Scanning transmission ion micro-tomography (STIM-T) of biological specimens

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Abstract

Computed tomography (CT) was applied to sets of Scanning Transmission Ion Microscopy (STIM) projections recorded at the LIPSION ion beam laboratory (Leipzig) in order to visualize the 3D-mass distribution in several specimens. Examples for a test structure (copper grid) and for biological specimens (cartilage cells, cygospore) are shown. Scanning Transmission Micro-Tomography (STIM-T) at a resolution of 260 nm was demonstrated for the first time. Sub-micron features of the Cu-grid specimen were verified by scanning electron microscopy.

The ion energy loss measured during a STIM-T experiment is related to the mass density of the specimen. Typically, biological specimens can be analysed without staining. Only shock freezing and freeze-drying is required to preserve the ultra-structure of the specimen. The radiation damage to the specimen during the experiment can be neglected. This is an advantage compared to other techniques like X-ray micro-tomography. At present, the spatial resolution is limited by beam position fluctuations and specimen vibrations.

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1. Introduction

The technique of Computed Tomography (CT) is used to determine the 3D distribution of a physical property in a specimen from a set of projections taken at different orientations [1]. The development of CT was mainly driven by medical applications. The first medical scanner used X-rays and was presented by Godfrey Hounsfield in 1973 (later he was awarded the Nobel Prize together with Allan Cormack). However, the theory of reconstruction from projections is universal and can be modified for other forms of radiation, such as focussed ion beams.

The physical quantity measured in scanning transmission ion micro-tomography (STIM) is the energy loss of the ions. This primary data can then be fed into a STIM-T 3D reconstruction process. Focussed ion beams were first used for tomographic analysis by Cormack and Koehler [2]. Their first test used a 128 MeV proton beam with a diameter of 2 mm. Pontau et al. [3] and Fischer [4] showed that ion-beam tomography is possible with a resolution of several microns. With further development, beams with smaller diameters became available, and sub-micron resolution was achieved [5]. This development led the way for practical biological applications [6].

This paper gives examples for high-resolution tomography, addresses the effects specific to ions like lateral beam broadening, energy straggling, detector damage and the energy dependence of the stopping power of the ions. The standard reconstruction method of backprojection of

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filtered projections (BFP) [1,7] was used and problems of beam position fluctuations and inaccuracies of the rotational movement at the sub-micron scale are discussed.

The advantages and drawbacks of STIM-T can be summarized as follows:

- (1) Non-transparent objects can be studied in contrast to confocal laser scanning microscopy; however, samples have to be in vacuum, i.e. living specimens cannot be studied. The focal range of STIM-T is large and of the order of 100 μm . STIM of living cells, however, is feasible without noticeable increase in apoptosis or other endpoints.
- (2) The sample preparation is easy: freeze-dried samples without fixation and dyes are adequate; this reduces the risk to study preparation artefacts. Electron tomography is an emerging new technique, however sample dimensions have to be far below 1 μm due to excessive lateral straggling.
- (3) The radiation dose is about two orders of magnitude lower compared to X-ray micro-tomography; thus no cryo-fixation is required and radiation damage plays little role.

2. Experimental setup

A schematic view of a STIM-T experiment is shown in Fig. 1. The ion beam is scanned across the specimen using magnetic coils. The angular displacement of the beam can be neglected since the distance between the coils and the specimen is $\sim 30\text{ cm}$. A windowless PIN photodiode (Hamamatsu 1223-01) with a resolution of 27 keV at full-width at half-maximum (FWHM) measures the residual energy of the protons after traversing the specimen. The FWHM value of roughly 35 keV for the energy straggling

is slightly larger than the FWHM of the detector, which means the effects of energy straggling can be ignored. Because of the statistical nature of the interaction between individual protons and a specimen, multiple energy loss measurements are made and are later median filtered to remove non-representative values. The detector suffers from damage during the experiment since the protons are implanted into the crystal. Thus, as soon as the deviation of the measured energy for protons in vacuum, i.e. outside the sample, decreases by 1%, the detector is shifted to expose a fresh region. This occurs every 10 to 20 projections.

2.1. Sample preparation

The cartilage specimens were prepared as follows: a small cylinder was punched from a knee joint (tibia) of a juvenile pig within 48 h after slaughter and plunged into liquid nitrogen. The frozen specimen was cut into slices of 20 μm thickness with a cryo-microtome and subsequently freeze dried. Under a microscope, rods of $\sim 15\text{--}20\text{ }\mu\text{m}$ in diameter were cut from the micro-sections using a scalpel. Another biological specimen, a cygospore, was simply dried in air. The test specimen was a portion of a 2000-mesh Cu-grid with known dimensions.

All specimens were mounted on a steel needle using carbon glue (Leit-C, Plano GmbH). The needle is attached to a goniometer head and, with the help of the integrated microscope in the specimen chamber, is shifted and tilted to align the rotational axis with respect to the long axis of the specimen. The projection angle of the specimen axis is controlled via a step motor during the experiments. A motorized x/y stage corrects the specimen position in case it moves out of the field of view during specimen rotation.

2.2. Data acquisition and pre-processing

The diameter of the incident proton beam was 80 nm. For all experiments a set of 361 projections, 250×250 pixels each, were recorded in steps of 0.5° . Depending on the proton count rate the acquisition of a single projection took about 30 to 60 s. Ideally, for a projection width of $N_x = 250$ pixels, a set of $N_x \pi / 2 = 392$ projections should be acquired [8]. However, only 360 projections are collected and we tolerate that the full resolution is only achieved within a central area of 229 pixels in diameter because the object is confined within this area. At each position of the scan, the proton beam dwells until five protons are collected before the scan proceeds to the next position. These five energy values are median filtered. Median filtering is the method of choice for STIM because it excludes spurious and random events from contributing to the measured energy loss [9].

The damage of the detector causes artefacts because the sensitivity is locally reduced and phantom energy losses next to the specimen's image may occur. This can be corrected for by thresholding the energy values. Since

Fig. 1. Schematic drawing of a STIM-T experiment. For the experiments described in this paper, the spot size of the incident beam at the specimen surface was adjusted to 80 nm. Energies of single protons are measured with a p-i-n photo diode. The co-ordinates of the scanning system are combined with the measured energy values to map the specimen since the detector has no spatial resolution.

Fig. 2. Monte Carlo simulation for the energy loss of protons in cartilage. The SRIM software package [15] was used for the simulation and the assumed composition of cartilage was $C_{10}H_{13}NO_{2.2}$. For the qualitative reconstructions of the specimen density no dependence of the stopping power on the energy was used. This amounts to the approximation that the linear stopping power is constant in the range between 1800 and 2000 keV.

thresholding can lead to an erosion of contours in regions of the specimen with very low energy losses a second method was applied in the case of the Cu-grid: the object contour was detected with a special algorithm and every energy loss value outside the object was set to zero. Furthermore, the projection data was aligned by fitting the centre of mass of the individual energy loss projections to a sine curve, using a curve fitting algorithm developed in Refs. [10,11] and described in Ref. [12]. This is an essential step since the bearings of the goniometer are not accurate at the μm -scale as required for the reconstruction.

In our experiments we used proton beam energies of 2 and 2.25 MeV. The maximum energy loss of the cartilage-specimen was 200 keV and the stopping power was approximated to be constant in that range between 1800 and 2000 keV (see Fig. 2). If the energy loss of the ion is independent of its instantaneous energy and the composition of the specimen is homogenous, the energy loss is proportional to the areal mass density. Because of these assumptions for our cartilage specimen, the qualitative reconstruction of the specimen morphology could be obtained directly from the energy loss values without a conversion to the areal mass densities. No a priori knowledge was used for the reconstruction of the biological specimen. For the copper grid we used the knowledge of the composition for the conversion of the energy losses to the areal mass densities.

2.3. Tomographic reconstruction and ion beam specific effects

The experimental procedure of measuring a set of projections within one slice of a specimen is mathematically

equivalent to sampling the Radon transform of the object slice [1,7]. There are two ways to invert the Radon transform. The first one is an iterative approach. It involves a forward simulation of the Radon transform which is then compared to the projections measured in the experiment so that iterative corrections are applied to the tomogram. These iterations continue until the simulated and measured projection data are sufficiently similar. Examples are the Algebraic Reconstruction Technique (ART-algorithms) [7,13] and the Discretized Image Space Reconstruction Algorithm (DISRA) [14]. The second approach is a direct approximation of the inverse Radon transform, and such methods are sometimes called transform-based techniques. The most common transform-based algorithm is the Backprojection of Filtered Projections (BFP) [1]. In this paper, BFP was used to reconstruct all sets of experimental data apart from the copper grid specimen, where DISRA was used. Both algorithms were implemented in the software package [11].

In STIM-T experiments, many deviations from the ideal projection process occur which need to be corrected. Alternatively, the experimental conditions can be adjusted to get an approximation closer to the ideal projection process. The specific ion beam characteristics of concern are the energy dependence of the stopping power, the beam broadening within the specimen (lateral straggling) and the energy straggling. These phenomena are discussed next.

The energy loss of an ion passing through a specimen can be described by the linear stopping power dE/dx which consists of a nuclear fraction, which is insignificant at the typical ion energies used for STIM, and a dominating electronic fraction. The electronic part of the linear stopping power is due to the elastic Coulomb interactions between the penetrating ion and the electrons in the specimen and is described by the Bethe–Bloch-equation [15], for ion energies above 200 keV. It is proportional to the electron density of the material. Usually the stopping power is defined as

$$S(E) = \frac{1}{\rho} \frac{dE}{dx} = \frac{dE}{d(\rho z)} = \frac{dE}{d\mu}, \quad (1)$$

where ρ indicates the volume mass density and μ the areal mass density. For a compound, the stopping power is calculated using the Bragg rule:

$$S_C(E) = \sum_Z c_Z S_Z(E). \quad (2)$$

Here, c_Z denotes the mass fraction for the element in the compound with atomic number Z and has a corresponding stopping power S_Z . The values for the stopping powers S_Z are tabulated [16,17]. An illustrative graph for the linear stopping power (dE/dx) of protons in cartilage is shown in Fig. 2.

If a quantitative reconstruction of the mass density in a specimen is required, the energy loss has to be converted to the projected areal mass density μ along this trajectory prior to the reconstruction [3]. The conversion at every

scan position t within the projection line is accomplished via

$$\mu(t) = \int_{E_0}^{E_R(t)} \frac{1}{S_{C(t)}(E)} dE, \quad (3)$$

where E_0 is the incident beam energy and $E_R(t)$ is the measured residual energy of the transmitted ion. Typically, quantitative reconstructions can only be performed when the specimen's composition $C(t)$ is known and Eq. (3) is applied. To obtain this information, other techniques like particle-induced X-ray emission (PIXE) or a priori knowledge may be used. Nevertheless, for biological specimens, the accuracy of the areal mass density can be as good as several percent with only an approximate knowledge of the composition [13,18]. Therefore, to obtain a specimen's 3D morphology, a qualitative reconstruction using only the energy loss projections is usually adequate.

Another point to be considered is the lateral beam straggling [19]. The resulting beam profile that defines the interaction volume within the specimen is a convolution of the incident beam profile and the broadening function due to the multiple scattering with the electrons in the specimen. A simulation of beam broadening using SRIM [17] revealed a quadratic dependence between broadening and depth of penetration. For a 2 MeV proton beam and a cartilage specimen of 15 μm thickness, a beam broadening of only 120 nm is calculated [17]. An investigation of lateral beam broadening [20] shows that the widely used SRIM-code may underestimate the experimental values of beam broadening by about 40%. It is important that the interaction volumes for each ion trajectory do not overlap otherwise the spatial resolution of the reconstructed specimen is degraded. In such a case, the effects of beam broadening must be accounted for in the reconstruction process. For a given specimen, the two parameters that control the broadening of the proton beam are the incident energy and the specimen thickness.

A further effect caused by the statistical nature of the multiple interactions between ions and the electrons of the material is energy straggling: a beam of defined energy will have a Gaussian energy distribution after interacting with matter [21]. A SRIM simulation of the energy distribution assuming 2 MeV protons and 20 μm of (freeze dried) joint cartilage yields a FWHM of 34.8 keV [12]. This is in good agreement with the theoretical predictions in Ref. [20].

All data sets but one were reconstructed qualitatively by reconstructing the sinogram data directly with a BFP program. Only the copper grid was reconstructed using the DISRA program in addition. A Ram-Lak apodizing function [22] with a bandwidth of 0.6 was used because it turned out to be a good compromise between the sensitivity to noise in the data and the attainable resolution. To reconstruct these $250 \times 250 \times 250$ voxel data sets on a 1 GHz Athlon PC running under Linux, the non-iterative qualitative BFP reconstruction takes 4 min, whereas the iterative quantitative DISRA reconstruction takes 2.5 h.

3. Results and discussion

Figs. 3–6 show transparent volume renderings of the tomograms, generated by the program drawer3D [11]. All reconstructed data sets have a size of $250 \times 250 \times 250$ voxels. In Figs. 3 and 4 (stereo-image pair), the internal structures of the cartilage cells embedded in a dense matrix are clearly visible. Since the time between slaughtering of the pig and the freezing of the specimens was 48 h, the cells seem to be necrotic and cell structures like the nucleus were therefore already disintegrated. In future experiments, the specimen preparation has to be improved, e.g. faster freezing with liquid nitrogen cooled isopentane. Some structures of the cell organelles are clearly visible at a sub-micron level as can be inferred from the scales shown in the figures.

Fig. 7 shows slice #150 of the Cu-grid tomogram. Note the well-resolved sharp structure at the bottom. This was the result of cutting the Cu-grid with the scalpel and is well resolved.

It should be pointed out that all the reconstructions of biological specimen shown here were performed without the use of any prior knowledge of the specimen. For the copper grid an additional quantitative reconstruction was done using the DISRA technique. The composition of the material was known (copper) and the accuracy of the reconstructed density agreed with the tabulated value of 8.96 g/cm^3 within 4%. The accuracy of the density values depends on the knowledge of the specimen's composition and physical dimensions of the scan area. Compared to the qualitative reconstruction, there was no noticeable difference in morphology. However, a slightly increased level of noise was observed.

Fig. 3. The tomogram of a cartilage cell from the knee joint (tibia) of a juvenile pig, shown as a semitransparent rendering, generated with drawer3D [11]. Cell structures like the nucleus were not preserved and the cell seems to be necrotic because of the delay between slaughter of the animal and freezing of the specimen. The data set of the reconstruction has a size of $250 \times 250 \times 250$ voxels and the spacing between scanned points in the projections was 140 nm. The arrow indicates a sub-micron structure, the small bar below indicates the size of 1 μm .

Fig. 4. The tomogram of a cartilage cell from the knee joint of a juvenile pig, showing some selected regions. The distance between sampled points in the projections was 117 nm. This stereopair may be viewed with the optical axis of your eyes almost parallel but still focussing to the paper.

Fig. 5. The tomogram of a cygospore, shown as a semitransparent rendering. An internal, clearly segmented structure of lower density can be seen.

In order to verify the accuracy of STIM-T, additional scanning electron microscopy (SEM) was performed on the Cu-grid. In Fig. 8, the surface rendering of the Cu-grid tomogram is compared to the electron micrograph of the specimen surface. The morphology and several sub-micron features present in the STIM-T tomogram were confirmed by the SEM images.

To determine the resolution of the reconstruction, one edge of the Cu-grid tomogram was analysed via a line scan. An error function was fitted to the intensity profile and yielded a resolution of 260 nm, as shown in Fig. 9. The edge definition of the copper grid is not exactly known. However, it is certainly better than 200 nm, judging from the SEM-images. Therefore, this figure seems a conservative estimate. Since biological specimens usually do not provide sharp edges or other test structures similar to the copper grid, it is difficult to extract a similar figure from line scans over fragments of the cartilage cell nucleus or the cygospore core structure. However, we expect a comparable resolution for biological specimens as for the copper

Fig. 6. The tomogram of a section of Cu-grid (2000 mesh per inch), shown as a semitransparent rendering. The spacing between the sampled points of the projections was 96 nm.

Fig. 7. A cross-section at slice 150 from the Cu-grid tomogram depicted in Fig. 6. One pixel corresponds to 96 nm. The bar next to the lower edge corresponds to 1 μm.

grid because of the absence of shrinkage at currents in the order of 0.1 fA (10^{-16} A). Repeated STIM-microscopy images yield identical results for the same projection. Furthermore, the doses in STIM-T experiments are too low to affect the morphology, as will be discussed below. Fluctuations of the beam position due to external stray magnetic fields in the order of 1 μT and specimen vibrations limited the attainable resolution thus far. The Leipzig Nanoprobe can produce beam cross sections below 50 nm [23], but the statistical fluctuations of the specimen and beam positions were measured to have a FWHM of 350 nm (vertical) and 290 nm (horizontal), respectively [12]. Therefore, the position uncertainty rather than the beam broadening or the diameter of the beam limited the spatial resolution of the tomograms. This problem has subsequently been alleviated by the installation of an active compensation system against stray magnetic fields [24]. However, the specimen vibration remains to be eliminated. A new target chamber with UHV-translation stages with shorter dimensions and a UHV-goniometer are under

Fig. 8. Left: Scanning Electron Microscope image of the Cu-grid specimen. Some distinct features are in the sub-micron range and are clearly reproduced in the STIM-T tomogram. Right: Iso-surface rendering of the Cu-grid tomogram shown in Fig. 6. One period of the 2000 mesh grid corresponds to 12.7 μm .

Fig. 9. Slice of the 3D dataset of the reconstructed density of the copper-grid. The arrow indicates the location where the profile data shown on the right was extracted. The solid line is an error function fitted to the edge. This yields a lateral resolution of 260 nm assuming an infinitely sharp edge definition of the copper-grid.

construction and should greatly reduce specimen vibrations.

We did not correct for beam broadening during the reconstruction since fluctuations of the beam position were found to be larger than the simulated beam broadening expected for these biological specimens. Hence, only a small improvement of the resolution would be expected for these data sets. Bench et al. [25] demonstrated an improvement in resolution by incorporating beam broadening into the reconstruction process of STIM-T, but the model was restricted to a similar geometry and composition over neighbouring slices. An implementation of a full 3D model for lateral beam broadening should be feasible for an iterative technique such as DISRA [14], but at the cost of increased computation time and the development of a more sophisticated model for beam broadening.

3.1. Comparison to other methods

There are other methods, for example X-ray microtomography, that can reconstruct the 3D volume of

biological specimens at a resolution down to 60 nm [26], but synchrotron radiation is required and the radiation dose of 10^8 – 10^9 Gy may damage the specimen. For STIM-T the dose is more than two orders of magnitude lower. This number can be estimated as follows: An upper limit for the linear energy transfer in a biological specimen for 2.25 MeV protons is about 15 keV/ μm . A cartilage specimen of $20\,\mu\text{m} \times 20\,\mu\text{m} \times 20\,\mu\text{m}$ (volume: $8000\,\mu\text{m}^3$) receives in the order of 200 (pixels) $\times$ 200 (pixels) $\times$ 5 (protons/pixel) = 2×10^5 protons per projection each of which deposits $15\,\text{keV}/\mu\text{m} \times 20\,\mu\text{m} = 300\,\text{keV}$, i.e. a total deposited energy of $300\,\text{keV} \times 2 \times 10^5$ (protons) = $6 \times 10^{10}\,\text{eV}$ per $1\,\text{g}/\text{cm}^3$ (density of water) $\times 8000\,\mu\text{m}^3 = 8 \times 10^{-12}\,\text{kg}$. This results in a dose of $0.75 \times 10^{22}\,\text{eV}/\text{kg} = 1.2 \times 10^3\,\text{J}/\text{kg}$ (or Gy). With a total of 360 projections we finally obtain a figure about 4×10^5 Gy. Thus, cryo-fixation is not required to preserve the morphology.

Another technique with 3D capability, high resolution and low radiation dose is Electron Tomography [27]. Unfortunately, because of the very small penetration depth of the electrons, the specimen size is below 1 μm , which

Table 1
Characteristics of common 3D micro-analysis techniques

	Resolution X/Y	Resolution Z	Specimen size	Spec. damage
STIM tomography	< 300 nm	<300 nm	<100 μm	Low dose
Electron tomography	Up to 5 nm	Up to 5 nm	<1 μm	Low dose
X-ray tomography	Up to 60 nm	Up to 60 nm	$\sim 10 \mu\text{m}$, more at lower res.	High dose
Serial block face EM	Approx. 10 nm	50–70 nm	>200 μm	Destructive

makes the specimen preparation difficult and restricts the applications.

One further 3D analysis technique is the Serial Block-Face Scanning Electron Microscopy reported recently [28] where a resolution of 50–70 nm in axial direction and about 10 nm in lateral direction was accomplished. This method is destructive since the respective top layers of the specimen are physically removed. If the loss of the specimen and the technical effort is not a problem, relatively large specimen areas can be analysed at high resolution. For a quick overview, the features of the mentioned 3D methods are compared in Table 1.

4. Conclusion

STIM-T can be used to image the 3D structure of biological specimens at a resolution better than 300 nm and does not require any staining techniques to enhance the contrast of the material density in the specimen nor does it require cryo-fixation.

Without prior knowledge of the specimen composition, accurate 3D morphological images are obtained. If the specimen composition is known, a quantitative reconstruction of the 3D density distribution may be calculated with a potential accuracy of 10% [9].

To our knowledge, the resolution of 260 nm is the best value obtained with the STIM-T method so far; it can still be improved. A resolution of 100 nm may be obtained if the stray magnetic fields in the lab are sufficiently compensated and specimens smaller than 15 μm in diameter can be prepared. In this case, simulations of the lateral beam broadening show that interaction volumes smaller than 100 nm in diameter can be accomplished and modelling of beam broadening in the reconstruction process is not necessary. STIM-T can be applied to analyse the 3D structure of a wide range of biological and technical specimens. A precondition is that the ion-beam has sufficient energy to traverse the specimen. The technique offers a combination of high resolution, simple specimen preparation and very low radiation damage.

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