APPLICATION OF PHASE-CONTRAST X-RAY MICROTOMOGRAPHY TO STUDY THE INTERNAL STRUCTURES OF *RHODNIUS PROLIXUS*

André P. de Almeida [1,2], José Soares[1], Simone C. Cardoso[3], Delson Braz[2], Eloi S. Garcia[4], Marcelo S. Gonzalez[5], Patricia Azambuja[4], Regina C. Barroso[1]

[1] Physics Institute/State University of Rio de Janeiro, Brazil
São Francisco Xavier St, 524
20550-900 Rio de Janeiro, RJ, Brazil
apalmeid@gmail.com
cely_barroso@hotmail.com

[2] Nuclear Engineering Program/COPPE/Federal University of Rio de Janeiro, Brazil
Centro de Tecnologia (CT), Bloco I, sala I-133
21941-972 Rio de Janeiro, RJ, Brazil
apalmeid@gmail.com
delson@lin.ufrj.br

[3] Physics Institute/Federal University of Rio de Janeiro, Brazil
Centro de Tecnologia (CT), Bloco A, sala A-307
21941-972 Rio de Janeiro, RJ, Brazil
simone@if.ufrj.br

[4] Laboratory of Biochemistry and Physiology of Insects/Oswaldo Cruz Institute/ FIOCRUZ, Brazil
Ave Brasil 4365
21045-900 Rio de Janeiro, RJ, Brazil
azambuja.p@gmail.com

[5] Department of General Biology/Federal University Fluminense, Brazil
marcelosalabert@gmail.com

ABSTRACT

The PhC-SR-μCT is a nondestructive technique that allows the microanatomical investigations and 3D images reconstructions within a short time. This technique performed in blood sucker, *Rhodnius prolixus* – one of the most important primary vectors of of Trypanosoma cruzi, ethiologic agent of Chagas' disease in Latin America and also the most well-know studied insect in terms of both physiology and vector-parasite interactions. However, little is known about the development and structure of its internal organs. The aim of this work is to provide a non-invasive option for studying the internal structures of the main vector of Chagas' disease, which should help to answer important questions concerning anatomy, development, structure and plasticity of insect in general. Three-dimensional rendering images can provide a detailed knowledge of the interior of the insect, which is crucial for a better understanding of its function and evolution.

1. INTRODUCTION

X-ray computed tomography (CT) is a widely used technique for imaging three-dimensional internal structures and it is based on the difference in radiation absorption by different tissues.

Recent application of synchrotron radiation to high-resolution CT (micro-CT) has resolved three-dimensional structures at micrometer [1] to submicrometer scales [2]. Micro-CT analysis is generally used for objects containing metal elements because they give an appropriate contrast even in hard X-ray regions. Its application to biological materials is problematic because they are composed of lighter elements. The contrast can be enhanced by using soft X-rays, which are effectively absorbed by biological materials [3], or by applying the phase-contrast technique to develop the interferometric images [4,5]. However, the application of these methods is limited to samples through which soft X-rays can pass or that give an inherent contrast in a phase-shift cross section.

The main requirement is a high quality source with high spatial coherence, which can be provided by microfocus X-ray tubes or by high brilliance and low emmitance third generation synchrotron radiation sources. High coherency of the beam provided by third generation of the synchrotron radiation allows phase contrast imaging in the very simple experimental setup [6, 7].

The development of synchrotron X-ray sources and phase contrast imaging techniques have resulted in major advances in both the microscopic imaging of preserved specimens and the real time X-ray video of the internal processes of living organisms [8]. Recently, a review on real-time phase-contrast X-ray imaging was given by [9] but even after [10] published an excellent review on synchrotron X-ray imaging and outlined the many uses for anatomical imaging of living organisms.

Application of X-ray microtomography (μCT) on insects is quite recent [11-15] and its transposition to use phase contrast synchrotron X-ray microtomography (SR-PhC-μCT) even more recent [9,10, 16-20]. A detailed knowledge of insect's anatomical structures is crucial for a better understanding of their function and evolution. Traditionally, in both morphology and anatomy, the internal structures of whole organisms or parts of them are accessed by dissecting or performing fixed tissues to histological serial sections. The main limitation of the two most frequently used techniques (stereomicroscope and SEM) is the fact that they allow only the acquisition of 2D images or of partial 3D data. Microtomographic scanning of specimens allows acquisition of real volumetric 3D data that can be then used for 3D rendering and virtual manipulation of the sample in dedicated software [21].

In this work we used the SR-PhC-μCT to study the microanatomy of *Rodhinus prolixus* which is one of the most important insect vectors of Trypanosoma cruzi, ethiologic agent of Chagas' disease in Latin America [22]. Chagas' disease [23,24], also called American trypanosomiasis, is a human tropical disease, which is endemic in large areas of South and Central America. Among the parasitic diseases, Chagas' disease is ranked as one of the most important in Latin America in terms of social and economic impact, affecting about 18 million people, with about 100 million people living in what are considered to be high risk zones, and approximately 300,000 new cases occurring every year with around 21,000 deaths annually [25-27].

Although *R. prolixus* is one of the most well-know model in terms of both physiology and vector-parasite interactions studies, little is known about the structures of its internal organs and how some morphophysiological aspects of mainly the retrocerebral complex in different time of the life cycle are related to the development and reproduction of this insect vector. We investigate the potential and advantages of SR-PhC-μCT with emphasis given to the 3D digital reconstruction of head and thorax structures in order to get preliminary information about density and plasticity of the neuroendocrine tissues, which is responsible for the development and reproduction of the insect.

2. MATERIALS AND METHODS

2.1. Sample preparation

Rhodnius prolixus (Hemiptera: Reduviidae) were reared and maintained at 28°C and between 60 and 70% relative humidity in a BOD incubator. Fifht-instar nymphs were feed on human blood using a membrane apparatus (Fig. 1) as previously described by [28]. Three days after blood meal, insects were immobilized at 4°C for 10 min and bounded on a polystyrene table with entomological pins and transversally cut at the junction between prothorax and mesothorax segments of body. The anterior fragments were fixed and maintained at room temperature in a solution containing 1% glutaraldehyde and 5% sucrose in 0.1 M cacodylate buffer, pH 7.2 until using (not more than one month).
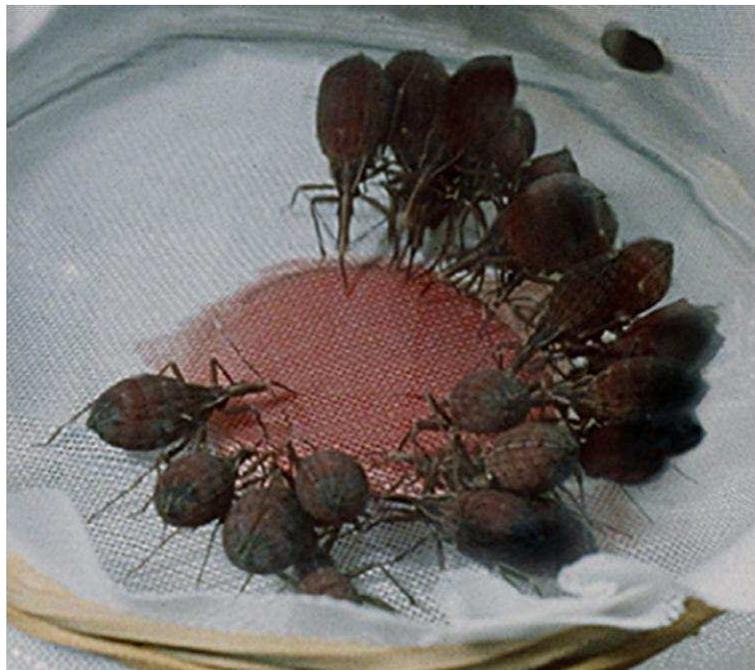

Figure 1. *R. prolixus* feeding through the membrane of a feeder apparatus.

2.2 SYRMEP beamline

The experiments were carried out at the third generation synchrotron radiation source of the ELETTRA synchrotron radiation facility at the SYRMEP (SYnchrotron Radiation on MEdical Physics) beamline. The beamline provides a monochromatic laminar-section X-ray with a maximum area of about 160×5 mm^2 at 20 keV, at a distance of about 23 m from the source. The system consists of a Si (1 1 1) crystal working at Bragg configuration. The useful energy range is 8–35 keV. The intrinsic energy resolution of the monochromator is about

10–3. Typical flux measured at the sample position at 17 keV is about 1.6×10^8 photons/mm^2 s with a stored electron beam of 300 mA as ELETTRA operates at 2 GeV [29]. A custom-built ionization chamber is placed upstream to the sample to determine the exposure on the sample. A micrometric vertical and horizontal translation stage allows the positioning and the scanning of the sample with respect to the stationary beam and a rotational stage allows CT acquisition with a resolution of 0.001°.

The detector system is comprised of a 12/16-bit CCD camera, with 4008×2672 pixels2, 4.5 micron pixel size CCD camera with a field of view of 18 x 12 mm^2, coupled to an intensifier screen with no magnification (1:1). The CCD camera can move along the sample-detector axis, in order to set the desired sample-to-detector distance d. According to the choice of the sample-to-detector distance, one may distinguish between the absorption and phase sensitive regimes. If the CCD is mounted very close to the sample we are in the absorption regime. For higher d values, free space propagation transforms the phase modulation of the transmitted beam into an amplitude modulation (Fig. 2a, b).
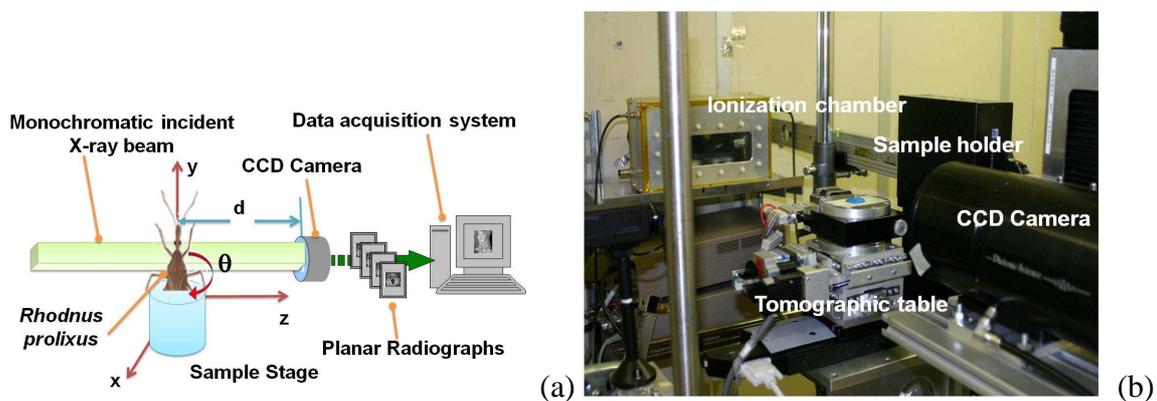

(a)   (b)

Figure 2. (a) Schematic and (b) experimental set-up used for PhC-μCT at SYRMEP beamline.

The detection system was positioned at 10 cm away from the sample position so that absorption technique could be performed.

2.3 Reconstruction

The tomographs were performed on a range from 0 to 180°, in steps of 0.2°, resulting in 900 projections. In a real X-ray imaging system the background signal is generally not uniform. This can be due to inhomogeneities in the X-ray beam intensity distribution or to non-uniform detector response, or both. It is therefore generally necessary to normalize each radiograph (Fig. 3a) by an image of the beam without the object (flat image, Fig. 3b). This is called a flat-field correction. Also, in each image recorded with the detector, the offset signal recorded when no photons hit the detector (dark image, Fig. 3c) should be subtracted from the image before further processing (dark-field correction). The corrected images are:

$$I_c(x, y) = \frac{S_0(x, y) - S_d(x, y)}{S_f(x, y) - S_d(x, y)} \quad\quad\quad (1)$$

where $S_0(x,y)$ is the image signal measured with the object in the beam, $S_d(x,y)$ is the dark signal, and $S_f(x,y)$ the flat signal. In order to keep the increase of statistical noise introduced by the correction to a minimum, it is preferable to take a series of flat-field and dark images and average these before using them for correcting the object images [30]. In our case, 5 dark and 5 flat images were taken before and after the 2D images acquisition. Fig. 4 shows the corrected image after flar-dark-field correction. The image is dominated by phase contrast. Edges and interfaces in the object are clearly visible.
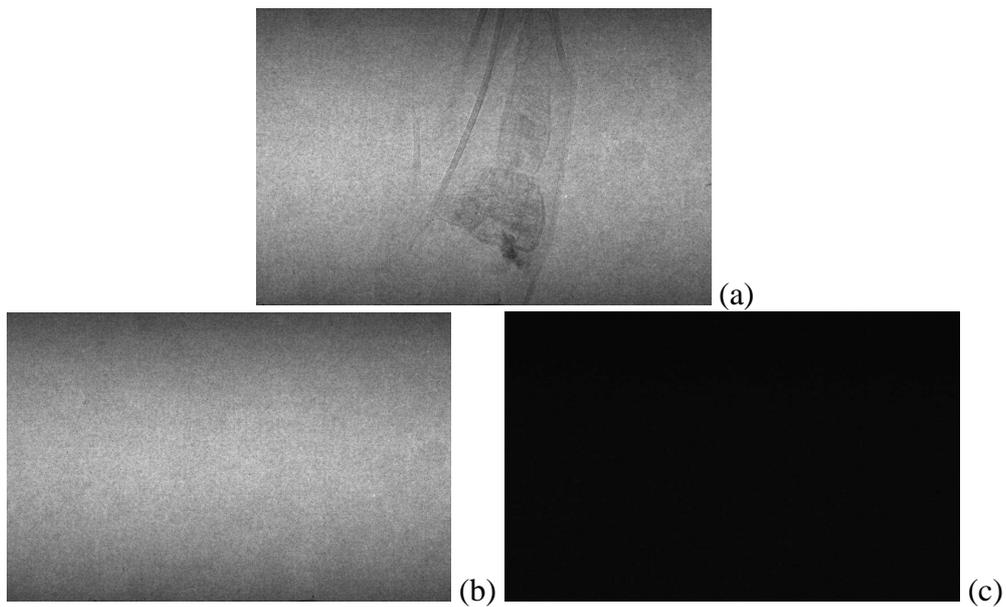

Figure 3. (a) Raw 2D image; (b) flat and (c) dark images.

Figure 4. One of the transmission radiographs recorded in the tomography scan, shown after flat-field normalization..

The facilities used for data collection provide stacks of images ("slices") from which a sub-volume (a Volume Of Interest, VOI) is cropped out for the analysis. For statistical purposes we analyzed the largest VOI obtainable from each different dataset. Once the VOI regions were obtained, the data needed some filtering to remove noise. Tomographic raw images were reconstructed using an imaging processing software (SYRMEP TOMO PROJECT) developed in the SYRMEP laboratory [31] which uses Interactive Data Language (IDL). The reconstruction was performed using filtered back projection with Shepp Logan filter. Fig. 5 shows slices obtained after reconstruction. To retrieve useful information from the 900 projections, it was necessary to reconstruct the data into a readable 3D structure. The volume size obtained from the reconstruction procedure was stored as 591 images.

The basic steps for reconstructing the slices through the depicted object were as follows [32]:

- Determine the centre of rotation from projection images at 0º and 180º.
- Read all 900 projection images one by one. Perform dark-current and flat-field normalization for each projected view, and rearrange the data into 591 sinograms by adding the respective pixel column to the respective 'raw sinogram'.
- Compute corrected sinograms by taking the negative logarithm of the ratio of the transmitted intensity to the intensity in the air outside the object for each view.
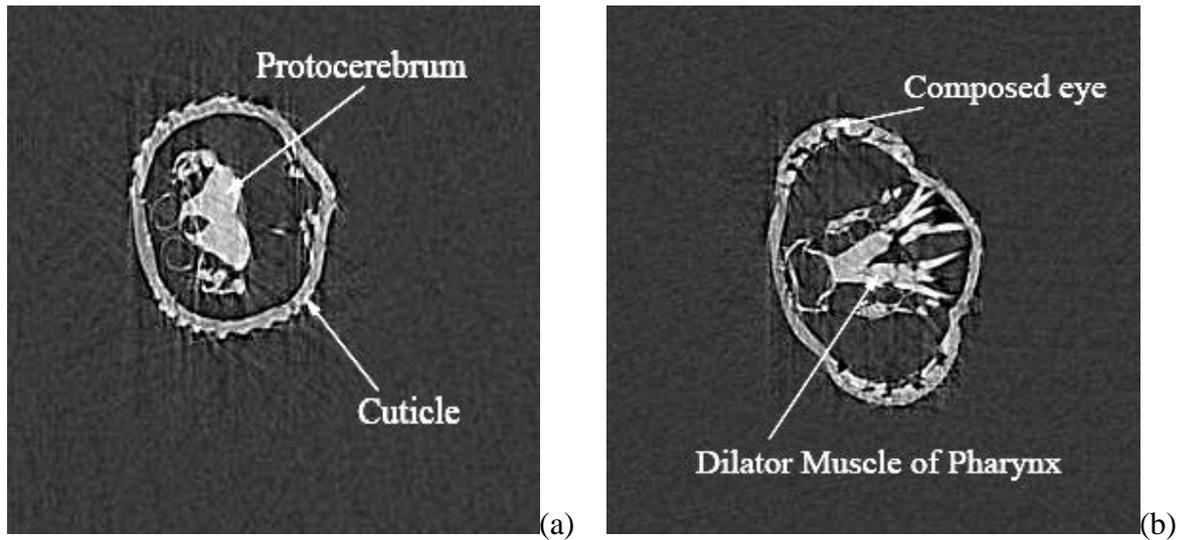
(a) (b)

Figure 5. Slices (a) and (b) obtained through direct tomographic reconstruction of the flat-field corrected transmission radiographs.

3. Results and Discussions

μCT can be used as qualitative imaging technique which permits to section tissues in their natural state virtually, in any direction, with sub micrometer resolution and without time-consuming elaborate dissections [16] The μCT approach allows observing and rotating the virtual reconstruction of the specimen It is then easy to orient it and to generate any interesting viewing angle in dedicated software (in the present case, ImageJ). The application of SR-PhC-μCT to insect anatomy demonstrated the ability to visualize fine details of the head and thorax of *R. prolixus* showing a 3D model external view of representative morphological characteristics, Fig. 6.
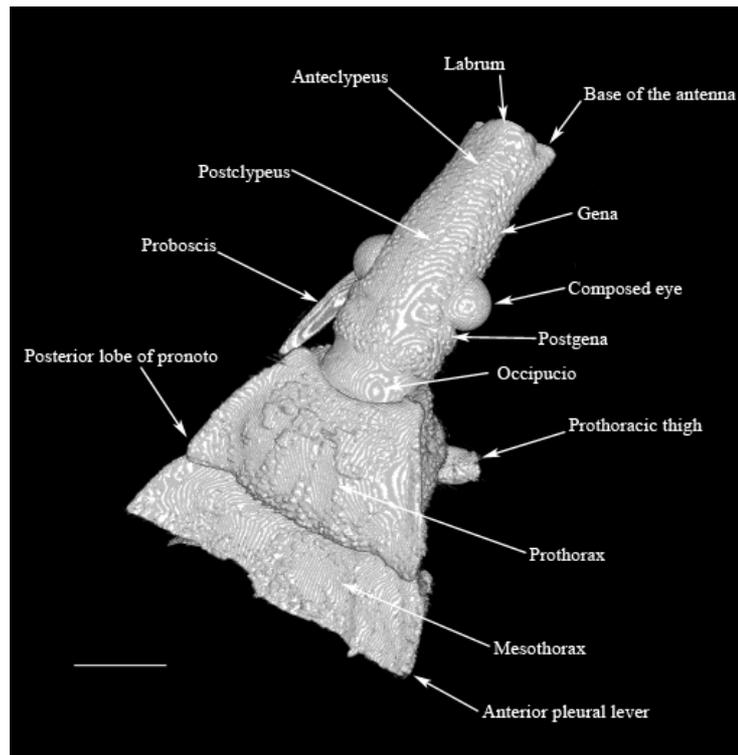
Figure 6. Volume-rendered PhC-µC image showing external aspect of head and thorax of *R. prolixus*. Bar = 1000µm

Synchrotron X-ray phase-contrast imaging is ideal for visualizing well defined internal structures that have different mass densities [9]. The contrast between the different internal tissues in the SR-PhC-µCT images was also sufficient to distinguish between cuticular structure and soft tissues such as muscle and nerve tissue. The observation of tiny details is easier and each part of the specimen is easily accessible and observable, which is particularly interesting for complex morphology specimens (Fig. 7).
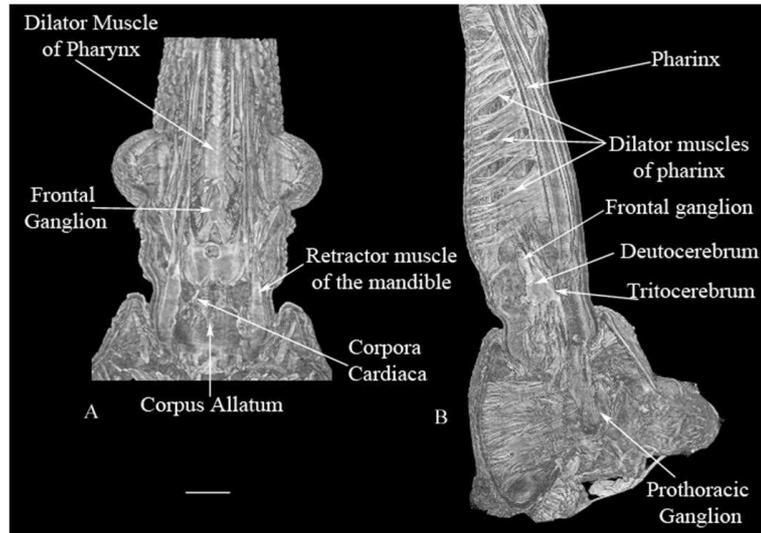
Figure 7. Virtual sections through the head of *R. prolixus*: horizontal (A) and sagittal (B) sectional planes. Bar = 500µm

4. Conclusions

Phase-contrast X-ray microtomography has been proved to be a good method for fundamental studies of organs syntopy and structure of *R. prolixus* without invading or disrupting them. This non-invasive, non-destructive technique provided a way to observe structures and organs preserving their original plasticity and development. In this perspective, here we present the first results of the high-resolution noninvasive investigation of the external structure and anatomy of one of triatomine insect vectors of Chagas' disease agent. This technique opens a window of opportunity to study models of interaction using insect vectors and parasites species. Thus, phase contrast synchrotron X-ray microtomography proved to be a very suitable technique to overcome the problem of generating 3D dataset from 2D data, typical of microscopy-based techniques, which should help to answer important questions concerning anatomy, development, structure and plasticity of insect in general.

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