The Role of Micro-CT in 3D Histology Imaging

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Key Words

3D reconstruction · 3D Slicer · High-resolution 3D histology · ImageJ · Micro-CT · Volume measurements

Abstract

Objectives: 3D histology tissue modeling is a useful analytical technique for understanding anatomy and disease at the cellular level. However, the current accuracy of 3D histology technology is largely unknown, and errors, misalignment and missing information are common in 3D tissue reconstruction. We used micro-CT imaging technology to better understand these issues and the relationship between fresh tissue and its 3D histology counterpart. **Methods:** We imaged formalin-fixed and 2% Lugol-stained mouse brain, human uterus and human lung tissue with micro-CT. We then conducted image analyses on the tissues before and after paraffin embedding using 3D Slicer and ImageJ software to understand how tissue changes between the fixation and embedding steps. **Results:** We found that all tissue samples decreased in volume by 19.2–61.5% after embedding, that micro-CT imaging can be used to assess the integrity of tissue blocks, and that micro-CT analysis can help to design an optimized tissue-sectioning protocol. **Conclusions:** Micro-CT reference data help to identify where and to what extent tissue was lost or damaged during slide production, provides valuable anatomical information for reconstructing

missing parts of a 3D tissue model, and aids in correcting reconstruction errors when fitting the image information in vivo and ex vivo.

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Introduction

The 3D reconstruction of whole-slide images has proven to be valuable in the visualization and diagnosis of disease beyond the 2D microscope slide [1]. 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 glass histology [2–6]. We are interested in using multimodality 3D imaging and 3D histology image data to accurately model, analyze, understand and test disease at high resolution in tissue samples imaged at $\times 20$ to $\times 40$ (0.46–0.23 $\mu\text{m}/\text{pixel}$). However, while high-resolution 3D histology is useful for 3D image analysis, including cell counting, volume-of-interest segmentation and volume measurement, its accuracy at the cellular level is unknown, and its relationship to fresh or embedded tissue has not been well studied. In order to conduct these basic analyses, accurately reconstructed histology models are required across all three spatial dimensions, yet inaccuracies within 3D histology recon-

Fig. 1. Current 3D histology workflow. Tissue samples (**a**) are fixed in formalin solution for preservation (**b**) and then embedded in paraffin wax tissue blocks (**c**). Tissue blocks are then sectioned by an automated sectioning machine (**d**), and the resulting slides undergo standard HE staining (**e**). Individual slides are then scanned to produce whole-slide images (**f**), aligned in an image stack with Voloom (microDimensions, Munich, Germany) software (**g**), reconstructed into 3D models (**h**) and lastly analyzed (**i**). Micro-CT imaging was incorporated into the workflow before and after paraffin embedding.

structions arise during tissue and slide preparation [7]. If we intend to improve the accuracy of 3D tissue models, we need to know precisely what happens to tissue between individual preparation steps. We believe that micro-CT technology can eliminate these inaccuracies and help us to better understand and improve the existing 3D histology workflow (fig. 1).

3D micro-CT has long been used as a high-resolution imaging technology in industries, such as aircraft engineering, fracking and microprocessor production, due to the X-ray-attenuating properties of dense materials such as rock and metal. However, micro-CT is an emerging technology within the biomedical field and holds great promise for imaging tissue specimens, like postoperative lumpectomy samples, for reconstruction, modeling and analysis in 3D [8]. The capacity of micro-CT to create high-resolution 3D models of ex vivo tissue, therefore, creates a unique opportunity to correlate image data with other imaging modalities. One of our target organs for multimodality image correlation, the human brain, was scanned in vivo and ex vivo by MRI in one of our previous studies and presents one such opportunity. However, we are particularly interested in correlating histology whole-slide images with micro-CT tissue scans. Correlating data from the two modalities could provide useful insight into how to improve the reconstruction and accuracy of current 3D histology models by providing information on where and to what extent tissue was lost or damaged during slide production or misaligned during

3D reconstruction [9]. Therefore, micro-CT technology has the capacity to fill in gaps in image data where the current 3D histology workflow is limited.

In this paper, we focus on the role of micro-CT within the 3D histology workflow in order to understand the morphological changes to tissue that take place during the process. We do this by measuring specimen volume from micro-CT data at two steps: formalin fixation and paraffin embedding. Comparing volume size of fresh tissue samples for 3D research is relatively uncommon, yet such an approach allows us to accumulate reference tissue data both to improve the accuracy of 3D histology models and to elucidate the relationship between cellular and radiological image information. Specifically, our objectives are: (1) to collect reference data to investigate tissue changes during the production of histology slides; (2) to implement quality control for formalin-fixed, paraffin-embedded tissue blocks in order to detect and prevent tissue-sectioning failures, and (3) to ultimately design an efficient and effective tissue-sectioning protocol for 3D histology reconstruction.

Methods

Specimens

We imaged and analyzed three types of tissue samples in this study: mouse brain, human lung tissue and human uterus tissue. All tissues analyzed were discarded materials from outside collaborators and the histology laboratory at our institution. The uterus

Fig. 2. Comparison of 2% Lugol-stained (**a**; 70 kV, 20 W, 250 ms) versus unstained (**b**; 50 kV, 20 W, 250 ms) lung tissue imaged with micro-CT.

and lung samples were each cut in half, and each half was analyzed separately, bringing the total number of samples examined in this study to five.

Fixation

All five samples had been fixed in formalin solution prior to our acquisition and remained in formalin before and after micro-CT imaging until they were embedded.

Staining

In order to correlate micro-CT data with 3D histology models and other imaging modalities, accurate and anatomically detailed images are required from each. However, soft biological tissues lack the high X-ray contrast properties that hard, dense materials like bone and metal possess. Most soft tissues, therefore, pose a challenge to standard X-ray imaging techniques. In order to enhance contrast in the tissues, we stained three out of our five samples with 2% Lugol's iodine solution for 1 week: the mouse brain, one uterus sample and one lung sample. Iodine's semimetallic properties have been shown to enhance nonhuman soft-tissue X-ray contrast and enable the high-resolution visualization of minute morphological details [10], a necessity for accurately correlating anatomical information between micro-CT tissue data, histology slides and 3D histology models. The remaining two uterus and lung samples were left unstained but remained in formalin fixative until they were embedded. They were analyzed in a comparative capacity to the stained samples in order to assess the usefulness of using Lugol's solution to stain human tissue samples. Differences in the micro-CT image quality and level of detail between stained and nonstained tissue samples can be seen in figure 2. We note that the scan parameters for these images were optimized on an individual basis. That is, scan parameters that produced the best results for Lugol-stained samples do not necessarily apply for unstained samples. The low-contrast micro-CT image of the unstained lung in figure 2b could be attributed to its low X-ray absorption properties.

Washing

All specimens were washed in distilled water before and after Lugol's staining in two separate 1-hour washes in order to remove

as much formalin from the samples before staining and to remove as much Lugol's solution as possible before embedding. Lugol's staining has been shown to have no apparent effect on the standard hematoxylin and eosin (HE) tissue staining process [11], but we sought to remove as much as possible from the tissue samples before embedding in order to remove unwanted sources of error during any future 3D histology reconstructions.

Embedding

All specimens were embedded in a TissuePrep ($58.0 \pm 0.5^\circ\text{C}$) high-melting-point paraffin wax tissue block at our institution's histology laboratory.

Micro-CT Imaging

All tissue samples and subsequent paraffin-embedded tissue blocks were imaged with micro-CT (Nikon XT H 225, Nikon, USA) at 50–70 kV and 10–30 $\mu\text{m}/\text{voxel}$ resolution. Scan parameters for each specimen type can be seen in table 1. Scan parameters for each sample were chosen on an individual basis in order to produce highest-quality 3D reconstructions. The mouse brain was mounted in 2% agarose gel for improved imaging stability, and the uterus and lung samples were imaged on the underside of a Falcon tube cap, which was taped down to the micro-CT imaging stage. All tissue blocks were also taped to the stage for improved scan stability.

Image Analysis

(Fiji Is Just) ImageJ (NIH, USA) was used to open the raw 32-bit micro-CT image files, assess scan quality and convert image stacks to TIF format for subsequent analysis. 3D Slicer (Brigham and Women's Hospital, Boston, Mass., USA) was used to segment tissue and conduct volume measurements. First, a Gaussian blur filter ($\sigma = 1-5$) was applied to all micro-CT images in 3D Slicer in order to reduce small-scale data heterogeneity and to ease the computational task of tissue segmentation. Segmentation was conducted by both intensity thresholding and simple region-growing algorithms, and 3D models were generated, compared and analyzed. All image scales were obtained from resolution data that were determined automatically by geometric magnification and stored within Nikon's .vgi micro-CT scan files. Final volume calculations

Table 1. Nikon XT H 225 micro-CT scan parameters

Sample	Projections, n	Frames, n/projection	Energy, kV	Power, W	Exposure, ms	Resolution, $\mu\text{m}/\text{voxel}$
Stained mouse brain	1,200	16	60	20	250	17.5
Stained mouse brain tissue block	4,500	2	50	30.7	250	32.5
Stained uterus	2,400	2	70	20	250	10.3
Stained uterus tissue block	4,500	2	50	30.7	250	29.2
Stained lung	2,400	2	60	20	250	10.9
Stained lung tissue block	4,500	2	50	30.7	250	29.2
Unstained uterus	2,400	2	60	20	250	23.2
Unstained uterus tissue block	2,400	2	50	30.7	250	29.9
Unstained lung	2,400	2	50	20	250	23.2
Unstained lung tissue block	4,500	2	50	30.7	250	29.9

(displayed in table 2) are all values generated by intensity thresholding segmentation. Intensity thresholding segmentation was ultimately deemed more accurate and reliable for our study than simple region growing for two reasons: (1) simple region-growing segmentation often resulted in ‘islands’ of missing data that had to be identified and filled in, which sometimes also filled in natural anatomical gaps in our tissue samples, and (2) because intensity thresholding was much faster and less computationally intensive than running the simple region-growing algorithm. All tissue block measurements were conducted using the line measuring tool on ImageJ.

Image Measurement Validation

We used the image resolution value calculated automatically by the Nikon XT H 225 micro-CT machine after every scan to set the image scale for the calculation of the sample volume. To assess the accuracy of the value, we tested the resolution of the machine by using it to set the image scale for measuring two known lengths. We measured the length of one of the tissue blocks again with the line measuring tool from a photograph of the tissue block next to a ruler, which was used to manually set the image scale. This measurement was compared to that of the micro-CT image of the tissue block where the scan resolution was used to set the image scale. The two measurements differed by 0.2%, suggesting that the micro-CT image resolution indeed could be used as a reliable image scale. The relation between the volume measurements of the micro-CT-scanned images and scanned histology slide images was also investigated. In this case, we compared the measurements between the length of the widest cross-sectional micro-CT diameter of a previously stained mouse brain and its later histology slide section. A summary of the results from these preliminary data can be seen in figure 3.

Results

Tissue Volume

We were able to assess volume changes in all five tissue samples. Among the samples analyzed, the unstained lung sample showed the largest decrease in volume from 650.61

Table 2. Volume change for each tissue sample

Sample	Volume, mm^3		Decrease, %
	fixed	embedded	
Stained mouse brain	648.99	461.95	28.8
Stained uterus	369.16	298.36	19.2
Stained lung	854.72	364.72	57.3
Unstained uterus	448.46	247.74	44.8
Unstained lung	650.61	250.40	61.5

to 250.40 mm^3 , a decrease of 61.5%. Next, the stained lung sample showed a decrease in volume from 854.72 to 364.72 mm^3 , a decrease of 57.3%. The unstained uterus showed the third largest volume decrease from 448.46 to 247.74 mm^3 , a decrease of 44.8%. The stained mouse brain showed the fourth largest volume decrease from 648.99 to 461.95 mm^3 , a decrease of 28.8%. Finally, the stained uterus sample showed the smallest volume decrease from 369.16 to 298.36 mm^3 , a decrease of 19.2%. These results are summarized in table 2 and figure 4. Pre- and postembedded micro-CT and 3D volume images may be seen in figure 5. We would like to note that the orientations of the postembedded samples did not perfectly align to their pre-embedded orientations as imaged by the micro-CT. This was likely through lack of sufficient tissue contrast in the unstained sample and a high degree of shrinkage and structural distortion in the stained sample due to the effects of tissue embedding. Together, both issues limited the 3D image correlation of the pre- and postembedded samples along the same sectioning planes and orientations. Furthermore, the images in figures 5a and b also demonstrate changes in the physical properties of the tissue after embedding.

Fig. 3. Differences in length measurement setting image scale with micro-CT resolution (**a, c**) versus known distance (**b, d**) for the ImageJ line measuring tool. The ruler was used to set the scale in image **b**, while the NanoZoomer native resolution was used to set the scale in image **d**. The measurement in image **d** was calculated using the NanoZoomer 'linear measure' annotation feature. A 0.2% difference in measurements was found for the tissue block, and a 15.7% difference was found for the brain slice.

Fig. 4. Graph of volume changes during embedding. Tissue volumes were analyzed with 3D Slicer. Segmentation was conducted by intensity thresholding, and tissue volume was calculated by the label statistics function. The 3D image scale was set using resolution values automatically generated by the Nikon XT H 225 machine.

Tissue Block Quality

We also examined tissue block quality using (Fiji Is Just) ImageJ from the reconstructed micro-CT data. We found that all five contained embedding artifacts, such as air bubbles and/or cracks in the paraffin wax block, which negatively affect the quality of serial sections and the ultimate outcome of histology slides and their reconstructed 3D tissue models. An example of a tissue block with both air bubbles and cracks may be seen in figure 6a.

Tissue Sectioning

Finally, we used the stained mouse brain micro-CT data to carry out tissue block measurements for designing an optimized tissue-sectioning protocol. We used the ImageJ line measuring tool to determine the thickness of wax in millimeters above the specimen (0.393 mm) and the distance from the top of the tissue block to the crack artifact near the bottom (9.633 mm). These measurements can be seen in figure 6. Poor infiltration and incomplete adhesion of the mounted blocks likely account for the observed defects in the paraffin blocks.

Fig. 5. Pre-embedded micro-CT images (**a**), postembedded micro-CT images (**b**) and 3D reconstructions of pre- (left) and postembedded (right) tissue samples (**c**). 3D volumes were segmented by intensity thresholding and generated with 3D Slicer.

Fig. 6. Cracks (green arrows; see online version for colors) and air bubbles (red circles) are common embedding artifacts that can be detected with micro-CT imaging (**a**). Measurements were taken to find the depth of the crack artifact (**a**) and to find the thickness of wax above the embedded specimen (**b**).

Histology and Micro-CT Image Measurements

We compared measurements between the length of the widest cross-sectional micro-CT diameter of a previously stained mouse brain and its later histology slide section, and found that the histology measurement was longer than the micro-CT measurement by 15.7%. Nevertheless, both lengths measured with the micro-CT resolution image scale were smaller than those measured with the known scale. This finding suggests that the reported postembedded volumes of our tissue specimens are likely underestimates of their true volumes.

Discussion

As table 2 shows, a sizable difference in volume exists between the pre- and postembedded tissue samples. We expected a small decrease in volume to occur during the paraffin-embedding process due to sample dehydration from exposure to high temperatures. However, a volume decrease of 20–60% was largely unexpected. Even if the tissue samples were stretched or otherwise physically distorted during paraffin embedding, we would expect roughly the same amount of tissue to remain within the tissue block, leading perhaps to a smaller but fairly comparable final volume.

Tissue volume may have decreased much more than expected for a number of reasons. (1) The resolution values that we used to set the image scale for our volume calculations may have been slightly inaccurate and may have introduced a degree of error into our final volume measurements. These resolution values were calculated automatically by the Nikon XT H 225 micro-CT machine though geometric magnification and were obtained from the vgi file created after every image scan. Specifically, we found a 15.7% difference in linear measurements between histology and micro-CT-scanned images. This difference could be due to the effect of tissue processing, i.e. tissue was distorted during embedding. Further study may help to elucidate the sources of this discrepancy in detail.

Contrast staining, or perhaps any intervening step that removes tissue from its fixative, also may have affected final volume size. Formalin was washed from samples prior to staining, and both stained uterus and lung samples showed a smaller decrease in volume than the unstained samples. If volume decrease may be primarily attributed to water loss during the paraffin-embedding process, perhaps the presence of a staining agent, the lack of a fixative or the combination of both helped stained tissue samples to retain a greater amount of fluid during embedding.

However, while both unstained samples showed a greater decrease in volume than the stained samples, the difference between unstained and stained volume changes was greater for the uterus than for the lung. The unstained uterus sample decreased in volume by 44.8%, while the stained uterus sample decreased by 19.2%. On the other hand, the unstained lung sample decreased in volume by 60.5% while the stained lung sample decreased in volume by 57.3%, which is consistent with the results presented previously [12]. This difference in volume change between the two tissue types is surprising and may be partially attributed to variations in the machine resolution for different scan parameters, segmentation errors, differences in tissue sample density and difficulty imaging the embedded, unstained lung tissue with micro-CT. Pre-embedded images of the unstained lung sample were acquired without problem, but the embedded sample poorly attenuated micro-CT X-rays, which made segmentation and volume measurements difficult and imprecise. Therefore, the embedded, unstained lung volume is likely smaller than measured due to a significant degree of excess segmentation from insufficient contrast between the tissue and the paraffin. This means that the true embedded, unstained lung volume change is likely greater than reported and more similar to that of the uterus.

Tissue volume changes also appeared to be inversely associated with tissue density. The lung, the least dense tissue analyzed, seemed to exhibit the largest decrease in volume, while the uterus, the most dense tissue analyzed, seemed to exhibit the smallest decrease in volume. The mouse brain's density and volume change were both intermediate to those of the other two samples. This relationship made sense because the lung tissue, balloon-like in structure, seemed to absorb the greatest amount of fluid when submerged in formalin and Lugol's staining, while the uterus appeared to absorb the least. Again, the mouse brain was intermediate. As a result, the lung tissue would have been more likely to lose more fluid during the embedding process than either the uterus or mouse brain tissue, leading to the greatest decrease in tissue volume among the three samples. Such variation in tissue shrinkage could impact the accuracy of quantitative measurements in tissue [12]. Information on the shrinkage characteristics of the different tissue types that we initially gathered in this work gives valuable inputs for designing an experimental setup involving a larger number of samples to come up with an effective embedding protocol for each tissue type, such as using wax for embedding [11, 13].

Finally, consistent with the findings reported [13], we found that micro-CT tissue block analysis is extremely useful in designing an effective tissue-sectioning protocol. Tissue block length measurements are useful in making the sectioning process more efficient for two reasons. (1) Because the machine can be programmed to cut away the excess wax above the specimen and (2) because the machine can be programmed to stop sectioning at a user-specified depth before an embedding artifact like an air bubble or crack is reached. Removing the top layer of wax can decrease tissue-sectioning time by eliminating the need for extra sectioning above the sample and can save money by producing fewer slides to be stained. Halting sectioning before an embedding artifact is reached can help prevent the production of low-quality or distorted tissue slides that complicate the reconstruction of accurate 3D tissue models. Furthermore, micro-CT tissue block analysis can provide information on the optimal angle at which to section tissue blocks based on the orientation inside of the embedded tissue. Having the capacity to measure distances within the tissue block as well as the best angle for sectioning, a given specimen greatly reduces the number of slides that must be stained and improves the overall efficiency of the sectioning process, saving researchers' time and money. We believe that the most effective tissue-sectioning technique, therefore, uti-

lizes individual micro-CT tissue block analysis prior to sectioning in order to both assess tissue block quality and to determine an appropriate and tissue-specific sectioning protocol for generating 3D histology reconstruction with the highest possible quality.

While some of our initial results were surprising, the data that we collected begin to elucidate elements of the 3D histology workflow that are seldom examined and facilitate the understanding of a pipeline to correspond in vivo and ex vivo imaging modalities [2–6]. Our results also indicate that micro-CT imaging technology is a very useful addition to the current workflow through its capacity to provide information on tissue morphology, volume of interest, tissue block quality and reference data for

3D histology models. Coupled with automated tissue-sectioning technology [14], we believe that incorporating micro-CT imaging into the existing workflow will help to reduce tissue slide errors and provide a foundation on which to greatly improve the quality and accuracy of 3D histology reconstruction capabilities. In the future, we hope to develop an automatic method of correlating histology and micro-CT data to create virtual histology slices that maintain original figures and sizes.

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