Application of the nano3DX X-ray microscope to biological specimens

Naoki Kunishima*

1. Introduction

Structural biology aims to understand life from observation of relevant biological structures and then to extend that knowledge to advance medicine, pharmaceutical development and so on. Because biological systems generally have hierarchical structures, the observation of biological systems varies in size range whether one is looking at tissue level structures, cellular structures or at molecular structures. Observation of these various biological samples is currently accomplished using various methodologies, including electron microscopy and visible-light microscopy. In this report, we will discuss an X-ray imaging of structures that range in size down to sub-micrometers. The nano3DX from Rigaku is a laboratory-based high-resolution X-ray microscope that uses a high-brilliance X-ray generator, a quasi-parallel beam technology and a high-resolution X-ray camera[1]. A major benefit of X-ray microscopes is their ability to examine thick specimens by taking advantage of the high permeability of X-rays to observe a sample's inner structure. X-ray microscopy bears a complementary relationship to other methodologies like electron microscopy that affords high resolution but is not suitable for thick specimens and light microscopy that allows live imaging of specimens. Further developments continue in the area X-ray microscopy, particularly for biological applications.

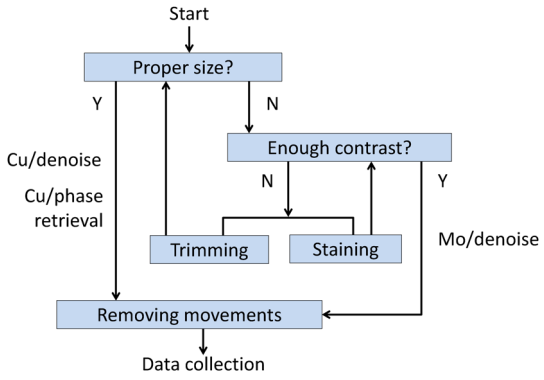

Fig. 1. Common workflow for X-ray microscopy.

2. Procedure for X-ray Microscopy Observation Using nano3DX

The X-ray microscopy technique measures two-dimensional projection images collected at a number of rotational angles. These 2D images are then used to reconstruct a three-dimensional image of the structure using a computational method called computed tomography (CT)[2]. The nano3DX offers an advantage over the other X-ray microscopes in that the system utilizes parallel beam geometry rather than X-ray divergence for magnification and is therefore immune to image blurring and loss of resolution caused by X-ray focus broadening, vibration and drift. This allows use of larger X-ray focus with high brightness

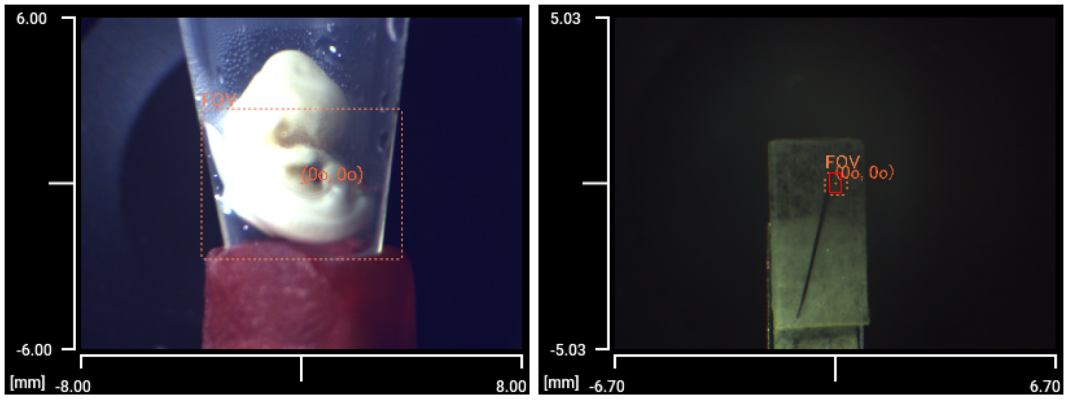

Fig. 2. Sample mounting methods.
Examples are shown for a stained mouse embryo (left; provided by Dr. Masaru Tamura of RIKEN) and an unstained human hair (right).

* X-ray Research Laboratory, Rigaku Corporation.

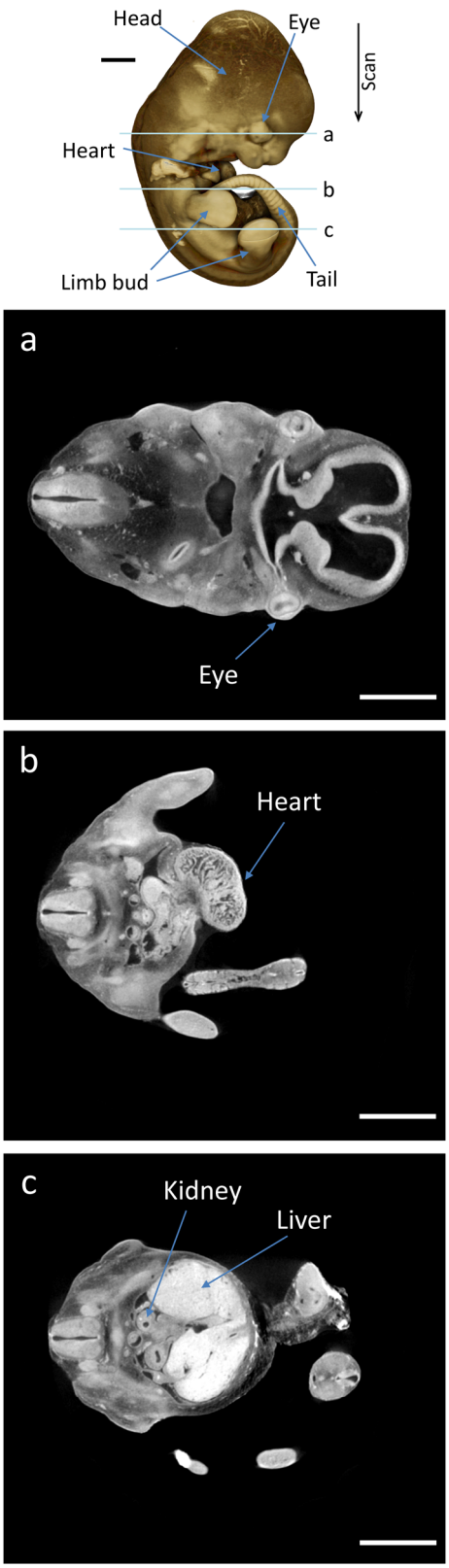

Fig. 3. Contrast improvement by absorption enhancement. A PTA-stained mouse embryo at 12.5 days post-fertilization (provided by Dr. Masaru Tamura of RIKEN) was observed using nano3DX. An overall 3D rendering (top) and three CT slices at corresponding positions indicated (bottom). Scale bars: 1 mm.

without compromising resolution to enable observation at submicron resolutions. In the nano3DX, characteristic or continuous X-rays from four different target materials (Cr, Cu, Mo, W) are available, although the Cu- and the Mo-targets are sufficient for most biological specimens. Although low contrast in X-ray images often hampers imaging for biological specimens, one can improve contrast by applying absorption enhancement or phase retrieval methods.

A common workflow for X-ray microscopy is shown in Fig. 1. First, one must prepare a specimen of suitable size using knowledge of the sample's chemical composition and the chemicals' relevant X-ray absorption. Online tools can provide information for X-ray attenuation lengths to help determine optimal sample size based on the X-ray energy used[3]. For instance, when using a Cu-target to observe unstained biological specimens composed of organic materials, ideal sample thickness is less than 2 mm. If the specimen is hydrated, ideal thickness is shifted to smaller in the range of 1–2 mm, depending upon the water content. Specimens stained with contrast reagents do not necessarily follow this rule. To evaluate attainable contrast for samples, first collect a projection image. Ideally, half of input X-rays should be transmitted through the specimen. If contrast is too low, the sample can be optimized by reducing the sample thickness or by staining with contrast reagents. In the case of unstained low-contrast specimens with small sample size, the contrast may be enhanced by applying a conventional median/Gaussian-based noise filter (denoise) or by phase retrieval. In the case of substances with strong absorption, such as stained specimens and bones, contrast can be enhanced by using a Mo-target and applying denoising filters, as needed. In cases where CT reconstruction fails due to sample movement during data collection, one must optimize the sample mounting procedure.

3. Sample Mounting Methods for X-ray Microscopy Measurement

Because current CT reconstruction methods do not consider sample drift during data collection, deterioration of image quality due to the drift cannot be avoided. Particularly with biological applications, sample mounting methods should be optimized carefully to minimize drift of the sample due to evaporation and/or thermal contraction/expansion. For example, using a plastic tube is recommended to mount wet specimens that are chemically fixed with formaldehyde (Fig. 2 left; stained mouse embryo). This method prevents evaporation-induced deformation by sealing the specimen container with a reduced volume of mother liquor. There are various commercially-available disposable tubes, however you should use a sample container of small enough size to avoid collisions with the instrument. Although the specimen in the tube is simply held in place by gravity and surface tension, CT reconstruction works in many cases. In the event that

sample does move during measurement, it can help to embed the sample in agarose[4]. In the case of specimens with low water content, such as hair and dry seeds, samples can be simply fixed on the sample stage using double-sided tape (Fig. 2 right; unstained human hair). When the double-sided tape does not work, dental wax (for instance, Utility Wax; GC Corporation) or glue may be used.

4. Contrast Improvement by Absorption Enhancement

X-ray contrast for biological specimens can be enhanced by staining with heavy-atom reagents. Figure 3 shows an example nano3DX measurement for a formaldehyde-fixed phosphotungstate (PTA)-stained mouse embryo at 12.5 days post-fertilization. In spite of the large size of this mouse embryo (～8 mm), the PTA stained images collected using a Mo-target show detailed structures for tissues throughout the embryo body. In this example, a CT reconstruction image of the whole embryo body was reproduced by combining two separate data sets.

5. Contrast Improvement by Phase Retrieval

In the nano3DX measurement with a hard X-ray source, a projection image of weakly absorbing specimens shows characteristic refraction-derived fringe-like contrast on the substance boundaries depending upon the sample to detector distance. Phase information can be calculated from the profile of the fringes using phase retrieval techniques[5, 6]. In the energy region of hard X-rays, the contribution of the phase to the X-ray contrast is much larger than that of the absorption, indicating that the contrast may be efficiently improved if phase retrieval succeeds[7]. Figure 4 shows an example for contrast enhancement of an unstained human hair by the phase retrieval. In this case, the sample to detector distance was 4 mm and data were collected with a voxel size of 0.63 μm. The algorithm used for phase retrieval was the Paganin method assuming that the sample was made of organic materials (δ/β＝600). While the original CT slice shows fringes on the substance boundaries, the fringes disappear in the phase-retrieved image with concomitant enhancement of the signal to noise (SN) ratio by about ten-fold. As a result, the low-density medulla structures at the core of the hair sample are clearer in the phase-retrieved image. This type of contrast enhancement would then allow for software-assisted image processing, such as automated segmentation for regions of interest. Another example is shown in Fig. 5. This is a live observation of an unstained pansy seed fixed on the specimen stage. The condition is the same as that in Fig. 4 except for a longer sample to detector distance of 7 mm. After

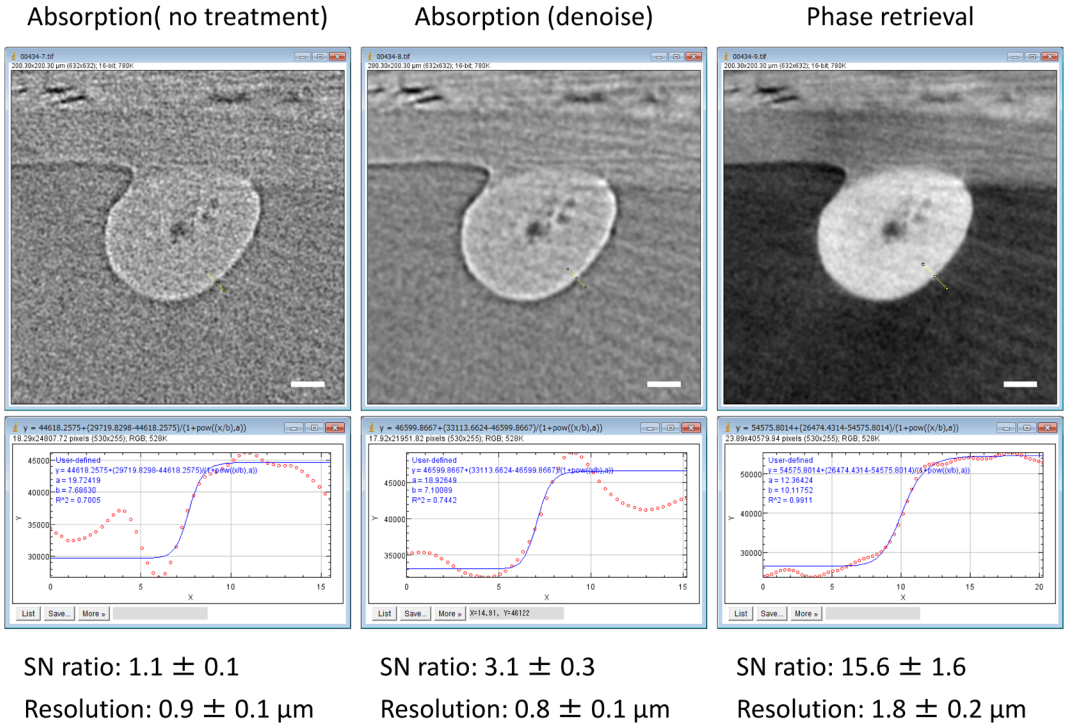

Fig. 4. Observation of human hair.
An unstained human hair (eyelash) was observed using nano3DX. Three CT slices from different treatments of imaging were compared at the same cross-section position of the hair: no treatment (left), general median/Gaussian-based noise filter (center), phase retrieval by the Paganin method (right). Scale bars: 20 μm. The graph represents an intensity profile along the yellow line in the CT slice and curve fitting to estimate the spatial resolution. The signal to noise ratio and resolution that were determined from each CT slice was given as the average ±95% confidence interval (n＝5).

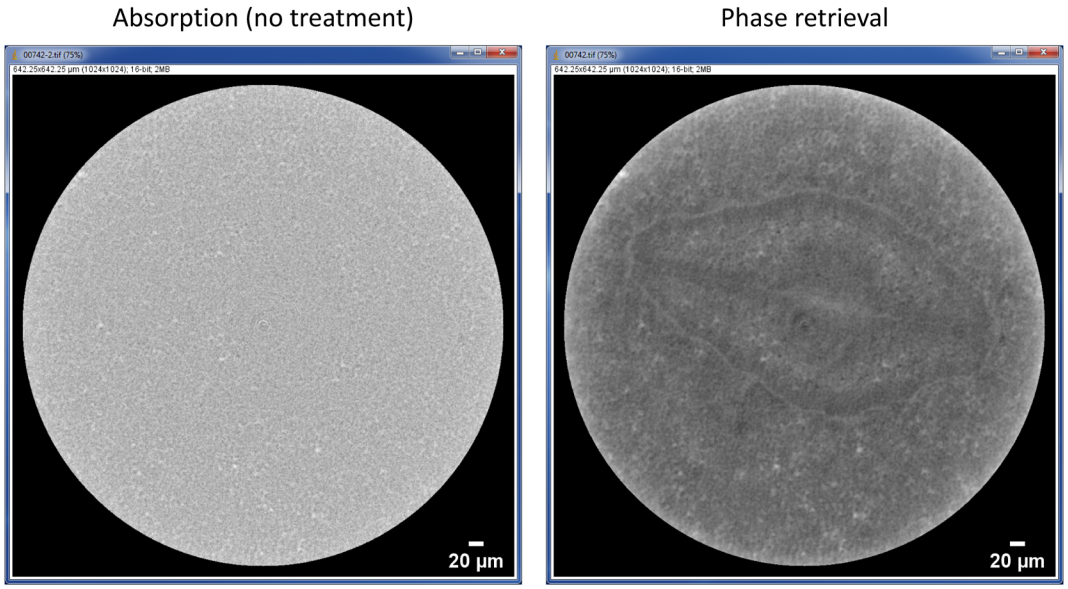

Fig. 5. Observation of pansy seed.
An unstained pansy seed was observed using nano3DX. Two CT slices from different treatments of imaging were compared at the same cross-section position of the embryo: no treatment (left), phase retrieval by the method of Paganin (right).

contrast enhancement by phase retrieval, the structure of embryo and its inner structures are clearly observed, while almost no structure is seen in the original CT slice.

6. Conclusion

While X-ray microscopy is commonly applied for industrial materials[8], its application in structural biology is still emerging. In terms of the hierarchy in structural biology, X-ray microscopy techniques are amenable for examination from size levels of tissue down to the cellular level. As introduced in this report, the technique can be applied to various biological specimens. The tissue-level large specimens, such as mouse embryos, are observed with proper absorption enhancement. On the other hand, the cellular-level unstained specimens, such as hairs and seeds, are examined by utilizing the phase retrieval method. At the Rigaku Corporation, relevant developments in X-ray microscopy are in progress for biological applications of nano3DX for use in medicine. A future goal for biological X-ray microscopy hopes to combine imaging results from X-ray microscopy, fluorescence light microscopy, electron microscopy and X-ray crystallography. Seamless observation with a variety of methods for hierarchical biological systems would be quite useful not only for structural biology but also in the medicine.

References

(1) *Rigaku Journal (Japanese version)*, **43** (2012), No. 2, 28−30.
(2) Y. Takeda and K. Hamada: *Rigaku Journal (English version)*, **31** (2015), No. 1, 10−15.
(3) For instance, http://henke.lbl.gov/optical_constants/atten2.html
(4) M. D. Wong, A. E. Dorr, J. R. Walls, J. P. Lerch and R. M. Henkelman: *Development*, **139** (2012), 3248−3256.
(5) D. Paganin, S. C. Mayo, T. E. Gureyev, P. R. Miller and S. W. Wilkins: *J. Microscopy*, **206** (2002), 33−40.
(6) D. Kalasová, T. Zikmund, L. Pína, Y. Takeda, M. Horváth, K. Omote and J. Kaiser: *IEEE Transactions on Instrumentation and Measurement*, (2019), in press. doi: 10.1109/TIM.2019.2910338
(7) A. Momose: *Houshakou (Japanese)*, **10** (1997), No. 3, 273−285.
(8) Y. Takeda: *Rigaku Journal (English version)*, **35** (2019), No. 2, 18−21.