Short communication

Micro-computed tomography with iodine staining resolves the arrangement of muscle fibres

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1. Introduction

Information on muscle geometry, particularly fibre architecture, is important for biomechanical modelling of musculoskeletal function. The established method for visualising and collecting these data is gross dissection (e.g. Friederich and Brand, 1990; Burkholder et al., 1994; Veeger et al., 1997; Kargo and Rome, 2002; Goetz et al., 2008). However, magnetic resonance imaging (MRI) techniques, such as diffusion tensor imaging (DTI), are now gaining in prominence for the application of this technique to describe the fibrous structure of skeletal muscle, and conclude that it has the potential to become a non-destructive and cost-effective method for investigating muscle fascicle architecture, particularly in comparative morphological studies.

3. Results

Six rodent heads, complete with skin and fur, were taken from 5 adult wild-type mice and for comparison 1 adult grey squirrel (Sciurus carolinensis). All 6 specimens had been previously fixed in phosphate buffered formal saline (PBSF) solution (polymerized formaldehyde dissolved as a 4% solution in phosphate buffered saline, allowing for long term storage with limited tissue shrinkage) for two years. Heads were then placed in varying concentrations of iodine potassium iodide (I2KI) in aqueous solution for different periods of time prior to imaging (incubation time; see Table 1). Samples were also taken from commercially available fixed foetal pig material (Carolina Biological Supply, Wisconsin). A 0.5 ml block of tissue from the distal part of the extensor digitorum longus (EDL) muscle was chosen, because in this area the tendons make their connections with skeletal muscle fibres, and so differentiation between muscle and connective tissue could be studied.

Each specimen was removed from the staining solution, rinsed with PBSF to remove excess stain and prevent surface saturation, and were imaged by microCT with the Metris X-tek custom 320 kV x-ray bay system at the EPSRC funded Henry Moseley X-ray Imaging Facility, University of Manchester. Imaging parameters were optimised for each specimen to maximise spatial and contrast resolution as well as data handling (details given in Table 1). Scan times ranged from 25 to 60mins. Image data were viewed and analysed with ImageJ 1.43 (http://rsbweb.nih.gov/ij/). To validate the microCT findings, the sample of EDL muscle was also sectioned for light microscopy by mounting on a cork disk and snap freezing in isopentane above liquid nitrogen. Ten micron sections were then cut in a cryostat. The isotropic microCT voxel data were reformatted with Imagej to match as closely as possible the plane of sections produced for light microscopy. Three-dimensional reconstructions of the squirrel microCT data were created in Amira 5.2 (Mercury Systems Inc., USA).

Results for the mice reveal improved soft-tissue contrast for I2KI specimens compared with the control (Fig. 1). The
improvement in contrast was dependent on both the concentration and incubation time with the specimen in 3.75% I₂KI over 7 days, giving the best result. The mouse masseter muscle, for example, showed clear definition of low attenuation septa, whose configuration suggested that the iodine stain was differentiating between muscle fibres and the epimysial connective tissue separating muscle components (e.g. deep and superficial layers of the masseter) and the perimysial tissue that delineates fascicles or parallel bundles of muscle fibres (Fig. 2). Initial findings for the squirrel using similar concentrations of agent and incubation periods to that for the mice were not satisfactory. The concentration and incubation period were gradually increased and assessed by repeated scanning, until a satisfactory result was achieved. This showed that the larger squirrel specimen (about 70 ml compared to 7 ml for the mice) required a considerably stronger solution of contrast agent and a longer period of incubation (see Table 1). The resulting images (Fig. 3a) also showed the bands of low attenuation, indicative of the epimysium and perimysium seen in the mouse specimens. The 3D reconstructions of the squirrel head demonstrate how muscle volumes, areas and fascicle directions could be mapped from the enhanced microCT data (Fig. 3b and c).

The enhanced microCT images of the pig EDL indicate that the low attenuation structures in the CT sections do indeed represent connective tissue septa dividing bundles of fibres (perimysium). This was confirmed by comparison with the histology sections of the sample (Fig. 4c). In some parts of the microCT scan in Fig. 4a, it was clear that the delineation of connective tissue and skeletal muscle tissue extended to the endomysial level. The endomysium is a very fine layer of connective tissue surrounding individual muscle cells.

**4. Discussion**

Our findings confirm that muscle structures, even down to the level of skeletal muscle fibres can be imaged non-invasively, with contrast enhanced microCT. Data such as muscle volumes and cross sectional areas can be easily derived from the microCT images and the 3D reconstructions demonstrate the possibilities for taking detailed measurements of fibre architecture, such as pennation angles and fascicle lengths.

The exact mechanism for the observed staining effect and the resulting contrast enhancement requires further investigation. One explanation for the retention of iodine in the muscle fibres compared with the connective tissues is that the compound becomes entrapped within the complex structure of glycogen found in muscle cells (Gage, 1906, 1917; Saenger, 1984; Yu et al., 1996; Lecker et al., 1997). The enhanced contrast on the microCT images would therefore correspond to a relative increase of X-ray absorption in these cells, due to the presence of iodine.

Metscher’s (2009a, b) earlier work on microCT imaging of embryos showed excellent penetration and stable contrast enhancement with 10% I₂KI solutions. Metscher (2009a) also demonstrates that vertebrate and invertebrate muscles can be visualised in small specimens with phosphotungstic acid (PTA). This much larger molecule required approximately 12 h incubation for specimens 2–3 mm thick, and is not practical for the size of specimens imaged in the present study. Indeed, findings for the squirrel indicate that we have almost reached the size limit for passive diffusion of iodine. Even larger specimens will no doubt require active perfusion by injection via the blood vessels or use of a pressure chamber. Presumably, large excised samples or

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**Table 1**

Sample and imaging details.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>ID</th>
<th>I₂KI (%)</th>
<th>Incubation (days)</th>
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<th>Voxel size (mm)</th>
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**Fig. 1.** Transverse microCT images of adult mouse heads showing (a) control; (b) 2.25% IKI solution for 7 days; (c) 3.75% IKI solution for 7 days and (d) 7.5% IKI solution for 1 day. Scale bar is approximately 3 mm.
organs (e.g., heart) that lack physical barriers to diffusion, such as skin and bone, would be easier to stain.

The technique described in the current paper has several potential advantages over existing methods used to study muscle architecture. Unlike gross dissection and light microscopy, the proposed method is non-destructive and according to Bock and Shear (1972) iodine staining is readily reversible. This means the same sample can be repeatedly scanned to optimise the imaging of different features (e.g., thin trabecular bone, and then with isotopes staining for muscle). The sample can then finally undergo destructive investigation, if necessary. The non-destructive nature of the method is an important consideration when dealing with scarce materials such as examples of unusual pathologies that can help contextualise our understanding of disease processes and museum specimens of an extinct or rare species that are important for resolving evolutionary trends. The method also has several possible advantages over DTI. Foremost, the spatial resolutions achievable with microCT are almost an order of magnitude greater than those practicable with MRI. Signal to noise diminishes more rapidly with decreases of voxel size in MR than in microCT. Hence, although in theory, MR can acquire voxels small enough to resolve individual muscle fibres, the data are often too noisy to actually delineate the fibres. Hence, in DTI, the arrangement of muscle fibres is not directly visualised, but rather mean vectors representing muscle fibre anisotropy, across a volume, are normally calculated. Another important consideration in comparison with DTI is cost. Cabinet and desktop microCT machines are now available for a fraction of the capital costs of

![Fig. 2. microCT images of adult mouse head in 3.75% IKI solution for 7 days: (a) transverse image at 0.033 mm showing position of resliced images b and c; (b) coronal image at 0.033 mm showing various muscles; (c) coronal image at 0.010 mm resolution showing thick dark bands of epimysium (black arrow) separating the muscles and the narrower dark bands representing perimysium (white arrow). AD, anterior digastric; ADM, deep masseter; ALT, anterior lateral temporal; AMT, anterior medial temporal; EP, external pterygoid; GH, geniohyoid; M, masseter; MH, mylohyoid; ZM, zygomatico-mandibular muscle. Scale bars are 3 mm.](image)

![Fig. 3. Images illustrating how the geometry and internal architecture of a muscle, in this instance the anterior deep masseter muscle in the squirrel, could be defined: (a) transverse enhanced microCT image showing the outline and internal features of the muscle; (b) 3D reconstruction of the skull showing the external geometry of the muscle (red); (c) 3D reconstruction of the skull demonstrating the arrangement and length of fibre bundles in the muscle. Volumes, areas as well as fascicle length and orientation can be taken, using the Amira toolkit. Not to scale.](image)
installing an MR facility and the running costs are considerably lower as well.

Conflict of interest statement
There are no conflicts of interest with regard to any of the authors, the subject of the paper and the organisations involved that could in any way bias this study.

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References