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Microscopic dual-energy computed tomography (microDECT) imaging of animal tissues: the colour of X-rays

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Abstract: In this view article, we present potential applications of laboratory-based spectral dual-energy X-ray microtomography imaging for *ex vivo* animal tissue samples. Technical details on this topic have been reported in

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previous work, so we focus explicitly on applications here, discussing both dual-energy imaging for the separation of mineralised tissue from one X-ray dense contrast agent, as well as the imaging of samples stained with two X-ray dense contrast agents. Examples are given for a variety of research areas, including preclinical biomedical phenotyping (vasculature, skeletal development), comparative vertebrate morphology, and invertebrate anatomy. Based on the data presented, we conclude that the use of spectral X-ray information can provide new insights into CT datasets. Although using the dual-energy approach initially requires more effort and cost in data acquisition, this additional effort is often worthwhile, as dual-energy datasets allow for more efficient data visualisation, segmentation, and analysis. Until now, software functions for acquisition and processing of dual-energy data have not been implemented in many of the software toolboxes provided by X-ray microtomography vendors with their scanners. We propose that a user-friendly implementation of software tools for acquiring and processing spectral CT data would be a big step towards a wider use of this promising and powerful imaging approach.

Keywords: X-ray microtomography; microCT; μ CT; DECT; basis material decomposition; diceCT

1 Background

1.1 Laboratory-based X-ray microtomography imaging of biological and biomedical samples

Over the last two decades, laboratory-based X-ray microtomography (microCT) imaging has evolved from an experimental technique to a routine method for *ex vivo* imaging of biological and biomedical samples at micron resolution. This is because microCT delivers genuine, isotropic 3D information from optically non-transparent samples. In

conventional microCT imaging, image contrast is achieved mainly based on differences in X-ray attenuation within the sample. This attenuation contrast depends on the photon energy, molecular density, and the atomic number of the elements found in different regions of the sample. However, biomolecules such as proteins and lipids mainly contain elements with a very low atomic number. Thus, they generally show low X-ray attenuation and limited contrast between tissue components. X-ray attenuation is much higher in mineralised biological tissues that contain high quantities of calcium ($Z = 20$) in the form of calcium phosphate or calcium carbonate. Therefore, when imaging biological tissue samples in an aqueous environment, such as buffer or agarose gel, high contrast is only achieved for mineralised tissue, while attenuation-based contrast in non-mineralised tissue is low (see [1] for a summary and references therein).

The contrast of soft (non-mineralised) biological tissues can be increased by staining the tissue with an X-ray-dense compound [2]–[5]. Staining improves soft tissue X-ray attenuation contrast, however, it also has drawbacks, such as tissue shrinkage [6]–[8]. Shrinkage can be minimised by refining the staining protocols. For example, shrinkage can be significantly reduced when staining with Lugol's iodine solution (I_2/KI) using buffered solutions [9] or by embedding samples in a hydrogel prior to staining [10]. Despite major advances in staining procedures, it is not always desirable or possible to stain samples with X-ray-dense compounds because staining protocols may not be fully compatible with other requirements, including downstream molecular analysis of samples. Soft tissue contrast can also be enhanced by replacing the water in biological tissue with media that have lower X-ray attenuation than water. This can be achieved through dehydration to ethanol [11], [12], paraffin embedding [13], [14], or drying [15]. For unstained biological tissue samples, propagation-based phase contrast (PBPC) computed tomography imaging improves image contrast. Thus, PBPC imaging is a suitable alternative to attenuation-based imaging of stained samples. PBPC imaging is feasible with several types of laboratory-based microCT scanners when using sufficiently long object-to-detector distances and sufficiently small pixel sizes. Dedicated phase-retrieval algorithms [16] have been shown to provide high image contrast, even in unstained biological samples with low attenuation [17], [18]. Thus, PBPC imaging potentially yields tomographic image data from biological tissues with a better structural preservation than stained samples, at least when the samples are imaged in an aqueous environment. However, samples dehydrated in ethanol or embedded in paraffin prior to PBPC imaging also undergo tissue shrinkage [19].

Despite the high contrast achieved through tissue staining or PBPC imaging, both approaches have limitations in identifying and separating different materials, i.e., parts of the sample with different elemental or molecular compositions. Due to their molecular composition and molecular density, different materials may exhibit similar or identical grey values in reconstructed CT volumes, complicating the interpretation, segmentation, and analysis of such datasets. Spectral X-ray imaging approaches can address this issue because they provide 3D information on the molecular composition of samples.

1.2 Conventional microCT versus energy-sensitive microCT

Transmission X-ray microscopy, or more generally microCT imaging, is remarkably similar to brightfield transmitted light microscopy. Both approaches use a broad polychromatic energy spectrum of electromagnetic radiation for imaging. In both approaches, a sample is positioned in between the energy source and the detector, and the image contrast is mainly generated by the absorption of photons within the sample. Finally, in both approaches, different regions of the sample – depending on a different molecular or elemental composition – may possess highly specific properties with respect to the absorption of the different photon energies. In summary, both approaches measure the absorption of polychromatic radiation in a potentially heterogeneous and thus colourful sample. The big difference between these imaging techniques is on the detection side. In transmitted light-microscopy, energy-sensitive digital detectors (RGB colour cameras) have been standard for decades. In contrast, X-ray microscopy has traditionally relied on energy-integrating detectors producing familiar grey-scale CT images (Figure 1A).

Two fundamentally different approaches exist for collecting X-ray spectral information from samples when using laboratory-based microCT setups equipped with a micro- or nanofocus X-ray source that emits a polychromatic X-ray spectrum. These approaches are summarised in Table 1. The first approach uses energy-sensitive X-ray detectors. These energy-sensitive X-ray detectors can be roughly grouped into two categories based on differences in implementation and energy resolution: (1) Single-photon-counting X-ray detectors allow multispectral X-ray tomography imaging [20]–[22], also known as photon-counting CT (PCCT). In PCCT, sample composition information is required because energy thresholds must be set before acquiring image data. The number of energy bins (channels) achievable in PCCT datasets is determined by the number of energy thresholds based on the detector design and is typically between two

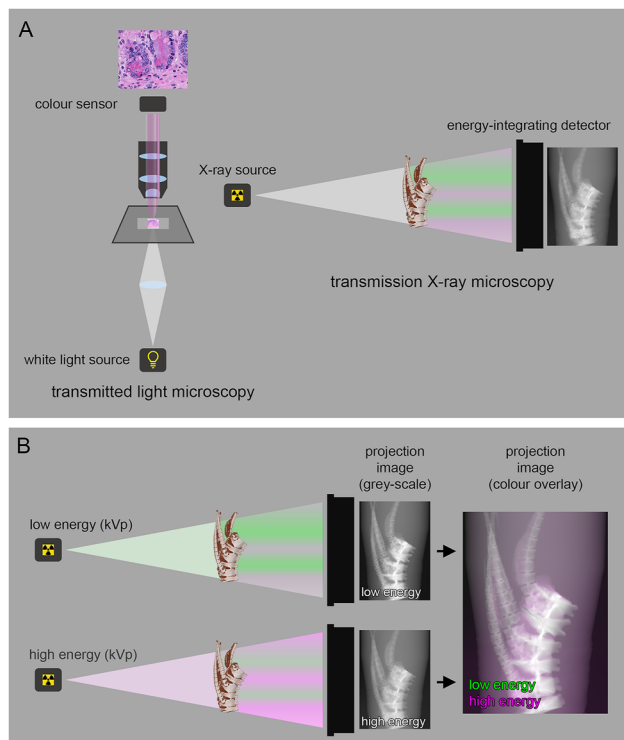


Figure 1: Limitations of conventional microCT imaging with energy-integrating detectors, and X-ray physics background of microDECT imaging. (A) In principle, the technical setup of X-ray microscopy (or microCT in general) is remarkably similar to that of transmitted light microscopy. In both modalities, a polychromatic spectrum is used for imaging, and different parts of the sample exhibit photon energy-dependent absorption properties. However, X-ray imaging has traditionally been limited to the use of energy-integrating detectors, which means that spectral information about the sample composition is lost on the detection side. (B) Dual-energy imaging allows spectral information to be collected. By imaging two materials (elements) with an appropriate energy pair, spectral information can be used to discriminate between materials as seen in the overlay projection image. Note that the chemical composition of the sample must be known *a priori* in order to select appropriate imaging settings.

and eight [23]–[25]. Single-photon-counting detectors can achieve energy resolutions below 5 keV [25], [26], which is adequate to distinguish elements such as iodine from gadolinium [27] or iodine from barium [21]. This makes PCCT a powerful tool for studying the distribution of one or more contrast agents [28], [29]. (2) Hyperspectral X-ray detectors can achieve higher energy resolutions of ~ 1 keV or lower [30]–[32] and several hundred energy bins. This allows them to record a complete X-ray absorption spectrum for any voxel in the sample over a large energy range [33]. Based on element-specific absorption edges, hyperspectral X-ray imaging provides information on the elemental composition of samples. Thus, prior knowledge of the sample

composition is not required for hyperspectral X-ray imaging, and high spectral contrast can be achieved even for elements that are close together in the periodic table [33]–[35]. Both single-photon-counting and hyperspectral detectors are well-suited to spectrally separate multiple elements or materials [33], [35]–[37], however, they are still quite rare in commercial X-ray setups. In principle, energy-sensitive detectors can achieve high spatial resolution when imaging with a micro- or nanofocus X-ray source, assuming a small focal spot size. In practice, however, the spatial resolution of datasets acquired with energy-sensitive detectors is limited by the comparatively low number of detector pixels for a given sample diameter (field of view, FOV). Spatial resolution for a given FOV can be increased by tiling multiple detector modules to form a larger area detector [38], [39] and by imaging the sample with higher geometric magnification. Tiling detector modules also enables imaging of larger FOVs at a given spatial resolution. Similarly, camera translation can extend the field of view [21], [23], [40].

The second approach to collecting X-ray spectral information from samples uses conventional energy-integrating detectors (EIDs). In this approach, spectral contrast is achieved by scanning a sample with two energy spectra resulting from different source peak voltages and X-ray filters (Figure 1B). This approach is known as dual-energy CT (DECT) [49], and can be extended to three or more scan energies (e.g., triple-energy CT [50]). With a dual-energy approach, prior knowledge of the sample composition is necessary to select suitable energy spectra for imaging. Additionally, spectral contrast is limited for elements close together in the periodic table when the goal is to extract volumetric material density maps using a basis material decomposition approach [41], [46]. Compared to imaging with energy-sensitive detectors, DECT has several drawbacks and limitations. When using sequential scan acquisition, scanning time is roughly doubled. Image quality in dual-energy datasets may be reduced due to artefacts arising from differences between the two scan energies, such as e.g. different levels of noise or beam hardening. Furthermore, the two datasets must be co-registered and resampled to achieve voxel-to-voxel correspondence between the two scan energies. However, DECT also has two distinct advantages. First, it can be performed on any commercially available microCT setup or X-ray microscope. Second, it can usually be performed with full detector resolution. Due to the large number of detector pixels in recent EIDs, DECT can yield high spatial resolution for a given sample diameter [41].

This view article has two main aims. First, we aim to showcase the diverse *ex vivo* applications of microscopic

Table 1: Comparison of dual-energy CT using energy-integrating detectors with true spectral X-ray CT using energy-sensitive photon-counting or hyperspectral detectors.

	Dual-energy CT (DECT)	Multispectral X-ray CT/photon-counting CT (PCCT)	Hyperspectral X-ray CT
Hardware requirements	Works with <i>energy-integrating detectors</i> (EIDs) on any commercially available microCT setup. A large energy range and a large number of filters increase flexibility [41].	Requires an energy-sensitive photon-counting X-ray detector [42].	Requires an energy-sensitive hyperspectral X-ray detector [33].
Detector pixel matrix	2048 × 2048 pixels or more in contemporary EIDs.	Typically much smaller than in EIDs (e.g. Medipix3: 256 × 256 pixels, 55 μm pitch [24]; Medipix4: 320 × 320 pixels, 75 μm pitch [43]; Pixirad-1/Pixie-III: 512 × 402 pixels, 62 μm pitch [25]).	Typically much smaller than in EIDs (e.g. Hexitec: 80 × 80 pixels, 250 μm pitch [30]; Mönch0.3: 400 × 400 pixels, 25 μm pitch [32], [44]).
Spatial resolution	Imaging can be performed at full detector resolution. A high number of detector pixels yields a high spatial resolution for a given sample diameter. Spatial resolutions below 10 μm are possible [41].	When imaging with a single detector module, spatial resolution is limited by sample diameter and the detector pixel matrix. For a given sample diameter, spatial resolution may be increased by tiling of detector modules into a large area detector array [39] and imaging at a higher geometric magnification.	When imaging with a single detector module, spatial resolution is limited by sample diameter and the detector pixel matrix. For a given sample diameter, spatial resolution may be increased by tiling of detector modules into a large area detector array [38] and imaging at a higher geometric magnification.
Chemical sample composition	Sample composition must be known in advance in order to select a suitable energy pair for imaging.	Sample composition must be known in advance to set suitable energy thresholds before acquiring the data.	Knowledge of sample composition is not required. Hyperspectral image data can be used to identify elements based on their absorption edges [33]–[35], [45].
Number of materials	Basis material decomposition typically extracts two materials plus a background fraction (e.g., water or air); it may also be used for three materials [41], [46], [47]. Dedicated algorithms can decompose up to five materials [48]. With 2D histogram segmentation, multiple materials can be separated if the sample contains distinct material phases. 2D histogram segmentation can also separate elements that are close together in the periodic table.	Multiple materials can be analysed. This works even for elements that are close together in the periodic table, such as iodine and barium [21].	Multiple materials can be analysed. This works even for elements that are close together in the periodic table, such as gold and lead [33] or iodine and barium [35].

dual-energy computed tomography (microDECT) for imaging animal tissues in various biological and biomedical fields. With this, we aim to raise awareness that this type of spectral CT data can be acquired using conventional equipment found in many laboratories and imaging facilities. We demonstrate that by selecting appropriate contrast agents and imaging protocols, one can obtain a significant amount of additional information compared to conventional grey-scale CT images. This additional spectral information can improve data visualisation and analysis, offering new possibilities for imaging biological samples. The second aim is to address microCT vendors. By demonstrating that microDECT imaging can benefit diverse biological and biomedical research fields, as well as material science and earth science, we hope microCT vendors will improve software tools to make acquiring and processing dual-energy data easier. Although several software tools for processing dual-energy data are currently available, software tools for basis material decomposition and providing quantitative maps of molecular density are particularly needed. This topic will be discussed in the ‘Conclusions and Outlook’ section.

1.3 Tissue staining and spectral contrast in microDECT

High-quality separation of two materials or elements based on microDECT using broadband polychromatic X-ray sources is only feasible for a limited number of element pairs. At first glance, this appears to be a major limitation of the approach. However, imaging of soft (non-mineralised) tissue is commonly performed on stained specimens, meaning that tissue absorption and contrast are enhanced by a chosen X-ray dense contrast agent. These contrast agents can be applied either by immersion in a staining solution or by perfusion through the vasculature. MicroCT imaging with immersion contrast staining was first described for osmium-stained mouse embryos in 2006 [51]. Since then, a number of X-ray contrast agents based on iodine [2], [3], tungsten [2], [3], molybdenum [52], [53], hafnium [54], ruthenium [55], [56], bromine [57], gadolinium [58], lead [59], [60], and others [4], [5] have been introduced for staining of different animal tissue components. Contrast agents for perfusion and vascular casting include iodine [61], [62], barium [8], [63], lead [50], [64], and bismuth [65], among others.

Typically, X-ray contrast agents are selected based on (i) their staining properties, i.e., their specific or non-specific binding affinities for various components of tissues; and (ii) their X-ray attenuation and the general or differential contrast they provide in stained tissue. However, when designing dual-energy imaging experiments, contrast agents may

also be chosen on the basis of their X-ray absorption spectra [66]. Some commonly used contrast agents have spectral properties that are ideal for dual-energy imaging with conventional polychromatic X-ray sources. In particular, elements whose K-shell electron binding energies fall in the range of laboratory X-ray sources show a sharp increase in X-ray absorption above this energy (the K-edge). When imaging small biological samples with laboratory microCT setups, imaging at peak source voltages of 40 kVp and 80 kVp typically provides good sample contrast and signal-to-noise ratio. The average photon energies for these source voltages (simulated for a tungsten target and a glass filter [67]–[69]) are ~ 27 keV (40 kVp spectrum) and ~ 42 keV (80 kVp spectrum), and the bulk of the beam X-ray energies are sufficiently separated to show useful differences in attenuation by different elements. Imaging with this energy pair provides optimal contrast for elements with K-edges between about 30 keV and 40 keV (atomic numbers between about $Z = 51$ and $Z = 57$). This range includes two elements which are components of popular X-ray contrast agents: iodine ($Z = 53$, K-edge at 33.17 keV) and barium ($Z = 56$, K-edge at 37.44 keV). In the following, we will use the term *K-edge stain* to refer to an element/stain whose K-edge absorption jump lies between the two mean photon energies of the dual-energy scan.

In principle, dual-energy imaging may be feasible even in the absence of a K-edge within the imaged energy range. However, the extraction of material fractions by material decomposition [46] works best in the presence of a K-edge stain [41]. The rationale for successfully separating two elements in this scenario is simple (Figure 2). (i) A K-edge stain is used as a reference. (ii) A beam-energy pair is then chosen for imaging so that the mean photon energy of the low-energy scan is below the K-edge stain’s absorption edge, while the mean photon energy of the high-energy scan is above this K-edge. The second stain or material must have a K-edge well above or below the range between the mean photon energies of the low and high energy scans, i.e. a relatively monotone absorption spectrum in the imaging energy range. With this arrangement, the absorption difference (or ratio) between the two elements changes substantially for X-rays above the K-edge energy. Thus, each scan energy will form images using mainly X-rays subjected to different spectral absorption – effectively two colour channels, analogous to making a colour photograph using black and white film and two colour filters. These channels can then be decomposed into material channels using the appropriate calibrations [41], [46].

Two application fields are common in microDECT imaging of biological specimens.

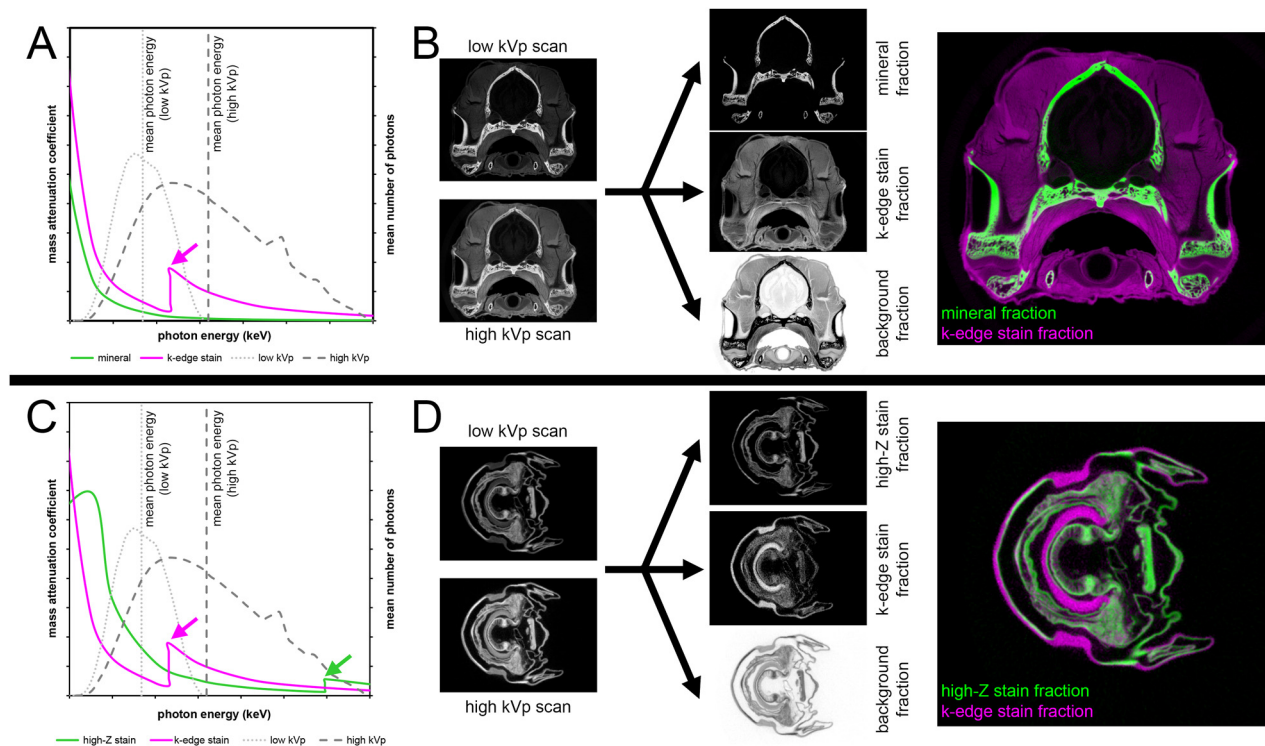


Figure 2: Rationale for microDECT imaging of biological samples. Two potential application fields are often encountered. (A) MicroDECT imaging can be used to distinguish between mineralised tissue and a K-edge stain. The two energy spectra are selected so that the mean photon energies are below and above the K-absorption edge (pink arrow) of the K-edge stain (iodine attenuation curve shown in pink). The K-edge of elements contained in biological minerals (attenuation curve of calcium shown in green) is much lower, so the mineral does not contribute to the spectral contrast with the given energy pair (here: 40 kVp/80 kVp). (B) Basis material decomposition is used to extract three material fractions from the two scans [41], [46], yielding one fraction for the mineral, one for the K-edge stain, and one for the background. (C) MicroDECT imaging is used to discriminate between a high-Z stain and a K-edge stain. The two energy spectra are selected to have mean photon energies below and above the K-absorption edge (pink arrow) of the K-edge stain (iodine attenuation curve shown in pink). The K-absorption edge (green arrow) of the high-Z stain (tungsten attenuation curve shown in green) is much higher compared to the mean photon energies of the two scans. Thus, the high-Z stain does not contribute to the spectral contrast with the given energy pair. (D) Basis material decomposition is used to extract three material fractions from the two scans, yielding a fraction for the high-Z stain, the K-edge stain, and the background. Displayed photon energy range: 10–80 keV. X-ray attenuation profiles were redrawn from data presented in [66]. X-ray spectra were simulated for 40 kVp and 80 kVp tube voltage (tungsten target, 0.8 mm glass PYREX filter) using the Siemens online tool for the simulation of X-ray spectra (<https://bps.healthcare.siemens-healthineers.com/booneweb/index.html>, accessed 16.01.2024), based on data from [67]–[69].

Application field 1: MicroDECT can be used to discriminate between mineralised biological tissue and a contrast agent [41], [70], [71]. Chemical elements that contribute most to contrast in biological minerals have low Z-numbers (e.g. hydroxyapatite: calcium = 20, phosphorus = 15) and thus show a continuous decrease in X-ray attenuation in the typical working range of laboratory X-ray sources. For small biological samples, spectral separation is therefore highly efficient when using K-edge stains such as iodine compounds (Figure 2A). Such samples can be easily imaged with an energy pair of 40 kVp/80 kVp [41], [71] or 50 kVp/90 kVp [70]. Separation of mineralised tissue from barium was successfully performed using a 45 kVp/80 kVp energy pair [71]. The separation of mineralised tissue from elements with very high atomic numbers is feasible but

requires much higher scan energies. For example, good spectral contrast for the separation of calcium and tungsten is achieved using a 140 kVp spectrum with tin filtration [47]. However, such high peak voltages are unsuitable for small biological samples, as the low attenuation of such samples at these high scan energies often leads to a decrease in signal-to-noise ratio. Furthermore, scan energies larger than 100 keV are not available on many lab-based microCT setups.

Application field 2: MicroDECT can be used to distinguish between two contrast agents [41], [71]. Double staining is most efficient when a K-edge stain is combined with a stain containing a high-Z element with a K-edge of ~70 keV or higher, hereafter referred to as *high-Z stains*. An ideal element pair is iodine/tungsten or any other combination of

iodine with a high-Z element. Due to the high K-absorption edge, the CT numbers of high-Z stains decrease from the low energy to the high energy scan, so that high-Z stains do not contribute to the spectral contrast when imaged with, for example, a 40 kVp/80 kVp or 50 kVp/90 kVp energy pair (Figure 2B).

2 Methods

Several fundamentally different approaches exist for acquiring and processing dual-energy scans. The data presented here were generated using a sequential scan acquisition approach and material decomposition, which extracts three material fractions from two scan energies, as described by Badea et al. [46], [72]. Details on all image processing steps, including intensity scaling, image registration, and material decomposition have been reported previously [41], [55], [71], so they will only be summarised briefly here. Technical details for all presented datasets, including sample preparation and image acquisition, are provided in the Supplementary Materials (Supplementary Tables 1 and 2).

2.1 Image acquisition

All datasets were acquired using an XRadia MicroXCT-400 (Carl Zeiss X-ray Microscopy, Pleasanton, CA, USA) equipped with a 150 kVp Hamamatsu microfocus source (Hamamatsu Photonics, Shizuoka, Japan) with a tungsten target and an operational range of 40–150 kVp. Tomographic dual-energy datasets were acquired using sequential scan acquisition with either the 0.4X or 4X detector assembly. Peak voltages and X-ray filters were selected according to sample composition to ensure adequate spectral contrast. The exposure time for each scan was adjusted to achieve a similar signal-to-noise ratio for both scan energies. Calcium/iodine and tungsten/iodine were imaged with a 40 kVp/80 kVp energy pair [41], [71] and calcium/ruthenium was imaged with a 40 kVp/60 kVp energy pair [55]. For the calcium/barium/tungsten triple-energy CT example, three scans were acquired using peak voltages of 45 kVp, 80 kVp, and 140 kVp. This followed previously published data on the spectral separation of calcium and barium using a 45 kVp/80 kVp energy pair [71], and data on the spectral separation of calcium and tungsten using 80 kVp and tin-filtered 140 kVp spectra [47]. Image volumes were reconstructed using XMReconstructor software and exported in *.TXM format. Depending on the sample size, the voxel size in the reconstructed datasets ranged from 4.33 μm to 60.62 μm (see Supplementary Table 2).

2.2 Processing of tomography data and basis material decomposition

The reconstructed microCT volumes were imported into Amira 3D software (FEI Visualization Sciences Group, part of Thermo Fisher Scientific, Mérignac Cédex, France, versions 2019–2023). Image intensities were standardised either to CT numbers (Hounsfield units, HU) using water and air as references [41], or to pseudo CT numbers (pseudo-HU) using the respective scanning medium and air as references [71]. CT numbers were used when samples were mounted in distilled water. When samples were mounted in a medium other than distilled water, such as absolute ethanol or agarose, pseudo CT numbers were used. Thus, the grey value for the respective mounting medium was always zero for both scan energies. Next, the datasets were oriented and co-registered using the *Register images* tool with a rigid transformation based on normalised mutual information. Then, the datasets were resampled using the *Resample Transformed Image* tool with Lanczos interpolation [41], [71]. Co-registration was necessary due to slight shifts in the order of a few pixels between the two scans, caused by sample drift [71]. After registration and resampling, a voxel-to-voxel correspondence was established between the two scan energies. The registered and resampled dual-energy scans were filtered to reduce image noise and saved in TIF format [41], [71]. The values for the material decomposition procedure were derived from the registered datasets based on the joint dual-energy attenuation profiles [41]. Subsequently, the dual-energy scans underwent basis material decomposition to extract three material fractions from the two scan energies [46]. Basis material decomposition was carried out using the custom MATLAB program DECTDec (<https://github.com/microDECT/DECTDec> [41]). The decomposed material fractions were saved in TIF format again.

2.3 Data segmentation and visualisation

Colour overlay images (slices, thick-slice maximum intensity projections) from scan energies and extracted material fractions were generated using FIJI ImageJ [73]. Volume renderings were created using Amira versions 2019–2023. Automated and semi-manual segmentation (Figure 5) was performed using the Amira *Segmentation editor*.

3 MicroDECT applications

The use of microDECT in addition to conventional microCT imaging can be advantageous for addressing a broad range of research questions. In image acquisition, dual-energy

imaging means that the scan time is approximately doubled (making image acquisition longer and more costly). Additionally, the processing of raw tomography data is more complex and time-consuming. However, once image acquisition and data processing are completed, dual-energy data offer two major advantages over conventional CT data. First, dual-energy data offer many additional possibilities for more efficient visualisation, segmentation, and data analysis. Second, information about the distribution of different elements in the sample can provide new insights into the material composition of the sample. All of this is discussed in detail in the following sections on various current and future microDECT applications in animal tissue imaging.

3.1 Biomedical research and preclinical phenotyping

3.1.1 Vascular phenotyping

For microCT-based detection of vessels in preclinical animal models, contrast enhancement by perfusion of the vasculature with an X-ray dense compound is of paramount importance. Several commercially available contrast agents facilitate high-resolution vascular imaging within soft tissues [50], [61]–[65]. A common challenge in vascular imaging occurs when blood vessels are located in close proximity to bone tissue. Limited differences in X-ray attenuation between perfusion contrast agents and mineralised bone impede their distinct segmentation in conventional microCT. Establishing a universal imaging approach or contrast agent that would provide sufficient radiopacity contrast relative to mineralised bone in preclinical research has proven challenging. The limitation arises from the variation in bone mineral density and therefore radiopacity in available small and large animal models [74], [75]. A decalcification step can partially mitigate this issue by facilitating segmentation of the contrast-enhanced vasculature. However, such a decalcification step prevents the simultaneous assessment of bone microstructure and vascular morphology, making it unsuitable for studies where both vascular and bone tissue are of interest. MicroDECT offers the possibility to distinguish and separate perfused vessels from closely adjacent bone based on their X-ray spectral properties (Figure 3A–D) [50], [71]. Two widely used contrast agents, the iodine compound μ Angiofil and the barium compound Micropaque, are K-edge stains and thus have ideal spectral properties for separation from bone [71]. Many research areas could benefit from an improved three-dimensional imaging of the vasculature within bone tissue, including bone biology, bone metastasis, tissue engineering,

implantology, guided bone regeneration, and reconstructive surgery [76]–[79].

As a specific example, microDECT imaging could improve the three-dimensional analysis of the vascular supply of the inner ear (Figure 3I–L). The mammalian inner ear is a complex sensory organ, with the spiral cochlea analysing auditory stimuli and the vestibular system sensing balance. Contrast-enhanced microCT imaging has been shown to visualise bone, fluid spaces, delicate membranes, and the vestibulocochlear nerve, as well as bionic implants, at high resolution [80]–[82]. The cochlea contains the densest capillary network in mammals. Age deteriorates systemic blood perfusion and may affect cochlear metabolism and hearing [83], [84]. Quantification of the vascular tree with fully automated tools [85] can enable to detect changes and correlate them with audiometric data. However, this requires high quality data sets. As sample preparation and contrasting procedures largely overlap with electron microscopic techniques [81], further correlative analysis allows histological processing down to the ultrastructural level [86] to detect e.g. a thickening of the capillary basal lamina [87] and arteriosclerotic changes [88].

MicroDECT can also be used to separate perfused vessels from tissues that have been contrasted with a high-Z stain such as phosphotungstic acid (PTA). Counterstaining provides an anatomical soft tissue context for interpretation and analysis of the microvasculature (Figure 3E–H) [71]. This allows, for example, analysis of the vasculature for specific brain compartments [71]. Other applications of microDECT for imaging the vasculature in the presence of two or more radiopaque materials remain largely unexplored. MicroDECT may offer advantages for imaging in guided bone regeneration studies, even when metal implants are involved. In the case of implants, there may be two distinct advantages. MicroDECT may allow better detection and analysis of blood vessels. In addition, microDECT reduces beam hardening artefacts by producing virtual monochromatic images (see the Outlook section for a more detailed discussion). This may provide improvement in quantitative bone analysis in the immediate vicinity of the implant, as metal-induced CT artefacts around implants can have a negative effect on such analyses [89], [90].

3.1.2 Phenotyping of skeletal development

Separating mineralised tissue from cartilage can be a common task in image data visualisation and segmentation. Gabner et al. [55] presented a protocol using an ethanol-based ruthenium red ($Z = 44$, K-edge at 22.12 keV) solution to stain cartilage matrix while preserving bone mineral.

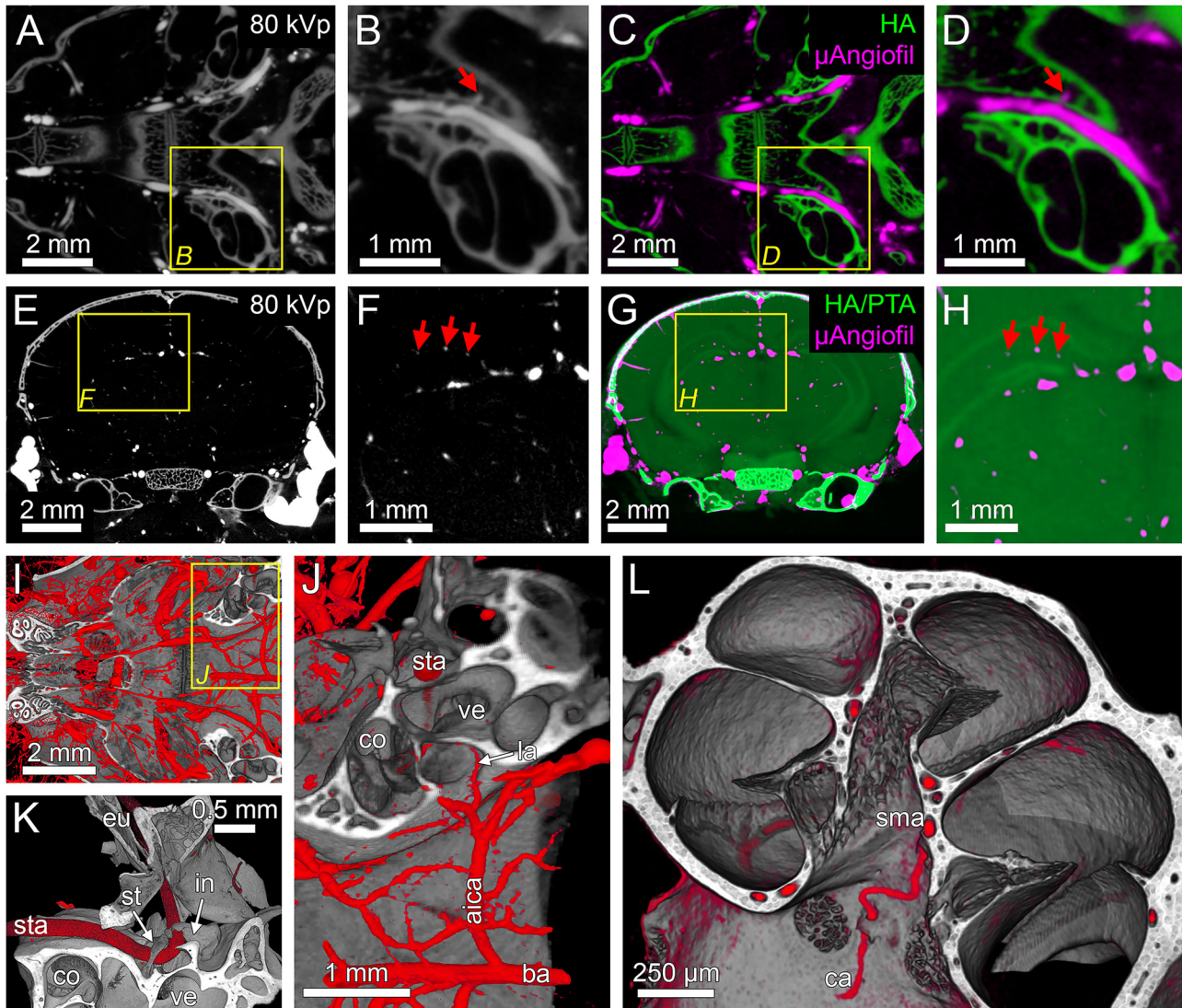


Figure 3: MicroDECT imaging of vascular perfusion specimens. (A–D) MicroDECT imaging allows spectral separation between bone mineral (hydroxyapatite, HA) and the iodine-based vascular casting agent μ Angiofil in the perfused mouse head (details on the sample and methods are reported in [71]). MicroDECT imaging enhances identification and segmentation of vasculature in the direct vicinity of bone (B vs. D, red arrow). (E–H) Counter-staining of soft tissue provides an anatomical context for the analysis of vasculature as exemplified in the hippocampus region of the brain. In a perfused sample without counterstaining (E, F), vessels are clearly visible, but due to the lack of anatomical reference information their precise localisation with reference to brain regions remains ambiguous. Phosphotungstic acid (PTA) counterstaining and microDECT imaging (G, H) provides the anatomical context, localizing the three highlighted vessels in the hippocampus (red arrows) (details on the sample and methods are reported in [71]). (I) Mouse skull base in a section at the level of the inner ear. (J) Higher magnified field of view of framed area in I show the inner ear blood supplying arterial branches. (K) Stapedial artery (sta) course through the middle ear. (L) Midmodiolar section through a mouse cochlea with the cochlear artery (ca) transit into the spiral modiolar artery (sma). Lateral wall capillary network was not infiltrated here and may require adapted perfusion techniques. aica, anterior inferior cerebellar artery; ba, basilar artery; ca, cochlear artery; co, cochlea; eu, Eustachian tube; in, incus; la, labyrinthine artery; sma, spiral modiolar artery; st, stapes; sta, stapedial artery; ve, vestibulum.

Using a 40 kVp/60 kVp energy pair, the stained cartilage can be spectrally separated from the intrinsic contrast of the mineralised tissue. In the original publication, data were reported only for comparatively small samples such as a mouse fetus at embryonic day 16.5 (E16.5) or a severed forelimb of an E14 chicken embryo. More recently, we have also explored the applicability of ruthenium red staining

to larger samples. Similar to traditional clearing and staining approaches, skinning, evisceration, and enucleation are recommended when staining larger samples. Current data, reported here for the first time, indicate that skinning allows at least staining of samples several centimetres in size, such as an E14 chicken embryo in toto, and the head and forelimb of a stillborn cat at postnatal day 0 (Figure 4).

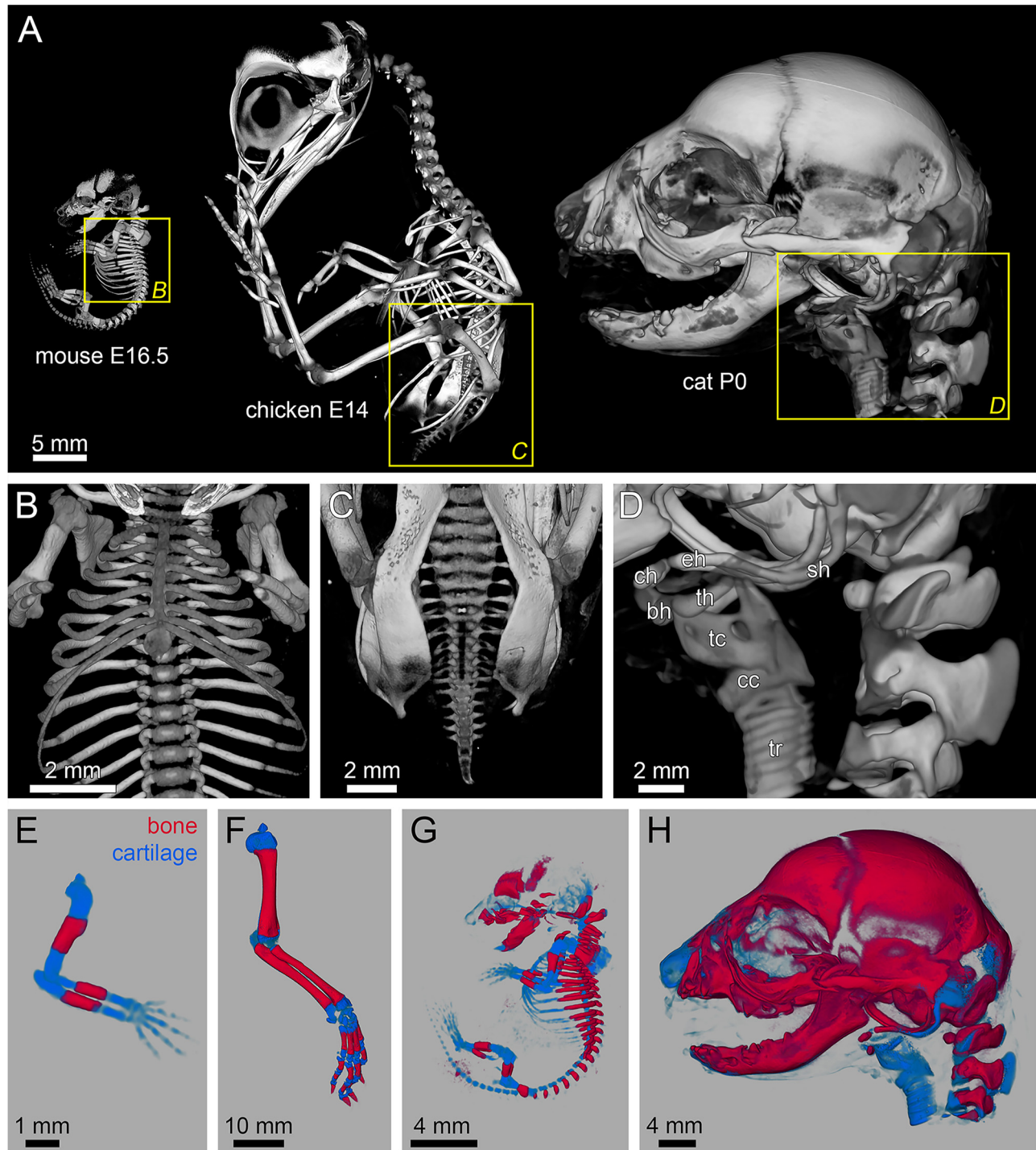


Figure 4: Cartilage staining and microDECT imaging for phenotyping of skeletal development. (A–D) Volume renderings of a mouse fetus at embryonic day 16.5 (E16.5, details on the sample are reported in [20]), an E14 chicken embryo, and the head of a stillborn cat at postnatal day 0 (P0) stained with ruthenium red and imaged with a 60 kVp/133 μ A X-ray spectrum. The E16.5 mouse was stained including the skin, while the chicken embryo was completely skinned, eviscerated, and enucleated before staining. The P0 cat head was also completely skinned before staining but not enucleated. Anatomical details, especially of the cartilaginous skeletal elements are exquisite, as shown on the mouse ribcage (B) and the developing synsacrum of the chicken (C). In the laryngeal region of the cat (D), in addition to the hyoid (sh, stylohyoid; eh, epihyoid; ch, ceratohyoid; bh, basihyoid; th, thyreohyoid), the thyroid cartilage (tc), the cricoid cartilage (cc), and the trachea (tr) are shown. (E–H) Two-channel volume rendering of bone and cartilage based on microDECT scans of the right forelimb of an E16.5 mouse (E) and a P0 cat (F), an E16.5 mouse in toto (G), and the head of a P0 cat (H). The two-colour maps were chosen to closely resemble the colours of the traditional clearing and staining method (bone, alizarin red; cartilage, alcian blue).

MicroCT and microDECT are valuable for studying normal skeletal development in vertebrates, and for the analysis of skeletal developmental defects in toxicity studies as well as the detection of malformations in knock-out models. Skeletal alterations are often described in the thorax with malformed elements of the rib cage, changes in the number of ribs, and delayed ossification using clearing and staining approaches [91]–[94]. While changes can be detected in the osseous parts of the hyoid [95], [96], analysis of the cartilaginous parts of the hyoid as well as the larynx is much more challenging. Especially for complex 3D structures and hidden skeletal elements (e.g., the arytenoid cartilages of the larynx), tomographic analyses that allow visualisation of the specimen from various angles as well as virtual sections are superior to traditional clearing and staining approaches.

While microDECT data on skeletal development generated so far are promising, image acquisition needs to be further improved. The currently used 40 kVp/60 kVp energy pair allows spectral separation, but at the expense of lower image quality and resolution in the ruthenium material fraction compared to the original 60 kVp scan. This loss in image quality is both related to the higher level of image noise when imaging with a 40 kVp spectrum filtered with a 0.1 mm molybdenum filter [55], and the suboptimal spectral contrast for calcium versus ruthenium when imaged with a 40 kVp/60 kVp energy pair. A 30 kVp/60 kVp setting may be more suitable for the separation of calcium and ruthenium, and more experiments are required to optimise scan settings for ruthenium red samples. In addition, the general staining intensity of ruthenium red is rather weak (400–900 HU when imaged at 60 kVp [55]). Other positively charged contrast agents should be evaluated for potential improvement of staining intensity, spectral properties, and thus image quality.

3.1.3 Other biomedical applications

There are many other potential applications for microDECT in three-dimensional imaging of tissue biopsies. As mentioned above, microDECT is well suited for distinguishing between mineralisation (intrinsic sample contrast) and soft tissue stained with a contrast agent such as iodine. Calcifications in tumour biopsies [97], [98] could be imaged in their *in situ* tissue context. Macro calcifications [99]–[101] in blood vessels could be analysed in relation to general soft tissue anatomy of the affected part of the vascular system. In addition, 3D cell culture models such as mineralised spheroids [102] could be examined in terms of both their mineralised and non-mineralised parts, combining a quantitative analysis of mineral distribution and concentration

with reference to the overall spheroid size and shape. All these applications have been unexplored until now.

In addition to analysing mineralisation in biomedical tissue samples, microDECT could be a generally useful tool for studying double-stained soft tissue biopsies. Different stains bind to different tissue components and these binding properties can be used to create spectral contrast in biopsy specimens. One case that has been studied involves the differences in binding properties between Lugol's iodine potassium iodide solution (I_2KI) and aqueous phosphotungstic acid (PTA) [41]. Double staining with I_2KI and PTA provides intriguing options for multilayered vertebrate tissues because I_2KI has a high affinity for binding to fat cells, which are not stained at all by PTA. Conversely, PTA has a high affinity for binding to collagen-rich connective tissue, which is typically very weakly stained by I_2KI . As mentioned above, the spectral properties of iodine and tungsten are ideal for microDECT imaging with conventional laboratory X-ray sources using a 40 kVp/80 kVp energy pair. This allows for the generation of very distinct colour images of heterogeneous tissue biopsies, such as skin samples [41].

Further testing and method development are required to optimise double staining approaches for microDECT. In tissue biopsies, including pathological samples such as tumour biopsies, the combination of a nuclear-specific stain with a cytoplasmic stain, analogous to H&E (haematoxylin & eosin) staining in histological sections, could be a highly attractive approach. Both, a modified lead-based haematein staining [59] and eosin Y (containing bromine) [57] are effective X-ray stains. However, their spectral separation is challenging, due to the very high (lead) and low (bromine) K-absorption edges. It remains to be tested whether an optimised microDECT imaging protocol can be established for this element pair. Alternatively, I_2KI may be established as a cytoplasmic X-ray counterstain for lead-based haematein labelling of cell nuclei, given its similar tissue-binding properties to eosin.

3.2 Comparative vertebrate morphology

Since the introduction of X-ray dense contrast agents, microCT has been the most widely used modality for high-resolution 3D imaging of vertebrate morphology. In particular, iodine-based immersion staining methods based on Lugol's iodine-potassium iodide solution (I_2KI) or elemental iodine in absolute ethanol (I_2E) [2], [3] have spread rapidly in the community. These staining techniques are inexpensive, have good tissue diffusion properties, and provide high tissue contrast. To date, they are widely used and summarised under the term diffusible iodine-based contrast-enhanced

CT [103]–[105]. Indeed, iodine-stained specimens provide exquisite anatomical information, facilitating comprehensive analysis of both the musculoskeletal system [106], [107] and internal organs [106]. However, iodine staining has limitations, including significant tissue shrinkage [6]–[8] and decalcification when applied in an aqueous medium [8], [108]. These adverse effects can be compensated for, as tissue shrinkage can be minimised by using buffered staining solutions [9] and decalcification can be minimised by using an ethanolic iodine solution [108].

There is another limitation with iodine samples, which is less problematic for biological interpretation of the images but can still complicate downstream processing of the acquired datasets. Iodine-stained tissue can attenuate X-rays as much as bone, leading to an intensity overlap (see, e.g., [41]) which complicates segmentation and visualisation. In unstained vertebrate specimens, the mineralised skeleton can be easily visualised and segmented using automated threshold segmentation [109], [110]. However, in iodine-stained specimens, this is often not possible and a lot of manual or semi-manual effort is required to obtain a clean segmentation mask of the mineralised skeleton. This limitation can be overcome by scanning the specimen in an unstained state, followed by a second scan after iodine staining [109]–[111]. However, this procedure can sometimes suffer from sample deformations that occur during the staining process, either due to repeated sample handling, manipulation, and mounting, or due to tissue shrinkage, or both. Finally, these deformations can lead to registration errors between the two datasets [112].

This is greatly facilitated by the use of microDECT imaging of iodine-stained samples. Basis material decomposition allows the extraction of a hydroxyapatite mineral fraction [41], which can be visualised and segmented like mineralised tissue in unstained specimens. In addition, basis material decomposition provides an iodine and a background fraction (‘water channel’ in [41]) allowing for improved tissue segmentation. All this information is available without registration errors. Musculature and internal organs can be segmented either from the original CT data or from the iodine and background fractions. Tissues with low iodine uptake, such as cartilage, are often best visible in the background fraction, which is similar to a brightfield image and typically shows minimal beam hardening artefacts. 3D software packages such as Thermo Scientific™ Amira allow simultaneous loading of the five relevant data sets (e.g. low energy scan, high energy scan, hydroxyapatite mineral fraction, iodine fraction, background fraction) and the experimenter can easily switch between them during the segmentation process. This facilitates effective segmentation of

different structures by combining automated, semi-manual, and manual segmentation tools (Figure 5).

MicroDECT data have been used in a variety of vertebrate taxa, including teleosts [113], frogs [114], salamanders [115], [116], lizards [117], and turtles [118]. Despite the doubled scanning time and the increased time and effort in processing the scan data, this extra effort is often worthwhile, as it substantially improves the downstream segmentation and analysis procedures, which are typically tedious and time-consuming.

3.3 Invertebrate morphology

Similar to vertebrate morphology, microCT imaging of iodine-, and PTA-stained samples has been also widely used for invertebrates [3], and critical point drying of stained specimens has been shown to further improve tissue contrast [119]. Because microDECT imaging with laboratory microCT scanners has not been used to study invertebrate morphology, this section will briefly discuss potential applications. Like vertebrates, many invertebrate taxa, such as molluscs, bryozoans, brachiopods, echinoderms, sponges, or arthropods may possess a mineralised skeleton. Invertebrate skeletons are mostly made up of materials such as calcium carbonate, which, like the calcium phosphate hydroxyapatite of vertebrate skeletons, are based on low-Z elements. The exoskeleton of arthropods (the cuticle) is a composite of proteins and chitin and may be calcified or reinforced by minerals. Mineralised invertebrate skeletons are therefore well suited for microDECT imaging when combined with a K-edge stain such as iodine and imaged with a 40 kVp/80 kVp energy pair. Two examples illustrate this.

The first example is a piece of a feather star (Echinodermata, Crinoidea, Comatulida) arm, stained with I_2E . Feather stars have five-ray symmetry and move freely with their arms. Their body is divided into a central trunk and arms. Their internal organs are mainly located in the trunk, but also radiate into the arms. The arms usually have branches called pinnulae, which in many species contain mature gonads [120], [121]. Sea feathers, like echinoderms in general, have a calcareous internal skeleton of individual ossicles, which are either plate-shaped (trunk) or form voluminous “vertebrae-like” elements (arms). These ossicles consist of a 3D trabecular network, called the stereome [122], and are covered by a thin layer of tissue toward the body surface. Muscles, allowing active movements, connect the ossicles of the arms. Due to the highly mineralised skeleton, microCT is a suitable technique for 3D imaging of crinoids (and echinoderms in general), allowing simultaneous assessment of skeletal and stained soft tissue structures [123]. Using microDECT imaging, the calcified ossicles can

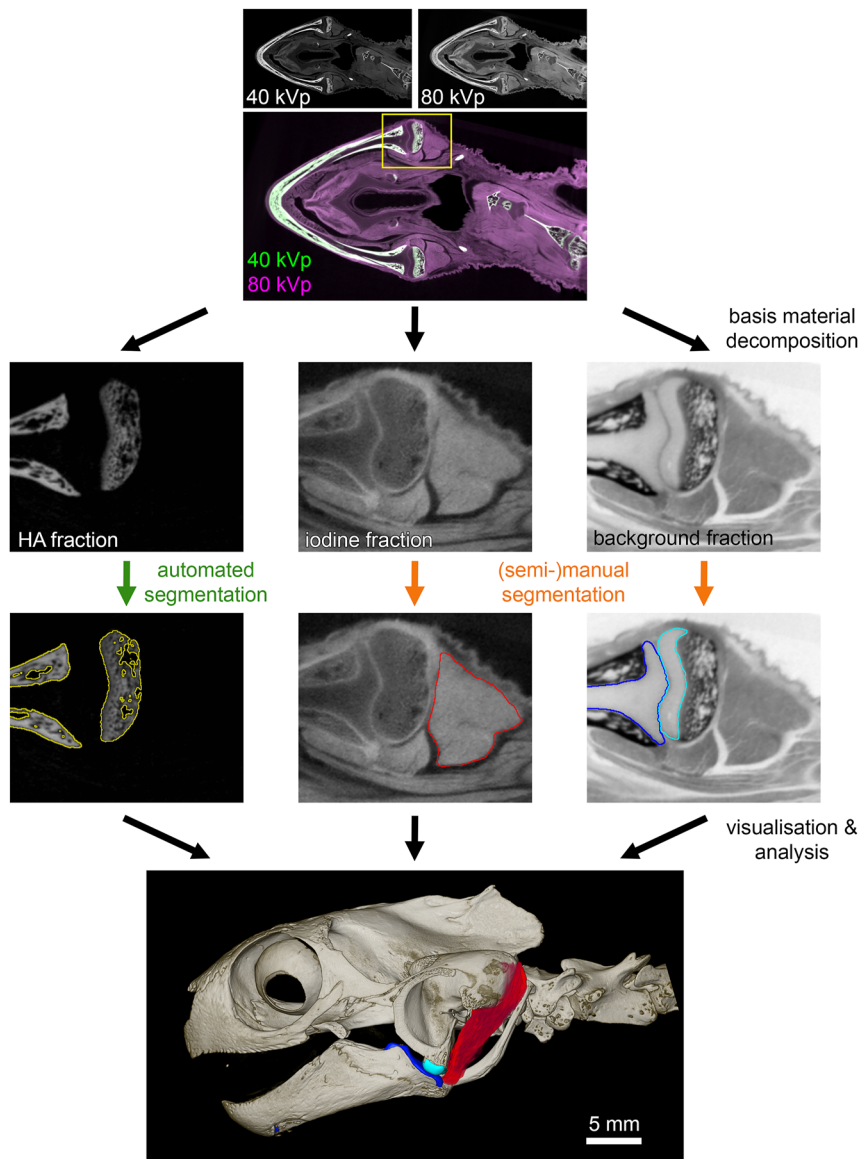


Figure 5: MicroDECT imaging in comparative vertebrate morphology. In iodine-stained specimens, such as the head of a turtle (*Cuora sp.*) stained with elemental iodine in absolute ethanol (I_2E), material decomposition allows extraction of a hydroxyapatite (HA), iodine and background (here: ethanol) fraction. This offers unique possibilities for segmentation and visualisation of the data sets. The mineralised skeleton (HA fraction) can be easily rendered or segmented by automatic thresholding. The iodine and background fractions can be used for manual and semi-manual segmentation of non-mineralised tissues such as musculature or cartilage. In particular, tissues that show little or no iodine uptake can often be segmented efficiently in the background fraction. Finally, segmentations from different material fractions are combined in a single rendering. In this case, the ossified skull is visualised together with the M. depressor mandibulae (red), Meckel's cartilage (blue), and the quadrate cartilage (turquoise).

be separated from iodine-stained tissues such as muscles or gonads (Figure 6A–D). Identification of soft tissue structures in close proximity to the ossicles is also improved. The calcified skeleton has been an obstacle to the study of soft tissue anatomy using physical sectioning techniques [120] as well as non-invasive tomographic methods. Therefore, the ability to separate calcified portions from soft tissue in morphologic analyses offers a significant improvement.

The second example is the mineralised cuticle of crustaceans. The arthropod cuticle consists of an endo-, exo-, and epicuticle that is hardened by the deposition of sclerotizing substances that form exoskeletal sclerites [124], [125]. While the epicuticle is usually composed of lipids, proteins, tanned lipoproteins and an outer wax layer, the endo- and exocuticle are composed of proteins and chitin [126]. The exocuticle is more sclerotised mainly due to fibre reinforcement and



Figure 6: MicroDECT imaging of invertebrate morphology. (A–D) Part of a sea feather arm (*Anthometra adriani*) stained with elemental iodine in absolute ethanol (I₂E). (A) Extracted material fractions allow to visualise the mineralised skeleton (top), stained soft tissue (middle), or both in a colour composite (bottom). Some arm ossicles (ao) show predetermined breaking points, the so-called syzygies (syz). (B) Volume renderings of the two material fractions with parts of the fractions longitudinally cropped. (C) Cross section of a single pinnula in an overview scan (top), high-res region of interest (ROI) scan (middle), and extracted material fractions (bottom). In the overview scan, anatomical features such as sacculi (pink arrowhead) and

cross-linking of proteins, whereas the endocuticle is more flexible. In addition, incorporation of e.g. calcium carbonate or metals can further increase cuticle hardening [127], [128]. The presence of calcium carbonate in the arthropod cuticle has previously been analysed with synchrotron-based microCT (SR μ CT) in crustaceans [129], [130]. Amphipods, which also incorporate calcium carbonate into their cuticle, are a perfect crustacean example for microDECT imaging. Combined with ethanolic iodine (I₂E) based soft tissue contrast, microDECT allows a clear separation of the mineralised cuticle from internal, non-mineralised and iodine-stained features such as musculature (Figure 6E–G).

MicroCT imaging of invertebrate soft tissue morphology has been used extensively to study anatomic details in groups such as arthropods (see e.g. [119], [131], [132]). In the future, microDECT imaging could be used to analyse double-stained invertebrate specimens to obtain more detailed information on tissue composition and to provide new options for data segmentation and visualisation. This is exemplified by two body regions of an insect with a non-mineralised cuticle stained with I₂KI followed by PTA. In the locust head, the highly sclerotised cuticle of the head capsule, the mouthparts, and the tentorium are only stained with I₂KI and thus visible in the iodine fraction, while all other tissues are predominantly stained with PTA (Figure 6H). MicroDECT imaging of this double-staining allows automatic separation of the highly sclerotised head capsule from non-sclerotised structures (Figure 6I), providing new options for imaging features such as the internal tentorium, to which musculature is attached [126].

The same iodine/tungsten double stain approach provides a similar pattern in the locust hindleg joint (Figure 6J). Their metathoracic legs are primarily used for jumping, which is achieved by rapid extension of the tibia [133]. It has been shown that energy is stored in deformable parts of the femur-tibia joint, specifically in the semilunar process [134], [135], which is a sickle-shaped, highly sclerotised and thickened cuticular area (Figure 6K). Comparing the iodine and tungsten material fractions gives strikingly different results. Iodine staining provides a homogeneous representation of the outer portion of the cuticle. In contrast, tungsten staining is restricted to the inner parts of the cuticle, providing much more details that are likely related to different cuticle properties that may correlate with different cuticular layers (compare arrows and sections and surfaces in Figure 6K). Stamm and Dirks [125] successfully visualised iodine-stained endo- and exocuticle using contrast stretching and multilevel Otsu-thresholding [73]. It would be worthwhile to compare our results with the data reported by [125], which may confirm the results of iodine and tungsten fraction imaging.

These preliminary data on double-stained invertebrate specimens show that microDECT imaging can successfully separate the outer parts of the arthropod cuticle from the inner cuticle layers and soft body tissues (Figure 6K–L). We hypothesise that this is due to the inability of the PTA stain to bind in the exocuticle and, more generally, in highly sclerotised parts of the cuticle. However, this observation requires further investigation and verification by complementary methods. Certainly, this staining pattern offers some clear

tube feet ossicles (green arrowhead) show similar X-ray attenuation and are therefore hard to distinguish. Due to higher spatial resolution in the ROI scan, the individual structures are easier to recognise. Extracted material fractions further improve the identification and visualisation of these structures. (D) Volume renderings of both material fractions from the high-res ROI scan of a single pinnula with parts of the fraction cropped (two lower images). Sectioning plane of the middle image shown in top image. (E–F) Anterior part of an amphipod crustacean (*Dikerogammarus villosus*) stained with I₂E. (E) Midsagittal section through head and pereopod (thick slice maximum intensity projection (MIP)). (F) Volume rendering of the two material fractions showing a virtual cross-section of the head. (G) Volume rendering of the two material fractions showing the musculature of the second gnathopods. (H–I) Head of a locust (*Schistocerca gregaria*) stained with I₂KI and PTA. (H) Midsagittal section through the head (thick slice MIP). Note that I₂KI staining is mainly present in sclerotised cuticular areas like the head capsule, the mandible and the tentorium. Weaker sclerotised areas and soft tissue like the brain and musculature are predominantly visible in the PTA fraction. Inset: Single virtual section of the dorsal mandible. Note the insertion (arrow) of the dma tendon (PTA fraction) to the mandible (iodine fraction). Here material differences occur, probably a softer insertion of collagen-rich connective tissue to the heavily sclerotised mandible. (I) Volume rendering of the ventral part of the head (I₂KI fraction in grey) and a sagittal section of the dorsal part of the head (PTA fraction in orange) in a frontolateral view. In the I₂KI fraction, sclerotised parts (head capsule, tentorium) are clearly separated. Besides soft tissue, the PTA fraction contains also the endocuticle. (J–L) Metathoracic leg with femur-tibia joint of *Schistocerca gregaria* stained with I₂KI and PTA. (J) Horizontal section through both podomeres (thick slice MIP (left) and single slice (right)). I₂KI stains the outer and presumably more sclerotised region of the cuticle (asterisks), while PTA stains the inner and presumably softer region of the cuticle (arrowheads). Internal structures like muscles and haemolymph are mainly visible in the PTA fraction. (K) Volume renderings of the two isolated material fractions show the femur-tibia joint in semilateral view. The I₂KI fraction provides a more or less homogeneous representation of the external cuticle, while the PTA fraction provides much more details that probably are linked to different endocuticular properties (compare arrows). (L) Volume rendering of the two material fractions at the femur-tibia joint. A virtual sagittal cut again allows to discriminate between the external I₂KI-stained (asterisks) and the internal PTA-stained (arrowheads) portions of the cuticle. a1, antenna 1; a2, antenna 2; ald, antennal levator and depressor; am, arm muscle; an, arm nerve; ao, arm ossicle; at, anterior tentorial arm; br, brain; cl, clypeus; cp, cover plate; da, dactylus; dma, dorsal mandibular adductor; eg, egg cells; ex, extensor; fl, flexor; gn1, gnathopod 1; gn2, gnathopod 2; lb, labrum; lr, labral retractor; md, mandible; pi, pinnulae; po, pinnular ossicle; pr, propodus; pt, posterior tentorial arms; sa, sacculi; slp, semi-lunar process; syz, syzygy; to, tube feet ossicle.

advantages for data segmentation and visualisation, potentially facilitating the creation of 3D models that can be used in biomechanical research. In addition, the ability to quickly analyse sclerotised structures such as the tentorium is helpful for data visualisation, as this would require tedious segmentation in conventional microCT datasets. While we have successfully applied microDECT imaging to echinoderms and arthropods, it remains to be tested on a variety of other taxa, especially those with calcified skeletons versus soft tissues, such as molluscs or brachiopods.

4 Conclusions and Outlook

Dual-energy CT imaging has been used extensively in medical imaging [136]–[140] and preclinical imaging of small animal models [46], [141]–[143]. Dual-energy imaging has also been used for microscopic applications [41], [55], [70], [71], [114], [116], [144]–[149], however, considering the potential of the method, it still seems to be underutilised in many research areas. Over the past decade, we have investigated sample preparation and imaging protocols for microDECT imaging of various types of animal tissues. Based on our findings and the data presented here, we propose that microDECT offers unique advantages over conventional microCT imaging in both data visualisation and analysis.

While the examples given here intended to highlight potential applications, the question remains as to why microDECT imaging to date is not yet widely used in the community. A key reason might be the lack of commercial microCT software to efficiently acquire and process dual-energy datasets. If microCT vendors would like to make dual-energy imaging more accessible to routine users, they should consider the following three points.

- (1) MicroCT image acquisition software should integrate dual-energy imaging protocols for common element pairs such as calcium/iodine, calcium/barium, iodine/tungsten, and several others. Based on the known X-ray attenuation profiles for each of these elements [66], the acquisition software should recommend optimal scan settings in terms of peak voltages and X-ray filters to achieve optimal spectral separation for two elements or materials. In principle, appropriate peak voltages and filters can be empirically determined. For element pairs that are difficult to separate, numerical simulation may be required to find optimised scan settings [50]. Additionally, vendors should also consider providing a wider range of X-ray filters, including not only common materials such as aluminium, copper, and brass, but also less familiar metal foils such as tin, silver, tungsten, lead, or molybdenum.
- (2) The image reconstruction software provided by vendors should include functions for accurate co-registration and resampling to achieve voxel-to-voxel correspondence between dual-energy data sets. Since the offset between the two scans is typically only a few voxels, this procedure can be fully automated. This functionality is already implemented in the software of some vendors.
- (3) Finally, vendors should consider providing more versatile software toolboxes to facilitate downstream processing of dual-energy data. Most importantly, in the future, this should include basis material decomposition of dual-energy scans into material fractions [41], [46]. This process could be fully automated for the most common element pairs for given dual-energy protocols (i.e., peak voltages, filters, etc.). The basis material decomposition is a matrix operation that includes the linear attenuation coefficients of the base materials [46], [50]. These values can be measured empirically, either from calibration phantoms or directly from samples [41]. Providing linear attenuation values for the most common elements and scan energies in vendor software would help scientists to perform successful dual-energy imaging experiments. Currently, vendor software available for processing dual-energy data mainly focuses on segmentation functions based on dual-energy 2D histograms. A dual-energy 2D histogram is a specific type of joint histogram that represents the distribution of intensity values in two registered scan energies. This plot can be used to specify regions of interest to create segmentation masks (see e.g. [150], [151]). Examples of software that implement dual-energy 2D histogram segmentation include the Zeiss Xradia Versa DSCoVer tool and the Bruker DEhist software. These tools provide powerful data segmentation options and have been shown to perform exceptionally well for samples containing distinct material phases. In such a scenario, 2D histogram segmentation can even separate elements that have very similar atomic numbers. However, 2D histogram segmentation has its limitations as it (i) does not provide true volumetric material density maps and (ii) has limitations when material fractions overlap. For example, in biological tissue samples, tissue components that bind different contrast agents may colocalize within the same voxel. For such data, basis material decomposition will outperform 2D histogram segmentation.

Currently, researchers must perform most of the experimental planning (choice of dual-energy scan spectra),

data processing, registration, and especially basis material decomposition (see e.g. <https://github.com/microDECT/DECTDec> [41]) themselves, which requires both specialised software and at least basic programming skills. In contrast, spectral unmixing tools have been available for many years in the acquisition software of e.g. confocal microscopes [152], [153]. A user-friendly implementation of these features would greatly help to make microDECT imaging more accessible to routine CT users and thus would be a big step towards a wider use of this promising approach.

Spectral information provides new insights into CT data sets. Dual-energy imaging initially requires more effort and cost in image data acquisition (scan time doubles and raw data processing becomes more time-consuming). However, this additional effort is often worthwhile, as dual-energy datasets allow for easier data visualisation, segmentation, and analysis. Dual-energy imaging allows the separation of two known materials (plus a background fraction). In

some cases, it may even be beneficial to add a third scan energy. Triple-energy CT can improve the spectral separation accuracy when three materials are present [50]. Thus, it may enable the extraction of three material fractions (plus a background channel) at the microscopic level. This concept is here demonstrated using a mouse leg sample containing bone mineral, barium-perfused vasculature, and tungsten-stained soft tissue (Figure 7). While these preliminary data on triple energy imaging at the microscopic scale are promising, further validation is needed to better judge the quality of basis material decomposition procedure in triple energy datasets. Recently, a multi-energy approach to discriminate up to four contrast agents using energy-integrating detectors has been discussed [154]. Furthermore, novel algorithms may be used to decompose up to five materials from dual-energy scans [48].

Our previous work and this view article have focused primarily on extracting material fractions (i.e., volumetric

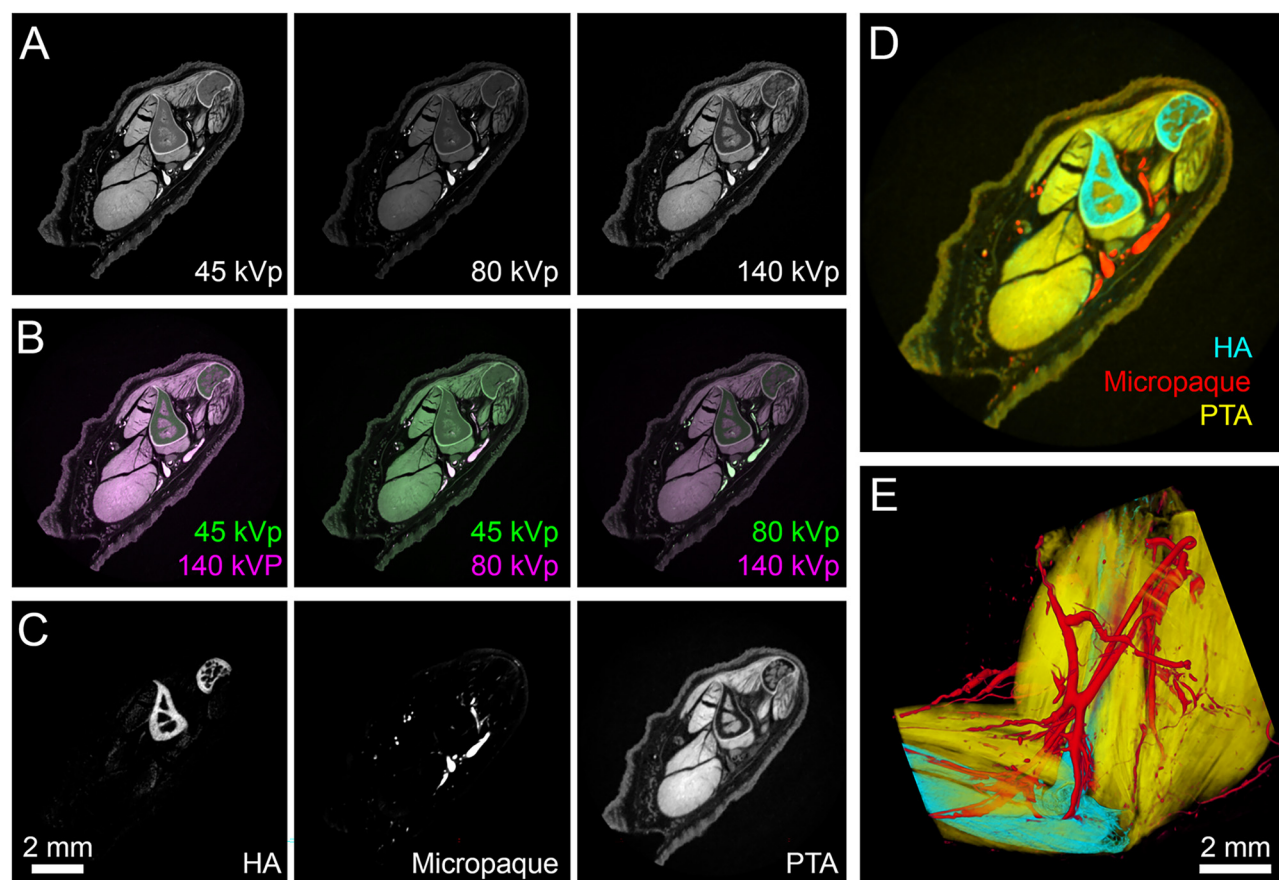


Figure 7: Microscopic triple-energy CT image of a Micropaque barium sulphate-perfused mouse limb counterstained with PTA. (A) Tomograms of the three scan energies. (B) Paired overlay images of two scan energies. In the 45 kVp/140 kVp overlay image, bone mineral (HA) contrasts with barium and tungsten. In the 45 kVp/80 kVp overlay image, barium contrasts with bone mineral and tungsten. In the 80 kVp/140 kVp overlay image, tungsten contrasts with bone mineral and barium. (C) Tomograms of the bone mineral (HA), barium sulphate (Micropaque), and PTA material fractions. Basis material decomposition can be performed either pairwise (dual-energy algorithm) or using a triple-energy algorithm. (D) Composite tomogram of the three extracted material fractions. (E) Volume rendering of the three material fractions.

maps of material density) from dual-energy scans. However, several other aspects of microscopic dual-energy X-ray imaging are worth further exploration. For instance, dual-energy CT scans can be used to generate virtual monochromatic images, which can help reduce beam hardening artefacts [155]–[157]. While virtual monochromatic spectral imaging has mainly been discussed in terms of improving data quality in clinical imaging, it also helps to reduce beam hardening artefacts in microCT datasets [148]. Furthermore, registered dual-energy datasets offer numerous new options for data segmentation, including direct segmentation on 2D histograms and machine learning pixel classification [41], [70], [71]. Most likely, dual-energy datasets will also provide new opportunities for segmentation using deep learning convolutional neural networks, as they provide richer information compared to conventional single-energy microCT scans.

We conclude that the future of CT is bright and colourful. For too long, we have neglected the hidden spectral dimension of our specimens. Spectral information collected using dual- or triple-energy approaches, together with the advent of commercial microCT scanners equipped with spectral detectors, such as the UniTOM XL (TESCAN, Brno, Czech Republic), which has a line detector delivering 128 energy bins over a range of 20–160 keV [37], or the MARS Spectral CT Scanner (MARS Bioimaging, Christchurch, New Zealand), which is equipped with a Medipix3RX photon-counting detector that allows for eight energy thresholds [158], will enhance our analysis of CT datasets and deepen our understanding of biological and biomedical samples in many applications.

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References

- [1] S. Handschuh and M. Glösmann, "Mouse embryo phenotyping using X-ray microCT," *Front. Cell Dev. Biol.*, vol. 10, 2022, Art. no. 949184.
- [2] B. D. Metscher, "MicroCT for developmental biology: A versatile tool for high-contrast 3D imaging at histological resolutions," *Dev. Dyn. Off. Publ. Am. Assoc. Anat.*, vol. 238, no. 3, pp. 632–640, 2009.
- [3] B. D. Metscher, "MicroCT for comparative morphology: Simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues," *BMC Physiol.*, vol. 9, p. 11, 2009.
- [4] R. Mizutani and Y. Suzuki, "X-ray microtomography in biology," *Micron*, vol. 43, nos. 2–3, pp. 104–115, 2012.
- [5] E. Pauwels, D. van Loo, P. Cornillie, L. Brabant, and L. van Hoorebeke, "An exploratory study of contrast agents for soft tissue visualization by means of high resolution X-ray computed tomography imaging," *J. Microsc. Oxford*, vol. 250, no. 1, pp. 21–31, 2013.
- [6] J. Buytaert, J. Goyens, D. de Greef, P. Aerts, and J. Dirckx, "Volume shrinkage of bone, brain and muscle tissue in sample preparation for Micro-CT and light sheet fluorescence microscopy (LSFM)," *Microsc. Microanal.*, vol. 20, no. 4, pp. 1208–1217, 2014.
- [7] P. Vickerton, J. Jarvis, and N. Jeffery, "Concentration-dependent specimen shrinkage in iodine-enhanced microCT," *J. Anat.*, vol. 223, no. 2, pp. 185–193, 2013.
- [8] P. Heimeel, *et al.*, "Iodine-enhanced Micro-CT imaging of soft tissue on the example of peripheral nerve regeneration," *Contrast Media Mol. Imaging*, vol. 2019, 2019, Art. no. 7483745.
- [9] Y. Dawood, *et al.*, "Reducing soft-tissue shrinkage artefacts caused by staining with Lugol's solution," *Sci. Rep. UK*, vol. 11, no. 1, 2021, Art. no. 19781.
- [10] M. D. Wong, S. Spring, and R. M. Henkelman, "Structural stabilization of tissue for embryo phenotyping using Micro-CT with iodine staining," *PLoS One*, vol. 8, no. 12, 2013, <https://doi.org/10.1371/journal.pone.0084321>.
- [11] M. Patzelt, *et al.*, "Ethanol fixation method for heart and lung imaging in micro-CT," *Jpn. J. Radiol.*, vol. 37, no. 6, pp. 500–510, 2019.
- [12] J. Dudak, *et al.*, "High-contrast X-ray micro-radiography and micro-CT of ex-vivo soft tissue murine organs utilizing ethanol fixation and large area photon-counting detector," *Sci. Rep.*, vol. 6, 2016, Art. no. 30385.
- [13] S. Handschuh, C. T. C. Okada, I. Walter, C. Aurich, and M. Glösmann, "An optimized workflow for microCT imaging of formalin-fixed and paraffin-embedded (FFPE) early equine embryos," *Anat., Histol., Embryol.*, vol. 51, no. 5, pp. 611–623, 2022.
- [14] O. Ermakova, T. Orsini, A. Gambadoro, F. Chiani, and G. P. Tocchini-Valentini, "Three-dimensional microCT imaging of murine embryonic development from immediate post-implantation to organogenesis: Application for phenotyping analysis of early embryonic lethality in mutant animals," *Mamm. Genome*, vol. 29, nos. 3–4, pp. 245–259, 2018.
- [15] M. Markel, *et al.*, "High resolution three-dimensional imaging and measurement of lung, heart, liver, and diaphragmatic development in the fetal rat based on micro-computed tomography (micro-CT)," *J. Anat.*, vol. 238, no. 4, pp. 1042–1054, 2021.

- [16] D. Paganin, S. C. Mayo, T. E. Gureyev, P. R. Miller, and S. W. Wilkins, "Simultaneous phase and amplitude extraction from a single defocused image of a homogeneous object," *J. Microsc. Oxford*, vol. 206, no. Pt 1, pp. 33–40, 2002.
- [17] P. Bidola, et al., "Application of sensitive, high-resolution imaging at a commercial lab-based X-ray micro-CT system using propagation-based phase retrieval," *J. Microsc.*, vol. 266, no. 2, pp. 211–220, 2017.
- [18] J. Romell, V. W. Jie, A. Miettinen, E. Baird, and H. M. Hertz, "Laboratory phase-contrast nanotomography of unstained *Bombus terrestris* compound eyes," *J. Microsc.*, vol. 283, no. 1, pp. 29–40, 2021.
- [19] G. Rodgers, et al., "Virtual histology of an entire mouse brain from formalin fixation to paraffin embedding. Part 1: Data acquisition, anatomical feature segmentation, tracking global volume and density changes," *J. Neurosci. Methods*, vol. 364, 2021, <https://doi.org/10.1016/j.jneumeth.2021.109354>.
- [20] J. P. Schlomka, et al., "Experimental feasibility of multi-energy photon-counting K-edge imaging in pre-clinical computed tomography," *Phys. Med. Biol.*, vol. 53, no. 15, pp. 4031–4047, 2008.
- [21] N. G. Anderson, et al., "Spectroscopic (multi-energy) CT distinguishes iodine and barium contrast material in MICE," *Eur. Radiol.*, vol. 20, no. 9, pp. 2126–2134, 2010.
- [22] J. Greffier, N. Villani, D. Defez, D. Dabli, and S. Si-Mohamed, "Spectral CT imaging: Technical principles of dual-energy CT and multi-energy photon-counting CT," *Diagn. interv. imaging*, vol. 104, no. 4, pp. 167–177, 2023.
- [23] M. F. Walsh, et al., "Spectral CT data acquisition with Medipix3.1," *J. Instrum.*, vol. 8, no. 10, p. P10012, 2013.
- [24] R. Ballabriga, M. Campbell, E. Heijne, X. Llopart, L. Tlustos, and W. Wong, "Medipix3: A 64k pixel detector readout chip working in single photon counting mode with improved spectrometric performance," *Nucl. Instrum. Methods Phys. Res. Sect. A Accel. Spectrom. Detect. Assoc. Equip.*, vol. 633, pp. S15–S18, 2011.
- [25] V. Di Trapani, et al., "Characterization of the acquisition modes implemented in Pixirad-1/Pixie-III X-ray detector: Effects of charge sharing correction on spectral resolution and image quality," *Nucl. Instrum. Methods Phys. Res. Sect. A Accel. Spectrom. Detect. Assoc. Equip.*, vol. 955, 2020, Art. no. 163220.
- [26] J. F. Marsh, et al., "Evaluation of a photon counting Medipix3RX cadmium zinc telluride spectral x-ray detector," *J. Med. Imaging (Bellingham Wash.)*, vol. 5, no. 4, 2018, Art. no. 43503.
- [27] S. Si-Mohamed, et al., "Improved peritoneal cavity and abdominal organ imaging using a biphasic contrast agent protocol and spectral photon counting computed tomography K-Edge imaging," *Invest. Radiol.*, vol. 53, no. 10, pp. 629–639, 2018.
- [28] M. Moghiseh, et al., "Spectral photon-counting molecular imaging for quantification of monoclonal antibody-conjugated gold nanoparticles targeted to lymphoma and breast cancer: An in vitro study," *Contrast Media Mol. Imaging*, vol. 2018, 2018, Art. no. 2136840.
- [29] V. Di Trapani, L. Brombal, and F. Brun, "Multi-material spectral photon-counting micro-CT with minimum residual decomposition and self-supervised deep denoising," *Opt. Express*, vol. 30, no. 24, pp. 42995–43011, 2022.
- [30] P. Seller, et al., "Pixellated Cd(Zn)Te high-energy X-ray instrument," *J. Instrum.*, vol. 6, no. 12, 2011, <https://doi.org/10.1088/1748-0221/6/12/C12009>.
- [31] R. Dinapoli, et al., "MÖNCH, a small pitch, integrating hybrid pixel detector for X-ray applications," *J. Instrum.*, vol. 9, no. 05, p. C05015, 2014.
- [32] A. Bergamaschi, et al., "The MÖNCH detector for soft X-ray, high-resolution, and energy resolved applications," *Synchrotron Radiat. News*, vol. 31, no. 6, pp. 11–15, 2018.
- [33] C. K. Egan, et al., "3D chemical imaging in the laboratory by hyperspectral X-ray computed tomography," *Sci. Rep. UK*, vol. 5, 2015, Art. no. 15979.
- [34] R. Warr, et al., "Enhanced hyperspectral tomography for bioimaging by spatio-spectral reconstruction," *Sci. Rep.*, vol. 11, no. 1, 2021, Art. no. 20818.
- [35] R. Warr, S. Handschuh, M. Glösmann, R. J. Cernik, and P. J. Withers, "Quantifying multiple stain distributions in bioimaging by hyperspectral X-ray tomography," *Sci. Rep.*, vol. 12, no. 1, 2022, Art. no. 21945.
- [36] J. R. A. Godinho, G. Westaway-Heaven, M. A. Boone, and A. D. Renno, "Spectral tomography for 3D element detection and mineral analysis," *Minerals*, vol. 11, no. 6, p. 598, 2021.
- [37] J. Sittner, et al., "Spectral X-ray computed micro tomography: 3-dimensional chemical imaging," *X-Ray Spectrom.*, vol. 50, no. 2, pp. 92–105, 2021.
- [38] M. D. Wilson, et al., "A 10 cm × 10 cm CdTe spectroscopic imaging detector based on the HEXITEC ASIC," *J. Instrum.*, vol. 10, no. 10, p. P10011, 2015.
- [39] D. Pennicard, S. Lange, S. Smoljanin, H. Hirsemann, and H. Graafsma, "LAMBDA — large area Medipix3-Based detector array," *J. Instrum.*, vol. 7, no. 11, p. C11009, 2012.
- [40] R. Aamir, et al., "MARS spectral molecular imaging of lamb tissue: Data collection and image analysis," *J. Instrum.*, vol. 9, no. 02, p. P02005, 2014.
- [41] S. Handschuh, C. J. Beisser, B. Ruthensteiner, and B. D. Metscher, "Microscopic dual-energy CT (microDECT): A flexible tool for multichannel ex vivo 3D imaging of biological specimens," *J. Microsc.*, vol. 267, no. 1, pp. 3–26, 2017.
- [42] X. Wang, D. Meier, K. Taguchi, D. J. Wagenaar, B. E. Patt, and E. C. Frey, "Material separation in x-ray CT with energy resolved photon-counting detectors," *Med. Phys.*, vol. 38, no. 3, pp. 1534–1546, 2011.
- [43] V. Sriskaran, et al., "High-rate, high-resolution single photon X-ray imaging: Medipix4, a large 4-side buttable pixel readout chip with high granularity and spectroscopic capabilities," *J. Instrum.*, vol. 19, no. 02, 2024, Art. no. P02024.
- [44] M. Ramilli, et al., "Measurements with MÖNCH, a 25 µm pixel pitch hybrid pixel detector," *J. Instrum.*, vol. 12, no. 01, p. C01071, 2017.
- [45] J. Dewanckele, W. de Boever, D. van Loo, B. Masschaele, and M. Boone, "Spectral Micro-CT imaging of minerals: Retrieving atomic information and density maps," *Microsc. Microanal.*, vol. 30, no. Supplement_1, 2024, <https://doi.org/10.1093/mam/ozae044.140>.
- [46] C. T. Badea, X. Guo, D. Clark, S. M. Johnston, C. D. Marshall, and C. A. Piantadosi, "Dual-energy micro-CT of the rodent lung," *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, vol. 302, no. 10, pp. L1088–L1097, 2012.
- [47] J. W. Lambert, Y. Sun, R. G. Gould, M. A. Ohliger, Z. Li, and B. M. Yeh, "An image-domain contrast material extraction method for dual-energy computed tomography," *Invest. Radiol.*, vol. 52, no. 4, pp. 245–254, 2017.

- [48] M. E. Lochschmidt, *et al.*, “Five material tissue decomposition by dual energy computed tomography,” *Sci. Rep.*, vol. 12, no. 1, 2022, Art. no. 17117.
- [49] T. R. Johnson, “Dual-energy CT: General principles,” *AJR Am. J. Roentgenol.*, vol. 199, no. 5 Suppl, pp. S3–S8, 2012.
- [50] P. V. Granton, S. I. Pollmann, N. L. Ford, M. Drangova, and D. W. Holdsworth, “Implementation of dual- and triple-energy cone-beam micro-CT for postreconstruction material decomposition,” *Med. Phys.*, vol. 35, no. 11, pp. 5030–5042, 2008.
- [51] J. T. Johnson, *et al.*, “Virtual histology of transgenic mouse embryos for high-throughput phenotyping,” *PLoS Genet.*, vol. 2, no. 4, p. e61, 2006.
- [52] R. E. Golding and A. S. Jones, “Micro-CT as a novel technique for 3D reconstruction of molluscan anatomy,” *Molluscan Res.*, vol. 27, no. 3, pp. 123–128, 2007.
- [53] R. E. Golding, W. F. Ponder, and M. Byrne, “Three-dimensional reconstruction of the odontophoral cartilages of caenogastropoda (Mollusca: Gastropoda) using micro-CT: Morphology and phylogenetic significance,” *J. Morphol.*, vol. 270, no. 5, pp. 558–587, 2009.
- [54] G. Kerckhofs, *et al.*, “Simultaneous three-dimensional visualization of mineralized and soft skeletal tissues by a novel microCT contrast agent with polyoxometalate structure,” *Biomaterials*, vol. 159, pp. 1–12, 2018.
- [55] S. Gabner, P. Böck, D. Fink, M. Glösmann, and S. Handschuh, “The visible skeleton 2.0: Phenotyping of cartilage and bone in fixed vertebrate embryos and fetuses based on X-ray microCT,” *Development*, vol. 147, no. 11, 2020, <https://doi.org/10.1242/dev.187633>.
- [56] H. Jahn, *et al.*, “Evaluation of contrasting techniques for X-ray imaging of velvet worms (Onychophora),” *J. Microsc.*, vol. 270, no. 3, pp. 343–358, 2018.
- [57] M. Busse, *et al.*, “Three-dimensional virtual histology enabled through cytoplasm-specific X-ray stain for microscopic and nanoscopic computed tomography,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 10, pp. 2293–2298, 2018.
- [58] M. D. Cockman, *et al.*, “Quantitative imaging of proteoglycan in cartilage using a gadolinium probe and microCT,” *Osteoarthritis Cartil.*, vol. 14, no. 3, pp. 210–214, 2006.
- [59] M. Müller, *et al.*, “Nucleus-specific X-ray stain for 3D virtual histology,” *Sci. Rep. UK*, vol. 8, no. 1, 2018, Art. no. 17855.
- [60] B. Metscher, “A simple nuclear contrast staining method for microCT-based 3D histology using lead(II) acetate,” *J. Anat.*, vol. 238, no. 4, pp. 1036–1041, 2020.
- [61] L. Schaad, *et al.*, “Correlative imaging of the murine hind limb vasculature and muscle tissue by microCT and light microscopy,” *Sci. Rep. UK*, vol. 7, 2017, Art. no. 41842.
- [62] R. Hlushchuk, *et al.*, “Cutting-edge microangio-CT: New dimensions in vascular imaging and kidney morphometry,” *Am. J. Physiol. Renal Physiol.*, vol. 314, no. 3, pp. F493–F499, 2018.
- [63] K. L. Sider, J. Song, and J. E. Davies, “A new bone vascular perfusion compound for the simultaneous analysis of bone and vasculature,” *Microsc. Res. Tech.*, vol. 73, no. 7, pp. 665–672, 2010.
- [64] S. Ghanavati, L. X. Yu, J. P. Lerch, and J. G. Sled, “A perfusion procedure for imaging of the mouse cerebral vasculature by X-ray micro-CT,” *J. Neurosci. Methods*, vol. 221, pp. 70–77, 2014.
- [65] R. Margolis, B. Merlo, D. Chanthavisay, C. Chavez, B. Trinh, and J. Li, “Comparison of micro-CT image enhancement after use of different vascular casting agents,” *Quant. Imaging Med. Surg.*, vol. 14, no. 3, pp. 2568–2579, 2024.
- [66] J. H. Hubbel and S. M. Seltzer, *Tables of X-Ray Mass Attenuation Coefficients and Mass Energy-Absorption Coefficients (Version 1.4)*, Gaithersburg, MD, National Institute of Standards and Technology, 2004 [Online]. Available at: [Http://Physics.Nist.Gov/Xaamdi](http://physics.nist.gov/Xaamdi) [Accessed: Nov. 01, 2016].
- [67] J. M. Boone and J. A. Seibert, “An accurate method for computer-generating tungsten anode x-ray spectra from 30 to 140 kV,” *Med. Phys.*, vol. 24, no. 11, pp. 1661–1670, 1997.
- [68] J. M. Boone, T. R. Fewell, and R. J. Jennings, “Molybdenum, rhodium, and tungsten anode spectral models using interpolating polynomials with application to mammography,” *Med. Phys.*, vol. 24, no. 12, pp. 1863–1874, 1997.
- [69] T. R. Fewell, R. Shuping, and K. Healy, *Handbook of Computed Tomography X-Ray Spectra, HHS Publication (FDA) 81-8162*, Washington, D.C., U.S. Government Printing Office, 1981.
- [70] D. Haberthür, *et al.*, “MicroCT-based imaging of microvasculature within bone and peri-implant tissues,” *bioRxiv*, 2024, Version 3, <https://doi.org/10.1101/2023.03.08.531678>.
- [71] S. Handschuh, U. Reichart, S. Kummer, and M. Glösmann, “In situ isotropic 3D imaging of vasculature perfusion specimens using X-ray microscopic dual-energy CT,” *J. Microsc.*, vol. 297, no. 2, pp. 179–202, 2025.
- [72] C. T. Badea, N. Befera, D. Clark, Y. Qi, and G. A. Johnson, “Dual-energy micro-CT imaging of pulmonary airway obstruction: Correlation with micro-SPECT,” *Proc. SPIE*, vol. 9038, 2014, <https://doi.org/10.1117/12.2043094>.
- [73] J. Schindelin, *et al.*, “Fiji: An open-source platform for biological-image analysis,” *Nat. Methods*, vol. 9, no. 7, pp. 676–682, 2012.
- [74] V. Entezari, *et al.*, “Further improvements on the factors affecting bone mineral density measured by quantitative micro-computed tomography,” *Bone*, vol. 50, no. 3, pp. 611–618, 2012.
- [75] A. Inui, *et al.*, “Age-related changes of bone mineral density and microarchitecture in miniature pigs,” *J. Vet. Med. Sci.*, vol. 66, no. 6, pp. 599–609, 2004.
- [76] H. Nyangoga, P. Mercier, H. Libouban, M. F. Baslé, and D. Chappard, “Three-dimensional characterization of the vascular bed in bone metastasis of the rat by microcomputed tomography (MicroCT),” *PLoS One*, vol. 6, no. 3, 2011, Art. no. e17336.
- [77] K. Setiawan, R. S. Primarti, S. Sitam, W. Suridwan, K. Usri, and F. D. E. Latief, “Microstructural evaluation of dental implant success using Micro-CT: A comprehensive review,” *Appl. Sci.*, vol. 14, no. 23, 2024, Art. no. 11016.
- [78] Y. Ren, J. Senarathna, W. L. Grayson, and A. P. Pathak, “State-of-the-art techniques for imaging the vascular microenvironment in craniofacial bone tissue engineering applications,” *Am. J. Physiol., Cell Physiol.*, vol. 323, no. 5, pp. C1524–C1538, 2022.
- [79] P. Carlisle, *et al.*, “Quantifying vascular changes surrounding bone regeneration in a porcine mandibular defect using computed tomography,” *Tissue Eng. Part C Methods*, vol. 25, no. 12, pp. 721–731, 2019.
- [80] B. Müller, *et al.*, “Anatomy of the murine and human cochlea visualized at the cellular level by synchrotron-radiation-based micro-computed tomography,” in *Developments in X-Ray Tomography V*, San Diego, California, USA, 2006, p. 631805.

- [81] R. Glueckert, *et al.*, “Visualization of the membranous labyrinth and nerve fiber pathways in human and animal inner ears using microCT imaging,” *Front. Neurosci.*, vol. 12, 2018, Art. no. 501.
- [82] T. Potrusil, *et al.*, “Finite element analysis and three-dimensional reconstruction of tonotopically aligned human auditory fiber pathways: A computational environment for modeling electrical stimulation by a cochlear implant based on micro-CT,” *Hear. Res.*, vol. 393, 2020, Art. no. 108001.
- [83] H. Jiang, X. Wang, J. Zhang, A. Kachelmeier, I. A. Lopez, and X. Shi, “Microvascular networks in the area of the auditory peripheral nervous system,” *Hear. Res.*, vol. 371, pp. 105–116, 2019.
- [84] K. K. Ohlemiller, “Mechanisms and genes in human strial presbycusis from animal models,” *Brain Res.*, vol. 1277, pp. 70–83, 2009.
- [85] P. Spangenberg, *et al.*, “Rapid and fully automated blood vasculature analysis in 3D light-sheet image volumes of different organs,” *Cell Rep. Methods*, vol. 3, no. 3, 2023, Art. no. 100436.
- [86] L. Johnson Chacko, *et al.*, “Growth and cellular patterning during fetal human inner ear development studied by a correlative imaging approach,” *BMC Dev. Biol.*, vol. 19, no. 1, p. 11, 2019.
- [87] B. Nguyen, G. Bix, and Y. Yao, “Basal lamina changes in neurodegenerative disorders,” *Mol. Neurodegener.*, vol. 16, no. 1, p. 81, 2021.
- [88] A. N. Berezovsky, M. Espahbodi, S. L. LaPrade, D. R. Friedland, and M. S. Harris, “Cardiovascular diseases and sensorineural hearing loss-A systematic review of the literature,” *Otol Neurotol. Off. Publ. Am. Otol. Soc. Am. Neurotol. Soc. Eur. Acad. Otol. Neurotol.*, vol. 46, no. 1, pp. 23–30, 2025.
- [89] S. Liu, J. Broucek, A. S. Viridi, and D. R. Sumner, “Limitations of using micro-computed tomography to predict bone-implant contact and mechanical fixation,” *J. Microsc.*, vol. 245, no. 1, pp. 34–42, 2012.
- [90] M. Schriber, A. W. K. Yeung, V. G. A. Suter, D. Buser, Y. Y. Leung, and M. M. Bornstein, “Cone beam computed tomography artefacts around dental implants with different materials influencing the detection of peri-implant bone defects,” *Clin. Oral Implants Res.*, vol. 31, no. 7, pp. 595–606, 2020.
- [91] R. Namuju and N. K. Chilaka, “Alpha-lipoic acid ameliorates cytarabine-induced developmental anomalies in rat fetus,” *Hum. Exp. Toxicol.*, vol. 40, no. 5, pp. 851–868, 2021.
- [92] N. R. Catlin, *et al.*, “The postnatal resolution of developmental toxicity induced by pharmacological diacylglycerol acyltransferase 2 (DGAT2) inhibition during gestation in rats,” *Toxicol. Sci. Off. J. Soc. Toxicol.*, vol. 189, no. 2, pp. 225–236, 2022.
- [93] M. Breton-Larrivière, E. Elder, L.-M. Legault, A. Langford-Avelar, A. J. MacFarlane, and S. McGraw, “Mitigating the detrimental developmental impact of early fetal alcohol exposure using a maternal methyl donor-enriched diet,” *FASEB J.*, vol. 37, no. 4, 2023, Art. no. e22829.
- [94] L. Álvarez-Barrera, R. A. Mateos-Nava, K. N. Hernández-Córdova, E. Lezama-Sánchez, V. A. Alcántara-Mejía, and J. J. Rodríguez-Mercado, “Transplacental and genotoxicity effects of thallium(I) during organogenesis in mice,” *Toxicol. Rep.*, vol. 14, 2025, Art. no. 101896.
- [95] J. M. DeSesso and A. R. Scialli, “Bone development in laboratory mammals used in developmental toxicity studies,” *Birth Defects Res.*, vol. 110, no. 15, pp. 1157–1187, 2018.
- [96] L. Álvarez-Barrera, J. J. Rodríguez-Mercado, R. A. Mateos-Nava, N. A. Ocampo-Aguilera, and M. A. Altamirano-Lozano, “Vanadium(IV) oxide affects embryonic development in mice,” *Environ. Toxicol.*, vol. 37, no. 7, pp. 1587–1596, 2022.
- [97] S. Dizbay Sak, S. Sevim, A. Buyuksungur, A. Kayi Cangir, and K. Orhan, “The value of Micro-CT in the diagnosis of lung carcinoma: A radio-histopathological perspective,” *Diagn. (Basel, Switz.)*, vol. 13, no. 20, 2023, <https://doi.org/10.3390/diagnostics13203262>.
- [98] R. Brahmetaj, J. Cornelis, and B. Jansen, “Micro-CT microcalcification analysis: A scoping review of current applications and future potential in breast cancer research,” *Tomogr. (Ann Arbor, Mich.)*, vol. 10, no. 11, pp. 1716–1729, 2024.
- [99] S. J. Borland, *et al.*, “X-ray micro-computed tomography: An emerging technology to analyze vascular calcification in animal models,” *Int. J. Mol. Sci.*, vol. 21, no. 12, 2020, <https://doi.org/10.3390/ijms21124538>.
- [100] J. Herrmann, M. R. Gummi, M. Xia, M. van der Giet, M. Tölle, and M. Schuchardt, “Vascular calcification in rodent models-keeping track with an extended method assortment,” *Biology*, vol. 10, no. 6, 2021, <https://doi.org/10.3390/biology10060459>.
- [101] L. Zhang, L. Li, G. Feng, T. Fan, H. Jiang, and Z. Wang, “Advances in CT techniques in vascular calcification,” *Front. Cardiovasc. Med.*, vol. 8, 2021, Art. no. 716822.
- [102] M. Koblenzer, M. Weiler, A. Fragoulis, S. Rütten, T. Pufe, and H. Jahr, “Physiological mineralization during in vitro osteogenesis in a biomimetic spheroid culture model,” *Cells*, vol. 11, no. 17, 2022, <https://doi.org/10.3390/cells11172702>.
- [103] P. M. Gignac and N. J. Kley, “Iodine-enhanced micro-CT imaging: Methodological refinements for the study of the soft-tissue anatomy of post-embryonic vertebrates,” *J. Exp. Zool., Part B, Mol. Dev. Evol.*, vol. 322, no. 3, pp. 166–176, 2014.
- [104] P. M. Gignac, *et al.*, “Diffusible iodine-based contrast-enhanced computed tomography (diceCT): An emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues,” *J. Anat.*, vol. 228, no. 6, pp. 889–909, 2016.
- [105] S. Callahan, J. M. Crowe-Riddell, R. S. Nagesan, J. A. Gray, and A. R. Davis Rabosky, “A guide for optimal iodine staining and high-throughput diceCT scanning in snakes,” *Ecol. Evol.*, vol. 11, no. 17, pp. 11587–11603, 2021.
- [106] L. B. Porro and C. T. Richards, “Digital dissection of the model organism *Xenopus laevis* using contrast-enhanced computed tomography,” *J. Anat.*, vol. 231, no. 2, pp. 169–191, 2017.
- [107] A. J. Collings and C. T. Richards, “Digital dissection of the pelvis and hindlimb of the red-legged running frog, *Phlyctimantis maculatus*, using diffusible iodine contrast enhanced computed microtomography (DICE μ CT),” *PeerJ*, vol. 7, 2019, Art. no. e7003.
- [108] C. M. Early, A. C. Morhardt, T. P. Cleland, C. M. Milensky, G. M. Kavich, and H. F. James, “Chemical effects of diceCT staining protocols on fluid-preserved avian specimens,” *PLoS One*, vol. 15, no. 9, 2020, Art. no. e0238783.
- [109] S. E. Santana, “Comparative anatomy of bat jaw musculature via diffusible iodine-based contrast-enhanced computed tomography,” *Anat. Rec. (Hoboken)*, vol. 301, no. 2, pp. 267–278, 2018.
- [110] R. Brocklehurst, L. Porro, A. Herrel, D. Adriaens, and E. Rayfield, “A digital dissection of two teleost fishes: Comparative functional anatomy of the cranial musculoskeletal system in pike (*Esox*

- lucius*) and eel (*Anguilla anguilla*),” *J. Anat.*, vol. 235, no. 2, pp. 189–204, 2019.
- [111] S. Handschuh, et al., “Cranial kinesis in the miniaturised lizard *Ablepharus kitaibelii* (Squamata: Scincidae),” *J. Exp. Biol.*, vol. 222, no. Pt 9, 2019, <https://doi.org/10.1242/jeb.198291>.
- [112] C. P. Orsbon, N. J. Gidmark, and C. F. Ross, “Dynamic musculoskeletal functional morphology: Integrating diceCT and XROMM,” *Anat. Rec. (Hoboken)*, vol. 301, no. 2, pp. 378–406, 2018.
- [113] T. Kaurin, *The Morphology of the Feeding Apparatus in Aphanis Mento*, Master’s thesis, Austria, University of Vienna, 2019.
- [114] S. Kunisch, V. Blüml, T. Schwaha, C. J. Beisser, S. Handschuh, and P. Lemell, “Digital dissection of the head of the frogs *Calyptocephalella gayi* and *Leptodactylus pentadactylus* with emphasis on the feeding apparatus,” *J. Anat.*, vol. 239, no. 2, pp. 391–404, 2021.
- [115] M. Weberndorfer, *Feeding in Ambystoma Andersoni*, Master’s thesis, Austria, University of Vienna, 2017.
- [116] D. Schwarz, M. T. Fedler, P. Lukas, and A. Kupfer, “Form and function of the feeding apparatus of sirenid salamanders (Caudata: Sirenidae): Three-dimensional chewing and herbivory?” *Zool. Anz.*, vol. 295, pp. 99–116, 2021.
- [117] T. Kinigadner, *Cranial Kinesis of the Leopard Gecko*, Master’s thesis, Austria, University of Vienna, 2023.
- [118] V. Blüml, *Feeding Kinematics and Skull Morphology of the Keeled Box Turtle Cuora Mouhotii (Gray, 1862)*, Master’s thesis, Austria, University of Vienna, 2019.
- [119] A. Sombke, E. Lipke, P. Michalik, G. Uhl, and S. Harzsch, “Potential and limitations of X-ray micro-computed tomography in arthropod neuroanatomy: A methodological and comparative survey,” *J. Comp. Neurol.*, vol. 523, no. 8, pp. 1281–1295, 2015.
- [120] T. Heinzeller and U. Welsch, “Crinoidea,” in *Microscopic Anatomy of Invertebrates, Vol. 14: Echinodermata*, F. W. Harrison, and F.-S. Chia, Eds., New York, Wiley-Liss, 1994, pp. 9–148.
- [121] T. Heinzeller and B. Ruthensteiner, “Treatise online no. 182: Part T, revised, volume 1, anatomy of internal organs: Crinoidea,” *Treon*, vol. 2024, nos. 178–184, 2024, <https://doi.org/10.17161/to.vi.22577>.
- [122] D. B. Macurda and D. L. Meyer, *The Microstructure of the Crinoid Endoskeleton*, Lawrence, Kansas, USA, The Paleontological Institute, The University of Kansas, 1975.
- [123] A. Ziegler, “Combined visualization of echinoderm hard and soft parts using contrast-enhanced micro-computed tomography,” *Zoosymposia*, vol. 15, no. 1, pp. 172–191, 2019.
- [124] R. E. Snodgrass, *Principles of Insect Morphology*, New York, McGraw-Hill, 1935.
- [125] K. Stamm and J.-H. Dirks, “Semi-automated differentiation of insect exo- and endocuticle in X-ray microtomography,” *Arthropod Struct. Dev.*, vol. 66, 2022, Art. no. 101139.
- [126] R. F. Chapman, *The Insects Structure and Function, 5th Edition*, Cambridge, Cambridge University Press, 2013.
- [127] H. W. Krenn, “Form and function of insect mouthparts,” in *Insect Mouthparts: Form, Function, Development and Performance*, H. W. Krenn, Ed., Cham, Springer International Publishing, 2019, pp. 9–46.
- [128] R. M. S. Schofield, et al., “The homogenous alternative to biomineralization: Zn- and Mn-rich materials enable sharp organismal “tools” that reduce force requirements,” *Sci. Rep.*, vol. 11, no. 1, 2021, Art. no. 17481.
- [129] A. Ziegler, F. Neues, J. Janáček, F. Beckmann, and M. Eppele, “Mineral in skeletal elements of the terrestrial crustacean *Porcellio scaber*: SR μ CT of function related distribution and changes during the moult cycle,” *Arthropod Struct. Dev.*, vol. 46, no. 1, pp. 63–76, 2017.
- [130] F. Neues, S. Hild, M. Eppele, O. Marti, and A. Ziegler, “Amorphous and crystalline calcium carbonate distribution in the tergite cuticle of moulting *Porcellio scaber* (Isopoda, Crustacea),” *J. Struct. Biol.*, vol. 175, no. 1, pp. 10–20, 2011.
- [131] B. Josten, S. N. Gorb, and S. Büsse, “The mouthparts of the adult dragonfly *Anax imperator* (Insecta: Odonata), functional morphology and feeding kinematics,” *J. Morphol.*, vol. 283, no. 9, pp. 1163–1181, 2022.
- [132] F. A. Rivera-Quiroz and J. A. Miller, “Micro-CT visualization of the CNS: Performance of different contrast-enhancing techniques for documenting the spider brain,” *J. Comp. Neurol.*, vol. 530, no. 14, pp. 2474–2485, 2022.
- [133] C. Wan, Z. Hao, and X. Feng, “Structures, properties, and energy-storage mechanisms of the semi-lunar process cuticles in locusts,” *Sci. Rep.*, vol. 6, 2016, Art. no. 35219.
- [134] W. J. Heitler, “The locust jump,” *J. Comp. Physiol.*, vol. 89, no. 1, pp. 93–104, 1974.
- [135] H. C. Bennet-Clark, “The energetics of the jump of the locust *Schistocerca gregaria*,” *J. Exp. Biol.*, vol. 63, no. 1, pp. 53–83, 1975.
- [136] J. Fornaro, et al., “Dual- and multi-energy CT: Approach to functional imaging,” *Insights Imaging*, vol. 2, no. 2, pp. 149–159, 2011.
- [137] I. Vlahos, R. Chung, A. Nair, and R. Morgan, “Dual-energy CT: Vascular applications,” *AJR Am. J. Roentgenol.*, vol. 199, no. 5 Suppl, pp. S87–S97, 2012.
- [138] T. Bongartz, et al., “Dual-energy CT for the diagnosis of gout: An accuracy and diagnostic yield study,” *Ann. Rheum. Dis.*, vol. 74, no. 6, pp. 1072–1077, 2015.
- [139] A. D. Sodickson, A. Keraliya, B. Czakowski, A. Primak, J. Wortman, and J. W. Uyeda, “Dual energy CT in clinical routine: How it works and how it adds value,” *Emerg. Radiol.*, vol. 28, no. 1, pp. 103–117, 2021.
- [140] T. J. Vrtiska, N. Takahashi, J. G. Fletcher, R. P. Hartman, L. Yu, and A. Kawashima, “Genitourinary applications of dual-energy CT,” *AJR Am. J. Roentgenol.*, vol. 194, no. 6, pp. 1434–1442, 2010.
- [141] E. J. Moding, et al., “Dual-energy micro-computed tomography imaging of radiation-induced vascular changes in primary mouse sarcomas,” *Int. J. Radiat. Oncol. Biol. Phys.*, vol. 85, no. 5, pp. 1353–1359, 2013.
- [142] R. Bhavane, et al., “Dual-energy computed tomography imaging of atherosclerotic plaques in a mouse model using a liposomal-iodine nanoparticle contrast agent,” *Circ. Cardiovasc. Imaging*, vol. 6, no. 2, pp. 285–294, 2013.
- [143] J. R. Ashton, et al., “Dual-energy micro-CT functional imaging of primary lung cancer in mice using gold and iodine nanoparticle contrast agents: A validation study,” *PLoS One*, vol. 9, no. 2, 2014, Art. no. e88129.
- [144] L. A. Fitri, et al., “Dual energy micro CT SkyScan 1173 for the characterization of urinary stone,” *J. Phys.: Conf. Ser.*, vol. 694, 2016, <https://doi.org/10.1088/1742-6596/694/1/012053>.
- [145] D. S. Maier, J. Schock, and F. Pfeiffer, “Dual-energy micro-CT with a dual-layer, dual-color, single-crystal scintillator,” *Opt. Express*, vol. 25, no. 6, pp. 6924–6935, 2017.

- [146] J. Martins de Souza E Silva, *et al.*, “Dual-energy micro-CT for quantifying the time-course and staining characteristics of ex-vivo animal organs treated with iodine- and gadolinium-based contrast agents,” *Sci. Rep.*, vol. 7, no. 1, 2017, Art. no. 17387.
- [147] P. M. Pires, T. P. D. Santos, A. Fonseca-Gonçalves, M. M. Pithon, R. T. Lopes, and A. D. A. Neves, “A dual energy micro-CT methodology for visualization and quantification of biofilm formation and dentin demineralization,” *Arch. Oral Biol.*, vol. 85, pp. 10–15, 2018.
- [148] T. Sellerer, *et al.*, “Quantitative dual-energy micro-CT with a photon-counting detector for material science and non-destructive testing,” *PLoS One*, vol. 14, no. 7, 2019, Art. no. e0219659.
- [149] R. Zboray, *et al.*, “Development of a Micro-CT scanner with dual-energy option and endovascular contrast agent administration protocol for fetal and neonatal virtual autopsy,” *J. Imaging*, vol. 10, no. 3, 2024, <https://doi.org/10.3390/jimaging10030060>.
- [150] Y. Zhu, S. L. Manske, and S. K. Boyd, “Cartilage imaging of a rabbit knee using dual-energy X-ray microscopy and 1.0 T and 9.4 T magnetic resonance imaging,” *J. Orthop. Transl.*, vol. 3, no. 4, pp. 212–218, 2015.
- [151] Y. Suzuki, D. S. Cousins, J. R. Dorgan, A. P. Stebner, and B. B. Kappes, “Dual-energy X-ray computed tomography for void detection in fiber-reinforced composites,” *J. Compos. Mater.*, vol. 53, no. 17, pp. 2349–2359, 2019.
- [152] R. M. Zucker, P. Rigby, I. Clements, W. Salmon, and M. Chua, “Reliability of confocal microscopy spectral imaging systems: Use of multispectral beads,” *Cytometry, Part A J. Int. Soc. Anal. Cytol.*, vol. 71, no. 3, pp. 174–189, 2007.
- [153] T. Zimmermann, J. Marrison, K. Hogg, and P. O’Toole, “Clearing up the signal: Spectral imaging and linear unmixing in fluorescence microscopy,” *Methods Mol. Biol.*, vol. 1075, pp. 129–148, 2014.
- [154] A. Kronfeld, *et al.*, “Quantitative multi-energy micro-CT: A simulation and phantom study for simultaneous imaging of four different contrast materials using an energy integrating detector,” *Heliyon*, vol. 10, no. 1, 2024, Art. no. e23013.
- [155] K. Matsumoto, M. Jinzaki, Y. Tanami, A. Ueno, M. Yamada, and S. Kuribayashi, “Virtual monochromatic spectral imaging with fast kilovoltage switching: Improved image quality as compared with that obtained with conventional 120-kVp CT,” *Radiology*, vol. 259, no. 1, pp. 257–262, 2011.
- [156] L. Yu, S. Leng, and C. H. McCollough, “Dual-energy CT-based monochromatic imaging,” *AJR Am. J. Roentgenol.*, vol. 199, no. 5 Suppl, pp. S9–S15, 2012.
- [157] E. Pessis, *et al.*, “Virtual monochromatic spectral imaging with fast kilovoltage switching: Reduction of metal artifacts at CT,” *Radiographics*, vol. 33, no. 2, pp. 573–583, 2013.
- [158] R. Ballabriga, *et al.*, “The Medipix3RX: A high resolution, zero dead-time pixel detector readout chip allowing spectroscopic imaging,” *J. Instrum.*, vol. 8, no. 02, p. C02016, 2013.

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