

Full length article

Evaluating non-destructive X-ray-based 3D histology using contrast-enhanced micro-CT to visualise and quantify vascular elastin and collagen

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ABSTRACT

Visualising and quantifying vascular 3D microstructure are essential for linking it to mechanics and disease. This study evaluated Phospho-Tungstic Acid (PTA) and 1:2 Hafnium-substituted Wells Dawson Polyoxometalate (1:2 Hf-POM) as Contrast-Enhancing Staining Agents (CESAs) for X-ray-based 3D Histology (X3DH) using Contrast-Enhanced Micro-focus Computed Tomography (CECT), as alternatives to classical 2D histology for visualising and quantifying collagen and elastin. Native and enzymatically digested porcine carotid artery segments, with varying elastin and collagen content, were CESA-stained and μ CT-imaged to generate X3DH data, used to calculate Elastin-Stained Volume Fraction (E-SVF). Equivalent CESA-stained and unstained sections underwent Verhoeff's (VH), Picrosirius Red (PR), and Haematoxylin–Eosin (H&E) histology to measure Elastin-Stained Area Fraction (E-SAF), Collagen-SAF (C-SAF), and cell density. X3DH results were compared with histology to evaluate correspondence and staining effects. Both CESAs produced strong signal colocalisation with elastic lamellae and weaker staining in the remaining ECM. E-SVF for both CESAs correlated strongly with E-SAF, but E-SVF values were about two times lower than E-SAF ($r^2_{\text{PTA}}=0.86$, $p < 0.0001$, $\text{slope}_{\text{PTA}}=0.42$ | $r^2_{1:2\text{-Hf-POM}}=0.86$, $p < 0.0001$, $\text{slope}_{1:2\text{-Hf-POM}}=0.49$). This highlights CECT as a non-destructive, accurate method for quantifying vascular microstructure with fewer artefacts than classical 2D histology. VH and PR staining were visually and quantitatively ($\pm 10\%$ E-SAF and C-SAF equivalence) unaffected by CESA-staining. PTA-staining with cryo-preservation caused loss of nucleic contrast, while 1:2 Hf-POM influenced ECM haematoxylin uptake, reducing nuclei visualisation and cell count accuracy in H&E histology. Both CESAs bind non-specifically but preferentially to elastin, enabling reliable quantification. However, vascular collagen remains difficult to visualise as ECM is non-specifically stained.

Statement of significance: Understanding the three-dimensional microstructure of vascular tissues is crucial for revealing how it governs mechanics and disease progression. This study investigates two contrast agents, phospho-tungstic acid and 1:2 hafnium-substituted Wells-Dawson polyoxometalate, for their potential to visualise and quantify two of the key constituents within arterial tissue, collagen and elastin, using Contrast-Enhanced Micro-focus Computed Tomography (CECT). By assessing these agents combined with CECT as

Abbreviations: (PTA), Phospho-Tungstic Acid; (1:2 Hf-POM), 1:2 Hafnium-substituted Wells Dawson Polyoxometalate; (X3DH), X-ray-based 3D Histology; (CESAs), Contrast-Enhancing Staining Agents; (E-SVF), Elastin-Stained Volume Fraction; (VH), Verhoeff's; (PR), Picrosirius Red; (H&E), Haematoxylin–Eosin; (E-SAF), Elastin-Stained Area Fraction; (C-SAF), Collagen-SAF; (SMCs), Smooth Muscle Cells; (GAGs), Glycosaminoglycans; (ECM), Extracellular Matrix; (SEM), Scanning Electron Microscopy; (μ -CT), Micro-focus Computed Tomography; (XPC)-CT, X-ray Phase Contrast; (CECT), Contrast-Enhanced Micro-focus Computed Tomography; (PLM), Polarised Light Microscopy; (VOI), Volume Of Interest; (ROI), Region Of Interest; (EL), Elastic Lamellae; (IEFs), Interlamellar Elastin Fibres; (IEL), Internal Elastic Lamina; (EEL), External Elastic Lamina; (GS), Grey Scale; (GV), Grey Value; (LUT), Look-Up Table.

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alternatives to gold-standard histological techniques for the visualisation and quantification of these key extracellular matrix components, this research advances CECT as a non-destructive alternative to traditional histological techniques. Improved three-dimensional imaging of vascular architecture deepens our understanding of arterial structure–function relationships and supports more accurate computational modelling, disease characterisation, and the development of next-generation cardiovascular devices.

1. Introduction

Blood vessels are organised into three principal layers: the intima, a thin inner layer composed of squamous endothelial cells and a basal membrane lining the lumen; the media, composed of concentric Elastic Lamellae (ELs), Smooth Muscle Cells (SMCs), and an interwoven collagenous network; and the adventitia, a fibrous outer layer rich in fibroblasts and collagen, with some elastin, that confers rupture resistance [1]. Glycosaminoglycans (GAGs) are also present within the intima and media, contributing to Extracellular Matrix (ECM) function [2]. The composition, quality, and spatial arrangement of microstructural components directly determine vascular mechanical properties and vary with anatomical location. Proximal elastic arteries, such as the aorta, are rich in medial elastin, accommodating pulsatile loading, whereas distal arteries exhibit a muscular phenotype with increased medial SMC and collagen content, providing contractile function and mechanical resistance [3].

This study focuses on elastin and collagen, the principal passive structural proteins governing arterial mechanics. Elastin dominates the low-strain regime, enabling compliance and reversible deformation under physiological pressures, while collagen contributes to nonlinear stiffening via fibre recruitment at higher strains, imparting tensile strength and the characteristic J-shaped loading curve [4]. GAGs facilitate fibre recruitment at lower strain levels [5], and although SMCs have a predominantly passive mechanical role, with their absence being associated with arterial stiffening [6,7], their principal function is active, regulating vascular tone through vasodilation and vasoconstriction [3]. Altered composition or organisation of these components is implicated in aneurysms [8], atherosclerosis [9], and dissections [10], underscoring the importance of understanding microstructure–function relationships.

Accurate imaging of the vascular microstructure is essential for elucidating these relationships, particularly in disease where architecture is disrupted. Classical two-dimensional (2D) histology, which involves embedding tissue, slicing it into thin sections and staining it for microscopy, remains the gold standard, offering sub-micrometre (0.25–0.5 μm) in-plane resolution and specific component visualisation [11]. It has enabled detailed characterisation of plaque composition [12–16] and elastin fragmentation in aneurysm models [17]. Classical histology is a widely used and continually evolving technique. Ongoing efforts aim to extend its capabilities, with one of the most significant recent advances being high-resolution whole-slide imaging combined with artificial intelligence–based analysis techniques. These approaches have led to some major developments in the histopathology of vascular tissue, enabling assessment of vascular tissue regeneration patterns in tissue-engineered vascular grafts [18], detailed histopathological characterisation of abdominal aortic aneurysms [19,20], and the generation of virtually-stained histological sections [21]. By digitalising histological slides and enabling automated analysis, these approaches aim to streamline histopathological workflows while improving the ability to visualise, quantify, and interpret vascular architecture across large datasets. However, despite recent developments, classical 2D histology remains destructive and planar, requiring multi-directional sectioning to infer 3D organisation, which is labour-intensive and prone to artefacts due to manual processing limitations and significant tissue shrinkage [22].

Several optical and electron-based methods address these limitations. Multiphoton, including auto-fluorescent second harmonic

generation imaging, has mapped collagen fibres, elastin, and cell arrangement in healthy arterial tissue [23] and collagen fibre organisation in human carotid plaques [24]. These techniques offer sub-micrometre resolution and 3D reconstruction but are limited by shallow imaging penetration depth ($\sim 200\ \mu\text{m}$ untreated, up to $\sim 1\ \text{mm}$ with clearing [25]), restricting volumetric analysis. Light-sheet fluorescence microscopy has been used to quantify arterial injury in pre-clinical models [26], overcoming penetration limits but lacking the required resolution for elastin and collagen microstructure visualisation. Scanning Electron Microscopy (SEM), including focused ion beam SEM, has provided 3D nanostructural detail in healthy [27] and diseased arterial tissue [28,29], but remains destructive and restricted to small interrogation regions.

Micro-focus Computed Tomography ($\mu\text{-CT}$) enables large-field 3D imaging and has been extensively used to identify and quantify calcifications in atherosclerotic plaques [24,30–33]. However, conventional $\mu\text{-CT}$ cannot readily visualise soft tissues due to low attenuation contrast, driving the adoption of X-ray Phase Contrast (XPC)-CT and Contrast Enhanced- μCT (CECT), which enable X-ray-based 3D Histology (X3DH) of soft tissues.

Synchrotron XPC-CT has been used to segment ELs in healthy and diabetic murine aortas [34,35], capture dissection propagation in pressurised rabbit aortae [36], and visualise plaque microstructure [37,38]. Laboratory-based systems employing propagation-based or Zernike phase contrast have imaged murine carotid arteries under varying pressure conditions [39]. However, access to synchrotron-based XPC-CT remains highly limited, and access to non-synchrotron XPC-CT setups is similarly constrained.

In CECT, soft-tissue contrast is achieved via diffusion of Contrast-Enhancing Staining Agents (CESAs) that bind to tissue components [40–42]. It offers micrometre-scale isotropic resolution, non-destructive 3D visualisation of large volumes, and compatibility with conventional laboratory X-ray sources. CECT facilitates detailed 3D histological characterisation and supports the generation of high-resolution microstructural datasets. Furthermore, it enables *in situ* mechanical testing, where digital volume correlation approaches can be applied to quantify internal deformation and strain fields [43]. As a result, CECT holds significant potential across a range of applications, including elucidating contrast mechanisms in other imaging modalities or informing and validating advanced computational modelling approaches [44]. In vascular research, CECT has mapped murine vasculature [45,46], characterised arterial and venous microstructure across species and in atherosclerotic specimens [22,47], and enabled real-time imaging during mechanical testing [48,49]. For comprehensive reviews of CECT, readers are referred to existing articles [40–43].

Despite its notable advantages and growing use, limitations remain when using CECT. The technique relies on the diffusion of CESAs into tissue, and staining protocols can influence tissue hydration, induce shrinkage, and alter mechanical properties. In addition, CESA diffusion can be slow and, depending on the tissue of interest, may require relatively long periods, on the order of days to weeks, to achieve sufficient penetration for imaging. These limitations can influence workflow planning and must be considered carefully, particularly when working with fresh tissue or time-sensitive experimental designs, such as *in situ* mechanical testing [43,50]. Lastly, contrast depends on the binding affinity of individual CESAs to different ECM components, which can limit the selective visualisation of specific components [41].

Numerous CESAs have been explored for cardiovascular imaging.

Lugol's iodine represents the most reported CESA [40–42], and was also applied to vascular tissue [51–53] while Phospho-Molybdic Acid (PMA) and Phospho-Tungstic Acid (PTA) were reported to offer improved contrast attributed to CESA binding to collagen-rich structures [47,54]. Osmium tetroxide targets unsaturated lipids and has been used to visualise lipid-rich plaques [55,56], whereas Sodium Polytungstate has been used to delineate ELs [48,49]. More recently, 1:2 Hafnium-substituted Wells Dawson Polyoxometalate (1:2 Hf-POM) and monolacunary Wells Dawson POM have been used to stain venous and arterial tissues, enhancing lamellar contrast [22,50]. Separately, whole-body perfusion with a modified Verhoeff's (VH) stain has also been used to visualise arterial wall architecture in murine models [45]. Current work increasingly examines diffusion behaviour, binding specificity, and mechanical effects of CESAs. Notably, Pétré et al. compared seven CESAs for vascular staining, assessing their diffusion and impact on tissue mechanics [50]. As collagen and elastin govern vascular mechanics, imaging approaches that enable their distinct visualisation and quantification are highly desirable. In this context, PTA and 1:2 Hf-POM have emerged as particularly promising CESAs for X3DH in vascular tissue. Prior studies in vascular tissue linked PTA to enhanced contrast in collagen-rich regions [47], while 1:2 Hf-POM has been shown to provide clear contrast enhancement of elastin lamellae [22].

PTA and 1:2 Hf-POM are believed to bind to proteins primarily through electrostatic interactions. Raman spectroscopy has been used to demonstrate that 1:2 Hf-POM binds electrostatically to collagen (types I and II) and tendon without altering their chemical structures, indicating stable yet non-destructive interactions. In contrast, while PTA shows a comparable affinity for collagen type I and tendon, it induces chemical modifications in collagen type II and fibrin [57].

PTA, widely used as a CESA since 2009 [58], has been used to image: tissue engineered bone constructs [59,60], cartilage [61–64], soft skeletal tissue [57] and vascular tissue [47,50,54], with contrast enhancement commonly observed in regions rich in ECM components, such as collagen and fibrin. For instance, Hanly et al. [47] observed higher attenuation in outer vessel regions than luminal areas when staining porcine carotid arteries with PTA, consistent with a collagen gradient, while Karhula et al. observed reduced PTA uptake following collagen digestion in articular cartilage [63]. However, the observed behaviours do not indicate specific binding to collagen.

In contrast, 1:2 Hf-POM was first reported for imaging in 2018 [57] as an alternative to PTA for soft skeletal tissues, and has since been used for kidney [65], cardiac [66], tendon [66], cochlear [67–69], brain [70], and more recently, vascular imaging [22,50]. Reports from Leyssens et al. [22] and Pétré et al. [50] demonstrate clear visualisation of ELs in vascular tissue using 1:2 Hf-POM.

However, as studies evaluating PTA and 1:2 Hf-POM were primarily performed on native vascular tissue containing high amounts of collagen, SMCs and elastin, this makes understanding the contrast-enhancing behaviour of each CESA challenging and it is unclear whether their uptake reflects cross-reactivity with other ECM components.

This study assesses the potential of PTA and 1:2 Hf-POM CECT for the visualisation of vascular elastin and collagen, to evaluate their utility for visualising and quantifying these components. Specifically, it evaluates their performance relative to established histological techniques for elastin and collagen assessment and examines whether CESA staining influences subsequent classical 2D histological analyses, including VH, Picrosirius Red (PR), and Haematoxylin-Eosin (H&E) staining.

2. Materials and methods

An overview of the methods is provided below in Fig. 1.

2.1. Porcine carotid artery-based microstructural models

Carotid arteries from 6-month-old large white pigs were collected

within 3 h of slaughter from an abattoir (Rosderra Meats, Edenderry, Ireland), cleaned of connective tissue, and cryopreserved at $-1^{\circ}\text{C}/\text{min}$ (Mr Frosty) from room temperature to -80°C in Tissue Freezing Media (TFM: RPMI, 1.8 M dimethyl sulfoxide, 0.1 M sucrose) to preserve integrity [71,72]. Carotids were chosen as their anatomical position places them at an elastic-muscular transition, resulting in variable microstructural characteristics. Following cryopreservation, samples were rapidly thawed at 37°C and transferred to PBS. Segments (7 mm, $n = 50$) were cut using a custom-made guillotine from 14 distinct carotids and randomised into treatment groups. Elastin and collagen degradation models were generated using native specimens, as well as purified- and crude- enzyme-treated specimens (Fig. 1(A)). To briefly describe this, enzymatic treatments involved incubation of the sample at 37°C in an enzymatic digestion solution. All samples were fixed immediately after treatment (10% formalin, HT501128, Sigma, 24 h, 4°C). Enzymatic treatment protocols were adapted from previous studies [73–76] and protocol details are included in Supplementary Materials (Table S.1).

2.2. Sectioning and histological ground truth preservation

To evaluate the effects of CESA-staining on classical 2D histology and preserve a 'CESA-unstained', referred to as 'ground truth', equivalent histological section to the CESA-stained section, in the event CESA-staining was found to affect histological visualisation and data extraction significantly, the following approach was adopted. 7-mm samples were incised at both ends using 3-mm round and oval biopsy punches (thin black dashed lines, Fig. 1(B)). A fine marker line was drawn along the sample surface connecting the two punch-incisions using a histology slide lab-marker, serving as a histology solvent-resistant cross-modality landmark. Each sample was then partitioned into two 3.5-mm segments (thick black dashed lines, Fig. 1(B)): one CESA-stained (black dot, Fig. 1(B)) and the other stored in PBS for ground truth comparison (white dot, Fig. 1(B)). Sectioning for classical 2D histology was initiated at the partition surface to improve comparison between corresponding CESA-stained and ground truth samples, which could be reduced by anatomical variation along the sample length.

2.3. Contrast-Enhancing staining

1:2 Hf-POM (1:2 Hafnium(IV)-substituted Wells-Dawson POM $\text{K}_{16}[\text{Hf}(\alpha_2\text{-P}_2\text{W}_{17}\text{O}_{61})_2] \cdot 19\text{H}_2\text{O}$) was synthesised as reported [77,78], and supplied by collaborators at UCLouvain, Belgium. PTA ($\text{H}_3\text{PW}_{12}\text{O}_{40}$) (10% solution, HT152-250ML) was purchased from Sigma-Aldrich. Staining solutions: 3.5% (35 mg/mL) 1:2 Hf-WD POM in PBS [22] and 2% (20 mg/mL) PTA in PBS [50] (P5493, Sigma-Aldrich, Wicklow, Ireland) were prepared. Using the 20 times sample volume rule [22], and assuming a maximum size of 8 mm diameter and 2 mm thick walls for each vessel sample, 3 mL of staining solution was used per sample. A 48-h optimal staining time was set after preliminary low-resolution tests showed near-complete staining at 24 h (see Supplementary Materials Figure S.1 and S.2). After 48 h, samples ($n = 26$ for PTA, $n = 24$ for 1:2 Hf-POM) were cryopreserved by immersion in isopentane at -78°C to standardise staining duration, then rapidly thawed at 37°C immediately prior to CT imaging.

2.4. Micro-focus computed tomography imaging

Samples were mounted in a custom holder to minimise movement and maintain hydration (immersed in PBS). Aluminium foil (high attenuation) and parafilm (low attenuation) were placed inside the holder within the vessel lumen to provide attenuation-based reference points and correct for dataset-to-dataset Grey Value (GV) variation. Image acquisition was performed using a Phoenix Nanotom M (GE Measurement and Control Solutions, Germany) with a 180 kV/15 W nanofocus X-ray tube, diamond-coated tungsten target, and data was reconstructed using Phoenix datos|x software. Identical acquisition and

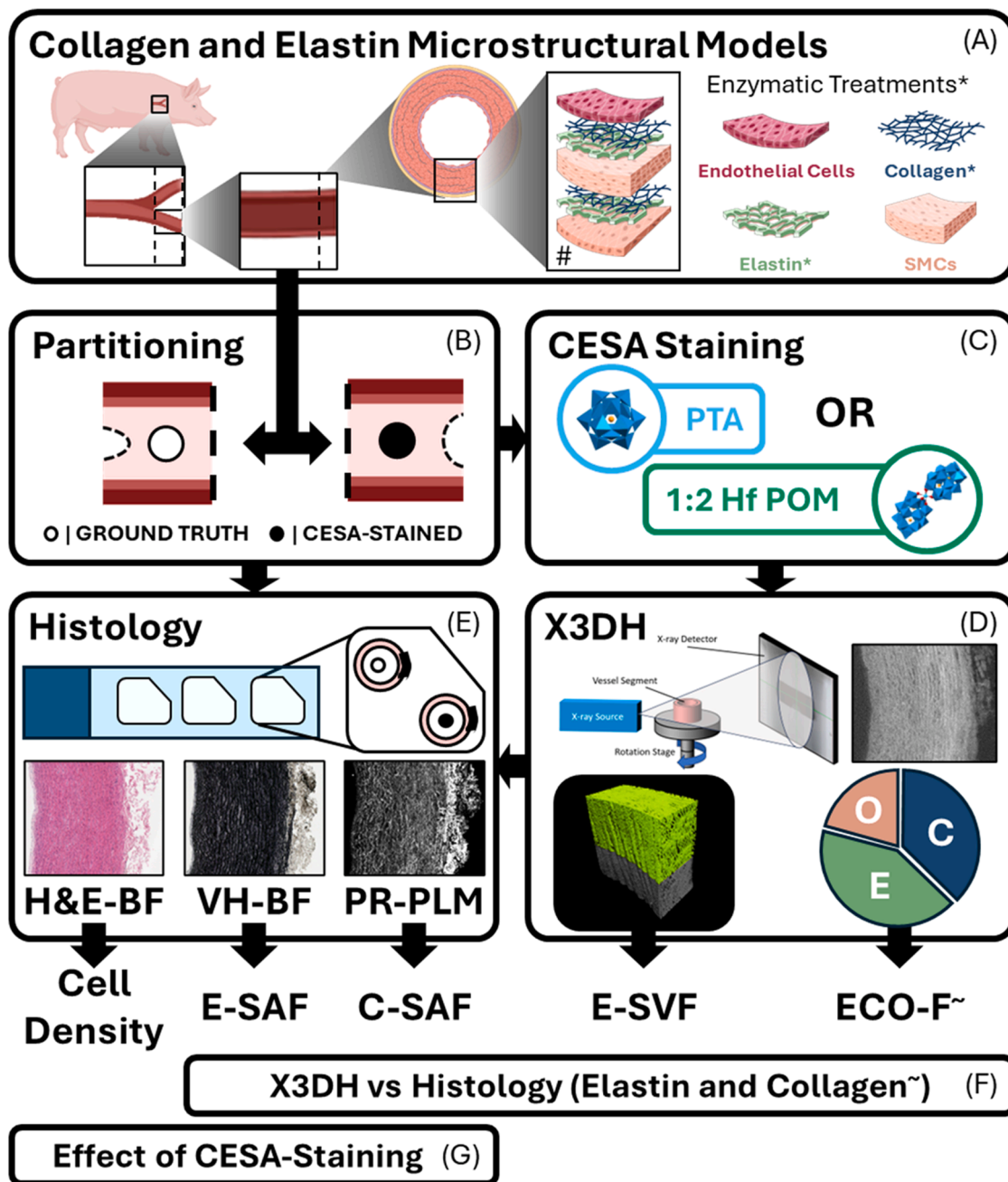


Fig. 1. Overview of methods: (A) Vascular models with varying collagen and elastin content are created from porcine carotids using enzymatic treatments, (*indicates treatments are primarily targeting elastin and collagen degradation, whereas # indicates that some of the arterial microstructural ECM components have been excluded from the schematic to simplify it), (B) Each model is partitioned into two halves: one as ground truth sample processed directly for classical 2D histology (white-filled dot), (C) the other stained with Contrast-enhanced staining agents (CESAs): PTA (Phospho-tungstic acid) or 1:2 Hf-POM (1:2 hafnium-substituted polyoxometalate) (black-filled dot). (D) X-ray-based 3D Histology (X3DH) via Contrast-Enhanced micro-focus Computed Tomography (CECT) is performed on the CESA-stained half, and visual and quantitative data (Elastin- Stained Volume Fraction (E-SVF) and Elastin-Collagen-Other Fraction (ECO-F~)) are extracted. (E) Haematoxylin and Eosin (H&E)-Brightfield microscopy (BF), Verhoeff's (VH)-BF, and Picrosirius Red (PR)-Polarised Light Microscopy (PLM) classical 2D histology are then performed. The visual and quantitative data (cell density, Elastin-Stained Area Fraction (E-SAF) and Collagen-SAF (C-SAF)) are extracted. These data are used to assess: (F) X3DH versus classical 2D histology for visualising and quantifying elastin and collagen (~ indicates that the corresponding results are included in the supplementary materials and not in the main manuscript), and (G) the effect of CESA staining on classical 2D histology. SMCs | Smooth Muscle Cells. The figure was partially created using BioRender.com.

reconstruction parameters (Table 1) ensured consistent visualisation across samples with varying contrast from stain type or degradation.

Acquisition parameters were optimised using native PTA-stained samples (highest contrast- enhancement) to ensure adequate flux through dense tissue. For high-resolution imaging, the field of view was

centred on the sample round incision mark to capture the radial Volume of Interest (VOI) (~5³ mm³). After imaging, stained samples were stored in PBS at 4°C until histological processing. PBS was replaced twice on the samples at 48 hours and two weeks post-imaging to ensure diffusion equilibrium of CESA between the tissue and the solution.

Table 1

Micro-focus Computed Tomography (CT) acquisition and reconstruction parameters used for X-ray-based 3D Histology.

Isotropic Voxel size (μm)	2	Skip	1
Source Voltage (kV)	100	Fast scan mode	NO
Tube current (μA)	84	Acquisition time (min)	1 hour, 1 min and 16 s
Filter	Yes (0.2 mm Aluminium)	Detector-shift	Yes
Mode	0	Sample Distance (mm)	11
Number of images	2300	Detector Distance (mm)	550
Image Width (pixels)	2500	Beam-hardening Correction	8
Image Height (pixels)	2400	ROI-CT Filter	YES
Exposure time (ms)	500	Inline Median Filter	YES
Average	2	Filter Volume Filter	YES

2.5. Classical 2D histology

Each sample's CESA-stained and ground truth 3.5-mm sections underwent histological processing: stepwise ethanol dehydration (Leica TP1020, Germany), paraffin embedding (Leica EG1150, Germany), and sectioning from the same plane for both segments at a thickness of 8 μm [75,79]. Sections were mounted sequentially, labelled in alternating stain order (VH, PR, H&E), stained using standard protocols, and cover-slipped using Pertex (Leica 3808707E, Germany).

Brightfield imaging (Leica Aperio AT2, Germany; ImageScope v12.4.6, Leica Biosystems, Vista, CA) was performed at 20 × (0.51 μm/px) to capture whole slides. Polarised light microscopy (PLM) was conducted on PR-stained samples (Olympus BX41 microscope; equipped with Ocular V2.0 software, Teledyne Photometrics, Tucson, Arizona) at 10 × (0.45 μm/px) over the radial ROI (FOV 1.22 × 1 mm²). One brightfield and two PLM images (45° apart [12,14,75]) were taken, with exposure time minimised to prevent 16-bit RGB sensor saturation.

2.6. Data analysis

2.6.1. Correlating different imaging modalities - region of interest selection

Lab marker ink on each sample (n = 100) provided a landmark for consistent co-localisation across imaging modalities. The VOI and Region of Interest (ROI) sizes were defined by the PLM field of view (1.22 × 1 mm²), the smallest area capturing full vessel thickness. All histological analyses used 1.22 × 1 mm² ROIs (n ≥ 3 for H&E; n ≥ 6 for VH and n ≥ 2 for PR-PLM) aligned with the sample marking. X3DH data were cropped to a matching 1.22 × 1 × 1 mm³ VOI (n = 1 for each of the n = 50 samples) in the same region.

2.6.2. Qualitative multi-modal imaging comparison

All acquired imaging datasets were visually assessed and compared following the interpretative criteria outlined below. In X3DH images, areas of higher signal intensity indicated increased stain binding. VH-BF imaging revealed elastin structures in black specifically, while PR-BF non-specifically stained collagenous tissue in red. GS PR-PLM selectively visualised collagen fibres, with brighter regions corresponding to thicker and denser fibre bundles. Finally, H&E staining enabled the visualisation of cell nuclei in purple and ECM in pink.

2.6.3. Quantitative X-ray-based 3D histology (X3DH)

All reconstructed 16-bit single-channel CT datasets (n = 50) were normalised using a custom MATLAB pipeline adapted from a previously utilised pipeline [66,80]. For each dataset, x, the lower (LL_x) and upper (UL_x) GV limits were obtained from histograms, while mean paraffin (a_{im,x}) and aluminium (b_{im,x}) intensities were obtained from line profiles across three slices. Dataset-specific reference values were then calculated, as described in Eq. (1):

$$a_{ref,x} = \frac{a_{im,x} - LL_x}{UL_x - LL_x}; \quad b_{ref,x} = \frac{b_{im,x} - LL_x}{UL_x - LL_x}; \quad (1)$$

Each dataset, y, was tested as a potential global reference for all datasets by computing for every other dataset x, lower (LL_{xy}) and upper

(UL_{xy}) GV limits, using Eqs. (2–4):

$$Factor_{xy} = \frac{|a_{ref,y} - b_{ref,y}|}{|a_{im,x} - b_{im,x}|}; \quad (2)$$

$$LL_{xy} = (LL_x \times Factor_{xy}) + a_{ref,y} - (a_{im,x} \times Factor_{xy}); \quad (3)$$

$$UL_{xy} = (UL_x \times Factor_{xy}) + a_{ref,y} - (a_{im,x} \times Factor_{xy}); \quad (4)$$

The dataset minimising spill-over beyond the [0–1] range was chosen as the global reference, and its a_{ref}, b_{ref} were slightly adjusted to avoid clipping. All datasets x were then normalised to this reference by linear rescaling, as described in Eq. (5):

$$GV'_x = (GV_x - a_{im,x}) \times \frac{b_{ref} - a_{ref}}{b_{im,x} - a_{im,x}} + a_{ref}; \quad (5)$$

where GV_x is the original- and GV'_x is the normalised GV of dataset x. This ensured consistent paraffin and aluminium foil intensities across datasets while preserving relative GV distributions.

2.6.3.1. Elastin-stained volume fraction (E-SVF). After MATLAB normalisation, datasets were imported into Dragonfly (v2024.1, Comet Technologies, Canada), cropped to 1.22 × 1 × 1 mm³ to match other images FOVs, and saved as *.ORSObjects. A segmentation pipeline was developed to isolate tissue and ELs. Parameters were manually optimised on representative datasets, then automated using Dragonfly's macro-builder. Tissue was segmented via Otsu thresholding with volumetric closing; ELs via an edge-enhancement workflow adapted from Pétré et al. [50]: (Median 7 × 7 → Cross 3D → Unsharp 7 × 7, SD 2.0, USF 1.00 → Frangi 7 × 7, σ 2) followed by thresholding at 2000.

All masks were visually checked; seven datasets were excluded due to poor segmentation, typically from overlapping ELs unresolved at 2 μm or thin distal structures. Remaining tissue and EL masks were used to compute Elastin-SVF, described below in Eq. (6):

$$Elastin\ SVF\ (\%) = \frac{\# \text{ of Voxels } (Stained\ Elastin\ Mask \wedge Tissue\ Mask)}{\# \text{ of Voxels } Tissue\ Mask} \times 100; \quad (6)$$

2.6.3.2. Collagen-elastin-other fraction signal model. An investigation was conducted to determine whether information on non-segmentable collagen could be extracted. This was based on the hypothesis that collagen might represent the second most prominent signal component, although this assumption may not be valid. To explore this possibility, an informed Look-Up Table (LUT) model was calibrated using No-Collagen (NC) and No-Elastin (NE) samples stained with both PTA and 1:2 Hf-POM. This approach was used to assess whether separation of elastin and collagen signals and quantification of non-segmentable collagen were feasible. Calibration and analysis details are provided in Supplementary Materials (Section: ECO-F Model Description & Results, Figure S.3–5, Table S.2–3). This approach performed poorly due to non-specific staining of other components (e.g., cells, non-fibrillar ECM), and is therefore reported only in the Supplementary Materials.

2.6.4. Semi-quantitative classical 2D histology

2.6.4.1. Elastin-stained area fraction (E-SAF). E-SAF (%) was quantified on VH-stained slides using QuPath (v0.6.0, Queen's University Belfast, UK). At least six slices per CESA-stained or ground truth sample were analysed within $1.22 \times 1 \text{ mm}^2$ annotations centred on the landmark. Tissue annotations were generated using a tissue thresholder, followed by a stained-elastin pixel classifier (resolution: full; channel: average; prefilter: Gaussian; sigma: 0.5; threshold: 1.6) to segment ELs. SAF was then calculated as described below in Eq. (7):

$$\text{Elastin SAF (\%)} = \frac{\# \text{ of Pixels (Stained Elastin Mask} \wedge \text{Tissue Mask)}}{\# \text{ of Pixels Tissue Mask}} \times 100; \quad (7)$$

2.6.4.2. Collagen-stained area fraction (C-SAF). C-SAF (%) was quantified from PLM data using a custom MATLAB script. At least two ROIs per CESA-stained or ground truth sample were analysed within $1.22 \times 1 \text{ mm}^2$ FOVs centred on the landmark. Brightfield and $0^\circ/45^\circ$ PLM images were imported as RAW 16-bit RGBs. A background (tissue-free) region and recorded Exposure Time (ET, ms) were used to normalise images using the following Eq. (8):

$$I_{\text{PLM-NORM (RGB)}} = (I_{\text{PLM-RAW (RGB)}} - \overline{I_{\text{PLM-Bg-ROI (RGB)}}}) / ET(\text{ms}); \quad (8)$$

This approach corrected for variable exposure times (longer in collagen-poor samples). The two-angle images were pixel-wise added (imadd), renormalised between 0 and 1 using global minimum–maximum values, and converted to single-channel 16-bit Grey Scale (GS) (rgb2gray) for SAF analysis. Tissue was segmented from GS brightfield images by thresholding, binarising regions significantly darker than the background as tissue. Threshold was defined according to Eq. (9):

$$\text{Tissue Mask} \leq \overline{I_{\text{BF-Bg-ROI (GS)}}} - 3 \times SD(I_{\text{BF-Bg-ROI (GS)}}); \quad (9)$$

The imclose operation was then applied to fill small noise-related holes in the tissue mask. The normalised, angle-combined GS PLM image was thresholded at 2000 A.U. to generate the collagen mask, and Collagen SAF was calculated according to Eq. (10):

$$\text{Collagen SAF (\%)} = \frac{\# \text{ of Pixels (Stained Collagen Mask} \wedge \text{Tissue Mask)}}{\# \text{ of Pixels Tissue Mask}} \times 100; \quad (10)$$

2.6.4.3. Cell density. Cell density (10^3 cells/mm^2) was quantified on H&E-stained slides using QuPath (v0.6.0, Queen's University Belfast, UK). At least three ROIs per CESA-stained or ground truth sample were analysed within $1.22 \times 1 \text{ mm}^2$ annotations centred on the landmark. A tissue thresholder was used to generate tissue annotations, and QuPath cell detection was applied using: Hematoxylin OD image at $0.51 \mu\text{m/px}$; nucleus settings—background radius $8 \mu\text{m}$, opening by reconstruction ON, median filter $0 \mu\text{m}$, sigma $1.5 \mu\text{m}$, area $10\text{--}400 \mu\text{m}^2$; intensity—threshold 0.2, max background 2, split by shape OFF; cell—expansion $5 \mu\text{m}$, include nucleus ON; general—smooth boundaries ON, make measurements ON. Cell density was then calculated according to Eq. (11):

$$\text{Cell Density} \left(\frac{10^3 \text{ cells}}{\text{mm}^2} \right) = \frac{\# \text{ of Cells Counted}}{\text{Area of Tissue Area (mm}^2\text{)} \times 10^3}; \quad (11)$$

As cell nuclei–ECM contrast differed between groups (ground truth, PTA-stained, 1:2 Hf-POM-stained), three cell detection parameter sets were visually optimised and tested. These did not notably improve agreement with the ground truth, so results are reported using the set of parameters set above for consistency.

All generated tissue, elastin-stained, collagen-stained, and cell-counting masks were visually checked against the original images to confirm consistent processing and absence of systematic errors.

2.7. Statistical analysis

All statistical analyses were conducted in GraphPad Prism 10 (GraphPad Software, San Diego, CA). Specific tests are listed in figure captions. Data are presented as mean $\pm$ standard error, with significance at $p < 0.05$, reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

X3DH (E-SVF) vs classical 2D histology (E-SAF) measurement correlations for the two CESAs were assessed via linear regression as variables were not fully independent, to evaluate bias (intercept 95% CI includes 0) and agreement (slope 95% CI includes 1). r^2 values (equivalent to squared Pearson's r) were interpreted as: <0.10 very weak, $0.10\text{--}0.30$ weak, $0.30\text{--}0.50$ moderate, $0.50\text{--}0.70$ strong, >0.70 very strong.

Equivalence between semi-quantitative histological measurements from CESA-stained and ground truth sections was tested using linear regression and the Two One-Sided Tests (TOST) procedure. Results were deemed equivalent if: (1) $r^2 > 0.7$; (2) slope and intercept 95% CIs included 1 and 0, respectively; and (3) TOST showed the mean difference was significantly below $+\Delta$ and above $-\Delta$. The equivalence margin (Δ) was $\pm 10\%$ for E-SAF and C-SAF, and $\pm 500 \text{ cells/mm}^2$ for cell density, reflecting typical measurement and biological variability.

3. Results

3.1. Modelled microstructural space

Semi-quantitative classical 2D histology results from ground truth sample sections are shown in Fig. 2. Enzymatic digestion treatments, together with inherent biological variability among samples, resulted in the developed vascular models covering a broad microstructural range. For the PTA-stained models ($n = 26$), E-SAF values ranged from 0.02 to 75.95% (mean 38.25%, median 42.31%, SD 20.55%), while C-SAFs extended from 0.00 to 72.25% (mean 32.57%, median 31.86%, SD 23.32%). The 1:2 Hf-POM-stained models ($n = 24$) followed a similar pattern, with E-SAF values between 0.04 and 63.46% (mean 34.29%, median 39.61%, SD 18.70%) and C-SAF ranging from 0.00 to 74.00% (mean 38.59%, median 37.46%, SD 20.53%). Using the median E-SAF (42.3%) and C-SAF (47.5%) across all samples, and $<10\%$ SAF threshold to indicate absence of elastin or collagen, six microstructural groups independently from enzymatic treatment, were defined. These are: High Collagen-High Elastin (HC–HE), High Collagen-Low Elastin (HC–LE), Low Collagen-High Elastin (LC–HE), Low Collagen-Low Elastin (LC–LE), No Collagen (NC) and No Elastin (NE) and are described in more detail in Fig. 2 below. The models covered nearly the full extent of this space, with at least two samples per stain represented in each group.

3.2. Qualitative comparison between X3DH and classical 2D histological imaging

Fig. 3 and 4 compare X3DH and histological imaging across the previously defined six microstructural groups (Fig. 2), each showing representative X3DH data and corresponding classical 2D histology from one representative sample per group.

Fig. 3 shows PTA-stained models. In the HC–HE sample (Fig. 3(A)), no degradation is visible, and intima, media, and adventitia are intact. VH-BF shows dense ELs and Interlamellar Elastin Fibres (IEFs), detectable as dark background between larger ELs; PR-BF and GS PR-PLM show abundant collagen in the media and collagen bundles in the adventitia; PTA-X3DH shows strong staining with distinguishable ELs but significant background signal. In HC–LE (Fig. 3(B)), marked degradation with adventitial loss and severe luminal elastin degradation can be seen. Although only the medial portion remains intact, collagen fibres are still dense; PTA-X3DH shows widespread staining, with higher intensity on the adventitial side, resembling the VH-BF negative pattern. In LC–HE (Fig. 3(C)), VH-BF shows dense ELs similar to HC–HE, but

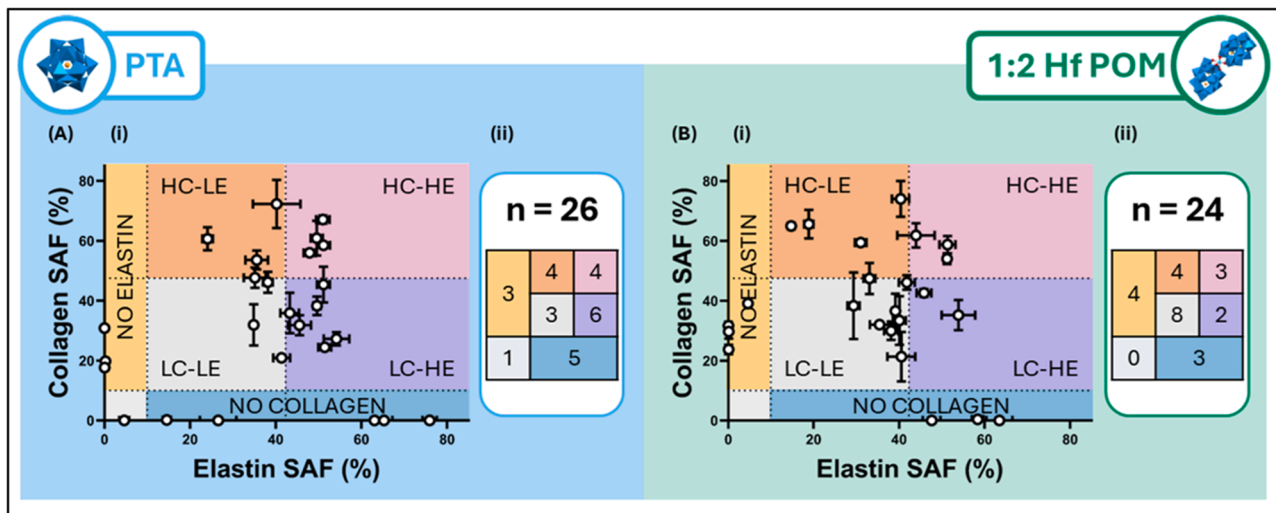


Fig. 2. Vascular degradation models showing Collagen (C-SAF) and Elastin (E-SAF) SAF distributions (%; $\mu \pm SE$) from semi-quantitative classical 2D histology of ground truth sections of: (A) Phospho-Tungstic Acid (PTA)-stained ($n = 26$) and (B) 1:2 Hafnium-substituted Wells-Dawson Polyoxometalate (1:2 Hf-POM)-stained ($n = 24$) samples. (i) C-SAF and E-SAF microstructural space plots; (ii) Break-down of sample number count in each microstructural group. Six microstructural groups are identified in both plots: (A-B): Pink | High Collagen-High Elastin (HC-HE) ($C-SAF \geq \bar{x}$ (C-SAF) & $E-SAF \geq \bar{x}$ (E-SAF)); Orange | High Collagen-Low Elastin (HC-LE) ($C-SAF \geq \bar{x}$ (C-SAF) & $10\% \leq E-SAF \leq \bar{x}$ (E-SAF)); Purple | Low Collagen-High Elastin (LC-HE) ($10\% \leq C-SAF \leq \bar{x}$ (C-SAF) & $E-SAF \geq \bar{x}$ (E-SAF)); Grey | Low Collagen-Low Elastin (LC-LE) ($10\% \leq C-SAF \leq \bar{x}$ (C-SAF) & $10\% \leq E-SAF \leq \bar{x}$ (E-SAF)); Blue | No Collagen (NC) ($C-SAF \leq 10\%$); Yellow | No Elastin (NE) ($E-SAF \leq 10\%$); $\bar{x}$ (C-SAF) and $\bar{x}$ (E-SAF) are the median C-SAF (42.3%) and E-SAF (47.5%) across all studied sections (ground truth and CESA-stained).

collagen is thinner and less abundant; PTA-X3DH produces high-intensity contrast, lower background signal, and sharper lamellae, especially in the lumen-facing region where collagen content is reduced. In LC-LE (Fig. 3(D)), VH-BF shows residual central elastin but near-complete loss on the luminal and adventitial sides; collagen is sparse, especially in the lumen-facing region and media, but with less loss on the adventitial side; PTA-X3DH shows strong uptake except in severely EL-degraded areas. In NC (Fig. 3(E)), VH-BF shows extremely dense elastin with minimal spacing, GS PR-PLM shows no collagen, and PTA-X3DH shows strong contrast with sharply defined but adjacent ELs and minimal background signal. In NE (Fig. 3(F)), VH-BF shows near-total elastin loss except for some scattered residues, GS PR-PLM shows a sparse swollen collagen network, and PTA-X3DH gives low staining except for small bright structures matching the elastin residues.

Fig. 4 shows representative 1:2 Hf-POM-stained models. Compared to PTA, 1:2 Hf-POM produced lower attenuation (images were windowed [0–46,260] versus [0–65,535]). In HC-HE (Fig. 4(A)), tissue architecture is intact with dense ELs, IEFs and abundant collagen; 1:2 Hf-POM-X3DH shows strong uniform staining, sharply defined ELs, low background signal, and better EL-contrast compared to PTA. In HC-LE (Fig. 4(B)), no degradation is seen, but the more muscular origin yields sparse thin lamellae - mainly Internal and External Elastic Lamina (IEL and EEL) - and dense collagen; 1:2 Hf-POM-X3DH shows intense binding to the sparse ELs, IEL and EEL with interlamellar saturation. In LC-HE (Fig. 4(C)), VH-BF shows dense elastin, and GS PR-PLM shows thinner collagen; 1:2 Hf-POM-X3DH shows high-intensity contrast like HC-HE but lower background and sharper EL visualisation. In LC-LE (Fig. 4(D)), both luminal and adventitial sides are degraded; VH-BF shows loose central elastin with minimal IEFs and scarce collagen; X3DH resembles the negative of VH-BF. In NC (Fig. 4(E)), VH-BF shows extremely dense elastin, and GS PR-PLM shows no collagen. 1:2 Hf-POM-X3DH shows strong contrast with sharply defined ELs and minimal background. Increased spacing between ELs can be noticed in X3DH compared to VH-BF. In NE (Fig. 4(F)), VH-BF shows near-complete elastin loss, leaving scattered fragments, GS PR-PLM shows a loose, swollen collagen network, and 1:2 Hf-POM-X3DH shows weak staining with small bright residues corresponding to remaining elastin.

3.3. Quantitative comparison between X3DH and classical 2D histological imaging

X3DH volumes enabled E-SVF extraction. An example of such extraction is summarised in Video 1 and 2. Fig. 5 shows E-SVF from X3DH versus E-SAF measurements from classical 2D histology. E-SVF correlated very strongly with E-SAF for both stains: for PTA ($r^2 = 0.861 > 0.7$, p -value < 0.0001), the slope was 0.42 (0.34–0.51 | 95% CI) and the intercept 4.27 (%) (0.46–8.08 | 95% CI), while for 1:2 Hf-POM ($r^2 = 0.858 > 0.7$, p -value < 0.0001), the slope was 0.49 (0.40–0.58 | 95% CI) and the intercept 4.35 (%) (0.70–8.00 | 95% CI).

3.4. Qualitative comparison Pre- and Post- CESA staining classical 2D histology

Fig. 6 (A–B) and 7 (A–B) compare pre- and post-CESA-stained VH-BF, PR-BF, GS PR-PLM, and H&E-BF (with zoomed cell nuclei views) of a representative HC-HE sample. In Fig. 6 (A–B), PTA-stained samples, VH-BF showed no noticeable changes between the ground truth and stained samples, with only minor variations typical of normal section variability. PR-BF and GS PR-PLM were similarly unaffected, consistent across all samples. In contrast, H&E-BF revealed an absence of haematoxylin-stained nuclei after CESA-staining, while eosin-stained ECM appeared visually unaffected. Zoomed views highlight clearly visible SMC nuclei before staining, but a loss of nuclear staining post-PTA-staining and cryopreservation, with only faint nuclear outlines visible in some tissue regions. In Fig. 7 (A–B), 1:2 Hf-POM-stained samples, VH-BF again appeared visually unchanged, while PR-BF and GS PR-PLM showed only a slightly lighter BF tone, consistent across all samples. H&E-BF, however, was significantly affected: nuclei stained purple as expected, but the ECM darkened considerably, showing a similar colour to cells. This was particularly noticeable in the ELs, whereby they appeared very dark.

3.5. Quantitative comparison Pre- and Post- CESA staining classical 2D histology

Fig. 6 (C–E (i–ii)) and 7 (C–E (i–ii)) compare pre- and post-CESA-stained E-SAF, C-SAF and cell density measurements obtained from

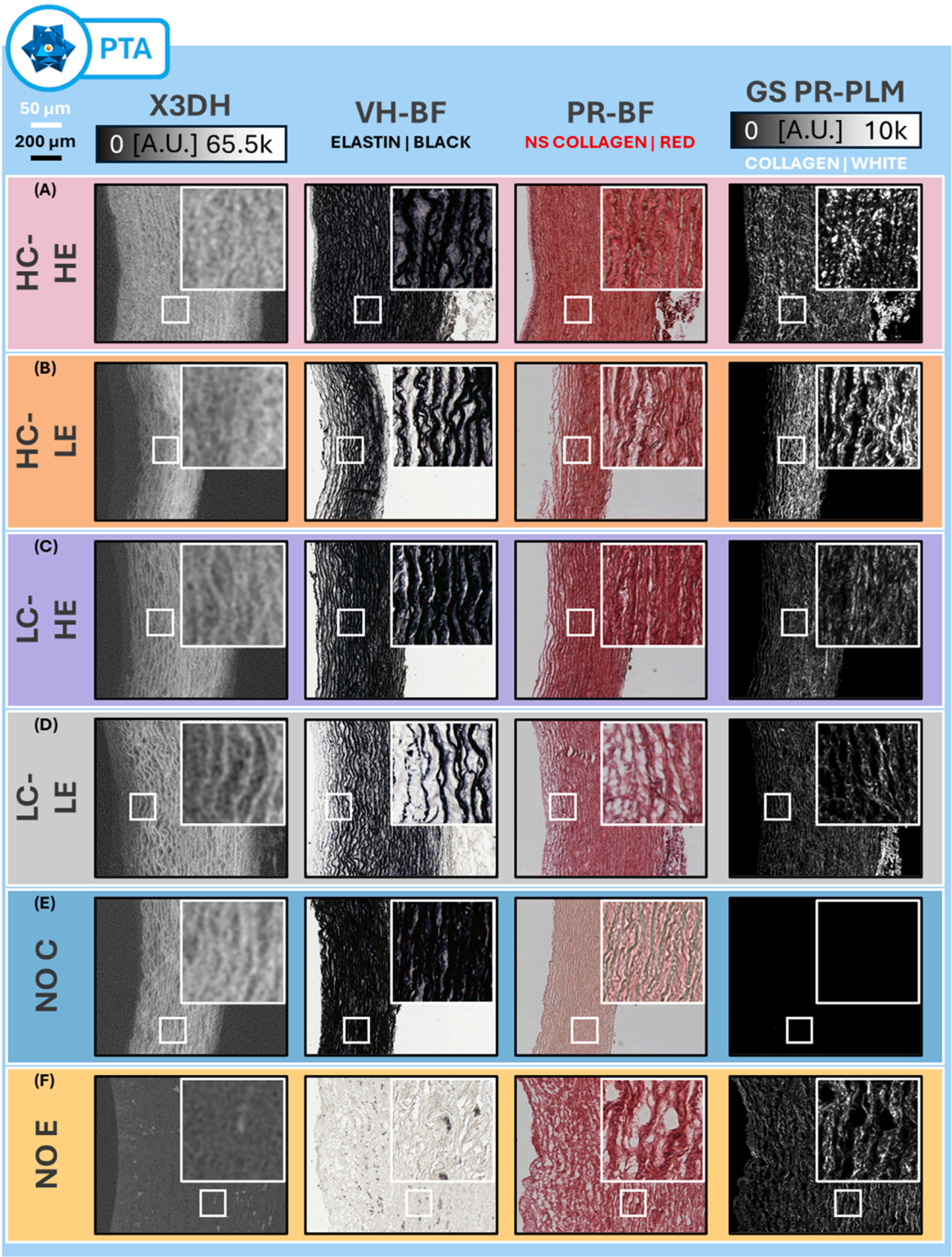


Fig. 3. Representative Phospho-Tungstic Acid (PTA)-X-ray-based 3D Histology (X3DH) axial slice and corresponding classical 2D histology, with internal zoom-in views, for the six degraded vessel model microstructural groups. Black bordered images - $1.22 \times 1.00 \text{ mm}^2$ ROI - black scale-bar (top left). White bordered images – zoom-ins as detailed - white scale-bar (top left). Columns (left to right): Normalised PTA-X3DH axial slice; Verhoeff's Brightfield; Picrosirius Red Brightfield; Grey scale combined & normalised PLM. Rows (top to bottom): Pink | High Collagen-High Elastin (HC–HE) ($\text{C-SAF} \geq \bar{x}(\text{C-SAF})$ & $\text{E-SAF} \geq \bar{x}(\text{E-SAF})$); Orange | High Collagen-Low Elastin (HC-LE) ($\text{C-SAF} \geq \bar{x}(\text{C-SAF})$ & $10\% \leq \text{E-SAF} \leq \bar{x}(\text{E-SAF})$); Purple | Low Collagen-High Elastin (LC–HE) ($10\% \leq \text{C-SAF} \leq \bar{x}(\text{C-SAF})$ & $\text{E-SAF} \geq \bar{x}(\text{E-SAF})$); Grey | Low Collagen-Low Elastin (LC-LE) ($10\% \leq \text{C-SAF} \leq \bar{x}(\text{C-SAF})$ & $10\% \leq \text{E-SAF} \leq \bar{x}(\text{E-SAF})$); Blue | No Collagen (NC) ($\text{C-SAF} \leq 10\%$); Yellow | No Elastin (NE) ($\text{E-SAF} \leq 10\%$); $\bar{x}(\text{C-SAF})$ and $\bar{x}(\text{E-SAF})$ are the median C-SAF (42.3%) and E-SAF (47.5%) across all studied sections (ground truth and CESA-stained). VH | Verhoeff's.; BF | Brightfield; PR| Picrosirius Red; NS | Not Specific; GS | Grey Scale; PLM | Polarised Light Microscopy.

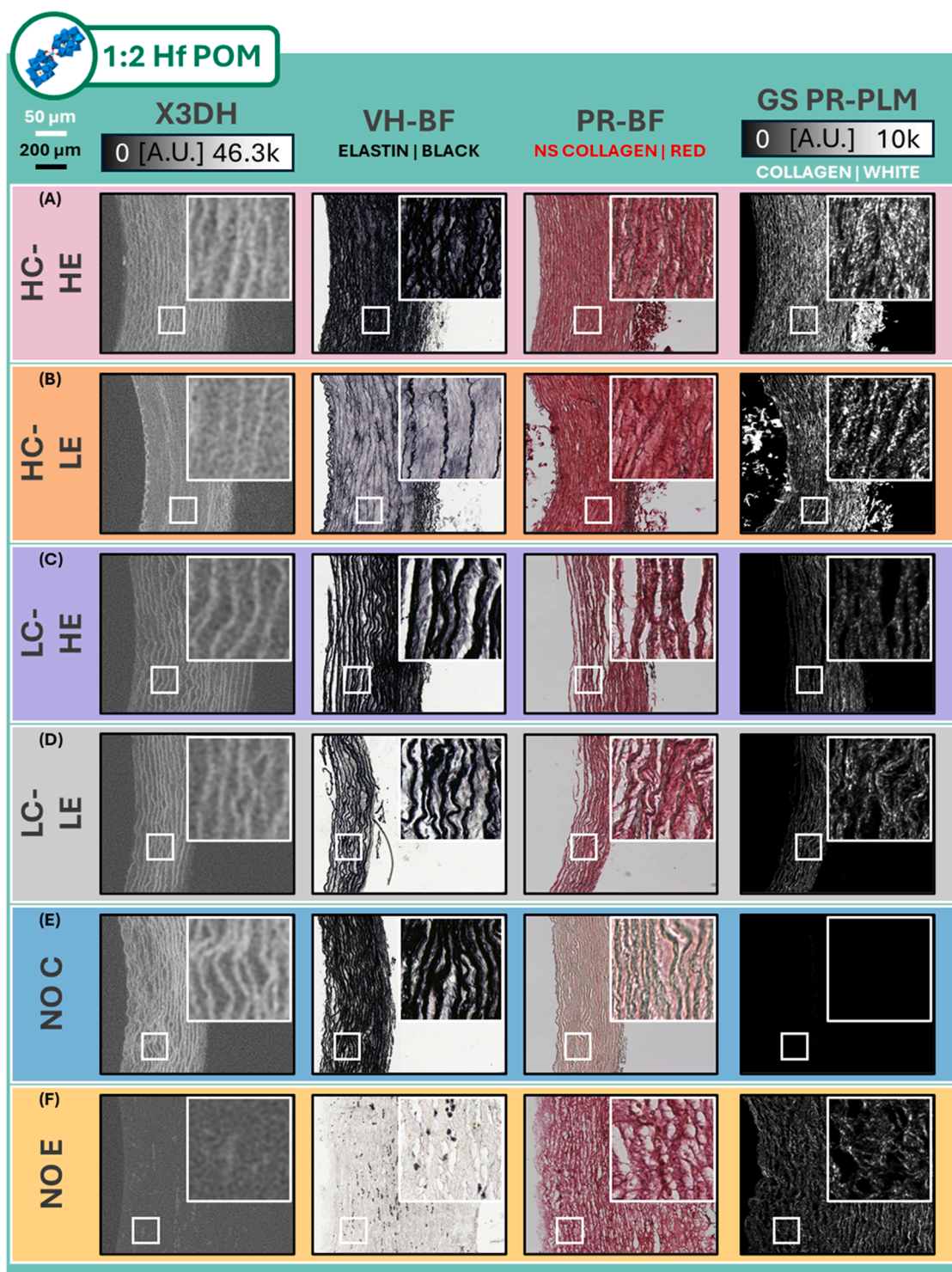


Fig. 4. Representative 1:2 Hafnium-substituted Wells-Dawson Polyoxometalate (1:2 Hf-POM)-X-ray-based 3D Histology (X3DH) axial slice and corresponding classical 2D histology, with internal zoom-in views, for the six degraded vessel model microstructural groups. Black bordered images - $1.22 \times 1.00 \text{ mm}^2$ ROI - black scale-bar (top left). White bordered images – zoom-ins as detailed - white scale-bar (top left). Columns (left to right): Normalised 1:2 Hf-POM-X3DH axial slice; Verhoeff's Brightfield; Picrosirius Red Brightfield; Grey scale combined & normalised PLM. Rows (top to bottom): Pink | High Collagen-High Elastin (HC-HE) ($C\text{-SAF} \geq \bar{x} (C\text{-SAF})$ & $E\text{-SAF} \geq \bar{x} (E\text{-SAF})$); Orange | High Collagen-Low Elastin (HC-LE) ($C\text{-SAF} \geq \bar{x} (C\text{-SAF})$ & $10\% \leq E\text{-SAF} \leq \bar{x} (E\text{-SAF})$); Purple | Low Collagen-High Elastin (LC-HE) ($10\% \leq C\text{-SAF} \leq \bar{x} (C\text{-SAF})$ & $E\text{-SAF} \geq \bar{x} (E\text{-SAF})$); Grey | Low Collagen-Low Elastin (LC-LE) ($10\% \leq C\text{-SAF} \leq \bar{x} (C\text{-SAF})$ & $10\% \leq E\text{-SAF} \leq \bar{x} (E\text{-SAF})$); Blue | No Collagen (NC) ($C\text{-SAF} \leq 10\%$); Yellow | No Elastin (NE) ($E\text{-SAF} \leq 10\%$); $\bar{x} (C\text{-SAF})$ and $\bar{x} (E\text{-SAF})$ are the median C-SAF (42.3%) and E-SAF (47.5%) across all studied sections (ground truth and CESA-stained). VH | Verhoeff's.; BF | Brightfield; PR | Picrosirius Red; NS | Not Specific; GS | Grey Scale; PLM | Polarised Light Microscopy.

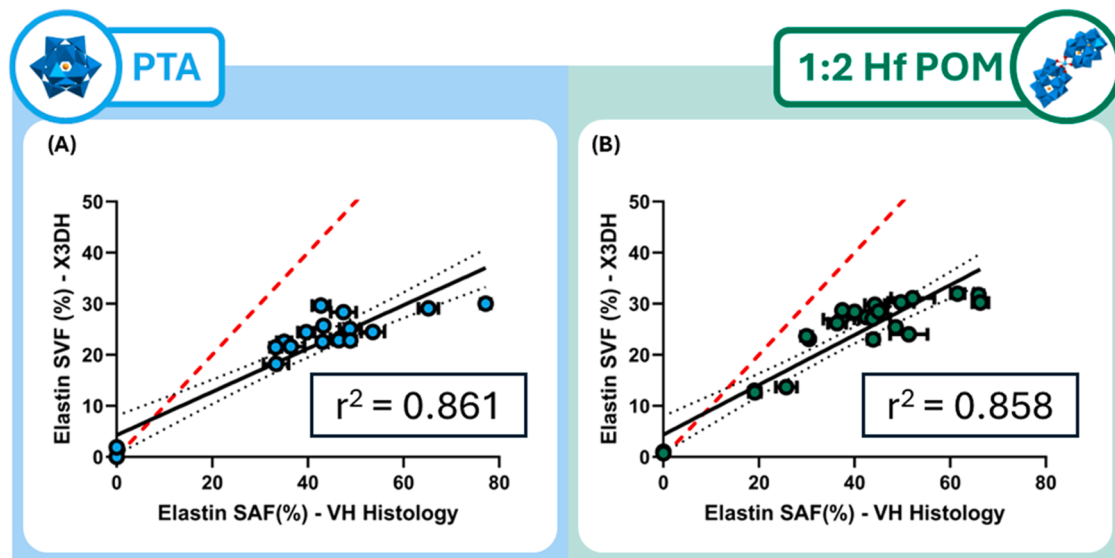


Fig. 5. Quantitative comparison of X-ray-based 3D Histology (X3DH) and classical 2D histology-derived stained elastin measurement is shown for (A) Phospho-Tungstic Acid (PTA) and (B) 1:2 Hafnium-substituted Wells-Dawson Polyoxometalate (1:2 Hf-POM). Plots show X3DH-based Elastin-Stained Volume Fraction (E-SVF) (single value taken from whole volume measurement) versus Contrast-Enhancing Staining Agent (CESA)-stained classical 2D histology Elastin-Stained Area Fraction (SAF) ($\mu \pm \text{SE}$) extracted from Verhoeff's (VH) stained sections, with linear regression fits (solid black), 95% confidence interval (dashed black), and the ideal 1:1 line (dashed red). SVF (%) was plotted against CESA-stained E-SAF (%) rather than ground truth E-SAF (%), as post-staining E-SAF matched ground truth, and using the same section better accounts for vessel variation.

VH, PR, and H&E histological analyses, as previously described, across all the analysed samples.

3.5.1. Elastin – stained area fraction (E-SAF)

Fig. 6(C)-(i) and 7(C)-(i) show pre- versus post- CESA-staining E-SAF for PTA- and 1:2 Hf-POM- stained samples, respectively. For both stains, CESA-stained E-SAF closely matched the ground truth values, with slopes and intercepts not significantly different from 1 to 0. For PTA ($r^2 = 0.92 > 0.7$, $p\text{-value} < 0.0001$), the slope was 0.92 (0.81–1.03 | 95% CI) and the intercept 4.44 (%) (–0.37–9.26 | 95% CI), while for 1:2 Hf-POM ($r^2 = 0.95 > 0.7$, $p\text{-value} < 0.0001$), the slope was 1.01 (0.90–1.12 | 95% CI) and the intercept 3.89 (%) (–0.26–8.04 | 95% CI). Fig. 6(C)-(ii) and 7(C)-(ii) display the paired difference between post- and pre- CESA-staining E-SAF measurements. For PTA, the mean paired difference (mean 4.29 (%), SD 4.43 (%)) was found to be significantly lower than $+\Delta$ ($p < 0.0001$) and higher than $-\Delta$ ($p < 0.0001$). Similarly, for 1:2 Hf-POM, the mean paired difference (mean 1.42 (%), SD 5.56 (%)) was found to be significantly lower than $+\Delta$ ($p < 0.0001$) and higher than $-\Delta$ ($p < 0.0001$).

3.5.2. Collagen – stained area fraction (C-SAF)

Fig. 6(D)-(i) and 7(D)-(i) show pre- versus post- CESA-staining C-SAF for PTA and 1:2 Hf-POM -stained samples respectively. For both stains, CESA-stained C-SAF correlated strongly with ground truth values, with slopes not significantly different from 1. For PTA ($r^2 = 0.96 > 0.7$, $p\text{-value} < 0.0001$), the slope was 0.87 (0.66–1.07 | 95% CI) and the intercept 8.23 (%) (0.14–16.33 | 95% CI), while for 1:2 Hf-POM ($r^2 = 0.91 > 0.7$, $p\text{-value} < 0.0001$), the slope was 0.91 (0.79–1.04 | 95% CI) and the intercept 5.74 (%) (0.23 – 11.25 | 95% CI). Fig. 6(D)-(ii) and 7(D)-(ii) illustrate the paired difference between post- and pre- CESA-staining C-SAF measurements. For PTA, the mean paired difference (mean 3.94 (%), SD 11.45 (%)) was found to be significantly lower than $+\Delta$ ($p < 0.05$) and higher than $-\Delta$ ($p < 0.0001$). Similarly, for 1:2 Hf-POM, the mean paired difference (mean 2.40 (%), SD 6.01 (%)) was found to be significantly lower than $+\Delta$ ($p < 0.0001$) and higher than $-\Delta$ ($p < 0.0001$).

3.5.3. Cell density

Fig. 6(E)-(i) and 7(E)-(i) show pre- versus post- CESA-staining cell density for PTA and 1:2 Hf-POM, respectively. For PTA, CESA-stained cell density showed a weak, non-significant correlation with ground truth values, with both slope and intercept differing significantly from 1 to 0 ($r^2 = 0.02 < 0.1$, $p\text{-value} = 0.53$). The slope was 0.10 (–0.22 – 0.41 | 95% CI) and the intercept 1.02 (10^3 Cells/mm^2) (0.22–1.82 | 95% CI). In contrast, 1:2 Hf-POM showed a strong correlation between pre- and post-CESA-staining cell density, though slope and intercept again differed significantly from 1 to 0 ($0.5 < r^2 = 0.51 < 0.7$, $p\text{-value} < 0.0001$). The slope was 0.62 (0.35–0.89 | 95% CI) and the intercept 1.14 (10^3 Cells/mm^2) (0.38 – 1.90 | 95% CI). Fig. 6(E)-(ii) and 7(E)-(ii) display the paired differences between post- and pre- CESA-staining cell density measurements. For PTA, the mean paired difference (mean –1.04 (10^3 Cells/mm^2), SD 1.30 (10^3 Cells/mm^2)) was found to be outside the $\pm\Delta$ boundaries. For 1:2 Hf-POM: the mean paired difference (mean 0.14 (10^3 Cells/mm^2), SD 0.66 (10^3 Cells/mm^2)) was found to be significantly lower than $+\Delta$ ($p < 0.05$) and higher than $-\Delta$ ($p < 0.0001$).

4. Discussion

In this study, the potential of PTA-X3DH and 1:2 Hf-POM-X3DH to substitute gold-standard classical 2D histology techniques, VH-BF for elastin and PR-PLM for collagen visualisation and quantification, was assessed by comparing imaging results across vascular models with differing collagen and elastin microstructural content.

4.1. Modelled microstructural space

Microstructural models exhibiting broad variations in elastin and collagen content were successfully produced. The models covered a broad E-SAF–C-SAF microstructural space, and although one group contained only two samples, the grouping was intended to illustrate how X3DH contrast varies with elastin and collagen composition rather than for statistical comparison between groups; thus, the sampling was sufficient to demonstrate the contrast enhancement behaviour for both CESAs. As shown in Fig. 2, despite originating from the same vascular region, a wide range of E-SAFs and C-SAFs can be observed across the models. The E-SAF

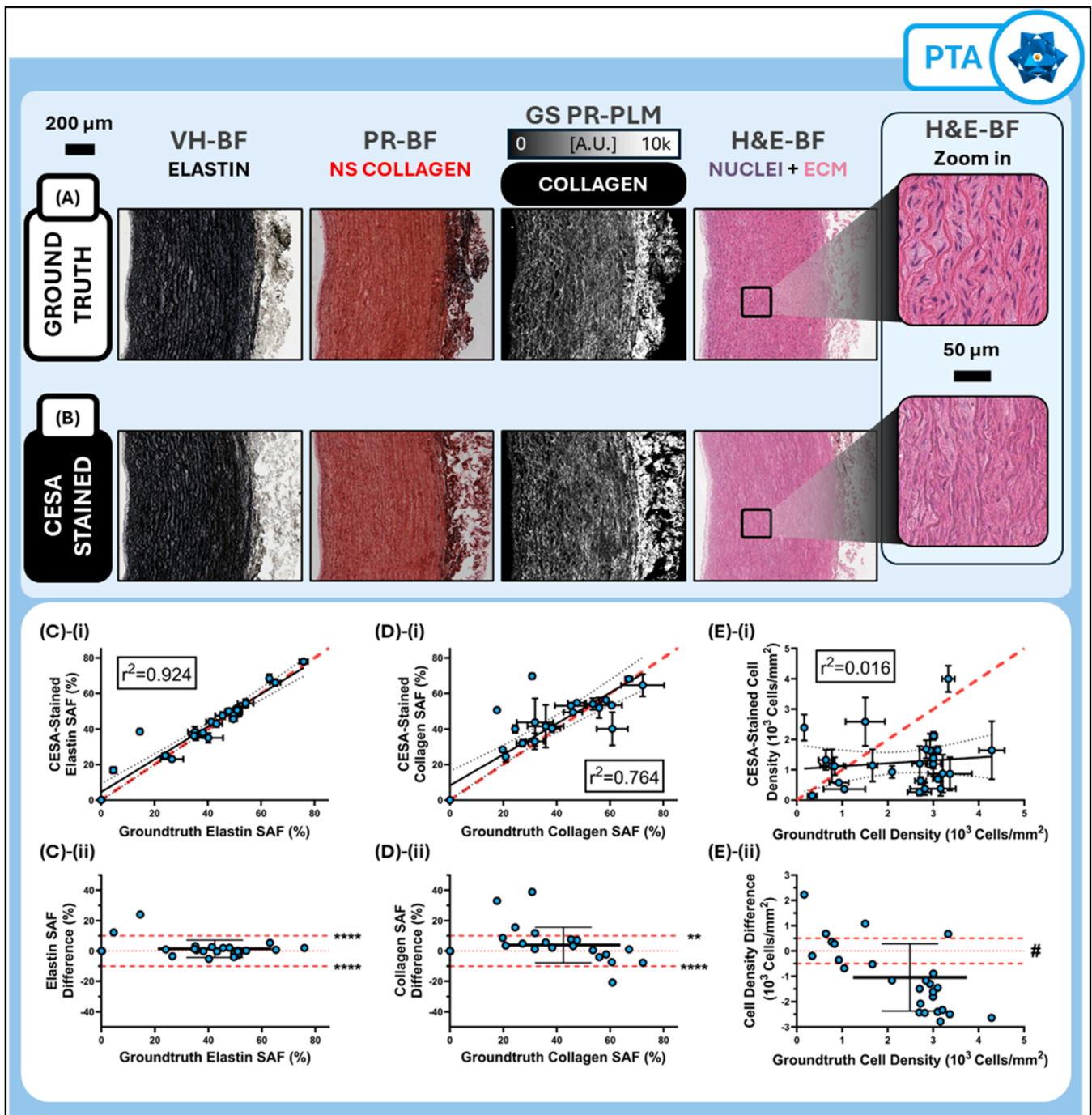


Fig. 6. Post Contrast-Enhancing Staining Agent (CESA)-staining versus ground truth classical 2D histology for Phospho-Tungstic Acid (PTA)-stained samples ($n = 26$). (A–B) from left to right: VH-BF, PR-BF, GS PR-PLM and H&E (with additional right-side zoom-in) classical 2D histology for: (A) A High Collagen – High Elastin (HC–HE) ground truth sample section, (B) corresponding CESA-stained sample section. (C)–(i) Plot of CESA-stained versus ground truth Elastin-Stained Area Fraction (E-SAF) ($\mu \pm \text{SE}$); (C)–(ii) Paired difference between CESA-stained and ground truth E-SAF vs ground truth E-SAF, with overlaid bar plot of paired difference ($\mu \pm \text{SD}$). (D)–(i) Plot of CESA-stained versus ground truth Collagen-SAF (C-SAF) ($\mu \pm \text{SE}$); (D)–(ii) Paired difference between CESA-stained and ground truth C-SAF vs ground truth C-SAF, with overlaid bar plot of paired difference ($\mu \pm \text{SD}$). (E)–(i) Plot of CESA-stained versus ground truth cell density ($\mu \pm \text{SE}$); (E)–(ii) Paired difference between CESA-stained and ground truth cell density vs ground truth cell density, with overlaid bar plot of paired difference ($\mu \pm \text{SD}$). For (C–E)–(i) plots: simple linear regression fit (solid black line), 95% CI lines (dashed black lines) and idealised 1:1 fit line (dashed red line). For (C–E)–(ii) plots: thin red dashed line indicates $y = 0$, thicker red dashed lines indicate $\pm \Delta$ used for the TOST test. Asterisks indicate significance between the mean value and $\pm \Delta$ using a one-sample t -test. # indicates that the test was not carried out, as the mean was outside the $\pm \Delta$ interval. VH | Verhoeff's; BF | Brightfield; PR | Picrosirius Red; H&E | Haematoxylin and Eosin; NS | Not Specific; GS | Grey Scale; PLM | Polarised Light Microscopy; ECM | Extracellular Matrix. Error bars may not be visible in instances where the bar was shorter than the datapoint outline radius.

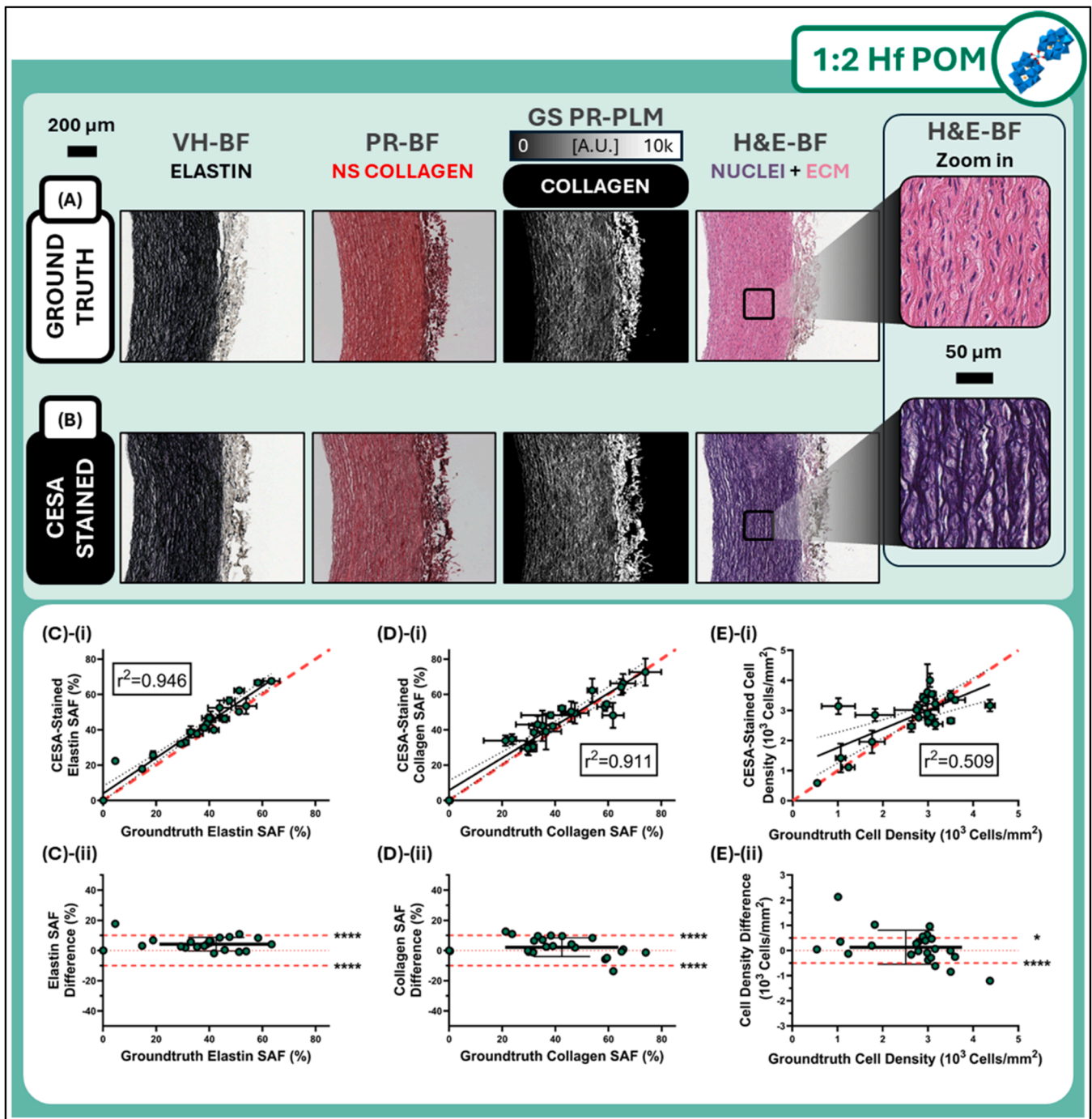
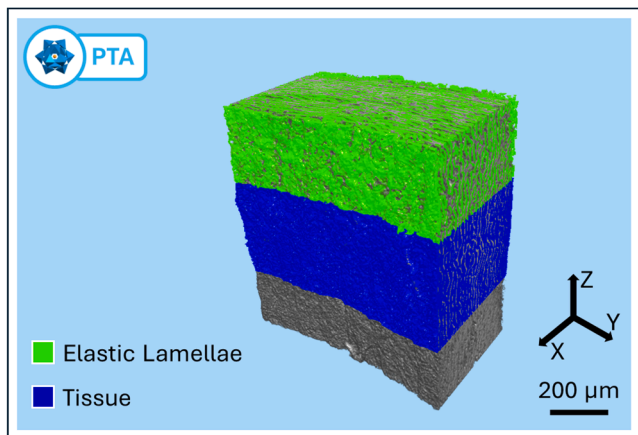
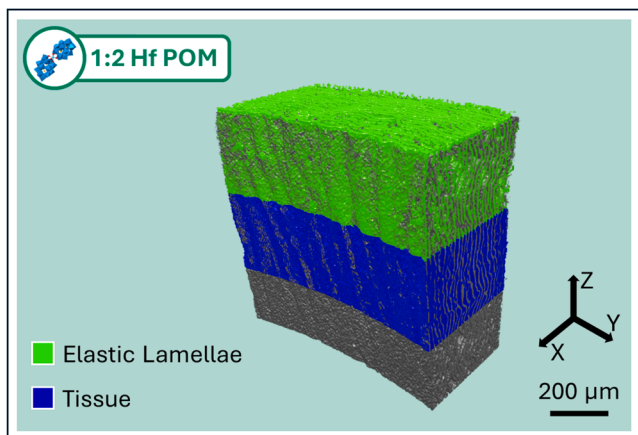


Fig. 7. Post Contrast-Enhancing Staining Agent (CESA)-staining versus ground truth classical 2D histology for 1:2 Hafnium-substituted Wells-Dawson Polyoxometalate (1:2 Hf-POM)-stained samples ($n = 24$). (A-B) from left to right: VH-BF, PR-BF, GS PR-PLM and H&E (with additional right-side zoom-in) classical 2D histology for: (A) A High Collagen – High Elastin (HC–HE) ground truth sample section, (B) corresponding CESA-stained sample section. (C)-(i) Plot of CESA-stained versus ground truth Elastin-Stained Area Fraction (E-SAF) ($\mu \pm SE$); (C)-(ii) Paired difference between CESA-stained and ground truth E-SAF vs ground truth E-SAF, with overlaid bar plot of paired difference ($\mu \pm SD$). (D)-(i) Plot of CESA-stained versus ground truth Collagen-SAF (C-SAF) ($\mu \pm SE$); (D)-(ii) Paired difference between CESA-stained and ground truth C-SAF vs ground truth C-SAF, with overlaid bar plot of paired difference ($\mu \pm SD$). (E)-(i) Plot of CESA-stained versus ground truth cell density ($\mu \pm SE$); (E)-(ii) Paired difference between CESA-stained and ground truth cell density vs ground truth cell density, with overlaid bar plot of paired difference ($\mu \pm SD$). For (C-E) -(i) plots: simple linear regression fit (solid black line), 95% CI lines (dashed black lines) and idealised 1:1 fit line (dashed red line). For (C-E) -(ii) plots: thin red dashed line indicates $y = 0$, thicker red dashed lines indicate $\pm \Delta$ used for the TOST test. Asterisks indicate significance between the mean value and $\pm \Delta$ using a one-sample t -test. VH | Verhoeff's; BF | Brightfield; PR | Picrosirius Red; H&E | Haematoxylin and Eosin; NS | Not Specific; GS | Grey Scale; PLM | Polarised Light Microscopy; ECM | Extracellular Matrix. Error bars may not be visible in instances where the bar was shorter than the datapoint outline radius.



Video 1. Thumbnail of video animation. Tissue & elastic lamellae segmentation example of a Phospho-Tungstic Acid (PTA)-stained sample. The video shows an animation of the segmented tissue in blue and segmented elastic lamellae in green within the tissue. The x, y & z directions correspond to the radial, circumferential and axial vessel directions.



Video 2. Thumbnail of video animation. Tissue & elastic lamellae segmentation example of a 1:2 Hafnium-substituted Well Dawson Polyoxometalate (1:2 Hf-POM)-stained sample. The video shows an animation of the segmented tissue in blue and segmented elastic lamellae in green within the tissue. The x, y & z directions correspond to the radial, circumferential and axial vessel directions.

range (0.02–75.95%) is representative of aneurysmal tissue, consistent with previous models [73], and of diseased atherosclerotic tissue [79]. Similarly, C-SAF values (0.00–74.00%) matched those reported in plaque caps (~40–80%) [14] and diseased human carotid arteries (~20–60%) [79] quantified using comparable PLM-based methods.

Controlling microstructural composition via partial enzymatic digestion proved challenging. In some cases, digestion advanced extensively from the luminal or adventitial sides, creating regions with native-like densities where some areas were fully digested while adjacent regions remained largely intact. Although protocols had been previously optimised on a subset of samples, the extent of digestion was difficult to predict due to strong dependence on initial tissue composition and enzyme potency, which varied between batches. This issue was particularly evident with crude enzyme preparations, where achieving controlled partial digestion was challenging. Enzymatic treatments also affected cellular content, as degradation of structural proteins caused partial cell dispersion. Despite challenges in controlling digestion and secondary effects like cell leaching, the resulting models remained representative and suitable for studying elastin and collagen microstructural variation.

4.2. Comparison between X3DH and classical 2D histological imaging

The contrast obtained with both PTA and 1:2 Hf-POM staining was broadly equivalent for visualising vascular microstructure. Both stains bind non-specifically but preferentially to elastin, allowing accurate depiction of ELs.

The PTA contrast ratio between ELs and the surrounding ECM was slightly lower than that of 1:2 Hf-POM due to a stronger background signal, particularly evident in HE-HC samples. This reduced contrast ratio could be due to greater non-specific PTA binding to collagen or other ECM components. Raman spectroscopy results show that PTA exhibits a similar affinity for collagen type I compared to 1:2 Hf-POM, while also inducing chemical alterations in collagen type II and fibrin, pointing to a binding that may be stronger but that also indicates a more disruptive and less selective interaction of PTA with these proteins [57], which may contribute to increased background staining.

The preferential, though non-specific, affinity of both stains for elastin arises from their chemical similarity. PTA (Keggin-type POM) and 1:2 Hf-POM (Wells–Dawson-type POM) are both negatively charged polyoxometalates that interact strongly with protoelastin [22], due to its high net positive charge ($pI \approx 10.40$, Exspasy). In contrast, arterial collagens, type I ($pI \approx 5.6$, Exspasy) and type III ($pI \approx 6.21$, Exspasy), carry lower positive charges under physiological conditions, resulting in weaker binding. Under experimental conditions, PTA was applied at $pH \approx 2.2$, where most proteins, including elastin and collagen, are positively charged and thus prone to anionic stain binding. Conversely, 1:2 Hf-POM was used at $pH \approx 7.4$, where elastin remains positively charged while collagen approaches or falls below its isoelectric point, potentially explaining 1:2 Hf-POM's higher contrast ratio between elastin and the surrounding ECM.

The performance of 1:2 Hf-POM staining aligns well with previous reports by Leyssens and Pétré [22,50], where 1:2 Hf-POM successfully enabled visualisation and segmentation of vascular ELs. In contrast, the present PTA results diverge from Hanly et al. [47], who reported preferential PTA binding to collagen. This discrepancy likely stems from experimental design differences. In their study, the authors utilised a lower resolution ($6 \mu m$ isotropic) to image native porcine carotid arteries. This precluded visualisation of individual ELs or collagen fibres, and the analysis focused on assessing regions closer to the adventitia versus the intima, with a stronger adventitial signal being attributed to collagen's radial distribution. While this interpretation is reasonable, it does not necessarily imply preferential binding of PTA to collagen, as an increased collagen content in the adventitial region would still contribute to the overall signal even with non-preferential binding.

E-SVF derived from X3DH was approximately two-fold lower than E-SAF values from classical 2D histology. Discrepancies between histological and X3DH elastin measurements are attributable to their methodological differences. Differences in section thickness ($8 \mu m$) versus acquisition resolution ($\sim 0.5 \times 0.5 \mu m^2$) can produce “over-projection” in histological images, artificially increasing apparent signal relative to volumetric data. To assess the “over-projection” effect, an ‘equivalent E-SAF’ was quantified from the corresponding X3DH data by projecting four slices of segmented elastin-stained lamellae. As shown in Supplementary Materials Figure S.6, strong correlations were maintained for both PTA and 1:2 Hf-POM ($r^2 > 0.86$), while the regression slope increased from 0.42 to 0.57 for PTA and from 0.49 to 0.60 for 1:2 Hf-POM. These findings indicate that, although over-projection contributes to the observed discrepancy, it does not fully account for it, as the slope is still significantly lower than 1.

The main source of the discrepancy can most likely be attributed to tissue shrinkage during histological processing. Leyssens et al. quantified volumetric shrinkage in porcine aortas across fresh, fixed, fixed-and-stained (1:2 Hf-POM), and paraffin-embedded states. Relative to fresh tissue, shrinkage after fixation and 1:2 Hf-POM staining was only about 4%, but after paraffin embedding it reached 45% [22]. The most relevant comparison here is between the fixed-and-stained and

paraffin-embedded states, where the volume difference was 43%, meaning the final volume after histological processing was 57% of the X3DH volume. Assuming isotropic shrinkage, and that shrinkage is driven mainly by tissue crosslinking and dehydration while elastin content remains unchanged, this gives a tissue area scaling factor of $0.57^{2/3} \approx 0.69$. Under these assumptions, the measured E-SAF would therefore be overestimated by approximately 45% ($0.69^{-1} \approx 1.45$). This is also likely to be a conservative estimate, as it does not include shrinkage in the sectioning direction, which would further increase the apparent signal through an effect analogous to “over-projection”. Although some shrinkage may be partly reversed by rehydration after wax removal on the slide, histology is still likely to substantially overestimate E-SAF.

Conversely, the lower resolution of X3DH, including partial-volume effects at a 2 μm voxel size, may blur elastin boundaries and lead to underestimation of E-SVF. The thresholding applied to the 3DXH data was not considered unduly conservative. However, the thresholding was based on the clearer, edge-enhanced contrast used to delineate ELs. As a result, very thin IEFs may still not be consistently resolved or segmented in the 3D analysis. This is especially true for fibres below the effective resolution limit due to the limited resolution and partial-voluming effects. By contrast, depending on their size, such fibres may be detected by histological segmentation because of histology’s higher spatial in-plane resolution.

Collectively, these effects would be expected to bias E-SVF downwards relative to E-SAF. However, the evidence presented suggests that, owing to its better volumetric agreement with fresh tissue and its isotropic sampling, X3DH provides an estimate that is less affected by processing artefacts and therefore offers a more reliable representation of elastin content.

Overall, X3DH via CECT provides a robust method for three-dimensional visualisation of vascular microstructure. Despite this, the technique currently remains constrained by the limited number of available CESAs, incomplete understanding of their staining specificity relative to well-established histology protocols, and spatial resolution. The 2 μm voxel size used in this study, while excellent for laboratory-based micro-CT, remains coarse relative to other imaging techniques and limits the visualisation of finer structures. In practice, even state-of-the-art laboratory-based systems are typically limited to voxel sizes of around 1 μm , which would still be insufficient to visualise, for example, individual collagen fibres in vascular tissue. At present, laboratory X3DH via CECT is therefore best suited to questions concerning three-dimensional tissue architecture, spatial relationships, and structural features larger than individual fibres, such as ELs, where this level of resolution can still provide biologically meaningful information.

Despite these limitations, X3DH via CECT may offer important advantages over classical two-dimensional histology for visualising vascular microstructure. Its three-dimensional, non-destructive, and less artefact-prone nature means that it can already support computational modelling by providing more faithful structural representations of vascular tissue. Moreover, the potential to combine CECT with *in situ* mechanical testing, provided that suitable staining protocols that preserve mechanical properties can be established [50], offers an opportunity to validate such computational models in a way that is not possible with conventional histology, thereby improving our understanding of tissue mechanics. More broadly, its inherently three-dimensional character also makes it well suited for correlative studies with other 3D imaging modalities, such as MRI, further expanding its potential as a complementary tool in vascular tissue characterisation.

4.3. Comparison pre- and post- CESA staining classical 2D histology

Although NaOH-based protocols (0.1 M NaOH [47,81]), have been used successfully to destain PTA-stained samples; protocols effects on fixed and stained tissue have not, to the authors’ knowledge, been

evaluated. This is important, as alkaline 0.1 M NaOH can lyse fixed tissue [82], raising concerns that prolonged exposure may cause similar damage and introduce artefacts between stained and destained samples. As this study aimed to assess whether classical 2D histology could be performed after CESA staining without altering results, any method that might compromise tissue or cellular integrity was avoided, and the samples were simply transferred back in PBS following staining. Future work should focus on evaluating the impact of destaining approaches with the aim of developing staining approaches that have minimal effects on tissue integrity.

As shown in the results (Fig. 6 and 7), CESA staining did not significantly affect PR or VH histological outcomes, as evidenced by both visual and quantitative agreement.

For E-SAF measurements, linear fits under both staining conditions produced slopes and intercepts not significantly different from 1 to 0, respectively, with very high correlations. Differences remained within a 10% equivalence range, considered acceptable given natural vessel variation and histological measurement repeatability. These findings are consistent with previous work using Verhoeff’s–Van Gieson staining on 1:2 Hf-POM-stained vascular samples, which showed no visible alterations despite the lack of direct comparison [22]. To the authors’ knowledge, this is the first evaluation of VH staining following PTA staining.

Similarly, for C-SAF measurements, the linear fit slopes did not differ significantly from 1, and correlations were high. Although intercepts were slightly above 0, this did not indicate systematic bias, as data points near the origin suggest the deviation likely reflects increased fitting error due to the higher variability of C-SAF measurements. As with E-SAF, C-SAF results remained within the 10% equivalence range. To the authors’ knowledge, this is also the first report on the effect of 1:2 Hf-POM staining on PR histology. Regarding PTA staining, while no direct comparative analyses have been reported, Hanly et al. observed no discernible differences in PR staining after PTA application [47]. Although their protocol included a destaining step, the present findings confirm that even without destaining, no differences were evident. Similarly, Nieminen et al. reported no apparent alterations in PR staining of cartilage following PTA staining [61].

Samples treated with 1:2 Hf-POM exhibited diffuse, non-specific haematin binding. Although the underlying mechanism remains unclear, it may relate to the chemistry of the alum-haematoxylin reaction. Haematoxylin itself is inactive and must first oxidise to haematein, which then complexes with metal mordants such as aluminium, iron, or tungsten to form the active dye. In Harris’ formulations, sodium iodate serves as the oxidant and aluminium salts provide the alum mordant [83]. The resulting haematein– Al^{3+} complex binds primarily to DNA via electrostatic attraction between the cationic complex and negatively charged phosphate groups. Given this mechanism, the presence of 1:2 Hf-POM in tissue following staining may modify the local ionic environment of the ECM and/or the tungsten present in 1:2 Hf-POM may compete with aluminium for haematein complexation. Residual 1:2 Hf-POM species could therefore promote additional, non-specific haematein binding, either through alternative metal–dye complex formation or by increasing tissue surface charge density. This may account for the diffuse background haematoxylin staining observed. Similar effects have been reported in bovine muscle [66] and can also be observed in a previous study in skeletal tissue [57]. The authors propose that this phenomenon is particularly pronounced in vascular tissue owing to its high elastin content, as elastin’s strong affinity for 1:2 Hf-POM likely contributes to marked haematoxylin overstaining. Importantly, notable discrepancies were observed between 1:2 Hf-POM-stained cell densities and ground truth cell densities, with 1:2 Hf-POM staining leading to inaccurate cell number estimation due to diffuse and non-specific haematoxylin staining. This indicates that 1:2 Hf-POM staining is not well suited for subsequent reliable cell density evaluation using H&E, and that improved washing protocols prior to H&E, or alternative techniques less influenced by the local ionic environment, such as DAPI fluorescent

nuclear staining, may be more appropriate.

Haematoxylin overstaining was not observed in PTA-stained samples, as PBS storage is more effective at displacing PTA compared to 1:2 Hf-POM. This is supported by some preliminary results in Supplementary Materials Figure S.1–S.2, where, following 24 h of staining and subsequent 24-hour PBS storage, attenuation levels in PTA-stained samples were markedly reduced, whereas 1:2 Hf-POM-stained samples remained unchanged. This behaviour can be attributed to the diffusion of PTA back into solution following incubation in PBS, the authors hypothesise that the diffusion may be driven by the higher pH of PBS (pH 7–7.4) relative to expected pH of the PTA solution (pH ~2.2 [50]), promoting its release. In contrast, this effect is not observed for 1:2 Hf-POM due to its near-neutral pH (pH ~7.2 [50]) in solution. Another possible hypothesis is that PTA undergoes dissociation into different POM species upon dissolution in PBS, which may be more readily removed during PBS storage.

The lack of cellular contrast in PTA-stained samples was initially attributed to impaired haematoxylin binding caused by residual PTA. However, subsequent H&E staining of vascular tissue treated equivalently but without a cryopreservation step did not exhibit this effect, showing strong haematoxylin nuclear uptake and clear cellular visualisation (Supplementary Materials, Figure S.7). This finding aligns with previous studies that performed H&E staining following PTA treatment without reporting reduced nuclear visualisation [62,84–87]. These results indicate that the reduced uptake is due to cryopreservation, indicating that PTA is unsuitable as a CESA for cryo-CECT, especially when visualising cellular structures is important.

4.4. Study limitations

This study presents several limitations. Although the models cover a broad microstructural range consistent with values reported for diseased tissue, they do not represent true pathological samples. Non-specific binding to other components, which may be present in higher proportion in diseased tissue, such as fibrin, cells and GAGs, should be considered when interpreting results in diseased contexts. Despite efforts to minimise discrepancies, minor variations may have been compounded by registration inaccuracies between classical 2D histological sections and 3D datasets, limiting the reliability of direct point-to-point comparisons. In addition, although E-SAF and C-SAF measurements derived from histological images (VH-BF and PR-PLM) currently represent the gold standard method for estimating relative collagen and elastin richness between samples, it is important to note that these measurements reflect stained area fractions rather than true percentage content.

For the ECO-F model, imaging resolution did not permit visualisation of individual collagen fibres in the no-elastin condition. Isolating the collagen-specific signal would have been beneficial for improving model accuracy. Additionally, background tissue contribution was assumed equivalent to PBS, as unstained samples imaged in PBS showed similar signal levels; however, some background signal originates from non-specific staining of cells or other ECM components is likely, constraining model accuracy and implementation. Moreover, degradation treatments likely altered cell density and induced swelling (elastin removal) or contraction (collagen removal), introducing variability in background signal contribution. Although generating a sample entirely devoid of both elastin and collagen could theoretically establish a baseline for background assessment and collagen-specific signal extraction, achieving such a condition while maintaining structural integrity for imaging is extremely challenging.

5. Conclusion

This study explored the potential of PTA-based and 1:2 Hf-POM-based X3DH using CECT as alternatives to the gold-standard techniques PR-PLM for collagen and VH-BF for elastin visualisation and

quantification in vascular tissue. Results showed that both PTA and 1:2 Hf-POM bind non-specifically but preferentially to elastin, enabling accurate quantification strongly correlating with VH-BF ground truth. Although showing high correlation with classical 2D histology, E-SVF underestimated elastin compared to classical 2D histology, likely due to limitations of histological methods, as X3DH provides a more accurate representation of elastin content and arrangement owing to hydrated and isotropically sampled imaging. However, meaningful collagen-related information could not be extracted from X3DH, as non-segmentable collagen signal could not be separated from elastin, which could result from a variety of factors such as low collagen signal, non-specific binding to other components and modelling limitations. No evidence suggested that CESA staining interfered with VH or PR histology, indicating that X3DH does not hinder subsequent histological staining for validating X3DH findings. The evaluated CESAs affected H&E staining, with 1:2 Hf-POM causing haematoxylin over-binding and PTA causing significantly reduced nuclear visualisation when combined with cryopreservation, both affecting cell density measurements. Future work should focus on developing collagen-specific CESAs to allow its visualisation and quantification in elastin-rich tissues and on creating washing protocols that prevent tissue damage and preserve histological staining compatibility for 1:2 Hf POM. Overall, this study highlights X3DH as a promising technique for fully three-dimensional imaging of vascular tissue, enabling non-destructive, quantitative, and histologically comparable analysis of tissue specimens.

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

During the preparation of this work, the authors used ChatGPT 5 and Microsoft Copilot to improve the text readability and language. After using the tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Francesco Digeronimo: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Robert Johnston:** Methodology. **Brooke Tornifoglio:** Writing – review & editing, Methodology, Conceptualization, Supervision. **Greet Kerckhofs:** Writing – review & editing, Supervision, Resources, Conceptualization. **Caitríona Lally:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2026.04.051.

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