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Nicolò Peruzzi\textsuperscript{a}, Béla Veress\textsuperscript{b}, Lars B. Dahlen\textsuperscript{c,d}, Tim S alditt\textsuperscript{e,f}, Mariam Andersson\textsuperscript{g,h}, Marina Eckermann\textsuperscript{e,f}, Jasper Frohnh\textsuperscript{n}, Anna-Lena Robisch\textsuperscript{n}, Martin Bech\textsuperscript{n} and Bodil Ohlsson\textsuperscript{i}

\textsuperscript{a}Division of Medical Radiation Physics, Department of Clinical Sciences, Lund University, Lund, Sweden; \textsuperscript{b}Department of Pathology, Skåne University Hospital, Malmö, Sweden; \textsuperscript{c}Department of Translational Medicine – Hand Surgery, Lund University, Malmö, Sweden; \textsuperscript{d}Department of Hand Surgery, Skåne University Hospital, Malmö, Sweden; \textsuperscript{e}Institute for X-Ray Physics, University of Göttingen, Göttingen, Germany; \textsuperscript{f}Cluster of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC), University of Göttingen, Germany; \textsuperscript{g}Department of Applied Mathematics and Computer Science, Technical University of Denmark, Lyngby, Denmark; \textsuperscript{h}Danish Research Centre for Magnetic Resonance (DRCMR), Center for Functional and Diagnostic Imaging and Research, Copenhagen University Hospital Hvidovre, Hvidovre, Denmark; \textsuperscript{i}Lund University, Skåne University Hospital, Department of Internal Medicine, Malmö, Sweden

**ABSTRACT**

**Objectives:** Light microscopical analysis in two dimensions, combined with immunohistochemistry, is presently the gold standard to describe the enteric nervous system (ENS). Our aim was to assess the usefulness of three-dimensional (3D) imaging by X-ray phase-contrast tomography in evaluating the ENS of the human bowel.

**Material and methods:** Myenteric ganglia were identified in full-thickness biopsies of the ileum and colon by hematoxylin & eosin staining. A 1-mm biopsy punch was taken from the paraffin blocks and placed into a Kapton\textsuperscript{®} tube for subsequent tomographic investigation. The samples were scanned, without further preparation, using phase-contrast tomography at two different scales: overview scans (performed with laboratory setups), which allowed localization of the nervous tissue (~1μm effective voxel size); and high-resolution scans (performed with a synchrotron endstation), which imaged localized regions of 320x320x320 μm\textsuperscript{3} (176 nm effective voxel size).

**Results:** The contrast allowed us to follow the shape and the size changes of the ganglia, as well as to study their cellular components together with the cells and cellular projections of the periganglionic space. Furthermore, it was possible to show the 3D network of the myenteric plexus and to quantify its volume within the samples.

**Conclusions:** Phase-contrast X-ray tomography can be applied for volume analyses of the human ENS and to study tissue components in unstained paraffin-embedded tissue biopsies. This technique could potentially be used to study disease mechanisms, and to compare healthy and diseased tissues in clinical research.

**Introduction**

Gastrointestinal diseases are common in all ages of the population. It is of great importance to improve the diagnostic accuracy, and to determine the etiology and affected cell types in various diseases. Enteric neuropathy with inflammatory and/or degenerative changes in theenteric nervous system (ENS) may be one causal factor to gastrointestinal dysfunction \cite{1,2}. The ENS is composed of two distinct components with different functions: the submucosal plexus and the myenteric plexus. The latter is situated between the inner and outer muscle layers and governs the peristaltic movements of the bowel \cite{3}. The plexus consists of ganglia built up by neurons, their axons and dendrites, glial cells and connecting neuronal fascicles between the ganglia. In addition, interstitial cells of Cajal (ICCs) and telocytes (fibroblast-like cells in previous literature) with their long and thin cytoplasmic projections surround and penetrate the ganglia and nerves in certain localizations \cite{4}.

Patterns and causes of dysmotility can be analyzed by different techniques in clinical practice \cite{5}. Tissue samples for diagnostic purposes of the ENS demand full-thickness biopsies, obtained by surgery in full anesthesia, with potential risks for the patient \cite{6}. Traditional histopathological studies of ENS are performed in two-dimensional (2D), 3–5 μm thick, sections using various tissue staining procedures combined with immunohistochemistry of several proteins. Due to the nature of this technique, only a small number of myenteric ganglia in a thin segment are available for evaluation \cite{1,2,6}. Three-dimensional (3D) reconstructions can be done only by...
building up images either from many serial sections, or in whole-mount specimen, with fluorescence confocal scanning microscopes; thus, with some difficulties to study the exact details of the various tissue components and their relationships [7–10].

X-ray computed tomography (CT) provides volumetric information in a non-destructive way and is widely used for medical applications. Standard X-ray attenuation-based microtomography would normally not show any contrast between different cell types or soft tissues, because of their low and relatively homogenous X-ray attenuation coefficient. Aside from attenuation, however, an X-ray wave that interacts with a material is also subject to a shift in its phase. Novel X-ray imaging techniques can obtain contrast from this phenomenon, in what is commonly called X-ray phase-contrast imaging (PCI). For hard X-rays and low atomic number materials, such as biological tissues, PCI provides the possibility to significantly increase the contrast (or alternatively decrease the radiation dose while keeping a comparable contrast) compared with attenuation-based imaging [11,12]. PCI can be performed with a wide variety of modalities and setups. Until recently, state-of-the-art results would require highly coherent X-rays only produced at synchrotron facilities [13]. Laboratory setups, of easier accessibility, are, however, starting to show comparable results [14].

Our hypothesis was that 3D PCI of unstained paraffin-embedded tissue from the bowel would allow to (a) give an overview and analysis of the cells within and around the ganglia and (b) determine the volume, shape and spatial change of the nervous myenteric plexus. The aim of the present methodologic study was to determine whether the method can be applied to study and describe the cellular morphology and possible changes of the my enteric plexus in the human ileum and colon.

Material and methods

The study was performed in accordance with the Helsinki declaration and approved by the Regional Ethics Review Board at Lund University (2012/527 and 2016/943, Date of approval 16/10/2012 and 15/11/2016). Subjects gave their written, informed consent before entering the study.

Sample preparation

Two full-thickness bowel samples were obtained; one from the colon of a 60-year old man, resected from the macroscopically normal part 5 cm above the carcinoma, and one biopsy from the ileum of a 56-year old woman suffering from Ehlers-Danlos syndrome and gastrointestinal dysmotility, with diagnosed atrophy of the myenteric ganglia in conventional immunohistochemistry [14,15]. The tissue samples were fixed in buffered formalin at room temperature and embedded in paraffin. The representative part of the ENS was chosen in a 4 μm thick hematoxylin and eosin-stained (H&E) section under the light microscope in both samples (Figure 1(A)). A biopsy punch of 1 mm diameter was taken from the paraffin block and placed into a Kapton® tube (Paramount, Indiana, US) for mounting in the subsequent tomographic investigation [16].

X-ray tomography

X-ray phase-contrast CT was performed on different instruments: two custom-designed laboratory μCT setups, located at the Institute for X-Ray Physics, University of Göttingen [16], and a dedicated synchrotron radiation nano-CT endstation (GINIX), installed at the P10/PETRAIII beamline, Hamburg [17]. All the setups use propagation-based, phase-contrast methods, in which the sample is placed between source and detector, and the phase information is obtained by free space propagation and self-interference of the coherent X-ray beam without any need of additional optical elements (Figure 1(B)). In all cases, phase retrieval is necessary to correctly extract the phase information before CT reconstruction [18].

The two laboratory setups allowed to image the whole 1 mm wide and 1.5-2.5 mm long samples with anisotropic...
effective voxel size of \( \sim 1 \mu m \) (Figure 2(A,B); \( \sim 15 \) h scan time per sample), enabling an identification of the neural tissue structure. The two systems could run in parallel, which was used to optimize time consumption.

Selected regions-of-interest (ROIs) of \( 320 \times 320 \times 320 \) \( \mu m^3 \) were scanned with an isotropic effective voxel size of \( 176 \) nm at the synchrotron-based endstation (Figure 2(C); \( \sim 2.5-3 \) h scan time), using inline holography based on a coherent divergent beam exiting from an X-ray waveguide [16,17]. Holographic phase retrieval based on the CTF-approach, ring removal and tomographic reconstructions were performed with in-house reconstruction pipelines [19].

Complete information regarding the experimental setups and the reconstruction pipelines is provided in the Supplementary Appendix. In the following, the scans obtained with the laboratory setups will be called overview scans, while the ones obtained in the synchrotron endstation will be named high-resolution scans.

**Image analysis**

All the obtained volumetric datasets could be digitally sectioned along any arbitrary slicing plane, enabling 3D virtual histology of the samples. In the histological evaluation, the term ‘spindle-shaped cells’ was used to describe the cells around the ganglia, which can represent either ICC, telocytes, or fibroblasts/cytes, because no differentiation could be made without immunohistochemistry.

In the case of the overview scans, the neural tissue was segmented using the Magic Wand tool of Amira™ (Thermo Fisher Scientific™, Waltham, Massachusetts, US), which uses a gray-value-based ‘region growing’ algorithm. This enabled a 3D rendering of the neural tissue structure, which was visualized with the same software. From the segmented data, the ratio of neural tissue volume over total examined tissue volume was also calculated. Finally, an evaluation of the neural tissue thickness was performed with the Filament tool in Amira™, which performs a skeletonization of the segmented structure and then measures a distance map on the voxels that are part of the skeleton.

**Results**

**3D Virtual histology**

In the high-resolution 3D virtual histology, the contrast was sufficient to follow and analyze the shape and the size changes of the myenteric ganglia, their various cellular components, i.e., neurons and glial cells, the periganglional space and the smooth muscle cells (Figures 3–6). For comparison, the same cell components are shown in traditional light microscopy (Figure 7).

In the healthy colon, long and very thin cytoplasmic projections could be observed from the periganglional spindle-
shaped cells on a tangential view of the periganglionic space (Figure 3). Small spindle-shaped cells were found at the border of the ganglion (Figure 4), from which projections could be found penetrating the ganglion (Figure 5). The details of the various components of the ganglion and periganglionic space can be followed through the volume, as exemplified by Video 1, provided as Supplemental Material. The first frame of the video corresponds to Figure 4, showing two very long cellular projections of the spindle-shaped cell A. The right upper projection runs on the surface of the ganglion at the border, whereas the lower left projection probably penetrates the ganglion. There are several neurons with or without nuclei; the small round nuclei, forming/gathering sometimes in small groups, are glial cells. At approximately

02 s in the video, there is a larger spindle-shaped cell in the middle with two projections running through the ganglion. The last frame shows the ganglion of Figure 5, with thin cellular projections situated both at the border and penetrating into the ganglion itself. Furthermore, one spindle-shaped cell (arrowhead on Figure 5) in the middle of the ganglion appears to almost be touching the plasma membrane of a neuron.

In the ileum from the patient, the ganglia were much smaller with a few neurons and glial cells without intraganglionic projections from the periganglionic cells (Figure 6). Spindle-shaped cells and projections were seen in the intermyenteric connective tissue plate and fine projections in the intercellular space between the muscle cells, but detailed analysis of the muscle cells was not possible (not shown).
Volumetric structure and quantification

In the colon, the 3D network was built up by the ganglia and the interconnecting nerve bundles, already noticeable with an appropriate slicing of the overview scan (Figure 8(A)). The network was even more discernible with the volume rendering of the segmented neural structure (Figure 8(B)). The segmented neural tissue had a volume of 0.04 mm$^3$, corresponding to a 7.2% ratio of neural tissue volume over total examined tissue volume. The thickness analysis showed two main trunks, identifiable as ganglia in the virtual histology, with thickness ranging between 80 $\mu$m and 130 $\mu$m along their length, connected by smaller structures (Figure 8(C)). A short video clip illustrating these concepts in the healthy colon sample is provided as Supplemental Material (Video 2).

In the ileum, the segmented neural tissue had a volume of 0.005 mm$^3$, corresponding to a 1.0% ratio of neural tissue volume over total examined tissue volume. The thickness analysis showed a main trunk, with thickness ranging between 60 $\mu$m and 70 $\mu$m, and several thinner branches, only in part connected to the main trunk within the examined volume (Figure 8(D)).

Discussion

The main finding of the present methodology description was that X-ray phase-contrast tomography could visualize and separate the different cellular components of the myenteric plexus in health and disease in unstained human ileum and colon. The 3D method could also provide the absolute and relative volume of the ENS compared with the whole sample volume.

The gold standard to describe pathological changes of the ENS in health and disease is by light microscopical examination, combined with immunohistochemistry, and quantitative analysis of neurons, glial cells, ICCs, telocytes and inflammatory cells, as well as up- or downregulation of diverse neuropeptides [14]. Despite the clear merits of routine immunohistochemistry in resolution and specific imaging of neuropeptides, this well-established method also has its shortcomings. Firstly, due to a large number of different neuropeptides, the combination and variation of the composition of neuropeptides may vary indefinitely, both within health and within the same disease entity. No pathognomonic changes of neuropeptides for enteric neuropathy have so far been described. Secondly, histology is based on 2D sections, and extension to 3D datasets based on parallel slices is very tedious, time-consuming and does not result in isotropic resolution due to slice thickness, alignment problems, and slicing artefacts. Fluorescence confocal microscopy with dual immunohistochemistry has been used with either serial thin cryostat sections with a maximal thickness of 15 $\mu$m [7–9,20], or serial optical sections of whole mount preparations [10]. In those studies, it is difficult to exactly...
That telocytes in the colon can both glial cells and spindle-shaped cells can be observed. In a pre-lution scan. Within and around the ganglion, the neurons, overview scan, and then focus on them with a high-resolution scan can be performed on the sample immediately after an overview scan, can be seen on Video 1. A high-resolution scan can be performed with a parallel-beam configuration, allowing for fast overview scans with slightly enhanced resolution and contrast compared to the ones presented in this work, but with scan times as short as $\sim 70$ s [24]. Beyond that, recent advances in computer architecture and software may allow the processing and analysis of data sets from numerous patients in the future. The spindle-shaped cells within the periganglionic connective tissue can represent either ICC, telocytes, or fibroblasts/cytes. These cells cannot yet be differentiated in the nano-tomographic pictures, although the ‘spindle-shaped cells’ (probably telocytes) can be very well followed within the ganglion, as shown in the high-resolution video clip (Video 1). We can expect that further advancements in X-ray sources, phase retrieval and segmentation methods will improve differentiation of various cells in the future. At present, double/triple immunohistochemistry, with or without confocal fluorescence microscopy [4,7,8,25–27], is a satisfactory, cheaper and quicker method for this purpose. However, staining and 3D visualization of myenteric ganglia by the new method of Graham and López et al. [21] takes several weeks, and demands many manual steps. Despite the disadvantages, our long-term goal will be to include more individuals to examine whether 3D X-ray phase-contrast tomography is applicable for the study of gastrointestinal disease mechanisms comparing healthy and diseased tissues. Although the small volume of ENS in our patient was in alignment with the ganglia atrophy observed in immunohistochemistry [15], one sample is not enough to state the difference between health and disease. Different methods are needed to evaluate tissue biopsies and may complete each other. While the approach is still user-dependent in order to identify the most representative area, once a ROI is chosen, the method is very reproducible from time to time and mainly automatic. It also does not require special sample preparation, as paraffin-embedding is already routinely used for histological studies. Furthermore, even though not as good as in paraffin-embedded samples, the same technique has been shown to still provide contrast in hydrated neural tissue [28], if the shrinking induced by paraffin-embedding is of major concern.

In conclusion, the present methodology paper shows that X-ray phase-contrast tomography can be applied to the analysis of the human ENS in health and disease and to study tissue components and the relationship between various cellular components of the ENS in full-thickness bowel...
biopsies, which is a promising novel technique for research and possible future clinical applications in selected cases.

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Disclosure statement

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ORCID

Lars B. Dahlin http://orcid.org/0000-0003-1334-3099

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