Feasibility and safety of synchrotron-based X-ray phase contrast imaging as a technique complementary to histopathology analysis

Running title: Feasibility and safety of X-PCI

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Author Contributions

H.D., A.B., V.DO., J.A.C., M.L., B.B. and A.D. designed the study.

V. DO., J. A. C. A. D., M. L., and K. D. W. collected the samples.

H.D. and A.B. performed the imaging experiments.

K.Y.C.L. and H.D. performed the image processing and interpretation.

K. D. W. provided technical and material support for the histochemistry and immunohistochemistry, gDNA isolation, dropletPCR, RNA sequencing analysis.

K.D.W., V.DO., J.C., M.L., and A.D. participated in the analysis and interpretation of the histochemistry and immunohistochemistry, gDNA isolation, dropletPCR, RNA sequencing analysis.

K.Y.C.L. performed segmentation of X-PCI datasets.

K.Y.C.L., H.D., K.D.W. and A.D. wrote the manuscript.

All authors read, revised, and approved the final paper.

Data Availability Statement

The majority of data generated or analysed during this study is included in this published article. The remaining data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

Samples were collected at Jessa Hospital in Hasselt (Belgium) in the context of a prospective observational cohort study which received ethical approval from Jessa Hospital and Hasselt University (ethical committee reference 20.36-infecti20.05). The samples subjected to technical analysis (histochemistry, immunohistochemistry, DNA isolation and RNA isolation) consisted of anonymously collected spare tissue from 3 clinically indicated lung, liver, and kidney surgery procedures at Jessa Hospital. No personal data was stored or processed with these samples. According to Belgian legislation, no informed consent is needed for use of spare tissue for technical validation of procedures implemented in a diagnostic pathology laboratory.

ABSTRACT

X-ray phase contrast imaging (X-PCI) is a powerful technique for high-resolution, three-dimensional imaging of soft tissue samples in a non-destructive manner. In this technical report, we assess the quality of standard histopathological techniques performed on formalin-fixed, paraffin-embedded (FFPE) human tissue samples that have been irradiated with different doses of X-rays in the context of an X-PCI experiment. The data from this study demonstrate that neither routine histochemical or immunohistochemical staining quality, nor DNA or RNA analyses are not affected by previous X-PCI on human FFPE samples. From these data we conclude it is feasible and acceptable to perform X-PCI on formalin-fixed and paraffin-embedded human biopsies.

INTRODUCTION

Imaging at the cellular level is essential for understanding biological processes in physiological and pathological states. Classically, two-dimensional (2D) imaging of microscopy slides is used to study individual cells and their arrangement into hierarchical structures or tissues. Use of 2D microscopy is widespread both in research and for clinical purposes.

In order to achieve 2D imaging of tissues, paraffin-embedding of formalin-fixed tissue (FFPE) is considered the gold standard for processing as it is practical in a wide range of settings and allows thin (typically between 2 to 5 µm) sectioning of tissue (Rosai 2007). An additional advantage is that protein or peptide epitopes can be searched for by immunostaining. Furthermore, DNA and RNA can be extracted for molecular analysis from FFPE blocks (Kokkat et al. 2013).

Although 2D imaging is still important in medicine, it is an incomplete visualisation of three-dimensional (3D) reality in which cells (mal)function. Histologic analysis damages the topology of structures due to its destructive nature, including processing and sectioning, which can lead to distortion and misinterpretation of true 3D structure (Xu et al. 2015).

In recent years, techniques have emerged for 3D light microscopic imaging of tissues in which light sheet microscopy of optically cleared tissues has gained the most interest (Renier et al. 2014; Klingberg et al. 2017; Glaser et al. 2017; Almagro et al. 2021; Merz et al. 2021). A critical factor for successful image generation with

this technique is effective tissue clearing that renders opaque tissues transparent, resulting in rather complex work-up and staining procedures.

An alternative approach to achieve 3D imaging is to digitally reconstruct 2D images. Although feasible, this technique easily leads to exhaustion of the paraffin-embedded biopsy because cutting of multiple serial sections is required. This is problematic when using invaluable human sample collections consisting of small biopsies, or from patients with rare diseases, where multiple research questions need to be answered on limited material. Additionally, algorithms for reconstruction might suffer from bias which can exacerbate the reliability of the final reconstruction, particularly when limited tissue is available (Pichat et al. 2018).

Micro-computed tomography (micro-CT) offers high-resolution (approximate range of 5 to 150 μm), 3D virtual slicing of samples in a non-destructive manner (Jeffery et al. 2011). Previous studies have used micro-CT to successfully image FFPE blocks in pathological tissue samples (Teplov et al. 2019; Yoshida et al. 2022). Micro-CT irradiation of fresh tissue, formalin-fixed tissue and FFPE blocks of pathological tissues has been shown to have no impact on protein expression (Teplov et al. 2019). The dose of synchrotron radiation micro-CT has been reported to be in the kGy range (Saccomano et al. 2018; Albers et al. 2021). However, quantification of the exact micro-CT radiation dose of each sample was not performed and would be advantageous to know. Although micro-CT is an invaluable technique, it is limited by its low contrast in soft tissues due to low X-ray absorption. The main approach to ameliorate contrast is the use of metal-based staining agents, for example, iodine (Degenhardt et al. 2010; Stephenson et al. 2012). Additionally, there are concerns

regarding the negative effects of staining involved in micro-CT on biological samples, for example, alterations in the structure due to tissue shrinkage which can cause abnormal histological appearance of tissue architecture and misleading diagnostic conclusions (Dawood et al. 2021; Lupariello et al. 2021).

X-ray phase contrast imaging (X-PCI) is a powerful technique for high-resolution, 3D imaging of soft tissue samples (Garcia-Canadilla et al. 2018; Dejea et al. 2019; Norvik et al. 2020; Borisova et al. 2021). Conventional X-ray imaging is based on absorption contrast in which the contrast relies on variation in X-ray absorption as a result of density differences and variation in thickness and composition of the sample (Bravin 2003). In soft biological tissue, the differences in absorption coefficients are very small for hard X-rays and the sensitivity is very low. This drawback can be overcome by using highly brilliant and coherent X-ray sources, such as synchrotrons, which exploit the refractive properties of X-rays and the phase change or phase shift transmitted through a sample. An attractive advantage of synchrotron radiation-based X-PCI is the high contrast offered which overcomes the requirement of contrast-enhanced agents which micro-CT is limited by. For soft tissues, phase contrast is approximately 3 orders of magnitude higher than absorption contrast (Grandl et al. 2013), which makes synchrotron radiation-based X-PCI an excellent technique for structural assessment of such samples. Furthermore, X-PCI enables 3D imaging of human samples in a non-destructive manner (Grandl et al. 2013; Walsh et al. 2021) and can supplement routine histology.

Nevertheless, irradiation releases energy into the tissue or FFPE block. Previous reports have documented the possibility to perform histology and

immunohistochemistry on previously irradiated paraffin-embedded blocks (Saccomano et al. 2018; Norvik et al. 2020; Zdora et al. 2020; Albers et al. 2021; Pinkert-Leetsch et al. 2023). However, it has not been investigated if DNA and RNA analyses are feasible on human tissue biopsies after synchrotron imaging. We hypothesised that the energy released through X-PCI is too low to cause significant damage to nucleic acids in human tissue biopsies. If our hypothesis is correct, this means FFPE blocks of human tissue can be safely subjected to 3D imaging by X-PCI whilst preserving the potential not only to perform (routine 2D) histochemistry and immunohistochemistry, but also molecular analyses.

MATERIALS AND METHODS

Sample description and preparation

Samples were collected at Jessa Hospital in Hasselt (Belgium) in the context of a prospective observational cohort study which received ethical approval from Jessa Hospital and Hasselt University (ethical committee reference 20.36-infecti20.05) (D'Onofrio et al. 2022). The samples subjected to technical analysis (histochemistry, immunohistochemistry, DNA and RNA isolation) consisted of anonymously collected spare tissue from 3 clinically indicated lung, liver, and kidney surgery procedures at Jessa Hospital. No personal data was stored or processed with these samples. According to Belgian legislation, no informed consent is needed for use of anonymous leftover tissue for technical validation of lab procedures.

Tissues were fixed in formalin after procurement for a minimum of 12 hours and a maximum of 48 hours, then processed further to paraffin blocks with an Excelsior AS processor (Thermo Fisher Scientific, Runcorn UK). In total, 6 blocks were made for technical analysis. Paraffin blocks contained tissue from one or more organs to minimise the number of blocks subject to synchrotron imaging and to work cost-effectively (Table 1). Laboratory procedures were performed according to national guidelines and subjected to ISO15189 accreditation; methods were standard for diagnostic pathology laboratories worldwide.

Synchrotron radiation-based X-ray phase contrast imaging

The tomographic imaging experiment was carried out at the TOMCAT X02DA beamline (Stampanoni et al. 2007) of the Swiss Light Source, Villigen, Switzerland. A multiscale propagation-based phase contrast imaging setup (Dejea et al. 2019) was mounted to image the paraffin blocks with different magnifications: 1x – 5.8 µm/voxel (PCO.Edge 4.2 camera, LuAg:Ce 300 µm scintillator, 240 cm propagation distance), 4x – 1.625 µm/voxel (PCO.Edge 5.5 camera, LuAg:Ce 20 µm scintillator, 20 cm propagation distance), and 10x – 0.65 µm/voxel (PCO.Edge 5.5 camera, LuAg:Ce 20 µm scintillator, 5 cm propagation distance), without sample manipulation between scans (Table 2).

Before imaging, wax pillars were manually placed on the paraffin blocks to aid sample alignment (Norvik et al. 2020). The blocks were then placed on the sample stage for tomographic acquisition at an X-ray energy of 21 keV (see Figure 1). To minimise dose deposition, samples were aligned using a set of optical cameras (Lovric et al. 2013) that allowed for the positioning of the wax pillar in the centre of

the field of view. Scans consisted of 1800 projections over 180°, as well as 100 flats and 30 darks to correct for X-ray beam inhomogeneities and detector noise (Table 2). Once the X-rays were transmitted through the samples, they were converted to visible light by a scintillator, magnified and recorded by different objective-detector configurations (Table 2).

All FFPE blocks were scanned using either a 1x or 4x configuration to obtain a general overview of all the contained samples. These scans were then used to select regions of interest to be imaged at the higher 10x magnification. The selection process was achieved with an in-house developed Fiji plugin that automatically computed the corresponding motor configuration. Thus, no sample manipulation was required between scans. Since some regions of interest were larger than the available field of view, several overlapping scans covering the whole area were acquired and stitched subsequently.

After performing synchrotron radiation-based X-ray phase contrast imaging, in-house scripts at TOMCAT beamline were applied to perform 3D reconstruction of all tomographic projections with Gridrec algorithm (Marone and Stampanoni 2012) using Paganin phase retrieval algorithm to recover phase information (Paganin et al. 2002). The δ/β value for the Paganin algorithm was tuned to 217.6.

Radiation dose calculation

Absorbed dose, D, is defined as the mean energy transferred to the sample per unit mass and can be described as:

$$D = E_{abs} \,/\, (V\varrho)$$

where D corresponds to the absorbed dose in Gray [Gy], and E_{abs} is the absorbed energy in Joule [J] by a sample of volume, V, and density, ϱ. E_{abs} can be further described by the mass energy absorption coefficient (μ_{en}/ϱ) and the number of absorbed photons (I_{abs}) with energy $h\nu$. I_{abs} will change exponentially with the sample thickness (z) following the Beer-Lambert law:

$$E_{abs} = I_{abs}\, h\nu \; = I_0\left[1 - e^{-(\mu_{en}/\varrho)\varrho z}\right]h\nu$$

where I_0 corresponds to the initial number of photons reaching the sample and directly relates to X-ray flux. To measure the total absorbed dose, D_T, during a scan, D needs to be multiplied by the number of projections (nproj) and exposure time (t) so that:

$$D_T = D * nproj * t.$$

For the purpose of this work, X-ray flux is measured with a PIPS diode (Lovric et al. 2016) and the samples were approximated as cylinders of soft tissue (ρ = 1.005 g/cm^3, μ_{en}/ϱ = 0.5258 cm^2/g at 21 keV [NIST]) with 2 mm diameter and height, surrounded by 10 mm of paraffin wax (ρ=0.9 g/cm^3, μ_{en}/ϱ = 0.42 cm^2/g at 21 keV (Scott et al. 2015). If the field of view was smaller than the sample size, D_T corresponds to the radiation dose within the scanned field of view. It should be remarked that this is an approximation and other regions of the sample will also receive extra dose as they cross the beam path during rotation.

The calculated dose per scan can be found in Table 2, while the accumulated dose in each sample can be found in Table 1. Note that in Table 1, we acquired different types and number of scans in each of the samples with the goal of having a wide spectrum of dose deposition and better understanding of how this affects classical histopathological analysis. One sample (Sample ID 23.8) was not irradiated and serves as a reference control (Table 1).

Histochemistry and immunohistochemistry

After the samples (FFPE tissue blocks) were returned from the synchrotron light source facility, 4 µm thick tissue sections were cut onto positively charged slides. No significant cracking, tearing, or breaking of slides was experienced during sectioning. Histochemistry (HC) analysis for periodic acid-Schiff (PAS), Jones, and Gordon Sweet (GS) were performed on the Ventana benchmark special stains system (Ventana Medical Systems, Tucson, AZ) according to manufacturer's guidelines.

Immunohistochemistry (IHC) analysis was performed on the Omnis instrument (Agilent, Santa Clara, CA). The following primary antibodies were used: PAX 8 clone SP348 (Abcam), CK7 clone OV-TL 12/30 (Dako), TTF1 clone SPT24 (Leica), and CD10 clone 56C6 (Dako). All IHC assays were performed using the target retrieval solution high pH for 30 minutes at 95°C and the EnVision FLEX detection DAB system (Agilent). The PAX8 antibody (dilution 1/100) was incubated for 20 minutes in combination with a mouse linker; the CK7 antibody (ready-to-use solution) was incubated for 10 minutes; the TTF1 antibody (dilution 1/400) was incubated for 20

minutes in combination with a mouse linker; and finally, the CD10 antibody (ready-to-use solution) was incubated for 12.5 minutes in combination with a mouse linker.

All slides were qualitatively evaluated by two pathologists (AD and ML). For the haematoxylin & eosin (H&E) stains, evaluation focussed on nuclear detail, artifacts, colour (contrast), and visibility of basal membranes and internal elastic lamina in vessels. For nuclear detail, colour and visibility of basal membranes and internal elastic lamina, there were 3 possible options to score their quality: good, sufficient or suboptimal. For artifacts, pathologists needed to score them as present or absent. For histochemistry, again slides were scored as good (+), sufficient, or suboptimal quality (-).

For immunohistochemistry, evaluation focussed on staining specificity, intensity, and background. All slides were first evaluated by pathologists separately and then discussed to come to an overall agreement. Pathologists were blinded to irradiation dose.

Genomic DNA isolation and integrity

From each sample, 5 consecutive formalin-fixed paraffin embedded (FFPE) tissue sections of 5 µm thickness were prepared. Genomic DNA (gDNA) isolation from these slides was performed using a QIAcube instrument using the QIAamp DNA Mini QIAcube Kit according to standard manufacturer's protocol (Qiagen, Venlo, The Netherlands). The concentration of gDNA was measured with a NanoDrop 2000 instrument.

DNA integrity of samples was determined using the Agilent 2200 TapeStation System in combination with the genomic DNA ScreenTape assay according to the manufacturer's instructions (Agilent Technologies, Waldbronn, Germany). The workflow automatically calculates the DNA integrity number (DIN): a high DIN indicates highly intact gDNA; a low DIN indicates strongly degraded gDNA. A DIN of more than 3 indicates sufficient DNA quality as defined by a >70% on-target rate in subsequent downstream sequencing pipelines (Agilent Technologies publication number G5991-5360EN).

DropletPCR

Epidermal growth factor receptor (*EGFR*) mutational analysis was performed by droplet digital PCR (ddPCR) (BioRad, Temse, Belgium). Two multiplex ddPCR assays were designed to detect exon 19 hotspot deletions and the T790M resistance mutation and the L858R, L861Q and G719A/C/S activating mutations, respectively. Detailed procedures and the in-house design can be found elsewhere (Sorber et al. 2019). Droplets were analysed using QX200 Droplet Reader (Bio-Rad). Data analysis was performed with QuantaSoft version 1.7.4.0917 (Bio-Rad), which uses the number of positive and negative droplets to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% CIs. Samples were evaluated based on maximal attainable sensitivity (reached assay sensitivity), which is dependent on the previously determined threshold of a positive target event (for example, T790M) and total DNA input (of sufficient quality to allow amplification). The required thresholds to detect clinically relevant DNA levels were based on in-

house data and literature (Lee et al. 2015). Hence, allowing the samples to be categorised as non-informative (>10%), restricted (≤1%), or adequate (<1%).

RNA sequencing

The Oncomine Focus Assay (Thermo Fisher Scientific) was used for the detection of gene fusions in 23 genes, including *ALK, ROS1, RET, BRAF*, and *NTRK1-3* following manufacturer's protocol. Briefly, RNA (from gDNA isolation) was reversely transcribed with SuperScript kit VILO cDNA synthesis kit (Thermo Fisher Scientific). Library preparation was carried out using RNA Oncomine™ Fusions assay (Thermo Fisher Scientific). The prepared libraries were partially digested using FuPa reagent, ligated to different barcode adapters and purified. Library quantification was carried out by qPCR and individual libraries were diluted to a final concentration of 30 pM. Finally, samples were pooled and processed using Ion Chef System (Thermo Fisher Scientific). Analysis of sequence data was carried out using Ion Reporter™ software 5.10.3 (Thermo Fisher Scientific). Fusions were detected using fusion detection module within the Ion Reporter workflow. To qualify a sample as valid and to proceed with the analysis, the minimum number of valid mapped reads was 20000.

Segmentation of X-PCI datasets

X-PCI datasets were visualised using ImageJ/Fiji (Schindelin et al. 2012). Manual segmentation of histologic substructures of interest in the heart, kidney, liver, and lung biopsies were performed using open-source software Seg3D (www.seg3d.org). Each biopsy X-PCI dataset consisted of 2160 images in TIFF format. A new label mask was created for each region of interest to be segmented. The 'Paint Brush' tool

was used and set to an appropriate diameter according to vessel size. The structures of interest were labelled every 1-10 slices. To fill between slices and smooth the isosurface, the ‘Smooth Binary Dilate Erode’ tool was used.

RESULTS

Visualisation of human tissue structures is feasible using synchrotron radiation-based X-ray phase contrast imaging

A proof-of-principle analysis with 3D segmentation of histologic substructures was performed to illustrate the potential of X-PCI for FFPE blocks. In kidney tissue, it was possible to render glomeruli in 3D, which gives an impression of the volume and position of glomeruli in relation to surrounding structures (Figure 2a). In heart biopsies, small vessels can be segmented and can allow for analysis of branching patterns (Figure 2b). In liver tissue, the interrelated course of hepatic artery, bile duct and portal vein can be illustrated (Figure 2c). Additionally, in lung tissue, vessels can be segmented and visualised using X-PCI (Figure 2d).

X-PCI is compatible with routine histochemistry and immunohistochemistry

In routine, haematoxylin and eosin (H&E)-stained slides are usually first assessed by a pathologist and results classically indicate the need for subsequent stains or molecular techniques; it is thus the mainstay of histopathological assessment.

In this study, for all evaluated parameters related to tissue and staining quality on H&E post-X-PCI (nuclear detail, basal membrane, and colour), the pathologists assessed all slides as having good quality. For artifacts, one slide showed minimal artifacts under the form of some folds in the tissue (sample 23.7). However, this did not influence the overall quality of the slide (Table 3).

For histochemistry, post-X-PCI PAS-stained slides were uniformly good, and GS-stained slides were most often of sufficient quality. However, one sample (sample ID 23.8) had suboptimal quality (Table 3). Sample 23.8 did not undergo synchrotron radiation X-ray phase contrast imaging, which suggests that the suboptimal quality was likely due to the staining procedure rather than the effects of synchrotron radiation. Jones staining was only performed on samples 23.7 and 23.10 (containing kidney tissue) and showed sufficient staining quality (not shown in table). Figure 3 shows representative images of staining quality for H&E and histochemistry.

For immunohistochemistry, CK7, TTF1, CD10, and Pax8 stainings were performed. CK7 is a cytoplasmic marker of simple non-keratinised epithelium in the human body and stains alveolar and bronchial epithelium in lung, bile ducts in liver, and segments of distal tubule in the kidney. TTF1 is a nuclear marker for bronchial and alveolar epithelium. CD10 stains bile canaliculi in liver and is positive on proximal tubular epithelium. Pax8 is a nuclear marker for kidney epithelium. All immunohistochemical stains demonstrated good results for specificity and intensity for all markers and all tissues performed (Figure 4). There was only slight background in the CK7 staining of sample 23.1, which did not hinder evaluation. The results for CK7 and TTF1 are shown in Table 3.

X-PCI does not impact quality of DNA and RNA isolated from tissue

Molecular analysis on DNA and RNA extracted from human paraffin samples is common practice in diagnostic pathology labs. We chose a ddPCR approach for

EGFR mutational analysis to test for ‘reached assay sensitivity’ in this study. As expected, all samples were wild-type for *EGFR* mutation. In all tested samples, EGFR analysis by ddPCR was possible, with a ‘reached assay sensitivity’ ranging from 0.28% to 1.1%. Additionally, targeted next generation sequencing (NGS) was performed for detection of fusions in 23 genes. No fusions were detected, as expected. All samples surpassed the minimum threshold of 20000 reads required for analysis, with absolute counts between 98597 reads and 203318 reads (Table 4). There was no clear correlation between number of reads and radiation dose.

DNA integrity results from TapeStation showed no detectable negative effect on DNA samples with increasing radiation dose (Figure 5). Smearing of DNA is evident in all sample lanes. No correlation between synchrotron radiation X-PCI dose and DNA concentration was observed. Sample 23.8 was not irradiated but still had low DNA concentration and DIN value (3.2) suggesting the effects are from formalin fixation rather than X-PCI (Figure 5 and Table 4).

DISCUSSION

In this paper we describe the feasibility and safety of applying synchrotron X-PCI to FFPE human biopsies for their posterior histological, histochemical and DNA/RNA analysis. We quantified the absorbed synchrotron radiation dose that the FFPE blocks were subjected to at 1x, 4x, and 10x magnifications (Tables 1 and 2). All doses were in the kGy range which has also been reported briefly in previous synchrotron radiation micro-CT studies (Saccomano et al. 2018; Albers et al. 2021). Our study goes further with radiation dose calculation to achieve the doses per scan and total dose for synchrotron radiation-based X-PCI.

DNA and RNA were successfully extracted from FFPE samples that had undergone X-PCI. A ddPCR approach was chosen to test for the capacity to do PCR-based mutation analysis. All samples except from one (1.1%), reached the threshold of <1% sensitivity. RNA sequencing analysis was performed and showed that all irradiated samples reached the threshold of >20000 mapped reads for analysis. Even for the blocks that were subjected to the highest irradiation dose of 2262.8 kGy (sample 23.3), there was no noticeable effect on DNA or RNA quality, and molecular analysis could be performed by both tested techniques (Figure 5 and Table 4). A possible explanation for this observation is that formalin fixation and paraffin embedding help protect DNA and RNA integrity of biological samples from radiation-induced damage (Bedolla et al. 2018). However, the low DNA concentration and DIN values (Table 4 and Figure 5) suggest that the effects from formalin fixation and/or paraffin embedding on DNA integrity are more significant than the effects from X-PCI. The minimum DIN value required for sequencing has been reported to be 3.0

(Jung et al. 2017) which all samples met. However, the values were generally low for all samples regardless of whether they were irradiated by synchrotron radiation or not, and regardless of the dose.

The data from DNA and RNA analyses suggest that it is safe to perform synchrotron radiation X-PCI on human FFPE tissue blocks without damaging the tissue so it can be used for routine analysis afterwards. Additionally, there was no visible effect of X-PCI on routine H&E and histochemical staining, nor with immunohistochemistry using a range of antibodies that are typically used in histopathological practice. This confirms what has been shown in previous reports (Saccomano et al. 2018; Norvik et al. 2020; Zdora et al. 2020; Albers et al. 2021; Pinkert-Leetsch et al. 2023).

As previously shown in the literature (Dejea et al. 2019; Norvik et al. 2020), X-PCI can be used to reconstruct 3D images of paraffin-embedded human tissues. Although this study was not designed for extensive showcasing of these possibilities, segmentation of specific substructures was perfectly feasible.

However, this proof-of-principle study has some limitations. Even though the variation between samples was low and no outliers were present, the sample size was relatively small. The study demonstrates that in principle, tissue quality and staining quality were not affected, but the number of tested immunohistochemical stains was modest. Here, only the Dako Omnis platform was tested, and it would remain good practice to monitor the effects of X-PCI on staining protocols with different primary antibodies and platforms. Molecular analysis focussed on DNA and RNA quality by PCR and NGS-based methods. The whole spectrum of molecular

techniques was not included. Finally, results should ideally be corroborated by different centres. To do this in a practical and cost-effective way, we suggest further X-PCI experiments should comment on the subsequent use of samples for routine pathology analysis to validate these findings.

In conclusion, subjecting histology FFPE blocks for prior X-PCI analysis using the range of radiation doses applied in this study does not compromise the quality of subsequent routine pathology evaluations including molecular analyses.

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Seg3D: Volumetric Image Segmentation and Visualization

FIGURES

Figure 1: Setup for synchrotron radiation-based X-ray phase contrast imaging of formalin-fixed paraffin-embedded blocks. On top of the block, three melted wax pillars were placed directly above the sample to aid sample alignment.

Figure 2: Three-dimensional reconstruction based on synchrotron radiation-based X-PCI can be used to segment histologic substructures in human tissue. Left panels depict conventional histology, middle panels depict 2D tomographic X-PCI images, right panels present 3D reconstructions from 2D images. The black scale bars represent 200 µm.
(a) Kidney biopsy from patient 453 S1: left panel shows haematoxylin and eosin (H&E)-stained kidney section with two glomeruli; middle panel shows X-PCI tomographic projection with two glomeruli labelled for manual segmentation with Seg3D software; right panel shows 3D volume rendered segmentation of glomeruli.
(b) Heart biopsy from patient 452: left panel shows H&E-stained heart section; middle panel shows X-PCI tomographic projection where small vessels have been manually labelled in red; right panel shows 3D volume of the segmentation of vasculature.
(c) Liver biopsy from patient 427 S2: left panel shows H&E-stained liver section, middle panel shows X-PCI tomographic projection where the bile duct (dark blue), portal vein (orange) and arteries (pink) have been manually labelled for segmentation with Seg3D software; right panel shows the 3D volume reconstruction of the histologic substructures.
(d) Lung biopsy from patient 451 S1: left panel shows H&E-stained lung section, middle panel shows X-PCI tomographic projection with small vessels manually labelled; right panel shows 3D volume of the segmented vessel.

Figure 3: Representative images of histochemical staining. Scale bars are indicated by bold black horizontal lines and represent 200 µm in length.
(a) H&E stain of kidney tissue from sample 23.7 (20S769/7) that was subjected to medium dose X-PCI radiation.
(b) H&E stain of kidney tissue from sample 23.10 (20S769/10) that was subjected to high dose X-PCI radiation.
(c) H&E stain of lung tissue from sample 23.1 (20S769/1) that was subjected to low dose X-PCI radiation.
(d) H&E stain of lung tissue from sample 23.3 (20S796/3) that was subjected to high dose X-PCI radiation.
(e) H&E stain of liver tissue from sample 23.5 (20S769/5) that was subjected to high dose X-PCI radiation.
(f) H&E stain of liver tissue from sample 23.3 (20S769/3) that was subjected to high dose X-PCI radiation.

Figure 4: Representative images of immunohistochemical stains. Scale bars are indicated by bold black horizontal lines and represent 200 µm in length.
(a) CK7 stain of kidney tissue from sample 23.10 that was subjected to medium dose X-PCI radiation.
(b) PAX8 stain of kidney tissue from sample 23.1 that was subjected to medium dose X-PCI radiation.
(c) CK7 stain of lung tissue from sample 23.1 that was subjected to low dose X-PCI radiation.
(d) CK7 stain of lung tissue from sample 23.3 that was subjected to high dose X-PCI radiation.
(e) CD10 stain of liver tissue from sample 23.3 that was subjected to high dose X-PCI radiation.
(f) CD10 stain of liver tissue from sample 23.5 that was subjected to high dose X-PCI radiation.

Figure 5: TapeStation DNA integrity results. DNA lanes are shown (left side) with their corresponding DNA integrity number (DIN) values. DIN and DNA concentrations are shown (right side) in relation to sample ID and X-PCI dose.