartilage*, Volume 28, Issue 1, 102 – 111 DOI: 10.1016/j.joca.2019.10.007

3.1 INTRODUCTION

Research into the structure of articular cartilage has been performed for over a century [163] but has historically been limited to destructive 2D imaging on a single plane [7]. There is potential for micro-computed tomography (micro-CT) to reveal full-field 3D information from intact segments of articular cartilage. Success could advance the study of osteoarthritis development and provide a natural pattern to enable full-field strain measurements of cartilage [61]. This could lead to advancements in chondral repair scaffolds that target biomimicry, as is possible with other tissues [164]. Articular cartilage is typically difficult to image by micro-CT due to poor absorption of X-rays owing to its composition of low-Z elements such as carbon, hydrogen, oxygen and nitrogen [109]. However, two methods that exist to increase contrast in cartilage are contrast agents [152] and phase contrast imaging [129].

Contrast agents, typically composed of high-density heavy metals, bind to extracellular components, increasing density and thereby increase the resulting signal [154]. Articular cartilage has been stained for micro-CT to study morphological changes [108, 165], glycosaminoglycan content [134, 144] and to infer the spatial distribution of collagen and assessment of degradation [143]. Pauwels *et al.*, conducted an investigation of 28 potential contrast agents and found that the most promising contrast agents for soft-tissue staining were phosphomolybdic acid ($\text{H}_3\text{PMo}_{12}\text{O}_{40}$, PMA), phosphotungstic acid ($\text{H}_3\text{PW}_{12}\text{O}_{40}$, PTA) and mercury chloride (HgCl_2) [152]. PTA and PMA have been used to indicate collagen distribution [155]. Whilst it is proposed that these stains may bind to various proteins [109], the underpinning mechanism(s) is not fully elucidated [133]. Many of these studies have involved the use of ethanol (EtOH) as the solvent to dissolve the contrast agent [155], whilst others fully desiccate the cartilage to enable the structure to be observed [10]. These changes in water content likely modify the mechanical properties of the material and thus may not be fully representative of the *in vivo* conditions. Imaging samples immersed in alternative liquids, altering less the physiological conditions, would be attractive.

Phase contrast imaging detects the phase shift that X-rays experience passing through matter, resulting from differences in refractive index or non-uniform thicknesses [166]. It is particularly useful for low-contrast biological samples in life-sciences [123, 125]. Three of the most common techniques are propagation phase contrast (PPC) [119], analyser-based imaging [115] and grating interferometry [167]. Many of these methods, including both analyser-based diffraction enhanced contrast and grating interferometry, require specialised and modified equipment. PPC requires no such modification to the equipment used for typical absorption contrast as long as the beam is highly spatially coherent and the scanner allows for the detector to be positioned sufficiently far from the sample. Various attempts have been made to image articular cartilage with phase contrast using

relatively common laboratory micro-CT [127, 128] as well as highly specialised synchrotron sources [122, 129]. Data indicates PPC in the synchrotron is able to detect the structure of cartilage [130], but lab micro-CT has not yet generated comparable images [122, 131]. The ability to image the cartilage structure in lab micro-CT would be highly advantageous, enabling wider access for researchers .

Herein we explore the hypothesis that a combination of staining and laboratory phase-contrast micro-CT can adequately visualise individual chondrocytes within intact samples of articular cartilage. Our primary aim was to develop a method to image chondrocytes with laboratory micro-CT. Our secondary aim was to achieve this whilst maintaining the tissue in conditions which deviate the least from those found physiologically.

3.2 METHOD

3.2.1 Sample Preparation

Fresh juvenile bovine (<2 years old) stifle joints (n = 3) from two animals were obtained from a slaughterhouse and stored at -25 °C until experimentation. The specimens were thawed at 4 °C, kept hydrated with Dulbecco's phosphate buffered saline solution (DPBS, #14190-094, Fisher Scientific, USA) and 3 mm cylindrical osteochondral plugs (n = 10) were taken from the femoral condyles using a hollow punch. Samples were prepared under four conditions (Figure 3.1). All were initially bathed for 30 minutes in PBS and the ethanol (EtOH) treated samples were immersed in step-wise increasing concentrations of EtOH [168]. From each of the PBS or EtOH treated groups, one osteochondral plug was maintained in the liquid without staining (denoted PBS or EtOH), whilst others (Table 3.1 and supplementary Table 8.1) were further processed with staining using a 1% w/v phosphotungstic acid solution (weight/volume, $H_3PW_{12}O_{40}$, PTA, #79690 Sigma-Aldrich, USA) in either PBS or 70% ethanol for 21 hours then rinsed in the medium prior to micro-CT scanning (denoted PBS+PTA or EtOH+PTA). The full description of each sample is included in the supplementary Table 8.1. Staining time was optimised in pilot experiments. All samples were stored in their liquid medium at room temperature prior to scanning.

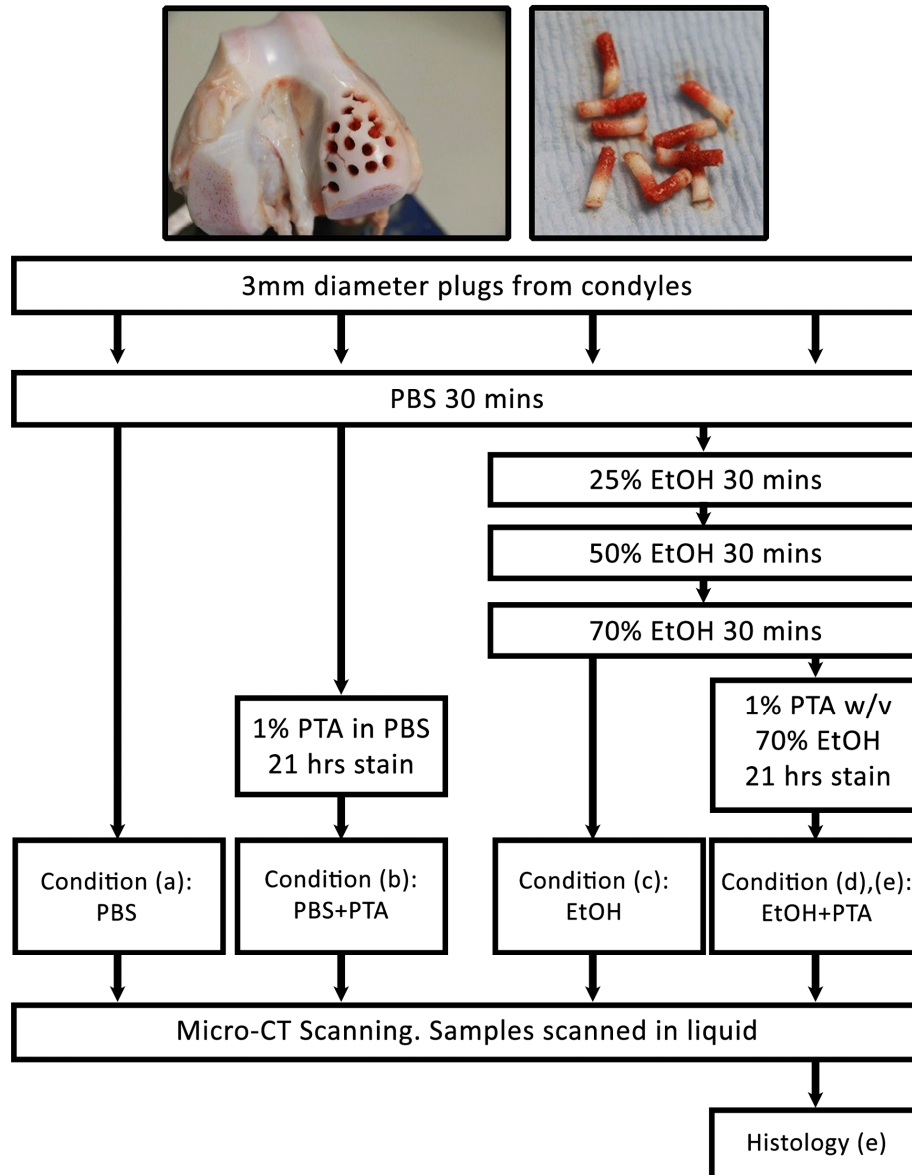


Figure 3.1. Sample preparation methodology for the four preparation methods using osteochondral plugs extracted from a similar position in the bovine condyles.

Table 3.1. Micro-CT scan parameters. All scans took place at 40kV and 75µA. AC = Absorption contrast, PPC = Propagation phase contrast signal. SOD = Source-to-Object Distance, ODD = Object-to-Detector Distance. No x-ray filters were used. The supplementary table provides a further breakdown of parameters for each individual sample and includes sources for each sample. Exp = Exposure time, Proj = Number of projections.

Sample	Method	Medium	Stain	n	Voxel (µm)	SOD (mm)	ODD (mm)	Exp (s)	Proj
(a)	AC	PBS	-	1	3.53	20.0	18.0	5	2401
	PPC			1	1.99	23.5	55.8	22	2401
(b)	AC	PBS	PTA	1	3.53	20.0	18.0	8	2001
	PPC			3	1.97 - 2.09	23.5 - 25 .0	55.0 – 60.0	30 - 34	2001 - 3201
(c)	AC	EtOH	-	1	3.53	20.0	18.0	6	2301
	PPC			1	1.99	23.5	55.8	25	2301
(d)	AC	EtOH	PTA	1	3.53	20.0	18.0	7	2001
	PPC			5	1.97 - 2.85	23.5 - 30	40 - 73	25 - 40	2001 - 3201
(e)				1	1.97	30	73	30	3201

3.2.2 X-ray Micro-Tomography Scanning

Samples were immersed in their corresponding liquid medium (PBS or 70% EtOH) and mounted in sealed plastic containers. All scans were carried out on a Versa 520 X-ray micro-CT scanner (Zeiss, Germany). For all scans, voltage and current were 40 kV and 75 µA respectively with no pixel binning. No x-ray filters were applied. To allow comparison of the signal between absorption and propagation phase signals, scans under different scanning protocols were taken. Image quality at different source-to-object (SOD) and object-to-detector (ODD) distances can be maintained by adjusting exposure time to ensure a sufficient photon count reaches the detector. The larger the SOD and ODD distances, the longer the exposure time required to maintain the photon count. Firstly the absorption signal was collected by minimising the SOD and ODD distances to reduce scanning time. This is the type of scan typically carried out with a micro-CT scanner. Next the phase contrast scan was taken with enlarged SOD and ODD, allowing implementation of propagation phase contrast (PPC). Using a larger SOD also

has the advantage of decreasing cone beam error[169]. Increasing the ODD for the PPC scan reduced the X-ray flux, resulting in exposure times typically four times longer than for the absorption scan. The full set of scan parameters for each scan are shown in Table 3.1 and in the supplementary Table 8.1. A volume of interest of approximately 2x2x2 mm was included for each set of conditions. For the bovine samples used during this study, the volume of interest did not constitute the entire cartilage height. For histological comparison, an additional sample (e) was prepared by the same method employed for the EtOH+PTA sample (d) and scanned to maximise image quality with a higher number of projections and further increased SOD and ODD. Reconstruction of the projection images to produce 3D volumetric data sets was performed using the Reconstructor Scout-and-Scan software (Zeiss, Germany). The reconstructed CT volumes were visualized and analysed using (Fiji Is Just) ImageJ software [170] (version 1.52g, NIH, USA). Two of the sample from groups PBS+PTA (b) and EtOH+PTA (d) were scanned twice using PPC to ensure scan repeatability and measure consistency in scan quality.

3.2.3 Histology

Following the micro-CT scan, one of the osteochondral plugs (e) was stored in 10% neutral buffered formalin (#HT501128, Sigma-Aldrich) for 24 hours. Before paraffin wax embedding, PTA was removed by ion-exchanging in a washing solution of 0.55 mM NaOH, 0.1 M of Na_2HPO_4 , 137 mM NaCl, and 2.7 mM KCl, pH 10 for 5 days following established protocol [156]. The sample was subsequently decalcified in 425 mM EDTA neutral solution for 7 days exchanged every day [171], paraffin-embedded and sections (5 μm) collected on Superfrost slides (Fisher Scientific, USA). Sections were dewaxed immersing twice for 5 minutes in Gentaclear (Genta Medical, UK), washed in tap water and subsequently stained with: (1) Alcian Blue at pH 2.5 with counterstaining of nuclei with Neutral Red [172]; (2) Masson's Trichrome[171]; (3) Picro-Sirius Red [173] or (4) Safranin O (0.5%)/Fast Green FCF(0.2%) with nuclei counterstained with Celestin blue and Harris Haematoxylin (all from Sigma-Aldrich, USA) [174]. All sections slides were cover slipped with DPX mountant (Sigma-Aldrich, USA). Colour micrographs were acquired using a Zeiss Axio Observer Inverted Widefield Microscope with an IC5 colour camera, and with a fully motorised stage controlled by ZEN Blue pro software capable of tiling and stitching, using a 20 $\times$ DIC Plan Apochromat air objective with numerical aperture of 0.8, and 2048 $\times$ 2048 resolution, with pixel size corresponding to 0.33 μm .

3.2.4 Image Analysis

3.2.4.1 *Contrast to Noise Analysis*

Of the constituent components within articular cartilage, the largest individual features are chondrocytes. Yet the chondrocytes have previously been difficult to visualise over large distances in

3D [132], owing to their small size, low spatial density and previously discussed low contrast. Typically each chondrocyte will occupy few voxels and noise can easily lead to erroneous segmentation. Therefore, as a measure of contrast and scan quality, the contrast to noise ratio (C/N) of individual chondrocyte features was calculated through the sample height for each sample and compared between the samples scanned under different conditions. Within each sample Figure 3.2A) a region was micro-CT scanned (Figure 3.2B). Eleven equally spaced layers per sample from the reconstructed image stack (Figure 3.2C) were analysed in ImageJ using the Plot Profile tool (Figure 3.2D and E) with a line length of 60 pixels to measure grayscale intensity across cells and their surrounding matrix ($n = 10$ per layer, $n = 110$ cells per sample)[119]. Chondrocytes were visualised with a higher grayscale value than the surrounding matrix. A higher C/N indicates more clearly visualised features. Care was taken to only include one chondrocyte per profile therefore only the highest peak characterised a cell, all others represented the surrounding matrix. A MATLAB script (R2016a, MathWorks Inc, Natick, MA, USA) computed the ratio of the amplitude of the maximum peak (A) above the background peaks for each plot, divided by the standard deviation of the surrounding noise peaks (σ_N) [175] (Figure 3.2E).

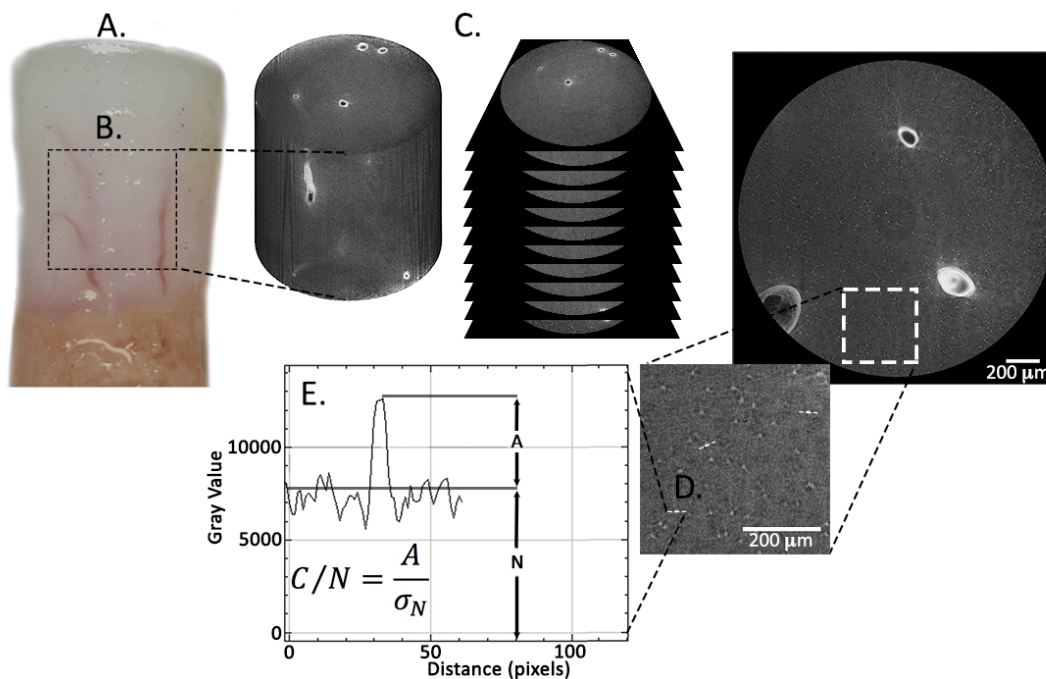


Figure 3.2. Methodology to extract contrast to noise (C/N) values in the micro-CT scans of osteochondral plugs. A volume of interest within the cartilage region of the osteochondral plug (A.) was scanned, as shown by the black dashed cube (B.). To measure changes through the cartilage height, eleven layers equally spaced 100 layers apart ($\approx 200 \mu\text{m}$; C.) were taken from within the 1000-layer thick z-stack and on each of these layers ten features were analysed with the Surface Plot Tool in ImageJ (denoted by dashed lines; D.) to extract the grayscale intensity as a function of distance from the cell (E.), resulting in 110 plots per sample. Greyscale values were processed in MATLAB: the amplitude of the signal ("A") was divided by the standard deviation of the noise (" σ_N ") to give the C/N. Images shown are for sample (d.1) EtOH+PTA with PPC.

3.2.4.2 *Histological Analysis*

The micro-CT and histological images for sample (e) were manually registered in ImageJ. Similarly located areas of 500 μm x 500 μm were selected in both modalities (micro-CT $n = 12$, histology $n = 3$ for each of the four stains) at three different heights through the sample ($n = 36$ in total each for micro-CT and histology). Three consecutive micro-CT slices were combined using the Max Intensity Z project process within Fiji ImageJ software to result in a comparable thickness (5.91 μm) to that of the histological slices (5 μm). The histological images were scaled to the same resolution as the micro-CT. Images were segmented using the Trainable Weka Segmentation [176] and the Analyze Particles tool was used with a minimum size of 5 μm^2 and circularity of 0.15-1.00 to measure density and roundness.

3.2.4.3 *Cellular Analysis*

Image stacks were imported to Fiji and to aid segmentation, the Subtract Background tool was utilised with a rolling ball radius of 10 pixels. Images were subsequently thresholded to segment the particles from the matrix. Three metrics were considered: volume fraction, for which the BoneJ plugin was utilised; lacunae volume and 3D cell count (frequency). The Particle Analyzer plugin was utilised for the latter two, with volume resampling of 2, and subsequently feature volumes of above 50 μm^3 were analysed. This was applied for two sets of analysis: firstly, 135 x 135 x 80 μm volumes ($n=6$) at three heights through the cartilage with renderings created with Fiji's Volume Viewer; and secondly, chondrocyte hypertrophy for which cubic volumes of 200 μm length were segmented in one sample (e) at similar distances from the subchondral bone in areas both in close proximity to vasculature ($n=5$) and far ($> 500 \mu\text{m}$) from vessels ($n=5$). Vascularity was measured using the Thickness Map command within Avizo (Thermo Fisher Scientific, USA).

3.2.5 **Statistical Analysis**

Contrast to noise ratio (C/N) data for each micro-CT scan was imported to SPSS (IBM SPSS Statistics, version 25, Armonk, NY). Students t-tests were run to understand the effects of liquid medium (PBS or EtOH), staining (with or without PTA) and scan-type (absorption and propagation imaging) on C/N, and between the two scan-types for each preparation technique (Table 3.1 a-d). Data are mean $\pm$ standard deviation unless otherwise stated. Contrast to noise values were normally distributed, as assessed by Q-Q plot inspection. By inspection of boxplots, 14 of the 20 groups contained a limited number of outliers further than 1.5 box lengths. These constituted at most 1.8% of all values per group and thus were included. There was non-homogeneity of variances for groups ($p < 0.0005$) as assessed by Levene's test for equality of variances therefore the Welch's t-test was chosen, for which equal variances are not assumed. Paired-sample t-tests were run on the C/N values for the two pairs of repeat scans of samples prepared with PBS+PTA and EtOH+PTA. There were no outliers in the PBS+PTA

groups and two significant outliers were found in the EtOH+PTA data (comprised of $n = 220$ data points) which were not excluded. Assessment by Q-Q plot inspection showed normally distributed differences in C/N scores for both conditions.

Student's t-test was used to determine if there were differences between chondrocyte lacunae close and far away from vasculature for two metrics tested independently: frequency and volume fraction. There was one non-extreme outlier in the frequency data, as assessed by inspection of a boxplot, and no outliers in the volume fraction data. Data for both metrics was normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$) and there was homogeneity of variances, as assessed by Levene's test: for frequency ($p = 0.474$) and for volume fraction ($p = 0.107$). Volume measurements were tested with a Mann-Whitney U test to determine if there were differences in lacunae volumes ($n = 3478$) between regions of interest close and distance to vasculature. Distributions of lacunae volume for both groups were similar, as assessed by visual inspection, satisfying test requirements.

3.3 RESULTS

3.3.1 PPC Imaging Following PTA Staining in EtOH Yields Greatest Chondrocyte Visualisation

Our data show that the most efficient combination of imaging modality, sample preparation medium and staining to resolve cellular details in articular cartilage was to use propagation phase contrast (PPC) scanning of samples stained with PTA in EtOH (Figure 3.3D). Cellular features in cartilage were also visualised in samples stained with PTA prepared in PBS as well as in unstained samples stored in EtOH. For both scan types, C/N for samples in ethanol were higher than PBS ($p < 0.0001$, Figure 3.3A), and higher for samples stained in PTA compared to unstained samples ($p < 0.0001$, Figure 3.3B). The C/N score for ethanol was higher than PBS for both unstained ($p = 0.001$) and stained samples ($p < 0.0001$, Figure 3.3C). Propagation scanning increased C/N for all sample preparation methods (Figure 3.3D). There was no difference between the pairs of repeat scans in either the PBS+PTA or EtOH+PTA samples ($p = 0.852$ and $p = 0.112$ respectively, Figure 3.3E), showing scan repeatability. Simultaneous imaging of subchondral bone and cartilage did not impede image quality.

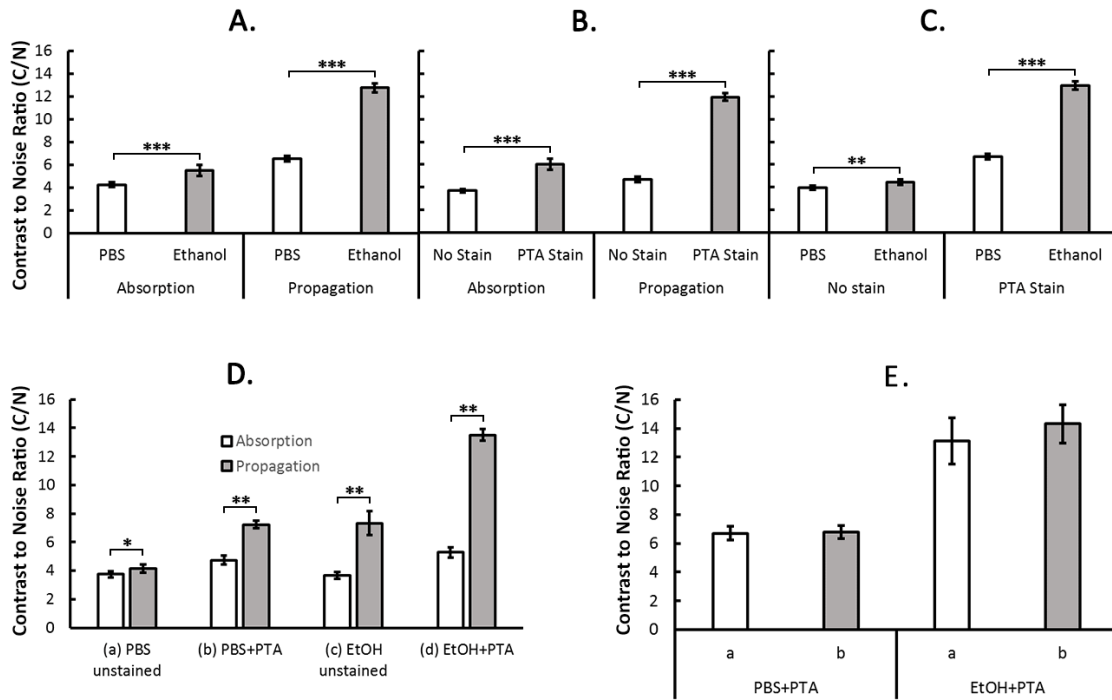


Figure 3.3. Contrast to noise (C/N) data, as described in Figure 2, was statistically analysed with t-tests in SPSS and displayed are mean contrast to noise ratio for the three variables: scan type (absorption and propagation), medium (PBS and EtOH) and staining (none and PTA-staining) with their 95% CIs. (A.) Between mediums for the two scan protocols C/N was greater for EtOH than the PBS in both scanning methods (both $p < 0.0001$) with mean differences of 1.243 (95% CI 0.709 to 1.778) for absorption contrast and 6.231 (95% CI 5.772 to 6.690) for propagation contrast. (B.) Between unstained and unstained samples for the two scan protocols. For absorption contrast, staining increased C/N by a mean value of 2.325 ($p < 0.0001$, 95% CI 1.824 to 2.837) and by 7.202 ($p < 0.0001$, 95% CI 6.786 to 7.618) for the propagation method. (C.) Between mediums with and without staining. Unstained, there was an increase in the C/N of 0.524 ($p = 0.001$, 95% CI 0.220 to 0.828) using ethanol rather than PBS and when using PTA staining there was an increase of 6.279 ($p < 0.0001$, 95% CI 5.83 to 6.730). (D.) Mean contrast to noise values for all scans divided by preparation technique. PPC increase C/N for all groups compared to AC: (a) PBS by 0.400 ($p < 0.038$, 95% CI (0.022 to 0.779)), (b) PBS+PTA by 2.490 ($p < 0.0001$, 95% CI 2.093 to 2.885), (c) EtOH by 3.654 ($p < 0.0001$, 95% CI 2.782 to 4.527) and (d) by 8.224 ($p < 0.0001$, 95% CI 7.686 to 8.762). (E.) Two repeat propagation scans were taken of samples from the PTA-stained groups. No difference in mean C/N was found with either medium: PBS had a mean difference of 0.058 ($p = 0.852$, 95% CI -0.560 to 0.676), EtOH had a mean difference of 1.183 ($p = 0.112$, 95% CI 0.281 to 2.648). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

3.3.2 Depth-Dependent Properties

Contrast to noise ratio values through the height of each sample are shown in Figure 3.4. A control was included as a measure of contrast variation in the surrounding matrix which was similar in both scanning methods. For most of the sample preparation techniques contrast improved using the propagation phase scanning method (Figure 3.4B) compared to the absorption scans (Figure 3.4A) and remained improved through most of the sample height.

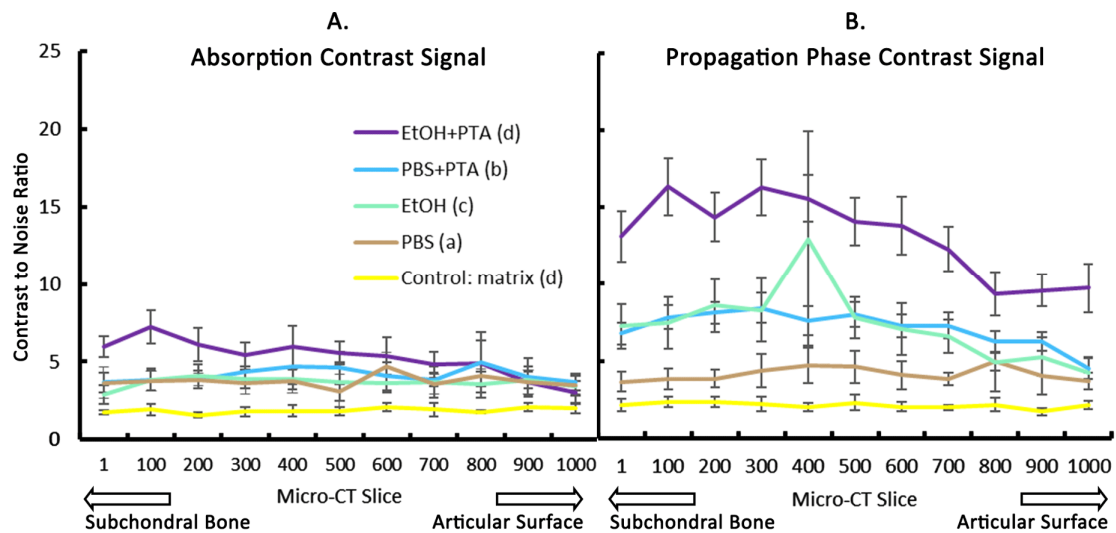


Figure 3.4. Mean contrast to noise ratio (C/N) values (with 95% CIs) through the height of osteochondral plugs prepared with various methods and micro-CT scanned using the two Absorption Contrast (A.) and Propagation Phase (B.) protocols. Chondrocyte grayscale intensity ($n = 10$) was plotted across 11 scan layers per sample as described in Figure 3.2. The scans included a depth of approximately 2 mm of articular cartilage mid-way through its height. 100 micro-CT slices equates to approximately 200 μm . For comparison, data collected without cellular features is given as a control for signal variation in the surrounding matrix.

Reconstructed z-slice images from the micro-CT scans for each sample preparation method (Figure 3.5) infer the same results as the C/N values suggest: that propagation phase contrast shows more clearly defined features (Figure 3.5B). Features are most clearly visualised with staining in the ethanol group. Features reduce in size and intensity throughout the sample height.

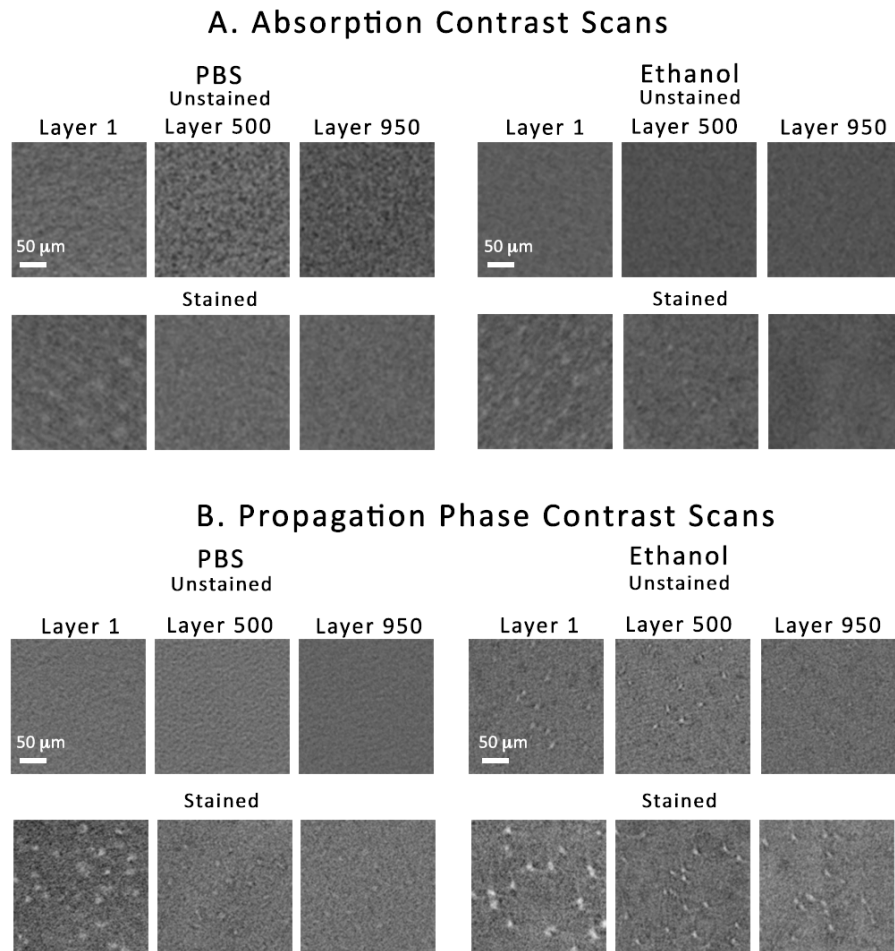


Figure 3.5. Comparison of image quality through the height of samples under the different sample preparation techniques and micro-CT protocols. Layer 1 is in the cartilage above the subchondral bone, and layer 950 is distally located closer to the articular surface. For illustrative purposes brightness was normalised between sets of images, all analysis was carried out on unedited images.

3.3.3 Histological Validation

In order to validate our findings from micro-CT analysis, we performed image registration between micro-CT and conventional cartilage histology images of the same sample (Figure 3.6A). Both techniques allowed observation and quantification of similar cellular features (Figure 3.6B, D, E) and cellular distribution (Figure 3.6C). We attempted to correlate cellular area between the two imaging methods, but the area of an individual cell was too sensitive to greyscale thresholding for this to be accurately feasible. However, the density of cellular features observed with micro-CT was between the values for chondrocytes and their lacunae in the histological images (Figure 3.6C) and cellular roundness was similar (Figure 3.6D).

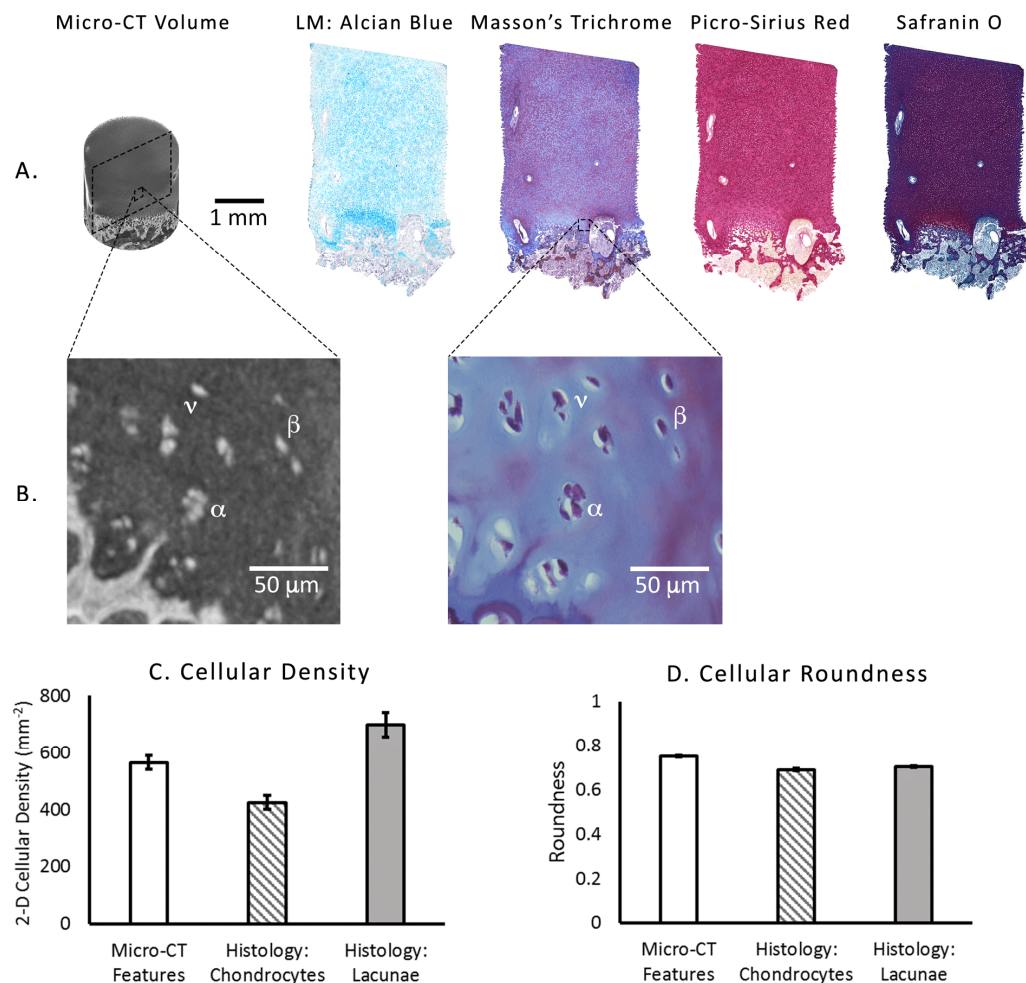


Figure 3.6. Micro-CT comparison with histology. (A.) Reconstructed micro-CT volume and light microscopy (LM) histological slices (Alcian Blue, Masson's Trichrome; Picro-Sirius Red and Safranin O) of sample (e): 70% EtOH and PTA staining. The scale of the micro-CT and histology images is comparable. (B.) A high magnification region of interest between the two techniques is shown with symbols denoting corresponding features. Equivalent $500 \times 500 \mu\text{m}$ areas were analysed between both methods (each $n = 36$) and mean values (with 95% CIs) are shown for cellular density (C.) and cellular roundness (D.). Measurements for lacunae encompass the chondrocytes and other cellular features.

Depth-dependent feature analysis ($n = 12$) was carried out between micro-CT and histology (Figure 3.7). Density of cellular features reduced with distance from the subchondral bone for both methods (Figure 3.7A-C). Roundness remained similar for both micro-CT and histology throughout the sample height (Figure 3.7E).

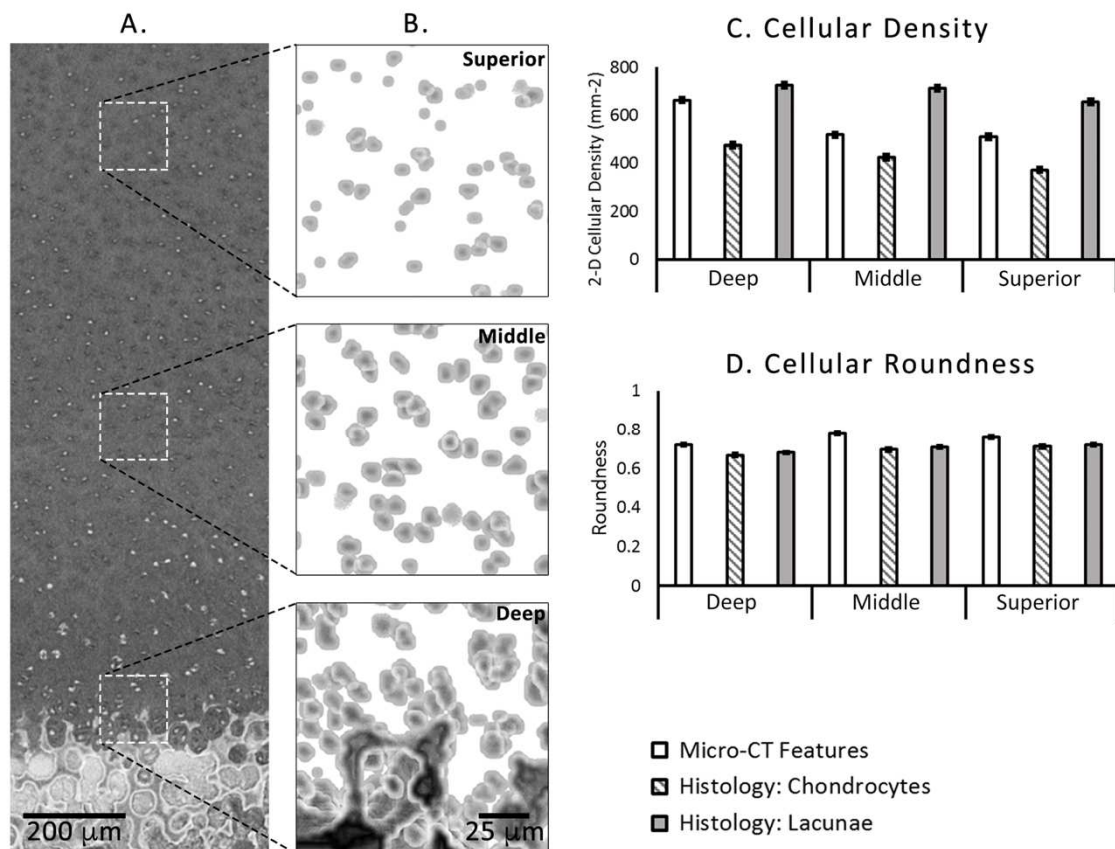


Figure 3.7. Reconstructed micro-CT slice of 1.97 μm thickness of osteochondral plug sample (e): 70% EtOH and PTA staining (A.), with higher magnification volume renderings of different regions through the height of the cartilage: deep, middle and superior (B.). Renderings are of a volume of 135 x 135 x 80 μm produced with Fiji's Volume Viewer. Measures of density (C.), and roundness (D.) were calculated over 500 x 500 μm areas at the three locations for both micro-CT and histology ($n = 12$). Measurements for lacunae encompass the chondrocytes and other cellular features. Displayed values are mean and 95% CIs.

3.3.4 Vascularity and Cellular Hypertrophy

Vascularity was discovered in the non-calcified cartilage in all samples (Figure 3.8), with canals progressing from the subchondral bone. For the displayed sample, across a total cartilage height of 3.8 mm, vessels permeated a height of 3.6 mm: 93% of the total height.

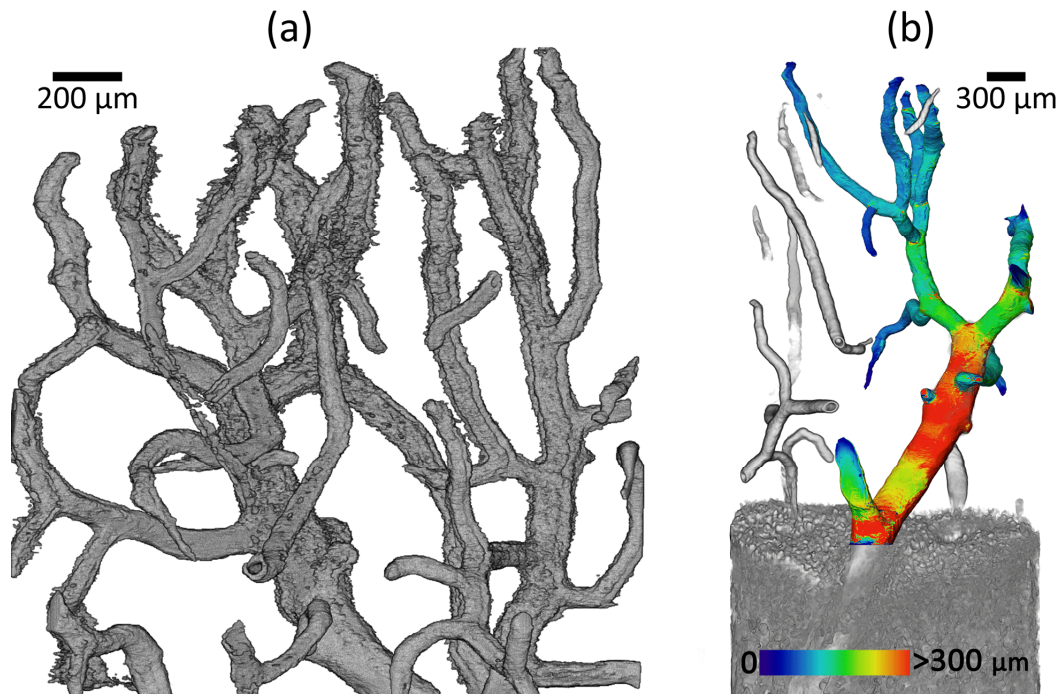


Figure 3.8. Vascular canals were observed in all samples, and are shown here two samples: sample (d) and an additional sample prepared in the same way as sample d. The vessels were extremely prevalent throughout the cartilage (A). A stitch scan was performed to include the total sample height and the thickness of the vessel has been calculated as shown (B).

An increase in mean chondrocyte lacunar volume indicates cellular hypertrophy [177], which governs bone growth but also is a characteristic of osteoarthritis. One potential application of the micro-CT method developed here is to measure three dimensional changes in chondrocyte morphology. The hypothesis was that lacunae close to the vessels would be smaller than those located further from the vessels. Differences were found between the two regions of the cartilage for all three metrics measured in the study of hypertrophy (Figure 3.9). As expected in healthy cartilage, the median lacunar volume was greater ($p < 0.001$) for chondrocytes remote from vasculature ($441 \mu\text{m}^3$) compared to those in close proximity ($137 \mu\text{m}^3$). Cellular density varied between $30,000$ - $60,000 \text{ mm}^{-3}$ depending on the region.

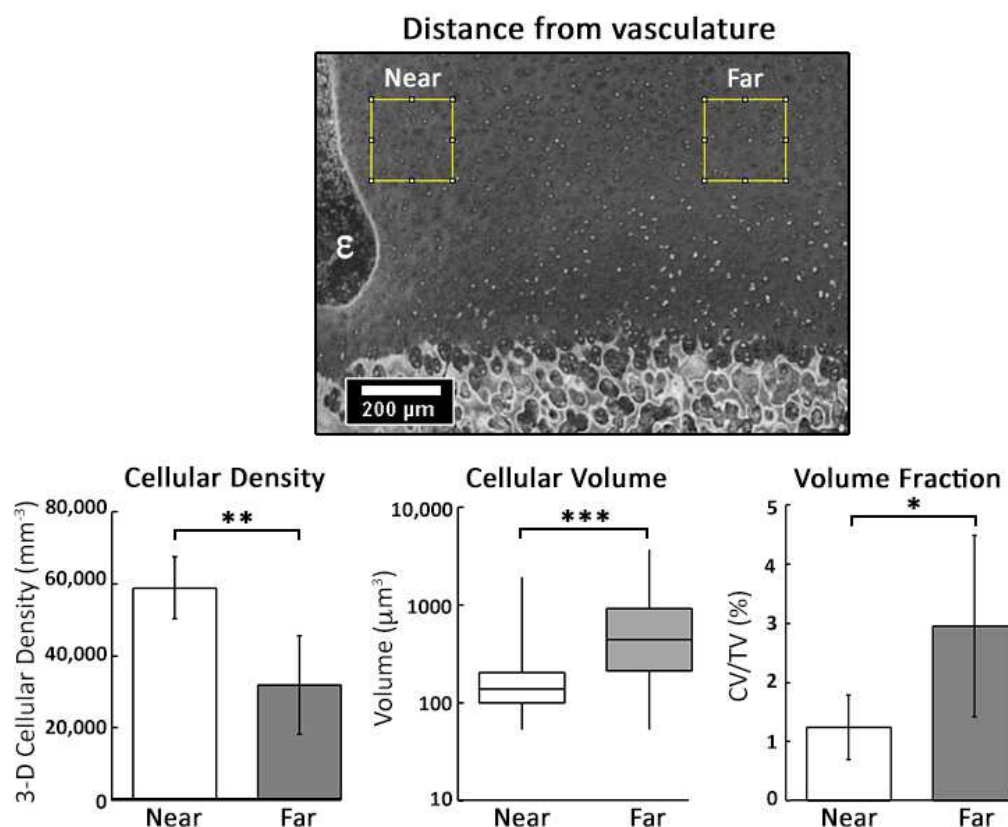


Figure 3.9. Comparison between 200x200x200 μm volumes of interest close to vasculature (marked with ϵ), and over 500 μm from the vasculature ($n=5$). Charts for the mean cellular density and volume fraction are shown with mean and 95% CI error bars. Box plots for cellular volume are plotted on a logarithmic scale. CV/TV = Cellular Volume/Total Volume. Cellular density and volume fraction were tested with Student's t-test and volume was tested with a Mann-Whitney U test. There was an increase in mean density of 27025 (95% CI, 13637 to 40412) near to vasculature compared to distant lacunae, $t(8) = 4.655$, $p = 0.002$. Median volume was higher in far lacunae compared to near $U = 2406966$, $z = 34.282$, $p < 0.0001$. There was a difference in mean volume fraction with lacunae far from vasculature being 0.017 (95% CI, 0.003 to 0.003) higher compared to lacunae near, $t(8) = 2.932$, $p = 0.019$. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$.

3.4 DISCUSSION

We report for the utility of a standard laboratory micro-CT scanner to visualise and quantify features of the chondrocyte population within intact articular cartilage in 3D. Histological staining was used to confirm these cartilaginous features observed by micro-CT at the cellular level. Images between both methods were successfully registered, confirming the location and distribution of features. Measurements of cellular density measured with micro-CT yielded values within the range of chondrocytes and their lacunae measured with histological images. Morphology was compared with cellular roundness, for which both techniques yielded similar values. Repeatability of measured C/N values was confirmed for both PBS and EtOH-stained samples. Imaging was successful using propagation phase contrast imaging with the sample maintained within a liquid environment and is compatible with either PBS or EtOH as a medium, achieving the aims of the study. It is pertinent also

that we find that simultaneous imaging of hard and soft tissues did not impede image quality. Propagation phase contrast increases the contrast of individual chondrocytes compared to using absorption contrast. This offers researchers the opportunity to image chondrocyte distributions in 3D without specialised synchrotron equipment, enabling investigations such as chondrocyte morphology across grades of cartilage damage, 3D strain mapping techniques such as digital volume correlation to evaluate mechanical properties *in situ*, and models for 3D finite element analysis *in silico* simulations. Vascularised cartilage canal networks were identified within imaged bovine samples though it should be noted that these did not appear in any mature human samples (chapter 4).

This study represents a complimentary addition to the growing body of evidence supporting the non-destructive imaging of the constituents of articular cartilage. Previous studies have differed in focus on other aspects of the cartilage structure [155]; involved the use of highly specialised synchrotron facilities [122, 131, 178]; or drying and dehydration that may change the organisation and mechanical properties of the tissue [10]. We have compared and quantified the output scans of samples prepared using different preparation techniques and scanning signals. As with previous studies it was found that heavy metal staining provided an improvement in signal attenuation [152, 155]. The use of PBS as the medium during sample preparation and subsequent staining is atypical in previous literature and provides a more physiological environment than EtOH or formalin fixation. The visualised features in this study are comparable to those achieved for similarly prepared samples in micro-CT and synchrotron facilities using a similar voxel size [122, 155], and additionally can image the adjacent subchondral bone. For sample (e) which was processed with EtOH and PTA staining we found the cellular density of chondrocyte features to reduce from 663 mm^{-2} to 511 mm^{-2} when approaching the cartilage surface. An earlier study using confocal microscopy and sectioning of bovine samples found cellular density to follow a similar trend with the lowest density furthest from the articular surface^[179]. We note that there was a discrepancy in density between the chondrocytes and their lacunae. A potential cause of this was damage incurred by sectioning and associated processing, this is avoided with non-destructive visualisation techniques such as micro-CT. Micro-CT values for roundness were approximately 7% higher than with histology. This may be due to partial volume effect artefacts observed with micro-CT scanning [180]. Prolific cartilage canals were discovered, covering the majority of the sample height. In mature human samples this would constitute a remarkable discovery since articular cartilage is unquestionably considered an avascular tissue. Previous studies have shown canals within young human [181] and non-human donors [182] yet all of these studies have taken place in 2D, relying on destructive sectioning [183, 184]. The ability to now investigate such structures non-destructively offers clear benefits.

Our study has several limitations. Host tissue was stored frozen at -25 °C and thawed for use. Successful histological staining of nuclei and cells post-scanning suggest that tissue disruption is not any more extensive than would be expected in samples prepared in this way. Previous studies have reported no difference in mechanical properties between fresh and frozen soft tissues [185] yet testing the method works with fresh tissue would be beneficial. Currently, this method has only been applied to a small number of juvenile bovine samples, and future studies are needed to increase the sample size and to confirm that the method works with human articular cartilage. Penetration issues were experienced by virtue of the low-energy X-rays being easily absorbed by the sample and surrounding container, and the method is limited by specimen size. It was found that the thickness of the sample container had an effect on the signal reaching the detector. Wall thickness was kept below 1 mm to reduce weakening of the signal. Given that samples were scanned within liquid, using containers of large internal diameter decreased the detected signal due to increased liquid volume. The largest samples we have scanned with this technique were 6 mm in diameter. This provided sufficient resolving power at the perimeters and centre of the sample but suffered from inconsistent signal quality in the intermediate region. The scans presented in this study include ≈ 2 mm of the bovine cartilage height in the volume of interest. Ideally the whole cartilage thickness would have been included yet owing to the large thickness of bovine cartilage we sought to preserve resolution where possible. For the absorption scans a pixel size of 3.5 μm was used, compared to 2 μm for the phase contrast scans. Ideally this variable would have been removed but limitations with the scanner access and parameters necessitated a larger pixel size. Scanning parameters could not be kept consistent between scans owing to differences in sample density due to the different preparation techniques. Attempts were made to keep the overall scanning time similar for all samples but differences in exposure time and number of projections may still have affected comparison between the scans. Previous studies have shown that ionizing radiation can have a significant effect on a range of measured properties in articular cartilage samples [186, 187], including its mechanical properties[188]; bone is also negatively affected [189]. Low energy X-rays interact with these low-density materials and cause more damage than high energy beams causing particular problems for the low voltages used throughout this study [190]. Further to this, the heating effect on the sample has been shown to affect protein structures and illicit physical shrinkage [64]. These effects could be reduced by limiting the number of projections, and therefore scanning time. Moini *et al.*, have reported colour changes in amino acids upon irradiation [190], and we observed that some of the samples stored in EtOH and stained with PTA had a temporary blue hue after scanning. Further work is necessary to determine whether these observations have negative implications for this mode of

imaging. Currently the method has been validated in 2D against histological sections, further work is recommended for validation against established 3D techniques such as confocal imaging.

Herein, we report a novel and validated non-destructive technique to visualise chondrocyte features through a region of several millimetres in articular cartilage. This enables an objective quantification of chondrocyte distribution and morphology in three dimensions allowing greater insight for investigations into studies of cartilage development, degeneration and repair. One such application of our method, is as a means to provide a 3D pattern in the cartilage which, when combined with digital volume correlation, could determine 3D strain gradient measurements enabling potential treatment and repair of cartilage degeneration. Moreover, the method proposed here will allow evaluation of cartilage implanted with tissue engineered scaffolds designed to promote chondral repair, providing valuable insight into the induced regenerative process.

4 3D STRAIN MAPS IN HUMAN ARTICULAR CARTILAGE

Chapter 4 utilises the bovine chondrocyte imaging method developed in Chapter 3 on human samples with the aim of creating a pattern to track sample deformation and measure strain in 3D.

The author wishes to acknowledge Geoffrey Ng for providing samples, the staff of the Student Teaching Workshop in the Department of Mechanical Engineering for assistance when manufacturing the bespoke loading rig, Brett Clark (Natural History Museum) for assistance operating the micro-CT scanner and Saman Tavana for fruitful DVC discussions.

4.1 INTRODUCTION

Articular cartilage is typically a robust soft tissue, transmitting forces during motion through to the subchondral bone over decades whilst maintaining excellent tribological properties. Yet articular cartilage can be easily damaged and is frequently damaged through sporting injuries, traumatic accidents and active lifestyles which are sustained long in to retirement [15]. Small injuries heal, yet larger damage, or regions not given sufficient rest, will tend to not regenerate correctly even with surgical intervention [191]. Many surgical interventions exist yet none have provided a suitable long term solution. The damage can proliferate, eventually leading to osteoarthritis which is the most widespread cause of physical morbidity and impaired quality of life in the western world[19]. There is huge appetite to treat these injuries early and to successfully regenerate articular cartilage back to its original quality.

Successful tissue regeneration is driven in part by providing appropriate mechanical stimuli [192, 193] which drives chondrogenic differentiation of progenitor cells [29]. A potential cause for the lack of efficacy in currently available interventions is poorly matching mechanical properties leading instead to growth of inferior fibrocartilage [191]. In the field of orthopaedics there is a transition towards stiffness-matching musculoskeletal implants to the surrounding tissue to provide comparable strain: most often bone since success for cartilage has been poor. This is to both overcome issues such as stress shielding which results from a strain-mismatch [194], and to positively affect tissue ingrowth [195]. Success requires intimate knowledge of the mechanical properties of the host tissue, chiefly its strain response. Although research has been carried out on the mechanical properties of articular cartilage they have not been well characterised in three-dimensions at the tissue level, an oversight in the route to successful biomaterial and implant development.

As with many biological tissues, articular cartilage is non-homogenous, anisotropic and multi-phase. It is composed largely of collagen, proteoglycans and a low density of chondrocytes, saturated with an interstitial fluid phase. The composition, and arrangement, of these components vary through the height of the cartilage in distinct zones [196]. Therefore traditional methods to extract bulk mechanical data for homogenous engineering materials such as universal testing machines can be useful for exploring strain-rate dependency in articular cartilage [197] yet providing only a singular value for the entire tissue is not particularly useful [39] when creating strain-matched implants which necessitate localised intra-tissue resolution.

Current efforts to produce local strain maps of cartilage have largely taken place in 2D, either on an exposed surface [35, 198-200], or within 100 μm of the edge of the sample [7, 201-203]. Two dimensional techniques do not account for out of plane deformation [49], damage during sample

extraction [204] including release of residual strain [205] and cannot be expanded to look at the tissue-biomaterial interface [52, 53]. Confocal laser scanning allows 3D measurements [11, 206] but the technique inherently lacks penetration depth due to diffusion and scattering [179] therefore again covers a very limited depth of the sample, likely not being representative of the interior structure[207]. Ideally the tissue should remain physiologically intact and imaged in 3D over a large field of view to represent the internal deformation accurately [208].

Digital volume correlation (DVC) offers the opportunity to create full-field three dimensional displacement and strain maps by using a combination of 3D imaging and in-situ loading. First proposed for bone [2], DVC has now been expanded to bone-biomaterial interfaces [52], implant micromotion [53] and soft tissues such as intervertebral disks [54, 92] and lungs [55]. The technique relies upon features within the tissue which can be imaged and the displacement of which subsequently tracked during loading. Trabecular bone is ideally suited to DVC because its porosity gives it a highly defined two phase appearance when micro-CT scanned providing an excellent pattern to correlate [100]. Articular cartilage is not easily imaged in 3D with the required spatial resolution and sufficient intra-tissue contrast. 3D strain maps have been produced using clinical MRI on living patients [94] yet lacked resolution to credibly inform implant design. The largest features within the tissue are the chondrocyte cells, yet these occupy only a small proportion (<10%) of the matrix, are approximately 10 microns in diameter and vary in density and volume through the cartilage depth providing additional challenges. We recently developed a method using staining and laboratory micro-CT to image the chondrocytes of bovine articular cartilage on a field of view of several millimetres [209]. We propose that this method could be applied in combination with in-situ mechanical loading and DVC to reveal strain maps within articular cartilage. Visualisation and quantification of the 3D strain response in articular cartilage could be of great benefit for understanding cartilage degeneration, improving in-silico models [210] and to inform the design of osteochondral implants for tissue regeneration.

4.2 METHODS

4.2.1 Specimen Preparation

Ethical approval for this study was sought from the Imperial College Human Tissue Bank (R15022-6A) and the work is covered by REC Wales approval 17/WA/0161. Following approval from the local Research Ethics Committee, 3 mm diameter osteochondral plugs were harvested from fresh-frozen right femoral condyles of two cadaveric human donors (Figure 4.1a). Plugs with the least curved articular surface were selected (n=4) with a thickness of approximately 1-1.5 mm to allow the required field of view to be captured in a single scan (Table 4.1). To maintain the natural boundary condition during testing, the cartilage-bone interface was left intact. The plugs were bathed in PBS for 30

minutes and transferred to increasing concentrations of ethanol (25, 50, 70%) for 30 minutes each then stained with a 1% w/v phosphotungstic acid (weight/volume, $\text{H}_3\text{PW}_{12}\text{O}_{40}$, PTA, #79690 Sigma-Aldrich, USA) solution in 70% ethanol for 21 hours following established protocol [211]. After staining, samples were rinsed and stored in 70% EtOH until scanning. The bone of each osteochondral sample was mounted to a polyoxymethylene platen with polymethyl methacrylate bone cement (Simplex, Kemdent, UK) with close observation of the orientation of the articular surface to maintain sample alignment. After which the sample was loaded in to a bespoke portable loading device equipped with a load cell (Figure 4.1b, LBS100, Interface Force Measurements Ltd, UK). The use of metal fixtures was avoided close to the field of view to ensure no artefacts were introduced during scanning. The sample chamber was filled with 70% ethanol, the top compression platen was seated and silicone grease (404-124, RS Components, UK) used around the circumference to limit evaporation of the ethanol during scanning.

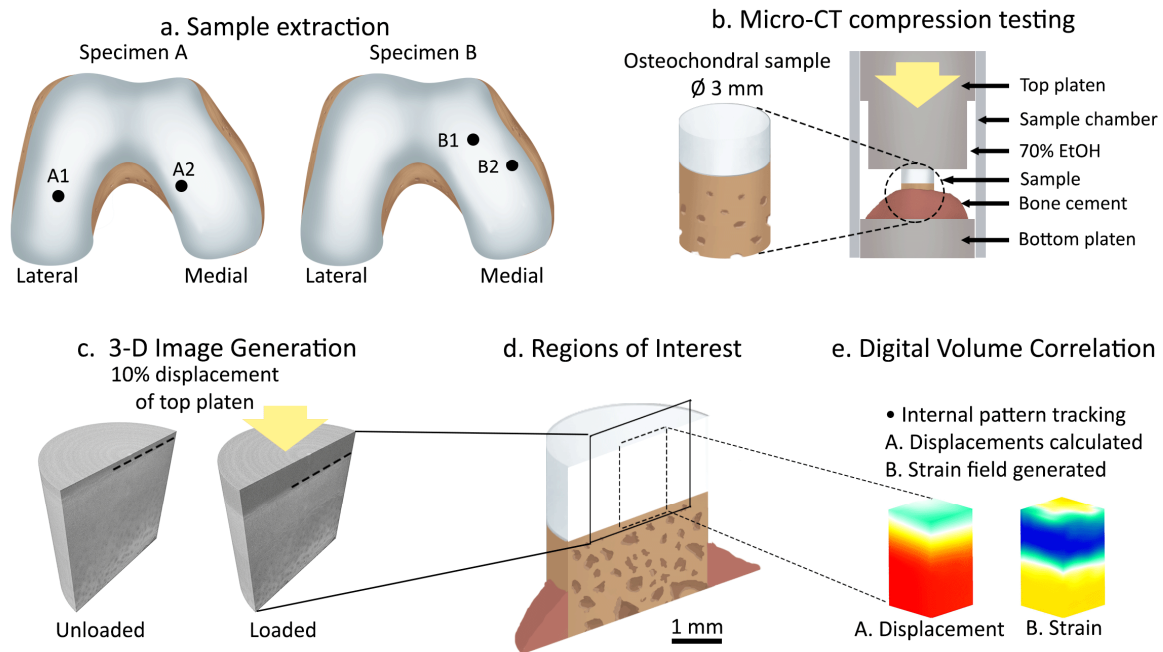


Figure 4.1. Human osteochondral samples were extracted from the femoral condyles of two cadavers (a). Each osteochondral sample was loaded in to the bespoke mechanical loading device (b) and physically attached to the bottom platen with PMMA bone cement. The samples were scanned inside the micro-CT scanner firstly twice with no displacement and then with a load applied under displacement control resulting in nominally 10% strain compared to the original cartilage thickness (c). The solid box of panel d represents the micro-CT field of view and the dashed box represents the core within the sample which was digitally extracted for DVC. DVC software tracks the displacement of internal feature patterns allowing displacement and strain fields to be generated, displayed here are axial displacement and strain for sample B2(e).

Table 4.1. Human donor and sample details utilised with in-situ testing and micro-CT as part of this study. BMI = Body mass index

Donor	Gender	Age	BMI	Sample ID	Cartilage thickness (μm)
A	F	46	24	A1	1580
				A2	1570
B	F	37	41	B1	900
				B2	1060

4.2.2 X-ray Micro Tomography Scanning

All scans were carried out on a Versa 520 X-ray micro-CT scanner (Zeiss, Germany) with the X-ray source set at 50kV and 80 μA with no filter applied. A 4 x optical magnification lens provided a field of view of approximately 2 mm. Projections were captured with binning of 2, resulting in a volume of dimensions of approximately 1000 x 1000 x 1000 voxels. Further imaging parameters are shown in Table 4.2. Samples A1 and A2 were prepared and stained at the same time yet sample A1 was stored in 70% ethanol after staining for 48 hours before scanning whilst sample A2 was stored for an additional 23 days. The difference in optimum exposure time between these two samples suggests that some of the heavy metal stain leached from the cartilage matrix sample A2 during storage, requiring a shorter scan whilst still providing good contrast.

Table 4.2. Micro-CT parameters utilised for the four samples. SOD = source to object distance, ODD = object to detector distance

Sample ID	Voxel size (μm)	SOD (mm)	ODD (mm)	Projections	Exposure time per projection (s)	Approximate length of scan (hours)
A1	1.869	27	70	1201	32	10.7
A2	1.820	26	70	1201	20	6.7
B1	1.867	27	70	1101	32	9.8
B2	1.867	27	70	1101	32	9.8

To quantify error, two scans of each sample were taken in series with an applied preload of 2 N to stabilise the sample. After these two scans a load was applied at a rate of approximately 10 $\mu\text{m/s}$ (~1 %/s) using displacement control to nominally result in an average of 10% strain through the cartilage height (Figure 4.1c). Each sample was held in the scanner prior to scanning for a minimum of 30 minutes for thermal and mechanical stabilisation, including stress relaxation, prior to subsequent scanning. One scan under load was taken for three of the samples. A series of loaded scans were taken of sample A2 to determine the effects of prolonged exposure to X-rays, resulting in a total of 110 hours exposure for this sample.

4.2.3 Image Processing

Reconstructor Scout-and-Scan software (Zeiss, Germany) was used on the micro-CT projections images to produce 3D volumetric data sets. The reconstructed CT volumes were visualized and analysed using (Fiji Is Just) ImageJ software (version 1.52g, NIH, USA) [170]. Image stacks for each sample were converted to 8-bit, rigidly registered at the lowest slice of the imaged bone and cropped to result in a region of interest of approximately 550 x 550 x 900 pixels (1 x 1 x 1.8 mm). No further image adjustment, enhancement or filtering was applied.

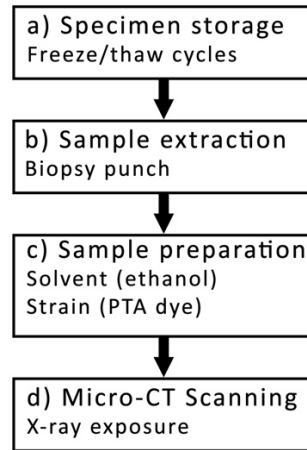


Figure 4.2 Sources of potential damage to the sample which may affect mechanical properties. Human cadaveric samples were delivered frozen and stored at -25°C (a). 3 mm sample plugs were extracted using a punch, this may have damaged the exterior of the plugs (b). Samples were prepared using both ethanol and a dye, both of which may affect mechanical properties (c). Finally, exposure to X-rays during scanning is known to affect sample integrity (d).

4.2.4 Digital Volume Correlation

4.2.4.1 Set Up

Image stacks were imported to the DVC software DaVis version 8.4.0 (LaVision, Germany). The pairs of repeat scan data were correlated using the three available processing options: 1) Direct correlation (DC) 2) Fast-Fourier transform (FFT) and finally a combination of both 3) FFT + DC. The operating principles and calculation process for the DC and FFT methods has been described previously [63] and a review of the method and applications recently published [60]. In essence, DVC tracks the deformation of microstructural features within the sample to result in a three dimensional strain field (Figure 4.1e). The technique relies upon a unique pattern to correctly correlate features and reduce spurious vectors. DaVis utilises a local-approach by splitting the volume in to smaller subvolumes. Subvolumes in the undeformed scan volume are deformed and translated over a determined search distance to correlate against the subvolumes of the deformed volume. A correlation map is formed and the highest correlating subvolume is chosen. A shape function characterises the required

deformation encompassing translation, rotation and both normal and shear strain [71]. Smaller subvolumes provide higher spatial resolution but accommodate fewer features thereby incurring higher errors. To investigate the error at across a range of sizes, subvolume sizes from 16 – 112 voxels ($\sim 30 - 205 \mu\text{m}$) were considered in increments of 16. For FFT+DC the FFT pre-shift was set at 12 pixels larger than the DC step and three calculation passes were utilised. Additionally a multistep approach was trialled using progressive steps of 176, 150, 112 and finally 80 voxels with 1, 1, 2 and 3 passes respectively. For all methods, overlap was set to 50%, peak search radius was 4 pixels, 50% required valid voxels and no binning was applied.

4.2.4.2 *Error Quantification*

Since no load was applied between the repeat scans, any deviation from constant-strain reveals the quantity of error in the system. Error can be quantified by calculating the mean absolute error (MAER), representing the systematic error, and the standard deviation of error (SDER, equation 2), representing the random error inherent within the system [67]. MAER and SDER of strain were calculated through the cartilage using a custom script in MATLAB (R2019a, The MathWorks Inc, USA). The random error was also calculated for the displacement components. The systematic error of displacement was not calculated because the real displacement is unknown.

Once the MAER and SDER were calculated for each subvolume size a compromise must be sought between spatial resolution and the maximum acceptable error for the compression study. For this study we are dealing with soft tissue undergoing a large strain of approximately 10% ($100,000 \mu\epsilon$). Previous DVC studies have set a maximum acceptable error as 10% of the nominal strain [69] therefore for this study the maximum acceptable error, used to decide upon the DVC calculation method, was set to 1% strain ($10,000 \mu\epsilon$). DVC output was quantified both across the whole cartilage height and for comparison with literature, the cartilage was split in to three zones: superficial 0 - 15 %, middle 15 – 50 % and deep 50 – 100% from the articular surface [3, 10, 11].

4.2.4.2.1 *Number of projections study*

In addition to the typical error quantification, in an effort to optimise scanning time the effects of projections quantity was considered. This was carried out by exporting image stacks from the Reconstructor Scout-and-Scan software with different numbers of projections for a pair of unloaded samples. These image stacks were then imported in to DaVis and analysed across a range of subvolume sizes and error quantified in the typical manner. Please note this was only carried out on bone and not cartilage.

4.2.4.3 *3D Strain Plot Generation*

The unloaded-loaded pair of volumes relating to the compression test (Figure 4.3b and c) were correlated in the same way and same settings as previously described, using the multistep subvolume approach. The applied displacement changed the vertical position of the tissue-platen boundary (Figure 4.1c) therefore the compression platen was geometrically masked to limit erroneous vector calculations.

4.2.4.4 Irradiation Damage

DVC was run for the unloaded scan (Figure 4.3b) of sample A2 and each of the four loaded conditions (Figure 4.3c,d,g,h). An imaging issue occurred with scan c and therefore it has been excluded and is discussed in the limitations section.

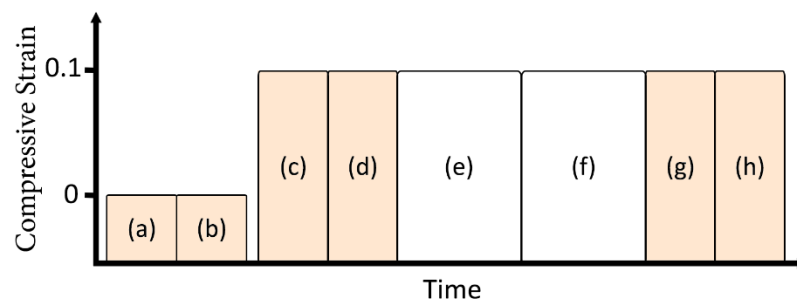


Figure 4.3. The loading schedule for sample A2. Scans (e) and (f) were carried out with similar settings except for no binning and an increased exposure time of 75 seconds per projection resulting. The purpose was to increase X-ray exposure to the sample and these scans are not included in this study. The other three samples (A1, B1 and B2) were subjected to the same repeat scan protocol (a and b) and then only one loading step at 10% compressive strain (c).

4.2.4.5 Apparent Strain Comparison

The axial cartilage deformation was manually calculated using two methods. Firstly, in Fiji the bulk cartilage thickness was measured manually ($n = 9$) for each sample in both the unloaded and loaded states. The change in thickness provided the technical strain. Secondly a grid overlay was used in Fiji and the change in vertical location of subvolumes in regions ($n = 9$) were measured continuously through the height of the samples A2 and B2. Manual tracking was carried out by an observer blinded to the DVC results. In the superficial zone it was more difficult to track the pattern and a minimum of $n = 3$ subsets were tracked at all times. To note, the DVC algorithm was able to perceive a pattern and maintained a high correlation coefficient through the sample height (Figure 4.4d).

4.3 RESULTS

4.3.1 Imaging Human Cartilage Using Laboratory Micro-CT

Micro-CT scanning permitted visualisation of cellular features within the human articular cartilage (Figure 4.4a) which were successfully located in successive scans (Figure 4.4c), creating a unique pattern for DVC. Error was quantified for each specimen at a range of different subvolume sizes.

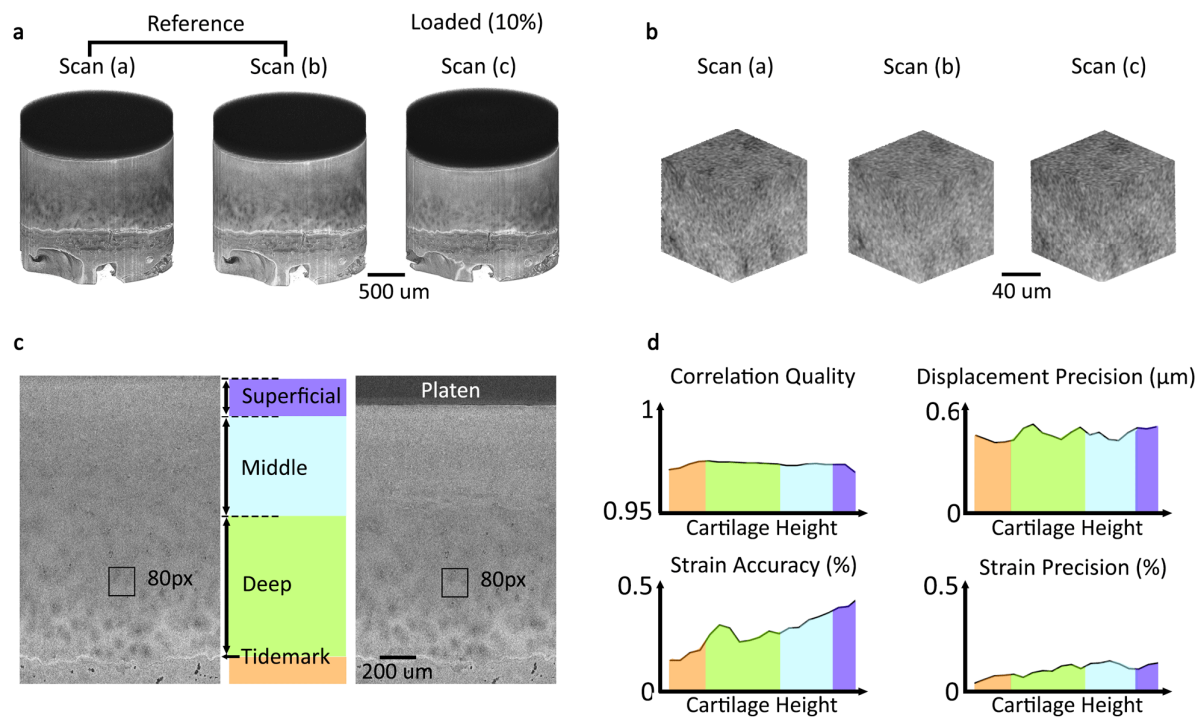


Figure 4.4. Successful 3D imaging of human articular cartilage across successive scans (panel a), shown here for sample B2. The samples underwent three micro-CT scans: two unloaded ((a) and (b)) and one with 10% strain applied (c). The volumes of each micro-CT scan were divided into smaller subvolumes, of edge length 80 microns (panels b and c), and these were correlated through the larger volume. The cartilage was split into three zones with distance from the tidemark: deep (0-50%), middle (50-85%) and superficial (85-100%), (panel c) with an overlay of subset size used for tracking patterns. Pattern tracking quality was quantified with correlation quality and error in displacement and strain, shown here averaged across the four samples (panel d).

A DVC approach tracking features within subsets of size 80 voxels (Figure 4.4b) with 50% overlap fulfilled the maximum acceptable error criteria ($< 1\%$ strain) for all strain components in all regions of the tissue (Figure 4.4d), providing a spatial resolution of $75\ \mu\text{m}$. On average this provided a systematic error (MAER) and random error (SDER) of 0.33% strain and 0.18% strain respectively throughout the cartilage and reduced further in the bone (Figure 4.4d, orange regions), representing 5.1% total error in comparison to the applied strain (10% strain). Cartilage strain systematic error (MAER) and random error (SDER) were best in the deepest zone (MAER: 0.22% strain, SDER: 0.13% strain) and worsened with distance from the subchondral bone. Among the six strain components in all regions of the cartilage, on average the highest uncertainty occurred for the axial strain component in the superficial zone in terms of systematic error (0.78% strain) and in the in-plane transverse

component in the superficial zone for random error (0.51% strain). Displacement random error was under 1 μm throughout the whole sample and correlation approach was excellent (> 0.93) for all samples and throughout the entire sample height (Figure 4.4d).

Strain error varied between the components and varied in magnitude between samples (Figure 4.5). The axial component (E_{zz}) harnesses the largest systematic error (MAER, Figure 4.5) for three of the four samples, and the largest random error for all four human cartilage samples. of error (SDER, Figure 4.5). Random displacement error (SDER, Figure 4.5) was relatively evenly distributed among components (SDER, Figure 4.5).

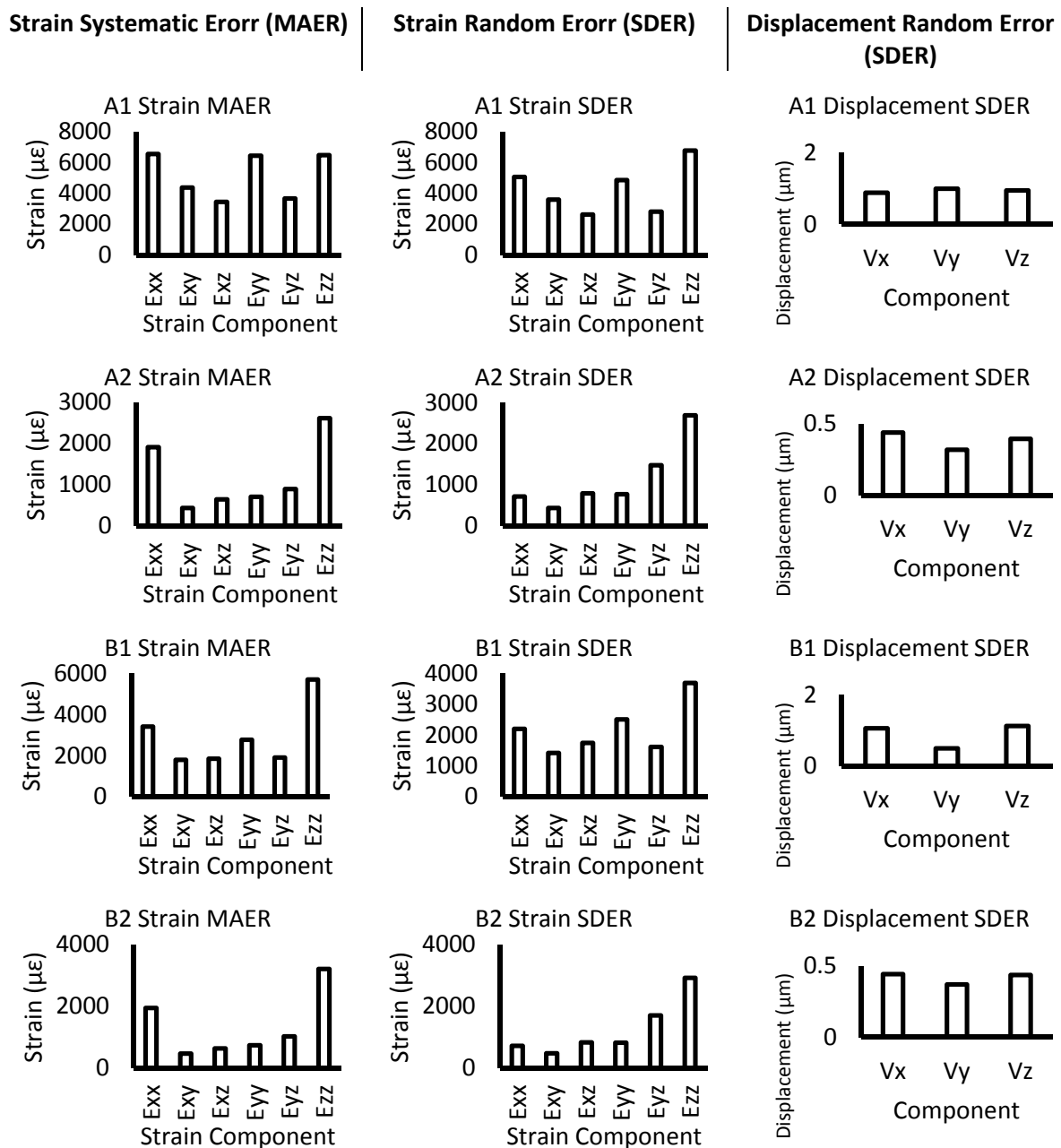


Figure 4.5. DVC error computed for each of human cartilage sample ($n = 4$) for each strain and displacement component at a subvolume size of 80 voxels. Note that these results are for a single step at 80 voxels rather than the multi-step approach as used to process the loaded sample data..

4.3.1.1 *Number of projections*

Image quality degraded with fewer projections. When a series of consecutive projections were removed, image quality quickly degraded (Figure 4.6, rows 1 and 2 a-f), even when the majority of the projections were left intact. However, when a larger number of projections were removed at regular intervals, image quality was largely preserved even when up to 75% of the projections were removed (Figure 4.6 and Table 4.3, row 3 g-i).

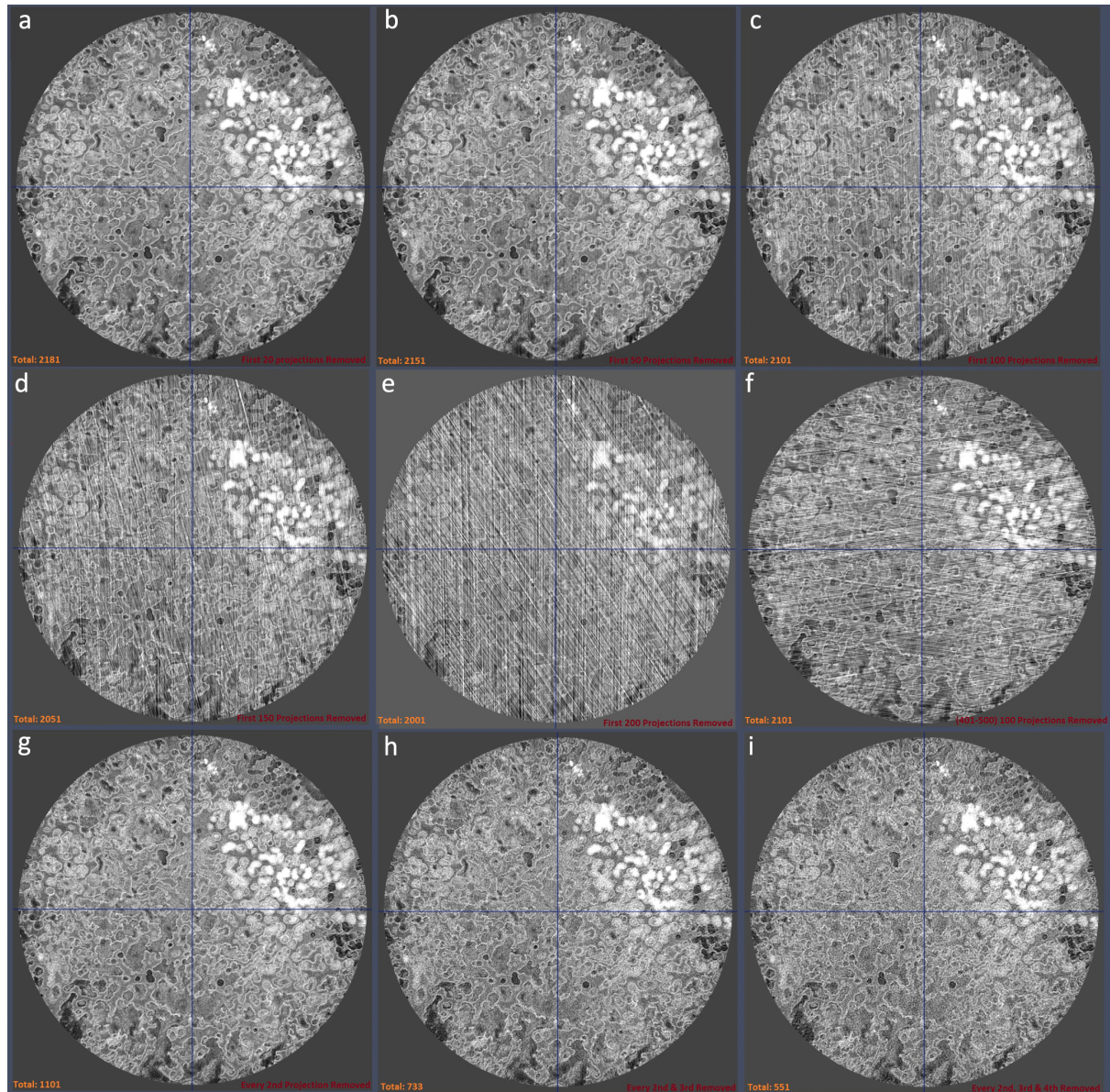


Figure 4.6. A micro-CT image stack was reconstructed using a different number of projections. Two image stacks were processed in the same manner to allow a constant-strain error quantification study to take place between them. The number of projections for each reconstruction is described in Table 4.3.

Table 4.3 A description of the processing applied to the reconstructions displayed in Figure 4.6, the number of projections removed was calculated in relation to the original number of projections acquired: 2201.

Reconstruction	Projections removed	Projections removed (%)
a	First 20	0.9
b	First 50	2.3
c	First 100	4.5
d	First 150	6.8
e	First 200	9.1
f	100 from region 401-500	4.5
g	Every 2 nd	50
h	Every 2 nd and 3 rd	67
i	Every 2 nd , 3 rd and 4 th	75

Correlation quality for any given subvolume size increased with the number of projections (Figure 4.7a). Displacement random error did not follow an obvious trend between the different numbers of projections across the range of subvolume sizes included in this study for this one sample (Figure 4.7b). Strain accuracy and random error followed the same trends as outlined for all the samples: decreasing error with larger subvolume sizes. The largest number of projections appeared to potentially incur the largest strain errors (Figure 4.7c and d).

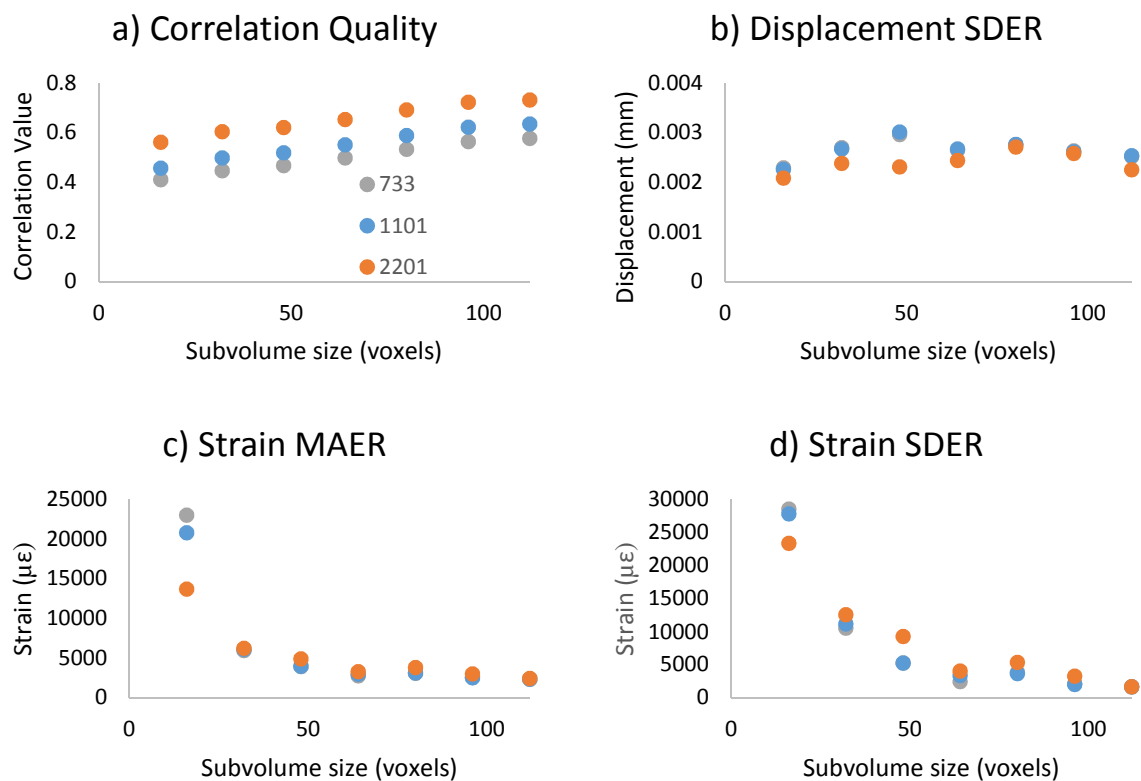


Figure 4.7. The principals of a constant-strain study were applied to one sample using two image stacks reconstructed using three different numbers of projections: 733 (grey, h in Figure 4.6 and Table 4.3), 1101 (blue g in Figure 4.6 and Table 4.3) 2201 (orange). Parameters considered were correlation quality (a), displacement random error (b), strain systematic error (c) and strain random error (d)

4.3.2 High Resolution Strain Patterns in Articular Cartilage

The developed method allows visualisation of the cartilage's mechanical response in three dimensions from the subchondral bone through to the superficial cartilage over a distance of several millimetres (Figure 4.8). The generated vector map allows determination of all displacement and strain components in 3D (Figure 4.8c and d). It is clear that there are anomalies at the edges of the sample, across both the external surface (Figure 4.8b), and close to the edge in the exposed slices (Figure 4.8c and d), highlighting one of concerns with 2D techniques which only analyse this region.

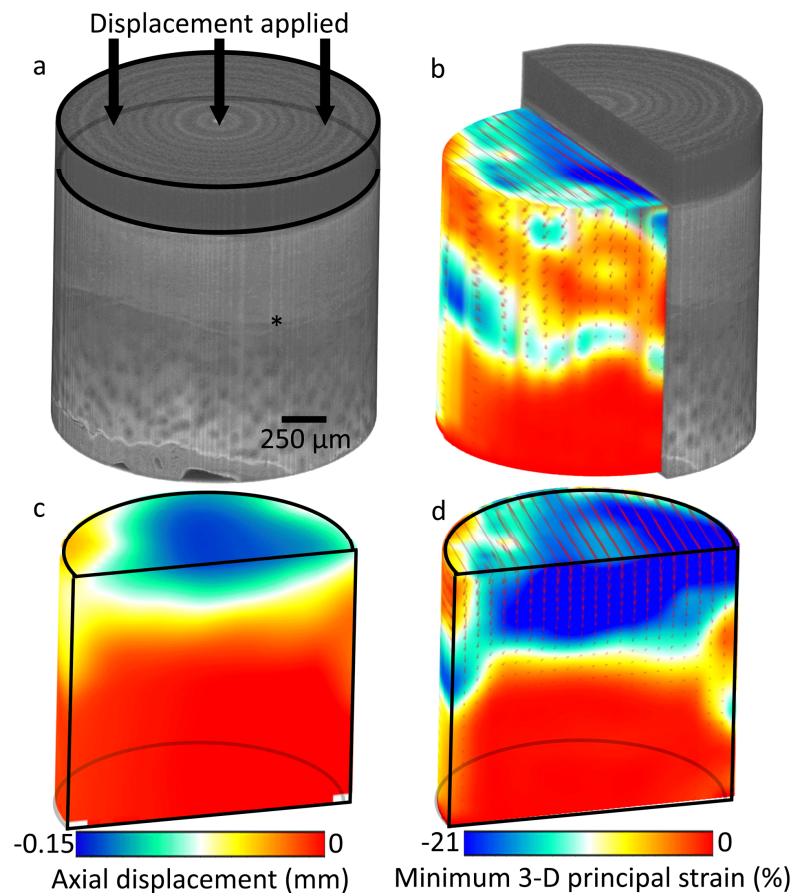


Figure 4.8. Generation of 3D displacement and strain plots. Displacement was applied during micro-CT (a), allowing DVC to track the movement of features within the 3D volume. Once correlated, it is possible to analyse the mechanical properties of the whole 3D volume (b, minimum 3D principal strain field overlayed). Axial displacement (c) and minimum 3D principal strain (d) are shown sliced through the centre of the sample. The total displacement vector map is overlaid in red on images b and d. Images shown are for sample A2. *denotes a clear zonal change in the structure of the cartilage in the middle zone.

Generating rich high resolution volumetric data enables non-destructive evaluation of localised inhomogeneity in the mechanical response of the tissue in all samples (Figure 4.9a-d). Displacement was higher for the two thicker samples (A1 and A2, Figure 4.9). The strain response was found to be highly non-linear, with certain regions of each specimen exhibiting much higher strains than the

average value. A steep strain gradient was observed and of note, the thicker cartilage samples (A1 and A2) accommodated the applied displacement with strain only occurring in the middle and superficial zones, in contrast to the thinner samples (B1 and B2) which were strained down to within the deep cartilage zone (Figure 4.10b-d). Samples A2 and B2, from different human donors, exhibited a large maximum principal strain response (Figure 4.9d) in the superficial zone compared to the other two samples.

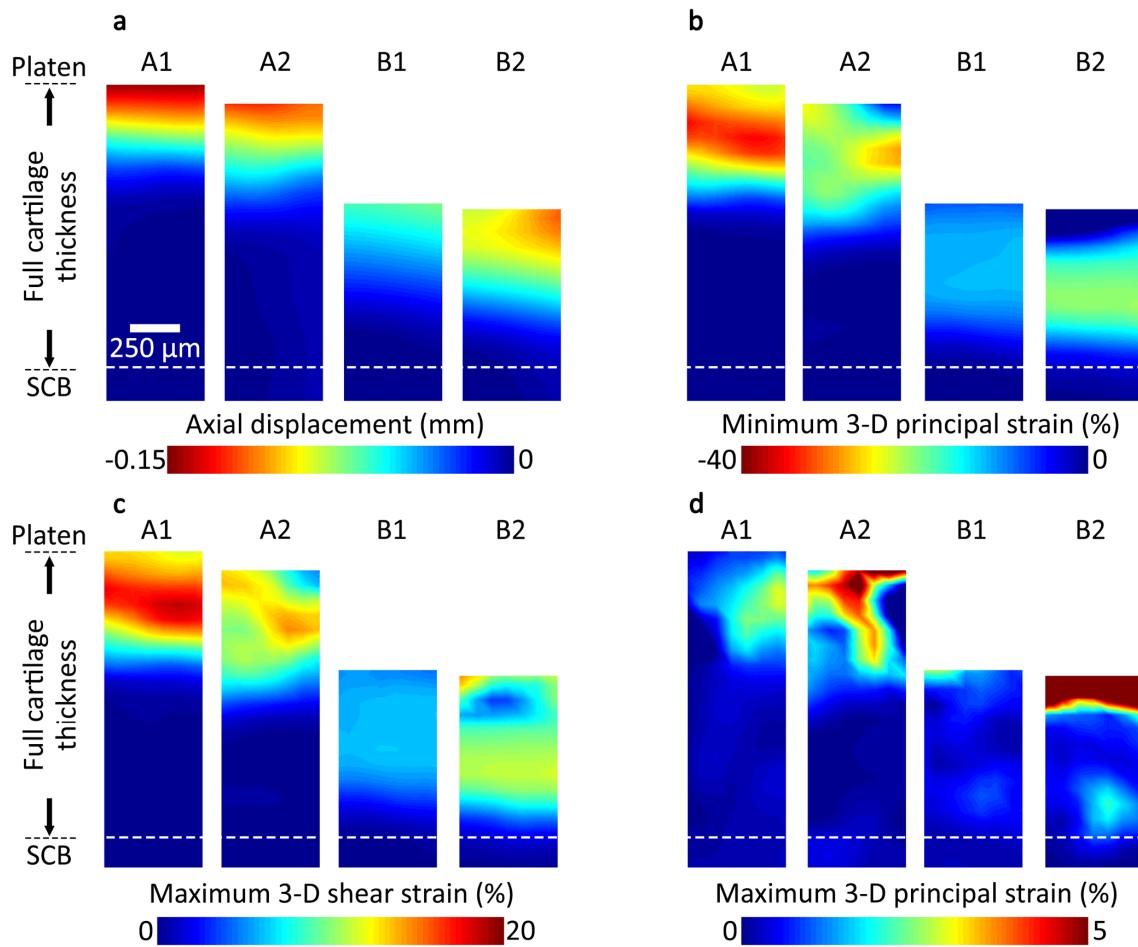


Figure 4.9. Visualisation of displacement (a) and strain components: minimum principal strain (b), shear strain (c) and maximum principal strain (d) in human osteochondral samples from a central slice of the DVC volume. SCB = subchondral bone. The top of each plot represents the top of the cartilage height i.e. the superficial region. Please note that the scale is reversed compared to other figures: i.e. red represents the highest magnitude.

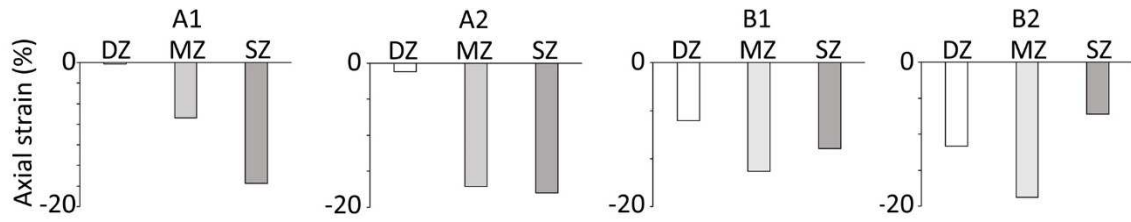


Figure 4.10 Axial (compressive) strain calculated through the three non-calcified cartilage zones for the four human osteochondral samples calculated using micro-CT and DVC., DZ = deep zone, MZ = Middle cartilage zone, SZ = Superficial cartilage zone.

4.3.2.1 Apparent Strain Comparison

Strain values calculated with DVC were comparable with those calculated manually, both as an average through the whole cartilage height ($R^2 = 0.931$, Figure 4.11a) and at the intra-tissue level (Figure 4.11b and c).

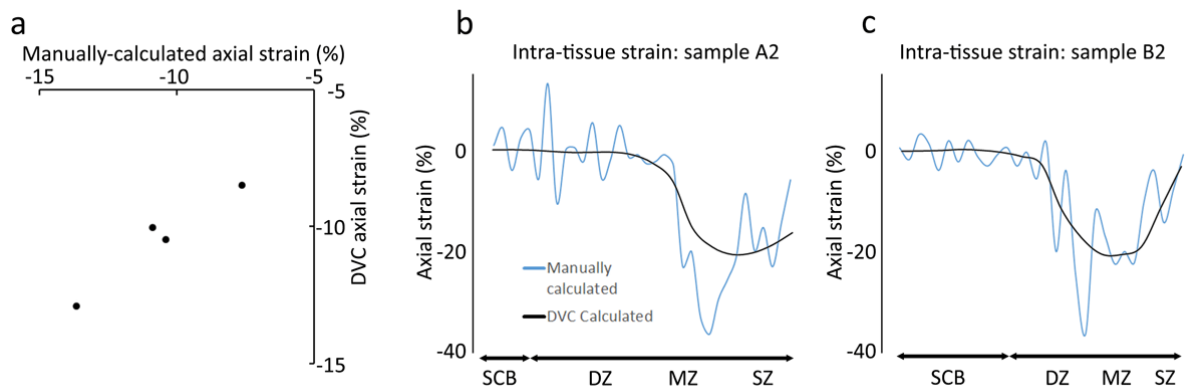


Figure 4.11. Strain values calculated by DVC were compared against values calculated manually with two methods: Firstly by measuring the change in height of the sample before and after compression (a, $R^2 = 0.931$, equation: $y = 1.002x - 0.0074$) and secondly, by manually tracking displacement of features within the cartilage, shown here for samples A2 and B2 (b and c). SCB = Subchondral bone and from left to right are the cartilage layers with increasing depth from the bone: DZ = deep cartilage zone, MZ = Middle cartilage zone, SZ = Superficial cartilage zone.

4.3.3 Irradiation Damage

Increasing length of exposure to X-rays during micro-CT was shown to increase the magnitude of strain in the articular cartilage (Figure 4.12). The subchondral bone and deep cartilage zones were largely unaffected, and the effect was most prominent in the superficial zone (SZ, Figure 4.12).

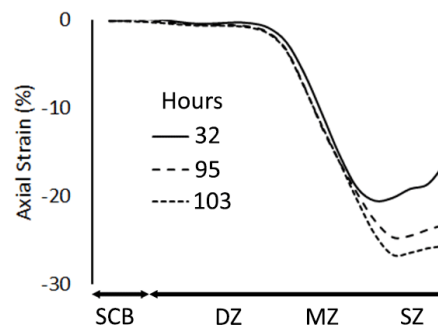


Figure 4.12. Axial strain through the articular cartilage (zones DZ to SZ) was compared during three cumulative X-ray micro-CT scans on sample A2, each therefore exposing the sample to higher doses of irradiation. The local intra-tissue strain levels for each of the three scans as introduced in Figure 4.3. SCB = Subchondral bone and from left to right are the cartilage layers with increasing depth from the bone: DZ = deep cartilage zone, MZ = Middle cartilage zone, SZ = Superficial cartilage zone.

4.4 DISCUSSION

4.4.1 High Spatial Resolution Strain Patterns in Articular Cartilage

We present for the first time high resolution three dimensional strain patterns within human articular cartilage. This represents a leap forward for osteoarthritis research, whereby studies into the mechanical properties of cartilage have previously been limited to 2D, low resolution or volumes of under 100 micrometres from the sample surface. The method achieves displacement random error of under one micrometre and strain errors of under 1% strain. This has been accomplished with laboratory equipment, avoiding use of poorly accessible synchrotron facilities which has often been a documented limitation [87]. DVC-calculated strain values have been compared against values calculated manually. Further work should be carried out to further optimise and quantify image quality whilst reducing scan time.

4.4.2 Comparison to Literature

The majority of studies of strain in cartilage have taken place in 2D on non-human species [7, 11, 199, 200, 202, 203, 212], with strain observed by slicing the sample and observing strain across the exposed surface. These studies showed the peak strain occurring in the superficial cartilage zone and most showing a non-linear decrease in observed strain through the middle zone to the deep zone. Our study showed the same trend in samples ($n = 2$) from one of the two human donors (A), but in the other donor (B) both samples showed peak strain occurring in the middle zone (Figure 4.10). It is possible

that the difference in boundary conditions, including more physiologically-appropriate constraint mechanisms, in our study may have accounted for differences in strain response (Figure 4.8). Two samples, A2 and B2, exhibited a large maximum principal strain in the superficial zone (Figure 4.9d). The magnitude of maximum principal strain was highest in the most superficial of the calcified cartilage in the most analogous previous study ([87]), potentially caused by lateral contraction and the Poisson effect during loading. One sample from each donor was successfully compared against manually-calculated strain results.

Inter-patient variability is well documented in the biology [213, 214] and mechanical response of human tissue [215, 216] and was observed in this study (Figure 4.9). It should be noted that donor B had a body mass index typically classified as extreme obesity (Table 4.1). Chan et al conducted an *in vivo* study using MRI, providing strain plots on living human participants [217]. They witnessed similar levels of inter-subject variability as in this study, with peak strain occurring in different regions between patients. Clinical MRI lacks the spatial resolution to provide rich intra-tissue strain measurements, therefore our study provides a high-resolution complimentary technique necessary for improving biomaterial implant design.

Bone is a good candidate for DVC because it is composed of highly defined feature boundaries with high porosity creating an excellent unique pattern[71]. Soft tissues create a challenge for DVC because of poor contrast [49] and particular to articular cartilage: low feature density. No other studies of DVC on the full height of articular cartilage exist for comparison. Recently a study on strain in whole mouse joints was conducted at a synchrotron, yet only the calcified cartilage was considered [87]. The displacement errors observed in our study are of sub-micron random error throughout all cartilage zones, with a similar magnitude to the aforementioned mouse joint study and to that of a study on rat intervertebral disks [54]. The strain errors calculated in this study were not dissimilar to those for other biological tissues using the same software [63] and given the low stiffness and therefore high strain imposed on cartilage, the errors satisfied the maximum acceptable error criteria of 10% of applied strain [87]. It may be feasible with further work to use filtering to further improve systematic error and random error [100].

Strain error varied between the six individual components (**Figure 4.5**). Previous studies have noted averaging error between the components underestimates the largest error by approximately 50% [63]. In this study the largest strain error component was a maximum of almost 300% larger. Further investigation is required and increasing the sample size would provide a further insight and allow statistical analysis between the components.

It was observed that excessive exposure to X-rays during scanning did appear to affect strain in the cartilage as previously documented [186] particularly in the superficial zone. It should be noted that the length of exposure for these additional scans was intentionally excessive to illustrate its effect. Now that error has been quantified it may be possible for future experiments to conduct only two scans per sample (one unloaded and one loaded) which brings the total scan time to under 24 hours. Nevertheless X-ray exposure remains a valid concern and care should be taken to reduce unnecessary dosage. The number of required projections was investigated and of note found that image quality could be maintained with fewer projections though irregular projection spacing had a large effect on image quality. Resulting images and DVC error did not appreciably degrade even when the number of projections was limited to 733: 33% of the original data set. It should be noted this was only carried out for bone, yet implementation in future studies could assist in optimising settings to reduce exposure to X-rays. The author is not aware of other studies which have quantified micro-CT system parameters in terms of the resulting DVC error.

The chapter builds on the imaging method developed in Chapter 3 which utilised bovine samples, applied now to human samples. Features were generally less well defined in the human samples with several likely explanations. Firstly scanning time was intentionally reduced for the human study in comparison to the bovine study. Secondly the bovine sample staining protocol was not altered for the human samples: it is possible that given the difference in species, age and sample freshness, that an amended preparation protocol would aid image quality and allow better strain tracking.

4.4.3 Limitations

The aim of this study was to develop a novel method for localised strain measurements in human articular cartilage. Since only four samples across two human donors were included limited conclusions can be drawn to generalise the strain maps we have found to the wider population. Additionally, it is known that biomechanics vary depending on the sample location in the human joint [218]. Further work is encouraged based on the outlined method to expand the sample size both for statistical purposes and to allow comparison of healthy tissue compared to the different stages of osteoarthritis.

Throughout each stage of experimentation damage may have been caused to the cartilage, resulting in changes to the mechanical properties (Figure 4.2). All samples were received frozen and therefore underwent a freeze-thaw cycle before use, as is often done in other DVC studies [52, 87]. Studies have found this to have little impact on the mechanical properties of cartilage [219, 220]. Processing the samples with the PTA heavy metal stain and 70% ethanol dehydration will have both likely altered the cartilage's properties [161, 162]. Whether or not this treatment has variably changed the response to

loading through the cartilage height is unknown. The aim should always be to keep the sample as physiologically unaltered as possible, and our previous work [209] has shown that preparation in PBS with PTA staining, or in 70% ethanol without staining, show promise and we suggest that this is explored by future researchers to limit potential alteration of strain response.

Prolonged high energy X-ray exposure can alter tissue such as bone [80] and cartilage [186]. We have evaluated changes in cartilage with prolonged low energy X-ray dosage yet it is possible that even a short dose of X-rays are changing the properties of cartilage in comparison to its natural state[190]. In an effort to reduce exposure, scans were only taken in two states: constant-strain and loaded to the chosen strain value (10%). Taking intermediary scans may aid pattern tracking and successful correlation, though we did find good agreement in our DVC values with the manually calculated results.

The strain response of cartilage has shown viscoelastic properties [221] and studies with dynamic loading have shown different results to static loading [203]. The required scanning time in this laboratory micro-CT study necessitated measurement of strain during static rather than dynamic loading. A complimentary study of great interest would be that of dynamic loading which may be possible with continuous data capture and short scan times, potentially at a synchrotron facility.

The DVC software utilised in this study uses only the small strain calculation, thereby omitting the quadratic terms of the Green stain tensor definition. The effect of this has been calculated to be approximately 5 % of the average reported strain values. To remove this uncertainty it is recommended for future studies to consider using a large strain Green strain tensor calculation either using suitable DVC software, or to export the displacement field and calculate strain with a home-written script.

Pattern quantity and quality appeared to reduce closer to the articular surface, likely due to the inherent change in cartilage structure and imaging limitations [209]. It is possible that further image processing including use of filtering could improve contrast and DVC results [100]. We have quantified error and correlation quality through the cartilage height to consider these effects on the DVC output and both were within the bounds expected for biological tissue. Additionally, the manually-calculated strains suggest that the strain profiles generated are accurate.

The scanning setup for this study necessitated the rig to be taken out of the scanner to apply load. Images were registered before DVC analysis to ensure alignment, the rig was placed in the scanner for a minimum of 30 minutes before scanning for mechanical and thermal stability [54] and the load was checked before and after scanning. A cable management system installed in the system would have allowed recording of strain throughout the duration of the experiment.

4.4.4 Conclusions

We present for the first time high resolution three dimensional strain maps of articular cartilage, providing sufficient spatial resolution for intra-tissue local strain measurements. The developed method is carried out non-destructively using laboratory equipment. This will enable future studies on cartilage biomechanics and osteoarthritis progression facilitating improved treatment with the potential for matching implants to the stiffness of the tissue at a local level.

5 VISUALISATION OF STRAIN IN HYBRID BIOMATERIAL IMPLANTS

The work in this chapter utilises materials and devices developed by colleagues in the Department of Materials, Imperial College London.

The degradable hybrid material was initially developed by Dr Francesca Tallia, including optimising the 3D printing approach. All hybrid scaffold samples utilised in this study were produced by Agathe Heyraud who also assisted with collection of images for DIC of the hybrid samples.

Dr Gloria Young further developed the hybrid material for tissue-replacement (non-degradable) applications, produced the non-degradable samples used in this study and assisted with collection of images and DIC processing of such samples.

The author carried out all micro-CT scanning and DVC analysis. The author is grateful to micro-CT support from Brett Clark (Natural History Museum).

5.1 INTRODUCTION

Previous generations of orthopaedic implants have suffered from a mismatch in mechanical properties in comparison to the local tissue [195, 222]. This compromised long-term success by causing issues such as stress shielding and micromotion, leading to degradation of remaining tissue, implant loosening and higher revision rates. Research suggests that for an implantable biomaterial to achieve success for its given role, suitable mechanical properties are paramount [223]. This is equally true both for tissue replacement biomaterials, whereby robust long-term fixation is paramount, and for tissue regeneration with degradable implants whereby desired cell pathway differentiation depends upon mechanobiological stimulation [28, 29].

A goal is often to match the stiffness of implants to the host tissue. This is non-trivial since human tissue is typically complex, exhibiting anisotropy, non-homogeneity and in the case of soft-tissues may oftentimes be multi-phase. Two soft-tissues with a high unmet demand in suitable implants are articular cartilage and the intervertebral disc (IVD). Articular cartilage has vertically graded mechanical properties to transmit forces to the underlying subchondral bone with stiffness increasing with distance from the bearing surface. The IVD is composed of two distinct zones, with the central nucleus pulposus surrounded by the annulus fibrosus, both of which have very different mechanical properties [92]. It quickly becomes apparent that typical materials testing equipment, which can only consider the bulk mechanical response, should not solely be relied upon in the task of tailoring the properties of biomaterials to tissues such as these.

Tools such as linear variable differential transformers (LVDTs) and strain gauges are widely used during materials testing but lack sufficient spatial resolution for this application [49]. Digital image correlation (DIC) improves on resolution, has been applied to biomechanical analysis [51] and is an established technique to quantify strain [224] yet it is still inherently limited to surface measurements. Surface strain measurements are useful yet are not necessarily representative of the mechanical conditions within the implant. This is a limitation, particularly for tissue regeneration applications whereby the implant design determines the mechanical loading experienced by cells in the interior of the scaffold therefore playing a role in the tissue type grown. Equally, surface measurements are not sufficient for tissue replacement implants with intricate designs, such as radially inhomogeneous properties as required for IVD-replacement applications.

The principles of DIC have been extended to the third dimension to consider full-field strain by way of digital volume correlation (DVC). Since its inception 20 years ago for measuring strain in bone [2], DVC

has been applied to soft tissues [54], implant micromotion [53] and strain across the interface of tissue – biomaterial systems [52]. In this study we implemented laboratory micro-CT and DVC as part of a process to inform implant design for two different soft tissue applications: degradable scaffolds for articular cartilage regeneration, and a non-degradable dual-phase solid material for tissue replacement. DVC performance was compared with the more established DIC technique, and compared against ground truth calculations.

5.2 MATERIAL AND METHODS

5.2.1 Device Manufacture

5.2.1.1 Degradable Scaffolds for Cartilage Regeneration

Hybrid SiO_2 -PCL-PTHF ink was synthesised using established protocol [41]. During synthesis the liquid solution can be cast into a mould, or as is used in this application, extrusion 3D printed. The ratio of inorganic (SiO_2 : silica) to organic (PCL: polycaprolactone and PTHF: polytetrahydrofuran) components defines the material properties of the implant and may be tailored over a wide range to a given application whilst behaving as one phase [45]. For this study a w/w ratio of 80:20 (inorganic:organic) was utilised. The ink was 3D printed using robocasting to produce scaffolds with strut width and pore size both of approximately 200 μm . To consider the localised strain mechanics of differently sized implants, implants of two sizes were produced: cubic implants of 5.5 mm edge length typically used for materials testing ($n = 8$ Figure 5.1a) and cylindrical implants of dimensions similar to the intended application: 5 mm diameter and 1.2 mm height ($n = 4$ Figure 5.1b).

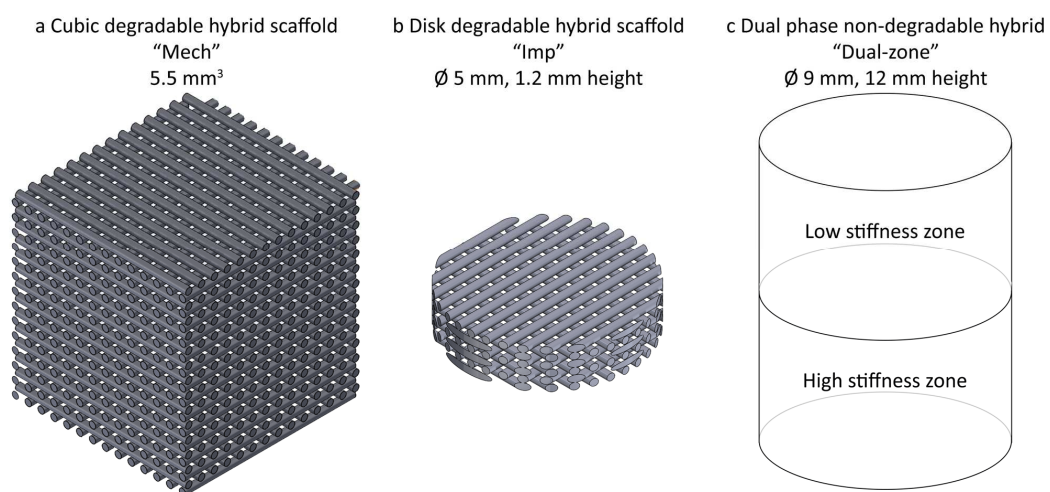


Figure 5.1. Three sample types tested in this study: two varieties of degradable hybrid scaffold intended for articular cartilage regeneration a) Cubic “Mech” samples typically used for device development and mechanical testing b) Cylindrical “Imp” samples of dimensions similar to those intended for implantable devices. c) Non-degradable “dual zone” hybrids have been developed for tissue replacement applications and this study included a sample composed of two different material compositions to provide a compositional gradient mechanical properties.

5.2.1.2 *Multi-zone Non-degradable Implants for Intervertebral Disc Replacement*

A modified version of the hybrid material can be synthesised with the omission of PCL, removing the ability for the implant to degrade. This provides a material with potential for tissue-replacement applications. Hybrid SiO₂-PTHF solutions, of varying inorganic – organic ratios were synthesised and cast in Teflon moulds to produce samples of approximately 9 mm diameter and 12 mm height (Figure 5.1c). Casting multiple compositions together allows formation of samples with spatially-graded compositions and mechanical properties.

The correlative imaging techniques employed in this study rely upon the existence of uniquely identifiable features within the images, the displacement of which can be tracked to infer local strain. The hybrid material itself was optically homogenous, lacking visible features. When 3D printed, the scaffold structure provided a unique pattern, however non-porous solid hybrids naturally lack unique local features to be tracked. The addition of particles to create contrast has previously allowed successful strain measurements with DVC [225]. One dual-zone hybrid material with a composition gradient was cast from multiple sols, with the addition of ZrO₂ particles (sieved to <38 µm) [226] at a concentration of 2 mg mL⁻¹ to provide contrast during micro-CT for DVC analysis [227].

5.2.2 Imaging

5.2.2.1 *Three Dimensional Micro-Computed Tomography*

A Zeiss Versa 520 X-ray micro-CT scanner (Zeiss, Germany) was employed with voltage of 70 kV and current of 86 µA. No filtering was applied. The number of projections, exposure time per projection and binning varied with sample size and type (Table 5.1). Binning for all samples was set to 2 except for the dual-zone sample, where no binning was applied. Each sample was loaded in to a custom-built *in-situ* mechanical testing rig equipped with a load cell (LBS100, Interface). A small preload (<5 N for scaffolds, 10 N for the dual-phase sample) was applied to all samples ensure good contact with the platens and the sample was allowed 30 minutes for thermal and mechanical equilibration. Two consecutive micro-CT scans were taken of each sample for quantification of error within the system. Displacement was then applied to each sample and rescanned again after a minimum of 30 minutes to avoid data capture during stress relaxation. Additional scans were taken of some of the samples with more displacement applied (Table 5.1).

Micro-CT projections were reconstructed using Reconstructor Scout-and-Scan software (Zeiss, Germany) to produce 3D volumetric data sets. The reconstructed CT volumes were visualised and

analysed using (Fiji Is Just) ImageJ software (version 1.52g, NIH, USA) [170]. Image stacks for each sample were converted to 8-bit, rigidly registered at the static lowest slice of the imaged scaffold and cropped to a volume interest approximately 550 x 550 x 900 pixels for the scaffold samples, and 700 x 700 x 1500 pixels for the dual-phase sample. Image stacks for the dual-phase material were binarised so ensure correct tracking of the ZrO₂ particles. No further image adjustment, enhancement or filtering was applied.

Table 5.1. X-ray micro-computed tomography settings for each sample studied using DVC, including the strain applied between scans. “Mech” samples were classified as samples typically used for mechanical testing and are of similar dimensions to those used for the DIC analysis. “Imp” samples are of dimensions similar to those intended for implantable devices. The dual-zone sample is made from the non-degradable composition and is intended for applications requiring a solid material with a gradient of properties, such as implants for intervertebral disc replacement.

Composition	Sample	Voxel size (μm)	Projections	Exposure time per projection (s)	Sample height (μm)	Strain (%) at loading steps
Degradable	Mech1	10.6	801	1	4750	-8, -18
	Mech2	4.1	1201	1.5	3375	-8
	Imp1	3.9	1201	1.5	930	-2, -12
	Imp2	3.9	1201	1.5	925	-7
	Imp3	3.9	1201	1.5	910	-4
	Imp4	4.1	1201	1.5	1200	-11
Non-degradable	Dual-zone	7.7	1201	6	11500	-6, -14

5.2.2.2 Two Dimensional Imaging

Cubic degradable scaffold samples ($n = 6$) with dimensions of approximately 5.5 mm were mechanically compressed up to a minimum of 20% axial strain, using a compression testing machine (Bose Electroforce Series III with 450N load cell, TA Instruments, USA) in displacement control at a rate of 1 mm min⁻¹. Solid non-degradable cylindrical samples were tested: firstly of one zone for error quantification, after which the dual-phase sample was tested following micro-CT and DVC analysis.

In combination with the mechanical loading, images were taken with a digital camera (750D, EF-S 60 mm lens and 34 mm extension tubes, Canon, Japan) mounted on a tripod (MKC3-P02, Manfrotto, Italy) at a distance of approximately 20 cm from the sample to provide localised strain measurements when combined with digital image correlation software. Image resolution was 6000 x 4000 pixels, providing an approximate pixel size of 4 μm for the scaffolds and 6 μm for the non-degradable solid

samples. A series of static images were taken of each sample prior to loading for error quantification. As with the micro-CT setup, the scaffold inherently provided a pattern and the addition of a pattern was required for the non-degradable cylindrical samples, for which a unique ink pattern was manually applied. Photographs were taken consecutively every two seconds with an exposure time of 1/60 s, aperture of f / 5.6 and ISO of 800. A lamp was placed close to each sample to act as a continuous light source for constant and even illumination.

5.2.3 Feature Correlation

5.2.3.1 *Digital Volume Correlation (DVC)*

Reconstructed image stacks were imported to DaVis (8.4.0, LaVision, Germany), sub-divided in to subvolumes and correlated between the sets of scans. As described in previous chapters, initially error was quantified across a wide range of potential subvolume sizes from 16 – 112 voxels using the static constant-strain data sets, for the three available DVC processing methods: 1) Direct correlation (DC), 2) Fast-Fourier transform (FFT), 3) A combined approach of DC and FFT. For the combined approach the FFT pre-shift window size was 12 voxels larger than the step size.

Following the constant-strain study, the optimised subvolume size was applied to the unloaded-loaded pairs of data for all samples ($n = 7$): FFT+DC with a subvolume size of 48 voxels, an FFT pre-shift window of 150 voxels, 50% overlap between subvolumes, a peak search radius of 16 voxels and 3 passes. This provided a spatial resolution of 24 voxels: approximately 100 μm for the majority of scaffold samples, and 185 μm for the dual-phase non-degradable sample. In the instance where multiple loading stages were available, the Sum of Differential correlation mode was applied. A geometric mask was applied to the top platen to eliminate the risk that including this movement in the DVC calculations could lead to erroneous vectors.

5.2.3.2 *Digital Image Correlation (DIC)*

The sequential images were imported to the DIC software (GOM Correlate 2018, GOM, Germany). Unlike the DVC software utilised in this project, which allows the user to collect results for all subvolumes with no filtering applied, the DIC software instead requires the minimum acceptable pattern quality to be decided as part of the calculation process. Therefore a range of minimum acceptable pattern quality values were considered to ensure the results were acceptable. For the degradable scaffold samples, pixel size was calculated and error was quantified across a range of subset sizes matched to the resolution used when DVC processing the micro-CT data. Minimum pattern quality was investigated and after which the loaded scenario data for each scaffold sample was processed using a minimum pattern quality of 0.4, a facet size of 48 pixels and 50% overlap:

matching the array used for the DVC analysis. The dual-phase non-degradable sample was subsequently analysed using a minimum pattern quality of 1.1, a facet size of 75 pixels and 50% overlap. Due to the cylindrical shape of the non-degradable implant, strain was measured only on a central plane for which optical focus was maintained.

5.2.3.3 *Error Quantification*

For both DVC and DIC, systematic error (MAER) of the calculated strain and random error (SDER) for displacement and strain were calculated using established protocol [67]. The systematic error of displacement vectors is not presented because the true displacement during acquisition is not known.

5.2.3.4 *Apparent Strain Comparison*

After compression testing, output images for degradable scaffolds from both 2D (DIC) and 3D (DVC) techniques were compared with strain values calculated manually. The length between the top and bottom platens was measured in ImageJ before and after applying a compressive force and the technical strain was calculated using equation (8):

$$\varepsilon = \Delta l / l_0 \quad (8)$$

where ε is the resulting strain, l_0 is the original length between platens and Δl is the change in length. Further to the bulk strain, for $n = 2$ of the scaffold DVC scans, strain was manually calculated using the described approach with a resolution matching the computational technique (i.e. every 24 pixels / voxels) through the height of the sample at $n = 9$ regions: providing intra-sample strain measurements.

5.3 RESULTS

5.3.1 *Implant Imaging*

All hybrid biomaterial samples, both degradable and non-degradable, were successfully imaged in three-dimensions with micro-CT (Figure 5.2). Figure 5.2 shows that the struts of scaffold samples were clearly defined and deformation during loading was clearly visible. The ZrO_2 particles were distributed well within the solid dual-zone sample and provided contrast within the sample (Figure 5.2, dual-zone).

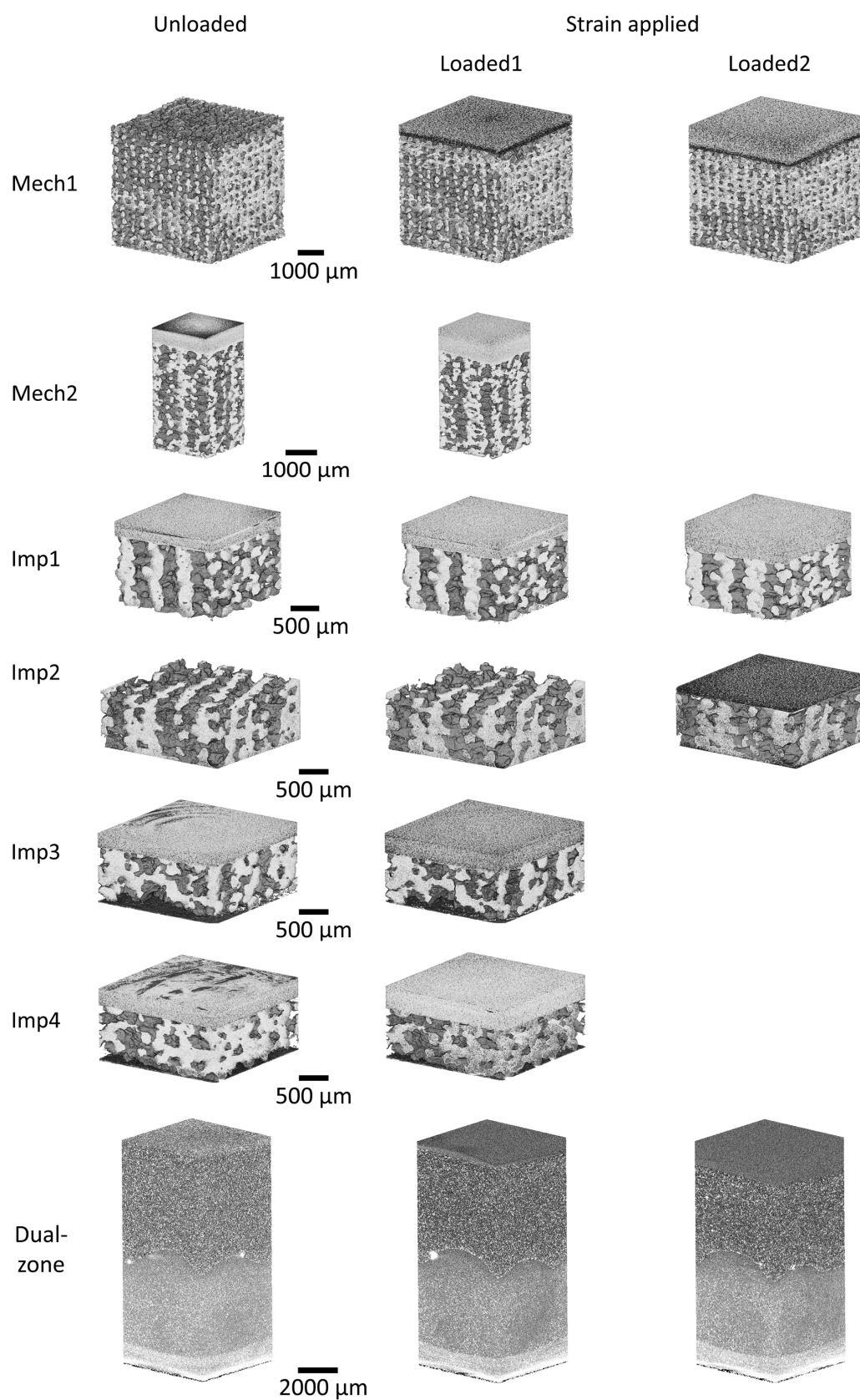


Figure 5.2. Micro-CT imaging of hybrid devices was carried out both unloaded and with mechanical loading applied. “Mech” and “Imp” devices were degradable scaffolds, the “Dual-zone” sample was a non-degradable sample with texture provided by ZrO_2 particles.

5.3.2 Degradable Scaffold Implant Testing

5.3.2.1 Imaging Error Quantification

5.3.2.1.1 Digital Volume Correlation

The magnitude of error reduced with increasing subvolume size, at the expense of worse spatial resolution, for both strain and displacement errors (Figure 5.3a-c). All three approaches followed a power law relationship (Figure 5.3d). The FFT+DC approach and a subvolume size of 48 voxels with 50% overlap gave a spatial resolution of 93-254 μm depending on the sample size and type. This protocol (FFT+DC: 48 pixel subvolume size) resulted in strain systematic error of 0.17% and a strain random error of 0.031%. Displacement random error was 0.97 μm (0.08 – 0.23 of voxel size).

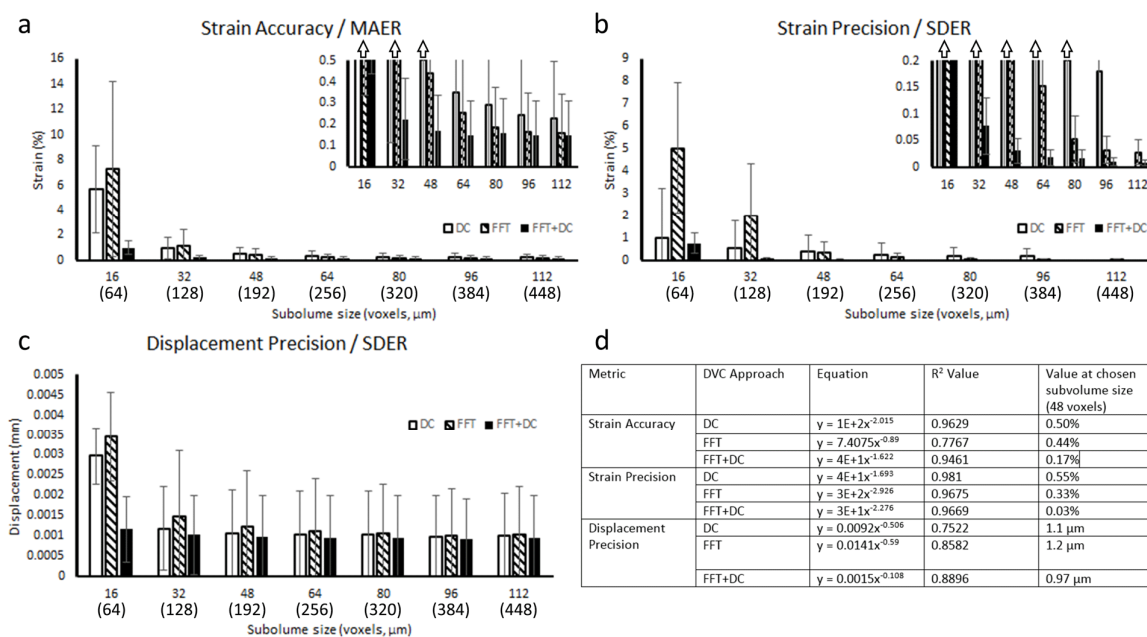


Figure 5.3. Strain systematic error (a) and random error (b) and displacement random error (c) for the hybrid scaffold samples ($n = 6$) for the three DVC approach. DC = Direct Correlation, FFT = Fast Fourier transform, FFT+DC = combined approach of FFT and DC. Inset is the same data with the y-axis scaled to better show values for the larger subvolume sizes. The x axis is labelled in voxels and in brackets is the equivalent approximate micron distance valid for all scaffolds except Mech1. Error bars show the S.D. between all samples. Table (d) shows the power law equations and R^2 values for each metric and DVC method.

Error across each of the six strain components, and three displacement components, is shown in **Figure 5.4**. Both systematic (MAER) and random (SDER) error was highest for the axial (E_{zz}) component.

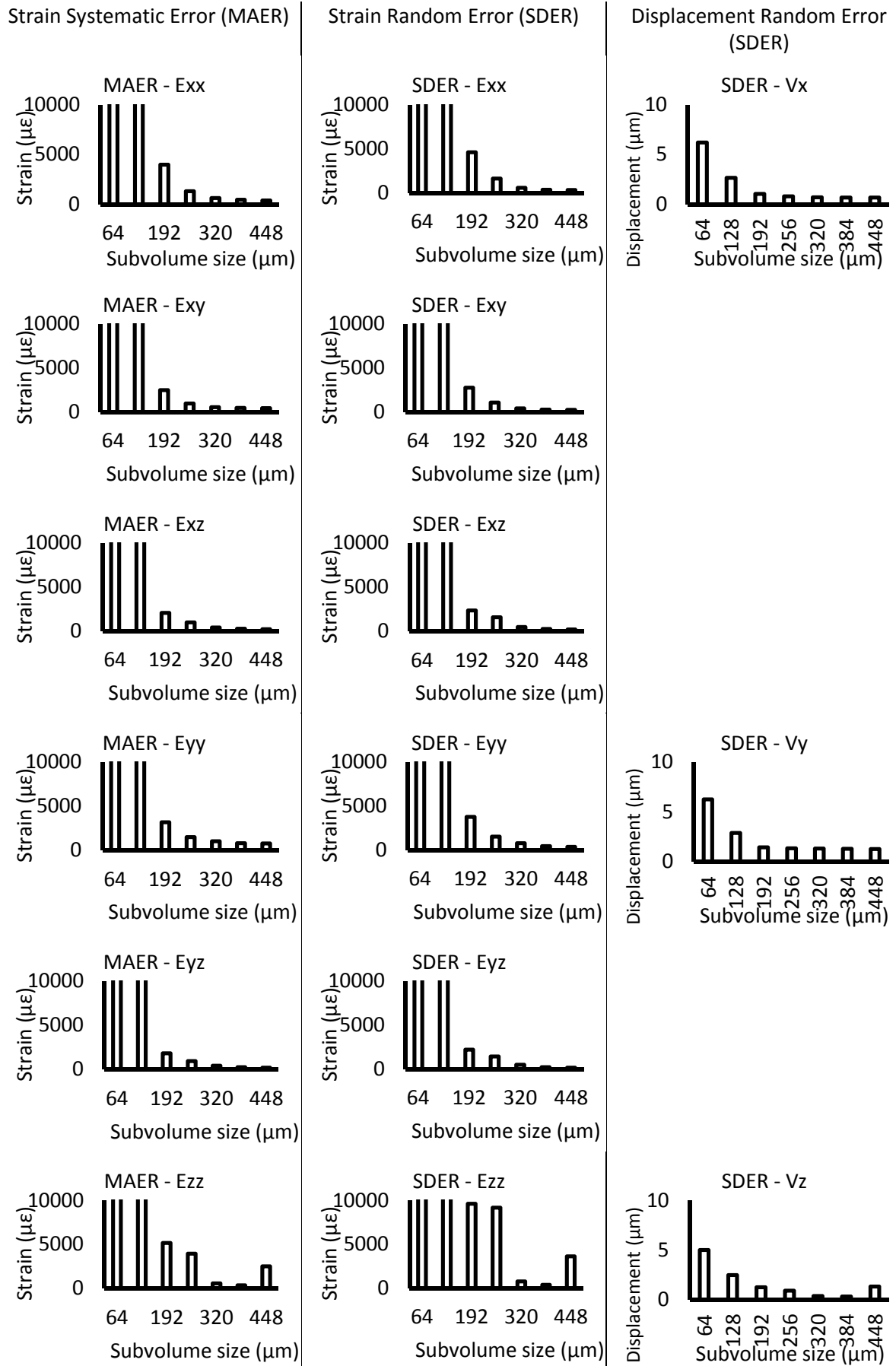


Figure 5.4. Strain and displacement components for each subvolume size tested for sample Mech1 during the constant strain test. Note that bars which continue above the shown range are indicated by omission of the top vertical line.

5.3.2.1.2 Digital Image Correlation

Minimum Pattern Quality

Pattern quality was assessed on the scaffold DIC images ($n = 12$), nominally at a facet size of 25 pixels. Strain systematic error (Figure 5.5a) and random error (Figure 5.5b) appeared to improve with increasing pattern quality threshold levels. The strain systematic error and random error were both worst with a pattern quality level of 0.2, rather than 0. Displacement random error (Figure 5.5c) remained relatively stable throughout the whole measured range. As would be expected the number of valid measurements decreased with higher pattern quality threshold levels (Figure 5.5d). A minimum pattern quality level of 0.4 was chosen for the constant-strain analysis, representing a compromise between the number of valid measurements and the error components.

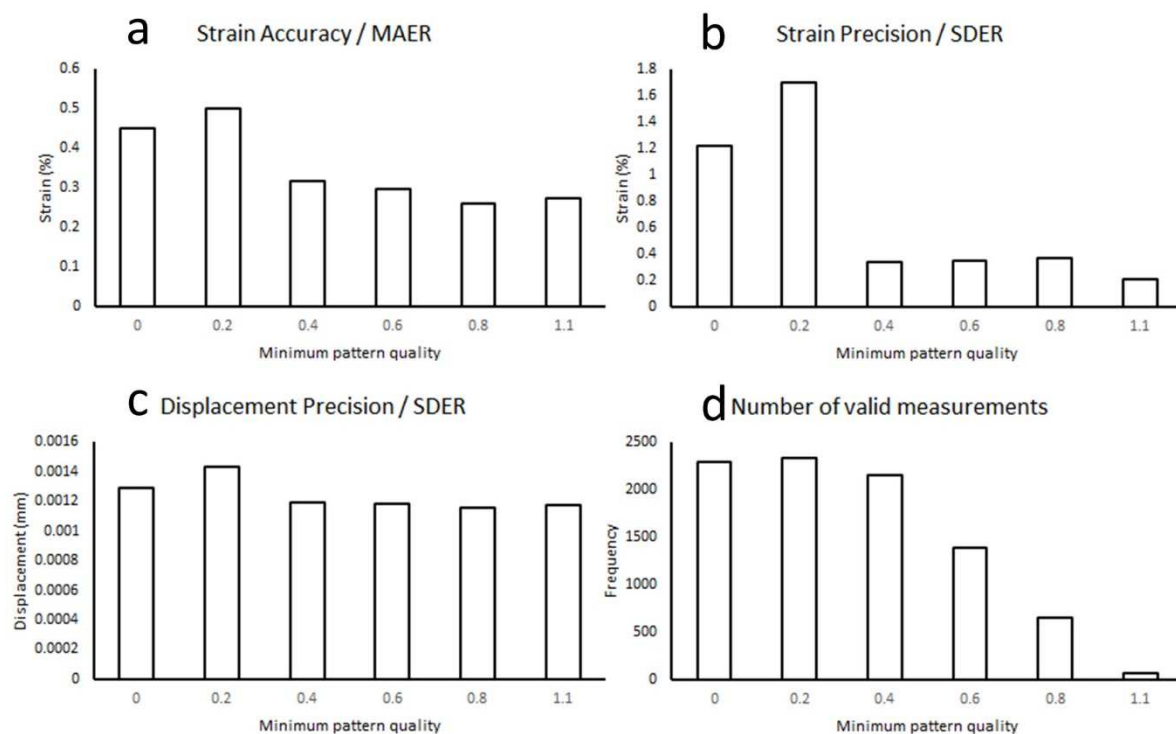


Figure 5.5. DIC analysis was carried out on images ($n = 12$) to determine the required minimum pattern quality. The effects of pattern quality on error components (a-c) and the number of valid measurements (d) were considered. Carried out on $n = 1$ samples.

Constant-Strain Error Analysis

Hybrid degradable scaffold DIC results showed a similar trend to the DVC data: increasing the facet size improved (decreased) the error for both strain systematic error (MAER, Figure 5.6a) and strain random error (SDER, Figure 5.6b). Displacement random error was stable at $11 \mu\text{m}$ ($2.75\times$ pixel size, Figure 5.6c) across the whole range of subsets: representing much higher error in comparison to the imaging resolution when compared to the 3D DVC. A subset size of $192 \mu\text{m}$ (48 pixels) represented a

good compromise between acceptable error and spatial resolution, providing errors of under 0.1 % strain for both systematic error and random error (Figure 5.6d).

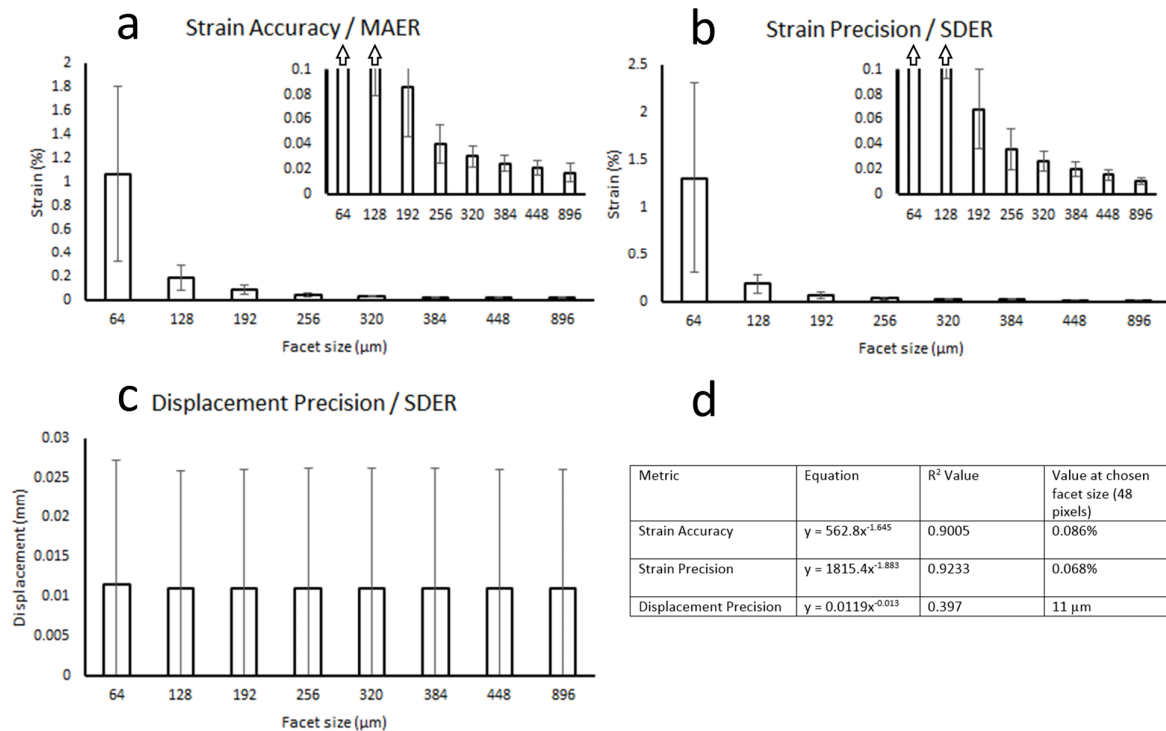


Figure 5.6. DIC analysis carried out on static photos of samples as a comparison to those collected during DVC and micro-CT. Constant-strain error analysis for the hybrid scaffold samples ($n = 6$). a) Strain systematic error (MAER), b) Strain random error (SDER), c) Displacement random error (SDER) and d) Power law equations and R^2 values for each metric. Error bars show the S.D. between all samples.

5.3.2.2 Strain Visualisation

5.3.2.2.1 3D DVC

High resolution micro-CT and DVC enabled three dimensional quantification and visualisation of displacement and strain within the degradable scaffold samples (Figure 5.7). This permitted measurements throughout the volumetric region of interest. Anomalies observed at the external surface, such as the low displacement and strain in the front right-hand edge of the implant (Load1, Figure 5.7), could be compared against internal measurements (Figure 5.8 and Figure 5.9) which avoided these surface defects.

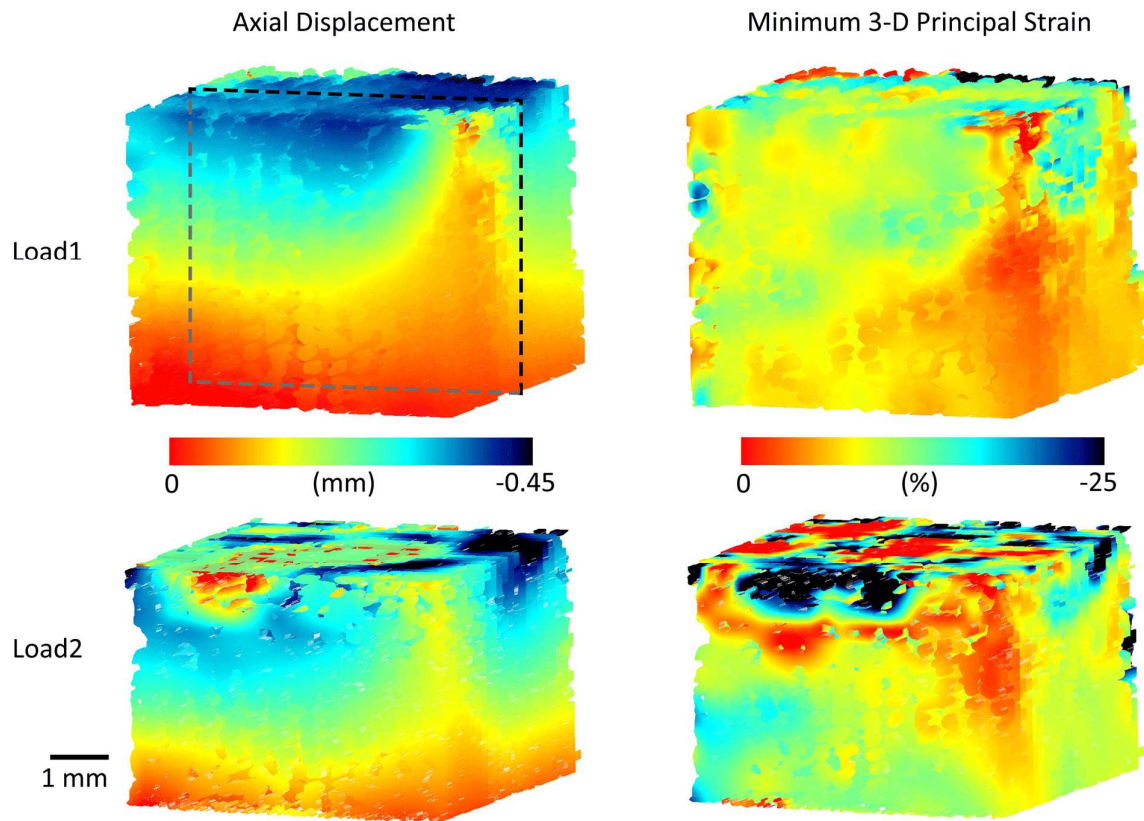


Figure 5.7. Three dimensional visualisation of displacement and strain for a scaffold sample (Mech1) following mechanical loading at two displacement levels using micro-CT, in-situ loading and DVC. The dotted box represents the approximate central region displayed in the following figures of each sample.

For all scaffold samples, intra-implant strain quantification was possible, providing rich 3D visualisation of deformation in different regions of the sample. For example observe the strain concentrations in the lower right-hand region of sample Imp under both loading levels (Imp 1, Minimum Principal Strain plots, Figure 5.8). Load was applied by a platen at the top of the sample, which is where the axial displacement was found to be highest for all implant “Imp” samples with lower magnitude displacement further from the top of the samples (Figure 5.8 Axial displacement column). With appropriate resolution it was possible to visualise deformation at the intra-strut level, for example the strain concentrations across struts near the centre of sample Imp3 (Figure 5.8).

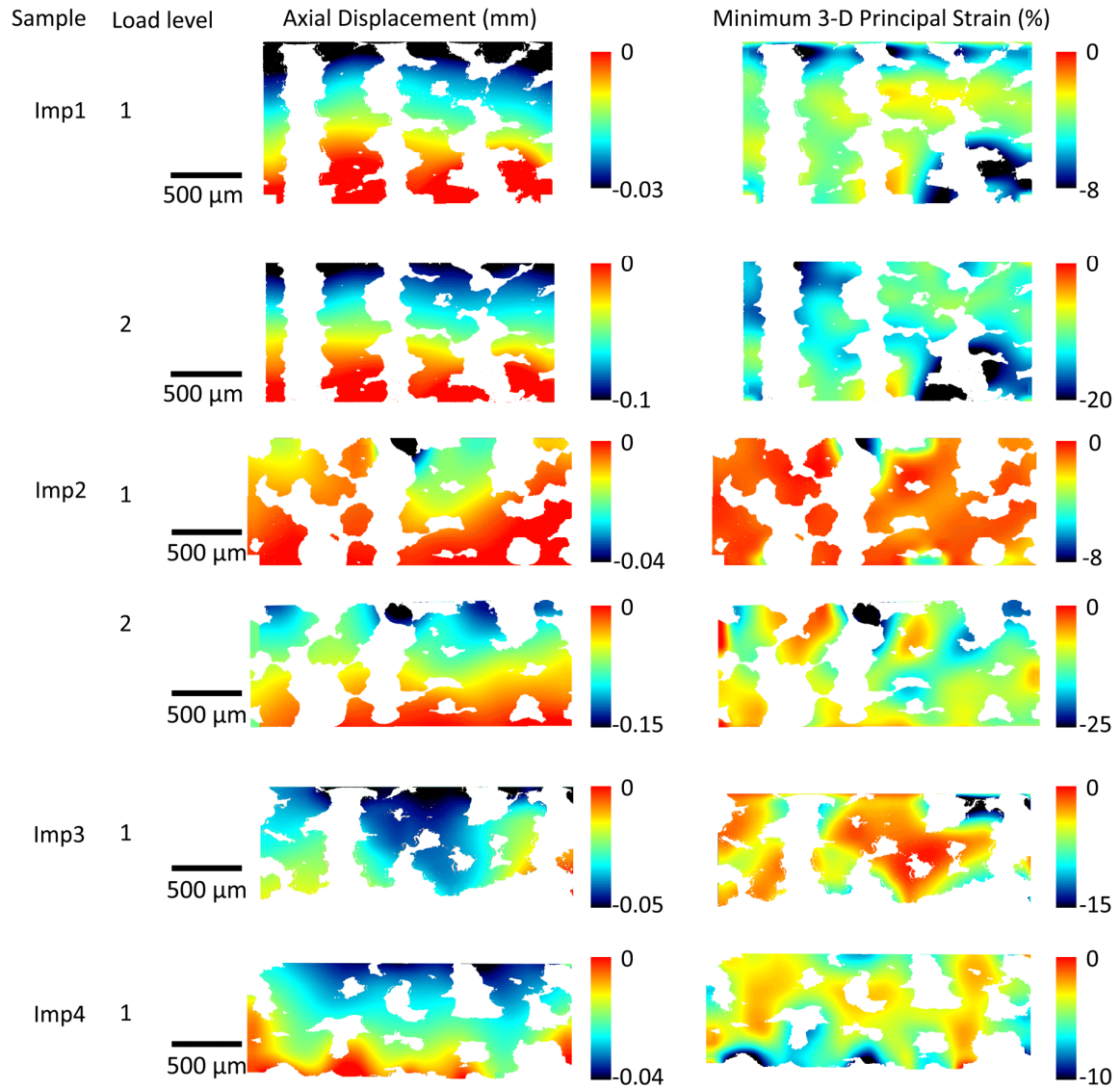


Figure 5.8. Displacement and strain calculated for thin hybrid implants calculated using DVC. Shown here is one vertical slice through the centre of each 3D sample volume.

Imaging of the taller “Mech” samples provided intra-implant strain measurements in 3D (Figure 5.9). The magnitude of displacement was higher than for the implant samples (Figure 5.8), owing to the larger dimensions of the “Mech” samples for mechanical testing. Minimum principal strain magnitude varied across the cross-section of implants (Figure 5.8 and Figure 5.9). For all samples except for Mech2, strain magnitude was highest at either the top or bottom of the sample, though was localised to one region rather than occurring uniformly across the whole surface: for example the top right-hand corner of Mech1 at both loading levels (Figure 5.9).

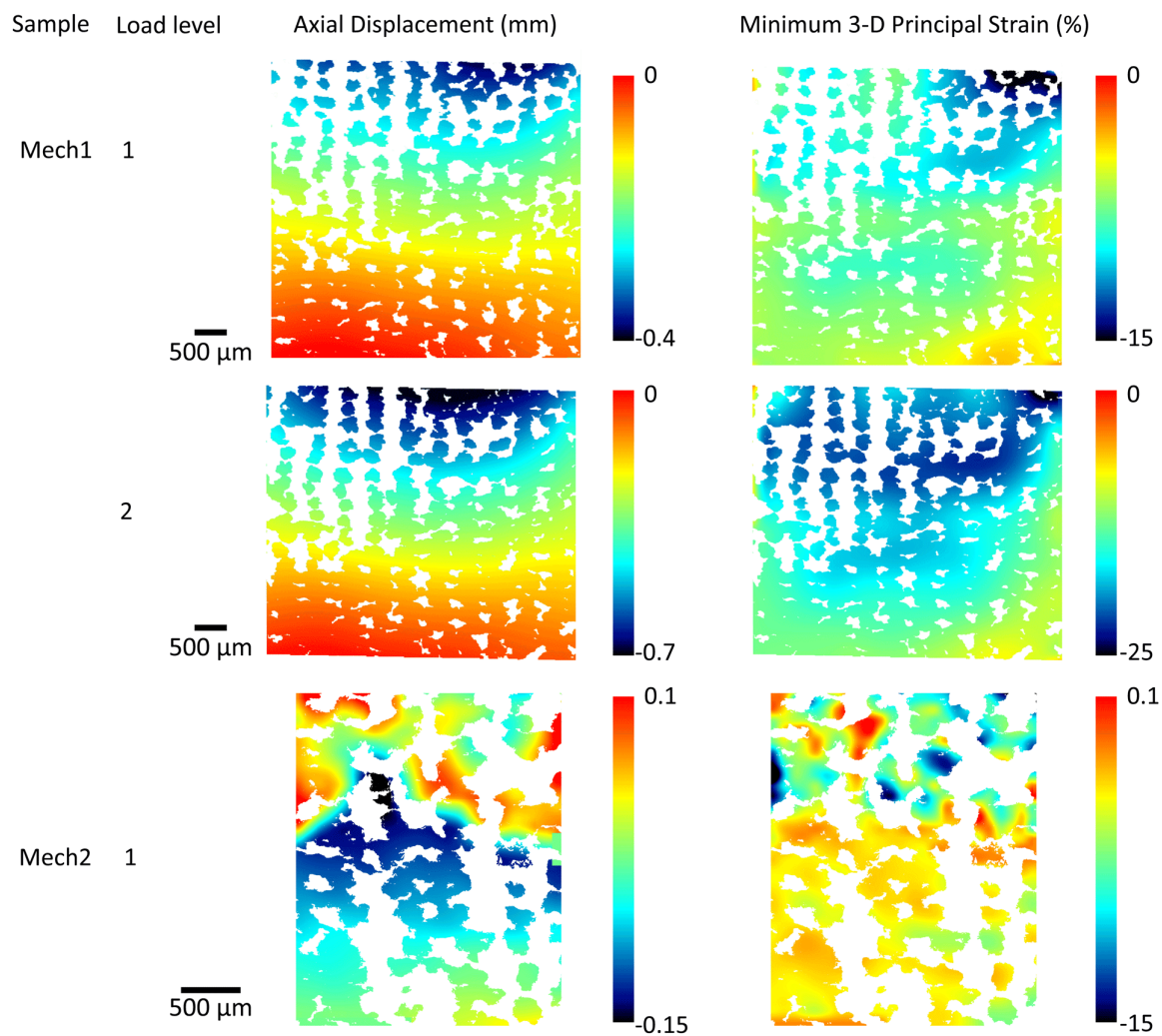


Figure 5.9. Displacement and strain calculated for cubic hybrid implants intended for mechanical testing and device development, calculated using DVC. Shown here is one vertical slice through the centre of each sample. Note that the scales for the Mech2 sample include positive values for both axial displacement and minimum 3D principal strain.

5.3.2.2.2 2D DIC

Visualisation of displacement and strain was successful across the exposed front surface of degradable scaffold implants (Figure 5.10). The displacement and strain plots displayed illustrate what was estimated to be 10% for the bulk sample (calculated with Equation 1), with the true average strain value across the face of each implant provided (Figure 5.10, Axial strain). The facet size of 48 pixels utilised during DIC processing allowed visualisation of localised strain concentrations within each sample. Strain magnitude appeared to be highest at regions close to the periphery of the sample. Please note that not all samples were perfectly cubic due to inconsistencies during the production process. Therefore, as load was initially applied to the samples, the bottom surface of some of them was not fully in contact with the platen until midway through the test: hence the actual strain

measurements are lower than predicted. This effect was successfully visualised and may be observed by comparing the displacement pattern seen for sample DIC_1 in comparison to DIC_5 (Figure 5.10).

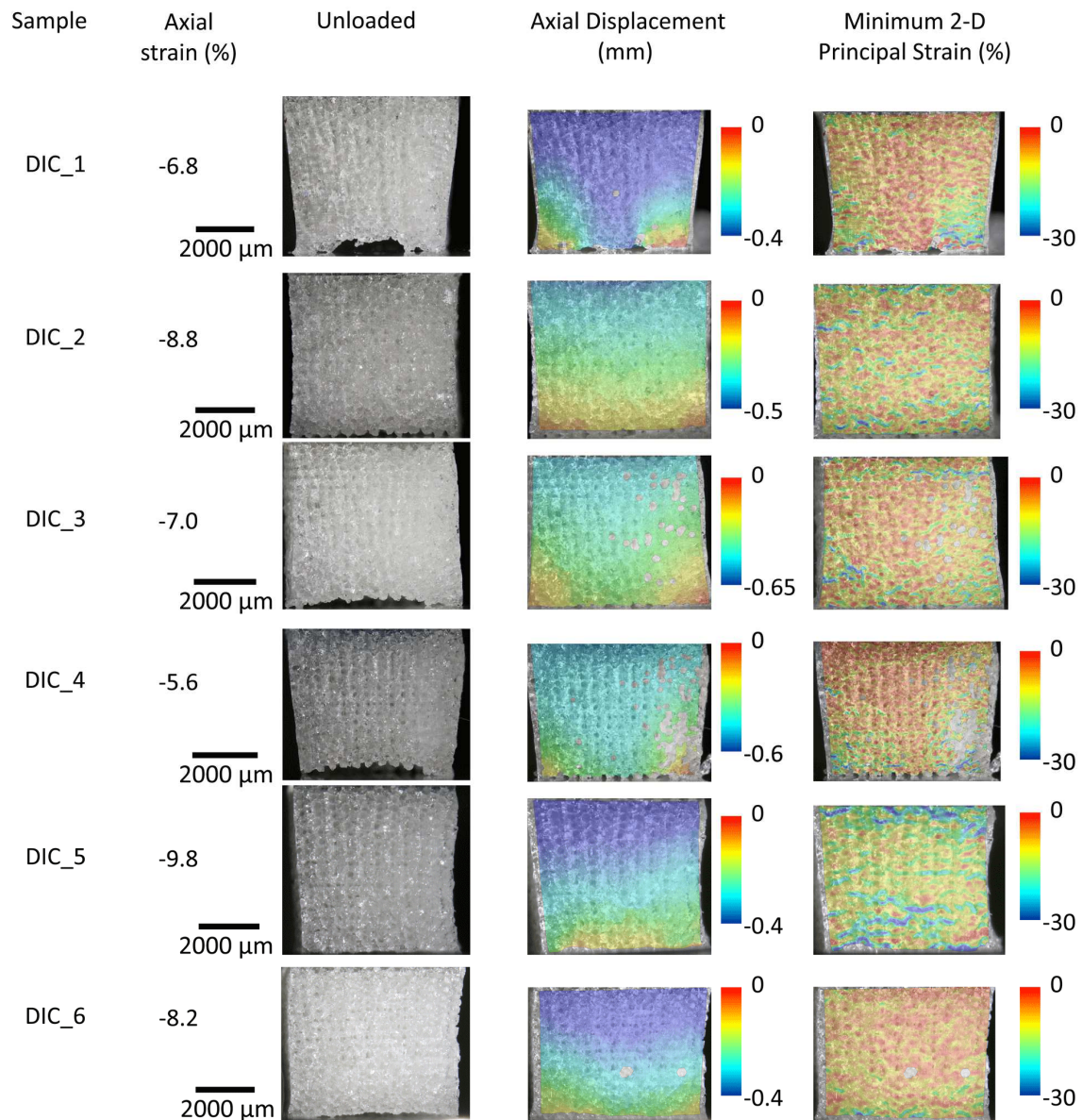


Figure 5.10. Displacement and strain visualisation using 2D DIC on the surface of hybrid scaffolds ($n = 6$), imaged using a digital camera with sequential images collected during laboratory mechanical testing. The axial strain measurements listed are from an image estimated to be at 10% axial strain.

5.3.2.3 Apparent Strain Comparison

Global Strain

Strain values calculated using the correlative software for both DVC and DIC techniques were compared against manually-calculated measurements for degradable scaffold samples (Figure 5.11a,b). A strong correlation was found for both DVC ($R^2 = 0.98$) and DIC ($R^2 = 0.97$), suggesting that

both techniques provide accurate strain measurements. More measurement points are presented for DIC owing to the continuous nature of data capture during the mechanical testing. It should be noted though that the DIC strain values are only of the surface of the implant and were compared only against images of the surface, where the volumetric data collected for DVC was compared against strain values throughout the entire sample volume.

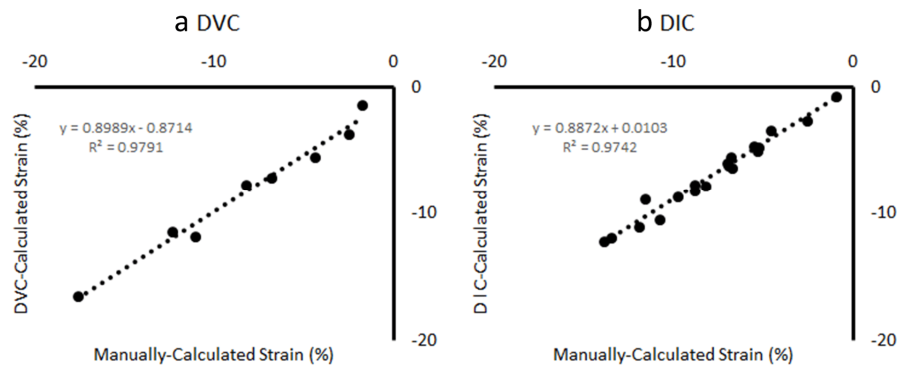


Figure 5.11. Computationally-calculated average global strain values were compared against manually-calculated values for both 3D DVC (a) and 2D DIC techniques (b) using degradable scaffold samples ($n = 6$ for both).

Local Intra-sample Strain

Further to the validation carried out with bulk-strain measurements, DVC measurements were compared against manually-calculated measurements at higher spatial resolution for two strain levels on one degradable sample (Figure 5.12). Anecdotally both methods produced similar strain profiles at both levels of strain. Midway through the height of the sample at the first loading step (Loaded1, Figure 5.12a) there is a deviation of approximately 2% in calculated strain values. At the higher loading level strain values deviation in strain values was under 1% between the two DVC and manually-calculated strains (Figure 5.12b).

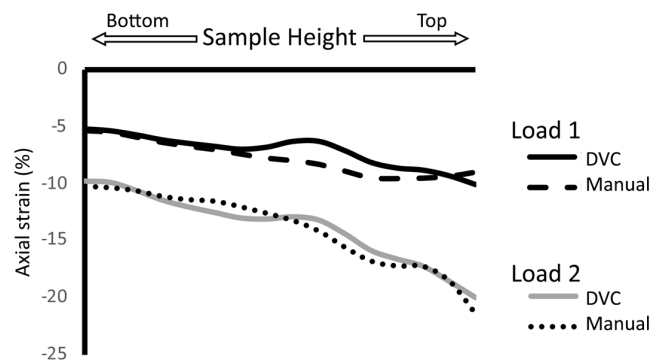


Figure 5.12. Intra-sample strain measurements calculated both using DVC software and manually calculated for one degradable scaffold sample which underwent two levels of compression with in-situ mechanical testing during micro-CT scanning.

5.3.3 Non-Degradable Implant Testing

5.3.3.1 Imaging Error Quantification

5.3.3.1.1 Digital Volume Correlation

The non-degradable dual-zone sample ($n = 1$) was divided in to three regions for analysis: the two zones of different composition and the transitional zone at the interface between these compositions (Figure 5.13a). The subvolume size utilised for analysis of the compression stages was of length 48 voxels (370 μm , Figure 5.13b).

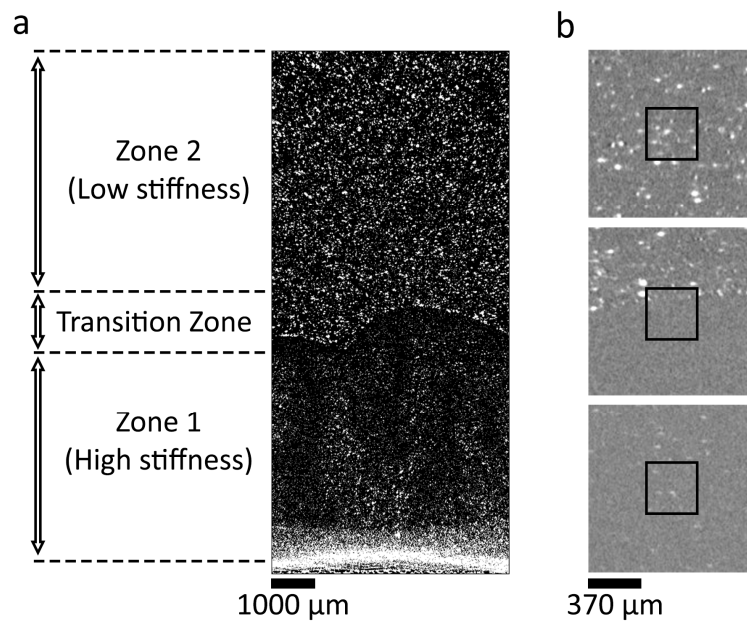


Figure 5.13. Micro-CT imaging of ZrO_2 particles within the solid dual-zone sample provided contrast and a pattern for tracking. The sample was cast with two different compositions of hybrid material, resulting in an in-homogenous mechanical properties (a). The final DVC analysis with compression applied was carried out with a subvolume size of 48 voxels, the size of which in relation to features is shown by the black box (b).

Errors followed the same relationship with subvolume size as observed with the scaffold samples: larger subvolume size resulted in lower error but at the expense of spatial resolution. The FFT+DC approach resulted in the lowest errors and for the same subvolume size as the scaffolds (48 voxels) with 50% overlap, the spatial resolution was 185 μm . Error varied through the height of the sample, strain systematic error was optimum in the transitional zone between the two compositions (0.02%, 197 $\mu\epsilon$, Figure 5.14a), strain and displacement random error were both best in zone 2 (0.006%, 56 $\mu\epsilon$ and 0.47 μm , 0.06x voxel respectively, Figure 5.14b and c).

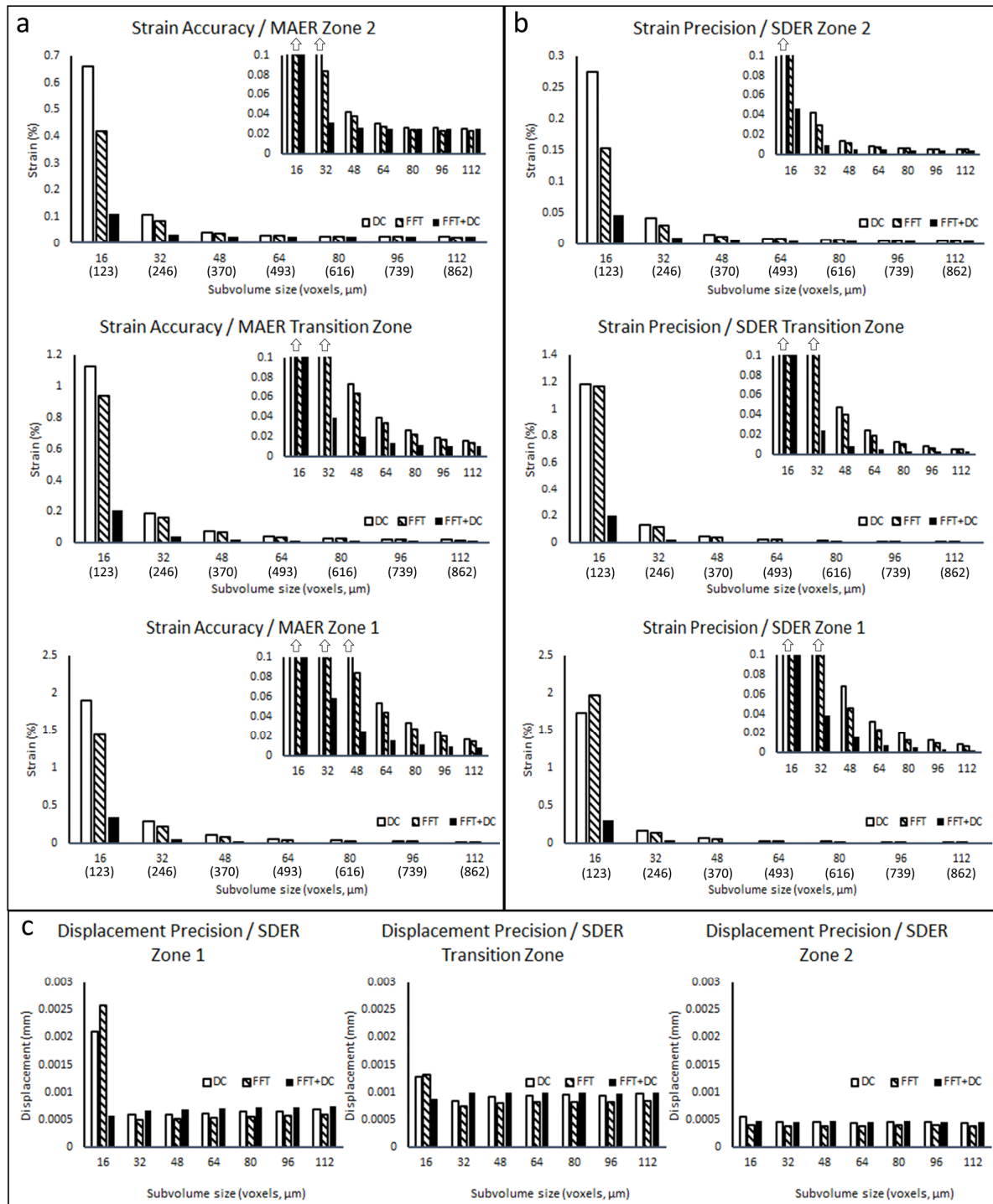


Figure 5.14. Constant-strain study for the dual-zone sample following repeat micro-CT imaging and DVC analysis. Strain systematic error (a), random error (b) and displacement random error (c) were quantified for a range of subvolume sizes in each of the zones of the sample as illustrated in Figure 5.13.

5.3.3.2 Strain Visualisation

5.3.3.2.1 3D DVC

Following static micro-CT of the sample, compression was applied to the dual-zone non-degradable sample at two levels of loading (Figure 5.15b and c). DVC analysis was carried out, providing 3D visualisation of deformation (Figure 5.15d and e). Minimum principal strain showed regions under high compression (negative values) but also high tension (positive values, Figure 5.15f and g). DIC results for the dual-zone non-degradable hybrid are shown in the appendix.

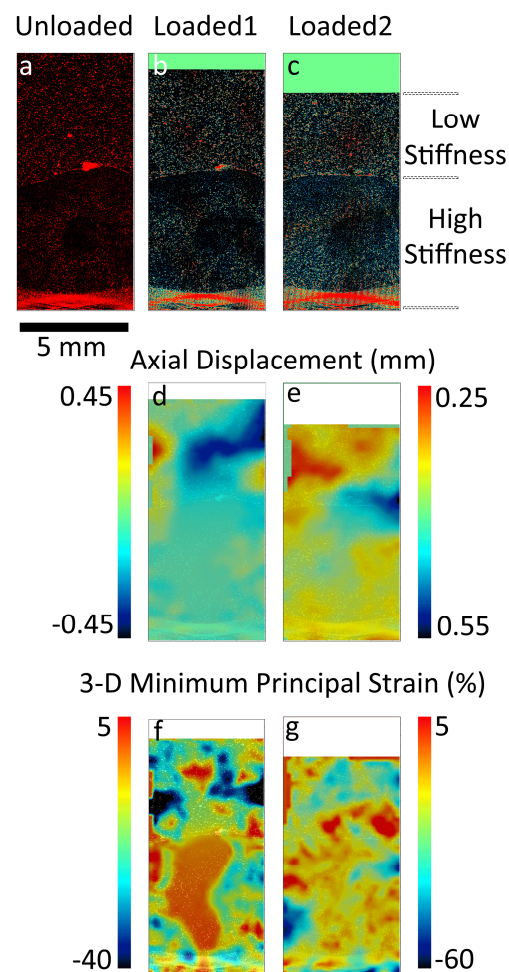


Figure 5.15. Following in-situ compression in combination with micro-CT scanning (a-c), image stacks for the dual-zone sample were analysed with DVC, providing visualisation of displacement (d,e) and strain (f,g) at both compression stages.

5.4 DISCUSSION

5.4.1 Key Message

The most important finding of this work was that three dimensional imaging and DVC permits visualisation of strain within the interior of hybrid implants. This is achieved with systematic error and random error within established acceptable levels for the applied deformation [87]. Features were successfully tracked enabling volumetric visualisation of displacement and strain through the entire sample (Figure 5.7). Visualisation allows comparison of deformation within samples of different morphology and dimensions to aid device design (Figure 5.8 and Figure 5.9). The solid dual-zone hybrid sample was successfully impregnated with ZrO_2 particles dispersed well through the dual-zone sample (Figure 5.13). This provided a pattern for tracking deformation without apparent deterioration of mechanical properties.

Strain concentrations were found to occur at the top or bottom of most of the samples. This was likely caused by the uneven sample height (Figure 5.10, DIC_1) and potentially the effects of friction at the platens which may be mitigated in future experiments with lubrication. DVC constant-strain and loaded results were compared with the more established DIC strain measurement technique. Strain errors for both methods followed power law relationships. The magnitude of error was higher for the DVC technique, potentially due to X-ray interactions causing larger strain errors [65]. Displacement random error was approximately one order of magnitude better in DVC than DIC despite a similar resolution. Both techniques showed similar displacement fields and for both the minimum principal strain appeared to be highest close to the edge of each sample. Care should be taken in interpreting results at the edge of the sample since the transition in pattern quality has been identified as an area of high error [105]. DVC and DIC both provided comparable strain values to manually-calculated strain measurements (both $R^2 > 0.97$). Further validation was successfully carried out for the DVC technique considering intra-sample strain systematic error. Carrying out analysis on further samples is recommended to enable statistical testing to be carried out robustly. DIC sometimes suffered from a loss of pattern tracking, in part due to out of plane movements, which inherently was not encountered with DVC. Unexpectedly, error in the degradable scaffold samples using the DIC technique was higher with a pattern quality of 0.2 than 0 before decreasing for higher pattern quality values (Figure 5.5a-c). Further work should be carried out to evaluate this with a larger sample size. Error within the dual-zone sample was the lowest of all samples and imaging techniques ($< 0.02\%$, Figure 5.14) and for which error was highest in zone 1: likely due to the lower density of ZrO_2 particles (Figure 5.13b) caused as part of the synthesis process. However, the DVC software struggled to successfully track the deformation of the non-degradable implant under loading which was also observed for the Mech2

scaffold sample: confirming that appropriate steps in compression are required for successful tracking. Owing to the combination of low and high stiffness components, the Poisson effect was observed as described in other DVC studies of soft materials [58] and caused out of plane movements which limited the available data collection region for the non-degradable sample with DIC. With further work to vertically optimise the strain response in the hybrid, the material may be a good candidate as a cartilage-regenerative implant.

One of the main advantages of DVC is the potential to consider strains throughout the volume of the sample providing excellent spatial resolution (Table 5.2). DIC may be used as a complimentary technique for its high temporal resolution, allowing fast acquisition at many levels of loading. Both techniques have respective advantages and the author suggests that utilising both when developing implants would provide an excellent suite of mechanical testing capabilities.

Table 5.2. A comparison of the advantages and disadvantages of 3D digital volume correlation (DVC) and 2D digital image correlation (DIC).

DVC		DIC	
Advantages	Disadvantages	Advantages	Disadvantages
High spatial resolution with full- field analysis: 3D & internal features	Requires access to costly equipment therefore usually offers low temporal resolution	Fast data capture allowing high temporal resolution	Out of plane movement artefacts and loss of tracking
Potential for complicated loading scenarios including tissue-implant systems	Caution must be observed, long exposure to X-rays may potentially cause damage	Accessible: low barriers to entry	Surface measurements only

5.4.2 Comparison to Other Studies

DVC studies have previously been conducted on soft materials [58, 228, 229] and scaffolds [76, 97] though none exist on similar hybrid biomaterials. The challenges of DVC have previously been highlighted, some of which were encountered during this study including long scan duration [60]. Following protocol established by other studies, largely in use on micro-CT of bone or bone-biomaterial systems [50, 59, 67, 105], a constant-strain error analysis was conducted. As found by previous studies, in this study error was observed to decrease with increasing subvolume size [63, 68, 230, 231], specifically noted to follow a power law relationship [98, 232]. Error was quantified for both sample types and was compared against the surface strain measurement DIC technique. For both

sample types the FFT+DC DVC calculation technique provided the lowest error. The FFT+DC approach is a unique feature of the LaVision DVC software utilised in this study and its use has not been widely discussed in the literature. Previous studies have not consistently found either FFT [92] or DC [63] approaches were better than each other. Potential causes may be that different methods suit different sample types and imaging modalities in generating the imaging pattern. Further work is encouraged to consider how the different DVC approaches are affected by differences in pattern.

DVC strain error for the scaffold samples using the FFT+DC approach had on average a systematic error of 0.2% and a random error of 0.03% strain with the optimised subvolume size. DVC displacement random error was 1.1 μm (0.09 – 0.26x voxel size), compared with 11 μm (2.75x voxel size) for the DIC analysis. DVC strain systematic error was in fact worse than for DIC, though both were well within acceptable limits. This difference may potentially be due to changes to the samples during the scanning process X-ray interactions causing morphological changes. Further work is encouraged to investigate these effects. For the dual-zone sample type, DVC error varied in the regions of the sample. Strain systematic error was below 0.03% and random error below 0.01% (300 and 100 $\mu\epsilon$) in all regions. Displacement random error was below 0.7 μm in all regions (0.09x voxel size). Previous studies using the addition of small particles to provide a pattern for DVC found displacement errors of 1.3 μm [226]. A potential reason for these results improving on those of the scaffold samples is that the high density of ZrO_2 particles provided a pattern with improved distribution and of smaller size therefore providing more features per subvolume. A large variation in error was observed between samples which has been observed previously [105]. The errors found in this study are within the range observed for bone [71]. It may be possible to reduce these errors further, either by image enhancement via filtering [100], or by using alternative DVC software [59].

5.4.3 Limitations

The sample size used in this study was relatively small, in particular for the non-degradable dual-zone portion of the study. The DVC results compared well with both the DIC analysis, and manual calculations yet future work should include testing of more samples to enable statistical analysis to take place.

Thermal error is a concern for both DIC and DVC techniques [65] yet changes to the sample are a particular concern in DVC and micro-CT due to X-ray exposure. The long data acquisition times required for volumetric imaging currently limit the ability for high temporal resolution during mechanical testing to all but synchrotron facilities. Optimisation is required to find the minimum

acceptable micro-CT to enable such experiments. Error was quantified and remained within acceptable limits for the application yet a useful future study would include successive imaging of a hybrid sample to measure the cumulative effects of X-ray dosage as has been carried out for bone [80].

Micro-CT settings were not kept consistent between samples which was not ideal for drawing comparisons. Future work should ensure that parameters are constant between all scans.

DVC requires tracking of pattern between successively loaded images. Due to limitations in the time provided to access the micro-CT facilities only a small number of loading stages were possible. For the dual-zone sample these loading stages were too far apart to provide successful tracking of features. Future studies should utilise smaller steps in loading to ensure successful tracking is possible.

5.4.4 Conclusions

Micro-CT and DVC have been successfully used to visualise displacement and localised strain concentrations within novel hybrid materials. The technique has been applied to both porous scaffolds, and complex solid multi-zone implants. The results are compared with the established DIC technique and successfully compared with manual calculations. The technique provides a complimentary method for device testing allowing improved design of medical implants.

6 3D MECHANICAL ANALYSIS ACROSS THE OSTEOCHONDRAL – BIOMATERIAL INTERFACE

Following analysis of the hybrid scaffold material in Chapter 5, the following chapter describes implantation of these materials in to an ovine model.

The samples analysed in this chapter were part of a pilot study and the results were fed in to a larger study taking place in February and March 2020.

The foundations of the work described in this chapter were very much collaborative and the author wishes to acknowledge the contributions of many other researchers. The hybrid material and scaffold optimisation were initially developed by Dr Francesca Tallia. All biomaterial implants were produced by Agathe Heyraud who also providing assistance managing the *in vivo* study. Development of the sterilisation protocol benefitted from the input of Dr Silvia Rodrigues Mendes Ferreira. Laboratory testing for development of the surgical technique was carried out by Agathe Heyraud, Mr Talal Al-Jabri and the author. Ovine surgery was carried out by Mr Talal Al-Jabri and Prof Gordon Blunn (University of Portsmouth) at the Royal Veterinary College. Assistance at the micro-CT imaging facility was provided by Brett Clark (Natural History Museum). Assistance optimising the DVC protocol was provided by Saman Tavana.

The author carried out all micro-CT imaging, *in-situ* testing, 3D image analysis and DVC analysis.

6.1 INTRODUCTION

Osteochondral injuries are increasingly prevalent and current early-stage surgical interventions do not provide satisfactory long-term outcomes to critically-sized defects [34, 233]. The problem is chiefly an inability to effectively regenerate articular cartilage [19] and the demand for osteochondral implants with tissue-regenerative capabilities has never been stronger. Devices are constantly being developed in an effort to address this currently unmet need with varying degrees of success [24, 25]. Effective design requires intimate knowledge of the local tissue environment and matching of these mechanical properties with the intended device [234]. Upon implantation, device suitability and long term success relies upon suitable interaction of the tissue and implant components as a complete system. For example, a strain mismatch may lead to progenitor cells not differentiating into the intended cell lineage [31, 32] and failure may occur if micromotion of the implant is above 40 – 150 μm [46, 235].

The development and optimisation of orthopaedic devices inevitably involves the use of animal models, especially on the pathway to regulatory approval. It is imperative to gather as much information as possible from these studies, both to test the product with maximum scrutiny, and to limit the number of animals utilised, due to financial and ethical considerations. In addition to scoring systems of the defect site, retrieved explants will be analysed under histological analysis to determine the type and quality of tissue formed [24]. Histology is time consuming and destructive and only small areas of 2D slices can be viewed at one time, under high magnification. With the advent of high resolution three dimensional imaging techniques, such as micro-computed tomography (micro-CT), has come the potential to carry out volumetric morphological analysis as a complimentary technique to histology. Beyond passive morphometric analysis, micro-CT imaging and analysis has now progressed to quantitative mechanical testing with the pairing of *in-situ* loading and digital volume correlation (DVC). This has provided the opportunity to characterise strains across the osteochondral interface [87] and examine *ex-vivo* explants to quantify bone-implant micromotion [53], bone cement implantation [59] and cementless implant press-fitting [69]. Following sample retrieval, *ex-vivo* analysis has taken place on bone-biomaterial systems for tissue regeneration [52], the stability of bone-screw systems [102] and newly formed bone tissue [85]. No studies thus far have considered DVC analysis of tissue-biomaterial systems aiming for articular cartilage regeneration.

This study aims to investigate the potential for three dimensional evaluation of strain and micromotion within osteochondral tissue-biomaterial systems. This was carried out using an ovine model. Analysis was carried out after six weeks of implantation using *in-situ* micro-CT mechanical testing and DVC on an explanted tissue-biomaterial sample including newly formed tissue ($n = 1$). Static micro-CT images were collected for all samples involved in the ovine study ($n = 6$). The limited sample size ($n = 1$) makes this an exploratory study. Success would provide a non-destructive

understanding of mechanical conditions within an osteochondral tissue-implant system across the interface with newly formed tissue and may enable development of successful osteochondral regeneration technologies to reduce osteoarthritis prevalence.

6.2 METHODS

6.2.1 Implant Production

Scaffold implants composed of a SiO_2 -PTHF-PCL hybrid material [41] were extrusion 3D printed in a repeating structure with struts of approximately 200 μm diameter and 200 μm spacing (Figure 6.1a). A second variety of implants were produced with the addition of a solid “cap” of the same material to act as a low-friction bearing surface to reduce tribological wear of the surrounding and opposing cartilage (Figure 6.1b). The cap was first made by pouring the liquid hybrid sol into a smooth mould and allowing it to partially gel. The scaffold part was then 3D printed and the two were joined before gelation was complete, allowing bonding of the two components. Following manufacture implants were washed twice in deionised water, cut to 6 mm diameter (hollow punch, Boehm, Germany), washed again in deionised water and then sterilised by 50 kGy gamma irradiation (King’s College London).

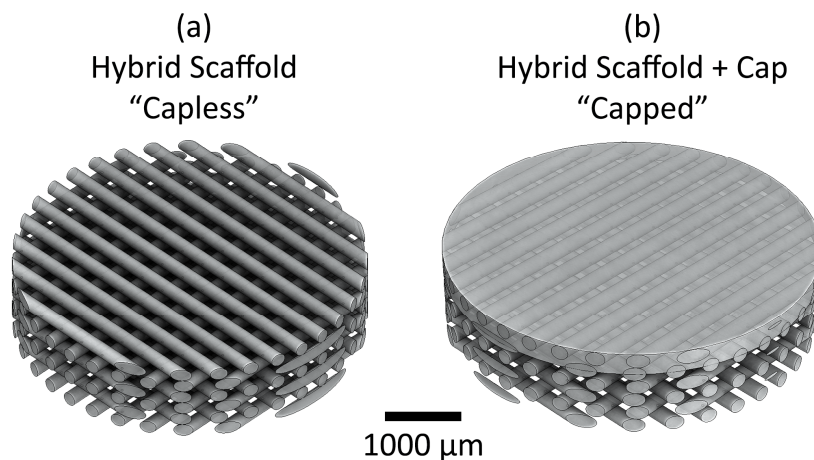


Figure 6.1. A schematic representation of the two hybrid implant designs a) 3D printed scaffold b) 3D printed scaffold with the addition of a dense low-friction cap of the same material to reduce wear to the opposing cartilage surface.

6.2.2 Surgery

Following ethical approval in compliance with Home Office regulations, three mature female sheep of age 24 - 48 months were anaesthetised and full-chondral defects were created in a load-bearing region in both femoral condyles of the left hind stifle joint using a 5 mm diameter biopsy punch (5 mm Disposable Biopsy Punch, Kai Medical, Japan). Access to the second condyle in each case was provided by dislocation of the patella. A 1.1 mm K-wire was drilled into the subchondral bone at the centre of the defect to release stem cells and to act as a guide to ream liberated cartilage from the defect (5 mm reamer with electric drill, Bosch, Germany). A micro curette (Arthrex, Germany) was utilised to ensure the defect perimeter was not fibrillated. Implants were cut to size during surgery as required to fit the defects, either using a 5 mm diameter biopsy punch (5 mm Disposable Biopsy Punch, Kai Medical, Japan) or manually using a scalpel (No.11 blade, Swann-Morton, UK). Three treatments (n = 2) were trialled: 1) scaffold only ("Capless", Figure 6.2a), 2) scaffold with cap ("Capped", Figure 6.2b), 3) scaffold without a cap but with a periosteal flap ("Capless with flap", Figure 6.2c). Each implant was placed in to the defect and secured to the surrounding cartilage with cyanoacrylate glue (derma+flex, Chemence Medical, USA) at four points on the perimeter of the top of the implant. Periosteal tissue was harvested from the periphery of the medial femoral condyle using a scalpel (No.11 blade, Swann-Morton, UK) and secured again with the cyanoacrylate glue. Implant placement was randomised between the sites (Table 6.1). Prior to suturing, the joint was articulated through ten flexion/extension cycles to ensure the implant was secure and to provide flow of synovial fluid. The incision was sutured and the animal transferred to a recovery area.

After six weeks, tissue-implant samples of 15-20 mm diameter and 5-10 mm height were extracted with an oscillating saw and transferred to PBS (DPBS, #14190-094, Fisher Scientific, USA) with antibiotics and antimicrobials. Samples from equivalent regions of non-operated joints were extracted for histological comparison. Images were taken of all samples with an optical microscope (RH-2000, HIROX, Japan).

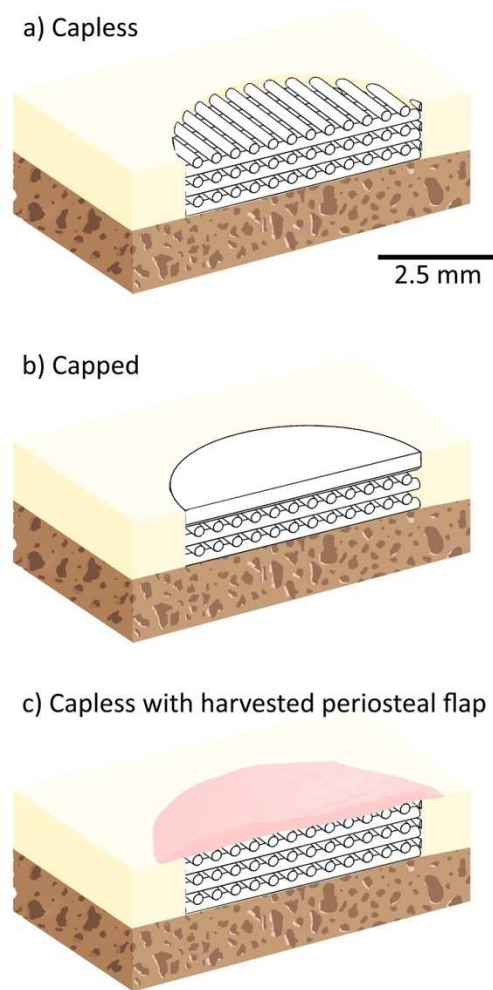


Figure 6.2. The surgical plan comprised of chondral defects created in femoral condyles of three sheep (total $n = 6$ defects), trialing three interventions (each $n = 2$): a) “Capless” hybrid scaffold, b) “Capped” hybrid implant, c) Capless implant with a periosteal flap “Capless with flap”. Shown here is a schematic representing a crosssection through the centre of each scaffold, with the implant resting on the subchondral bone.

Table 6.1. Device implantation of the three different interventions. All six samples underwent micro-CT scanning in PBS. The two samples from Sheep A were retained for further micro-CT analysis and “Capped1” underwent in-situ mechanical testing*. All four samples from Sheep B and C were processed for histological analysis.

Sheep	Medial femoral condyle	Lateral femoral condyle
A	1. Capless1	2. Capped1*
B	3. Capless with flap1	4. Capped2
C	5. Capless with flap2	6. Capless2

6.2.3 Micro-CT

All explants ($n = 6$) were micro-CT scanned in PBS on the day of retrieval using a Versa 520 micro-CT scanner (Zeiss, Germany) with coarse settings to provide a rapid evaluation of the defect site (Table

6.2). Each projection was of 2048 x 2048 resolution with no binning or filtering applied. Both implants from sheep A (Capless1 and Capped1) were retained for further imaging whilst four of the samples were then transferred for histological analysis. The two retained samples were stained in 1% w/v phosphotungstic acid (weight/volume, H₃PO₁₂O₄₀, PTA, #79690 Sigma-Aldrich, USA) solution in 70% ethanol for 21 hours following established protocol [209]. *In-situ* mechanical testing was performed on the Capped1 sample, with two unloaded scans taken consecutively. Further scans were taken at two levels of applied compression after 30 minutes to ensure the sample was thermally and mechanically stable. Samples were kept in liquid at all times, including during scanning.

Table 6.2. Micro-CT parameters for scanning ex-vivo samples. A coarse scan was taken of all samples (n = 6). *In-situ* testing was then carried on the Capped1 sample with scans taken under successive load to permit study of deformation mechanics using DVC.

Scan Protocol	Sample	Voltage	Current	Voxel size (μm)	Number of projections	Exposure time per projection (s)
Preliminary	All (n = 6)	80	87.5	10.2	2401	5
DVC study	Capped1 (n = 1)	60	83	4.5	1201	16

Reconstruction of the projection images to produce 3D volumetric data sets was performed using the Reconstructor Scout-and-Scan software (Zeiss, Germany). The reconstructed CT volumes were visualized and analysed using (Fiji Is Just) ImageJ software [170] (version 1.52g, NIH, USA).

6.2.4 DVC

As described in previous chapters, constant-strain error analysis was carried out on the repeat unloaded scans of sample Capped1. Within Fiji, two image stacks of dimensions 450 x 450 x 450 voxels were extracted to include all constituent parts of the tissue-biomaterial system (Figure 6.3). Images were imported in to DaVis version 8.4.0 (LaVision, Germany) and the constant-strain study was conducted in the usual manner: subvolume sizes from 16 – 112 voxels (~30 – 205 μm) were considered in increments of 16 for all three DVC processing methods: 1) Direct correlation (DC) 2) Fast-Fourier transform (FFT) and finally a combination of both 3) FFT + DC. For FFT+DC, the FFT pre-shift was set at 12 pixels larger than the DC step and three calculation passes were utilised. Error was quantified by calculating the mean absolute error (MAER), representing the systematic error or systematic error, and the standard deviation of error (SDER), representing the random error or random error inherent within the system [67]. MAER and SDER of strain were calculated through the cartilage using a custom script in MATLAB (R2019a, The MathWorks Inc, USA). The random error was also calculated for the

displacement components. The systematic error of displacement was not calculated because the real displacement is unknown.

Following optimisation of the DVC calculation technique and subvolume size using the error quantification, the unloaded-loaded1-loaded2 image stacks were processed in accordance to the maximum acceptable error. This was carried out with two volumes of interest: firstly on a region at the edge of the defect of dimensions 821x821x401 voxels, and then on the whole sample with dimensions 2048x2008x587 voxels. For the latter, greyscale values below 1 were algorithmically masked: removing black voxels outside of the volume of interest. Typically, the maximum acceptable error is considered to be 10% of the nominal strain [87]. For this osteochondral system the highest nominal strain applied was approximately 2.5% therefore the maximum acceptable error was set as 0.25% strain (2500 $\mu\epsilon$). Using this threshold the compression DVC study was carried out using the FFT+DC method and a subvolume size of 80 voxels, providing a spatial resolution of 181 μm .

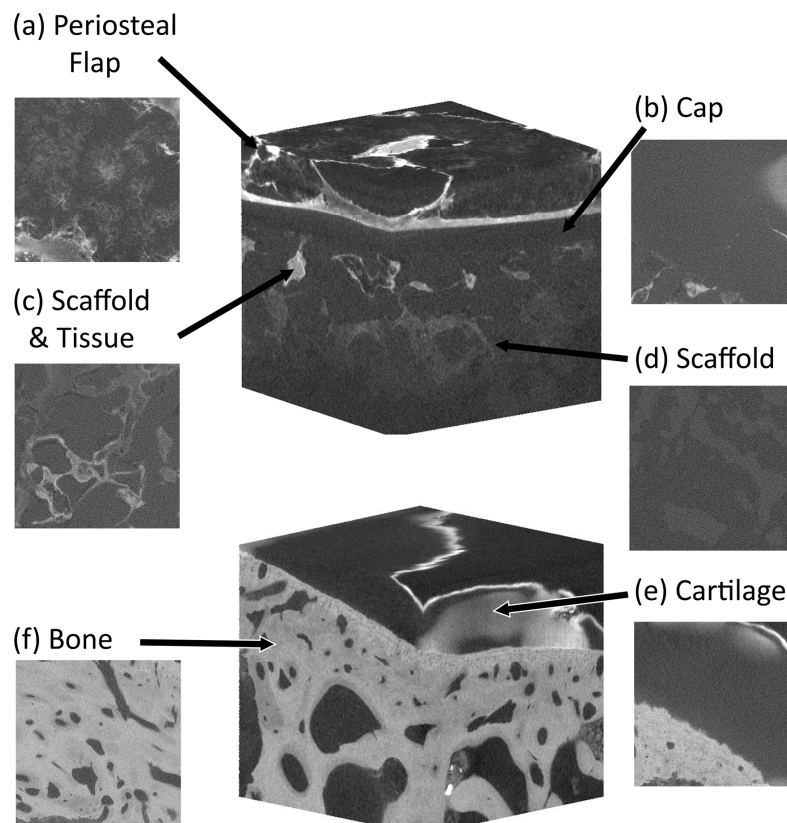


Figure 6.3. Micro-CT image of an ex-vivo sample; the intervention components (a-d) and local tissue (e and f) were analysed with DVC software between two repeat unloaded micro-CT scans with no load applied. Three dimensional micro-CT imaging error was calculated for sample “Capped1” by way of a constant-strain study for error analysis on all components of the tissue-biomaterial system. No image processing has been applied. Please note that due to morphology, the cartilage volume of interest (e) included a small region exterior to the tissue composed of PBS.

6.3 RESULTS

6.3.1 Surgical Procedure

Defects were created and devices were successfully implanted (Figure 6.4a). After six weeks the implants were retrieved. The implants were visible within some of the defects (Capped1, Figure 6.4b) whilst others were covered in a layer of biological tissue (Capless2, Figure 6.4b). Optical microscopy was carried on the *ex-vivo* samples, showing status of the exposed articular surface at a higher magnification (Figure 6.4c) and micro-CT allowed visual interrogation of the whole 3D explant (Figure 6.4d).

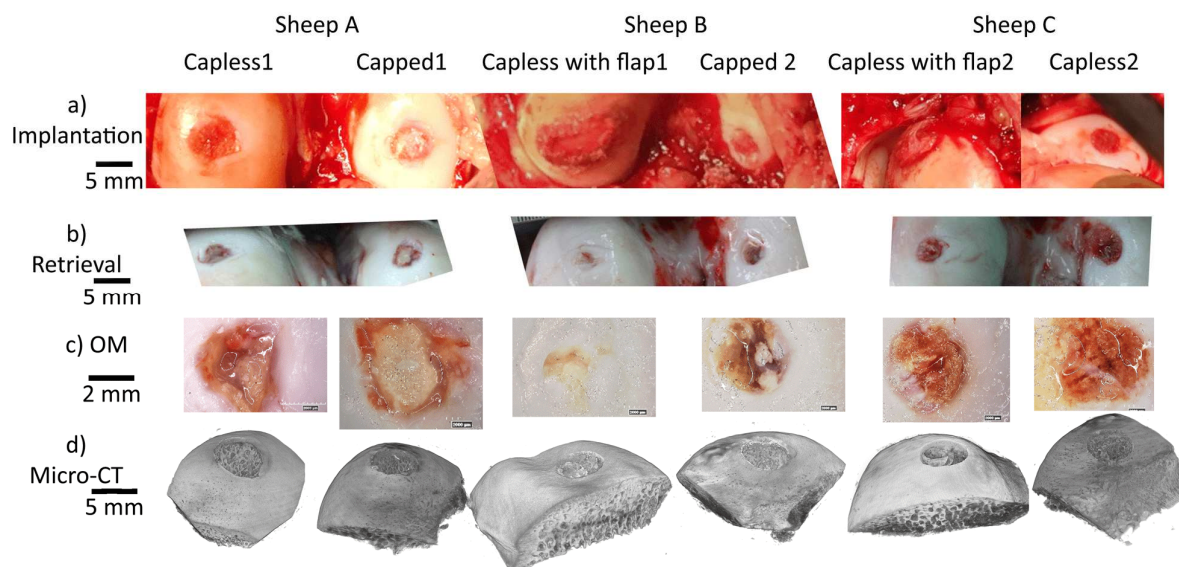


Figure 6.4. Samples were implanted into 5 mm defects within the medial condyles in three sheep, a photograph showing the surgical site at implantation (a). After six weeks the study was concluded and a photograph was taken of the surgical site (b). Explants were retrieved of the implant and surrounding cartilage and bone tissue, optical microscopy of the implant articular surface (c) and micro-CT of the entire *ex-vivo* sample (d).

Defect sites were visualised non-destructively in 2D with optical microscopy (Figure 6.5a and b) and in 3D with micro-CT (Figure 6.5c-e). Features were located between both imaging modalities (Figure 6.5b,e*). The surgical procedure was intended to create a full-thickness defect in the cartilage, to the level of the subchondral bone. Analysis of micro-CT established that the defect was drilled in to the underlying bone, to a depth of approximately 3-3.5 mm deeper than intended (Figure 6.5e). The implants were therefore seated within a void surrounded by bone.

The morphology of the created defects were confirmed for all six implant sites (Figure 6.6), verifying that the implants were seated within the subchondral bone rather the articular cartilage.

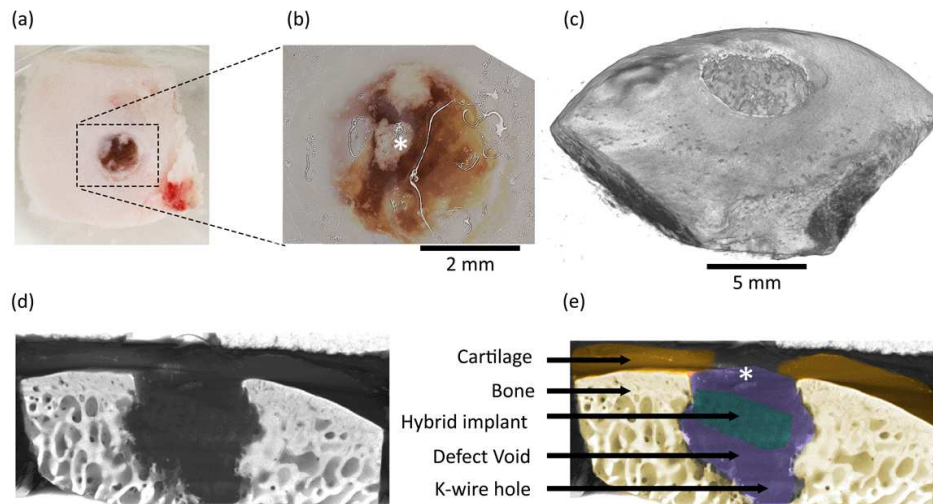


Figure 6.5. Imaging was carried out on all samples ($n = 6$). a) optical microscopy of Capped2) and b) at higher magnification, showing growth of tissue denoted by *. c) 3D micro-CT of the sample, with no staining applied d) A transverse slice through the micro-CT volume and the same slice was annotated with each of the tissue-biomaterial components (e). * Denotes the tissue visible in both optical microscopy (b) and micro-CT (e). The scale of all micro-CT images (c-e) was as displayed for panel c.

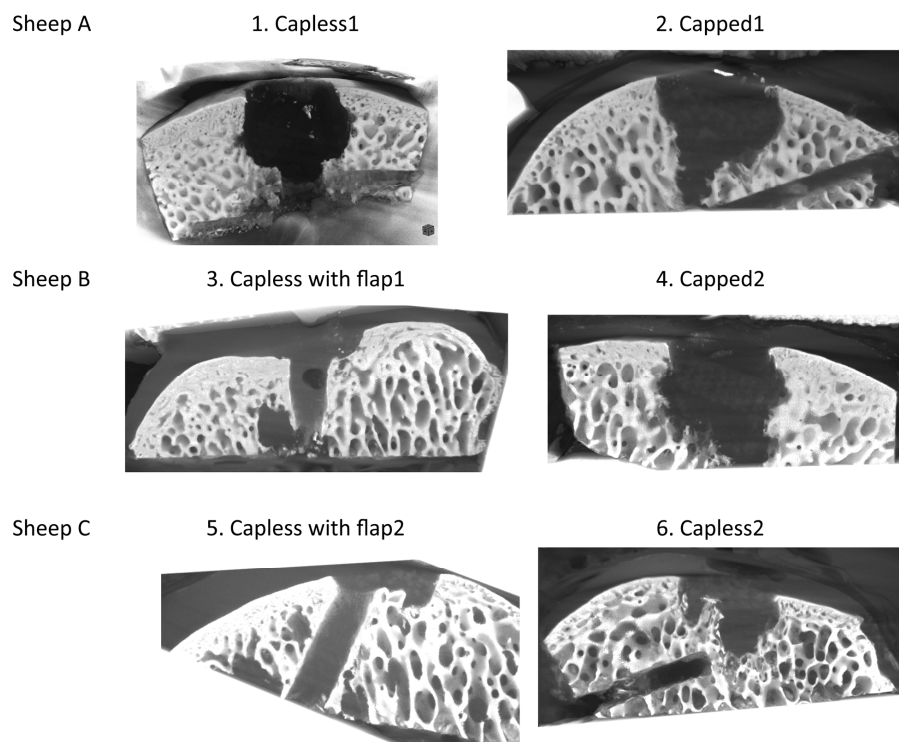


Figure 6.6. Non-destructive 3D micro-CT was performed for all six implant sites. A transverse slice through the 3D micro-CT volume for all six samples with no staining applied, illustrating the depth of the defects created. The defects were created deeper than planned, and the implant is visible within the bone defects for some of the explants, for example Capped1 and Capped2.

6.3.2 Error Quantification

Error was quantified for all tissue (Figure 6.7) and biomaterial (Figure 6.8) components of the system across a range of subvolume sizes and for the three DVC methods available. These have been summarised for the chosen subvolume size of 80 voxels using the FFT+DC DVC method (Figure 6.9). Both strain errors were below 0.1% strain for all components except for the cartilage which incurred a strain systematic error of 0.12% strain. Displacement error for all components was below 8 μm .

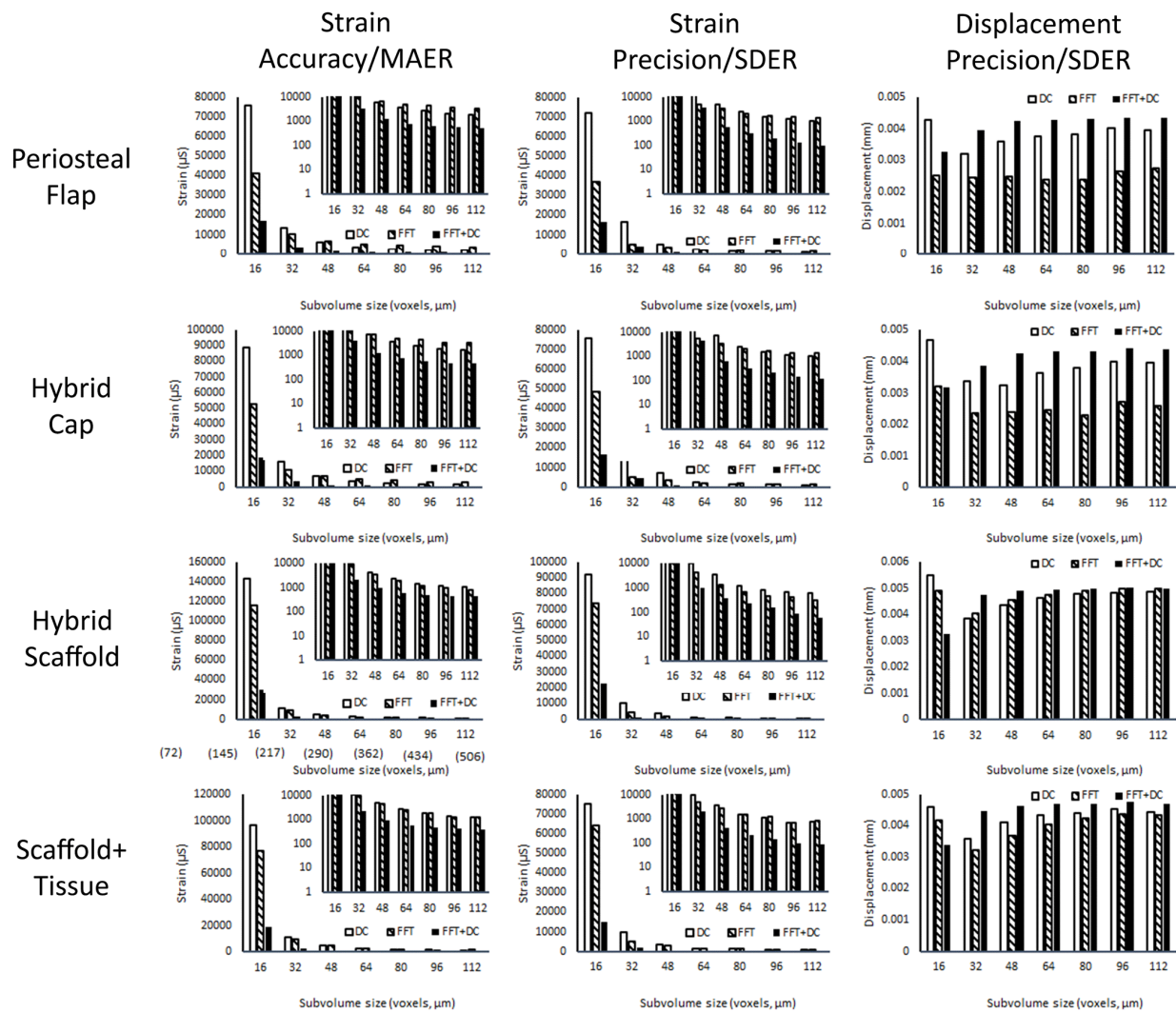


Figure 6.7. Strain and displacement errors, across a range of subvolume sizes, for each of the tissue components. Please note the logarithmic scale for the inset panels.

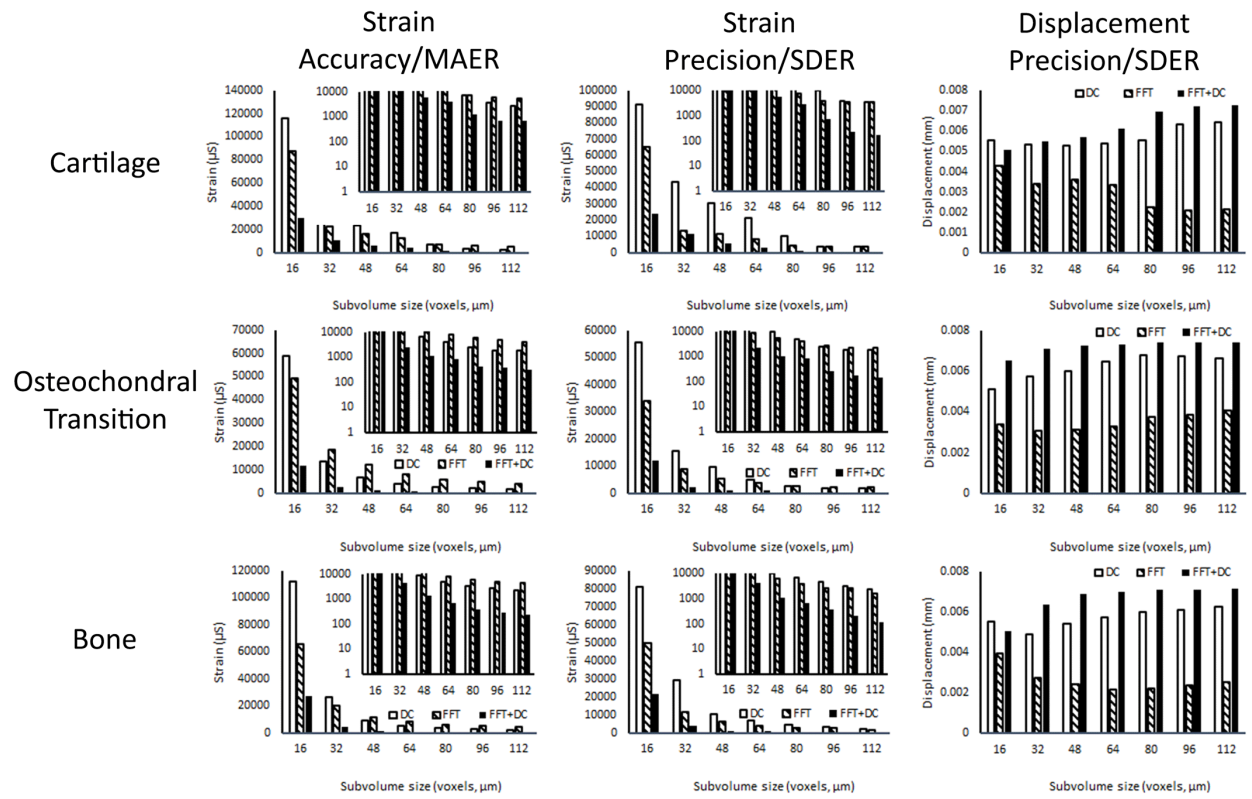


Figure 6.8. Strain and displacement errors, across a range of subvolume sizes, for each of the implant components. Please note the logarithmic scale for the inset panels.

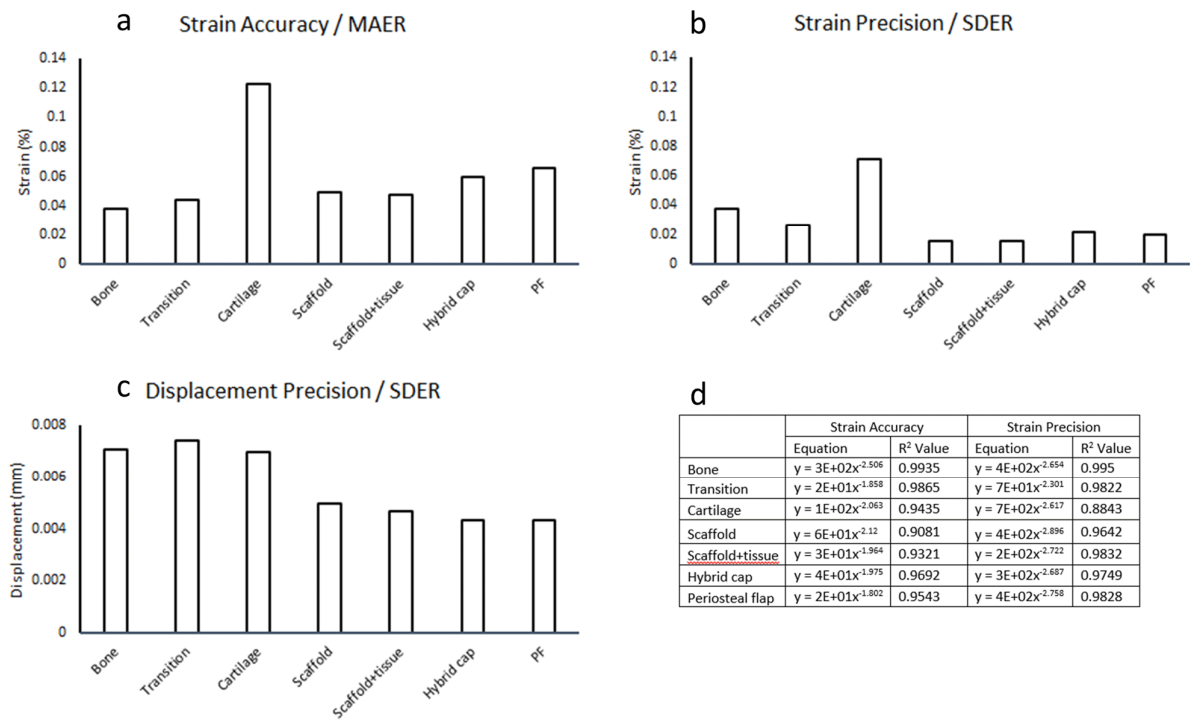


Figure 6.9. Quantification of error for seven components of the tissue-biomaterial system for sample Capped1 ($n = 1$) at a subvolume size of 80 voxels (360 μm) using the FFT+DC method: a) Strain systematic error b) Strain random error c) Displacement random error d) Power law relationships computed for the FFT+DC method across all tested subvolume sizes for the system component. PF = Periosteal Flap.

6.3.3 Deformation Visualisation

Load was successfully applied between successive scans of sample “Capped1” (Figure 6.10) by way of the *in-situ* mechanical testing rig during micro-CT scanning. The topography of the bone created an uneven bone-cartilage interface height which impeded compression of the cartilage (Figure 6.10a *). The placement of the implant, at the level of the subchondral bone, did not permit compression of the implant-cartilage interface as planned (Figure 6.10b and c).

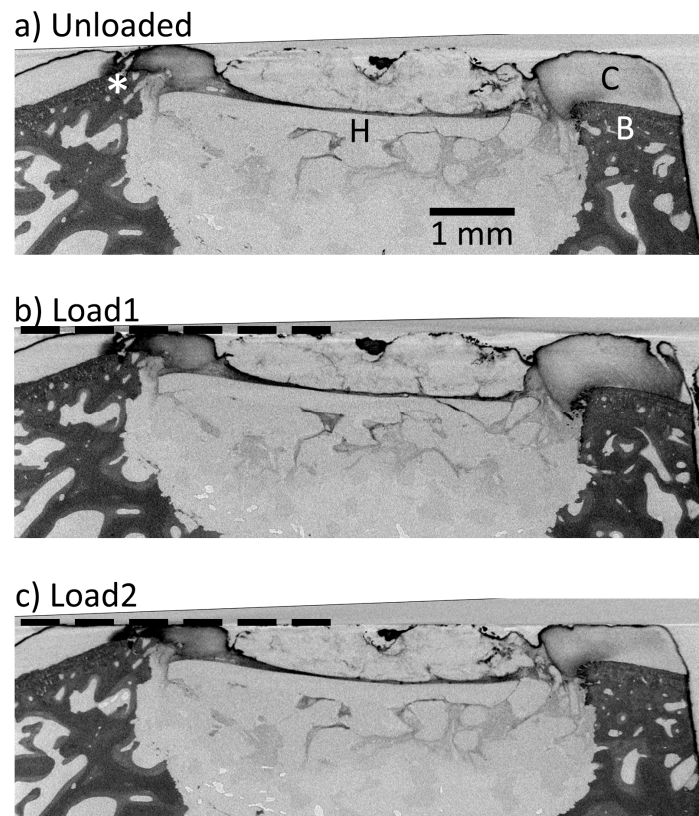


Figure 6.10. A transverse slice of the micro-CT scan of sample Capped1 with successive levels of load applied: a) the unloaded reference scan; b) The first level of compression; c) The second level of compression. The system components are labelled in panel a: C = Cartilage, B = Bone, H = Hybrid implant. A peak in the height of the bone, seen on the left hand side of the defect (*), impeded loading of the cartilage and hybrid as anticipated. The depth of the defect created, in to the subchondral bone, as shown by the large region below the hybrid implant, was unplanned. The dashed line represents displacement of the compression platen.

Displacement and strain were successfully calculated and visualised in three-dimensions (Figure 6.11). Displacement of the implant compared to the surrounding tissue was observed, giving a measure of relative micromotion (Figure 6.11b and e). Caution should be observed in interpreting the strain results in regions with poor correlation quality. Strain within the implant was low, of a similar magnitude to the surrounding tissue, yet high strain was measured in the defect void at both loading levels (Figure 6.11c,f). Of note, the correlation value was high (> 0.8) for the bone, cartilage and hybrid

components, yet the defect void surrounding the implant lacked sufficient pattern, hence only limited correlation was possible in these regions (Figure 6.11d,g).

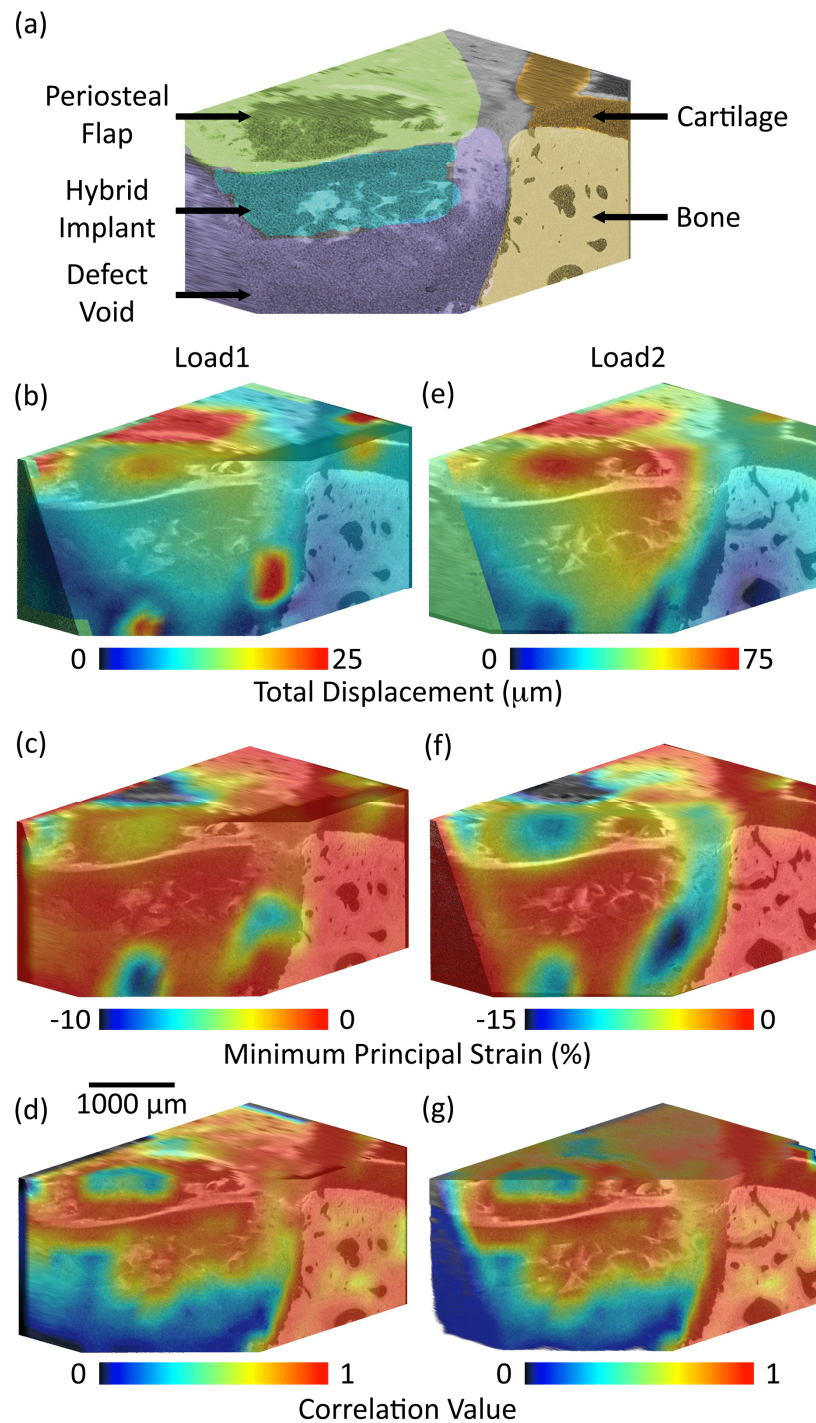


Figure 6.11. Combining three dimensional imaging with in-situ mechanical testing and DVC enabled visualisation of local displacement (b,e) and strain (c,f) for each component of the system (a) at both levels of loading. Also presented is the correlation value (d,g): 0 = no correlation 1 = perfect correlation.

Each slice from the three dimensional volume provides rich localised data points of the displacement from which components of strain may be extracted as required. Total displacement and minimum principal strain were plotted (Figure 6.12) between the key components of the system. As observed visually, displacement was highest in the hybrid implant, with an average of 40 μm relative to the surrounding bone (Figure 6.12a). However, when considering strain, the hybrid implant experienced little mechanical strain compared to both the cartilage and underlying void (Figure 6.12b).

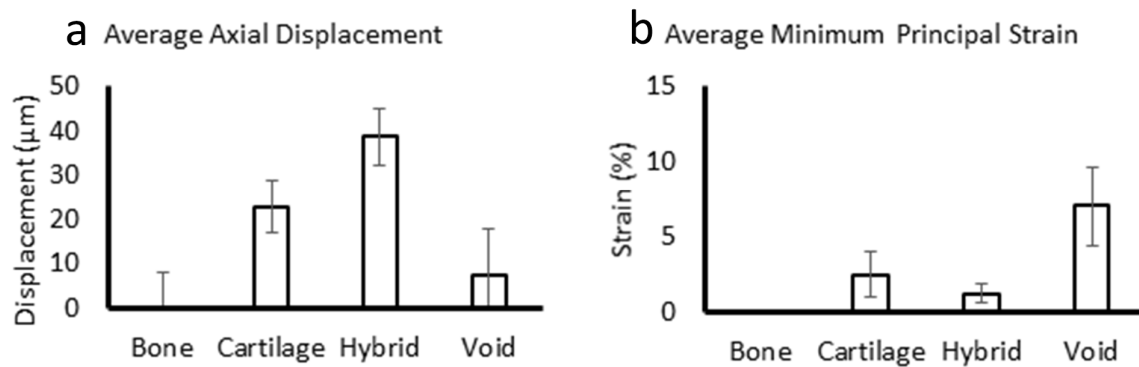


Figure 6.12. Data collected from subvolumes ($n = 3$) at slices ($n = 5$) throughout the 3D volume for total displacement (a) and minimum principal strain (b). Total displacement was normalised against bone. Average minimum principal strain in bone was less than 0.1%. Values are mean and error bars represent S.D. Data presented here represents Load2: the higher level of compression applied.

6.4 DISCUSSION

6.4.1 Most Important Finding

Laboratory micro-CT and DVC enabled non-destructive mechanical testing of biomaterial-osteocondral tissue samples retrieved following implantation within an ovine model. Visualisation of displacement and strain within the system was possible including quantification of micromotion (Figure 6.12) and local strain concentrations (Figure 6.11). This could enable optimisation of fixation methods to limit micromotion, and of ensuring implant behaviour is compatible with surrounding tissue at a local level ultimately increasing the chances of success. Quantified displacement and strain errors within all tissue and biomaterial components of the system were within acceptable limits. It is possible that the magnitude of errors could be further reduced by further optimising the DVC steps [52] or image filtering [100].

The defects created during surgery were deeper than intended with removal of both cartilage and bone tissue (Figure 6.5). This resulted in the implants being seated within a void of bone, with little contact to the proposed cartilage region (Figure 6.10). Feedback was provided to the surgical team to ensure that results from this pilot study informed technique for the larger study. DVC results of the

explanted sample (Figure 6.11 and Figure 6.12) illustrate this: the implant, and superficial periosteal flap, experienced displacement yet this was largely rigid body motion due to the underlying void rather than intended deformation. For this reason, average displacement appeared to be higher than the cartilage (Figure 6.12), yet resulted in very little strain compared to the surrounding tissue void. It could be inferred that limited mechanical strain was likely transduced to cells within the scaffolds during the *in vivo* study. The micromotion of the implant (40 μm) in comparison to the surrounding bone was within acceptable limits but in light of the surgical technique further work is required to confirm these results within the intended chondral defect site.

6.4.2 Comparison to Literature

The strain components of the error analysis follow a power law relationship with decreasing error with increasing subvolume size, as previously described [98, 232]. Strain errors were approximately similar between all components of the system except for cartilage which incurred errors 2 – 4 times higher than the other components. Strain errors of approximately 1000 $\mu\epsilon$ (0.1% strain) or less were possible with a subvolume size of 80 voxels. When combined with 50% overlap this effectively provided a spatial resolution of 180 μm . DVC has previously been carried out on bone and biomaterials tested after 6 weeks of implantation in sheep [52]. The study reported strain systematic error and random error of 200 $\mu\epsilon$ and 100 $\mu\epsilon$ respectively with a spatial resolution of 125 μm , yet was carried out on a bone model at a synchrotron with approximately 50% higher resolution. The same authors carried out a separate study to consider the properties of regenerated bone tissue using DVC [85]. Our study provides a natural progression to include cartilage as part of the osteochondral tissue region. No studies exist for comparison against either cartilage DVC analysis, or of osteochondral tissue-biomaterial systems. We were able to use laboratory equipment, avoiding the need to access synchrotrons which has been noted as a limitation [87].

Of note, the displacement errors observed in this study were higher than would be expected. Displacement random error was calculated to be between 4-8 μm for the different components at the chosen subvolume size, equating to 0.9-1.8x the voxel size. The previously described sheep study observed displacement random error of 0.22 μm [52], and a laboratory micro-CT study using the same software package observed random error of less than 2 μm [59]. Although suitable for scenarios including cartilage which typically experience high strain and displacement (approximately 10% and 150 μm), further work should be carried out to investigate why displacement random error was comparatively poor compared to strain errors.

6.4.3 Limitations

The cartilage of this study did not appear to show obvious features unlike those previously documented in this thesis for bovine and human samples. This would explain why the cartilage incurred the highest errors in the constant-strain study, and further work is required to improve the cartilage imaging. Our previously documented success with cartilage imaging were carried out on smaller explants (3 mm diameter) in different species to that of this trial [209]. The same protocol was followed, yet further optimisation is required to ensure successful imaging. Staining may affect mechanical properties [161, 162], previous findings suggest that it may be avoidable [209] which should be considered for future work.

The surgical technique during this study created defects deeper in to the tissue than intended. This resulted in the implants receiving reduced mechanobiological stimuli than intended. Further studies, with improved implant placement, are being planned. This study illustrated that the described technique is able to successfully illustrate the unintended micromotion of the implant within the surrounding defect void.

In-situ mechanical testing and DVC analysis was carried out on a very limited sample size ($n = 1$) therefore the study is intended as an exploratory pilot study. Studies of large animal models inherently involve small sample sizes and likewise DVC studies are usually limited to small sample sizes [85, 87]. The developed technique ensures maximum data can be collected from each sample to inform development of successful devices. Furthermore the protocol is compatible with histological processing after micro-CT scanning [209].

6.4.4 Conclusions

The combination of laboratory micro-CT, *in-situ* testing and DVC provides a means to non-destructively evaluate biomechanics within osteochondral tissue-biomaterials explant systems. Visualisation of displacement and strain provides a measure of load-bearing capacity between the system elements. Engineers and materials scientists can use this data to strain-match implants ensuring suitable mechanobiological stimuli to cells for successful cartilage regeneration. Additionally, the technique allows implant micromotion to be measured, which was previously impossible non-destructively. Having the ability to non-destructively test the entire system as one and yet quantify local micro-level deformations provides a compelling case for wide-spread application of the technique.

7 DISCUSSION

7.1 RESEARCH FINDINGS

The most important finding of this PhD is that the chondrocyte cells within articular cartilage can be visualised in 3D and permit measurement of localised strain within the tissue. Both visualisation and strain have been compared against established techniques. DVC has successfully been implemented in novel biomaterials, providing intra-strut strain measurements within scaffolds. Finally, the tissue-biomaterial system has been examined as a whole, permitting measurement of micromotion and strain across the interface between the tissue and implant.

The motivation for the thesis was to apply DVC to articular cartilage and novel biomaterials, in the hope that improved device design could lead to successful regeneration of articular cartilage. To this end progress has been made, and the work must be further developed to ensure the findings are implemented to achieve clinical outcomes.

7.1.1 Progress Towards the Outlined Aims

This thesis comprised four aims. The progress towards which is now discussed in turn:

7.1.1.1 *To develop a protocol to image the cells within articular cartilage in three-dimensions across a distance of several millimetres, hence providing a pattern for DVC.*

As illustrated in Chapter 3 cells within 3 mm bovine articular cartilage plugs were successfully imaged with the underlying subchondral bone. All scanning was carried out on samples maintained within a liquid environment. Cellular features were confirmed against 2D histology. Of the four sample preparation techniques trialled, the most successful images resulted from contrast agent staining in an ethanol solution. Features were imaged using alternative techniques which avoided either staining or ethanol and these should be further pursued to ensure conditions are maintained as close to physiological as possible.

A surprising finding of this work was the highly vascularised canal networks within juvenile articular cartilage. Cartilage vascularisation has previously been observed in certain species but never in three-dimensions to the author's knowledge.

7.1.1.2 *To evaluate 3D strain maps within human articular cartilage by applying in-situ mechanical testing and DVC.*

Following the protocol developed in Chapter 3, an *in-situ* loading rig was built and micro-CT scans were carried out on loaded human osteochondral samples. Previous studies have been limited to surface measurements [35], 2.5-D [7] or low spatial resolution [94]. Features were successfully tracked with

DVC between the scans, permitting visualisation of 3D strain maps both within the height of the cartilage, and across the cartilage-bone interface. This was achieved with a resolution of 75 μm through a cartilage height of approximately 1-1.5 mm, enabling high-resolution localised gradients to be measured. Strain measurements were compared against manually-calculated values both against the total cartilage strain ($R^2 = 0.93$) and local measurements throughout the cartilage. Please note that the method developed in Chapter 3 was optimised for bovine samples, further work may be recommended to conduct this optimisation on human samples.

7.1.1.3 *To evaluate 3D strain maps within novel biomaterials to inform device development.*

Two hybrid biomaterial systems were evaluated with in-situ testing during micro-CT and compared against the established DIC surface strain technique. The first, degradable hybrid scaffolds for cartilage regeneration, permitted successful evaluation of 3D strain maps. DVC-calculated strains were comparable with manually calculated measurements ($R^2 = 0.98$) with high enough resolution for intra-strut strain measurements to enable tailoring of device properties at a local level. The second device system, non-degradable implants for tissue replacement, was impregnated with ZrO_2 particles for pattern tracking. Evaluation under constant-strain conditions revealed promising systematic error and random error, yet under loading conditions the results were not convincing. It should be noted the focus of the project is on tissue regeneration and therefore aligned with the scaffold-type devices tested which was successful with a more thorough analysis carried out on this. The scope was widened to include the feasibility study presented by the solid tissue-replacement material.

7.1.1.4 *To assess mechanics across the tissue-biomaterial interface.*

Finally, building on the work presented by the findings of Chapters 4 and 5, the entire tissue-device system was considered by way of an ovine model. It should be noted that in this chapter only one sample was evaluated with in-situ testing and DVC hence it remains an exploratory pilot study. Two studies of a similar nature have recently been performed by other researchers on bone-biomaterial system [52] and on the newly formed bone material [85]. No studies have considered osteochondral systems. An issue with the surgical implantation resulted in the implants being improperly located hence the cartilage-biomaterial interface could not be evaluated as planned. However, analysis of the *in-situ* testing data showed promise, providing measurement of micromotion and strain within the system components. A further ovine model study is in progress which may provide samples for future researchers to evaluate the interface as planned.

7.1.2 Clinical Relevance

The ability to create high-resolution 3D strain maps within articular cartilage may provide target mechanical properties for the development of new devices. Further to this, the ability to create

cartilage strain maps provides the potential to non-destructively study the effect of degeneration of the biomechanics of articular cartilage.

Matching the strain within cartilage and biomaterials may harness the mechanobiological response within the human body for successful regeneration of damaged cartilage. With this comes improving the lives of millions of people with matching economic benefits for society. The work outlined in this thesis lays only the groundwork to this goal being achieved, yet from this preliminary study the framework has been created for future success. Much work is required and is outlined in section 7.4 Future Work.

7.1.3 Research Impact

The method for imaging cartilage (Chapter 3) was accepted for publication in *Osteoarthritis and Cartilage* [209]. This method, and strain measurements within articular cartilage (Chapter 4), were presented as a rapid fire presentation and poster, at ToScA (Tomography for Scientific Advancement) 2019 in Southampton and was awarded first prize for the rapid fire presentation. Strain measurements within hybrid scaffolds (Chapter 5) were presented as a podium presentation at the 9th International Workshop on Interfaces: New Frontiers in Biomaterials in Santiago de Compostela, Spain, April 2018 and was awarded the Journal of the European Ceramic Society Trust student podium prize. Four further publications resulting from this thesis are currently under preparation for submission: High resolution three-dimensional strain measurements in human articular cartilage (Chapter 4), drafted for submission to *Nature Biomedical Engineering*; Vascular articular cartilage canals: three dimensional visualisation in juvenile bovine tissue (Chapter 3), currently being drafted for submission to *Journal of Anatomy*; Quantifying strain in hybrid scaffold implants in 3D using digital volume correlation (DVC) (Chapter 5), drafted for submission to *Acta Biomaterialia*; Full-field strain and micromotion analysis across the osteochondral tissue– biomaterial interface in an ovine model currently being drafted for submission to *Journal of Microscopy*.

The hope is that by presenting and publishing this work, engineers, materials scientists and clinicians will pursue the design of strain-matched implants and thus develop devices to ultimately regenerate articular cartilage.

7.1.4 Continuity Between Studies

The chapters presented in this thesis were performed as discrete studies; however three of the results chapters include a constant-strain error analysis and the results of which have been summarised (Table 7.1). For studies involving tissue a large subvolume size was used than for the hybrids. This was for two reasons: firstly, the voxel size for the tissue studies was generally smaller, therefore the resulting subvolume resolution was relatively similar. Secondly, this was to ensure sufficient cellular

pattern was included in each subvolume, the size and density of which were considerably smaller than the ~200 μm struts and pores of the hybrid scaffold. For the chosen subvolume size the strain errors were a maximum of 0.33% and 0.18% strain for systematic error (MAER) and random error (SDER) respectively, representing an acceptable signal to noise ratio for the applied load. Error was lowest for the non-degradable dual-phase hybrid material, for which the pattern was created by addition of ZrO_2 particles. The well dispersed nature of these particles provided an excellent pattern in the top half of the sample, though was less well distributed in the lower composition. Displacement random error (SDER) was under 1 μm for all samples except for the ovine study within Chapter 6 for which the error was of similar magnitude to the voxel size. Despite excellent error results, DVC of the dual-phase material did not result in excellent loaded results. Further samples should be analysed to verify this is repeatable. A multi-step approach was only considered in Chapter 3, and utilising it in the other chapters may have resulted in improved systematic error, random error and correlation value.

Table 7.1 A summary of the constant-strain error analyses carried out in three of the results chapters of this thesis. “FFT+DC” is the combined DVC approach available in DaVis software, “MS” = Multistep approach for which the final subvolume size is displayed, “MAER” = mean absolute error, and is synonymous with systematic error, “SDER” = Standard deviation of the error and is synonymous with random error. CValue = Correlation value whereby 1 is a perfect match.

Chapter & sample type	Chosen subvolume size (voxels)	Subvolume resolution (μm)	Strain MAER (%)	Strain SDER (%)	Displacement SDER (μm , /voxel)	CValue
4 - Human cartilage (n = 4)	FFT+DC MS: 80	75	0.33	0.18	0.74, 0.4x	0.97
5 -Degradable scaffold (n = 6)	FFT+DC: 48	93-254	0.17	0.031	0.97, 0.08- 0.23x	0.72
5 - Non- degradable device (n = 1)	FFT+DC: 48	185	0.02	0.006	0.47, 0.06x	0.65
6 - Hybrid scaffold (n = 1)	FFT+DC: 80	180	0.049	0.015	4.6, 1.0x	0.57
6 - Ovine cartilage (n = 1)	FFT+DC: 80	180	0.12	0.071	7.0, 1.6x	0.91

7.2 STRENGTHS OF WORK

7.2.1 Laboratory Equipment

All micro-CT imaging involved in this thesis was carried out on a laboratory micro-CT scanner. The *in-situ* testing rig was designed and made in-house with widely available equipment. Many similar studies have been limited to synchrotron X-ray sources. The ability to carry out these experiments in a lab-setting opens up the possibility of imaging and *in-situ* testing to many researchers. Additionally there is the possibility to carry out longitudinal studies in this setting which may not be compatible with resource allocation at a synchrotron.

7.2.2 Human Tissue

The cartilage DVC results presented in Chapter 4 were performed on cadaveric human tissue accessed through the Biomechanics Group at Imperial College London. Often preliminary studies of this nature are often limited to non-human tissue and a strength of this work is the ability to directly apply our findings to the intended human application. I remain thankful for the fortunate position having access to this precious resource has provided to this study.

7.3 LIMITATIONS OF WORK

7.3.1 Micro-CT Access and Sample Size

Throughout all results chapters a smaller than ideal sample size has been utilised. This has primarily been a limitation in access to the micro-CT facilities. The largest utilised sample size was with the degradable hybrid devices for which six scaffolds were evaluated under a limited number of loading conditions. Work was carried out to reduce the scanning time for higher throughput. Wherever possible validation has been implemented against more established techniques to ensure robust conclusions. Nevertheless the sample size has generally not permitted statistical analyses, therefore one of the main suggestions of further work is to simply follow the established method on more samples.

7.3.2 X-ray Damage

A concern for all experiments involving X-rays is the damage they may cause to samples, which have been observed both in bone [80] and cartilage [186]. The effect of prolonged X-ray exposure on strain was evaluated on human cartilage within Chapter 3. The preliminary results suggest that exposure does affect mechanical properties though as a slow pace and therefore for the lengths of time typically required for experimentation the effects may not be significant and can be accounted for. Further

work should be carried out to evaluate the effects both on more samples, and on the biomaterial devices.

7.3.3 Tissue Processing

The most successful cartilage images were observed with both staining and dehydration in ethanol, both of which can affect cartilage's properties [161, 162]. The author deemed it prudent to follow this protocol to ensure successful imaging and hence all tissue strain maps presented in both Chapters 4 and 6 followed this protocol. Other studies which have resulted in successful imaging have involved aggressive full desiccation [10]. Samples in this study were maintained within a liquid environment at all times including during scanning. As shown in Chapter 3, it may be possible to avoid either staining or ethanol treatment to maintain conditions more close to physiological.

7.3.4 DVC Calculations

The version of DaVis software utilised for all DVC analysis only included the small strain theory, omitting quadratic terms and thereby causing discrepancies when large deformations are studied. For a nominal strain of 10% the associated error is approximately 6% of the measured value. For the purposes of this thesis which involved the preliminary development of protocols DVC on materials for the first time, this error is acceptable though it should be considered for future studies.

7.4 FUTURE WORK

7.4.1 Further Human Cartilage Scanning

The current study only includes $n = 4$ samples from two donors. From the limited samples tested so far there was intra-patient similarity yet inter-patient variability. It is necessary to increase the sample size, both for statistical purposes but also to build a database of strain profiles across donors and demographics including across donors of varying levels of cartilage degeneration. This could provide enhanced understanding of cartilage biomechanics and inform device design.

7.4.2 Device Development

The architecture of the tested degradable device comprised a simple repeating design. It has been shown through this study and others that the strain profile within cartilage is highly non-linear therefore ultimately the final device will likely be required to increase in complexity to match the strain gradient. Evaluating different pore architectures should be carried out on the path for an optimised strain-matched implant. Additionally, sample size should be increased to accommodate statistical analysis.

7.4.3 X-ray Scanning Optimisation and Evaluation

Further work should be carried out to further optimise the scanning protocol. The current study was limited in access to micro-CT facilities and it may be possible to improve image quality and/or reduce scanning time with further testing. Chiefly the number of required projections for each sample type, and potentially changing the source to object and object to detector distances. This could improve throughput, enable studies with more loading stages and reduce the effects of X-ray damage.

The effects of X-ray damage on the mechanical properties of the different sample types must be evaluated. Following the method shown in Chapter 4, through the use of a longitudinal constant-strain study it should be possible to quantify the effects of X-ray damage on strain which can then be accounted for.

7.4.4 Constant-Strain Simplification

The current study uses a constant-strain study for error analysis in all three results chapters involving DVC: Chapters 4, 5 and 6. Although this approach does address error due to the microscopy setup, it does not fully capture error associated with correlation between reference and deformed scans.

Future work to include a constant-strain study with the addition of virtually deforming one of the scan volumes would allow quantification of error in line with a more realistic loading scenario.

7.4.5 DVC Method Development

Image filtering has been shown to decrease DVC error in bone [100] and therefore it should be evaluated in both cartilage and biomaterials. This may enable smaller subvolume sizes to meet the requirements of the maximum acceptable error, therefore improving the spatial resolution. Furthermore, a multi-step approach was trialled only in chapter 4, it is possible that utilising it in the later chapters could have resulted in lower error. As discussed in the limitations section, the strain maps included in this thesis use strains calculated using the small strain theory. It is recommended in future work to use the software only for generating the displacement fields and calculating strain separately, for example in MATLAB, or to use software which does not omit the quadratic terms.

7.4.6 Further Ovine Model Testing

The ovine study in this thesis laid the groundwork for successful visualisation of mechanics across the tissue-biomaterial interface. Yet the aim was to evaluate micromotion and strain between the cartilage and biomaterial which was incompatible with the implemented surgical technique. Further work should be carried out utilising explanted samples testing with the intended surgical technique to fully evaluate the proposed system-wide DVC method.

7.5 CONCLUSION

Three dimensional strain maps have successfully been created in human articular cartilage, novel biomaterials and across the tissue-biomaterial interface in an ovine model. Laboratory equipment has been utilised, ensuring the potential for widespread implementation. Applying the developed methodology for future device development enables strain-matching of implants with the potential for successful articular cartilage regeneration.

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8 APPENDICES

CHAPTER 3 - SUPPLEMENTARY TABLE

Table 8.1 Comprehensive breakdown of the source, preparation method and scanning protocol for each specimen used in the study of Chapter 3. Exp = exposure time, Proj = Number of projections.

Sample	Ani	Stifle	Sample	Method	Medi	Stain	Voxel	SOD	ODD	Exp	Proj
	mal		code		um		(μm)	(mm)	(mm)	(s)	
(a)	A	I	a	AC		-	3.53	20.0	18.0	5	2401
	B	I		PPC			1.99	23.5	55.8	22	2401
(b)	A	I	b.1	AC	PBS	PTA	3.53	20.0	18.0	8	2001
	A	I					1.99	23.5	55.8	33	2001
	B	I	b.2	PPC			1.97	25.0	60.0	30	2601
		I					1.97	25.0	60.0	30	2601
	B	II	b.3				2.10	25.0	55.0	24	3201
(c)	A	I	c	AC		-	3.53	20.0	18.0	6	2301
	A	I		PPC			1.99	23.5	55.8	25	2301
(d)	A	I	d.1	AC	EtOH	PTA	3.53	20.0	18.0	7	2001
	A	I					1.99	23.5	55.8	35	2001
	A	I					2.48	23.5	40.0	25	2001
	B	I	d.2	PPC			1.97	25.0	60.0	28	3201
	B	I	d.3				2.09	25.0	55.0	34	3201
	B	II	d.4				2.08	27.0	60.0	40	2901
							2.08	27.0	60.0	40	2901
(e)	B	I	e				1.97	30	73	30	3201

CHAPTER 5 – DUAL COMPOSITION DIC

Minimum Pattern Quality

Following the method for the degradable scaffold samples, minimum pattern quality was assessed on solid cylindrical non-degradable implants ($n = 3$) using a facet size of 100 pixels (approximately 600 μm). Of note, strain error was found to increase with increasing pattern quality (**Figure 8.1.a and b**), following an opposite trend to that of the other samples involved in this study. Displacement random error was relatively stable at 0.05 mm (50 μm , **Figure 8.1.c**). The number of valid measurements decreased with increasing pattern quality as would be expected and as seen in the scaffold samples (**Figure 8.1.d**).

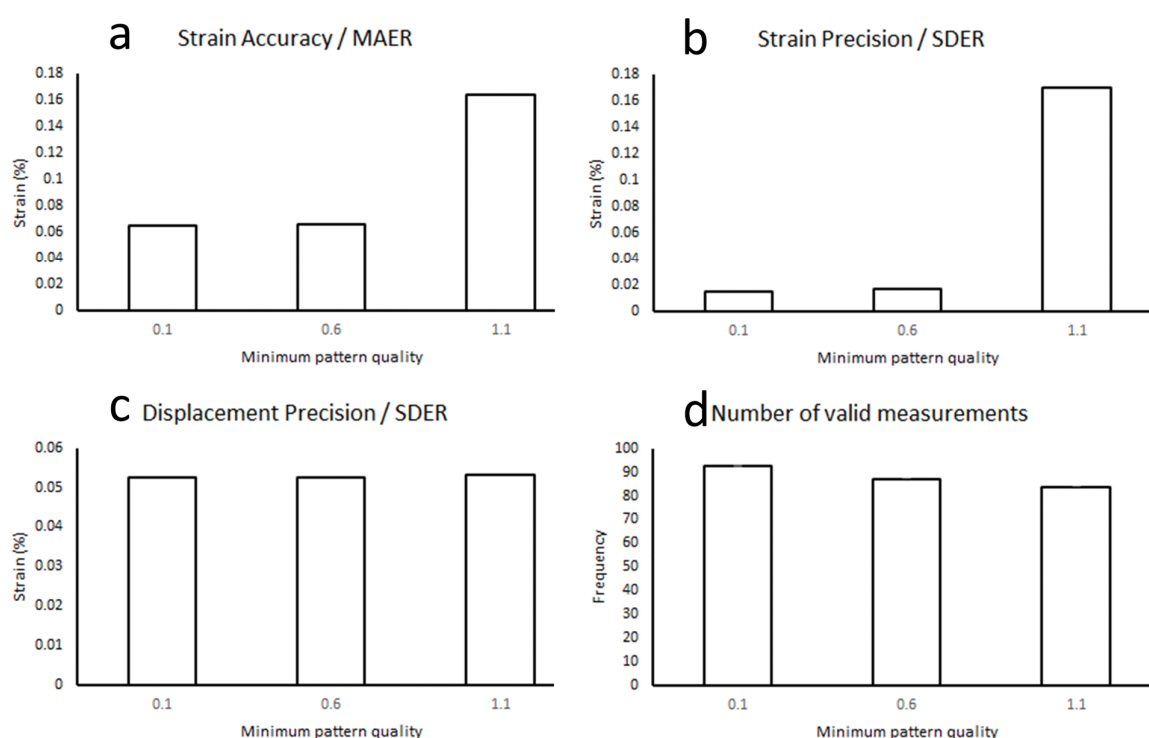


Figure 8.1. Minimum pattern quality was assessed on non-degradable solid samples ($n = 3$), providing quantification of strain systematic error (a), random error (b) and displacement random error (c). The number of valid measurements provides a measure of measurement point density (d). Error bars show the S.D. between all samples. Data was collected in collaboration with Dr Gloria Young with data processing for these samples also carried out by Dr Young.

Constant-Strain Error Analysis

Error was assessed for hybrid non-degradable samples ($n = 3$) using the DIC technique. Strain systematic error and random error decreased with increasing facet size (**Figure 8.2.a** and **b**) following a power law relationship ($R^2 > 0.98$, **Figure 8.2.d**). Displacement random error was stable at 0.05 mm across the range of facet sizes tested (8x pixel size, **Figure 8.2.c**): higher than observed for scaffolds under both DIC and DVC, and for the non-degradable dual-zone sample under DVC. A facet size of 75 pixels was chosen as an appropriate compromise between error and spatial resolution: providing a strain systematic error of 0.1% and random error of 0.02% (**Figure 8.2.d**).

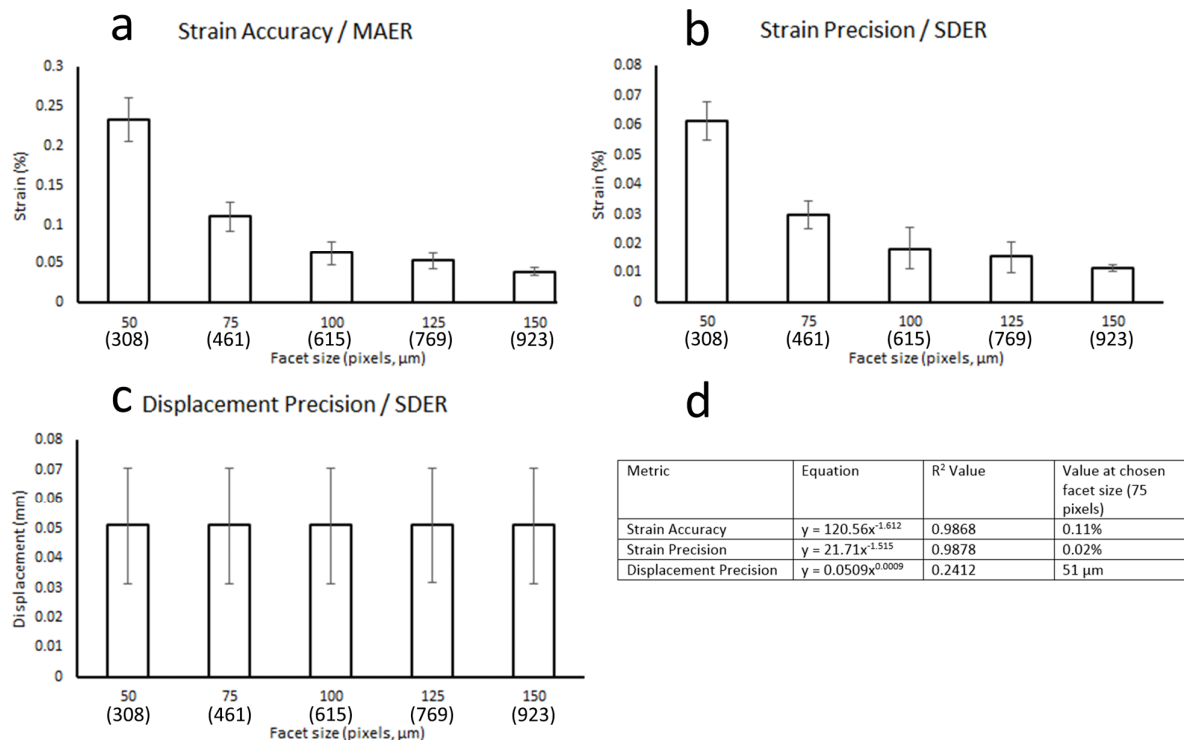


Figure 8.2. Error between consecutive static images was analysed across a range of facet sizes for non-degradable samples ($n = 3$). Strain error was quantified in terms of systematic error (a) and random error (b). Additionally displacement random error (c) was quantified. Power law equations of best fit, associated R^2 values and the metric quantity at the implemented facet size were measured (d). Error bars show the S.D. between all samples. Data was collected in collaboration with Dr Gloria Young and Dr Young carried out data processing for these samples.

2D DIC Loaded Results

Following micro-CT and DVC analysis a pattern was applied with ink to the dual-zone sample for interrogated using laboratory mechanical testing and DIC (Figure 8.3a-c). Displacement was highest in the low-stiffness region with a gradient visible across the interface between the two zones (Figure 8.3d and e). Minimum principal strain magnitude was highest across the interface between the zones (Figure 8.3f and g). Notice the low-stiffness region significantly deforming with lateral displacement

(Figure 8.3c) causing out-of-plane movements and depth-of-field limitations using the DIC technique. This restricted measurements to a region only at the centre of the observable sample surface.

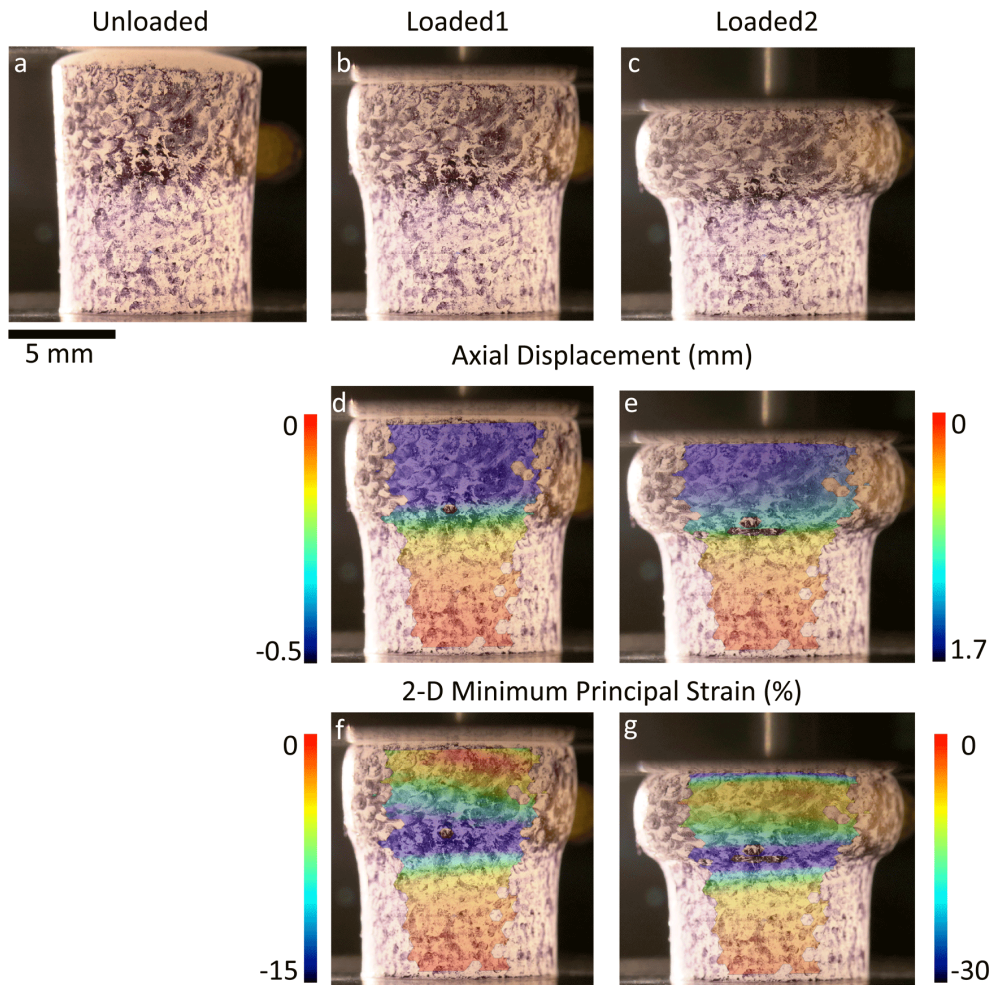


Figure 8.3. Two-dimensional DIC analysis using a digital camera and a mechanical compression machine was performed on the dual-zone sample (a-c), with an ink pattern randomly applied to the surface of the sample. Displacement (d and e) and strain visualisation (f and g) were permitted across a region at the exposed surface of the sample.

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Determining collagen distribution in articular cartilage using contrast-enhanced micro-computed tomography

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