



Exploring contrast-enhancing staining agents for studying adipose tissue through contrast-enhanced computed tomography

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Abstract Contrast-enhanced computed tomography offers a nondestructive approach to studying adipose tissue in 3D. Several contrast-enhancing staining agents (CESAs) have been explored, whereof osmium tetroxide (OsO₄) is the most popular nowadays. However, due to the toxicity and volatility of the conventional OsO₄, alternative CESAs with similar staining properties were desired. Hf-WD 1:2 POM and Hexabrix have proven effective for structural analysis of adipocytes using contrast-enhanced computed tomography but fail to provide chemical information. This study introduces isotonic Lugol's iodine (IL) as an alternative CESA for adipose tissue analysis, comparing its staining potential with Hf-WD 1:2 POM and Hexabrix in murine caudal vertebrae and bovine muscle tissue strips. Single and sequential staining protocols were compared to assess the maximization of information extraction from each sample. The study investigated interactions, distribution, and reactivity of iodine species towards biomolecules using simplified model systems and assesses the potential of the CESA to provide chemical information. (Bio)chemical analyses on whole tissues revealed that differences in adipocyte gray values post-IL staining were associated with chemical distinctions between bovine muscle tissue and murine caudal vertebrae. More specific, a difference in the degree of unsaturation of fatty acids was identified as a likely contributor, though not the sole determinant of gray value differences. This research sheds light on the potential of IL as a CESA, offering both structural and chemical insights into adipose tissue composition.

Supplementary key words adipose tissue • muscle • adipocytes • bone marrow • lipids/chemistry • 3D histology • DICECT • Lugol's iodine

Adipose tissue (AT) is a type of loose connective tissue located in distinct regions throughout the body (e.g. intraperitoneal, subcutaneous, visceral, within the bone marrow, intermuscular, and intramuscular) (1–6). Even though AT was first mentioned in a publication in 1837, it was not until the 1980s that the first secretory function of adipocytes (Ads) was recognized (6). Due to the increased interest of the scientific community in this specialized tissue, the physiological functions of Ads have been widely explored and four main types have been determined: white, brown, beige, and pink Ads (6). In addition to these four main types of AT, two additional distinct types could be identified inside the bone marrow (BM), namely the constitutive and regulated bone marrow adipocytes (BMAds) (7). Nowadays, it is well-established that Ads play a major role in multiple physiological processes (e.g. thermogenesis, lipid metabolism, energy storage, bone homeostasis, and glucose homeostasis) and that dysregulation of healthy Ads can consequently contribute to several severe pathologies (e.g. type II diabetes, obesity, atherosclerosis, osteoporosis, and cancer) (1, 8–19).

To fully understand the role of Ads in (patho)physiological processes, it is imperative to study their spatial distribution within a tissue (e.g. proximity between Ads

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and other cells and tissue constituents) and to thoroughly characterize their structural parameters (e.g. volume, volume fraction, Feret diameter, equivalent diameter (20)) combined with chemical information on the Ad composition (e.g. types of lipid species, saturated vs. unsaturated). To this end, multiple imaging modalities, both 2D and 3D in nature, have been applied: optical and fluorescence microscopy, microMRI and microCT. The microscopical techniques rely on colorimetric stains (e.g. Oil Red O (hydrophobic), Haematoxylin & Eosin Y (hydrophilic)), fluorescent stains (e.g. Nile Red (hydrophobic)) and immunohistochemical staining methods to visualize Ads in a 2D (e.g. bright-field microscopy) or 3D (e.g. light-sheet microscopy) fashion (21, 22). Despite the high spatial resolution and discriminative power, sample preparation is laborious and destructive for the tissue (e.g. sectioning, clearing). Moreover, the size of the specimen to be imaged is limited (23, 24). However, efficient, automated analysis protocols have mitigated analysis time substantially (25–27). MicroMRI can nondestructively visualize bulk AT in 3D and can determine molecular properties of AT based on chemical shifts of the molecules present in the matrix (28–30). However, the spatial resolution of microMRI is insufficient to visualize individual Ads, hence their structural properties cannot be computed. Moreover, it only allows obtaining averaged information on lipid molecular structures and is expensive. MicroCT can achieve higher spatial resolution than microMRI, but due to the weak X-ray attenuating properties of soft tissues (e.g. hematopoietic tissue, AT), segmentation of AT is impossible or requires subjective threshold determination (31).

To complement the above-mentioned imaging techniques, researchers have investigated contrast-enhanced computed tomography (CECT) imaging. This technique can generate 3D images with the same spatial resolution as microCT, while visualizing soft and dense tissue constituents. The current standard contrast-enhancing staining agent (CESA) for visualization of Ads using CECT is osmium tetroxide ($\text{Os}^{\text{VIII}}\text{O}_4$) (32–37). 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 (38). OsO_3 being unstable in aqueous and alcoholic solution disproportionates back to OsO_4 and the black insoluble $\text{Os}^{\text{IV}}\text{O}_2 \cdot 2\text{H}_2\text{O}$ (39). As OsO_4 is a highly toxic and quite volatile compound (0.93 kPa at 20°C) (33, 40, 41), a nontoxic alternative with similar detection potential is desired. Also, tissue decalcification is required to obtain homogeneous staining and accurate visualization of the OsO_4 -stained Ads. Moreover, homogeneous staining of the tissue might be compromised due to insufficient decalcification and high density of adipocytes (42). Additionally, post-processing of the tissue sample is limited with respect to antibody-based staining methodologies (33, 39).

To overcome these limitations, new CESAs have been introduced to the field of bone marrow adipose tissue imaging, namely the Hf-WD 1:2 POM (43, 44) and Hexabrix® (45, 46). Due to their hydrophilic nature and negative charge at physiological pH, these CESAs appear to be unable to enter Ads and instead reveal them on CECT images by increasing the gray value (GV) of the surrounding tissue. The Hf-WD 1:2 POM can differentiate between empty vasculature within the BM and other tissue constituents, which is not the case for Hexabrix®. While this might be advantageous if a researcher desires to study the vascularization of the BM, it might complicate the segmentation protocols and subsequent image analysis, since empty vessels and Ads develop similar GVs. Despite these benefits of Hexabrix® and Hf-WD 1:2 POM compared to OsO_4 in staining for visualization of applications for visualization of individual Ads, they both generate contrast by avoiding interaction with Ads. As a result, they can provide structural information but no chemical information on Ads. Finally, Lugol's iodine was evaluated in our search for a CESA that can penetrate Ads, potentially provide chemical information, and allows computation of structural properties, without the toxicity profile linked to OsO_4 . Lugol's iodine is a well-known CESA solution in diffusible-iodine contrast-enhanced computed tomography (47–49) but has not yet been studied in the context of CECT on AT. The main components of Lugol's iodine solution are molecular iodine (I_2), iodide (I^-), and triiodide (I_3^-). In the present study, we compared Lugol's iodine, Hf-WD 1:2 POM, and Hexabrix® for the staining potential of AT in murine caudal vertebrae (MCV, bone marrow adipose tissue) and bovine muscle tissue strips (BMT, intermuscular AT). To avoid osmotic effects as much as possible, we used an isotonic Lugol's iodine solution (measured osmolality = 303 mOsm, IL). Since the three evaluated CESAs could provide different information, we also evaluated whether sequential staining is possible and yields consistent results. Furthermore, the interactions of the iodine species in terms of distribution between phases and reactivity towards biomolecules were investigated. Finally, using (bio) chemical analyses on whole tissues, we investigated the potential origins of the different staining behavior of Lugol's iodine towards AT in BMT and MCV.

MATERIALS & METHODS

Tissue samples

Bovine muscle tissue. Samples of the psoas major muscle from Hereford cows between 18 and 24 months old were collected from a nearby farm (Jos Theys Boerderij, Belgium). Care was taken to consistently choose a cut along the length of the muscle at the same central location across many animals. Smaller pieces with an approximate diameter of 5–10 mm and an approximate length of 5–10 mm were used as samples.

After dissection, samples were preserved by overnight immersion in a 4% formaldehyde (FA) solution in PBS (10 mM, pH = 7.4) overnight at 4°C. Samples were put on a shaking plate during fixation and washing. After dissection, the control samples were stored in PBS at 4°C until the start of the experiment. Before the experiment, fat depots and muscle fibers were dissected for the solid-state NMR analysis.

Fat depots (n = 14) and muscle fibers (n = 9) from fresh, unfixed samples were dissected and weighted for the lipidomics analysis. Samples were stored in the −80°C freezer until initiating the lipidomics analysis.

Murine caudal vertebrae. Mice (C57BL/6J, 8-week old, female, n = 4 and male, n = 4, housed in the animal facility of the Institute of Physiology, Czech Academy of Sciences) were fed normal diet after 6 months of dietary intervention sacrificed by cervical dislocation under diethyl ether anesthesia (FGU, CAS, Prague, Lab of Dr Michaela Tencerova). Tails of mice were collected for subsequent bone analysis of the caudal vertebra. Female mice samples were used for the CECT experiments and male mice samples for the solid-state NMR experiments. After dissection, tissue samples were fixed for 24 h in a 4% FA solution in PBS and subsequently washed in PBS overnight. After washing the samples overnight, they were transferred to tubes containing fresh PBS. After initiating the experiment, the tails were stripped of the skin and the transitional vertebrae (7, 8, and 9) were collected. Samples were stored in PBS at 4°C. Muscle surrounding the bone was removed using tweezers prior to staining.

For the lipidomics analysis, caudal vertebrae were freshly harvested (n = 7) and stored in the −80°C freezer until initiating the lipidomics analysis. All experiments were performed according to the Institute of Physiology of the Czech Academy of Sciences guidelines and were approved under protocol 81/2016.

Contrast-enhancing staining agents and staining solutions

Hexabrix® was commercially available (Guerbet, 320 mgI/ml, 39.3 m/V% Ioxaglate meglumine, and 19.6 m/V% Ioxaglate sodium). The Hf-WD 1:2 POM ($K_{16}[Hf(\alpha_2-P_2W_{17}O_{61})_2] \cdot 19H_2O$) was synthesized in-house according to literature procedures (50–52). Purity was confirmed by ^{31}P NMR

(calibration of the spectrum with a 85 V% H_3PO_4 solution external reference and comparison of NMR chemical shifts with literature (51)). The IL solution was prepared by dissolving KI (12.95 g) and I_2 (6.47 g) in 500 ml MilliQ water (measured osmolality = 303 mOsm/kg). In the case of the NMR measurements, H_2O was replaced by D_2O . In what follows, if D_2O was used, it is explicitly mentioned by adding “deuterated” in front of the CESA (e.g. Deuterated IL).

Staining solutions were always prepared by diluting (i.e. IL or Hexabrix®) or dissolving (i.e. Hf-WD 1:2 POM) the CESA in PBS. The concentrations and properties of the CESAs are listed in Table 1. All stock solutions had a clear appearance. The Hexabrix® and Hf-WD 1:2 POM solutions were colorless, and the IL solution was deep brown/red. pH measurements were performed using a FiveEasy pH meter F20 (Mettler Toledo) with pH microelectrodes (InLab® Micro).

CESA staining procedure

Single staining. Prior to staining, the BMT (n = 3 per CESA) and MCV (n = 3 (transitional vertebra 7, 8, and 9) coming from one tail per CESA) samples were gently dried on a piece of paper and transferred to Eppendorf tubes containing 5 ml of the staining solution. The samples were incubated at room temperature while gently shaking, for 5 days (Hexabrix® and Hf-WD 1:2 POM) or 16 days (IL). A white precipitate was formed during the staining process in the Eppendorf tubes containing the Hf-WD 1:2 POM. After image acquisition, one sample per CESA was washed in PBS for 20 days, followed by image acquisition.

Sequential staining. Prior to staining, the BMT (n = 3) and MCV (n = 3 (transitional vertebra 7, 8, and 9) coming from the same tail per CESA) samples were gently dried on a piece of paper and transferred to Eppendorf tubes containing 5 ml of the staining solution. The following order of staining-washing cycles was applied to all samples: Hexabrix® (5 days) → Wash PBS (3 days) → Hf-WD 1:2 POM (5 days) → Wash PBS (3 days) → IL 16 days → Wash PBS (3 days). After each step, CECT data was acquired. Also, in these Eppendorf tubes containing the Hf-WD 1:2 POM, a white precipitate was formed during the staining process.

MicroCT image acquisition and reconstruction

Image acquisition was performed using a Phoenix Nano-Tom M (GE Measurement and Control Solutions, Germany),

TABLE 1. Overview of CESA and staining solution properties

CESA	Molecular weight (g/mol)	Heavy atom	Charge
Hexabrix®	1,291 (Na^+)/1,463 (NMDG $^+$)	Iodine (I)	−1
Hf-WD 1:2 POM	9,473	Tungsten (W) and Hafnium (Hf)	−16
Lugol's iodine	• I_2 : 253.8 • KI: 166.0 • KI_3 : 419.8	Iodine (I)	• I_2 : 0 • I^- : −1 • I_3^- : −1
CESA	pH of solution (in PBS)	Concentration (m/V% or V/V%)	Concentration (mmol/L)
Hexabrix®	7.1	20 V/V%	84
Hf-WD 1:2 POM	6.7	3.5 m/V%	3.69
Lugol's iodine	7.4	• I_2 : 0.65 m/V% • KI: 1.29 m/V%	• I_2 : 25.5 • KI: 77.9

In all cases, the solvent is PBS and the total volume of the solution is 5 ml. Charge value assumes full dissociation and concerns the heavy atom(-containing ion). Note that if the preparation of staining solution requires dilution of a CESA with PBS, the PBS itself is also diluted. The final molarity of PBS in IL = 5 mM and in Hexabrix = 8 mM.

NMDG $^+$, N-methyl-D-glucamine cation.

TABLE 2. CT data acquisition parameters

Parameter	Fast acquisition mode – stained	Fast acquisition mode – washed
Focus-detector distance	350 mm	350 mm
Focus-object distance	7 mm	7 mm
Voxel size	2 μ m	2 μ m
Tube voltage	70 kV	70 kV
Tube current	120 μ A	120 μ A
Focal spot size	1.99 μ m	1.99 μ m
Mode	0	0
Filter	0.1 mm Al	0.1 mm Al
Exposure time	500 ms	500 ms
Number of images	2,000	1,500
Averaging	1	1
Skip	0	0
Scan duration	16 min 40 s	12 min 30 s

with a diamond-coated tungsten target. Before image acquisition, the samples were removed from the Eppendorf tube, gently dried using a piece of paper, and wrapped in parafilm. A reference material (Al_2O_3 , ceramic bead) was added on top of the parafilm. Acquisition parameters can be found in Table 2. The reconstruction was performed using Datasx GE Measurement and Control Solutions software (version 2.7.0 – RTM) where the image was cropped to reduce the size of the dataset and the inline median, ROI CT filter, and Filter volume algorithms, implemented in the software, were applied.

Image processing and analysis

Automatic histogram windowing and GV normalization. One reconstructed dataset of an IL-stained MCV (single staining) was converted from 16 bit Tiff files to 8 bit bmp files with automatic histogram windowing. Based on the GVs of the

reference materials in this dataset, a GV normalization was performed on all other datasets (washing, single, and sequential staining), which allowed for quantitative comparison of GVs. Both operations were performed using in-house developed Matlab (MATLAB R2021b) scripts (53).

Gray value measurements. Gray value measurements were performed using Fiji software (54). Where possible, the rectangle tool was used to draw areas where the mean GV was recorded; if not, the segmented line tool was applied. For Ad GV measurements, the GVs of 10 Ads were recorded in random locations per sample. Five regions were recorded per sample for all other tissue constituents (muscle fibers and bone). Table 3 shows an overview of the measured GVs with their respective standard deviations. Note that upon staining with Hf-WD 1:2 POM, the outer edge of the bone is characterized by very high GVs relative to the rest of the bone (Fig. 1A, yellow arrow) and was excluded during the GV measurements.

Volume analysis of sequentially stained MCV constituents. Volume analysis of bone, AT, BM, and vasculature and the total sample volume were performed on the normalized 8 bit bmp datasets of the sequential stained MCV samples. All manipulations were performed using Avizo (version 2021.1, Thermo Fisher Scientific). The dataset was filtered using a nonlocal means filter (Spatial standard deviation = 5, Intensity standard deviation = 0.2, Search window = 10 px, Local Neighborhood = 3 px). Then, the vertebra was segmented from background by using a series binarization of the bone, followed by dilation of the bone and filling of the binary mask. An erosion module was applied to reduce the size of the mask to the size of the filled vertebra. The GV thresholds

TABLE 3. GVs of Ads and other tissue constituents after staining following the single staining protocol or the sequential staining protocol, GVs for the same samples after a washing step and the controls (untreated samples)

	Single staining protocol		Sequential staining protocol	
	GV Ads	GV Muscle fiber	GV Ads	GV Muscle fiber
CESA (BMT)				
Hexabrix	24.8 $\pm$ 1.2	52.3 $\pm$ 1.3	26.8 $\pm$ 1.8	55.1 $\pm$ 3.7
Hf-WD 1:2 POM	25.8 $\pm$ 1	76.1 $\pm$ 1.2	30.7 $\pm$ 1.6	72.9 $\pm$ 1.9
IL	67.8 $\pm$ 5.8	147.4 $\pm$ 6.1	84.1 $\pm$ 4.7	174.9 $\pm$ 8
CESA (MCV)				
Hexabrix	30.2 $\pm$ 1.7	92 $\pm$ 1.9	26.5 $\pm$ 1.6	85.3 $\pm$ 1.9
Hf-WD 1:2 POM	33.1 $\pm$ 1.1	86.6 $\pm$ 3.7	28.1 $\pm$ 1.7	84.4 $\pm$ 2.6
IL	207 $\pm$ 11.9	111.6 $\pm$ 2.2	226.5 $\pm$ 3	110.6 $\pm$ 4.8
	Washing (single staining, n = 1)		Washing (sequential staining)	
	GV Ads	GV Muscle fiber	GV Ads	GV Muscle fiber
CESA (BMT)				
Hexabrix	16 $\pm$ 3.3	34.2 $\pm$ 2.1	27.4 $\pm$ 0.3	31.4 $\pm$ 0.8
Hf-WD 1:2 POM	28.9 $\pm$ 2.9	62.6 $\pm$ 1.5	28.4 $\pm$ 1	57.5 $\pm$ 2.9
IL	68.1 $\pm$ 3.5	105.1 $\pm$ 5.7	59.1 $\pm$ 4.8	85.5 $\pm$ 23.8
CESA (MCV)				
Hexabrix	27.5 $\pm$ 1.3	89.04 $\pm$ 2.3	29.7 $\pm$ 1.1	98.8 $\pm$ 0.8
Hf-WD 1:2 POM	34 $\pm$ 1.3	98.4 $\pm$ 2.5	30.4 $\pm$ 0.7	84 $\pm$ 0.7
IL	145.3 $\pm$ 2.2	91.2 $\pm$ 2.7	140.6 $\pm$ 10	89.7 $\pm$ 1.6
Controls (n = 1)				
BMT	26.55 $\pm$ 0.98	30.6 $\pm$ 0.12	/	/
MCV	29.6 $\pm$ 1.2	/	/	80.3 $\pm$ 1.1

All conditions, except washing (single staining) and control samples, were performed in triplicate. For the conditions with n = 1 (indicated in the table), the given SD is the deviation within the sample.

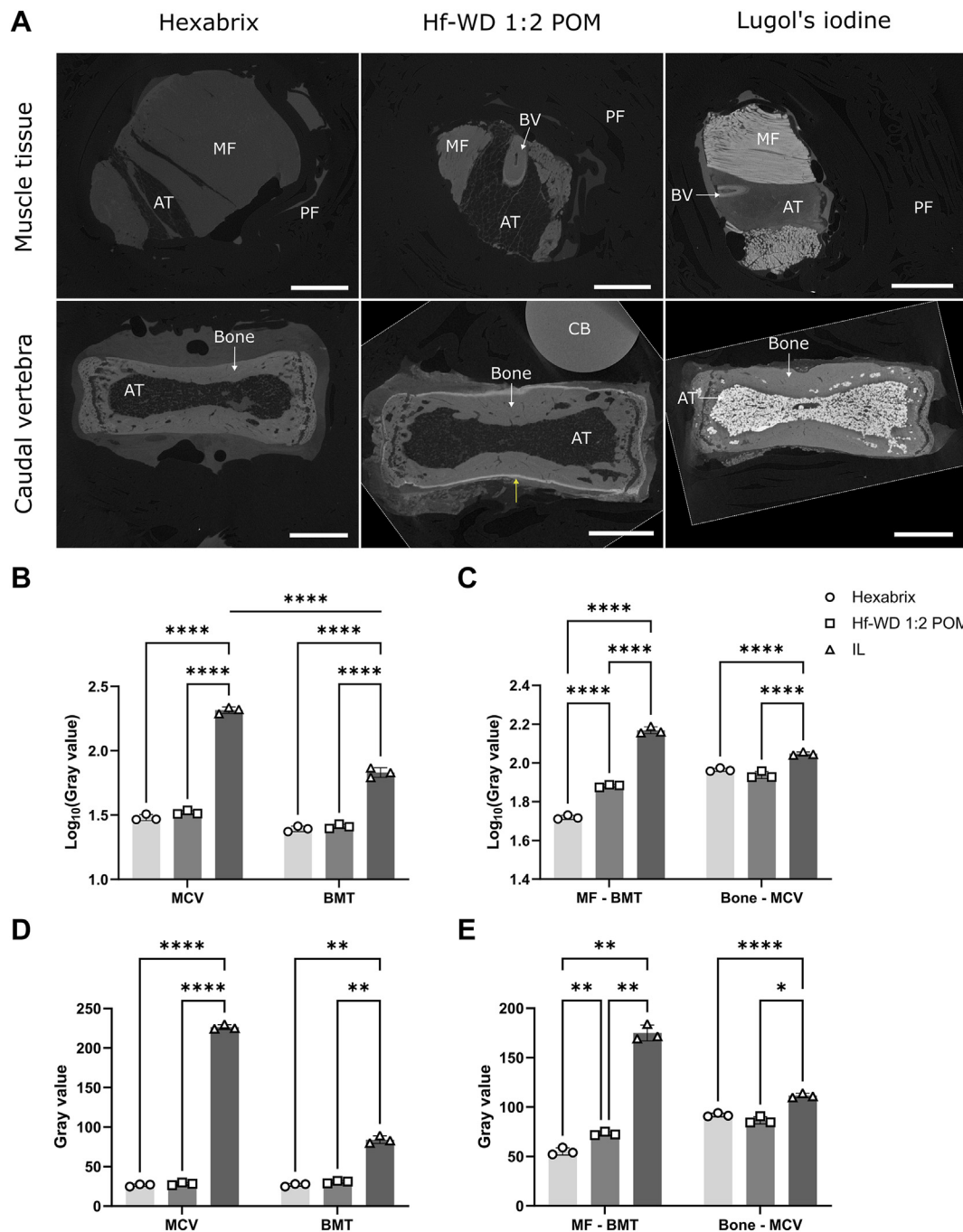


Fig. 1. Comparison of staining behaviour of the evaluated CESAs (single and sequential staining). A: Representative CECT images of BMT and MCV, stained with the three CESAs (single staining protocol). Scale bar = 1 mm. B: Comparison of adipocyte gray values between the CESAs per species (BMT & MCV, single staining). Two-way ANOVA followed by Tukey's multiple comparison test ($n = 3$, $\alpha = 0.05$). C: Comparison of gray values of other tissue constituents present in the samples between the CESAs per tissue (single staining). Two-way ANOVA followed by Tukey's multiple comparisons test ($n = 3$, $\alpha = 0.05$). D: Comparison of adipocyte gray values between the CESAs per species (sequential staining). Two-way ANOVA with repeated measures followed by Tukey's multiple comparison test ($n = 3$, $\alpha = 0.05$). E: Comparison of gray values of other tissue constituents present in the samples between the CESAs per tissue (sequential staining). Two-way ANOVA with repeated measures followed by Tukey's multiple comparisons test ($n = 3$, $\alpha = 0.05$). AT, adipose tissue; BV, blood vessel; CECT, contrast-enhanced computed tomography; CESA, contrast-enhancing staining agent; CM, ceramic bead; MF, muscle fiber; PF, parafilm. * P -value ≤ 0.05 , ** P -value < 0.01 , and *** P -value < 0.0001 .

were applied to segment the bone and BMAd. These thresholds were unique for each CESA (Table 4) due to their variety in staining behavior. Next, a despeckle module (3D kernel size: 7 px $\times$ 7 px $\times$ 7 px) was applied to the binary

masks to remove leftover noise. If the vessel, present in the vertebrae, was segmented with the bone due to overlapping GVs, it was removed before volume analysis. Finally, the volumes were determined using the label analysis module.

TABLE 4. Ratios of AT volume with respect to bone volume, total vertebra volume, and bone marrow (including vasculature) volume

CESA	AT/Total	AT/Bone	AT/BM& Vasculature
Hexabrix	0.177 ± 0.00896	0.281 ± 0.0222	0.932 ± 0.0769
Hf-WD	0.195 ± 0.017	0.292 ± 0.0384	1.479 ± 0.0781
1:2 POM			
IL	0.177 ± 0.019	0.252 ± 0.0291	1.521 ± 0.325

(Bio)Chemical analyses

Total reflection X-ray fluorescence. Concentrations of the element iodine (I) were determined using total reflection X-ray fluorescence (TXRF) spectroscopy (S4 T-STAR, Bruker). The tungsten L-line was used. The machine was operated at 60 kV and 600 μ A. Acquisition parameters were the following: excitation: W_L 8.5, atmosphere = N₂, acquisition time = 500s, cycles = 100, deconvolution = Profile bayes (optimized), step width = 50, maximum cycles = 1,000. The measured values deviated slightly from the real values and hence a calibration curve was used with barium as an internal standard to compute the correct ppm values (supplemental Fig. S1A). Sample preparation was performed as follows. TXRF sample holders were coated with SERVA solution (25 μ l) and dried in an oven (60°C) for at least 15 min. Solutions to be measured were prepared by combining the following: 5 μ l sample solution (1-octanol) or 3 μ l sample solution (octanoic acid) + 50 μ l Ba standard solution (1,000 ppm/ml) + 945 μ l or 947 μ l of stock solution (Milli-Q water + 5% Triton-X 100 + 5% NH₃). Prepared samples were homogenized by using the vortex machine. Two microliters of the prepared samples were added to the center of the TXRF sample holder, followed by drying in the oven (60°C) for exactly 30 min, after which the samples were ready to be measured. For each measurement, technical replicates (n = 4) were used. The measurement was acceptable if the coefficient of variation was below 5%. If an outlier was identified, based on Dixon's Q test, it was discarded from the dataset.

To determine the distribution of iodine between two phases over time (supplemental Fig. S1B), the IL stock solution was diluted (1:1 ratio) with PBS in glass vials to obtain a total volume of 1.5 ml. To this vial, the hydrophobic phase was added (1-octanol or octanoic acid, 1.5 ml). Automatic vigorous shaking (speed 1,500 rpm) was applied to accelerate the distribution of iodine species between both phases. Prior to taking aliquots, the glass vial was centrifuged (1 min, 3,000 rpm, 2,084 g) to enforce clear phase separation (no entrainment visible anymore). Aliquots of 30 μ l from the aqueous phase were collected immediately after the addition of a stock solution of IL (i.e. after one manual shake), after 5 min + 1 min centrifugation, 10 min + 1 min centrifugation, 20 min + 1 min centrifugation, 30 min + 1 min centrifugation, and 45 min + 1 min centrifugation. Sample preparation and measurements were performed as described above.

Knowing the minimal time required to reach equilibrium, two groups of four glass vials were prepared containing IL diluted with PBS (1:1 ratio, 1.5 ml total volume). To the first group, 1-octanol (1.5 ml) was added. To the second group, octanoic acid (1.5 ml) was added. The first vial of each group was used as a control vial. The other three vials were charged with L-glycine (10.3 mg, 91.5 mM considering only aqueous phase), Hf-WD 1:2 POM (35 mg/ml, 3.69 mM), or palmitic acid (34 mg, 88.4 mM considering only organic phase), respectively. Automatic vigorous shaking was applied for 1 h. Sample preparation and measurements were performed as described above.

UV-VIS measurements. UV-VIS measurements were performed to investigate the nature of iodine species in the aqueous or organic phase (i.e. 1-octanol and octanoic acid). An Agilent Cary 60 UV-Vis spectrophotometer recorded absorption spectra in quartz cuvettes. Blank spectra were recorded with the neat solvent. Interpretation of the spectra was only attempted in the region where the absorbance of the solvent is smaller than 1. The following dilutions were applied to the samples: PBS phases were diluted 201 times to acquire information on I₃⁻ and 4,221 times to acquire information on I⁻. The octanol phase was diluted 201 times and the octanoic acid phase was diluted 101 times. Stock solutions of I₂ in 1-octanol (19.4 mM) and octanoic acid (21.3 mM) were prepared before initiating the experiment.

Nuclear magnetic resonance.

Liquid-state NMR: Iodination of Amino Acids. Three glass vials were charged with either L-tyrosine (0.02 mmol, Sigma Aldrich, BioUltra $\geq$ 99%), L-phenylalanine (0.02 mmol), or L-histidine (0.02 mmol, Sigma Aldrich, BioUltra, $\geq$ 99.5% (NT)) dissolved in a PBS/IL (3 ml total, 1:1, in D₂O) mixture. Note that not all L-tyrosine was dissolved. Hence, prior to performing the experiment, the stock solution of L-tyrosine in deuterated PBS was centrifuged and the supernatant was used. We assumed a maximal 2.5–3 mM concentration in PBS at pD 7.66 and at room temperature (55). The pD was measured each time when samples for NMR measurements were taken, using a FiveEasy pH meter F20 (Mettler Toledo) with pH micro electrodes (InLab® Micro). Before acquiring NMR spectra, the signal-to-noise ratio was confirmed to be high enough (>150) for correct quantitative measurements.

The effect of the presence of Hf-WD 1:2 POM on the reaction between IL components and L-tyrosine was investigated. For this experiment, we also used the same stock solution of L-tyrosine in deuterated PBS and assumed a maximal concentration of 2.5–3 mM. Control samples did not contain Hf-WD 1:2 POM, while other samples contained Hf-WD 1:2 POM (17.5 mg, 0.002 mmol, 0.5 ml total volume). Prior to initiating the measurement, deuterated IL (250 μ l) was added to the solution.

¹H NMR spectra were recorded over time (between one and five days) on a Bruker Avance II+ HD 600 (probe: BBO 600S3 BB-H-D-05 Z BTO, basic transmitter frequency: 600.13 MHz) spectrometer with the following parameters: NS = 32, pulse angle = 30°, D1 = 6 s. All NMR spectra were analyzed using TopSpin (version 4.1.3). Before integration, spectra were processed using zero-filling (512k), automatic phase correction, and automatic baseline correction. If spectra were not well-phased automatically, manual phase correction was performed.

Iodination of Palmitoleic Acid by IL in PBS or by I₂ in Dichloromethane. Small-scale (0.02 mmol palmitoleic acid) reactions were used to determine the I₂ incorporation over time in palmitoleic acid. Four glass vials were prepared: three containing PBS (0.25 ml, pH = 7.3) and one containing dichloromethane (DCM, 0.5 ml), followed by the addition of palmitoleic acid. IL (0.25 ml) was added to the vials containing PBS, and to the vial containing DCM, a molar excess of I₂ (6.6 mg, 0.026 mmol) was added, followed by vigorous stirring at room temperature. Reactions were stopped by the addition of a saturated sodium thiosulfate (Na₂S₂O₃) followed by 5 min of vigorous stirring, after 30 min, 1 h, and 3 h in the case of the PBS samples and after 3 h for the DCM sample. ¹H NMR spectra were acquired on a Bruker Avance III 400 MHz

spectrometer (probe: BBO 400S1 BBF-H-D-05 2 SP, basic transmitter frequency: 400.17 MHz, NS = 32, D1 = 5 s, pulse angle = 30°). NMR spectra were analyzed using TopSpin (version 4.1.3). Before integration, spectra were processed using zero-filling (512k), automatic phase correction, and automatic baseline correction. If spectra were still not phased well, manual phase correction was performed. To determine the conversion of the starting material, the terminal $-\text{CH}_3$ signal was integrated with respect to the $-\text{CH}=\text{CH}-$ signal.

A large-scale (0.105 mmol palmitoleic acid) reaction was performed to obtain high-resolution NMR spectra. IL was added to a glass vial filled with PBS (1 ml, pH = 7.3) and palmitoleic acid, followed by vigorous stirring. The reaction was stopped by adding saturated $\text{Na}_2\text{S}_2\text{O}_3$ solution after 3 h followed by 5 min of stirring. Before acquiring NMR spectra, an extraction step with DCM was performed. Finally, DCM was not completely removed ([supplemental Fig. S5](#)) before the sample was dissolved in CDCl_3 . High-quality 2D (HSQC-TOCSY and DOSY) spectra were acquired on Bruker Avance Neo 600 MHz spectrometer (probe: PI HR-TBO600S3-BBF/H/F/D-5.0-Z FB, basic transmitter frequency: 600.35 MHz).

Solid-state NMR: High-resolution Magic Angle Spinning. MCV and BMT control samples were randomly divided into two groups ($n = 4$ per tissue). The control samples were stored in deuterated PBS (3 ml, D_2O , pD = 7.66). The other samples ($n = 3$ per tissue) were stored in a 1:1 mixture of deuterated IL/PBS (1.5 ml/1.5 ml, D_2O) for two days in the fridge at 4°C. The solutions were refreshed after two days of incubation, to maximize the exchange of H_2O with D_2O (total time in solution was four days). Before performing the high-resolution magic angle spinning (HR-MAS) measurements, the samples were incubated for one minute in a solution of $\text{Na}_2\text{S}_2\text{O}_3$ in D_2O to remove residual I_2 adsorbed on the tissue surface and prevent damage to or discoloration of the rotors. Immediate removal of the brown colour on the tissues was observed. Acquisition of ^1H NMR spectra was performed on a Bruker Avance II+ HD 600 spectrometer using 4 mm rotors, a spinning frequency of 5 kHz, calibration of the magic angle was performed using KBr, temperature was controlled at 25°C, $n_s = 64$, pulse angle = 30°. NMR spectra were analyzed using TopSpin (version 4.1.3). Before integration, spectra were processed using zero-filling (512k), automatic phase correction, automatic baseline correction, and zero-filling. If spectra were still not phased well, manual phase correction was performed. Integration was performed by peak-picking relevant peaks and computing the full-width at half maximum. Then two times, the full-width at half maximum was added to and subtracted from the peak value, defining the integration range.

Liquid-chromatography mass spectrometry lipidomics. Global lipidomic profiling of dissected adipose tissue and muscle fibers from BMT and whole MCV samples was conducted using a biphasic solvent system of methyl *tert*-butyl ether and 10% methanol in water ([56](#)), followed by liquid chromatography-mass spectrometry (LC-MS) analysis.

An amount of 18–35 mg of each sample was homogenized (1.5 min) with 275 μl of methanol containing internal standards using a grinder. Then, 1 ml of methyl *tert*-butyl ether with an internal standard was added and shaken (30 s). Finally, 275 μl of 10% methanol with internal standards was added, and after vortexing (10 s), the tubes were centrifuged (16,000 rpm, 5 min, 4°C). An aliquot of 10 μl of the upper phase was collected and evaporated for adipose tissue samples. The dry extracts were resuspended in 1 ml of methanol containing

an internal standard (CUDA), shaken (30 s), centrifuged (16,000 rpm, 5 min, 4°C), and used for the analysis of high abundant triacylglycerol (TG) species using LC-MS analysis. An aliquot of 100 μl of the upper phase was collected and evaporated for muscle fiber samples. The dry extracts were resuspended in 100 μl of 80% methanol containing an internal standard (CUDA), shaken (30 s), and centrifuged (16,000 rpm, 5 min, 4°C). An aliquot of 100 μl of the upper phase was collected and evaporated for muscle fiber samples. The dry extracts were resuspended in 300 μl of methanol containing an internal standard (CUDA), shaken (30 s), centrifuged (16,000 rpm, 5 min, 4°C). An aliquot of 100 μl of the upper phase was collected and evaporated for caudal vertebra samples. The dry extracts were resuspended in 400 μl of methanol containing an internal standard (CUDA), shaken (30 s), and centrifuged (16,000 rpm, 5 min, 4°C).

The LC-MS system comprised a Vanquish UHPLC system (Thermo Fisher Scientific, Bremen, Germany), a heated electrospray ionization (HESI-II) probe (Thermo Fisher Scientific), and a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) ([56](#)). The ACQUITY Premier BEH C18 column (50 mm $\times$ 2.1 mm i.d.; 1.7 μm particle size) equipped with a VanGuard FIT cartridge (5 mm $\times$ 2.1 mm i.d.; 1.7 μm particle size) (Waters) was used to separate complex lipids. For lipidomic analysis in positive ion mode, the mobile phase included (A) 60:40 acetonitrile/water with 10 mM ammonium formate and 0.1% formic acid and (B) 90:10:0.1 isopropanol/acetonitrile/water with 10 mM ammonium formate and 0.1% formic acid. For lipidomic analysis in negative ion mode, the mobile phase included (A) 60:40 acetonitrile/water with 10 mM ammonium acetate and 0.1% acetic acid and (B) 90:10:0.1 isopropanol/acetonitrile/water with 10 mM ammonium acetate and 0.1% acetic acid. Other LC-MS parameters can be found in the following references ([45](#), [57](#)).

The LC-MS instrumental files were processed using MS-DIAL v. 4.8 ([58](#)). Complex lipids were annotated using in silico MS/MS spectra available in MS-DIAL software. Exported data sets for each matrix and platform as signal intensity from the detector (peak heights) were normalized using the amount of sample, injection volume, and extraction aliquot to provide comparable data among all matrices. The interest of the study was mainly in the AT of both samples; hence, the analysis of the muscle fibers from BMT served as a control of proper dissection of the BMT AT ([supplemental Fig. S2](#)).

Statistical analyses

All statistical evaluations were performed using JMP (JMP® Pro, version 16.0.0), RStudio (version 2023.03.0), and GraphPad (GraphPad Prism, version 9.3.1). Normality and homoscedasticity were evaluated using the respective plots and, if possible, the tailored statistical tests (e.g. Shapiro-Wilk test, Levene's test). To ensure that we meet the normality and homoscedasticity assumptions of the used statistical tests, we sometimes had to transform the data using a logarithmic (base 10) transformation. In all figures, error bars represent the standard deviation of the triplicates unless stated otherwise. For all statistical tests, we used $\alpha = 0.05$, not significant (no star): P -value > 0.05 , * P -value ≤ 0.05 , ** P -value < 0.01 , *** P -value < 0.001 , **** P -value < 0.0001 .

RESULTS

The CESAs show similar staining patterns when comparing single and sequential staining protocols

To evaluate the affinity of the three CESAs (i.e. Hexabrix (5 days staining), Hf-WD 1:2 POM (5 days

staining), and IL (16 days staining)) for distinct tissue constituents (Ads, muscle fibers, and bone) in both BMT and MCV, GVs were statistically compared on normalized GV images (Fig. 1), using both single and sequential staining protocols. In general, similar staining patterns were observed when comparing the single and sequential staining protocols. Note that the GVs had to be log₁₀-transformed in the case of single staining, due to the heteroscedasticity of the data, while this was not the case for the sequential staining group.

Staining the samples with Hexabrix® and Hf-WD 1:2 POM did not lead to an increased GV of the Ads compared to an unstained sample. However, the GVs of Ads were significantly different between species (i.e. MCV and BMT) in the case of single staining, but not in the case of sequential staining (Fig. 1B, D). Next, IL staining significantly increased the GV of Ads within both BMT and MCV, in comparison with Hexabrix®- and Hf-WD 1:2 POM-stained samples and showed different GVs between the BMT and MCV samples with both staining protocols.

The CESAs interacted differently with the other tissue constituents (bone for MCV and muscle fiber for BMT), showing the same trends for both staining protocols (i.e. single and sequential staining, Fig. 1C, E). Within BMT, visually, all CESAs were able to increase the GV of muscle fibers, with Hexabrix showing a smaller increase, compared to IL and Hf-WD 1:2 POM. IL showed the highest GV for the muscle fiber. Within MCV, IL seemed to increase the GV of the bone.

GV analysis reveals small, but consistent differences between staining protocols

While similar staining patterns were observed for both single and sequential staining protocols, it remains important to pinpoint whether significant differences in GVs for Ads or other tissue constituents could be detected. To perform this comparison statistically, a repeated measures mixed model was required to estimate the relevance of each parameter evaluated. The model was composed of the following main effects: CESA (i.e. Hexabrix, Hf-WD 1:2 POM, or Isotonic Lugol), tissue (i.e. MCV or BMT), and staining protocol (i.e. single or sequential staining) and their respective statistical interaction effects (i.e. the influence of main effects on each other, e.g. CESA*Tissue).

The analysis revealed a significant dependency of the CESA and tissue but no significant effect of the staining protocol on the measured GV (Fig. 2B and supplemental Table S1). Further, the outcomes of Hexabrix staining and Hf-WD 1:2 POM staining were independent of the applied protocol, since no statistical interactions were found between these CESAs and the staining protocol. In the case of Hexabrix, this highlights the experiments' reproducibility, since Hexabrix was the first CESA applied in the sequential staining protocol and should hence give the same outcome as the single staining protocol. In the case of the Hf-WD

1:2 POM, this indicates that prior Hexabrix staining does not affect the inability of the Hf-WD 1:2 POM to penetrate the Ad. However, the statistical interaction between the staining protocol and the GV obtained by IL staining was significant, indicating that IL staining was affected by the prior staining with Hf-WD POM or Hexabrix in the sequential staining process. A significant statistical interaction between the staining protocol and the tissue was found, showing that the staining process was impacted differently by the staining protocol for BMT compared to MCV. Specifically, the GV difference between the single and sequential staining protocol for BMT Ads was larger upon IL staining than for the MCV Ads. Finally, a significant statistical interaction was found between all CESAs and the tissue, revealing that the GV of the Ads is inherently lower in BMT compared to MCV for all CESAs evaluated.

To determine the effect of the investigated parameters (i.e. CESA, staining protocol and their statistical interaction) on the observed GV of the other tissue constituents (i.e. muscle fiber and bone), we also used a repeated measures mixed model, but separately for BMT and MCV (supplemental Table S2). Both the main effects of CESA and staining protocol were significant for the BMT muscle fibers, with the CESA having the highest estimated influence on the GV. Next, the statistical interaction between the staining protocol and the CESA showed a significant GV difference between single and sequentially IL-stained BMT muscle fiber samples (Fig. 2C). For the MCV bone, the CESA significantly influenced on the GV, revealing that the bone of the MCV samples stained with IL had higher GVs. The staining protocol had a borderline significant influence (*P*-value of the main effect = 0.0262) on the GV. Further investigation is required to verify this influence.

Next, we again used a mixed model analysis to investigate the effect of washing by simply incubating the tissue samples in PBS (10 mM) on the measured GV (Fig. 2D, E and supplemental Tables S3 and S4). It was found that only Ads stained with IL showed significantly decreased GVs upon washing the sample with PBS. This is in agreement with the fact that Hf-WD 1:2 POM and Hexabrix do not interact with Ads and subsequently do not increase their attenuation, reaffirming an inherent AT GV difference between both species. Within the BMT muscle fibers, a significant decrease in GV was observed after washing for Hexabrix- and IL-stained samples, while no significant removal of the Hf-WD 1:2 POM from the muscle fibers was observed. Finally, no significant differences were observed in the MCV bone upon washing the samples. Hence, significant differences observed concerning the GV measurements of the bone should be considered with care.

Volumetric analyses of MCV bone, BM & vasculature, and BMAds showed a clear correlation between total amount of AT and the total vertebra size for all

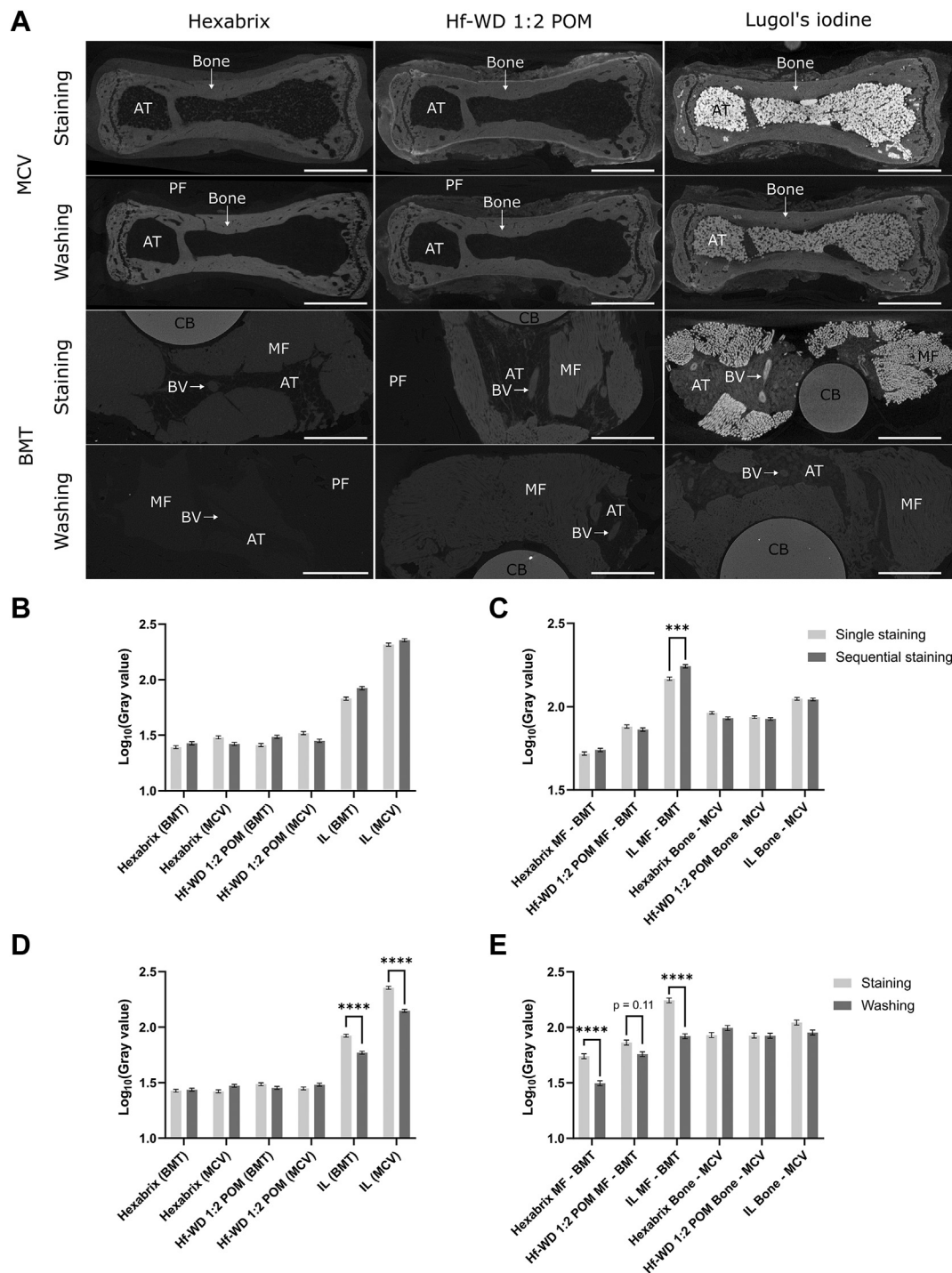


Fig. 2. Comparison between single and sequential staining protocols and the effect of washing. A: Representative CECT images of bovine muscle tissue and murine caudal vertebrae during the staining-washing cycles. Scale bar = 1 mm. B: Comparison of adipocyte gray values between single and sequential staining per CESA and tissue. C: Comparison of gray values of other tissue types present in the samples between single and sequential staining per CESA and tissue. D: Comparison of gray values of adipocytes during staining-washing cycles per CESA and tissue. E: Comparison of gray values of other tissue types present in the samples during staining-washing cycles per CESA and tissue. Plotted values on graphs are based on least squares (LS) means and standard error (SE) derived from the repeated measures mixed models ($n = 3$, $\alpha = 0.05$). Significance stars are based on Tukey's multiple comparisons test on the LS means and SE's on the means. AT, adipose tissue; BV, blood vessel; CECT, contrast-enhanced computed tomography; CESA, contrast-enhancing staining agent; CM, ceramic bead; MF, muscle fiber; PF, parafilm.

CESAs ($r_p = 0.87$, P -value = 0.0021, 95% CI [0.49, 0.97], [supplemental Fig. S3](#)), which is in agreement with literature (27). Therefore, instead of absolute volumes,

relative volumes were compared ([Table 4](#)). The ratios between AT volume with respect to bone volume was smallest for IL and similar for Hexabrix and Hf-WD 1:2

POM, potentially indicating shrinkage of the IL-stained Ads.

The ratios between AT volume with respect to BM (including vasculature) volume showed a higher ratio for Hf-WD 1:2 POM and IL than Hexabrix, potentially because of a partial volume effect (PVE). While Hf-WD 1:2 POM and Hexabrix are both unable to increase the GV of the Ads, we visually see that the BM surrounding the Ads is less clear in the Hf-WD 1:2 POM sequentially stained sample (Fig. 2A). However, potential shrinkage and PVE are confounded within this experiment and additional experiments are required to estimate the effect of both parameters.

Breaking down the IL staining process into simplified model systems

IL was found to be the only CESA able to increase the GV of Ads in both BMT and MCV and with both staining protocols, albeit in a different way. To investigate the staining behavior of IL more in-depth, we performed several follow-up in vitro experiments. Reactivity was evaluated between iodine components of the IL solution and the following amino acids (AAs): L-tyrosine (Y), L-histidine (H), and L-phenylalanine (F), under staining conditions using ^1H -NMR measurements (Fig. 3A and supplemental Fig. S4). Full conversion of Y towards the diiodinated product was observed, while only partial conversion towards the monoiodinated product was observed for H. No iodinated derivatives were observed for F. The reaction between iodine species and Y or H ceased after one day and was accompanied by an acidification of the medium (Fig. 3B) as expected from the reaction mechanism in which a H^+ is replaced by an I^+ on the aromatic ring (59). Finally, it was also shown that the kinetics of the iodination of the conversion of Y towards iodinated derivatives were decreased in the presence of Hf-WD 1:2 POM (Fig. 3C).

Additionally, the reactivity of iodine species towards palmitoleic acid was determined with NMR (supplemental Fig. S5). In the biphasic system, approximately 25% of the palmitoleic acid had converted to new products and no difference was observed between 30 min, 1 h, and 3 h. Hence, similar to the AAs, this seems to be a rather quick process. Noteworthy is that the conversion after 2 h of reacting with a molar excess of I_2 in a monophasic (DCM) system was approximately 20%.

Prior to TXRF analysis, the inertness of the hydrophobic phases (i.e. 1-octanol and octanoic acid) in the biphasic systems was confirmed through ^1H - or ^{13}C -NMR (supplemental Fig. S6). TXRF measurements showed that iodine species obtained higher concentrations in the aqueous phase in all studied systems (Fig. 3D). Perturbation of the system (addition of Hf-WD 1:2 POM, L-glycine or palmitic acid) did not significantly affect the distribution of iodine species. Iodine species obtained consistently higher

concentrations in 1-octanol than octanoic acid. Identification of iodine species in the unperturbed systems was achieved through UV-VIS measurements (Fig. 3E–H and supplemental Fig. S3 – PBS samples). The spectra show that in all cases, I_2 could be identified as the main component of the organic phases (Fig. 3G, H), while the aqueous phase mainly contains I^- and I_3^- (Fig. 3E, F) (60). In the case of octanoic acid, a higher concentration of I_3^- was found in the aqueous phase, in agreement with the higher concentration of iodine species as determined by TXRF. Given the relative intensity of the I^- and the I_3^- bands (taking into account the dilution factors) and that considering the extinction coefficient of I_3^- is higher compared than for I^- , we can conclude that in both cases, the main component of the aqueous phase is I^- after equilibrium was reached (60, 61).

The results showed that passive distribution of iodine species, reactions with Y and H residues, and reactions with unsaturated fatty acids, among others, contribute to the increase in GV. These processes depend on the chemical environment and physical parameters of the system. However, only iodination of Y was found to achieve full conversion. None of the other processes resulted in complete conversion or unidirectional movement of iodine species. The GV of protein-rich constituents, like muscle fibers, is among others determined by reactions with Y and H residues. In fatty acid-rich constituents, such as Ads, the GV is, among others, a result of the dissolution of hydrophobic I_2 and subsequent reactions with unsaturated FAs.

Investigation of the IL staining process by HR-MAS and lipidomics analysis

To further investigate the hypothesis that the amount of unsaturated fatty acids could play a role in the final GV of Ads (in BMT and MCV) after IL staining, we evaluated the degree of saturation of the AT in both tissues in two independent ways (Fig. 4): LC-MS-based lipidomics analysis and HR-MAS solid-state nuclear magnetic resonance (ssNMR) analysis. Using HR-MAS, we investigated whether an actual reaction between unsaturated double bonds and I_2 was observed or whether the GV was mainly linked to the solubility of I_2 in the hydrophobic phase.

Through principal component analysis, the LC-MS-based lipidomics analysis revealed a clear distinction between the Ads originating from BMT or MCV (Fig. 4A). Evaluating the sum of lipids from lipid classes revealed that BMT were consistently richer in free fatty acids, TGs, and oxidized triglycerides, while the MCV contained a plethora of other, more complex lipid classes. Partial least squares discriminant analysis further showed that the lipid species, obtaining high variable importance in the projection (VIP) scores, were saturated, mono-, di-, and tri-unsaturated in the BMT samples. In contrast, higher degrees of unsaturation were found in the MCV samples (supplemental Figs. S7

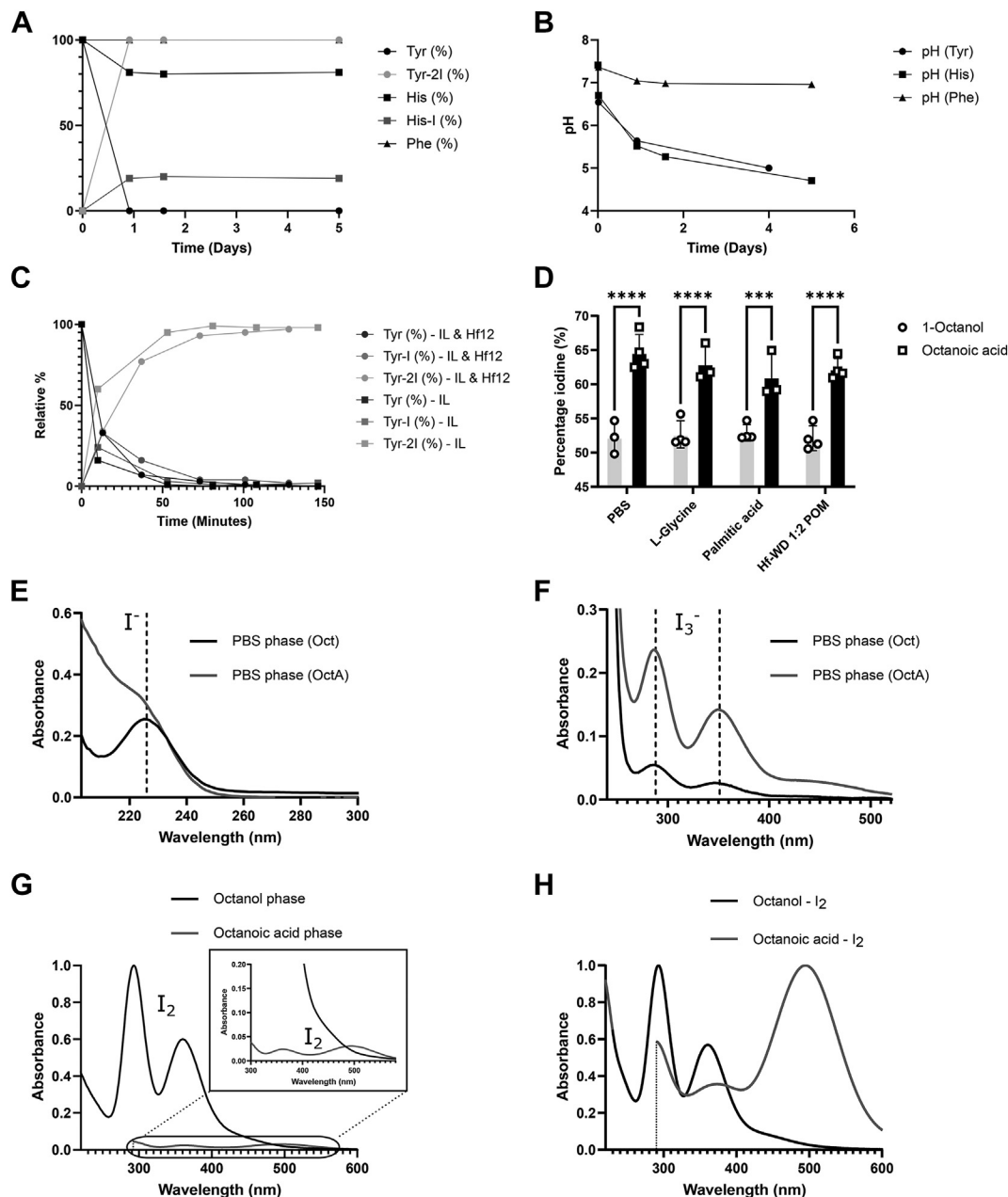


Fig. 3. Breaking down IL into simplified model systems. A: Iodination of L-tyrosine, L-histidine, and L-phenylalanine over time expressed as a relative percentage of each product in the NMR tube. B: Change of pH over time during the iodination of L-tyrosine, L-histidine, and L-phenylalanine. C: Iodination of L-tyrosine with and without Hf-WD 1:2 POM expressed as a relative percentage of each product in the NMR tube. D: TXRF-based evaluation of the percentage iodine species in the aqueous phase under different conditions relative to the total amount of iodine species in the system. E: UV-VIS spectra of PBS phase after extraction with 1-octanol and octanoic acid (diluted 2,211 times). *Dashed line* = 226 nm. F: UV-VIS spectra of PBS phase after extraction with 1-octanol and octanoic acid (diluted 201 times). *Dashed lines* = 288 nm and 352 nm. G: UV-VIS spectra of hydrophobic phases after extraction with PBS (diluted 201 times) and zoom on the octanoic acid signal. H: Reference spectra of I₂ dissolved in the organic phases without extraction.

and S8). Grouping TGs and phosphatidylcholines with respect to the number of double bonds per lipid group confirms that MCV has a higher frequency of polyunsaturated side chains in these two classes of lipids (Fig. 4B, C).

Annotation of HR-MAS ssNMR signals was based on literature (62) (Fig. 4D). Analysis of the signals for both species (BMT and MCV) revealed that on

average, the Ads in MCV contain significantly more unsaturated double bonds per FA chain than those in BMT (Fig. 4E). To validate that reactions due to the presence of IL are occurring within the Ads, tissues were incubated with the staining solution. A significant decrease in double bonds was observed in both cases, which was most pronounced for the BMT samples (Fig. 4F).

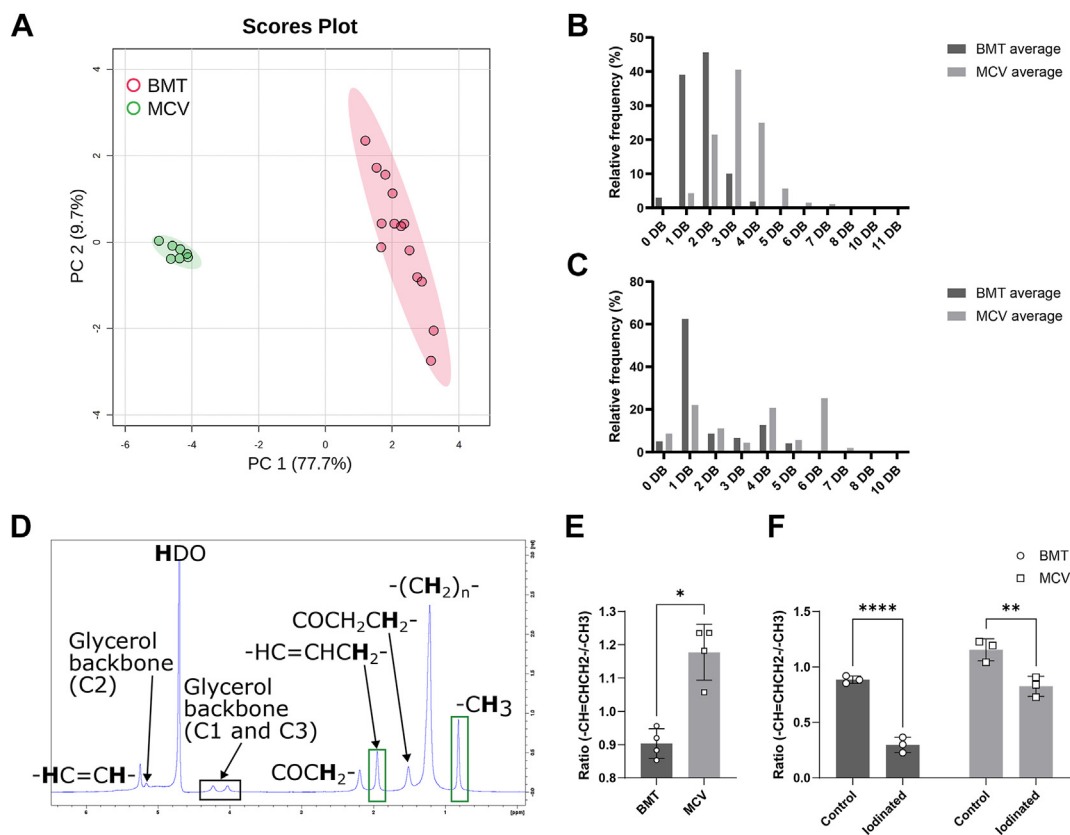


Fig. 4. Investigating IL staining on whole tissue samples. A: Scores plot after PCA analysis on AT from MCV and BMT. B: Average frequency distribution of triacylglycerols by number of double bonds for BMT and MCV. C: Average frequency distribution of phosphatidylcholines by number of double bonds for BMT and MCV. D: HR-MAS spectrum of MCV sample with annotation of signals. Green rectangles indicate integrated signals and are compared in (E) and (F). E: Comparison between the number of double bonds per fatty acid side chain. Mann-Whitney test ($n = 4$, $\alpha = 0.05$, exact two-tailed P -value = 0.0286). F: Comparison between the number of double bonds per fatty acid side chain before and after iodination of the tissue sample. Note that AT was separated from muscle fiber in the case of BMT. Two-way ANOVA followed by Bonferroni's multiple comparisons test ($n = 3$, $\alpha = 0.05$). BMT: Control – Iodinated: P -value < 0.0001, 95% CI [0.4137, 0.761] and MCV: Control – Iodinated: P -value = 0.0016, 95% CI [0.1558, 0.503]. AT, adipose tissue; PCA, principal component analysis.

The abovementioned results indicate that there is a difference in the expected degree of saturation between BMT and MCV. Moreover, loss of intensity of the ^1H NMR signal of unsaturated double bonds in the presence of IL was shown, indicating that iodine species in IL migrate within the Ads and subsequently react with the unsaturated double bonds. This shows that the degree of saturation of the lipids in the Ads indirectly contributes to the measured GV by reacting with I_2 species and hence trapping extra I_2 in the Ads in bound form.

DISCUSSION

To uncover the role of the AT in a 3D tissue environment, it is imperative to collect high-resolution, nondestructive 3D images and study its spatial distribution and structural properties. To this end, researchers have used, among others, CECT mainly in combination with OsO_4 as the preferred CESA (33, 34, 37). However, this CESA is inconvenient to use due to its toxicity, slow diffusion, and required decalcification of

mineralized tissues. Therefore, in this study, we compared three CESAs that have (Hexabrix (63, 64) and Hf-WD 1:2 POM (44)) or have not (IL) been used previously to study AT for their staining potential of MCV AT and BMT AT. The CESAs were evaluated using a single and sequential staining protocol. Due to the positive but different interaction of IL components with Ads, we focused on a more in-depth examination on the nature of the interactions using spectroscopic and spectrometric techniques.

Single and sequential staining revealed no staining of Ads by Hexabrix and Hf-WD 1:2 POM, but the data revealed a potential difference between the base level of the GV of these unstained Ads. This was also reflected after IL staining, which increased the GV of the Ads, albeit different in BMT compared to MCV. The staining of Ads by IL is in agreement with the hydrophobic nature of I_2 , confirmed to be present in the hydrophobic layers using UV-VIS measurements (Fig. 3G, H). I_2 readily dissolves in the hydrophobic environment and subsequently interacts with its components. In a control experiment, the distribution of

iodine species depended on the nature of the hydrophobic phase. In all conditions, less I_2 was distributed from the aqueous phase to octanoic acid relative to 1-octanol. Since the pK_a of octanoic acid is estimated to be around 5 (65), the pH of the aqueous solution might influence the distribution of iodine species in this model system, apart from affecting the iodine speciation equilibria (66). The difference in staining intensity is, among others, linked to the reaction between I_2 and (un)saturated fatty acids in adipocyte lipid droplets. Our data show that BMT AT contains more unsaturated lipids than MCV AT (Fig. 4E, F). However, this reaction is thermodynamically unfavorable and complete conversion is not expected and was not observed with palmitoleic acid or any unsaturated FA under the staining conditions (67). Moreover, it is also known and confirmed (Fig. 3A) that iodine species react with histidine and tyrosine (59, 68, 69).

We have shown that the residual presence of Hf-WD 1:2 POM is likely not catalyzing the iodination of Y (Fig. 3). However, the increased GV could also be the sum of the GV induced by IL staining and the GV caused by the residual presence of Hf-WD 1:2 POM after washing if the presence of iodine species does not affect the stability of the Hf-WD 1:2 POM. The significant effect of the staining protocol on IL-stained Ads for both MCV and BMT could also be linked to the potential charge diffuse (i.e. chaotropic) nature of the POM species in solution (70). Charge diffuse ions are known to disrupt the cell membrane structure (71), and as a result, the influx of iodine species into the cells might be enhanced.

Evaluating the segmented volume ratios of MCV AT versus total vertebrae volume, bone, and BM (including vasculature) for the different CESAs in combination with the images (Fig. 2A – MCV, staining, Table 4) provides insights into the segmentation quality. First, the Ads in a Hexabrix-stained vertebra are more easily discernible from the background than those in a Hf-WD 1:2 POM-stained vertebra. The simple segmentation of adipocytes hence seems to benefit from Hexabrix staining, as corroborate by previous research (63). Nonetheless, if researchers require information on the vasculature, staining with Hexabrix is not recommended, as the vasculature is not easily discernible from the extracellular matrix. In IL-stained samples, the PVE might play a role; however, this is not expected to have an enormous impact on the volume ratio, in this study, given the voxel size and isolated Ad size ($2\ \mu m^3$ vs. $20,000\ \mu m^3$). Note that for OsO_4 staining, the PVE should also be taken into account. Also, it is possible that our IL solution induced soft tissue shrinkage (72), which complicates the interpretation of these ratios and their differences. To address the issue of shrinkage, a stronger buffer could be used (73). However, this will likely impact the equilibria of the pH-dependent IL staining processes (66).

The staining process of the tissue samples involves two key steps, both impacting the equilibrium

concentration (i.e. GV) of the iodine species in the sample. First, passive diffusion will distribute the iodine species throughout the tissue, achieving different concentrations at different locations according to their preferred environment. I_2 being more hydrophobic in nature will preferably, although not exclusively, concentrate in hydrophobic environments, while I^- and I_3^- will be entirely excluded by hydrophobic phases. Second, the iodine species can react and interact with the molecules in their environment during the diffusion process, such as olefins (e.g. unsaturated fatty acids) (74), amino acids (59, 68, 69, 75–77), and polysaccharides (76, 78, 79), altering the concentrations of free species and redistributing them accordingly. The equilibrium concentration of iodine species is highly dependent on I^- concentration and pH of the solution (66). Since we have a limited concentration of iodine species in IL, the order in which tissue constituents are encountered during diffusion could impact the GV.

The difference in reduction of the amount of double bonds (Fig. 4F), measured by HR-MAS, could be related to the penetration speed of iodine species through both tissues, since it is known that BMT samples are already fully stained within nine days (80). Another experimental differences resides in the sample preparation since the AT was dissected from the muscle fibers in BMT tissue samples prior to staining the tissue, while the MCV was evaluated in its entirety. The bone in the MCV could serve as a physical barrier, decreasing the diffusion of iodine species towards the AT. For these reasons, we cannot infer that unsaturated double bonds react faster in BMT than MCV based on the data in this study.

Future work should build further on the fundamental knowledge of the staining process and focus on applying the investigated systems to answer biological research questions. To further elucidate the effect of the presence of Hf-WD 1:2 POM on the IL staining process, the Hf-WD 1:2 POM could be completely destroyed by incubation in a basic aqueous solution (81–83). Next, one sequential staining protocol was evaluated in this study. However, in the future, multiple staining protocols could be evaluated by using a Latin square experimental design (i.e. $A \rightarrow B \rightarrow C$, $B \rightarrow C \rightarrow A$ and $C \rightarrow A \rightarrow B$) (84). Additionally, since Hexabrix is not commercially available, similar molecules, such as Telbrix®, that are still available could be evaluated. From a chemical point of view, this work did not evaluate the interactions between negatively charged iodine species (e.g. I^- and I_3^-) with biomolecules using similar model systems. Furthermore, two biomolecule classes were not investigated: carbohydrates and nucleic acids. Future research should try to elaborate on these interactions.

CONCLUSION

Three known CESAs (Hexabrix, Hf-WD 1:2 POM, and IL solution) were evaluated for their staining potential in

the CECT of adipocytes using a single or sequential staining protocol. Hexabrix and Hf-WD 1:2 POM could not increase the GV of adipocytes, but an increase was observed upon staining with IL, albeit different for BMT and MCV. The GV of adipocytes and muscle fibers was increased when applying the sequential staining protocol compared to the single staining protocol, presumably due to the residual presence of Hf-WD 1:2 POM in the tissue. Hence, the potential change in the measured GVs should be considered when analyzing tissues stained via the sequential staining protocol for the IL-stained samples. Chemical model systems allowed us to monitor the main iodine species (i.e. I_2 , I^- , and I_3^-), present in the IL solution. These species have different affinities for hydrophilic and hydrophobic phases and can iodinate biomolecules, such as L-tyrosine, L-histidine, and palmitoleic acid, to different extents. Further analyses of the tissue samples using HR-MAS ssNMR and LC-MS-based lipidomics showed that on average, the BMT adipose depots contained less unsaturated fatty acids compared to the MCV adipose depot. This likely contributes to the observed GV difference but certainly should not be considered the only contributor. This work highlights that alternative CESAs to the toxic OsO_4 can stain the adipocytes and the surroundings, revealing the adipocytes either in positive or negative contrast.

Data availability

The original contributions presented in this study are included in the article/Supplemental material. The microCT datasets generated and/or analyzed during the current study are not publicly available due to their considerable size. Further inquiries can be directed to the corresponding author. 

Supplemental data

This article contains [supplemental data](#).

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

Ads, adipocytes; AT, adipose tissue; BM, bone marrow; BMAd(s), bone marrow adipocyte(s); BMT, bovine muscle tissue; CECT, contrast-enhanced computed tomography; CESA, contrast-enhancing staining agent; DCM, dichloromethane; FA, formaldehyde; GV, gray value; Hf-WD 1:2 POM, Hafnium-substituted Wells-Dawson polyoxometalate; His (H), histidine; HR-MAS, high-resolution magic angle spinning; IL, isotonic Lugol's iodine; LC-MS, liquid chromatography-mass spectrometry; MCV, murine caudal vertebra; OsO_4 , osmium tetroxide; Phe (F), phenylalanine; PVE, partial volume effect; ssNMR, solid-state nuclear magnetic resonance; TG, triacylglycerol; TXRF, total reflection X-ray fluorescence; Tyr (Y), tyrosine.

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