

Contrast-enhancing staining agents for *ex vivo* contrast-enhanced computed tomography: A review

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ABSTRACT

Ex vivo microCT imaging has emerged as a powerful tool for 3D histology of biological tissues, offering significant advantages in terms of spatial resolution, simplicity of protocols and acquisition speed. Among the various techniques available, contrast-enhanced computed tomography (CECT) is particularly favored for its ability to simultaneously visualize soft and mineralized tissue types through the use of contrast agents (CAs), making it suitable for laboratory-based microCT devices. This review focuses on contrast-enhancing staining agents (CESAs), a subclass of CAs, which enrich the X-ray attenuating atom content in soft tissues through interactions. Within this review, CESAs are categorized based on their chemical composition into organic, mixed (i.e. heavy metal and organic ligand) and inorganic compounds, each with specific properties and applications. Despite the growing interest and numerous studies on CESAs, the selection process often relies on trial-and-error, anecdotal knowledge, or commercial availability. This review aims to enhance understanding of the chemical interactions and distribution patterns of CESAs within biological tissues, by discussing a selection of studies grouping observations by tissues and organs, to gain a better understanding of consistent affinity patterns. The findings highlight the complexity and accompanying challenges of predicting CESA distribution. This review will provide a foundation for both intelligent CESA selection and design, tailored to specific research needs as well as a guide for the application expert in choosing relevant literature for designing their experiments.

1. Introduction

Ex vivo microfocus X-ray computed tomography (microCT) imaging is an interesting tool for 3D histology of biological tissues in terms of spatial resolution, cost and acquisition speed. Within the realm of microCT imaging, contrast between neighboring elements can be obtained directly (e.g. phase-contrast imaging) and indirectly (e.g. through the use of contrast agents). Two primary contrast mechanisms are employed for direct visualization of small biological samples: attenuation contrast and phase contrast. Attenuation contrast, also known as absorption contrast, relies on the attenuation of X-rays by the sample, which is dependent on the density and elemental composition of the sample. This method is commonly used in conventional X-ray imaging

and is sensitive to variations in attenuation coefficients within the bulk of the sample. It is suitable to investigate strongly attenuating samples, such as mineralized tissues, but struggle with discriminating non-mineralized or soft biological tissues that have similar or weak attenuating properties. The introduction of contrast agents (CA) improves the attenuation properties of the samples, and hence, the applicability of attenuation contrast to biological tissues. However, usage of CAs renders this an indirect method of visualization. Nowadays, contrast-enhanced computed tomography (CECT) is the preferred techniques for lab-based microCT devices, which is also reflected by its increased popularity (Fig. 1).

Phase contrast, on the other hand, is based on the refractive properties of X-rays upon interaction with biological tissues [1]. This method

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is particularly useful for imaging low-density materials, which consists of components of similar attenuation coefficients. Phase-contrast computed tomography (PCCT) highlights edges and internal boundaries of a sample, making it complementary to conventional attenuation contrast computed tomography. As a result, PCCT is able to produce high-resolution datasets of both soft and dense biological tissue without the need of contrast agents. The advancement of high-brightness, tunable X-ray sources, coupled with improvements in X-ray detectors and computational techniques, is driving the emergence PCCT for direct visualization of biological tissues. Researchers have made efforts to create hardware capable of producing coherent X-ray beams in laboratory-based CT devices or to exploit Paganin filtering methods, even though data acquisition times are generally quite long [2–5]. Therefore, in many cases, synchrotron facilities are still required to conduct such experiments. Unfortunately, the necessity of synchrotron facilities incurs significant costs. Additionally, the need for complex iterative reconstruction algorithms and the limited size of specimens further restricts the practicality of PCCT as a technique for everyday use [6]. Other highly significant techniques, such as dual-energy computed tomography [7] (DECT), single-photon counting computed tomography [8] (SPCCT) and dark-field X-ray microCT [9] (DFXCT), are expected to receive increasing interest within the scientific community. In a laboratory setting, the selection of the appropriate method is contingent upon the characteristics of the sample as well as the capabilities of the instrumentation employed. This study centers on CECT, which is currently the most widely utilized X-ray based technique for soft-tissue visualization.

The CAs contain electron-rich elements (e.g. Br, I, Os, W, ...), commonly referred to as heavy elements, which contribute strongly to the attenuation of X-rays. The two main classes of CAs are perfusing contrast agents (PCAs) and contrast-enhancing staining agents (CESAs). Note that nanoparticles can be used both as PCA or CESA [10–13]. An extensive discussion of this type of CA falls outside of the scope of this review and the reader is referred to the following accounts and references therein [11,12,14]. PCAs are liquid formulations of heavy element containing substances that are perfused in the vascular system, potentially followed by solidification or gelation (e.g. Microfil, μ Angiofil) [15, 16]. In case of solidification or gelation, these CAs are more appropriately termed casting contrast agents (CCAs). PCAs allow detailed visualization of the circulatory system and quantification of the spatial distribution of the vessels. However, this technique is delicate due to the relatively high viscosity of commonly used formulations. Therefore, care must be taken upon perfusion of the vascular system in order to avoid vessel rupture. Also, in some cases the surrounding tissue is dissolved

leaving only the cast of the vasculature, without any possibility of further experiments. These limitations raise concerns of metrological accuracy of the reported measurements. Furthermore, no information concerning the surrounding soft tissue constituents is obtained.

For a more comprehensive visualization regarding soft tissues encompassing the vasculature, the use of CESAs is deemed more suitable. These molecules contain heavy atoms, similar to CCAs, but additionally interact with the tissue through (non-)covalent interactions. Herein, biological tissues are stained through passive diffusion by immersion in a solution containing a CESA. An effective CESA should efficiently attenuate X-rays and exhibit favorable diffusive properties to achieve practical staining times. To achieve an excellent discriminative power, it should demonstrate different interaction modes and affinity patterns towards distinct tissue constituents. The CESAs can be further divided in two main classes: specific and non-specific CESAs. While non-specific CESAs might exhibit increased affinity towards certain tissue constituents, they will not specifically stain a single protein as is the case in immunohistochemistry (e.g. a nucleus-“specific” haematoxylin-derived stain that also reveals the general tissue microstructure [17]). In contrast, the specific CESAs are designed to have a pronounced specificity towards certain biomolecules. Although literature on specific CESAs is considered limited, protein-specific CECT gained considerable attention as exemplified by the increasing number of publications on this topic [10,18–21]. In the last decade, metal nanoparticles with surface modifications have emerged as a powerful tool for targeted CECT. Excellent reviews by the groups of Cormode and Barber have recently summarized the latest advances in the application of nanoparticles as specific CESAs [14,16]. Meanwhile, a plethora of non-specific CESAs have been explored and developed in order to increase the discriminative power of microCT for soft tissues, evidenced by the numerous published reviews [1,12,15,22–36].

CAs for *in vivo* imaging in a preclinical context, typically iodine-based or barium-based, are used for imaging vasculature (angiography), the gastrointestinal (GI) tract, or other internal organs [37,38]. In angiography, iodinated CAs are mostly used, which allow for high-resolution mapping of blood vessels, aiding studies on vascularization, tumor angiogenesis, or cardiovascular diseases. For GI imaging, oral or rectal administration of CAs can enhance the visibility of the digestive tract, helping to assess gut motility, absorption, or pathology. Perfusion-based staining, where CAs are injected into the circulatory system prior to animal sacrifice, maximizes contrast penetration and staining uniformity, creating detailed 3D images of vascular networks. This approach is particularly useful for post-mortem microCT imaging as it enables high-definition visualization without the motion artifacts

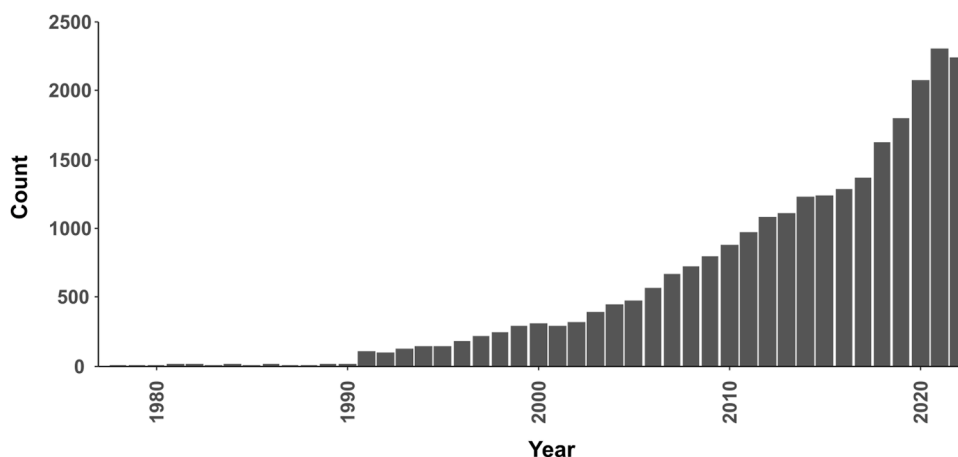


Fig. 1. Trend in absolute number of publications concerning CECT per year. This graph illustrates the increasing popularity of CECT between 1978 and 2023. This graph was based on query search in Web of Science with the following keywords: contrast-enhanced computed tomography, contrast-enhanced CT, CECT, CE-CT, contrast-enhanced microfocus computed tomography, contrast-enhanced microCT and contrast-enhanced μ CT. Note that both preclinical and clinical research papers are represented within this search.

associated with live imaging [39]. These techniques are powerful tools, but will not be further discussed. The present study serves to provide a summary of non-specific CESAs for *ex vivo* microCT imaging, which are classified based on their chemical structure. Reports until the end of 2023 are considered.

2. Non-specific contrast-enhancing staining agents for CECT imaging

Nowadays, the selection of a CESA frequently relies on trial-and-error approaches and anecdotal knowledge. Yet, enhancing our understanding of the basic interactions and anticipated distribution of CESAs within tissues would enable the selection of suitable CESAs tailored to address specific biological research questions, while reducing the required number of tissue samples. Fundamental studies on the staining mechanisms of the CESAs are imperative to bring CECT to the next level, as oftentimes correlated observations are mistaken for causation. Additionally, the literature should be thoroughly reviewed in order to outline staining patterns, taking into account the chemical aspects of the staining process. The present review discusses a selection of studies grouping observations by tissues and organs, to gain a better understanding of consistent affinity patterns. Emphasis will be directed towards the chemical classification and properties of CESAs and their use in 3D X-ray based histology of biological tissues.

2.1. CESAs: general properties and chemical classification

The most important property of a CESA is its ability to interact with biological tissues and subsequently attenuate X-rays (*vide supra*). This is achieved by including heavy atoms in the molecular structure. The number of heavy atoms per molecule is highly important, because the molecular sensitivity of microCT is in the mM range. Hence, a substantial concentration of CESA should be obtained within the specimen in order to achieve sufficient contrast, especially when compared to microscopy-based techniques. [27] Nevertheless, Pauwels et al. demonstrated that not all molecules incorporating heavy atoms were found to be suitable CESAs [40]. In their study, 28 commercially available inorganic compounds, with variable molarities in aqueous solution, were evaluated for their staining potential on store-bought bacon (*i.e.* mainly muscle, adipose and connective tissue). While all of the evaluated compounds were able to attenuate X-rays, certain ones (such as lead citrate and potassium permanganate) were deemed unsuitable for use as a CESA for CECT imaging. This was primarily because they could not effectively distinguish between different types of tissues. It is worthwhile to briefly reflect on the word “distinguish”, which implies limitations to what can be observed (*i.e.* limitations to the resolution). Two different types of resolution, spatial and contrast resolution, should be considered when performing a CECT experiment. The spatial resolution is mainly determined by the CT system, acquisition (*e.g.* the voxel size) and reconstruction parameters and does not depend on the CESA that was applied. It gives an indication of the smallest details that could be observed within the images. The contrast resolution gives an indication of which grey value differences can be detected with respect to the noise in the images. This measure depends, among others, on the CESA. More specifically, it depends on the concentration of heavy atoms (linked to the affinity of the CESA towards different tissue constituents) and the type of heavy atoms. The combination of both aforementioned measures will determine whether a particular feature can be discriminated from its surrounding structures/features.

When developing or assessing CESAs, multiple additional factors need to be considered. First, it is imperative that the compound dissolves in the solvent of choice, which is in most cases a buffered aqueous solution (*e.g.* phosphate-buffered saline (PBS)), a low molecular weight alcohol (*e.g.* methanol) or a mixture of both. Second, the use of a CESA should not interfere with the non-destructive nature of CT imaging. The CESA should preferably not irreversibly change the tissue

microstructure or chemical composition, as this could affect subsequent analyses (*e.g.* immunohistochemistry, mechanical testing). Third, these compounds should exhibit varying affinities towards distinct tissue components, allowing easy differentiation and structural analysis. Fourth, the interactions between the CESA and the tissue should not be extremely strong, as this could lead to prolonged duration to achieve staining equilibrium, which would result in impractically long staining times. Fifth, the CESA should be non-toxic for researchers to ensure broad applicability. Furthermore, microCT setups are not equipped with standard precautionary measures that allow working with toxic substances (*e.g.* good ventilation) and could be contaminated. Sixth, the CESA should preferably be easily synthesizable or commercially available, as substantial amounts are required for staining a tissue due to the relatively low sensitivity of the technique. Additionally, subsequent analyses or valuable samples (*e.g.* natural history museum specimens) that are needed to remain intact may sometimes require the removal of the stain after CECT. Throughout the following sections, most discussed compounds only satisfy a subset of all criteria, despite their regular use as CESAs.

Non-specific CESAs can be classified in three main groups based on their chemical composition: organic compounds, mixed compounds and inorganic compounds (Fig. 2). The organic compounds can be subdivided into ionic compounds (*e.g.* CA4+) and non-ionic compounds (*e.g.* Iomeprol). Note that a non-ionic compound is not necessarily hydrophobic. The group of mixed compounds contains both an inorganic and an organic part. The inorganic part typically is a metal that is responsible for X-ray attenuation (*e.g.* Gd^{3+}), while the organic part consists of chelators that coordinate with this metal (*e.g.* tetraazacyclododecane tetraacetic acid (DOTA)). The charge of these CESAs depends both on the charge of the ligands and the metal. The group of inorganic molecules can be subdivided in simple salts (*e.g.* lead acetate ($Pb(OAc)_2$) and more complex nanoclusters, *i.e.* the polyoxometalates (*e.g.* phosphotungstic acid (PTA)).

Table 1 presents a comprehensive list of successfully applied CESAs, encompassing various molecules and formulations. For a more detailed perspective tailored to applications experts, Table S1 provides an extensive overview of biological tissues stained and imaged using *ex vivo* CECT. This Supplementary table includes information such as the type of CESA, sample and sample size, fixation method and time, staining duration, acquisition medium, stain removal process, subsequent (immuno)histochemistry and any observed tissue damage resulting from the CESA. Table S1 serves as a valuable resource for readers, aiding them in selecting pertinent literature for designing their experiments.

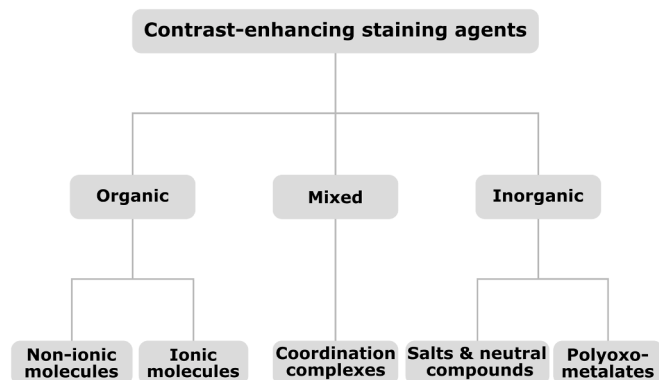


Fig. 2. Chemical classification of non-specific CESAs: the first classification is based on the nature of the elements that constitute the CESAs: organic molecules, mixed molecules and inorganic molecules. The second classification further subdivides each class in more niche groups.

Table 1

Non-exhaustive list of CESAs used in *ex vivo* 3D X-ray based histology by CECT imaging. Type refers to the chemical classification, in which ‘mixed’ indicates CESAs containing both an organic and inorganic part. Herein, the inorganic part is not merely a counterion but rather a transition metal with ligands. Bold number in potential charge range indicates charge of species at physiological pH (7.4). Note that the charges that can be populated within the charge range depend on the chemical properties. For organic CESAs and ligands of mixed CESAs, the charge was determined using MarvinSketch (Calculator Plugins were used for structure property prediction and calculation, Marvin 20.8.0, 2020, ChemAxon (<http://www.chemaxon.com>)). Chemical structures of CESAs can be found in Fig. 3, Fig. 12 and Fig. 16

CESA	Type	Heavy atom	Charge range	Target tissue
Hexabrix® (Ioxaglate)	Organic	Iodine (I)	– 1:0	Hominine cartilage [41–46] Hominine bone TE ^a construct [47] Bovine cartilage [41, 48–56] Leporine cartilage [51, 56–58] Caprine cartilage [59] Murine ^b cartilage [45, 60–64] Murine brain [65] Murine adipose tissue [66, 67] Murine bone [68] Canine cartilage [69] Porcine cartilage [70] Rabbit cartilage [58] Bovine cartilage [71,72] Leporine cartilage [73] Leporine brain [74] Murine brain [74] Bovine cartilage [48,51, 52,73,75–78] Leporine cartilage [51,58, 73] Murine brain [65] Murine cartilage [62,64, 79,80] Hominine cartilage [42–44,81–88] Hominine cartilage TE construct [89] Equine cartilage [90–92] Leporine cartilage [58] Leporine cartilage [58,73] Bovine cartilage [73] Murine cartilage [93–96] Bovine cartilage [93] Murine cartilage [93] Bovine cartilage [93] Porcine cartilage [70] Piscine brain [97] Murine brain [98] Murine spinal cord [99] Cephalopod brain [100] Murine spinal cord [101] Bovine cartilage [52,54] Ovine cartilage [102] Murine adult [103] Murine kidney [104,105] Murine whole body [104] Murine kidney [106,107] Murine brain [108] Hominine kidney [109] Bovine cartilage [48,53] Hominine cartilage [42] Hominine cartilage [81, 86–88] Bovine cartilage [75,77] Hominine cartilage [41] Murine heart [110] Murine lung [110] Hominine cartilage [42] Murine adult [103] Murine liver [17] Murine vascular system [111] Hominine bone TE neo-tissue [47]
Cysto Conray II® (Iothalamate)	Organic	Iodine (I)	– 1:0	
Hypaque® (Diatrizoic acid)	Organic	Iodine (I)	– 1:0	
CA4+	Organic	Iodine (I)	0:+ 3/+ 4	
CA2+	Organic	Iodine (I)	0:+ 2	
CA1+	Organic	Iodine (I)	0:+ 1	
Optiray® (Ioversol)	Organic	Iodine (I)	0	
Iomeron® (Iomeprol)	Organic	Iodine (I)	0	
Ultravist® (Iopromide)	Organic	Iodine (I)	0	
Iopamiron® (Iopamidol)	Organic	Iodine (I)	0	
Visipaque® (Iodixanol)	Organic	Iodine (I)	0	
Omnipaque® (Iohexol)	Organic	Iodine (I)	0	
XlinCA (<i>in situ</i> polymerization)	Organic	Iodine (I)	0:+ 2	
Eosin Y	Organic	Bromine (Br)	– 2:0	
Magnevist® (Gadopentetate)	Mixed	Gadolinium (Gd)	+ 3 (Gd) and – 5:0 (L) ^c	
ProHance® (Gadoteridol)	Mixed	Gadolinium (Gd)	+ 3 (Gd) and – 3:0 (L)	
MultiHance® (Gadobenate)	Mixed	Gadolinium (Gd)	+ 3 (Gd) and – 5:0 (L)	
Gadovist® (Gadobutrol)	Mixed	Gadolinium (Gd)	+ 3 (Gd) and – 3:0 (L)	
Gd(DTPA ^d)Lys ₂	Mixed	Gadolinium (Gd)	+ 3 (Gd) and 0:+ 1 (L)	
Bismuth-DTPA	Mixed	Bismuth (Bi)	+ 3 (Bi) and – 5:0 (L)	
Haematoxylin (haematein)	Mixed	Lead (Pb) or aluminum (Al) or Iron (Fe)	+ 2 (Pb), + 3 (Al), + 2 (Fe) and – 1:0 (Haem) ^e	
Phosphotungstic acid (PTA, [PW ₁₂ O ₄₀] ³⁻) in water or alcohol (MeOH or EtOH)	Inorganic	Tungsten (W)	– 3	

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Table 1 (continued)

CESA	Type	Heavy atom	Charge range	Target tissue
				Hominine cartilage [112, 113] Bovine cartilage [55] Murine bone [114,115] Murine kidney [115,116] Murine brain [117] Murine embryonic whole body [118–120] Murine vasculature [111] Murine lung [121] Murine heart [122] Glirine cartilage [123] Equine cartilage [113] Dipterous thorax and head [124] Leporine brain [125] Leporine muscle [125] Apiarian brain [126,127] Cephalopod brain [100] Porcine ligaments [128] Porcine vasculature [129, 130] Ranine larva [120] Lumbricine adult [131] Equine cartilage [113] Murine embryo [120] Piscine head [120] Ovine heart [132] Porcine ligaments [128] Porcine vessel [129] Porcine heart [132] Porcine vessel [130,133, 134] Murine kidney [115] Murine bone [115] Murine bone [115] Murine kidney [115] Murine vasculature [135] Porcine vasculature [130, 135] Hominine vasculature [135] Murine brain [65] Murine bone [114,115, 136] Murine vasculature [135] Murine kidney [115] Murine heart [137] Bovine muscle [137] Hominine cochlea [138–140] Hominine vasculature [135] Porcine vasculature [130, 135] Porcine tendon [137] Murine placenta [141]
Phosphomolybdic acid (PMA, $[\text{PMo}_{12}\text{O}_{40}]^{3-}$) in water or alcohol (MeOH or EtOH)	Inorganic	Molybdenum (Mo)	– 3	
Sodium polytungstate (SPT, $[\text{H}_2\text{W}_{12}\text{O}_{40}]^{6-}$)	Inorganic	Tungsten (W)	– 6	
Wells-Dawson phosphotungstate (Parent-WD POM, $[\alpha/\beta\text{-P}_2\text{W}_{18}\text{O}_{62}]^{6-}$)	Inorganic	Tungsten (W)	– 6	
Monolacunary Wells-Dawson phosphotungstate (Mono-WD POM, $[\alpha_2\text{-P}_2\text{W}_{17}\text{O}_{61}]^{10-}$)	Inorganic	Tungsten (W)	– 10	
1:2 Hf-substituted Wells-Dawson phosphotungstate (Hf-WD 1:2 POM, $[\text{Hf}(\alpha_2\text{-P}_2\text{W}_{17}\text{O}_{61})_2]^{16-}$)	Inorganic	Tungsten (W) and Hafnium (Hf)	– 16	
1:2 Zr-substituted Keggin phosphotungstate (Zr-K 1:2 POM, $\text{Zr}(\alpha_2\text{-P}_2\text{W}_{17}\text{O}_{61})_2]^{16-}$)	Inorganic	Tungsten (W) and Zirconium (Zr)	– 10	
Preyssler anion (PA, $[\text{NaPW}_{30}\text{O}_{110}]^{14-}$)	Inorganic	Tungsten (W)	– 14	
Osmium tetroxide (OsO_4)	Inorganic	Osmium (Os)	0	
Silver nitrate (Ag_2NO_3)	Inorganic	Silver (Ag)	+ 1 (Ag)	
Lugol's iodine (I_2/KI) dissolved in water or mixed with alcohol (EtOH or MeOH)	Inorganic	Iodine (I)	0 (I_2 = molecular iodine) and – 1 (I^- = iodide and I_3^- = triiodide) ^f	
				Dipterous larvae [153] Leporine brain [125] Leporine muscle [125] Leporine heart [154] Lumbricine adult [131] Apiarian brain [126] Anguine adult [155] Crocodilian head [156] Accipitrine forelimb [157] Cetacean fetus [158]

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Table 1 (continued)

CESA	Type	Heavy atom	Charge range	Target tissue
				Murine brain [159–163] Murine nerve (PNS) [164, 165] Murine heart [154,162, 166] Murine muscle [162] Murine head [167,168] Murine embryo [169,170] Murine vasculature [111] Murine adult [158] Porcine ligaments [128] Porcine vessel [129,130] Porcine fetus [158] Hominine fetal brain [171] Hominine fetus [172] Hominine brainstem [173] Cephalopod brain [100] Glirine muscle [174] Bovine muscle [137] Avine head [175,176] Avine embryo [177] Echidna muscle [178] Pteropine head [179] Dipterous thorax and head [124] Eusuchian head [180] Struthionine head [180] Passerine adult [181] Piscine head [182] Piscine adult [183] Mammalian tooth [184] Sciurine head [168] Murine placenta [185]
Aqueous iodine (I ₂)	Inorganic	Iodine (I)	0 (I ₂ = molecular iodine) and – 1 (I [–] = iodide and I ₃ = triiodide) ^g	
Alcoholic iodine solutions (I ₂ in EtOH/MeOH)	Inorganic	Iodine (I)	0 (I ₂) ^h	Avine adult [186] Cetacean fetus [158] Leporine brain [125] Leporine muscle [125] Murine brain [117,146, 187,188] Murine heart [110] Murine lung [110] Murine adult [158] Murine kidney [189] Murine liver [189] Cephalopod brain [100] Dipterous thorax and head [124] Passerine adult [181] Piscine head [190] Porcine fetus [158] Apiarian brain [126] Murine nerve (PNS) [191] Murine spinal cord [191] Ovine brain [192] Murine intestine [193] Murine embryo [193] Murine limb [193] Axolotl forelimb [193] Avine embryo [193] Chicken embryo [194] Murine embryo [194]
Uranyl acetate (UA)	Inorganic	Uranium (U)	0	
Potassium dichromate (K ₂ Cr ₂ O ₇)	Inorganic	Chromium (Cr)	– 2	
Lead acetate (Pb(OAc) ₂)	Inorganic	Lead (Pb)	+ 2	
Ruthenium Red [(NH ₃) ₅ Ru ^{III} ORu ^{IV} (NH ₃) ₄ ORu ^{III} (NH ₃) ₅]Cl ₆	Inorganic	Ruthenium (Ru)	+ 6	

^a TE = tissue engineered.^b Murine refers to the subfamily “murinae” and contains both mice and rats.^c L = ligand.^d DTPA = diethylenetriaminepentaacetic acid.^e Haem = haematein, *i.e.* the oxidized form of haematoxylin.^f These are the only relevant iodine species in an aqueous Lugol’s iodine solution if pH < 6.^g These are the only relevant iodine species in an aqueous Lugol’s iodine solution if pH < 6.^h Ignoring the formation of charge-transfer complexes between I₂ and alcohols.

2.2. Organic CESAs

The current library of organic CESAs comprises compounds with highly similar molecular motifs, featuring a halogen (mainly iodine) covalently bound to an aromatic ring system. Typically, the halogen incorporated in these molecules is predominantly iodine or bromine, conferring the X-ray attenuating characteristics (Fig. 3). With the exception of Eosin Y, these molecules likely originate from the same commercially available starting material, 5-amino-2,4,6-triiodoisophthalic acid. Consequently, within this group of molecules, differences in affinity patterns are anticipated to primarily stem from variations in size, hydrophilicity and net charge. Analysis of Table 1 indicates that this set of CESAs has predominantly been utilized in investigating the microstructure of cartilage. Furthermore, they have found applications in the examination of tubular structures (e.g. blood vessels), nervous tissue, kidney, and adipose tissue.

2.2.1. Applications in cartilage imaging

Understanding the staining patterns observed in cartilage tissue using an organic CESA requires a basic understanding of cartilage properties, functions, and composition. Several types of cartilage exist, including hyaline, elastic, fibrous, regeneration and degeneration cartilage each having unique compositions and functions within an organism. A specific type of cartilage can be made out of multiple layers, differing in chemical composition and properties [195]. Cartilage is well-known for its role as a template for bone formation during the embryonic stage of development [196,197]. Furthermore, it plays a role in providing the bone with flexibility, support and resistance to external forces [198].

In the field of CECT, the most well-studied type of cartilage is articular cartilage (AC), since it is the predominantly affected type of cartilage in patients suffering from osteoarthritis (OA). AC is a specialized type of hyaline cartilage, which covers the end of bones in the joint. Its major role is facilitating the transmission of loads and helping to move the joint smoothly. AC is mainly composed of a dense ECM in which chondrocytes are distributed. The ECM principally consists of water, collagen and aggrecan (cartilage-specific proteoglycan core protein) [199]. Collagen fibers are the most abundant macromolecules in the ECM, of which type II collagen is the most prominent. Aggrecan aggregates consist of a central hyaluronan (hyaluronic acid backbone) with multiple aggrecan monomers non-covalently attached [200]. Each aggrecan monomer consists of a core protein with covalently attached glycosaminoglycan (GAG) chains. These GAGs are long unbranched polysaccharide chains that consist of repeating disaccharides units, which contain at least one sulfate group per disaccharide. The most abundant disaccharide types present in GAGs are chondroitin sulfate and keratan sulfate. Due to the presence of both sulfate and carboxylate groups, aggrecans carry a fixed negative charge density at physiological pH (≈ 7.4 [201]). The distribution of GAGs within the AC is recognized as non-uniform, exhibiting a gradual increase in concentration towards the deeper zone [202]. The distribution pattern is contingent upon the tissue's state (e.g. healthy vs osteoarthritic [203]). The concentration of GAGs in the ECM matrix has been accordingly seen as a biomarker for AC health [204]. Hence, CESAs that are capable to detect changes in the GAG content are of significant interest to study disease progression and support diagnosis of OA.

Initially, negatively charged CESAs (i.e. ioxaglic acid (Hexabrix®) and iothalamic acid (Cysto Conray II® or CC2)) have been evaluated by the CECT community because of their commercial availability. This approach is known as equilibrium partitioning of ionic contrast microCT (EPIC-microCT) [56]. Cartilage is visualized as a negative contrast due to the inverse distribution of the negatively charged CESAs and the GAGs within AC after reaching equilibrium concentrations. While most of the studies confirmed the negative correlation between GAGs and grey value conferred by anionic organic CESAs statistically [46,49,50,52,56,62,71–73], some studies involving Hexabrix failed to do so

[42–44,48] (Fig. 4). Researchers postulated that this inconsistency might be due to variations in cartilage types used, the applied CESA concentration and the tissue condition (fixed vs fresh vs degraded vs mechanically injured). In addition, this inconsistency might be influenced by the imaging system (hardware), the acquisition and reconstruction parameters (software) and the administration method (e.g. injection, passive diffusion, ...).

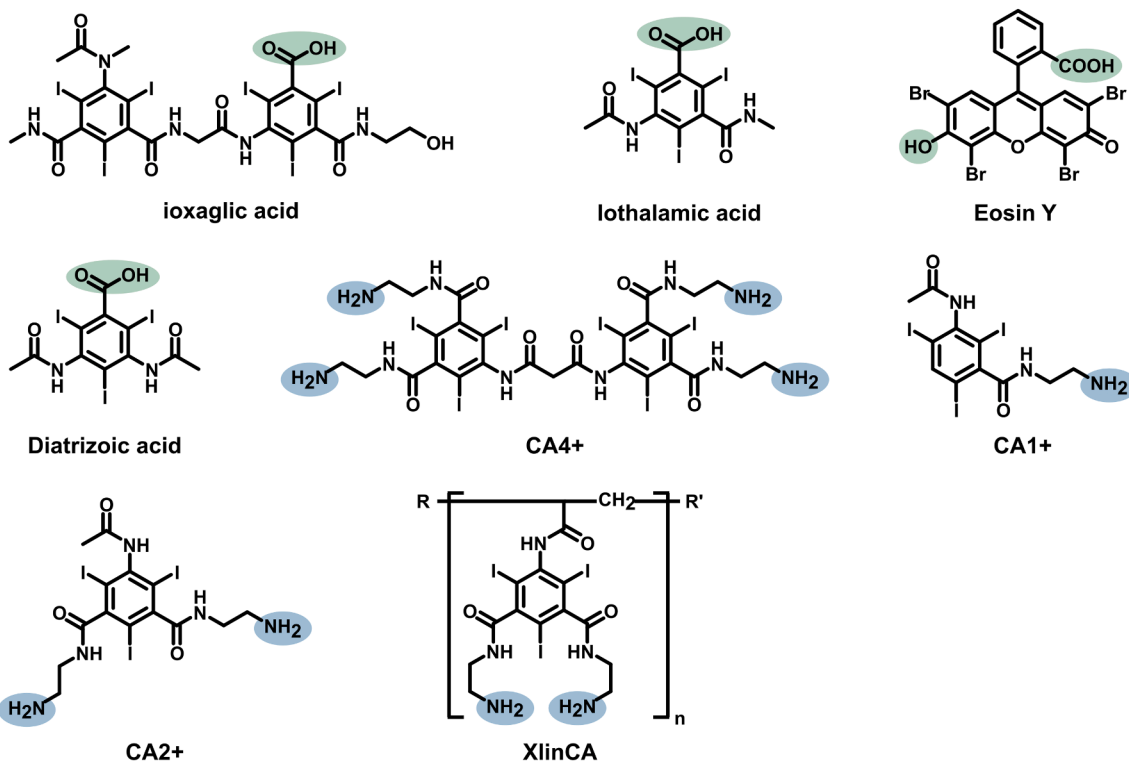
Despite numerous publications showcasing both significant and non-significant negative correlations, it is reasonable to assume that the proposed inverse relationship due to electrostatic repulsion holds true. Another issue arising from the repulsive interaction between GAGs and negatively charged organic CESAs involves visualization of GAG content in osteoarthritis (OA) tissue samples. In these samples, the GAG concentration near the subchondral bone is so low that both cartilage and bone display similar grey values after reaching equilibrium partitioning [45]. This similarity hampers accurate segmentation and volumetric analysis. The above-mentioned shortcomings were addressed by Baer et al. [41], who implemented photon-processing spectral CT in their workflow (Fig. 5), combined with biochemical analyses to prove a strong negative correlation between the concentration of GAGs and the concentration of iodine within the cartilage tissue. Additionally, segmentation was considerably optimized by filtering on specific heavy elements like iodine from the CESA and calcium from the bone.

Although anionic organic CESAs were commonly used to study the properties of cartilage via CECT imaging, CESAs with a similar structure but positive charge were developed by the Grinstaff group. Their aim was to improve the sensitivity of detecting changes in the GAG concentration based on attractive electrostatic interactions.

Soon after their first publication that compared cationic (CA1+, CA2+ and CA4+) with anionic (CC2 and ioxaglic acid) CESAs [58], other research groups also started evaluating and validating the potential of the most promising CESA, CA4+. In 2017, the Grinstaff group published a five-step, large-scale synthetic procedure of CA4+ and compared CA4+ with an anionic (i.e. ioxaglic acid) and uncharged (i.e. iodixanol) organic, iodinated CESA [52]. Fig. 6 clearly shows the time- and charge-dependent behavior of CA4+ diffusion in cartilage tissue, which was compared with the diffusion of iodixanol and ioxaglate. The synthesis was further optimized by Fantoni *et al.*, who claimed to combine the final two steps (acidic Boc-deprotection and ion exchange (trifluoroacetate $\rightarrow$ chloride)) by performing the Boc-deprotection step using an HCl solution in dioxane [76], however without properly describing the methodology. Multiple independent studies have shown strong and significant correlation between the cartilage's GAG content and the attenuation provided by the CESA based on comparison with biochemical assays. Alternatively, the sensitivity of cationic CESAs to the cartilage's GAG content was demonstrated by attenuation pattern that aligns with the GAG distribution, such as the increased GAG content going from the superficial cartilage towards the osteochondral interface. These patterns were evident in healthy, mechanically injured and PG-depleted tissue samples [42–44,48,52,62,64,71,75–77,80–82,84–88,205].

Even at lower concentrations, CA4+ produced increased X-ray attenuation of the cartilage tissue compared to anionic CESAs [52,62,206–208]. Furthermore, the higher equilibrium concentration of CA4+ contributed to improved sensitivity towards GAG content. This led to more precise imaging of the cartilage tissue and improved differentiation of tissue structures [42,43,64,73,78,85,209]. Karhula *et al.* investigated CA4+ using high-resolution datasets and noted that due to its heightened affinity for GAGs, CA4+ accumulates in higher concentrations in chondrons (chondrocytes and pericellular matrix (PCM)) with increased GAG concentrations compared to the interterritorial matrix (ITM) [82]. However, a consistent secondary delayed increase in attenuation, independent of the concentration used, suggests a potential additional slow diffusion process. This increased attenuation might be related to an influx of CA4+ inside the cell and subsequent interaction with negatively charged cell components (e.g. nucleic acids). A similar

Ionic organic CESAs



Non-Ionic organic CESAs

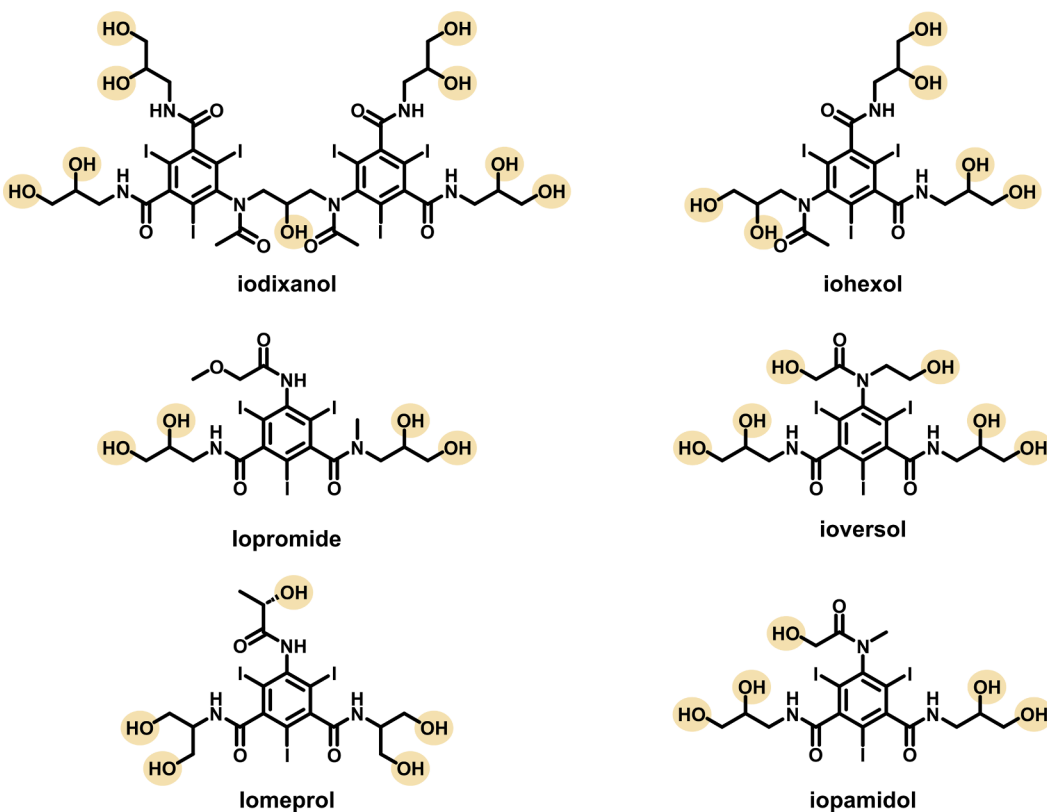
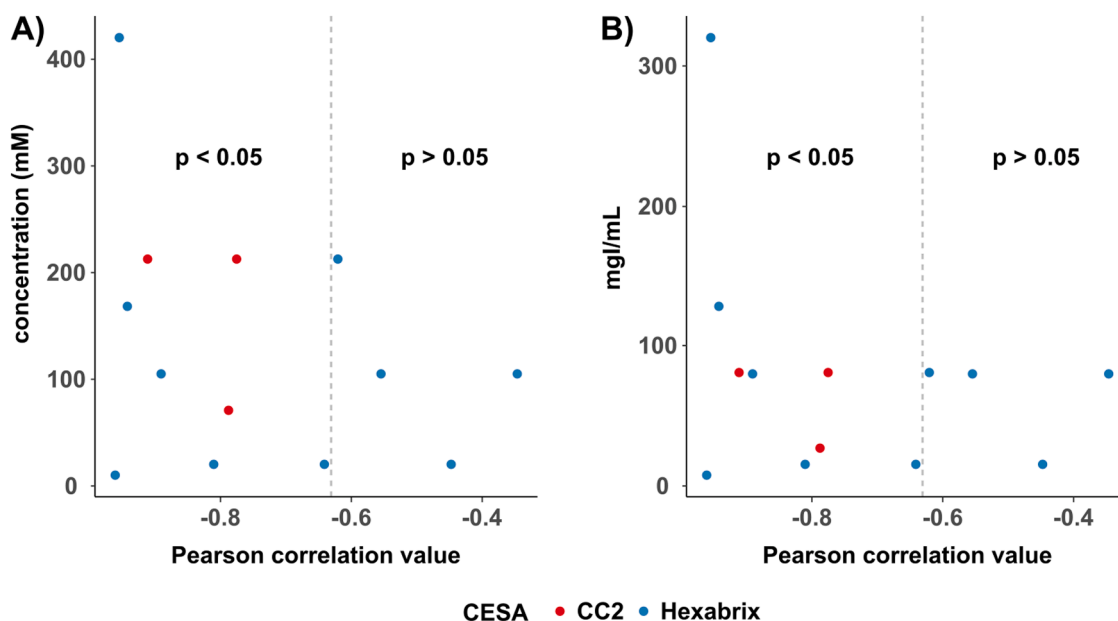


Fig. 3. Chemical structure of organic CESAs divided in the ionic and non-ionic molecules: Green = terminal functional groups that are likely negatively charged in physiological aqueous solution (isotonic (≈ 312 mOsm) and $\text{pH} \approx 7.4$), blue = terminal functional groups that are likely positively charged in physiological aqueous solution and yellow = terminal functional groups that will contribute to the hydrophilicity of the molecule, but will not be charged in physiological aqueous solution.



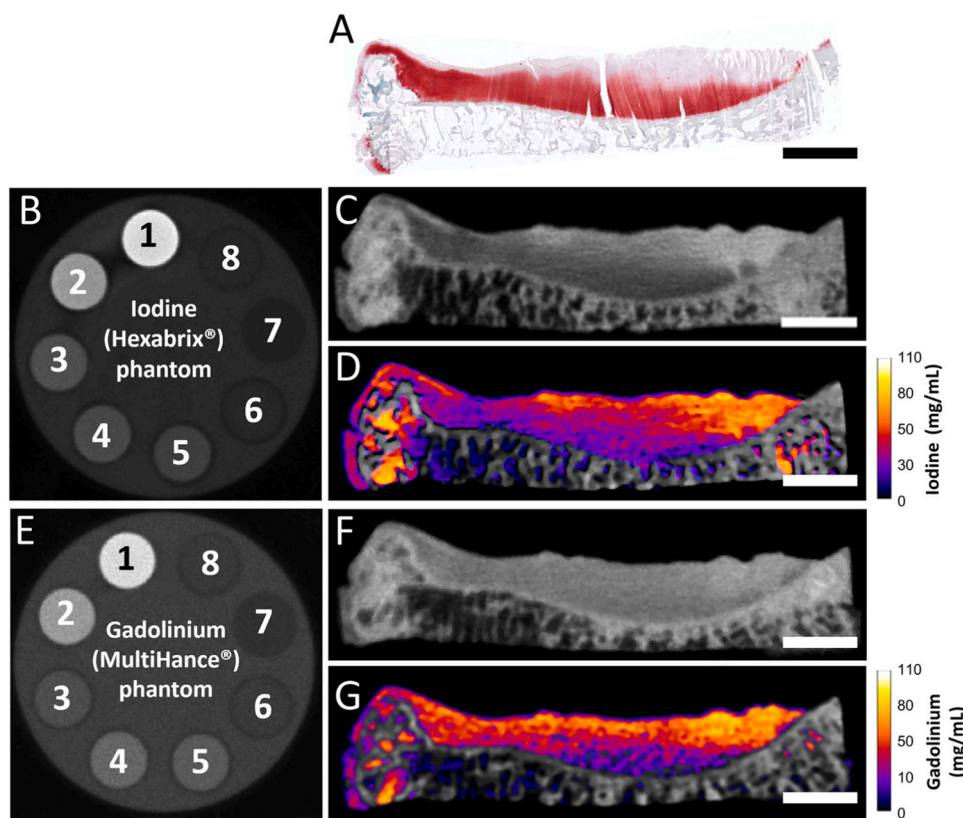


Fig. 5. Comparison of equilibrium distribution of Hexabrix, MultiHance with colorimetric staining using spectral CT. A) Histological image of a lateral human tibial plateau, stained with Safranin O/fast green/hematoxylin. Red color from Safranin O stain indicates the presence of GAG. B) Iodine phantom (1: 160 mg/mL I; 2: 96 mg/mL I; 3: 32 mg/mL I; 4: 700 mg/mL CaCl_2 ; 5: 500 mg/mL CaCl_2 ; 6: 15 mg/mL CaCl_2 ; 7: Oil; 8: water). C) Single-energy image (to mimic a conventional CT image) slice of the sample incubated in ioxaglate. D) Material-decomposed MARS image obtained from the same sample shown in panel A. The image shows the concentration of iodine from the anionic contrast agent (shown in the heat map as per bar on right). Calcium is shown on the greyscale. E) Gadolinium (Gd) phantom (1: 79 mg/mL Gd; 2: 47.4 mg/mL Gd; 3: 15.8 mg/mL Gd; 4: 700 mg/mL CaCl_2 ; 5: 500 mg/mL CaCl_2 ; 6: 15 mg/mL CaCl_2 ; 7: Oil; 8: water). F) Single-energy image (to mimic a conventional CT image) slice of the sample incubated in gadobenate dimeglumine. G) Material-decomposed MARS image obtained from the sample shown in panel A. The image shows the concentration of gadolinium from the anionic contrast agent (shown in the heat map as per bar on right). Calcium is shown on the greyscale. Note: purple layer on the top articulating surface of cartilage D) and G), indicating the low Gd/I (high GAG) concentration, is an imaging artifact due to the partial volume effect. Scale bar = 5 mm.

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combination with trithiocarbonate. Their novel method was successfully validated by whole mouse imaging [104]. Additionally, they acquired a high-resolution dataset on the mouse brain perfused with XLinCA (Fig. 8). Despite the success of XLinCA, it is important to acknowledge that perfusion of excised *ex vivo* tissues still present significant challenges, particularly when the vascular network is compromised or lacks a definitive entry point.

2.2.3. Applications in nervous tissue imaging

Six different organic CESAs have been evaluated for imaging nervous tissue, specifically the brain and the spinal cord [65,74,97–101,108,212]. These CESAs include CA4^+ , ioxaglic acid, diatrizoic acid, Eosin Y (used in conjunction with PCCT), iopamidol and iodixanol (used in conjunction with PCCT). The distribution of non-ionic and anionic, organic CESAs throughout the brain is consistent between different species belonging to the subphylum of vertebrates. Upon staining, grey matter regions show higher X-ray attenuation compared to white matter regions. This phenomenon is likely explained by the fact that grey matter generally contains a higher amount of mobile water content than white matter [213]. Moreover, negatively charged and uncharged CESAs are found to be repelled by white matter [212]. Consequently, more CESA tends to accumulate in grey matter regions. On the contrary, CA4^+ , a cationic organic CESA, showed higher X-ray attenuation of white matter regions due to electrostatic attraction. Besides molecular

interactions, it is important to mention that the tissue architecture and cell density influences the CESA distribution within the tissue. In comparison with CA4^+ , ioxaglic acid is preferred due to its relatively fast penetration rate, its superior contrast between grey and white matter, as well as its ability to provide clearer visualization of the brain's internal structures [212]. In the same study, cuprizone-induced demyelination could be visualized and quantified after staining murine brain with ioxaglic acid (Fig. 9). Interestingly, de Crespigny et al. also evaluated diatrizoic acid on a mouse glioma model and found that the glioma region was hyperintense compared to the surrounding grey matter [74].

In contrast, invertebrate species, which do not belong to the subphylum of vertebrates, utilize different mechanisms apart from myelin encapsulation to facilitate rapid conduction of electrical signals across axons (e.g. thicker axons) [214]. Iopamiron, a non-ionic, iodinated CESA was evaluated on the oval squid at a high concentration without success. The staining did not distinguish known anatomical features after achieving the equilibrium distribution [100]. This further shows that besides the tissue's mobile water content, properties associated with myelin, tissue architecture and cell density might also influence the final distribution of the CESA within the tissue.

2.2.4. Applications in kidney imaging

Only a small number of reports have explored the utilization of an organic CESA in examining the kidney microstructure. Busse et al.

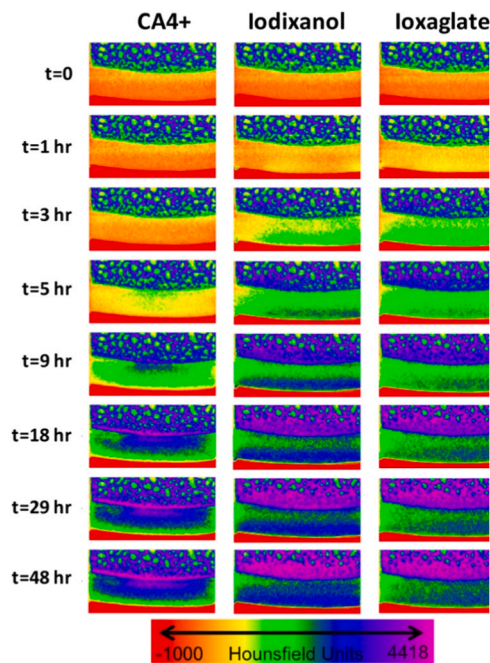


Fig. 6. μ CT images of a bovine osteochondral plug visualized in color maps. All images are taken of the same plug cross section. The color map ranges from air in red (each image bottom) to cartilage in yellow, green, and blue and bone in dark blue and purple (each image top). The contrast agents diffuse into cartilage over time, and the color turns from yellow to green to blue, indicating higher CT attenuation.

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developed a CECT staining protocol, in which they used Eosin Y, a well-known colorimetric stain. Eosin Y, containing four bromine atoms covalently bound, required relatively high concentrations (0.43 M) due to bromine's limited efficiency in X-ray attenuation to ensure sufficient contrast [106]. A crucial step in their protocol involved acidic tissue fixation (4 % formaldehyde in Dulbecco's phosphate-buffered saline with 5 % glacial acetic acid). This process protonated amino acid side chains, which improved electrostatic interactions between Eosin Y and cytoplasmic proteins. Subsequently, a PBS washing step neutralized the medium, enabling the deprotonation of Eosin Y's carboxylic acid and alcohol groups [215]. The resulting images showed higher concentrations of Eosin Y molecules in the cytoplasm, while excluded from the nuclei, as expected from known staining pattern in colorimetric staining experiments. To highlight the efficacy of this staining protocol, Eosin Y was successfully applied as a CESA for studying human renal cell carcinoma [109]. To address limitations associated with the commercial Eosin Y disodium salt in terms of contrast enhancement, Busse et al. optimized the salt [107]. They discovered that substituting Na^+ counterions with Ba^{2+} increased X-ray attenuation upon staining with Eosin Y but significantly reduced its solubility. To overcome the solubility issues, the number of bromine atoms on the fluorescein core was reduced to two, allowing a higher maximal concentration and enhanced contrast. Fig. 10 shows a high-resolution dataset of murine kidney stained with the dibromo fluorescein barium salt. To obtain more information on the vasculature and tubular structures within the kidney, Kuo et al. successfully applied XLinCA (*vide supra*), confirming the added value of a water-soluble, monomeric CESA that can undergo *in situ* polymerization [216].

2.2.5. Applications in bone marrow and adipose tissue imaging

Recently, ioxaglic acid has been introduced as a CESA for studying the structural properties of adipocytes (Ads) within the bone marrow.

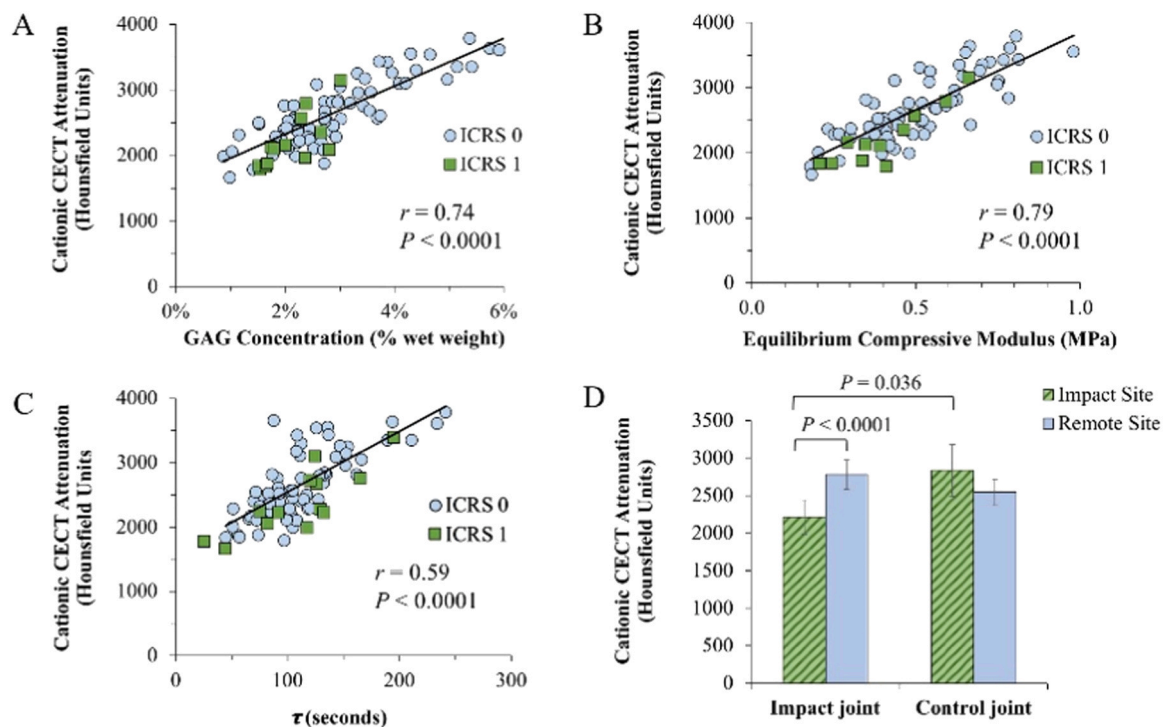


Fig. 7. Comparison of cationic contrast-enhanced computed tomography (CECT) (microCT) with glycosaminoglycan (GAG) concentration (image A), equilibrium compressive modulus (image B) and permeability (τ , image C). Data points in each image are additionally identified by their macroscopic scoring on the International Cartilage Repair Society (ICRS) scale. Correlation coefficients (r) and P -values identify the strength of correlation between variables using a Bland correlation controlling for repeated measures between subjects. Mean cationic CECT attenuation at the impacted and remote cartilage sites within the impact and control joints are shown in image D (error bars: 95 % confidence interval).

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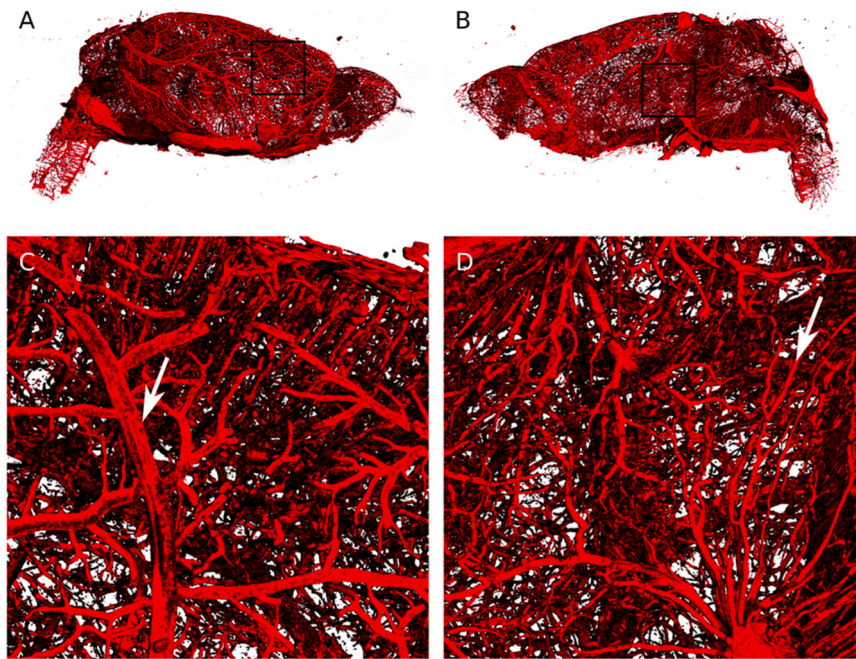


Fig. 8. 3D rendering of the brain hemisphere vasculature perfused with XlinCA. A) Outside view. B) Inside view. C) Magnified view of the region indicated by the black square in A). Diameter of blood vessel indicated by the white arrow: 70 μm . D) Magnified view of B). Diameter of blood vessel indicated by the white arrow: 15 μm .

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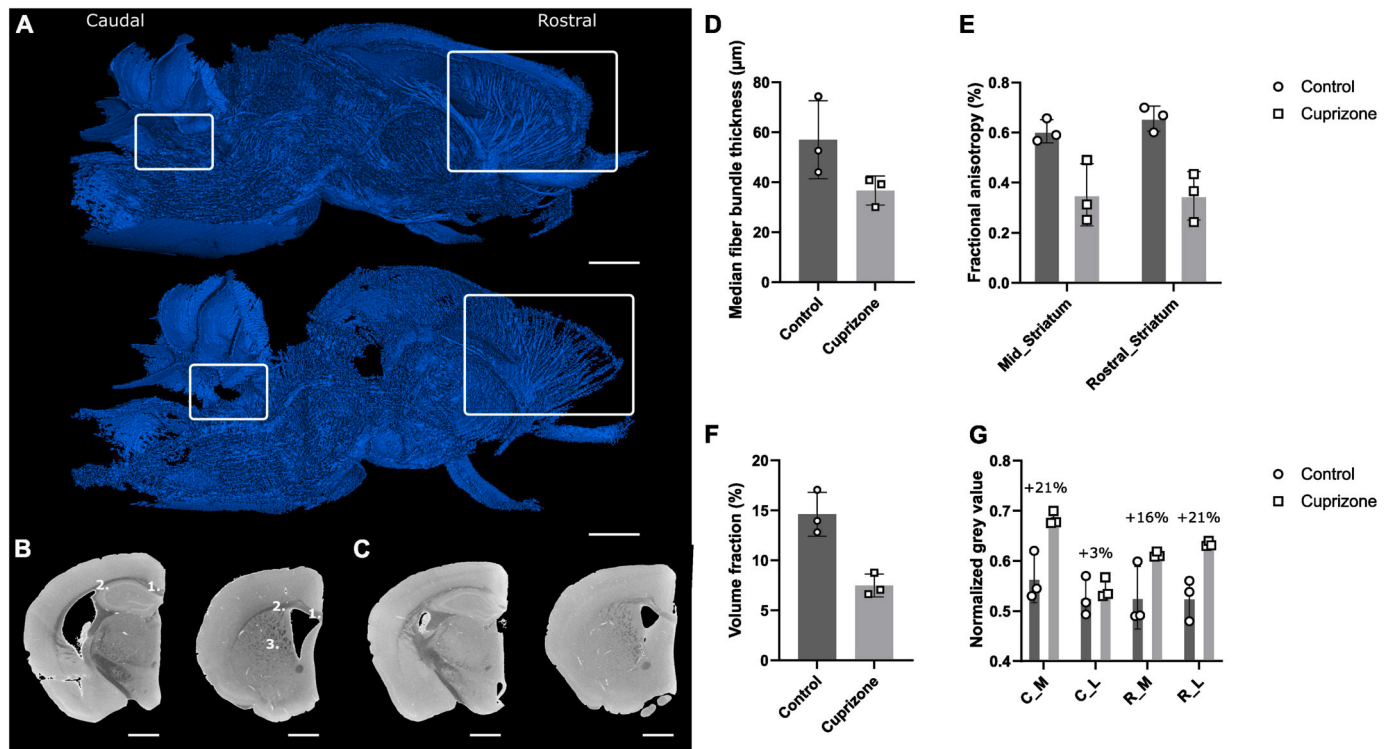


Fig. 9. Effect of cuprizone treatment on myelination using Hexabrix staining and quantitative white matter parameters. A) 3D rendering of segmented white matter of a cuprizone-treated hemisphere (top) and a healthy hemisphere (bottom). White rectangles indicate regions where demyelination was expected (left: white matter near cerebellar nuclei, right: corpus callosum and corpus striatum). B) Caudal (left) and rostral (right) coronal gray scale cross-sections of a healthy hemisphere with medial (1) and lateral (2) corpus callosum and corpus striatum (3). Scale bar = 1 mm. C) Caudal (left) and rostral (right) coronal gray scale cross-sections of a cuprizone-treated hemisphere. Scale bar = 1 mm. D) Median fiber bundle thickness of healthy and cuprizone-treated hemispheres. E) Computed fractional anisotropy of healthy and cuprizone-treated hemispheres on the rostral part of the striatum and the middle part of the striatum. F) Volume fraction of white matter fiber bundles in the striatum. (G) Normalized gray values of healthy and cuprizone-treated corpus callosum. C_M, caudal, medial; C_L, caudal, lateral; R_M, rostral, medial; R_L, rostral, lateral.

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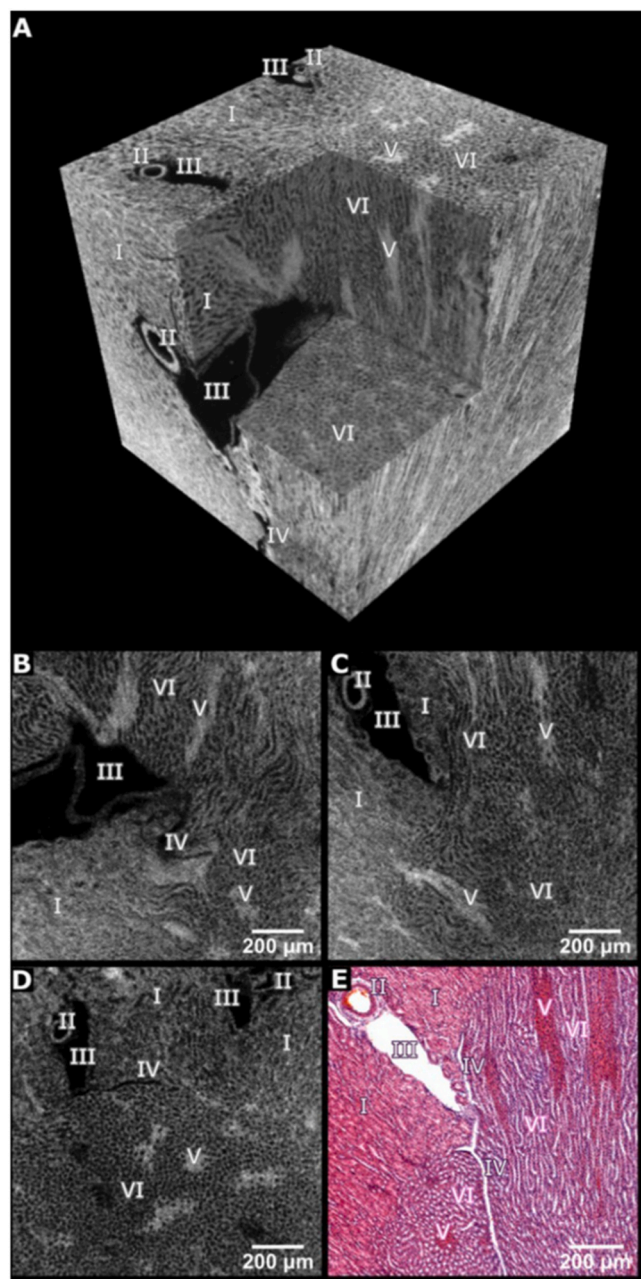


Fig. 10. High-resolution μ CT data of the cortex and outer medulla region of a mouse kidney after application of the dibromo fluorescein barium salt. (A) Representative μ CT volume of $1.20\text{ mm} \times 1.20\text{ mm} \times 1.20\text{ mm}$ (effective voxel size $\approx 3.3\mu\text{m}$). (B–D) Individual μ CT slices derived from the orthogonal planes through the volume shown in (A). (E) Representative X-ray barium eosin Y-stained histological microscopic slide. The cell nuclei were counter stained with Mayr's sour hematoxylin. I proximal convoluted tubules, II: artery, III: vein, IV: capillary, V: medullary rays, and VI: loop of Henle. Adapted from [107] and reused under Creative Commons license (CC-BY-NC-ND).

The anionic and hydrophilic nature of ioxaglic acid prevents its diffusion into Ads, yet it effectively stains the surrounding tissue in a homogeneous way. Upon staining with ioxaglic acid, three distinct phases can be distinguished in bone samples: bone, bone marrow and Ads. This distinct contrast facilitated image processing and structural analysis, which was exploited in several studies. Marin et al. studied the impact of diabetes mellitus and aging on bone fracture healing [68]. In this study, changes in bone, cartilage and adipocytes were evaluated by comparing the

fracture callus of young mice, diet-induced mice and low-fat diet age-matched mice. Benova et al. studied the influence of anti-diabetic drugs and diets enriched with omega-3 polyunsaturated fatty acids on several bone parameters, such as Ads, in mouse models of obesity [66, 217]. In this work, Hexabrix® was used to visualize bone marrow Ads in whole proximal tibia of mice that were treated with anti-diabetic drugs (Fig. 11).

2.3. Mixed CESAs

The group of mixed CESAs contains coordination complexes of ionic ligands that chelate a metal center (Gd^{3+} , Pb^{2+} , Al^{3+} , Fe^{2+} and Bi^{3+}) (Fig. 12). The Gd-based CESAs are the most common, since they are adapted from the pool of MRI contrast agents [218]. Within the subset of Gd-based CESAs for CECT imaging, the main differences arise from the total charge of the complex. The CESAs derived from haematoxylin consist of two variations: one where haematein chelates Pb^{2+} , and another where it chelates $\text{Al}^{3+}/\text{Fe}^{2+}$. Additionally, there exists a single example of a Bismuth-containing mixed CESA [103]. Here, Bi^{3+} is chelated by a diethylenetriaminepentaacetic acid (DTPA) ligand, which is the same ligand as used in gadopentetic acid (Magnevist®). Since the latter study focuses on *in vivo* contrast-enhancement and pharmacokinetics of the CESA, it will not be discussed in depth in this work. However, due to the similarity in structure and charge as gadopentetic acid, it would be an interesting candidate to evaluate for *ex vivo* CECT imaging. These CESAs have been used to study cartilage, heart, lung, liver and vasculature.

2.3.1. Applications in cartilage imaging

Several Gd-based mixed CESAs have been used for studying cartilage, often in comparison [41,42,48,53] or in combination [75,77,81, 84,86–88] with an organic CESA.

Upon comparing anionic mixed and anionic organic iodinated CESAs, the results across different studies exhibited inconsistencies. In the comparison, it was revealed that gadopentetic acid displayed a similarly weak correlation with GAG content in comparison to ioxaglic acid [42,53,73]. When comparing gadobenic acid with ioxaglic acid, Baer et al. found that both could provide equally strong inverse significant correlations with GAG content [41]. Upon comparing $\text{Gd}(\text{DTPA})\text{Lys}_2^{3+}$ with CA4^{+} , the sensitivity towards GAG content of CA4^{+} was shown to be higher, but for both a positive correlation was observed [42].

The research group of J. Töyräs investigated the use of dual CESA solutions combined with DECT for studying cartilage tissue. These formulations combined CA4^{+} (cationic) with gadoteridol (neutral) with the aim of improving sensitivity and information that can be extracted per sample. This was based on the hypothesis that CA4^{+} will primarily partition according to the GAG content, while gadoteridol will partition according to the porosity and water content of the sample. However, not all studies were able to show a clear correlation between the gadoteridol concentration and the water content in the cartilage [75]. Additionally, Bhattarai et al. found an inverse relationship between the collagen content and the gadoteridol distribution [88].

In an effort to enhance the visualization of tissue borders, Honkanen et al. included a third element into the staining solution, polyethylene glycol-coated Bi-nanoparticles [77]. The nanoparticles were not able to penetrate the tissue, but were able to give a very clear delineation of the cartilage-solution interphase and revealed potential mechanical defects on the cartilage surface (Fig. 13).

2.3.2. Applications in vasculature imaging

In an attempt to develop a staining procedure for visualizing elastin, which is present in the vascular wall, Dunmore-Buyze et al. developed a modified Verhoeff's solution (56 V/V% aluminum (Harris) haematoxylin, 2.2 m/V% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.6 m/V% KI and 0.3 m/V% I_2) [111]. The main difference with well-known Verhoeff's-Van Gieson elastin stain is

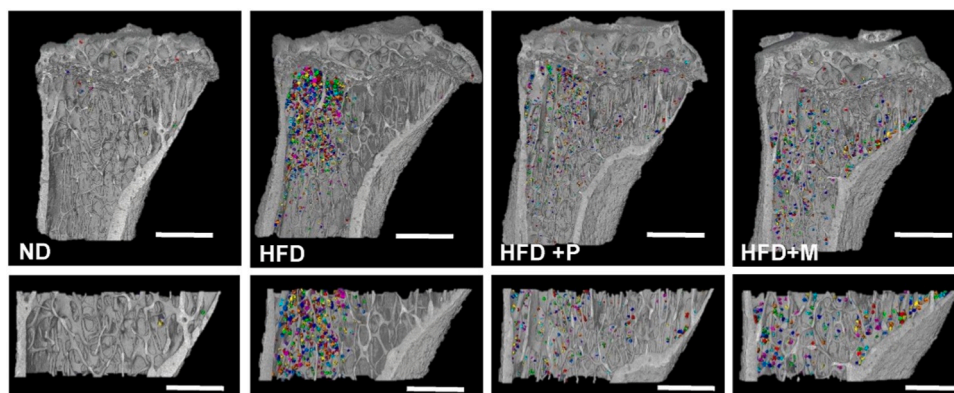


Fig. 11. Bone marrow adipose tissue composition is differently affected in obese mice treated with pioglitazone and MSDC-0602K. Representative pictures of bone marrow adipocytes (BMAds) stained with contrast agent Hexabrix in whole proximal tibia and zoomed pictures of BMAds in selected region of interest in proximal tibia (defined in Material and Methods) scanned by contrast-enhanced CT (CECT). Pictures were created using Avizo Software (scale bar 1000 μm). Conditions from left to right: normal diet (ND), high-fat diet (HFD), high-fat diet treated with pioglitazone (HFD + P) and high-fat diet treated with MSDC-0602K (HFD + M). Colors of adipocytes are arbitrary.

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the exchange of the alcoholic haematoxylin solution by an aluminum haematoxylin solution (also known as Harris haematoxylin) to prevent precipitation of the heavy-atom containing components within the vessels. Even though the staining mechanism is yet to be understood, the staining components showed an enhanced affinity for the vascular wall and successfully revealed the vasculature of an adult whole mouse (Fig. 14).

2.3.3. Applications in heart and lung imaging

Silva et al. evaluated gadobutrol (neutral), which is also adapted from MRI, for its potential in heart and lung imaging [110]. It was found that this CESA was able to provide a general overview of the organ morphology, without affecting tissue integrity. The lung tissue showed higher grey values compared to the esophagus and heart after equilibrium was reached. One drawback noted in the staining protocol was the incomplete removal of the staining solution from the heart's ventricles, which could potentially increase the labor intensity involved in segmentation.

2.3.4. Applications in liver imaging

One report exists in which a mixed CESA has been used to visualize the liver microstructure. The aim of the study by Müller et al. was to introduce a haematoxylin-derived CESA, chelating Pb^{2+} [17]. The authors emphasized the importance of the acid fixation step during the sample preparation, similar as previously reported for the Eosin Y staining methods [107]. However, it should be noted that the presence of acetic acid during the fixation could cause coagulation of nucleic acids. Further investigation is required to determine the precise interaction mechanism. Furthermore, the CESA was not selective for nuclei, but showed increased affinity for nuclei. Interestingly, the nuclei of different cell types (e.g. hepatocytes, Kupffer cells and endothelial sinusoidal cells) could be differentiated based on variations in grey values (Fig. 15).

2.4. Inorganic CESAs

Inorganic CESAs can be divided in two subgroups based on the size of the molecules (Fig. 16). The first subgroup contains salts (e.g. potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$)) and neutral compounds (e.g. molecular iodine (I_2)). While the number of studies that evaluated simple salts is limited, the number of studies that focuses on inorganic iodine molecules in both aqueous or alcoholic solutions is enormous. Given the widespread use of inorganic iodine-based formulations within the CECT field, they delineate a specific subclass known as diffusible iodine-based contrast-enhanced computed tomography (DICECT). Despite their seemingly

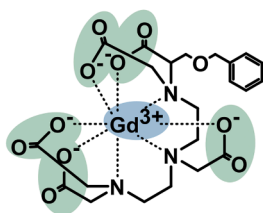
simple nature, inorganic iodine molecules (e.g. I_2 , KI, ...) are known to exhibit complex behavior in aqueous and alcoholic solutions [219]. The second subgroup contains the family of the anionic polyoxometalate (POM) nanoclusters. These molecules feature transition metals (e.g. W, V, Mo, ...), in their highest oxidation states (+V or +VI), also referred to as addenda atoms. These addenda atoms are bridged [M-O-M] and terminated [M=O] by oxygen atoms. The POMs relevant for this work, belong to the class of iso- (e.g. sodium polytungstate), hetero- (e.g. phosphotungstic acid), lacunary polyoxoanions (e.g. monolacunary Wells-Dawson phosphotungstate) and derivatives (e.g. 1:2 hafnium-substituted Wells-Dawson phosphotungstate). While a detailed description of POM chemistry is outside of the scope of this review, a general misconception about POMs in the CECT community will be briefly addressed.

Often in CECT literature, POMs are considered as highly negatively charged molecules. Even though the chemical formula might give this false impression, the true charge at the surface of the POM molecules is divided over the entire volume of this molecule, making some of these molecules charge diffuse (i.e. (super)chaotropic) [229–232]. The chaotropic nature of the POMs is less pronounced for metal-substituted POM derivatives compared to their hetero- or iso anion counterparts. This unique nature of POMs, as compared to simple, small anionic molecules, bestows upon them a complex interaction profile with biological tissues, which should not be neglected when discussing the staining behavior of POMs [233–236]. Furthermore, POMs are known to exhibit an extremely complex solution behavior in aqueous solutions [237,238]. Consequently, an observed staining pattern should not be linked to a single POM specie, without proper confirmation of the structure. To obtain a deeper understanding of the fundamentals of POM synthesis and chemistry, the reader is referred to the following literature [239,240]. The inorganic CESAs have been evaluated for imaging of cartilage, bone, nervous tissue, kidney, vasculature, heart, lung, muscle tissue, cochlea and placenta.

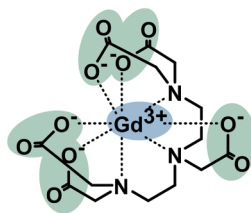
2.4.1. Applications in cartilage imaging

The group of S. Saarakkala, evaluated PTA and phosphomolybdic acid (PMA) with the aim of visualizing the collagen distribution within articular cartilage. A preliminary study revealed that the attenuation profiles after PTA or PMA staining showed a similar trend as the collagen distribution, determined through Fourier-transform infrared imaging (FTIRI), in the middle and deep layers of the cartilage [113]. However, a large deviation was found near the superficial layers. Please note that while PTA and PMA are used in multiple well-known colorimetric trichrome stains for traditional 2D histology to reveal connective tissue,

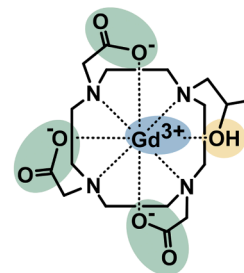
Gd-based



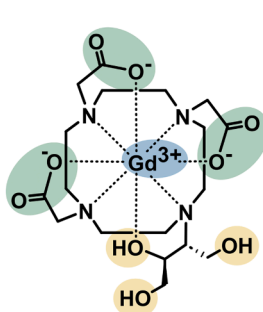
gadobenic acid



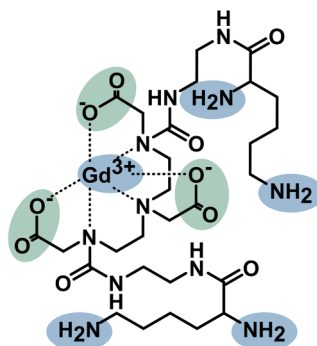
gadopentetic acid



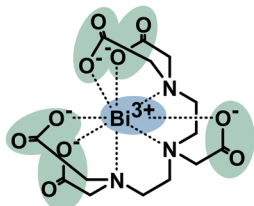
gadoteridol



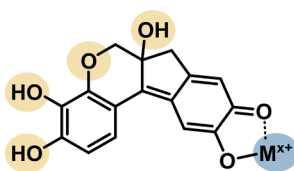
gadobutrol

Gd(DTPA)Lys₂

Other



Bi-DTPA



Haematein complex

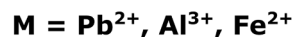


Fig. 12. Chemical structure of mixed CESAs divided in Gd-based and other molecules: Green = terminal functional groups that are likely negatively charged in physiological aqueous solution, blue = metal cations or terminal functional groups that are likely positively charged in physiological aqueous solution, and yellow = terminal functional groups that will contribute to the hydrophilicity of the molecule, but will not be charged in physiological aqueous solution.

these POMs are not designed or known for targeting a specific protein [241,242]. The sensitivity of PTA towards the collagen content in cartilage was found to be superior to PMA, which was therefore excluded from future experiments. To further explore the influence of cartilage parameters (i.e. collagen and PG content) on PTA staining, staining experiments on enzymatically degraded (i.e. collagenase and chondroitinase ABC) samples were performed [55] (Fig. 17). As expected, a decrease in collagen content by collagenase resulted in a decrease in PTA staining intensity, mainly near the superficial layers. Interestingly, in this case no large deviation was found between FTIRI and CECT. Note that the collagenase treatment also induced a significant depletion of PGs. In addition, a decrease in PG content by chondroitinase ABC resulted in a slight increase in PTA staining intensity, near the superficial layer. Hence, these results show that both POM-protein interactions and, to a lesser extent, electrostatic interactions with fixed charges from PG molecules, affect the distribution of PTA throughout

the tissue. The smaller effect of the fixed charges on PTA distribution could be associated with its charge diffuse nature. Despite the remaining questions on the staining mechanism of PTA, Nieminen et al. were able to successfully apply PTA-based CECT in the development of a 3D histopathological grading system of osteochondral tissue [112].

Further, Davis et al. found PTA could be a suitable CESA to study the mechanical properties of AC through digital volume correlation (DVC), since it induced sufficient contrast-enhancement and did not alter the stiffness of the cartilage [123]. Dissolution of PTA in PBS (10 mM) was preferred over an aqueous 70 % ethanol (EtOH) solution, due to the dehydrating nature of EtOH. However, this could lead to either to formation of a plethora of other POM species derived from PTA [243], complete nullification of the buffer [115] or a combination of both. To address the pH-lability of PTA, it would be interesting to investigate the comparable silicotungstic acid (STA), given its greater resilience to fluctuations in pH [244].

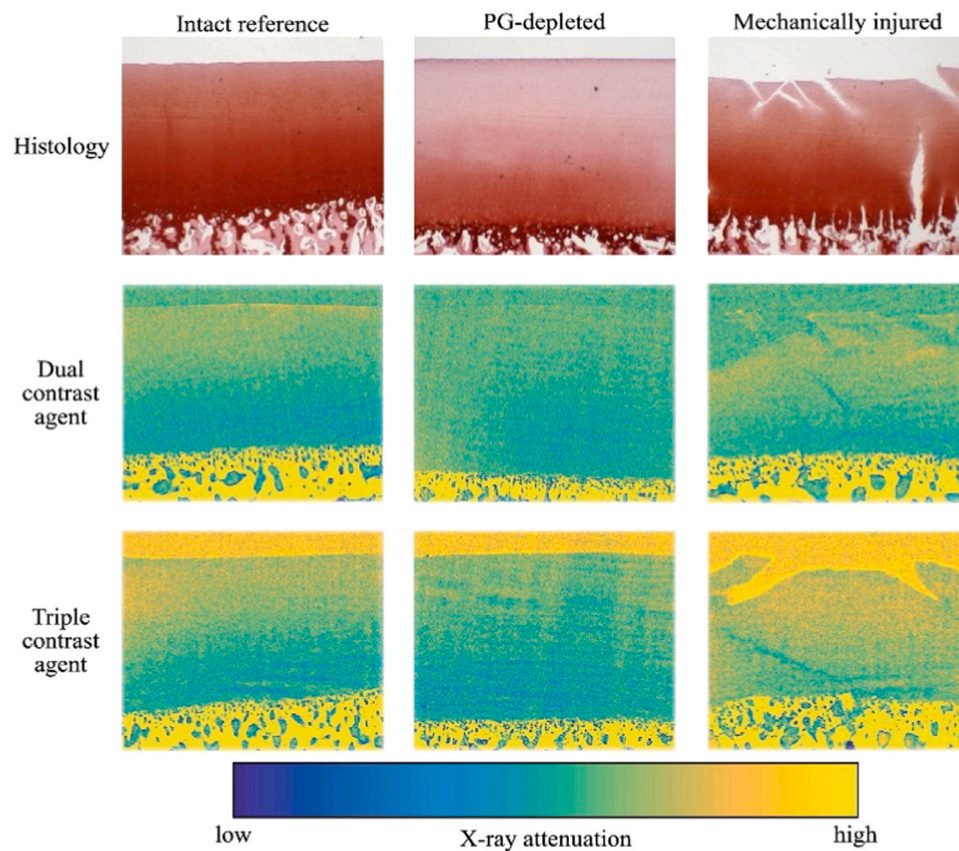


Fig. 13. Safranin-O stained histological sections and synchrotron microCT (32 keV) images (average of five consecutive 6.5 μm thick slices) of the intact reference, PG-depleted, and mechanically injured samples imaged with dual and triple contrast agents 2 h after the contrast agent immersion. Articulating surface and cracks are better visualized with the triple contrast agent owing to better contrast induced by bismuth nanoparticles (BiNPs) that, due to their size, are too large to diffuse into cartilage. The enhancement caused by the BiNPs was similar for 34 keV synchrotron microCT images (not shown). CT images were selected to closely match the locations of the histological sections.

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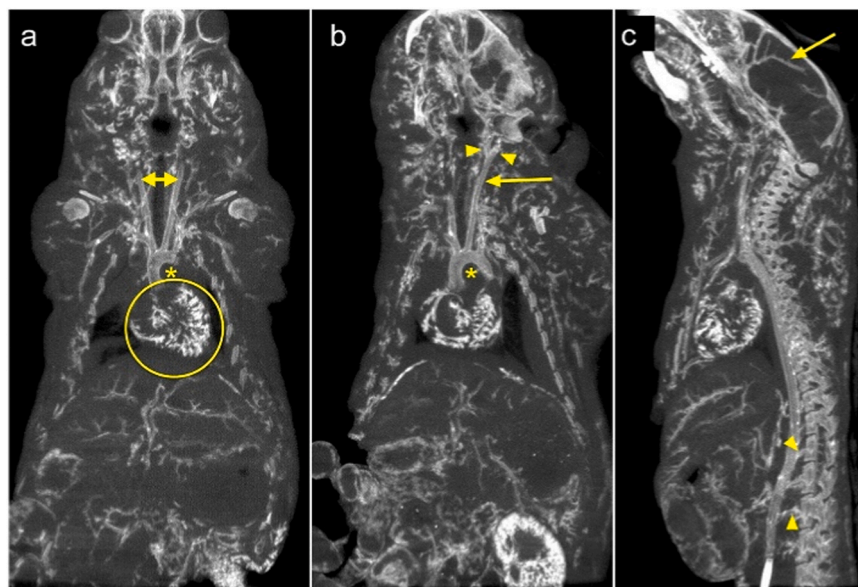


Fig. 14. Coronal (a,b) and sagittal (c) slices of an AlumHemFeI-T whole-body perfused mouse. Slices are taken from images acquired with a 5-minute scan protocol (90 kVp; 50 μm voxels). The images are 1-mm maximum intensity projections selected to highlight the coronary vasculature (circle in a), aortic arch (* in a,b), common carotid arteries (arrows in a,b), the internal and external carotid arteries (arrowheads in b), the cerebral vasculature (arrow in c), and the small lumbar vessels (arrowheads in c).

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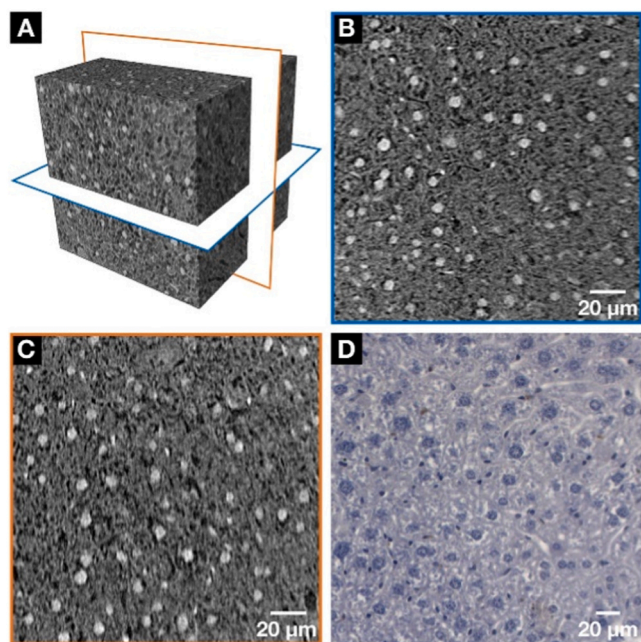


Fig. 15. NanoCT data (A–C) in comparison with the histological microscopic slide (D) derived from the same mouse liver lobule after application of the hematein-based X-ray staining protocol. Clear visualization of the larger hepatocyte cell nuclei and the smaller cell nuclei such as Kupfer cells and SECs in white (A–C) or dark purple (D) and the BC network displayed in black (A–C) or white (D) was achieved, respectively. (A) The volume of interest (VOI) highlighting the two nanoCT slices shown in (B, blue frame) and (C, orange frame). (B, C) Representative individual nanoCT slices as indicated in the VOI from (A). (B) and (C) are positioned orthogonal to each other. The orientation of the BC network, which is formed by the hepatocytes is seen, i.e. more horizontal arrangement is seen in (B) and a more vertical alignment in (C). The nanoCT slice thickness is 580 nm. (D) Representative histological microscopic slide with a thickness of 3 μm obtained from the same mouse liver lobule sample after the applied hematein-based staining and embedding in a paraffin block. Adapted from [17] and reused under Creative Commons license (CC BY 4.0).

Finally, Gabner et al. developed a new protocol for imaging cartilage using Ruthenium red in ethanol as CESA [194]. The approach enabled clear differentiation of cartilage matrix due to its high affinity for Ruthenium red, which interacts with the proteoglycans in the cartilage matrix. Importantly, the protocol preserved bone mineral, allowing simultaneous visualization of both cartilage and bone, albeit with similar grey values. Using DECT, they achieved spectral separation of cartilage and bone based on the distinct X-ray attenuation properties of the Ruthenium red-stained cartilage and the bone.

2.4.2. Applications in bone, bone marrow and adipose tissue imaging

Prior to the establishment of ioxaglic acid as a CESA for studying AT using CECT (*vide supra*), inorganic CESAs have been evaluated. Osmium tetroxide ($\text{Os}^{\text{VIII}}\text{O}_4$), the first CESA used to study AT in 3D using CECT, was introduced by Scheller et al. [148], and is currently still considered the gold standard in the field of AT research (Fig. 18) [149–152]. This molecule readily dissolves in the hydrophobic fat reservoir of the Ad and reacts with double bonds of unsaturated fatty acids, forming an osmate ester, which can be hydrolyzed further giving rise to a vicinal diol and the reduced $\text{Os}^{\text{VI}}\text{O}_3$ species [245]. OsO_3 , being unstable, disproportionates back to OsO_4 and the black insoluble $\text{Os}^{\text{IV}}\text{O}_2 \cdot 2\text{H}_2\text{O}$ [246]. Despite its specific interaction with unsaturated double bonds, it has been found that the use of OsO_4 is limited to small, decalcified samples and that post-processing for classical 2D histological assessment is not possible. Hence, due to these limitations, combined with the high toxicity and volatility of OsO_4 [148,247,248], a non-toxic alternative with similar detection potential was desired. To this end, Kerckhofs et al.

introduced the 1:2 Hafnium-substituted Wells-Dawson polyoxometalate (Hf-WD 1:2 POM). This CESA could simultaneously reveal bone, bone marrow, vasculature and adipocytes [114]. After staining, the bone remained the highest attenuating tissue, followed by the bone marrow, vasculature and the adipocytes obtained the lowest grey value. In their study, it was found through Raman spectroscopy that PTA chemically alters several ECM proteins (i.e. collagen type II and fibrin), which was not the case for the Hf-WD 1:2 POM, highlighting the non-destructive character of the Hf-WD 1:2 POM. Frangi et al. demonstrated the efficacy of the Hf-WD 1:2 POM in addressing a biological question concerning PIT2-deficiency in a mouse model [136]. A noteworthy limitation, hampering other research groups from exploiting this POM, is a meticulous three-step synthesis to obtain the final product [227, 240]. This problem was confronted by de Bournonville et al., who conducted a comparative study among various other POMs (i.e. parent Wells-Dawson POM (Parent-WD POM), monolacunary WD POM (Mono-WD POM), trilacunary WD POM (Tri-WD POM) and PTA), that required less synthetic steps in contrast to the Hf-WD 1:2 POM (Fig. 19) [115]. Because of an enhanced diffusion, but similar contrast-enhancement potential in murine tibia samples, the Mono-WD POM was identified as an excellent alternative, even though its presence in CECT literature remains scarce. While being one of the few studies exploring the chemistry of CESAs, some remarks must be made. The concentrations of evaluated CESAs range from 3.69 mM to 12.15 mM. The concentration could affect contrast differences and hence this should be considered when interpreting the data. Next, it was found that the Mono-WD POM precipitated while this was not the case for the Hf-WD 1:2 POM. Even though this was elegantly solved by adding Li^+ ions to the solution [231], it should be noted that the molar concentration of the Mono-WD POM was approximately double of the Hf-WD 1:2 POM molar concentration. Thus, this is not necessarily a limitation of the POM, but possibly a consequence of the design of the experiment.

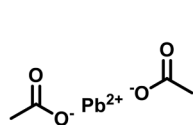
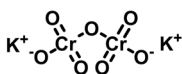
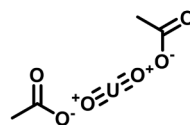
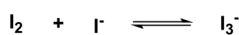
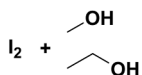
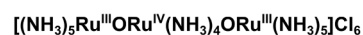
Another study evaluated DICECT for studying mammalian tooth development of the wallaby [184]. It was found that within the soft tissues of the specimen, the epithelial tissue reached highest attenuation values, followed by muscle and tongue. No extensive shrinkage was observed, which could have been a result of prior storage in methanol (MeOH) or EtOH, or due to regular refreshment of the staining solution. While unreacted iodine (I_2) was cleverly reduced to iodide (I^-) using a sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) solution, preventing the samples from having a deep brown color, this is unlikely the reason that shrinkage or damage to the tissue was prevented. Moreover, while it might appear that the iodine was removed from the tissue, $\text{Na}_2\text{S}_2\text{O}_3$ is unable to reduce covalently bound iodine. Furthermore, if no further washing steps are undertaken, I^- anions will still remain present in the tissue.

2.4.3. Applications in nervous tissue imaging

Currently, the inorganic CESAs are the most popular group of CESAs to study nervous tissue with CECT, based on the number of publications. Seven CESAs have been evaluated: uranyl acetate (UA), OsO_4 , PTA, Hf-WD 1:2 POM, Preyssler anion, Lugol's iodine and I_2 dissolved in alcoholic solutions. Their affinity patterns when staining nervous tissue will be discussed per CESA. UA has been adopted from electron microscopy (EM) as a heavy-element containing stain [249]. However, the two studies that applied UA did not unambiguously reveal the staining properties of UA. Smith et al. found that, within their timeframe, UA was unable to completely stain a bumblebee brain and was hence not further explored [126]. Parlanti et al. succeeded in obtaining fully stained murine spinal cord samples, showing white matter (WM) as hyperintense compared to grey matter (GM). However, in their procedure several molecules were used that contained heavy atoms (e.g. OsO_4 and $\text{K}_3\text{Fe}^{\text{III}}(\text{CN})_6$ during lipid fixation) [191], which makes it impossible to determine the affinity pattern of UA.

OsO_4 was introduced by Ribí et al. as a CESA for nervous tissue imaging of the honey bee [142], in which it was postulated that the concentration of OsO_4 depends on the lipid content of the sample. White

Salts and neutral compounds

**Lead acetate****Potassium dichromate****Uranyl acetate****Silver nitrate****Lugol's iodine solution****Alcoholic iodine****Osmium tetroxide****Ruthenium red**

Polyoxometalates

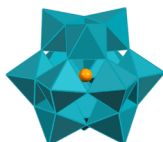
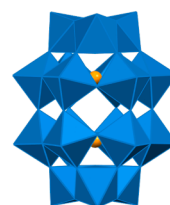
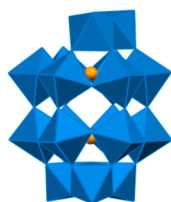
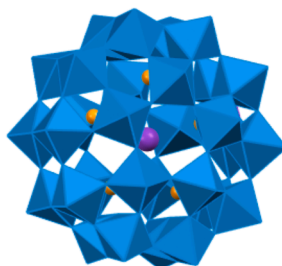
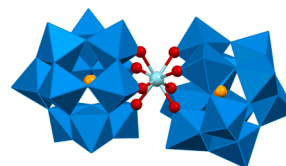
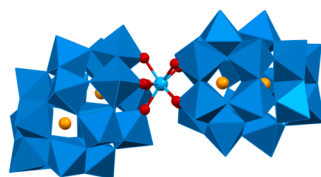
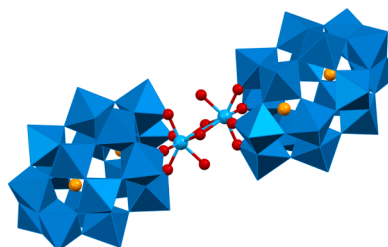
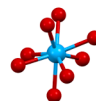
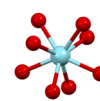
**PTA****PMA****SPT****Parent-WD POM****Mono-WD POM****Preyssler anion****Zr-K 1:2 POM****Hf-WD 1:2 POM****Hf-WD 2:2 POM****[PO₄]****Na⁺****[WO₆]****[MoO₆]****[HfO₈]****[ZrO₈]**

Fig. 16. Chemical structure of the inorganic CESAs divided salts and neutral compounds and polyoxometalates: oxidation states of salts: Pb = +II, Cr = +VII, U = +VI, Ag = +I. Polyoxometalates have a net negative charge at physiological pH. POM figures are made using Mercury software [220] and based on the following references: PTA [221], PMA [222], SPT [223], Parent-WD POM [224], Mono-WD POM [225], Preyssler anion [226], Zr-K 1:2 POM [227], Hf-WD 1:2 POM [227], Hf-WD 2:2 POM [228].

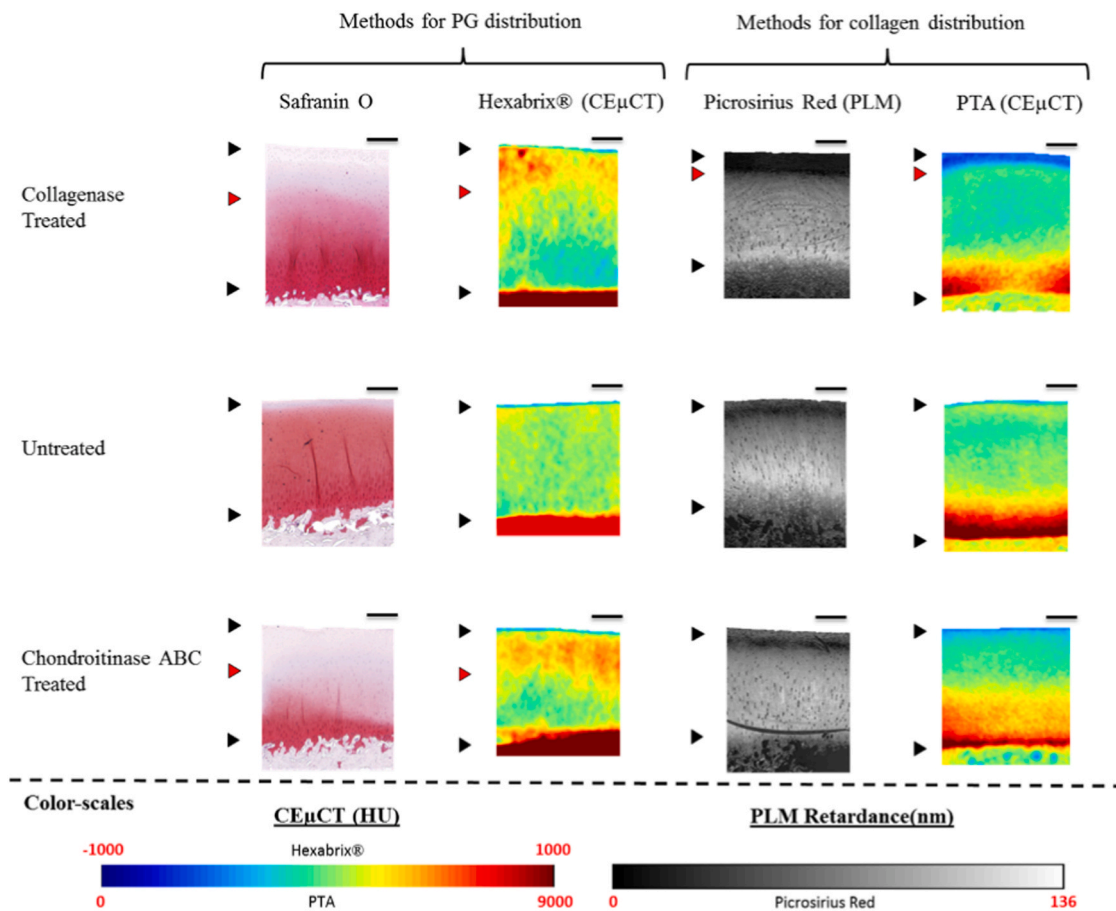


Fig. 17. 2D-slices of PTA- and Hexabrix®-labelled ACs (CEμCT) compared to the corresponding histological collagen and PG stains. Birefringence of Picrosirius red stain was imaged with polarized light microscopy (PLM) to visualize the collagen fiber content and distribution. The histological stains showed clear effects of the enzymes, which can be seen as an insufficient staining in the surface of the sample as compared to the control samples (the depth of the enzyme effect indicated with red arrows). The Picrosirius red stain and the PTA showed highly similar collagen distributions. Also Safranin O and Hexabrix® showed highly similar PG distributions. Color-scaled Hounsfield unit (HU) -values of the CEμCT and retardance (nm) of PLM are presented in the bottom. Different scaling between the PTA- and Hexabrix®-enhanced images were used to better visualize the distributions of the contrast agents in the cartilage. Black arrows indicate the cartilage surface and the uncalcified cartilage-CC interface. Scale bars on the right corner of each sample equal to 500 μm.

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et al. showed that OsO_4 was also a suitable CESA to study the brain volume of fish species [143]. Given the good contrast provided by OsO_4 staining, Masis et al. evaluated its potential to quantify lesions in the brain on a rat mouse model [145]. Due to the high quality of their images, it can be concluded that OsO_4 achieves higher concentrations in WM compared to GM, which could be linked to the hydrophobic nature of OsO_4 . Further, the molecular layer (ML) of the cerebellum is hyperintense compared to the granular layer (GL). Within the hippocampus, regions with high cell density in both cornu ammonis and dentate gyrus were hypointense (Fig. 20). Additionally, they found that penetration of OsO_4 through the tissue was accelerated by changing the cacodylate buffer with demineralized water. However, the hypothesis that water could act as a mild detergent and permeabilize the cell membranes is questionable [144]. Similar affinity patterns were observed by Pinto et al. who demonstrated that OsO_4 can be used to study a mice stroke model using CECT [146]. However, the aforementioned toxicity concerns regarding OsO_4 have also lead researchers to avoid this CESA [126].

PTA was evaluated as another well-known CESA in the field of CECT imaging, and was shown to be preferred when investigating the bumblebee brain [126]. The excellent contrast provided by PTA lead to the development of a CECT-based bumblebee brain atlas [127]. On the contrary, PTA was not preferred as CESA for studying the murine [117, 146, 187] or oval squid [100] nervous tissue, when compared to

inorganic iodine-based CESAs, since it was found to be less potent in terms of contrast resolution and required longer diffusion times. The affinity patterns of this POM with respect to nervous tissue could not be easily deduced from existing literature and require additional research.

Hf-WD 1:2 POM and Preyssler anion have been applied as CESA for staining murine brain [212]. Both POMs were able to discern between different type tissues and the surrounding and showed a higher affinity towards grey matter compared to white matter (Fig. 21). Although Preyssler anion showed a distinct contrast difference between the corpus callosum (white matter) and the surrounding tissue, it was more difficult to differentiate between the layers of the hippocampus. Interestingly, upon staining the tissue, the POMs did not induce any volume change during the process.

Inorganic iodine-based CESAs have been broadly explored to visualize nervous tissue using CECT [100, 117, 126, 146, 159–161, 163, 164, 167, 171, 173, 187, 188, 191]. The affinity patterns of both alcoholic iodine as well as Lugol's iodine components when staining murine and hominine nervous tissue are found to be similar upon evaluating the existing literature. Generally, it is observed that WM is hyperintense compared to GM. Furthermore, the GL seems to show slightly higher grey values compared to the ML and in some cases the Purkinje cell layer separating ML and GL could be seen as hypointense. The dense cell layers in the hippocampus were also hypointense, while the stratum moleculare seems to obtain a slightly hyperintense grey value. The

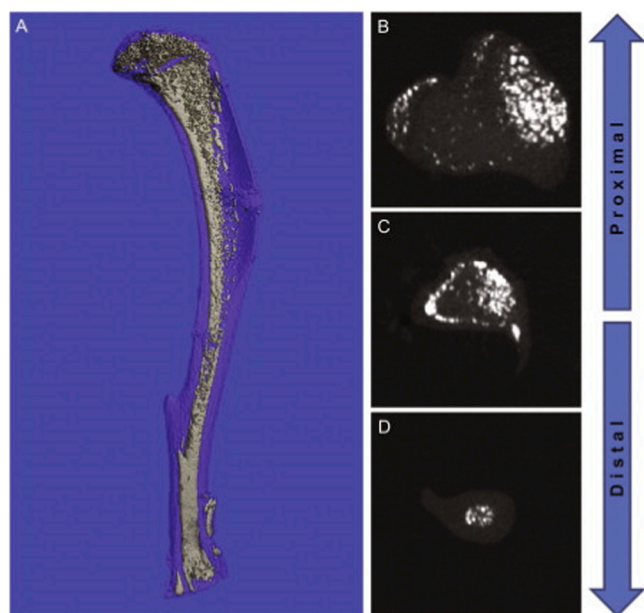


Fig. 18. Distribution of MAT in the medullary canal. Adipocytes in the BM of the mouse are unevenly distributed throughout the medullary canal. They are most densely clustered in the epiphyses. In the metaphysis and diaphysis, adipocytes are most numerous near the central vascular canal and adjacent to the cortical bone. (A) Three-dimensional reconstruction of a 16-week-old C3H mouse osmium-stained tibia. Light blue, bone; white, MAT. (B–D) Transverse sections of the same bone from more proximal (toward the knee) to distal (toward the ankle) showing defined regional clustering of the marrow adipocytes (white).

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forementioned studies have attempted to describe the staining behavior of Lugol's iodine and alcoholic iodine solutions, but often neglecting the intricate equilibria that exist in both solutions [219]. Even though the staining mechanisms of the different iodine species present in solution still require fundamental research, one observation by Heimel et al. can be addressed [164]. After washing the brain specimen with $\text{Na}_2\text{S}_2\text{O}_3(\text{aq})$, it was expected that a reduction in opacity of the sample is linked with a reduction in attenuation of the solution. However, when I_2 is reduced by $\text{S}_2\text{O}_3^{2-}$, it forms $\text{S}_4\text{O}_6^{2-}$ and I^- , which is still able to attenuate X-rays. Moreover, when iodine is covalently attached to proteins and lipids, it does not produce the intense brown coloration in the tissue, yet it retains the ability to attenuate X-rays. Inorganic iodine-based CECT has been successfully applied to quantify amyloid

beta plaques in an Alzheimer's disease murine model [188], a mouse stroke model [146], as well as to study cavernous malformations [160] and peripheral nerve regeneration [164]. The confirmation of amyloid beta plaques identity in murine brain stained with Lugol's iodine is shown in Fig. 22.

Finally, $\text{K}_2\text{Cr}_2\text{O}_7$ has also been investigated on a sheep cerebellum, providing excellent contrast between GM and WM. While the ML and GL obtain similar grey values, the Purkinje cell layer could be seen as hypointense [192]. The affinity for WM was hypothesized to result from an *in situ* reduction of Cr^{VI} to Cr^{V} and Cr^{III} , while oxidizing lipids. This preferably occurred in WM, which is more lipid-rich compared to GM. Abovementioned affinity patterns, in combination with what was discussed in 3.2.3, show several key factors affecting the distribution of CESAs through the nervous tissue: cell density and packing (e.g. hippocampus), lipophilicity (e.g. WM vs GM) and the presence of myelin (e.g. invertebrate vs vertebrate species).

The most well-documented limitation of inorganic iodine- and PTA-based methods concerns the extensive tissue shrinkage that has been observed in numerous tissues [125,162]. Several solutions have been proposed in the field of nervous tissue imaging for iodine-based methods, such as prior fixation of the tissue with a hydrogel formulation [159], frequent refreshment of the staining solution combined with longer fixation of the tissue [171] and performing the staining experiment in a buffer with higher concentration of buffering ions [172]. These respective studies have been able to show a reduction in shrinkage, but this is often at the cost of a change in the staining behavior of the inorganic iodine-based solution.

2.4.4. Applications in kidney imaging

Silva et al. reported a kidney staining procedure applying two fixatives (i.e. formaldehyde fixation followed by graded EtOH fixation) subsequently. Tissue staining was performed with I_2 in EtOH (absolute), which allowed clear differentiation between the renal cortex, vasculature and inner and outer medulla [189]. After complete staining was achieved, iodine distributed throughout the kidney with a gradient decreasing from the cortex towards the inner medulla. A similar staining pattern was observed by Missbach-Guentner et al. who used PTA staining to study the murine kidney microstructure [116]. It was postulated that the staining intensity correlated with the compactness of the tissue, decreasing from the cortex towards the inner medulla. Interestingly, in contrast with the majority of studies, it was found that the volume of the kidney increased after PTA staining with no apparent explanation. This has to be interpreted with caution, since no graphs or images are given and no other research groups have reported this behavior. Moreover, de Bournonville et al. observed a strong, expected shrinkage of the murine kidney after PTA staining [115]. This was not

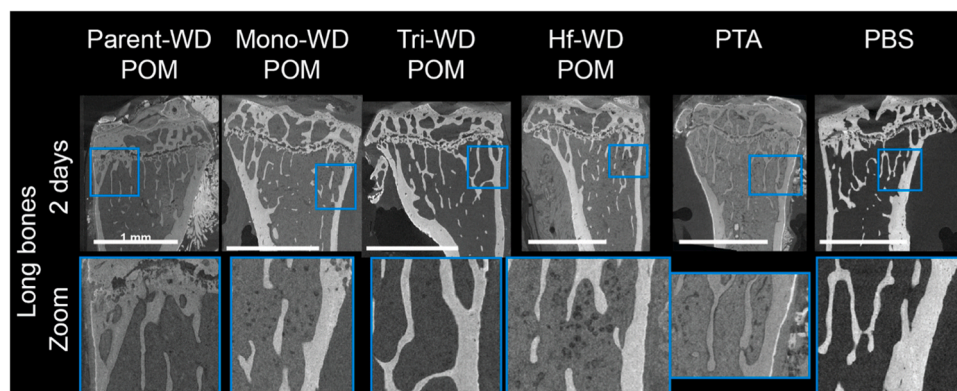


Fig. 19. CE-CT images of murine tibiae and kidneys stained with the different POMs. Typical longitudinal CE-CT slices of tibiae stained with different POM species (columns: Parent-WD POM, Mono-WD POM, Tri-WD POM, Hf-WD POM and PTA (positive control)) and PBS (negative control) for different staining times. Scale bar = 1 mm.

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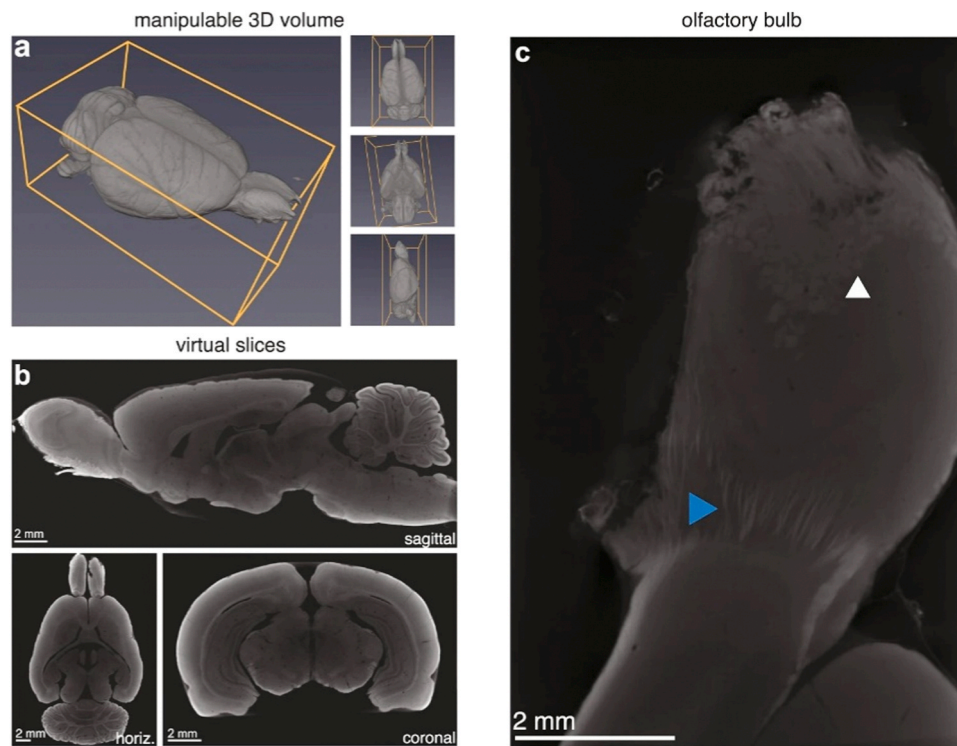


Fig. 20. Capabilities of lesion characterization through micro-CT imaging. (a) Manipulable 3D volume and (b) virtual slices (13.9 μm voxels) at arbitrary orientations from a micro-CT scan of a Long-Evans rat. (c) Olfactory bulb scanned at higher resolution (4.9 μm voxels) revealing glomeruli (white arrowhead) and individual nerve fibers (blue arrowhead).

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observed for other POMs (*i.e.* Tri-WD POM, Parent-WD POM, Mono-WD POM and Hf-WD 1:2 POM) that were synthesized as the salts instead of the acid, as can be seen in Fig. 23. Non-destructive POMs that were able to adequately stain the kidney (*i.e.* Mono-WD POM and Hf-WD 1:2 POM) also showed a decreasing grey value gradient from the cortex to the inner medulla, after staining was completed. The results of their study showed that, within the set of CESAs that were evaluated, the Mono-WD POM was preferred because of its chemical stability, ability to provide good contrast within the tissue, swift penetration through the tissue, ease of synthesis and non-destructiveness.

2.4.5. Applications in imaging the cardiovascular system

Inorganic CESAs used to study the heart and vasculature using CECT include: PMA, PTA, Lugol's iodine, SPT, Mono-WD POM Hf-WD 1:2 POM and OsO_4 . OsO_4 has been applied once, with the aim of visualizing the wall of the coronary arteries of a murine heart [147]. It was found that the arterial wall showed up hyperintense compared to surrounding tissues and could hence be segmented and analyzed. However, the low resolution of the images impeded any further deduction of affinity patterns. Stephenson et al. were interested in visualizing the cardiac conduction system (CCS) of rat and rabbit hearts, by staining the tissue with Lugol's iodine, as shown in Fig. 24 [154]. While successful in staining and segmenting the CCS, their results contradicted that Lugol's iodine components mainly distribute through the tissue according to the glycogen content. After all, the CCS achieved lower attenuation values compared to the myocardium, but was found to be strongly periodic-acid Schiff (PAS) positively stained, indicating a higher carbohydrate content. These findings clearly highlight the complexity of biological systems, and the inability of correlating staining patterns of non-specific CESAs to the distribution of a single molecule, such as glycogen. Due to the promising properties of Lugol's iodine as a CESA for heart imaging using CECT, Butters et al. optimized the staining process, albeit only for one specific concentration of iodine components [166].

Several additional studies were not only interested in the anatomy and microstructure of the heart, but also how these are affected by diseases. To this end, Dullin et al. applied PTA to study a high-fat diet induced atherosclerosis mouse model. Unexpectedly, they found that the heart swelled after PTA staining. However, it should be noted that the concentration of PTA in their system was quite low (2.43 mM), which is not able to nullify the PBS buffer (10 mM) completely, resulting in a non-ideal pH for PTA and the formation of a plethora of other POM species. Furthermore, they found that tissue stiffness was increased, and they hypothesized that this was related to the fixation procedure that was applied. Despite the potential effect of fixation on mechanical properties of the tissue [250], more research is required to prove this statement. Given the effectiveness of tungsten to attenuate X-rays, Pallares-Lupon et al. preferred the use of PMA, containing the less effective X-ray attenuating element, Mo, on larger samples in which the X-rays need to travel further through the sample [132]. Their results indicate that PMA has a high affinity for collagen-rich regions. Additionally, it is also able to provide a general overview of the heart anatomy, even though it seems from their images that in some samples homogeneous staining was not yet achieved.

None of the abovementioned methods was able to provide microstructural information on the single muscle fiber level. This was addressed by Maes et al. who introduced an extension of conventional microCT sample preparation, termed cryogenic contrast-enhanced computed tomography (cryo-CECT) [137]. Two CESAs, namely Hf-WD 1:2 POM and isotonic Lugol's iodine solution were evaluated for their staining potential of muscle fibers using cryo-CECT. They found that Hf-WD 1:2 POM in combination with freezing allowed visualization of individual heart muscle fibers due to the desalinating properties of crystallizing water [251], rendering muscle fibers strongly X-ray attenuating, while the ice phase was weakly X-ray attenuating. By using their methodology, they were able to demonstrate that transverse aortic constriction (TAC) surgery did not impact the orientation of the fibers,

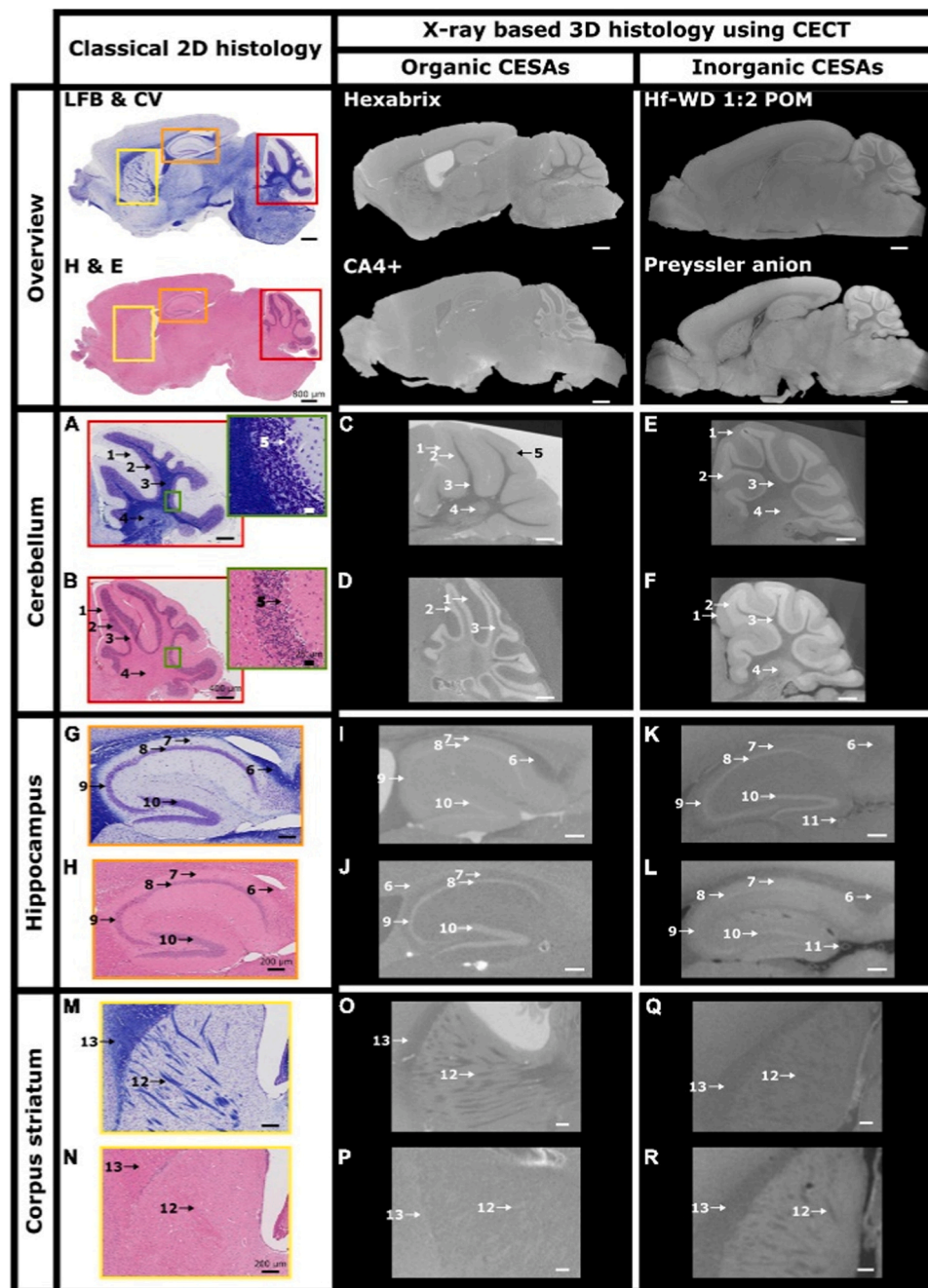


Fig. 21. Investigation of CESA distribution throughout the murine hemisphere. Overview and zoom on cerebellum, hippocampus and corpus striatum of cross-sections of murine hemispheres stained by LFB & CV (A,G,M), H&E (B,H,N), Hexabrix (C,I,O), CA4+ (D,J,P), Hf-WD 1:2 POM (E,K,Q), and Preyssler anion (F,L,R). 1 = molecular layer, 2 = granular layer, 3 = white matter, 4 = cerebellar nuclei, 5 = Purkinje cell layer, 6 = corpus callosum, 7 = stratum oriens, 8 = stratum pyramidale (CA1), 9 = stratum pyramidale (CA2 and/or CA3), 10 = stratum granulare (Dentate gyrus), 11 = blood vessel, 12 = Striatopallidal fiber bundles, 13 = corpus callosum.

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slightly, but non-significantly, impacted the diameter of the fibers and resulted in formation of interstitial fibrotic regions (Fig. 25). Interestingly, the fibrotic regions containing a high collagen content, shown by picrosirius red staining, obtained hypointense grey values compared to the surrounding tissue.

Since the heart and vasculature are inherently interconnected within their role in an organism, efforts have been made from the CECT community to better understand the microstructure of vessels. Brunet et al. and Helfenstein-Didier et al. used SPT as a CESA to study the tensile rupture and aortic dissection using CECT [133,134]. Their results

indicate that SPT can highlight the elastin sheets. Furthermore, the adventitia obtains a hypointense grey value compared to the media. Pétré et al. reported similar staining patterns of SPT and no change in mechanical properties after staining porcine aorta with SPT up to 17 days. However, fuzziness was observed in the CECT images in both studies [130]. Leyssens et al. found that the Mono-WD POM and Hf-WD 1:2 POM showed a similar affinity pattern (i.e. elastin-rich regions such as the media were hyperintense compared to collagen-rich regions such as the adventitia) towards different vessels (porcine vena cava, femoral artery and aorta, rat aorta and human aorta) (Fig. 26) [135]. Pétré et al.

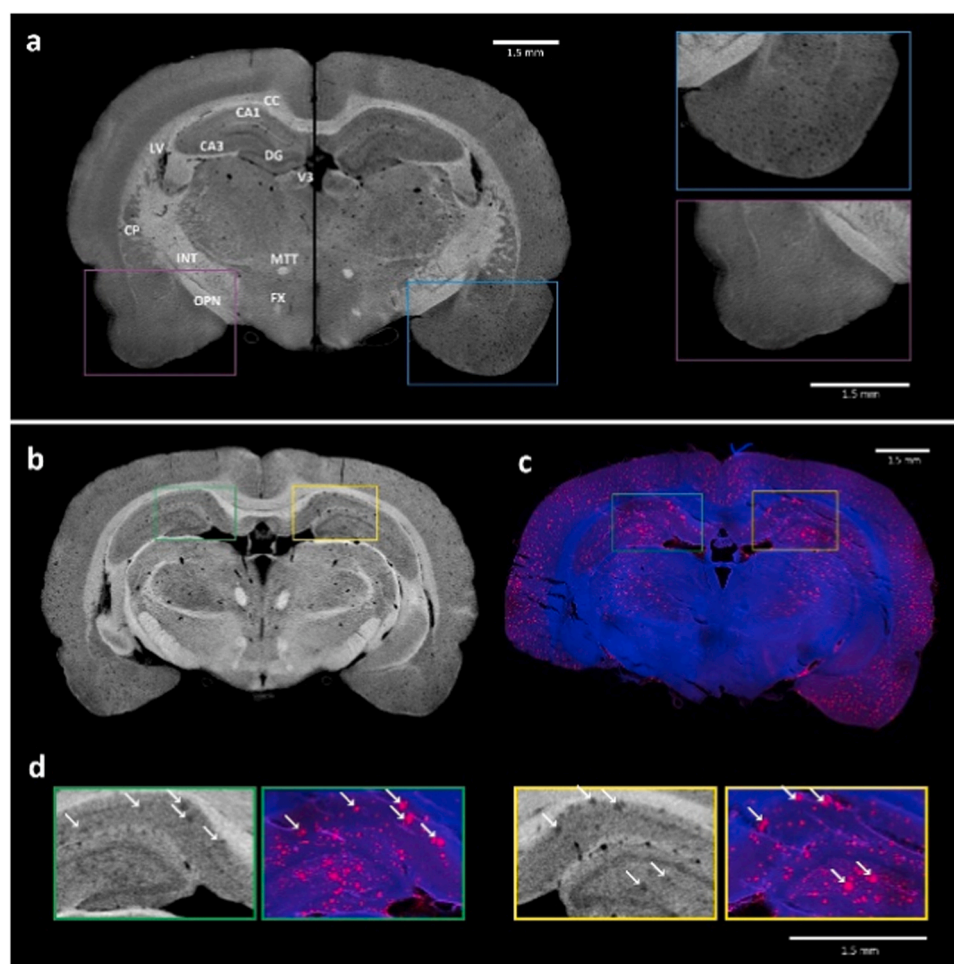


Fig. 22. Confirmation of plaques identity in microCT data: (a) Composed picture of the microCT scan sections from control (left side) and transgenic (right side) rat brain. The transgenic tissue exhibits a large amount of suspect amyloid plaques (right part of the brain). Selected detail image of corresponding areas that are shown in pink and blue frames. Basic anatomical structures visualized in microCT scan: CA1 and CA3 cornu Ammonis 1 and 3 of hippocampus, CC corpus callosum, CP caudoputamen, DG dentate gyrus, FX fornix, INT internal capsule, LV lateral ventricle, MTT mammillothalamic tract, OPN olivary pretectal nucleus, V3 third ventricle. Micro CT image (b) and immunohistology section with detected plaques (c) of corresponding coronal brain sections from the transgenic rat no. 60 confirms the presence of plaques in microCT scan. Selected plaques are indicated by white arrows in enlarged slice windows (d). Adapted from [188] and reused under Creative Commons license (CC BY 4.0).

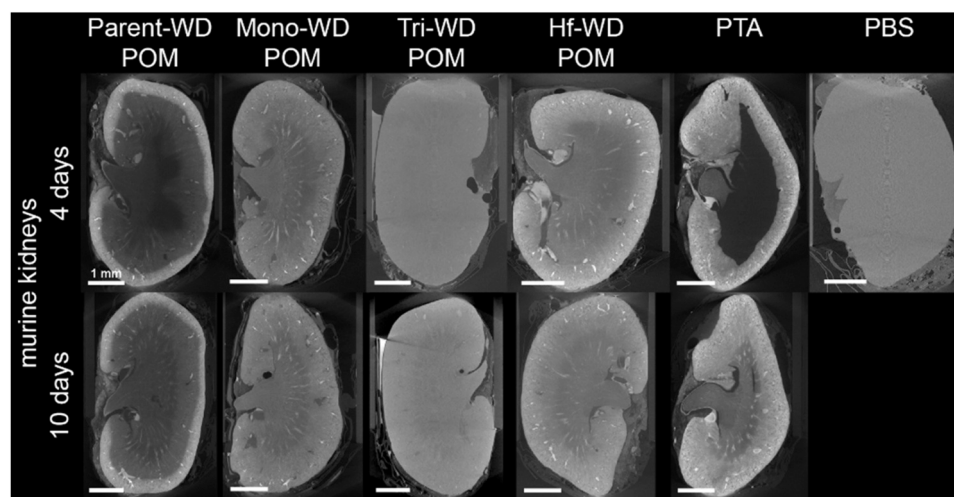


Fig. 23. CECT images of murine kidneys stained with the different POMs. Typical coronal CECT slices of kidneys stained with different POM species (columns: Parent WD POM, Mono-WD POM, Tri-WD POM, Hf-WD POM and PTA (positive control)) and PBS (negative control) for different staining times. Scale bar = 1 mm. (a) Reprinted and limited adapted from [115], Copyright 2020, with permission from Elsevier.

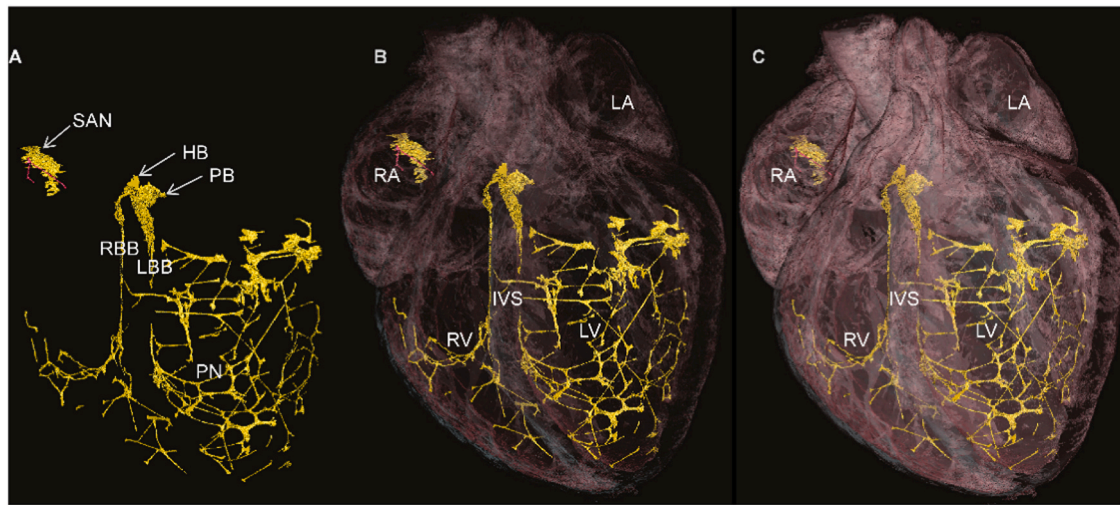


Fig. 24. 3-D representation of the CCS in failed rabbit heart. Segmented 3D surface from showing the three major components of the CCS (yellow) (A). Overlaid with cardiac mass (pink) at decreasing levels of transparency (B,C) showing the penetrating bundle emerging from the right atrium and transversing the IVS as the His bundle (in true in situ position). HB- His bundle, LA- left atrium, LBB- left bundle branch, PB- penetrating bundle, PN- Purkinje network, RA- right atrium, RBB- right bundle branch, RV- right ventricle, SAN- sinoatrial node, IVS- interventricular septum. Adapted from [154] and reused under Creative Commons license (CC BY 4.0).

reported no significant change in mechanical properties after staining porcine aorta with Mono-WD POM and Hf-WD 1:2 POM [130]. In contrast, when Nierenberger et al. evaluated PTA, PMA and Lugol's iodine as potential CESAs for imaging the porcine iliac vein, it was observed that the adventitia obtained higher grey values for PTA and PMA, while no strong difference was observed for Lugol's iodine staining [129]. Hanly et al. claimed a preferential affinity of PTA towards collagen-rich tissues, consistent with previous studies. However, the distribution of PTA throughout the sample was different, since the adventitia layer was hypointense compared to the media layer [252]. The potential origin of these differences can however not be deduced from their CECT datasets. Based on abovementioned studies, it seems that acidic POMs, such as PTA and PMA will preferably distribute according to the collagen content, while salts of POMs, such as SPT, will more readily enrich elastin-rich regions. Moreover, the presence of the Hf atom in the Hf-WD 1:2 POM seems to enhance the affinity towards the elastin sheets. However, more parameters will probably influence the distribution, which require well-designed experiments in order for them to be studied.

2.4.6. Applications in lung imaging

Silva et al. investigated the potential of Lugol's iodine on imaging lungs, still attached to the heart and esophagus. Their results show that the esophagus was hyperintense compared to the lungs. In addition, the staining process induced severe shrinkage of the samples (-25 %) [110]. This was attributed to the presence of EtOH, but is known to also be related to the presence of Lugol's iodine components [162]. Further, a linear increase in attenuation of their samples with respect to time was found, potentially indicating that staining has not yet reached an equilibrium (Fig. 27). Dullin et al. expanded the scope of CESAs for lung imaging with PTA, which, in combination with phase contrast-based contrast-enhancement, provided a facile tool towards guided-sectioning of biological tissues [121]. Contrast differences were observed for different constituents of the lung tissue, but these require more in-depth evaluation. Limitations mentioned of PTA-based CECT include the slow diffusion of PTA through the tissue samples and the loss of sharpness near the edges of the tissue.

2.4.7. Applications in skeletal muscle, tendon and ligament imaging

Given the affinity of inorganic CESAs for protein-rich tissues, inorganic CESAs have also been explored to study skeletal muscle tissue and

collagen-rich tendon and ligaments. Balint et al. evaluated PTA, PMA and Lugol's iodine for the visualization of porcine ligaments [128]. Their results showed that PTA staining resulted in the highest attenuation, but Lugol's iodine and PMA penetrated the tissue fastest. POM staining resulted in a clear diffusion front, but this was not the case for Lugol's iodine. Additionally, after homogeneous diffusion was reached, the POMs gave the most anatomical information, which could be related to increased tissue shrinkage. Interestingly, even though the molar concentration of PTA was lower compared to PMA, PTA staining resulted in the most drastic tissue shrinkage. This could be related to the higher acidity and more chaotropic nature of PTA [229,232]. Maes et al. also evaluated the abovementioned cryo-CECT protocol also successfully for the visualization of tendon using the Hf-WD 1:2 POM [137]. Furthermore, they investigated the effect of Lugol's iodine or Hf-WD 1:2 POM staining on the relative volume change of bovine skeletal muscle tissue. While, lugol's iodine shrunk the tissue, as expected based on literature [125,162], the Hf-WD 1:2 POM seemed to swell the tissue. However, to explain this phenomenon, additional research is required. DICECT has also successfully been applied to study biomechanics and evolutionary biology of the naked mole-rat masticatory muscles [253] and the echidna forelimb [178].

2.4.8. Applications in cochlea imaging

Given the seemingly non-destructive properties of the Hf-WD 1:2 POM, assuming a potential slight swelling is not considered destructive, Starovoyt et al. used this CESA for the imaging of the human cochlea [138–140]. First, CECT enabled the study of the optical coherence tomography-guided electrode insertion [139]. Herein, CECT was crucial to confirm the small impression trauma of the osseous spiral lamina due to contact with the optically-guided sheath. This was followed by a more in-depth investigation of the cochlear microstructures, which were at risk of trauma during an electrode insertion procedure (Fig. 28). Thorough examination of these datasets lead to the discovery of the round window arch, a new structure previously considered to be part of the secondary spiral lamina [138]. Finally, the excellent datasets of the human cochlea, obtained by CECT imaging after Hf-WD 1:2 POM staining, were used to develop a 3D-printed model of the scala tympani, which will be crucial to further perform electrode insertion experiments [140].

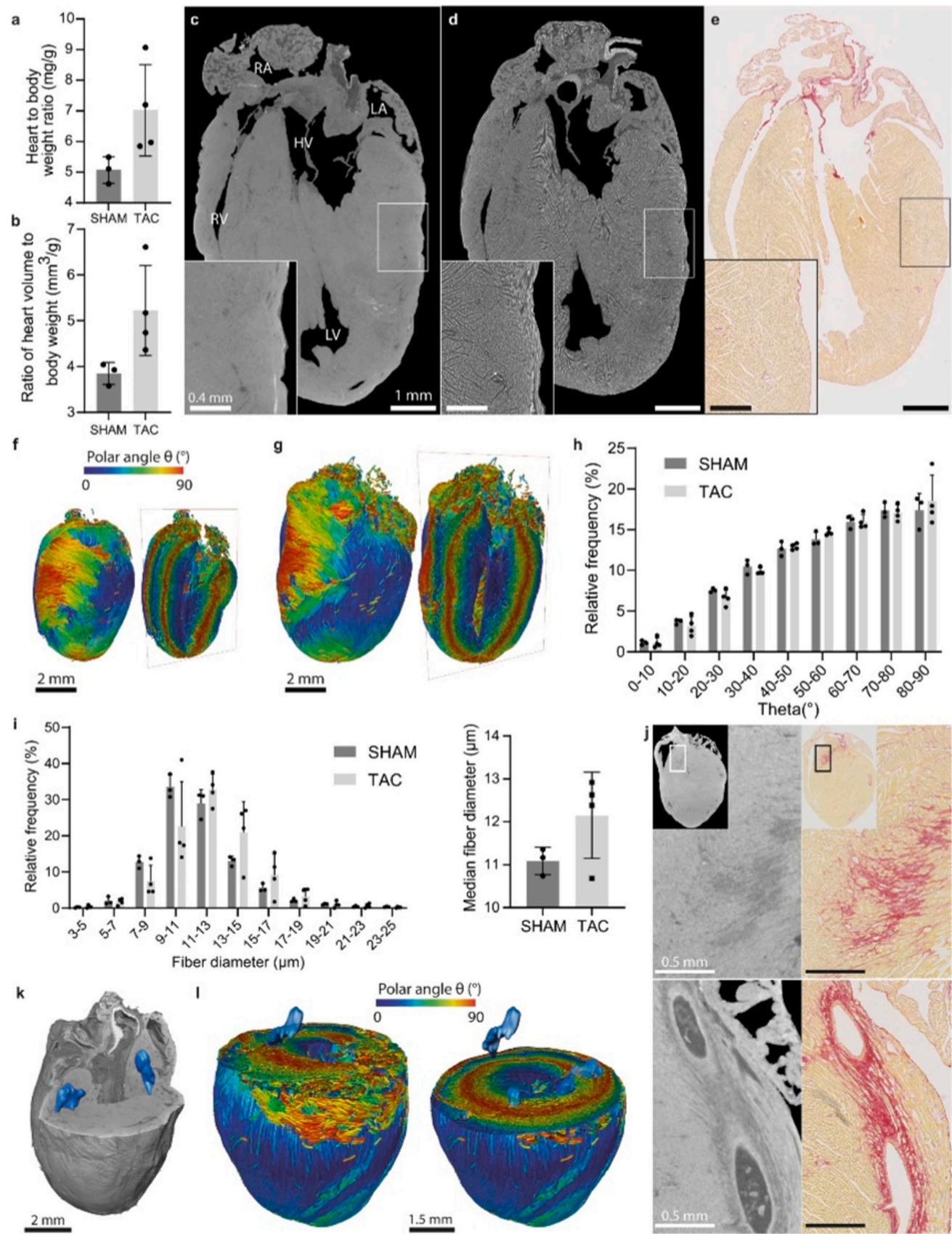


Fig. 25. 3D histopathology of pressure overload-induced hypertrophic murine hearts following TAC surgery. Bar graphs showing the mass (a) and volume (b) of the murine hearts of the sham and TAC group. Longitudinal sections of the same heart through the left and right ventricle, obtained by conventional Hf-WD POM-based CECT (c), cryo-CECT (d) and classical 2D histological sectioning followed by picrosirius red staining (e). RA right atrium, RV right ventricle, LA left atrium, LV left ventricle, HV heart valve. 3D spatial graph of the cardiac muscle fiber orientation of a sham heart (f) and a TAC heart (g). Fiber orientation is represented by the inclination, or polar angle θ and is indicated by the color scale. h Average histogram of the cardiac muscle fiber inclination comparing the sham and the TAC group. i Average histogram of the cardiac muscle fiber diagram (left) and a bar graph of the median fiber diameter (right) comparing the sham and the TAC group. j Sections obtained with CECT (left) and classical 2D histological sectioning followed by picrosirius red staining (right), showing the interstitial (top) and perivascular (bottom) fibrosis in a TAC heart. The rectangles in the inset indicate the location of the section. k Volume rendering of the CECT data of a TAC heart, showing the location of the regions that were severely affected by interstitial fibrosis (blue regions). l The regions of severe interstitial fibrosis (blue regions) overlaid with the fiber orientation map, clipped at two different heights. The fiber orientation is indicated as in f. Bars represent the mean, and error bars indicate the standard deviation; $n = 4$ for the TAC group and $n = 3$ for the SHAM group. To compare TAC and SHAM, two-sided unpaired t-testing was conducted on the data shown in the bar graphs of (a, b, i). $ns\ p > 0.05$.

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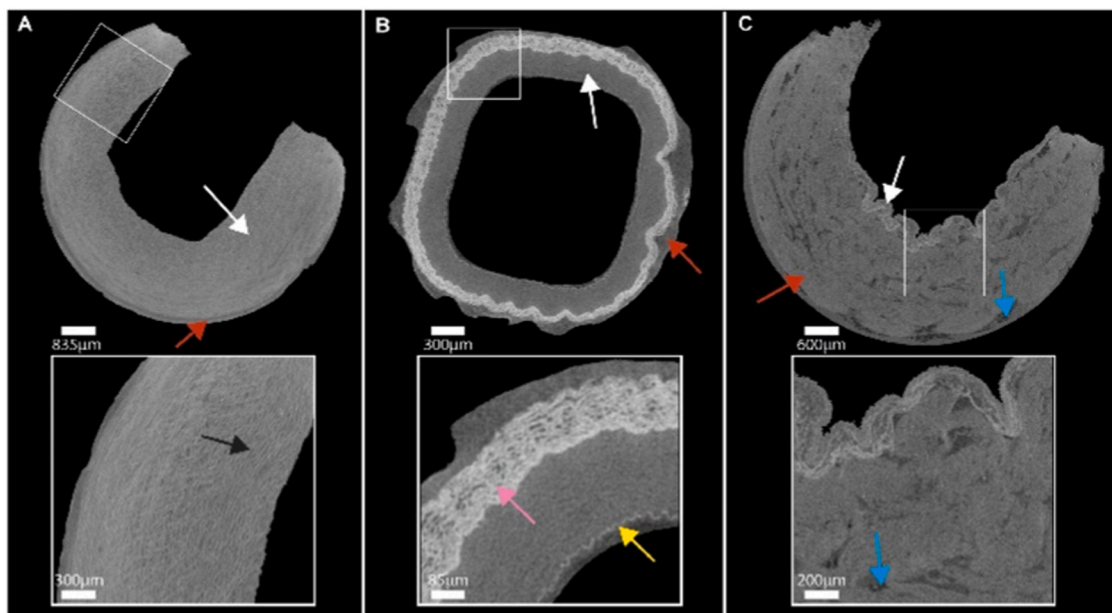


Fig. 26. Microstructural differences between the porcine aorta, femoral artery, and vena cava. CECT-based representations of the porcine (A) aorta, (B) femoral artery, and (C) vena cava stained with Hf-WD POM. Red arrow = adventitia, white arrow = media, yellow arrow = internal elastic lamina, pink arrow = external elastic lamina, black arrow = elastic lamella, blue arrow = adipocytes.

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2.4.9. Applications in placenta imaging

Hsu et al. pioneered simultaneous visualization of the murine embryo within the placenta using DICECT [185]. In contrast with general DICECT staining procedures, they did not use the Lugol's iodine solution, but rather an aqueous solution of I_2 . Note that I_2 will hydrolyze to a certain extent, forming the hypoiodous acid (HOI) and hydrogen iodide (HI) [254]. The I^- anions can subsequently react with I_2 forming I_3^- . As a consequence, while concentrations of I^- , I_2 and I_3^- likely differ from the ones in the Lugol's iodine solution, the same species can be present. Their staining protocol allowed differentiation between large vessels, decidua basalis (i.e. side of embryo implantation), decidua capsularis (i.e. opposite side of embryo implantation) and the myometrium (i.e. muscle wall surrounding the uterus consisting of smooth muscle cells). The decidua basalis was hypointense compared to the myometrium. The decidua capsularis attenuated X-rays the most. Limitations include potential shrinkage and visualization of smaller vessels. De Clercq et al. evaluated the 1:2 Zr-substituted Keggin polyoxometalate (Zr-K 1:2 POM) for revealing the microstructure and vascularization of the murine placenta by CECT [141]. Their results showed that the Zr-K 1:2 POM can be used to study placental development, differences between strains and pathology-induced placental and vascular defects, without inducing tissue shrinkage. Similarly to the I_2 -based CECT, the general placenta anatomy was clearly visible, with a comparable trend in attenuation for the different main structures, except for blood-filled vessels and the

maternal canal, which attenuated X-rays very strongly when using the Zr-K 1:2 POM (Fig. 29). Due to the high resolution of the images, other anatomical features could be distinguished. Within the placenta, the junctional zone was found to be hyperintense compared to the labyrinth.

2.5. Note on the removal of CESAs after staining

Stain removal in the context of CESA staining for *ex vivo* microCT imaging is an important subject, especially when subsequent experiments on the tissue assume a native state of the tissue. In some cases, the CESA can be sufficiently removed by simple incubation of the sample in a PBS solution [73]. In other cases, treatment of the sample with specific chemicals (e.g. $Na_2S_2O_3$ solution for reduction of I_2 to I^-) is required [252,255]. Importantly, it should be noted that a visual, macroscopical disappearance of the color is not a guarantee of destaining (e.g. I^- will also attenuate X-rays, even though it does not render the tissue sample colored) [26]. Hence, to remove CESAs from a 3D tissue, one should consider the physical and chemical factors that caused the CESAs to accumulate in that specific location in the first place.

First, chemical interactions between biomolecules and CESA molecules dictate whether a CESA will accumulate due to the formation of a covalent bond, or rather due to a (slightly) thermodynamically favorable interaction. If covalent bonds are formed during the staining process, these bonds have to be broken before any removal of the CESA is

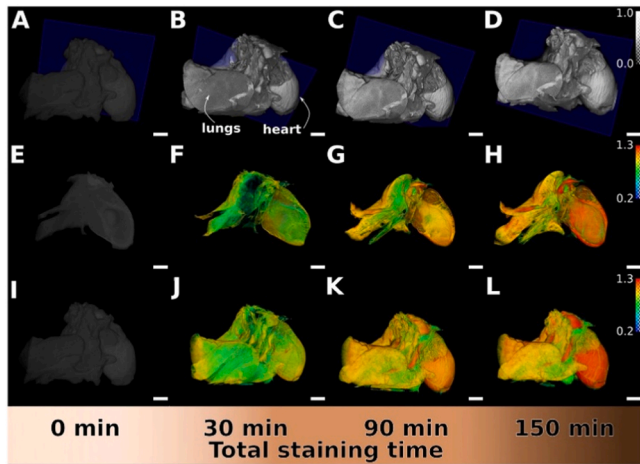


Fig. 27. Volumetric renderings and iodine maps of non-stained and I2E-stained heart and lungs with increasing staining times. Samples are shown according to increasing staining times of 0, 30, 90 and 150 min, from left to right. Using the same gray values threshold, comparable tomographic slices of the non-stained and the stained organs are shown for the 40 kVp measurement (A–D). Iodine distribution in the organs was obtained after processing the data obtained with two different X-ray energy spectra. Iodine distribution is visible in the virtual cuts (E–H, corresponding to the blue planes in the images immediately above) and reconstructed volumes (I–L) of the heart and lungs with increasing staining times, with the red-colored areas indicating the higher amount of iodine and green-colored the lower, quantified with dual-energy experiments. Adapted from [110] and reused under Creative Commons license (CC BY 4.0).

possible. If the bonds are of the weaker kind (e.g. aliphatic C-I bond) some of these bonds will spontaneously break under typical washing conditions (i.e. PBS or saline wash). However, if these bonds are of the stronger kind (e.g. aromatic C-I bond), significant removal by means of simple washing of the tissue is deemed not possible. If electrostatic interactions are considered, removal of the CESAs by means of simple washing is likely an equilibrium process, implying that multiple rounds of washing might be required depending on the concentration of CESA, ionic strength of the washing solution, etc. For example, Bansal et al. reported that still 50 % of the maximal X-ray attenuation conferred by CA4+ was observed after immersion of the plugs in a saline solution for 24 h [68,199]. Next, several CESAs interact with the tissue based on Van Der Waals forces that are weaker compared to ionic/charged interactions or covalent bonding. These offer the highest potential for facile removal after staining. Examples of such agents are uncharged iodine-based organic compounds (e.g. iodixanol). Nevertheless,

complete removal is not always guaranteed, as prolonged exposure or high concentrations can lead to entrapment within the tissue, complicating stain removal efforts.

Second, if we only consider CESA removal by means of passive diffusion, physical parameters influencing the ease of diffusion will dictate if and how easily the CESA can diffuse out of the region where it has accumulated. The effectiveness of agent removal is influenced by several key factors, including, but not limited to, the specific type of CESA used, its concentration, the sample size, the duration of staining, and the tissue's permeability characteristics, such as density and porosity.

It is important to note that in most cases, researchers rely on the visual removal of the stain, as inferred by comparing the appearance of a tissue from which the stain has been removed to that of an unstained tissue. This visual assessment is determined by the sensitivity of the imaging system, and the absence of significant visual (or even measured grey value) differences does not necessarily indicate that the staining molecules have been sufficiently removed from the tissue such that no impact on subsequent analyses can be guaranteed.

2.6. Recommendations and general challenges

This review offers a comprehensive examination of CESAs for *ex vivo* imaging of biological tissues. The literature overview provided in this review can serve as a valuable resource for selecting appropriate conditions when formulating protocols for staining experiments. It is crucial to recognize that the diversity of experimental parameters and conditions necessitates the optimization of each protocol to suit specific experimental contexts and answer specific research questions.

Despite the numerous studies discussed above, delineating the precise affinity mechanisms between CESAs and biological tissues remains a tedious task, because of several reasons.

1. The nested nature of the entire staining system, including the tissue sample, CESA and solvent, combined with the biological variability, variation in sample preparation protocols (e.g. fixation, storage, ...), staining protocols (e.g. solvent, concentration, time, volume, ...) and analysis protocols (e.g. software, parameters, ...) hampers an unconfounded description of chemical interaction mechanisms between the CESA and the biomolecules. In such nested and interconnected systems, modifying the characteristics of the staining solution may lead to changes in both the chemical properties of the tissue and the potential interaction mechanisms with the CESAs. For example, changing the pH of the staining solution may alter the charge (e.g. CA4+) and speciation (e.g. PTA) of CESAs. However, when striving to elucidate staining mechanisms, the analysis often

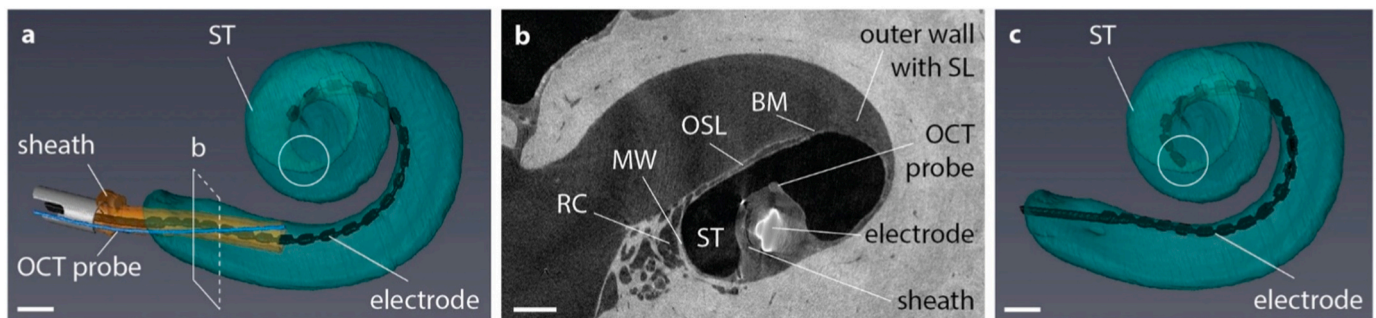


Fig. 28. Intracochlear position of the optically-guided sheath and the slim modular electrode. (a) CECT-based 3D rendering of the optically-guided sheath together with the slim modular electrode inside the ST in sample #8. The rectangle indicates the position of the CECT section in (b). Circle indicates the final position of the electrode array tip, as shown in (c). (b) CECT cross-section showing the position of the optically-guided sheath within the ST with respect to the three critical cochlear walls. (c) CECT-based 3D rendering of the final electrode position inside the ST, after extraction of the optically-guided sheath. Note that all electrode contacts are perfectly positioned inside the ST in close proximity to the modular wall. The position of the electrode array tip is encircled. BM basilar membrane, MW modular wall, OSL osseous spiral lamina, RC Rosenthal's canal, containing auditory nerve fibers, ST scala tympani. Scale bar: 1 mm. Adapted from [138] and reused under Creative Commons license (CC BY 4.0).

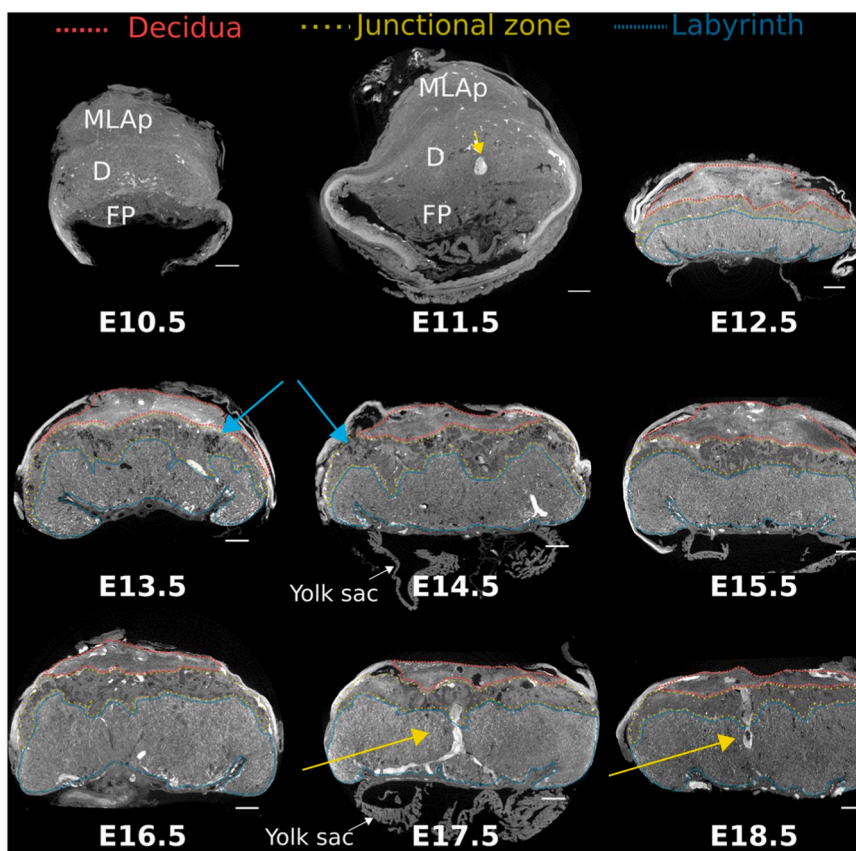


Fig. 29. Cross-sectional CE-CT images from E10.5–E18.5 placentas, with the different layers indicated. MLAp, mesometrial lymphoid aggregate of pregnancy; D, decidua (red); FP, fetal placenta; junctional zone (yellow); labyrinth (blue); blue arrow, lacunae in junctional zone; yellow arrow, maternal canal. Samples obtained from 129S6 mice. (Scale bar, 500 μm .)

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simplifies biological systems by disregarding factors such as chemical equilibria, molecular stability and physicochemical properties. This simplification may also involve focusing on a restricted set of proteins or a limited scope of potential interactions. Moreover, interactions with nucleic acids, and to a lesser extent carbohydrates, have not yet been investigated thoroughly and are hence not taken into account. These simplifications have led to the development of important insights in terms of non-covalent interactions (e.g. ion-ion interactions between CA4+ and GAGs, hydrogen bonding interactions between iohexol and the solvent, ...). However, care must be taken to not oversimplify the system, as this might lead to variable results and potentially irreproducible data or inconsistent conclusions. This has been illustrated by the inconsistent statistical descriptions of the distribution of anionic organic iodinated CESAs through cartilage tissue (Fig. 4). While the sign of the correlation was always the same, the magnitude of the correlation varied between studies. Additionally, several claims on specificity (e.g. specificity of PTA for collagen) require nuance as none of the abovementioned CESAs are developed to specifically target a biomolecule and certainly do not possess the specificity antibodies have for their respective antigens. From this perspective, it would be more correct to discuss the staining properties of a CESA in terms of affinity. Ideally, affinity of CESAs towards biomolecules should be studied in a series of systems, increasing in complexity, rather than only deducing affinity patterns based on oversimplifications of highly complex biological matrices. Although the model systems are not flawless replicas of biological tissues, they will allow to independently study different variables (e.g. pH, ionic strength, ...) and their effect on chemical interactions between biomolecules and CESAs.

These model systems could further be used to make inferences about larger biological systems. Alternatively, oversimplification of the affinity patterns might be a consequence of a suboptimal experimental design (too small sample size, no adequate control samples, confounded variables, ...).

2. Almost consistently, CESA solutions are compared based on the mass percentage of heavy atoms or molecules in solution. However, this method of comparison is only feasible when comparing molecules with similar molecular weights, which is rarely the case. Consequently, the relevant comparison to draw from such staining experiments should revolve around the systems under study, encompassing the tissue, solution, and CESA, rather than solely focusing on comparing the CESAs themselves.
3. This review distinctly indicates an uneven distribution regarding the exploration of different types of CESAs concerning specific tissue types. For instance, investigations involving organic CESAs have predominantly focused on studying cartilage, whereas studies involving inorganic CESAs have encompassed a wide range of other tissue types.
4. Expanding the library of CESAs would facilitate the assessment of how minor alterations in the chemical structure of these agents influence their affinity towards tissue constituents. While closely related CESAs such as PTA and PMA can be accurately compared when equal molar concentrations in solution are employed, the literature often presents cases where multiple discrepancies exist in molecular structure, impeding such a direct comparison.

2.7. Conclusions

The literature presented in this review has highlighted the potential of CECT to be used as a 3D virtual histology tool, due to the minimally destructive and relatively less labor-intensive character, while providing a 3D overview of the full tissue of interest. Additionally, it has also underscored the immense complexity of the studied systems. The interest of the scientific community for this technique has grown over the years, evidenced by the growing number of publications. While plenty of CESAs have been explored by the CECT community, the final choice of CESA is often based on a trial-and-error approach, on literature, on anecdotal knowledge, on commercial availability or simply random. Yet, enhancing our understanding of the basic interactions and anticipated distribution of CESAs within tissues would enable the selection of suitable CESAs tailored to address specific biological research questions. Furthermore, this would allow the intelligent design of novel CESAs, based on or derived from the known CESAs. The present review served to provide a classification of CESAs based on their chemical nature, along with a selection of studies focusing on specific tissues and organs, to gain a better understanding of the chemical aspects of the staining process. Each category of CESAs was subjected to a comparative investigation concerning the chemical nature, staining procedures and distribution within the tissue, separated by tissue type. The findings from these individual comparisons were compiled and used to discuss interactions between CESAs and biological tissues. Current CESAs predominantly exploit ionic interactions to regulate their equilibrium distribution within tissues. Nevertheless, a thorough investigation of existing literature has shown that other, weaker interactions, such as hydrogen bonding, also contribute to this distribution. A subset of CESAs can form covalent bonds with tissue biomolecules, with the stability of these bonds varying significantly depending on the specific biomolecule involved. The majority of CESAs, however, distribute throughout the tissue due to a combination of these interactions, rendering the prediction of their distribution highly complex. The review concludes by outlining the challenges associated with this complexity and proposing potential solutions or methodologies to address them. Addressing these challenges will prove invaluable for the future advancement of ex vivo CECT imaging of biological tissues.

CRedit authorship contribution statement

Greet Kerckhofs: Writing – review & editing, Supervision, Funding acquisition. **Wim M. De Borggraeve:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Sarah Vangrunderbeeck:** Writing – review & editing, Formal analysis, Conceptualization, Data curation, Funding acquisition, Investigation, Writing – original draft. **Tim Balcaen:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used OpenAI (2024), ChatGPT in order to improve the readability and language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.tmater.2025.100052](https://doi.org/10.1016/j.tmater.2025.100052).

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