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Research

Development of Standard Operating Procedure (SOP) of Micro-computed tomography (micro-CT) in Pathology

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Abstract

Background and goal: Micro-computed tomography (micro-CT) is an emerging technology in the biomedical field and enables us to analyze 3D structures non-destructively and observe these structures in various directions, thus enabling innovation in this area of pathology. However, application of micro-CT for medicine has just started and optimization per purpose has not yet been done. The purpose of this study is to 1) demonstrate the potential utility of micro-CT in pathology; 2) optimize micro-CT imaging technology and develop a standard operating protocol and; 3) investigate whether micro-CT incurs any radiation damage to pathological tissue samples.

Material and methods: The samples of fresh tissue, formalin fixed tissue and formalin fixed paraffin-embedded (FFPE) tissue blocks were scanned using a custom-built Nikon Metrology micro-CT system with a variety of parameters then evaluated with histology correlation in detail. Radiation damage to tissue samples was also evaluated. Through our study, we have established the scanning protocol and workflow for each type of sample.

Results: For fresh/fixed tissue, the house made polystyrene foam container was most ideal and the scanning time for fresh tissue was six minutes at as shortest, in which it is

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possible to detect neoplastic lesions in the tissue. In case of FFPE blocks, 10 - 17 hours scanned images were corresponding with histology specimens in 4-10x under the microscope. Regarding radiation damage to protein expression, no impact was found.

Conclusions: Micro-CT imaging of tissue specimens can be of clinical utility in fresh, formalin fixed tissues and FFPE blocks. We also found many additional valuable uses of micro-CT images in translational research.

Keywords: Micro-CT imaging, fixation, 3D-reconstruction, virtual microscopy, tumor boundary analysis.

Introduction

Micro-computed tomography (Micro-CT) was originally developed and employed in industries for non-destructive evaluation such as motor vehicle engineering and clucking because of the X-ray attenuating properties on dense materials such as metal. It is an emerging technology in the biomedical field that holds great promise for imaging pathology tissue specimens like post-operative lumpectomy samples for reconstruction, modeling, and analysis in 3 dimensional (3D) structures. Micro-CT's capacity to create high resolution 3D models of ex vivo tissue therefore offers a unique opportunity to correlate micro-CT image data with other imaging modalities and bring additional information for future diagnoses in pathology. Previous work for breast cancer suggested that micro-CT images of fresh tissue showed structures indicating vessel network, metastatic tumor, and lymph node tissue (1) (2) (3).

The 3D reconstruction of Whole Slide Images (WSIs) has proven to be valuable in the visualization and diagnosis of disease beyond the 2D microscope slide (6, 8). High-resolution 3D histology imaging is particularly advantageous to the discovery of diagnostic patterns in its capacity to improve correlation between imaging modalities, such as MRI, conventional CT, and histology [2, 3, 4, 5, 6]. However, while high resolution 3D histology is useful for 3D image analysis, including cell counting, volume of interest (VOI) segmentation, and volume measurement, its accuracy at the cellular level is unknown because tissue distortion and deformation could occur during the slide preparation process. In one of our previous studies we reported how the size and shape of tissue changes during the pathological process and how micro-CT technology can eliminate these inaccuracies and help us to better understand and improve the existing 3D histology workflow. (4).

Thus micro-CT enables us to analyze 3D structures non-destructively, observe these structures in various directions freely, and facilitates innovation in this area of

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pathology. However, the application of micro-CT in medicine has just started and optimization of usages per application has not yet been done.

There are many important parameters that have an effect on the final image quality such as beam energy (kV), beam current (µA), exposure time (msec), number of projections, frames per projections and distance between the x-ray source and sample. For example, (1) the beam energy (kV) allows for the penetration of denser or larger samples, although it increases noise; (2) Higher beam power (W) also allows for better penetration with lower noise but the micro-focused x-ray beam damages the X-ray source target sooner; (3) The number of projections relates to the artifact of projection lines; (4) the exposure time effects on the noise and contrast, and lastly, (5) the distance between source and sample effects on the resolution and scan area.

Our concern is that use of micro-CT in pathology may cause some kind of damage in the protein structure of the tissue samples (5).

Therefore, the aim of this study was to develop standard operating procedures (SOP) for the intra/post operative margin evaluation i.e. scanning of fresh and fixed tissue and for improving histology 3D imaging, and formalin fixed paraffin embedded (FFPE) tissue block scanning. For the fresh tissue samples, our aim is to utilize micro-CT scanning for intra-operative tumor margin detection. The targeted scanning duration was to complete entire process in 15min to 30 min. For the FFPE blocks, our aim is to utilize micro-CT to aid in structural detection such as tumor vs normal and histological correlation.

The SOP includes instructions for sample handling, and detailed parameters for scanning optimization such as how to position the sample to stabilize scanning; how to protect the sample during scanning from X-ray damage; and an investigation guide for the detection of radiation damage to the protein structure of the fresh tissues, formalin fixed tissue, and formalin FFPE tissue samples to ensure that no damage was caused by micro-CT scanning using a variety of immunohistochemistry (IHC) stains.

Materials and Methods

Materials

The materials we assessed were fresh tissue samples, formalin-fixed tissue samples and FFPE tissue blocks (1x1.5 inch and 3x2 inch). A total of 179 samples were scanned 550 times (63 fresh tissue samples scanned 185 times; 32 fixed tissue samples scanned 83 times; 91 FFPE block samples scanned 297 times) between February 2017 and December

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2017 from within our Institution. The details of these samples are described in Supplementary 1. The study was approved by the Institutional Review Board.

Micro-CT Imaging System

The micro-CT imaging system consists of a custom built micro-CT scanner (Nikon Metrology NV, Leuven, Belgium) and data reconstruction software CT Agent Medical Alpha version XT 5.1.4.2 MedX1 (Nikon Metrology NV). Scanned image data is stored in a data storage server in our institutions data center. Re-constructed imaging data is then visualized and analyzed by using commercially available software such as VG Studio Max 2.2.6 (Volume Graphics GmbH, Heidelberg, Germany), Dragonfly 3.1 (Object Research Systems, Montreal, Quebec, Canada) and Imaris 9.0 (Bitplane Inc., South Windsor, CT, United States).

Investigation of sample handling

Fresh tissue samples in plastic containers, plastic dishes and polystyrene foam containers were investigated along with the position of the samples within their containers. For 1x1.5 inch FFPE tissue block samples, a prototype modular sample handling system kinematic FFPE block holder set was available. For 2x3 inch FFPE block samples we used Megablock holder with x-y positioning table. Also, we have tried the carbon fiber paddles in different sizes for holding both 1x1.5 and 2x3 inch FFPE tissue block samples.

Investigation of scanning parameters

Investigated scanning parameters include beam energy (kV), beam current (uA), exposure time (msec), number of projections, frames per projections and the distance from the x-ray source to the sample. Multiple combinations of scanning parameters were applied to the scanning of all samples. The experimentation parameters were determined based upon the Manufacture's recommendation and the previous experiences of one of the authors. For fresh tissue, scanning duration was set at a 15 minute maximum and the best combination of each scanning parameter was investigated.

Histology Correlation

In order to assess correlation between micro-CT and histology, at least one H&E slide was processed from all FFPE block samples. Selected fresh tissue samples (all breast, lung, prostate, bone, colon polyp and lymph node, Figure 4, 5 and 7) were processed to make FFPE blocks. H&E stain slides were then prepared from each block and digitized with WSI scanners, Aperio AT2 (20x, 0.75NA, 0.50um/pixel) (Leica Biosystems Inc.,

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Buffalo Grove, IL, United States) and NanoZoomer2.0HT (20x, 0.75 NA, 0.46um/pixel) (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan). The reasons why two image sets were prepared was to ensure flexibility for reviewers and to support any possible scanning errors from causing unnecessary re-scanning. All histology slides in this research were scanned with these two scanners.

One or two pathologists and one or two image specialists reviewed all images scanned with both MicroCT and WSI scanners. MicroCT images are reviewed with either VG Studio Max 2.2.6 (Volume Graphics GmbH, Heidelberg, Germany), or Dragonfly 3.1 (Object Research Systems, Montreal, Quebec, Canada). The software was selected based upon user preference. Pathologists and image specialists identified each tissue component in MicroCT images. Correlated areas with histology slides were searched and showed on the monitor for detail observation.

Evaluation of radiation damage

In advance of micro-CT scanning, we divided the fresh tissue samples into 4 categories; 1) tissue scanned by micro-CT twice - when it was fresh and as an FFPE block after embedding, 2) the tissue scanned by micro-CT once, when it was fresh, 3) micro-CT scanned once, when it was an FFPE block, and 4) the tissue that was not scanned using micro-CT [Figure 1]. Our optimized scanning conditions were used for the scanning of the tissue. After scanning, fresh samples were fixed then processed to make FFPE blocks and later, H&E slides for histology correlation. 15 antibodies consisting of CD68, CD34, smooth muscle actin, vimentin, TTF-1, thyroglobulin, pan-cytokeratin AE1/AE3, Ki-67, estrogen receptor, progesterone receptor, HER2/neu, MLH1, MSH2, MSH6, and PMS2 were selected at random considering expression type (membrane, cytoplasm, or nuclei), tissue type (epithelium, stroma, or hemocyte), availability for therapy, and screening of cancer. All stains were applied to the slides and protein expression was investigated amongst a variation of scanning times.

All H&E and IHC slides were evaluated by pathologists using both a microscope and WSIs.

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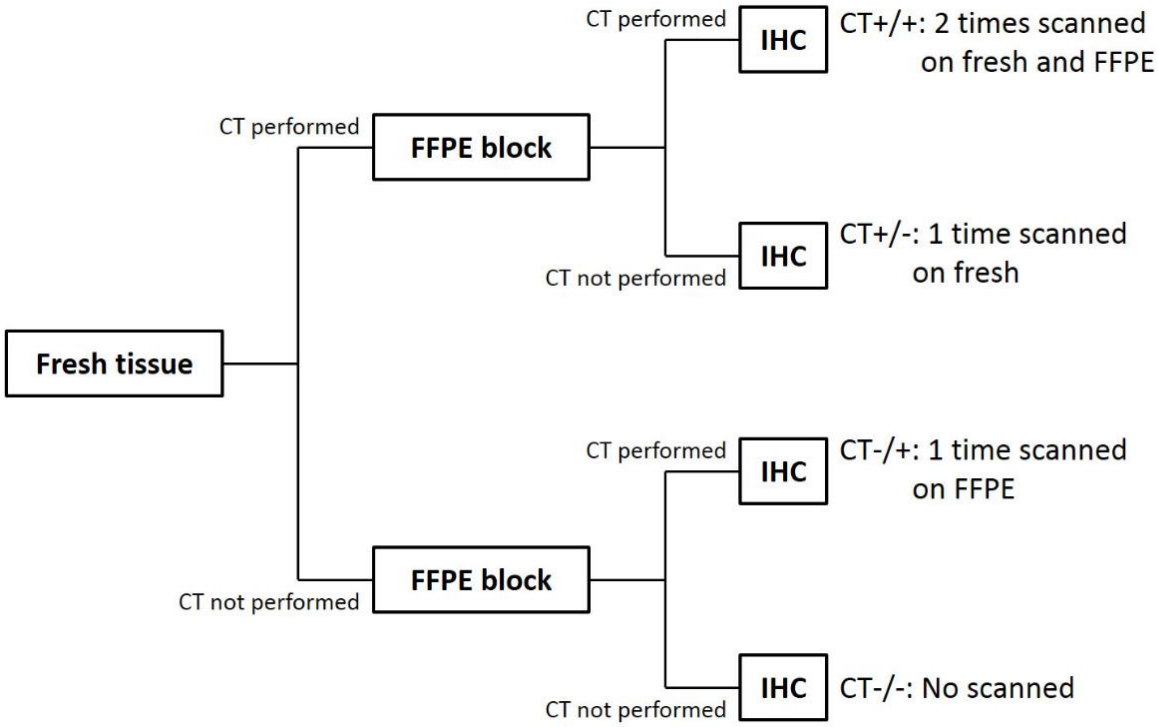

Figure 1 Algorithm of evaluation of radiation damage

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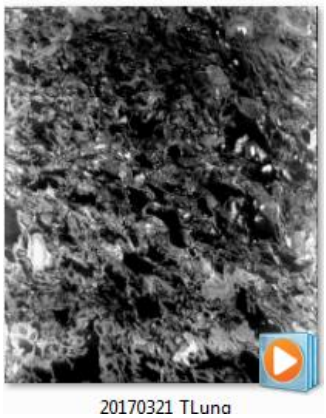
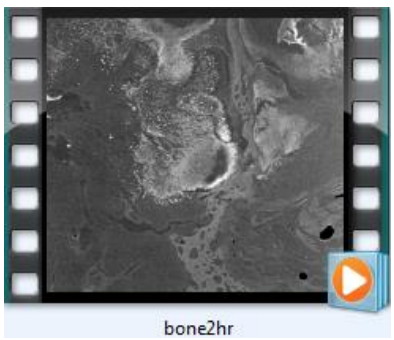
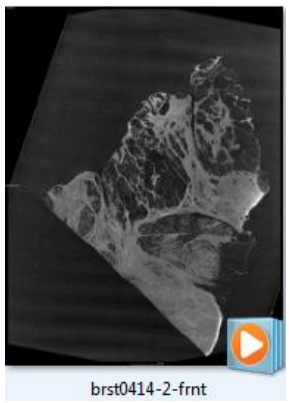
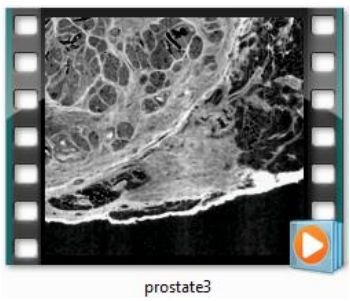
Results

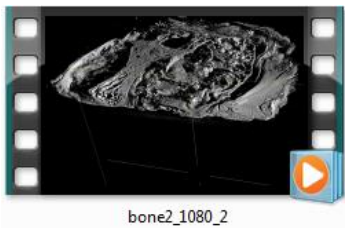
Examples of the obtained micro-CT image sequences are shown in series 1-5: (click the thumbnail to download).











Their details are discussed below.

Sample handling in Fresh tissue scanning

The plastic container (for clinical use), plastic dish, and foam container (created in-house) were used. The images which highlight how each container influenced the resulting micro-CT images is shown in Figure 2 [Figure2A-F]. Resulting images from tissue scanned within the plastic container and plastic dish showed an artifact the shape of the bottom part of each container (See Figure 2D and E) and hid some part of the tissue. The polystyrene foam container, created in-house, produced the highest quality of image with the smallest amount of artifact (Figure 2F). X-ray beams can penetrate plastic easily, but plastic had a similar signal to the scanned tissue and/or reflected the x-ray, which made it difficult to analyze any of the resulting images. On the other hand, x-ray beams easily penetrate polystyrene foam with less absorption because its composition is comprised of more than 90% air. Thus, it enabled the reduction of artifacts and their effect on the image quality of the micro-CT images.

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Evaluation of tissue sample positions in the MicroCT device was conducted. (Figure 2 G and H). Samples that were scanned in a laying (horizontal) position (G), did not show the detailed structure of the sample well. Samples that were scanned in a standing (vertical) position (H) showed the internal structure of the specimens clearer than in a laying position. This is because when scanning flat-lying tissue, a longer distance from the x-ray beam is required for penetration than when the same tissue is scanned in a vertical position.

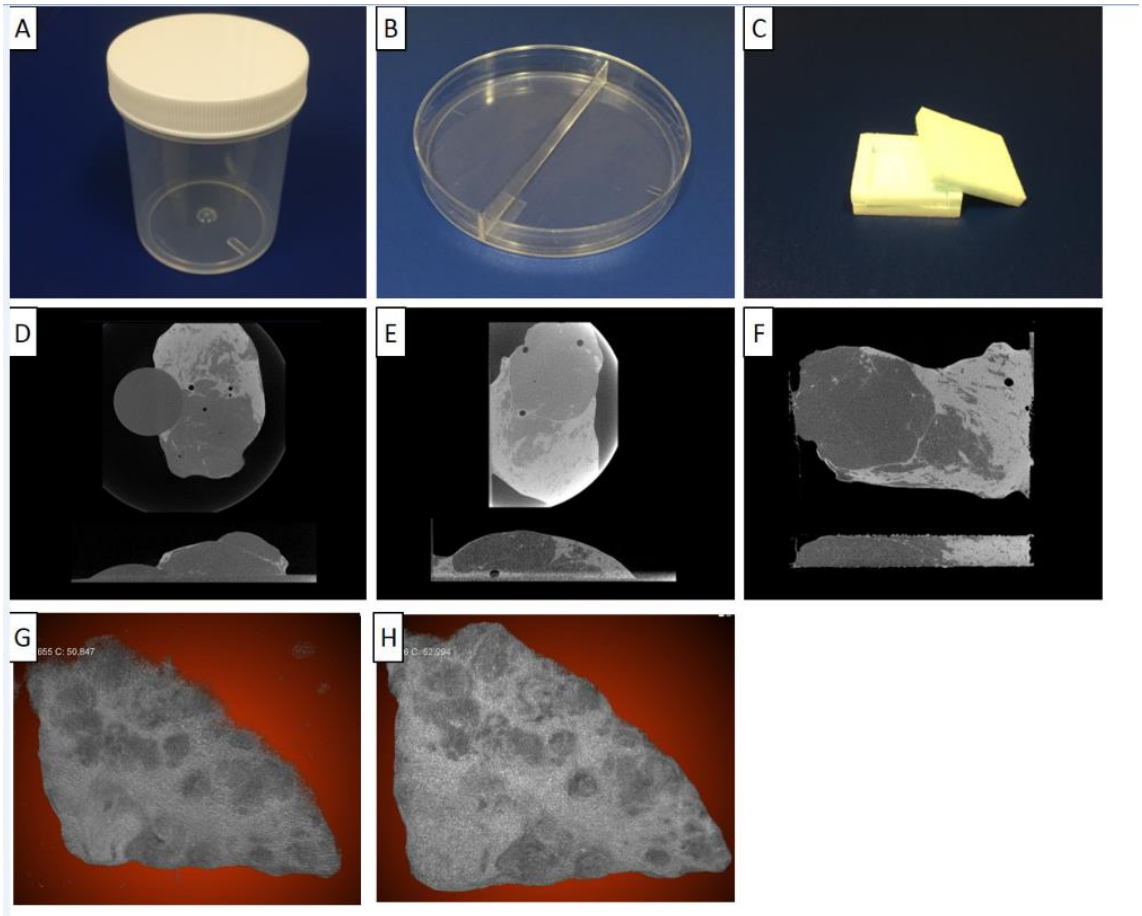

Figure 2: Comparison of fresh tissue scanning with container and positioning. Pictures of plastic container (A), plastic dish (B), and polystyrene foam container (C). Micro-CT image of plastic container (D) and plastic dish (F) had similar or high density compared with tissue samples, and difficult to avoid influence to sample image, but polystyrene foam (F) had quite low density not to impact to sample image.Scanned with laying position (G) was difficult to understand structure, but scanned image with standing position (H) as same sample as (G) was able to observe internal structure in specimen.

Sample handling for FFPE tissue block

The prototype modular FFPE block handling system, Megablock holder, x-y stage table and carbon fiber paddles were tested for 1x1.5 inch and 2x3 inch blocks. Examples of

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tested sample holders were shown in Figure 3(A-E). A-C are for 1x1.5 inch, normal size of block and D-E are for 2x3 inch block. The modular sample handling system A-B is easier to use, but carbon fiber paddles C are more compact and allow slightly higher scan resolution. All these holders are stable enough for 5-24 hours of scanning and all except the Megablock holder are good for batch mode scanning.

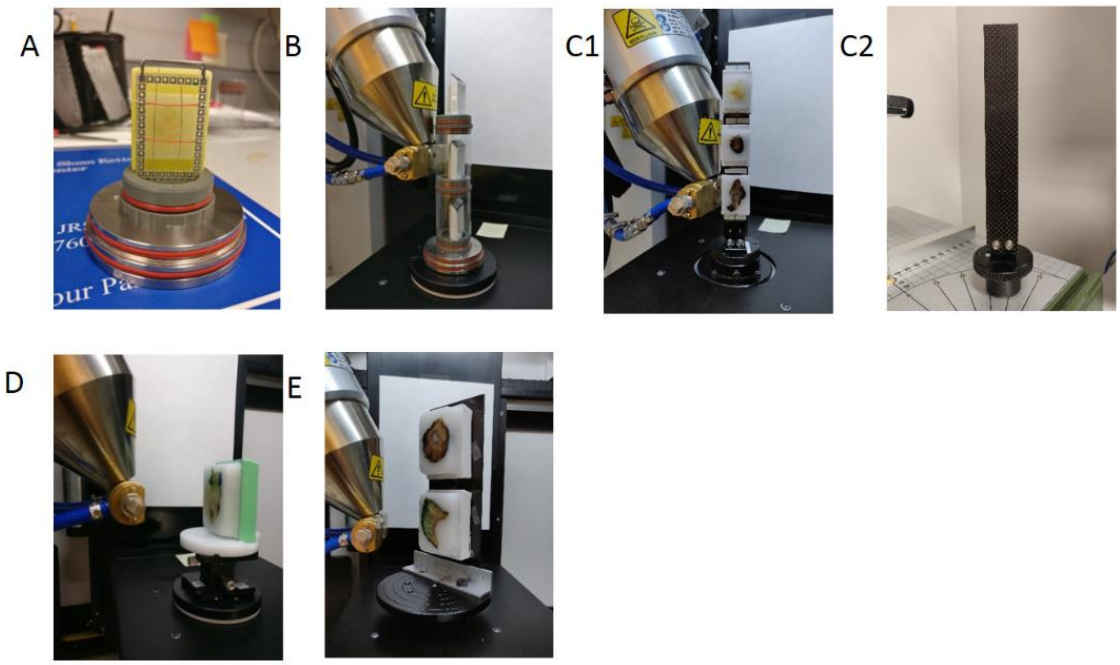

Figure 3: Used sample holders. A, B: Kinematic FFPE tissue block holder set (Nikon modular sample handling system), A: sample holder heavy kinematic base, B: thin stuck tube on rotational table with kinematic mount, C: small carbon fibre paddle, D: mega block folder on x-y stage with rotational table E: large carbon fibre paddle.

Scanning protocol optimization for Fresh tissue

To optimize the scanning protocol, we have tested a variation of scanning parameters such as beam energy, beam current, exposure time, number of projections, and so on. Our aim of optimization was to minimize scanning duration to 5-20 minutes, and to produce images of sufficient quality to detect tumor margins during the intraoperative process. An optimized protocol is shown in Table 1. One of common issues that arise when using the Micro-CT scanning system is the presence of ring artifact which is a CT phenomenon due to mis-calibration or the failure of one or more detector elements in a CT scanner. We often see a ring shaped shadow in the images. Scanning time can be estimated by combination of the number of projections and exposure time. Strong beam energy and beam current improves x-ray penetration and the contrast of the scanned image, however the images looked more coarse and granular. Initially, we tested

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different parameters per tissue type. For example, breast tissue had a high-quality of distinguishable contrast. The reconstructed micro-CT image of breast samples enabled the detection of adipose tissue which appeared as a lower density area and stromal or neoplastic lesion which appeared as a high density area [Figure4A]. Figure4 showed that an extensive high density mass at the center of the specimen was present [Figure 4B]. Adjacent to this mass, partitioned by fine linear moderate density, a ground glass opacity lesion was visible [Figure4C]. Additionally, a reticular shadow with broad band-like high density was seen [Figure4D]. In correlation to a histology specimen, a high density mass indicated expansive and solid proliferation of tumor cells [Figure4J]. Tumor cell proliferation was also seen in ground glass opacity [Figure4K] and reticular lesions [Figure4L]. After 59 tissue samples and 178 scans, we decided to use a Beam energy of 90kV, Beam current 133 µA, Beam Power 12.0 W, Exposure time, 250 msec, Number of projections 1501, and Frame per projection 1 as our optimization parameter for fresh tissue.

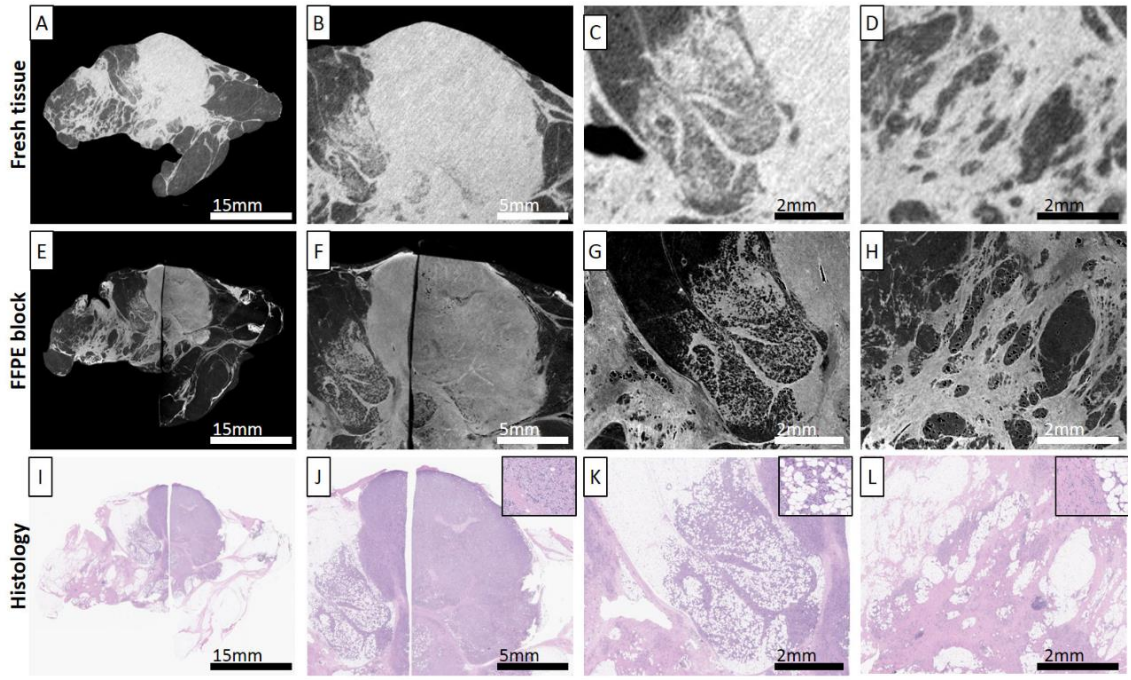

Figure 4: Correlation among micro-CT of fresh tissue, FFPE block, and HE histology of breast. Micro-CT of fresh tissue (A-D), micro-CT of FFPE block (E-H), and HE histology (I-L). (A), (E), (I) Whole image. (B), (F), (J) Expansive mass indicated main tumor lesion. (C), (G), (K) Cancer cells infiltrated into fat. (D), (H), (L) Cancer cells infiltrated into collagenous stroma. Abbreviation: CT, computed tomography; FFPE, formalin fixed paraffin embedded.

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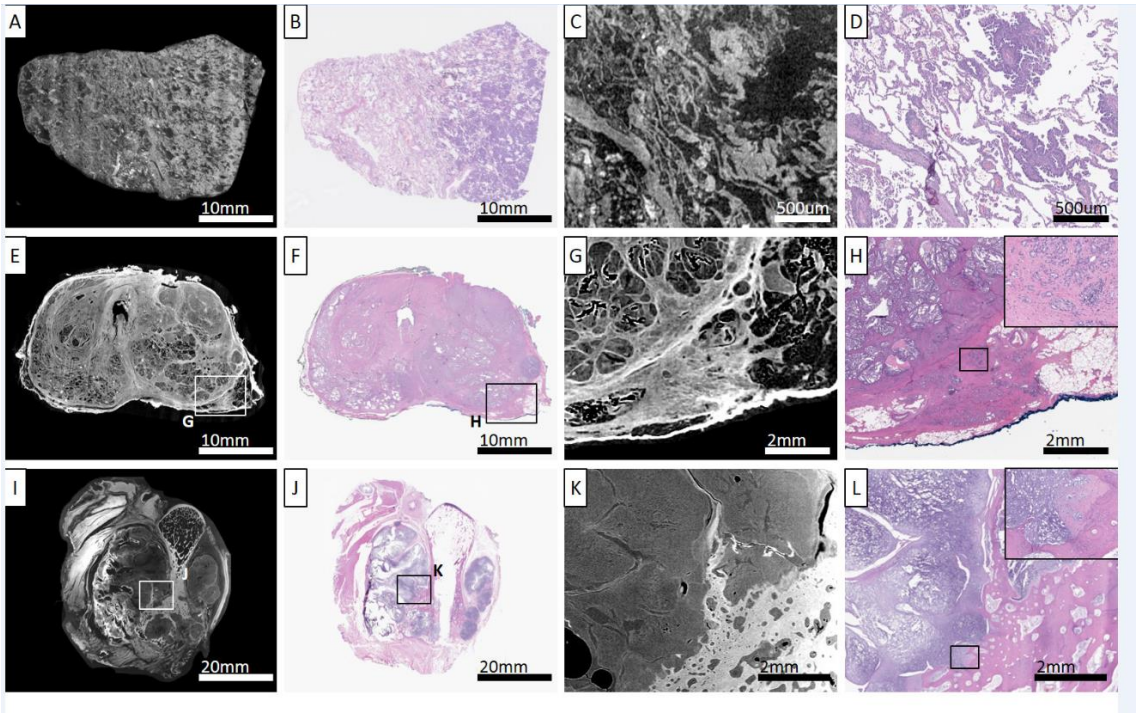

Figure 5: Correlation between micro-CT and histology in various organs. (A-D) Lung. Entire image of block in micro-CT (A) and histology (B). Border area included adenocarcinoma with lepidic growth and normal area was shown in micro-CT (C) and histology (D). (E-H) Prostate. Entire image of micro-CT (E) and histology (F). Extracapsular invasion of cancer showed by micro-CT (G) and histology (H). Abbreviation: CT, computed tomograph.y

Scanning protocol optimization for FFPE tissue block

Protocol optimization for the Scanning of FFPE tissue block was evaluated in the same manner as fresh tissue. The settings for most parameters were the same for all tissue types and both block sizes (1x1.5 and 2x3). The beam energy, beam current, number of projections and frames per projection [Table2] were the same. Only the exposure time had to be modified by tissue type. For example, micro-CT images of breast blocks indicated that tumor cells proliferated expansively and diffusely [Figure4 E-H]. The images of lung blocks were feasible to investigate and understand growth patterns [Figure4A-D]. Prostate CT images suggested localization and the extracapsular invasion of tumor components [Figure4E-H]. Furthermore, CT images of bone tumors enabled the investigation of the relationship between tumor and bone density and tumor type [Figure4I-L]. These images were correlated to histology specimen in 4-10x magnification.

Evaluation of radiation damage

IHC for all antibodies was performed and evaluated in four sample categories: 1) scanned twice, once fresh and once as FFPE block, 2) scanned one time as fresh sample,

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3) scanned one time as FFPE block, and 4) never scanned. We have evaluated all slides with both a microscope and WSIs. Some of IHC stains were required to evaluate stained intensity. To avoid any influence by color fidelity of an imaging device or monitor, we have confirmed each WSI evaluation with a microscope as well. We did not find any effect on protein expression after applying IHC staining to all of the samples from all four categories [Figure 6].

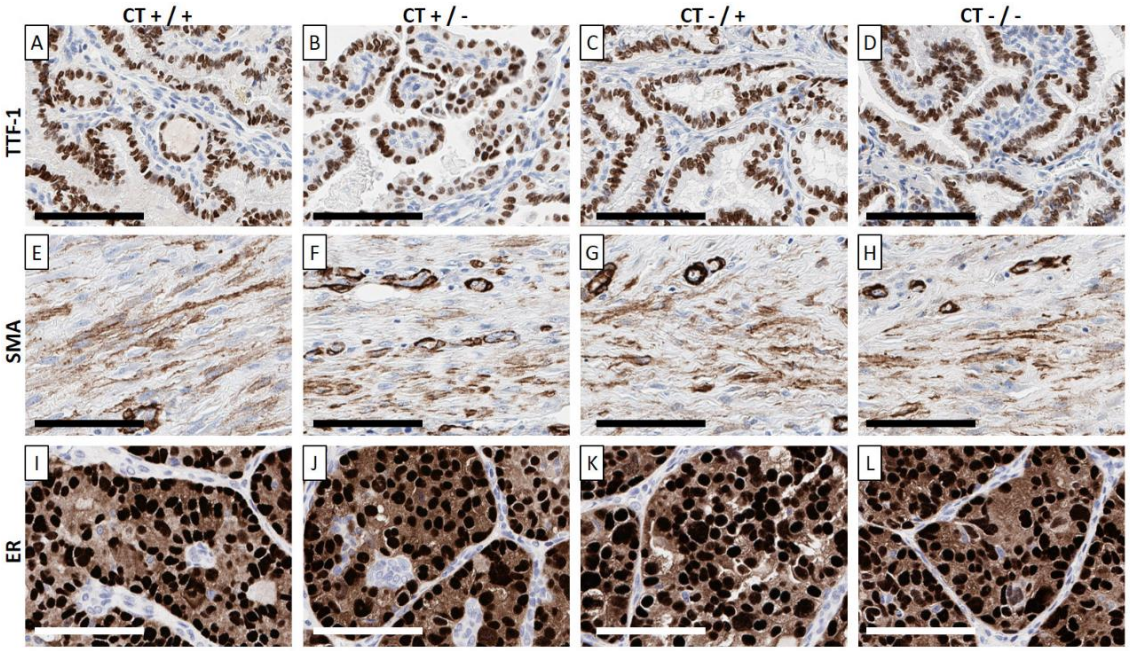

Figure 6: Checking of radiation damage by IHC. (A-D) TTF-1 is positive for nuclei of papillary carcinoma of thyroid gland. (E-H) SMA is positive for cytoplasm of fibroblasts and capillary wall in stroma of breast. (I-L) ER is positive for nuclei and cytoplasm of invasive ductal carcinoma of breast. Status of micro-CT scan in each column are (A), (E), (I) were scanned in fresh and block; (B), (F), (J) were scanned in fresh only; (C), (G), (K) scanned in block only; and (D), (H), (L) not scanned. All bar scales are 100um. Abbreviation: IHC, immunohistochemistry; CT, computed tomography; TTF-1, thyroid transcription factor 1; SMA, smooth muscle actin; ER, estrogen receptor.

Discussion

Micro-CT technology was originally developed for applications which required a non-destructive examination technique. However, we identified that with some technological modifications, its potential application in Pathology was not only possible, but could improve current patient care. Several reports suggested that micro-CT was used in areas of pathology for the analysis of neuron networks, identification of breast cancer, and the steric structure of idiopathic pulmonary fibrosis (1) (2) (3) (6) (7).

Challenges that arise when assessing the micro-CT's utility in pathology are due to the variety of organs and sample types analyzed by Pathologists when making a diagnosis.

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Depending on the organ such as lung or liver, the structure and components of the tissue are different. An additional challenge arises from the various sample types analyzed i.e. fresh, fixed and FFPE blocks. The appropriate scanning conditions have to be decided to ensure that images of the highest quality are produced from micro-CT scanning. Since a standard scanning protocol was not available, our SOP would provide guidance to whomever would like to scan pathological samples in any form, with micro-CT.

Fresh tissue samples

After scanning approximately 170 samples hundreds' times, we were able to create an optimized scanning protocol (SOP), which requires that only a few parameters are changed with each condition setting.

For fresh tissue, only one setting can be used for all 6 different organ systems we have tested. Scanning duration was always less than 15 minutes which proved suitable for use in the detection of intraoperative tumor margins. The most important factor which brought us meaningful optimization was in identifying the ideal position for the scanning of tissue and its container for tissue. The polystyrene foam which was created in-house reduced the presence of artifacts, thus improving image quality and the protection of samples from X-rays. In addition to the polystyrene foam container, the samplers were scanned standing in an up-right position. This produced significantly less "noise" in the resulting images. These conditions enabled the investigation of many sample properties such as density, compressive strength, tensile strength, etc. We found that the polystyrene foam was the most effective despite the facts that sample size and figure may vary by sample.

To minimize the amount of time needed for sample scanning, we have created polystyrene foam containers in several different sizes. Cutting the polystyrene foam was not as easy as we expected. An electric polystyrene foam cutter was selected to cut the foam into the form and sizes we designed. Because of tissue handling optimization, we could detect tumor margins in samples after only 15 minutes of scanning. In fact, the average scanning duration for fresh tissue was 6 minutes. The micro-CT images resulting from our optimized SOP enabled us to understand the location and/or spreading of a lesion before cutting a surgical specimen, even though the scanning was finished in just 6 minutes. It was true that scanning within a short time frame had limitations, such as reduced recognition of fine structures, but our SOP established that this time was sufficient for intra-operative diagnosis and to make frozen section slides from accurate sites of lesion and surgical margins. Lumpectomy patients will be identified with a surgical margin that is positive for tumor and requires re-excision in 18-59% of cases (10)

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(11) (12), so that micro-CT may contribute to a reduced the risk of breast cancer recurrence.

FFPE blocks

When scanning FFPE blocks in the MicroCT scanner, we have found universal values in most of the key parameters, except exposure time for both 1x1.5 and 2x3 inch blocks. The exposure time should be altered in each organ to account for differences in x-ray penetration and contrast. Thus, only adjusting the exposure time for each sample allows for stable and high-quality scanning. Though this SOP can be applied to other sample types, our guidelines provide for analysis of location, spreading, growth pattern, and the physical relationship of surrounding tissue to a tumor lesion. This means that micro-CT can find the most diagnostic area in the block and indicate the relevant plane for making a diagnosis, eliminating the risk of insufficient sectioning and needless deep sectioning. It can also contribute to saving turnaround time and cost.

The conditions outlined in this SOP allow for the scanning of the entire tissue in a block at once. It is possible to scan at a higher resolution (4-10x), focusing on the areas of interest which were identified in the initial scanning. Currently we are working on automatically scanning FFPE blocks multiple times to create a higher resolution of images, which is the same principal utilized for the whole slide imaging scanner. It is suggested that micro-CT imaging technology could be utilized for clinical pathology diagnosis. Our micro-CT has the unique technology necessary to have an exceptional field of view using a back projection cone beam reconstruction technique. It allows for the scanning of a wide variety of specimens regardless of their size and shape such as biopsy tissue and blocks to resection.

To mimic micro-CT FFPE block images reviewed by pathologists, we digitally generate H&E stains that resemble histology images [Figure 7]. Digital staining is based on differing intensity levels within the images. Pathologists prefer to see the digital H&E images instead of gray scale images. Although digital staining is not exact it does make it easier to see the histological features within the micro-CT image and is useful when we correlate the image with actual H&E stained slides.

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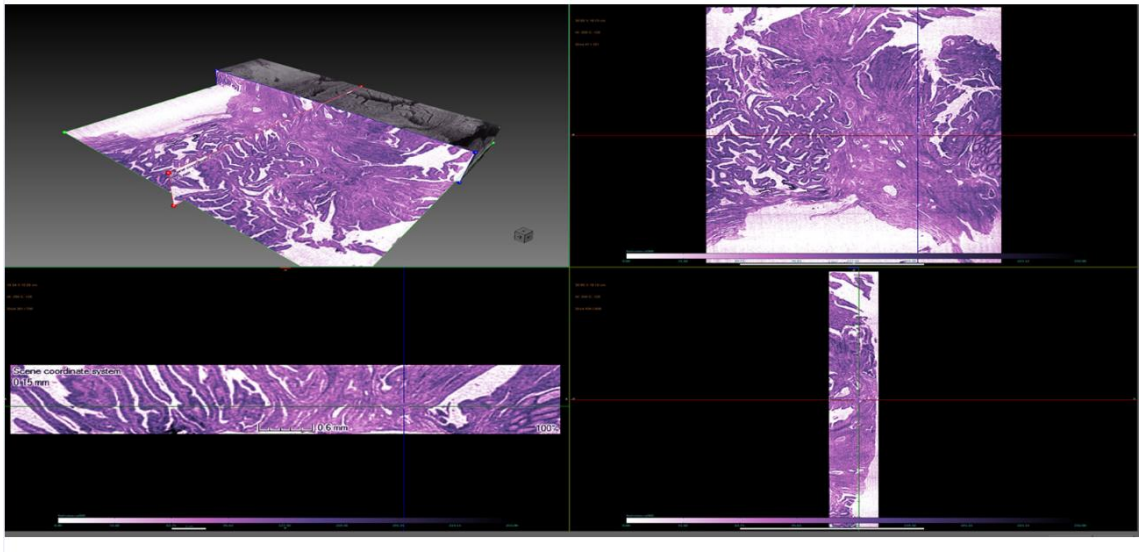

Figure 7: Colon polyp FFPE block. Digital stained H&E like appearance.

It was well known that radiation exposure has an impact on organisms, and that their protein structure is also affected by x-ray crystallography (5). A pathological diagnosis should be decided not only by morphological features, but also by the expression of protein and genes. Furthermore, molecular targeted therapy also needs the results of this diagnosis. Micro-CT's potential to influence pathological diagnosis and therapeutic strategy cannot be ignored. Needles to say, radiation damage for DNA sequencing should be evaluated, regardless of how expensive it is. Thus, our examination of IHC was just performed as a preliminary study with a small number of antibodies, and it showed that micro-CT had no influence on the protein expression of the tissue. Our trial played an important role for clinical introduction of micro-CT. Moving forward, further IHC testing is necessary in addition to the validation of gene expression by in situ hybridization and next-generation sequencing.

We are confident that micro-CT is going to play an important role in medicine as a new imaging modality in the near future. However, there are still many things we have to overcome, such as scanning speed, developing phantoms to have stable data, establishing file format including meta-data, developing a simple and fast viewer for image analysis, etc. We expect that our 20 years of experience in WSI makes our team the best suited to accelerate the speed of micro-CT development.

Conclusion

Micro-CT imaging is a promising new technology in medicine for intra-operative tumor margin detection and also to evaluate whole block histology characteristics without sectioning. It can help to re-construct accurate histology 3D models. It can also

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reconstruct these models despite any possible histology errors such as missing tissue, distortion, etc., in 2D WSIs as well. We also found micro-CT to be safe, as it does not effect protein expression. Micro-CT is still in the development stage however, we have been finding new uses for micro-CT in medicine and have many collaborations with other departments such as the department radiology, surgery, and more, to find additional uses for micro-CT in future patient care.

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Supplementary figures

S1: Configuration of scanned samples

Organs	Sample type Number of scan (number of sample)				Total Number of scan (number of sample)
	FFPE		Fresh	Fixed	
	Usual size	3×2inch			
Breast	75 (30)		130 (45)	64 (26)	269 (101)
Lung	113 (17)		3 (1)		116 (18)

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Liver	14 (4)		11 (4)	9 (1)	34 (9)
Kidney	13 (4)		11 (2)	1 (1)	25 (7)
Bone		14 (5)			14 (5)
Uterus	6 (1)		5 (1)		11 (2)
Lymph Node	3 (2)		7 (2)		10 (4)
Pancreas	9 (2)				9 (2)
Prostate		9 (5)			9 (5)
Adrenal	1 (1)		5 (1)		6 (2)
Thyroid	2 (2)		3 (2)		5 (4)
Upper GI	4 (1)				4 (1)
Gist Recurrence	3 (1)			1 (1)	4 (2)
Lower GI	4 (2)				4 (2)
Soft Tissue	1 (1)		3 (1)		4 (2)
Spleen	3 (1)				3 (1)
Total	251 (69)	23 (10)	178(59)	75(29)	527(167)

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Table

Table 1. Final scanning condition

		Beam energy (kV)	Beam current (uA)	Beam Power (W)	Exposure time (msec)	Number of projections	Frames per projections	Scan Time hh:mm
Fresh tissue		90	133	12.0	250	1501	1	00:06
FFPE block	Lung	70	100	7.0	2500	4821	4	16:50
	Breast	70	100	7.0	2500	4821	4	16:50
	Prostate	70	100	7.0	3500	4821	4	23:30
	Bone and soft tissue	70	100	7.0	3000	4821	4	20:10

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