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Does Micro-CT scanning damage DNA in museum specimens?

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Abstract

X-ray micro-computed tomography and DNA sequencing are useful and increasingly common tools in taxonomy and collections research. Whilst the benefits of each method are continually evaluated and debated individually, how the methods impact each other requires more attention. We compared DNA fragment length and the barcode sequence CO1 in samples throughout a CT-scanning protocol, for a range of X-ray exposures and energies. We found no evidence of DNA damage, but advise caution when using precious or archival material, highlighting the need for further investigations and considering potential areas for research.

Keywords: Micro-computed tomography; X-ray microtomography; DNA fragmentation; PCR; Sanger sequencing; Barcoding; *Lumbricus terrestris*; Oligochaeta

Introduction

The taxonomist's tool kit is ever expanding with new technologies, posing new challenges to curators and institutions charged with safe-guarding collections. Whilst most concerns focus on how these new technologies affect the morphology and physical integrity of the specimen - little attention has been paid to how these new methods impact upon one another.

Current calls for a more comprehensive and integrated approach to species identification - including images, scans and genetic analyses (Deans, *et al.,* 2011; Butcher, *et al*., 2012; Wheeler, *et al*., 2012; Edmunds, *et al*., 2013; Faulwetter, *et al*., 2013b; 2014, Riedel, *et al*., 2013b; Stoev, *et al.,* 2013; Faulwetter, *et al*., 2014), highlight the need for this fundamental question to be answered - how best to use specimens, integrating these new technologies, while safeguarding collections for the future? In this study, we look at just one aspect of this issue - does X-ray micro-computed tomography (micro-CT) affect the integrity of DNA within a preserved specimen?

DNA sequence data is now routinely used for taxonomic studies (Cook, *et al.,* 2010, Riedel, *et al.,* 2013b) and has had a huge impact on our view of relationships within the animal kingdom (Giribet, 2010). They are regularly cited as a key to solving the taxonomic impediment (Rougerie, *et al*., 2009; Riedel, *et al.,* 2013b) not least because "DNA barcoding" creates a framework for describing large numbers of taxa in a relatively short time - so called "Turbo-taxonomy" (Monaghan, *et al*., 2005; Rougerie, *et al*., 2009; Butcher, *et al*., 2012, Riedel *et al*., 2013a) as well as revealing otherwise cryptic or ambiguous taxa (Hebert, *et al.,* 2002, Perez-Iosada, *et al.,* 2009; James, *et al*., 2010). Sequence data are useful at every level of taxonomy - from individuals (Sharma, *et al*., 2011) to high level, deep divergences (Dunn, *et al*., 2008, Edgecombe, *et al.,* 2011). Edgecombe, *et al.,* (2011) describe it as "the most ground shaking innovation in modern phylogenetics".

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Micro-CT uses X-rays to generate 2D images of a specimen. As the specimen is rotated within the X-ray beam, hundreds or even thousands of images are collected at different rotational angles. Cross-sections are computed from these "shadowgrams" to build up a 3D model of the specimen. From this model, the specimen can be examined from any angle, sliced along any plane, or digitally dissected through a process of segmentation that can be used to reveal complex internal anatomy in ways that are exceedingly demanding through other techniques (for example serial sectioning, dissection or skeletonisation). Since the process is carried out with the specimen intact, usually still within its storage box or jar, it is often described as non-destructive.

Its widespread adoption (Giribet, 2010) has been credited with causing a renaissance in morphology (Budd & Olson, 2007). Since the technique allows detailed studies of the internal anatomy of specimens, without damaging morphology (when compared to traditional histological techniques or dissections), there have been calls for large-scale scanning of whole museum collections (Faulwetter, *et al.,*2013b).

However, X-rays (and other types of ionising radiation) cause damage to DNA such as Double Strand Breaks (DSBs) Single Strand Breaks (SSBs), abasic sites, intra-strand crosslinks, inter-strand crosslinks, oxidation and the deamination of cytosine to uracil (Brotherton, *et al.,* 2007, Dexheimer, 2013). Such damage may cause a blocking lesion (preventing polymerase from acting on the strand and halting PCR) or a miscoding lesion, where the DNA is sequenced incorrectly (Heyn, *et al.,* 2010). Obviously, DSBs fragment the DNA into smaller pieces which can affect the suitability of DNA for both Sanger and Next-generation sequencing.

Micro-CT is ideal for imaging hard, calcified structures such as bone, but the low X-ray absorbtion of low density non-mineralised tissues means that samples must be stained if soft tissues are to be imaged. Table 1 shows commonly used contrast stains for soft tissues and typical protocols. Iodine is a simple, effective and non-toxic stain which provides good contrast for alcohol preserved specimens (Metscher, 2009; Faulwetter, *et al.,* 2013a). However, Iodine stains have been shown to inhibit PCR (Marin, *et al.,* 2000; Auinger, *et al.,* 2008) and the staining and rinsing process (soaking the specimen in stain solution at room temperature for hours/days, then washing post-scan) may leave DNA vulnerable to decay by hydrolysis.

Previous studies have used PCR success to assess DNA damage in specimens analysed by X-ray radiography (Gotherstrom, *et al.,* 1995) and micro-CT scanning (Faulwetter, *et al*., 2013a). PCR may be inhibited by iodine stain, blocking lesions, or severe fragmentation, but miscoding lesions would not be detected by a simple "Will it amplify?" approach. PCR is an extremely powerful technique which can amplify pieces of DNA from just a single strand. All that is needed is one strand of intact, undamaged DNA from the area of interest. Any damage to other copies, or the rest of the genome, will go unnoticed. Also, PCR products may be generated from damaged DNA by "Jumping PCR" (Pääbo, *et al*., 1990). The result is chimeric fragments made up of a number of different sequences joined together. Such fragments would only be discovered by sequencing. Viable DNA and expected sequences have been obtained from micro-CT scanned gastropods (Suzanne Williams, *pers comm.* 2014) and polychaetes (Faulwetter, *et al*., 2014). However, optimal exposures and energies were used to obtain clear images. It has been the case that museum specimens on loan have

Stain	Stock solution	Staining procedure
PTA	1% (w/v) phosphotungstic acid in water	Mix 30 ml 1% PTA solution + 70 ml absolute ethanol to make 0.3% PTA in 70% ethanol. Keeps indefinitely. Take samples to 70% ethanol. Stain overnight or longer. Change to 70% ethanol. Staining is stable for months. Scan samples in 70% – 100% ethanol
IKI	1% iodine metal (I2) + 2% potassium iodide (KI) in water	Dilute to 10% in water just before use. Rinse samples in water. Stain overnight. Wash in water. Can be scanned in water or dehydrated to alcohol.
I2E, I2M	1% iodine metal (I2) dissolved in 100% ethanol (I2E) or methanol (I2M)	Use at full concentration or dilute in absolute alcohol. Take samples to 100% alcohol. Stain overnight or longer. Wash in alcohol. Stain does not need to be completely washed out before scanning.
Osmium tetroxide	standard EM post-fixation	Same as routine EM processing. Osmium-stained samples can be scanned in resin blocks, with some loss of contrast.

Table 1. Contrast stain formulations and protocols, from Metscher, 2009.

been Micro-CT scanned at unnecessarily high energies and exposures, leading to DNA damage (Isabelle De Groote, *pers comm.* 2014). For this reason, we wanted to test a greater range of scanning protocols.

It should be noted that the 2 extremes of our chosen dose range are not expected to produce optimal images. The purpose of this study is to seek evidence for damage to DNA as a result of micro-CT scanning and to establish guidelines for collections staff and not to provide methodologies for effective micro-CT analyses. The lowest voltage / lowest exposure scan was chosen as image quality may need to be compromised in order to safeguard collections. The highest voltage / highest exposure scan was chosen to maximise potential damage to DNA.

Hada & Sutherland (2006) demonstrated that X-rays induced DSBs, reducing the average length of DNA strands. However they irradiated DNA in solution and found that damage correlated strongly with microenvironment, suggesting that the interior of a cell would provide a radiation quenching microenvironment thus protecting the DNA within tissues. Paredes, *et al*., (2012) used a bioanalyser to analyse fragment length of DNA extracted from bird skins, comparing before and after micro-CT fragmentation profiles. They found no difference in profile and thus no evidence of DSBs. However, the DNA was highly fragmented to start with, probably as a result of preservation techniques. They are clear that their results might not be applicable to other tissue types or organisms such as invertebrates.

Evidence of DNA damage in calcified structures such as teeth and bone has been found following X -ray radiography (Gotherstrom, *et al.,*1995, and Knapp, 2013) and following Micro-CT (Grieshaber, *et al.,* 2008) but so far as is known, there is no evidence of damage in soft tissues.

As discussed, micro-CT of soft tissues may require staining, which could itself cause damage to DNA. The possible effects of Iodine stain (Marin *et al.,* 2000; Auinger, 2008) and chemical drying (Austin & Dillon, 1997) on PCR have been assessed but, as far as is known, no study has yet considered the effects of each stage of staining and scanning. Here, we test for miscoding lesions and DSBs in fresh samples before processing, after staining, after scanning, and after stain removal (washing).

We chose to focus on the mitochondrial gene Cytochrome Oxidase 1 (CO1) due to the availability of robust protocols and comparable sequences on Genbank. As a "DNA barcode" (Hebert, *et al*., 2003) it is also the focus of much of the museum's requests for molecular analyses.

Earthworm identification normally requires detailed dissections and the use of micro-CT has been proposed as a potential non-destructive method (Fernàndez, *et al.,* 2014). We therefore anticipate an increasing number of requests to scan such material. Difficulty of identification (particularly for novel or cryptic species) and controversies over taxonomic grouping, means there is also demand for molecular analyses on this group (Huang, *et al.* 2007, Perez-Losada, *et al.* 2009, Rougerie, *et al*. 2009, James, *et al.* 2010, James & Davidson, 2012). We chose the lobworm, or nightcrawler, *Lumbricus terrestris* Linnaeus (1758) for this trial due to its availability and ease of storage. The specimens in the collections at the Natural History Museum vary widely in their tissue type, age and preservation. To control for variability in quality of material (and to safeguard collections against unnecessary risk) this initial study used fresh material, euthanised on the first day of testing. All individuals were the same species, of the same age and from the same source.

Materials and Methods

14 adult clitellate *L. terrestris* purchased from Worms Direct UK were starved overnight on wet tissue paper. They were anaesthetised in 30% ethanol for a few minutes, then 100% ethanol for 10mins. Worms were washed in 2 changes of 100% ethanol before being cut into 3 body segments - head, clitellum and approx. 3cm of the body, from the tail-end. Body parts were stored in 100% ethanol at 4°C overnight and labelled 1-14, depending on which worm they came from.

Worms were treated as three separate pieces in case of variation between body segments caused by different tissue types or thickness, and between worms. Therefore each scan was assigned one head, one clitellum and one tail, but from different worms.

A tissue sample consisting of a single cross-section, one body segment in width and weighing approximately 15mg was taken from each body part at the following stages:

A) Post -euthanising
B) Post - staining
C) Post - scanning
D) Post – washing

All samples were stored in 100% ethanol at -20°C until DNA extraction.

X-ray micro-computed tomography

Each body part was stained by soaking in a solution of 1g crystalline iodine (VWR) in 100ml of 95% ethanol, for 4 hours at room temperature before being transferred to absolute ethanol. Body parts were secured for scanning by sliding them into plastic tubes embedded in Oasis floral foam (Oasis floral products) in a plastic beaker. The tubes were sealed with cling film to prevent evaporation, and the body parts were scanned in air rather than ethanol to provide greater contrast than would be possible between soft tissue and ethanol.

X-ray micro-CT scans were performed using a Nikon Metrology HMX ST 225 (Nikon metrology,

Tring, UK) micro-CT scanner. All scans were carried out at 150µA with a molybdenum target and 3,142 projections were taken over a 360° rotation with no frame averaging.

Samples were CT scanned at exposures of 354ms, 708ms and 2000ms, with accelerating voltages of 50, 100, 160 and 220 kV. A no-scan control sample was treated the same as 2000ms samples, but was left on the bench instead of being placed in the scanner. These parameters were chosen to represent a wide range of potential doses, from a 'safe' scan of short scan duration and low voltage (20 minutes for a low exposure of 354ms at 50 kV), to a maximum exposure scan of a long scan time and high voltage (two hours for a high exposure of 2000ms at 220 kV).

After scanning, body parts were transferred to 100% ethanol. Destaining should ideally be carried out immediately after scanning, but due to time constraints, specimens were stored at -20°C for three weeks. They were washed by soaking in several changes of 70% ethanol at room temperature, until the solution no longer changed colour. This process took over a week and samples were stored in the fridge over the weekend. They were then transferred through a series of washes, (80%, 90% and 100% ethanol) for long term storage at -20°C.

Data treatment

The 3D volumes were reconstructed using CT Pro (Nikon metrology, Tring, UK) using a modified Feldkamp back-projection algorithm (Feldkamp, et al., 1984). The 3D data sets were then rendered using VG Studio Max (Volume Graphics, Heidelberg, Germany) to produce visualisations (based on the density of the material) and to analyse the quality of the scans and produce virtual cross sections of samples.

DNA analyses

DNA extractions were performed using a Qiagen DNeasy blood and tissue kit, as per manufacturer's protocol "Purification of Total DNA from Animal Tissues" with the following modifications:

1. Tissues were washed in 500µl 1xTE twice to remove gut contents and residual ethanol.
2. Samples were digested for 2 hours at 56°C. Many samples were difficult to lyse and required grinding with a micropestle, or addition of another 20µl proteinase K. Samples were not vortexed post lysis.

DNA was eluted in 100µl of buffer AE and concentration estimated using a Qubit 2.0 fluorometer (Invitrogen).

Strand length analysis

DNA was diluted to give a final concentration of 10-100ng and analysed using an Agilent 2200 TapeStation with Genomic DNA ScreenTape, as per manufacturer's instructions.

PCR

A 658bp fragment of the CO1 gene was amplified using the barcoding primers (after Folmer, *et al.,*1994):

LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3')
HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3')

Each reaction consisted of 0.4mM total dNTPs, 2mM $MgCl_2$, 1.5u Bio-Taq DNA polymerase (Bioline), 0.04µM each primer and 1x reaction buffer (67mM Tris-HCL, 16mM $(NH_4)2SO_4$, 10mM KCl). Cycling conditions were: initial denaturation 94°C for 1 min followed by 30 cycles of 94°C for 30s, 45°C for 30s and 72°C for 15s, with a final elongation of 3mins at 72°C.

Sanger Sequencing

PCR product from post-euthanising and post-washing samples (A and D) were cleaned using Millipore multiscreen PCR 96 filter plates, as per manufacturer's instructions and sequenced bi-directionally using BigDye terminator reaction mix v3.1, in a 3730xl DNA analyser (Applied Biosystems). Sequences were aligned using Geneious pro version 5.4.6 (Biomatters). PCR clean-up and sequencing were carried out by the NHM sequencing facility.

Results

Micro-CT

The low kV/ short exposure scans produced data that wasn't ideal because the signal to noise ratio in the scan was poor, meaning that some features could not be identified in the data. The short exposure time also produced lower contrast between features of different densities (Fig 1, left). The high kV /long exposure scans demonstrated better signal to noise ratio in the scan, making features more easily discernible. However, some features are still lost, in this case, due to the high energy of the X-rays saturating the detector panel in regions of low density material since the X-ray beam was only lightly attenuated (Fig, right). The best quality scans were obtained using 100 kV and 708ms exposure times. These conditions gave a long enough exposure to produce good contrast, but without saturating the detector panel in regions of low density. These scans had the most clearly discernible features (Fig 1, middle).

PCR and sequencing

DNA extraction got progressively easier with treatment (A was the hardest and D the easiest) with many samples requiring physical disruption by grinding and vortex mixing or extra proteinase K during lysis. Some samples (particularly treatment A) were very difficult to lyse. All samples were successfully amplified by PCR. There were no sequence differences between before and after samples for any treatment .There was considerable sequence variation between individuals, but no other factor affected the DNA sequence, or the DNA amplifiability. There was therefore no evidence of miscoding or blocking lesions.

Fragment length analysis

If staining, scanning and washing all caused DSBs, the electropherogram peaks produced by the Tapestation would be expected to spread out and move down the x axis from treatment A through to D

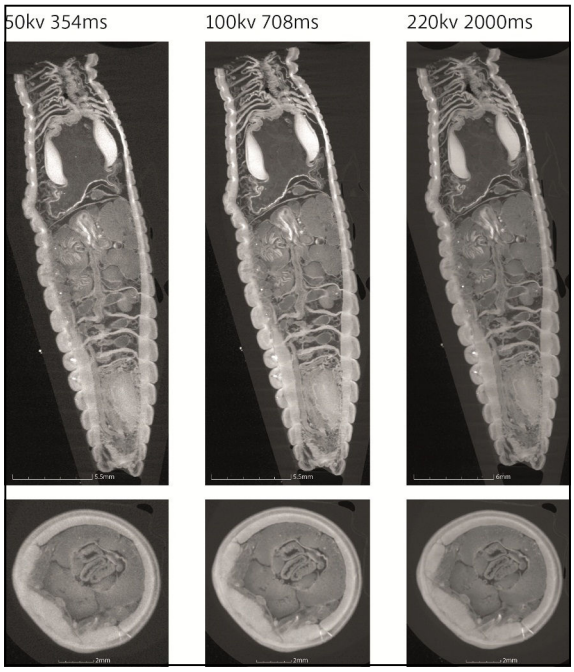

Fig. 1. CT scans of the anterior section, cut just before the clitellum, of specimen one *Lumbricus terrestris*. Top: longitudinal sections, Bottom: cross sections. Left images: Scanned at 50kv, 354ms, Middle images: Scanned at 100kv, 708ms, Right images: Scanned at 220kv, 2000ms. NB these images are from repeat scans taken after sampling, for illustrative purposes.

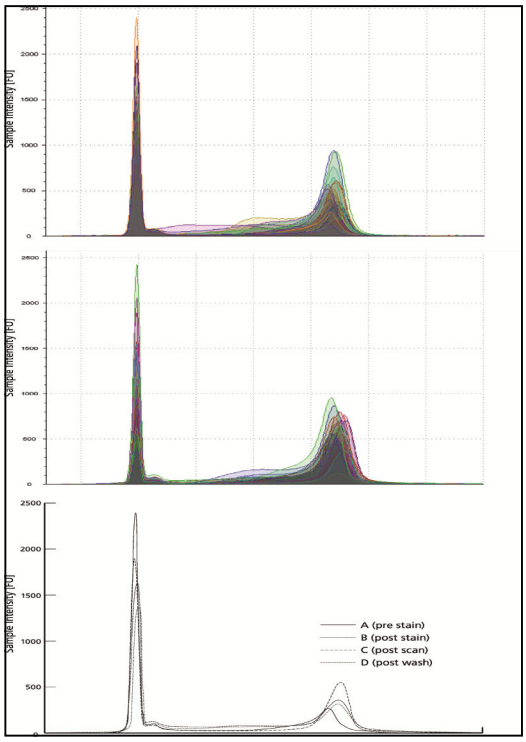

Fig. 2. Example of overlaid electropherograms showing DNA fragment sizes (x axis) against frequency (sample intensity in fluorescence units). The first peak is an internal marker at 35 bases. Comparison of Pre-stain/Group A (Top image) and Post wash/Group D (Middle image) shows variation within groups, but not between groups. The bottom image shows electropherograms for the Clitellum of scan 220kv 2000ms. There was little change in strand length distribution from pre-scan (Group A) through to post-wash (Group D).

(because the broken DNA would be shorter). Overlaying the electropherograms revealed no such pattern. Variation was marked across the samples but was no worse for treatment D than A. I.e. there is no appreciable difference between the electropherograms of stained, scanned and washed samples and those of fresh samples (Fig 2).

Comparison of modal strand length (as reported by the Tapestation) in Group A (Pre-stain) against D (Post wash) using a paired Ttest showed a significant *increase* in fragment size (T= 4.75 P= 1.3x10-5). T tests for each scan showed a significant increase for Scans160kv/354ms (T=3.86 P=0.03), 20kv/708ms (T=10.03 P=0.004), 220kv/2000ms (T=3.61 P=0.03) and the no scan control (T=4.62 P=0.002) (Fig 3).

If the treatments tested induced DSBs, a decrease in modal strand length would be expected. This does not happen when comparing all body parts together for each treatment (Figure 2 top and middle, Fig 3 top) nor for any of the body parts when considered individually. Fig 2 bottom and Fig 3 bottom show an example. In fact, samples in treatment D were significantly longer than those in treatment A. Therefore there is no evidence of DSBs induced by any treatment.

Discussion

Micro-CT did not affect modal length of DNA fragments in any of the samples, therefore we find no evidence of X-ray induced DSBs. Fragment length was highly variable across all samples, but generally increased with processing. The DNA extraction method is column based and therefore causes shearing as DNA passes through the filter. A number of methods were trialled (Gentra Puregene and QiaAmp mini kits from Qiagen, Free-it and CA solution from Clent Biosciences and DNAzol from Life Technologies), but none was consistently better than any other. Thus the DNeasy kit was chosen for its ubiquity in molecular biology. We noted that tissue lysis (the first step in DNA extraction) became progressively easier after staining, then scanning, then washing, with some samples requiring extra proteinase K and grinding or vortex mixing to breakdown tissues. Faulwetter, *et al.,* (2013a) found that Iodine inhibited lysis in polychaetes (Katerina Vasileiadou *pers comm.* 2014) and although our washed samples were the easiest to lyse, our unstained samples were the most problematic. Ethanol toughens Oligochaete tissues. We suggest that the process of staining and washing softened the tissues, allowing easier lysis and less physical disruption which would break the DNA. It is therefore likely that DNA shearing was primarily caused by the DNA extraction process rather than

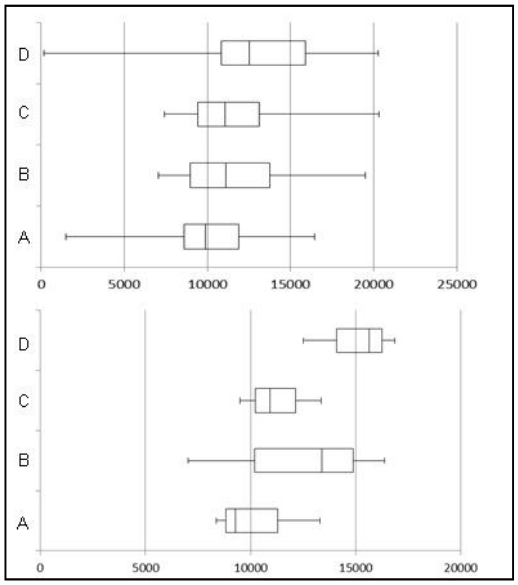

Fig. 3. Box and whisker plot showing the distribution of modal strand length (x axis) for Top: all samples. Bottom: Scan 220kv, 2000ms. The modal strand length increased with treatment, rather than decreased which would be expected if staining, scanning or washing induced DSBs.

anything else, as results were inconsistent with any other variable.

Stains may also be tissue specific (Faulwetter, *et al*. 2013b; Sykes, *et al.* 2013) meaning that, over time, one museum specimen may be subject to a number of different stains and repeated scanning. Since processing the samples seemed to affect the tissues, it may be argued that repeated staining, scanning and washing of the same specimen could cause degradation, particularly of soft tissues. Metscher (2009) stated that "each new type of sample must be tested with different fixations and stains to find the best treatment for the imaging required". We cannot say what effect other stains would have, or how iodine would affect other tissues or organisms. Indeed, even protocols for the same stain vary widely; our oligochaetes were soaked in Iodine for 4 hours, Metscher (2009) suggests overnight, whilst Faulwetter, *et al.,* (2013b; 2014) soaked polychaetes for up to 5 days. Optimisation of protocols before working on collections materials (to minimise manipulation of specimens i.e. staining and exposure) would be prudent.

There was no variation in amplifiability across the samples, all DNA extractions gave a distinct band of the expected size, and the DNA sequences remained unchanged. We therefore detected no miscoding or blocking lesions. However, we only looked at one mitochondrial gene and as already noted, PCR is an extremely powerful technique which can amplify pieces of DNA from just a single strand. It is possible that DNA was damaged, but in insufficient amounts to be detected by our method. Also, lesions induced by ionising radiation tend to be clustered (Nikjoo, *et al.,*1997; Sutherland, *et al*.,2000; Nikjoo, *et al.,* 2001, Semenko & Stewart, 2004; Hada & Sutherland 2006) rather than spread, so whilst no damage was seen in the CO1 gene, we cannot rule out damage to the rest of the genome.

We found no evidence of DSBs, blocking lesions or miscoding lesions induced by micro-CT. However, due to the issues outlined above, other techniques should be employed to verify our results before micro-CT can be declared safe for precious material. A number of techniques have been used to measure DNA damage, such as HPLC (Pääbo, *et al.,*1989), Single primer extension SPEX (Brotherton, *et al.,* 2007) NGS/sequencing by synthesis (Gilbert, *et al*., 2007), Polymerase Extension Profiling PEP (Heyn, *et al*., 2010) and DNA profiling (Knapp, 2013). These techniques are more expensive, time consuming and/or less robust than the methods employed here, so were not included in this initial analysis.

Conclusions

We found no evidence of DNA damage derived from micro-CT scanning or associated staining. Whilst (as far as is known) all studies have recovered viable DNA from scanned specimens, it is not clear whether the DNA has been damaged in other ways, due to the limitations of the detection methods used. Collections managers must consider future uses for specimens: Whilst Micro-CT scanning does not appear to hinder current DNA analyses, future technologies may be hampered by as-yet undetected damage. Comparing the entire genome of a specimen both before and after scanning using NGS is suggested as a next step in considering these difficult points.

Also, due to the vast range of methods, organisms, tissue types and variability in quality and quantity of DNA (fresh vs archival or ancient specimens for example) it is impossible to predict the effects of micro-CT on museum specimens in general. We therefore echo the comments of Paredes, *et al.,* (2012) that "Users seeking curatorial permission to scan rare specimens ...should carry out a [pilot] study on less valuable material."

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