**Supplementary material**

Methodological considerations of 3DR of small structures of interest

In the course of the present analysis several methodological problems occurred concerning the true visualization and representation of structures of interest (SOI) small in relation to section thickness, which showed a wide variability in the histological material (both modern and historic) available for this study. Previous principal considerations of stereology and 3DR (Weibel et al. 2007; Boyce et al. 2010; Tilic et al. 2015) helped to incorporate the section thickness and, hence, section volume as a crucial parameter into the following models visualizing these problems.

As microtomes may produce sections with a thickness (e.g. 10 µm) considerably larger than the size of small structures of interest (SOI), in this case the lumen of the notochordal canal (measuring 5 µm in width, cf. Fig. 19 Heuser 1932), different situations may arise in relation to the position of the SOI within the volume of any histological section. Four principal situations which were most likely to occur amongst the wide range of histological methods used for the specimens analysed here were modelled using wooden building blocks of the cuboro® marble track system (cuboro AG, Hasliberg Reuti, Switzerland). To this end, the blocks were aligned to represent a central wide longitudinal section of the embryonic disc containing a hollow internal structures resembling the lumen of a neurenteric canal running diagonally from its dorsalcaudal to its ventrocranial openings, Fig. 1A-E): (1) a SOI (Fig. 1A) with a diameter measuring less than the whole, but more than half, the section thickness and which lies centrally within the section will appear semi-translucent in the microscopical analysis of the section (Fig. 1B). (2) A SOI which has a diameter of about twice the section thickness and which does not lie centrally within the section will appear semi-translucent in the microscopical analysis of the section (Fig. 1C). (3) A SOI which has a diameter of about twice the section thickness and which lies centrally within the section will appear fully translucent in the microscopical analysis of the section (Fig. 1D). (4) A SOI which has a diameter of more than twice the section thickness will always appear fully translucent in the microscopical analysis of the section (Fig. 1E). Which of the four situations applied to which of the specimens analysed here was determined prior to segmentation and 3DR.

Additionally, some cellular structures such as the cellular nucleus or membranes may be smaller than the section thickness, too, and may be visible in selected planes of optical focus within the depth of that histological section, only. To visualize this situation, different wooden building blocks were aligned to represent the width of a histological section which contains the right half of notochordal canal as the “closest relative” to the neurenteric canal. Schematic sectioning images of cellular structures typically lining a narrow epithelial tubular structure were drawn onto relevant surfaces of the building blocks (Fig. 1F). Microscopic images of three different focal planes from the same 10 µm histological section containing a longitudinal part of notochordal canal (Fig. 1G-I) show cellular structures such as the apical cellular cross sections in the vicinity of the notochordal canal either as web-like structures (Fig. 1G) or as out-of-focus background (Fig. 1I). By correlating these images with the schematic drawings on the model the respective positions of the optical sections within the the model were marked with a black line in the dorsolateral view of the model (Fig. 1F).

On the basis of (a) the highly variable ratio between section thickness and size of SOI and (b) cellular structures visible in some optical sections of a histological section the following two approaches were applied (and especially highlighted as such with any of the reconstructions shown) to render SOIs which are smaller than the section thickness visible in 3DR (Fig. 2): (1) ´narrow’ segmentation and reconstruction meaning that only fully translucent areas of a section are segmented and reconstructed as an extracellular space (Fig. 2 B, D). (2) ´wide’ segmentation and reconstruction meaning that also semi-translucent areas of adjacent sections are segmented and reconstructed as an extracellular space (Fig. 2 C, E). As a consequence, ´wide´ reconstructions also consider visible plasma membranes of apical cells undergoing involution and differences in density of (extra-) cellular material when examining small structures in presomite embryos.

Additional considerations concern the fact that structures lying between two closely apposing differently stained areas were rarely seen in 3DR since the smoothening algorithm recognizes regions of the same colour only and distorts or even disguises structures in between. Also, thin planar SOIs showing a slight misalignment in one of the three axes are difficult to reconstruct, as well, because the smoothening algorithm is unable to connect these tissues and, thus, leads to disconnected and perforated SOIs in the 3DR. As a consequence, the basement membrane was left “unsmoothed” giving it a polygonal appearance. To avoid other less prominent smoothening artifacts final reconstruction images were modified using Adobe Photoshop (see [www.adobe.com](file:///C%3A%5CUsers%5CAlex%5CDesktop%5Cwww.adobe.com)) to fit the situation found in the histological sections.

A further reconstruction principle concerns the ‘third’ (left-right) body axis which cannot always be determined morphologically at early embryonic stages. This axis may even turn out to be inverted during the later reconstruction when sections which show an asymmetry on the transverse axis (Fig. 3 A) are joined either in an ascending order (Fig. 3 B, C) or in a descending order of slide numbers (Fig. 3 D). Realistic 3D models can be obtained, however, when starting the 3DR with tissues within the first section. Unfortunately, this is not always documented in historic publications. In cases of incomplete documentation it may be assumed that, normally, the microtomist begins the numbering of glass slides with ´1´. Consequently, choosing slide no. 1 as the start of the reconstruction should lead the natural left-right orientation. The plausibility of this method was proven when reconstructing the embryo Ludwig ´Da1´ for which a former analog reconstruction showed the same left-right orientation as the one created in this study.

The significance of 3D-reconstruction in embryology.

It has been suggested that that results of embryological findings should be implemented in medical education and taken into account in clinical practice, especially when concerned with obstetrics and pediatrics (Carlson 2002; de Bakker et al. 2016). 3DR is hereby viewed to be one of the most promising techniques in doing so. While later somite embryonic stages can be reconstructed with MRI or automatic scanning, alignment and segmentation algorithms, presomite embryos need the better solution of histology and the cellular information hereby gained, and an alignment plus segmentation carried out by hand to avoid morphologic distortions and an overlook of small structures. Thin plain SOIs and such in between boundary surfaces need special attention during the process of reconstruction. Nevertheless quality checks of the reconstructed specimens are obligatory, to avoid a severe, even unconscious bias of a manual reconstruction. All 3DRs need some sort of subjective interpretation, meaning they can only with reservations be seen as absolutely objective reproductions of the embryos. It showed to be easier to reconstruct specimen processed sectionized and photographed by the reconstructor himself, thus avoiding any confusion about exact protocols or left right orientation. When structures smaller than usual section thickness itself are of interest, considerations of either sectioning the embryo semi-thin, implementing a wide manner of reconstruction or using alternative methods like X-ray microscopy (Nazaretski et al. 2015) seem justified. Additionally, each relevant embryonic structure including lumen has to be reconstructed with a separate texture, preventing its elimination through the smoothing algorithms. In the future, embryologic reconstructions may – like they do in engineering science already (Kim et al. 2015) – as well include the time scale, resulting in 4D embryology, that could be implemented in virtual training settings (Fellner et al. 2017). If needed, a 3D printer can be used to turn digital 3D reconstructions into physical objects again, making microscopic structures touchable.

Supplementary figure 1: Modelling the appearance of a small structure of interest (SOI) in histological sections of different thickness and using different planes of focus. A Dorsal view of wooden building blocks containing a canal-like SOI starting in the centre of a trough-like depression and running from dorsal-right to ventral-left (indicated by a red tape). For the purpose of comparing canal diameter and section thickness the diameter of the canal is set to 1.0. B top: dorsal view with superposition of a central sagittal (longitudinal) section (light blue) with a thickness ‘d’ greater than 1.0. Bottom: lateral view of trough and canal as a low-density structures (light blue) positioned in the centre of the section thickness. C top: dorsal view of two sagittal sections (light blue and pink) with a section thickness ‘d’ between 0.5 and 1.0 and sectioning trough and canal tangentially. Bottom: lateral view of trough and canal as low-density structures when sectioned tangentially (light blue and pink). D top: dorsal view of three sagittal sections (light green, light blue and pink) with a thickness ‘d’ between 0.5 and 1.0, the median section (light blue) sectioning trough and canal concentrically. Bottom: absence of trough and canal in the two lateral sections (green and red) and lateral view of trough and canal as unstained areas (white) in the median section. E top: dorsal view of three sagittal sections (light green, light blue and pink) with a thickness ‘d’ between 0.25 and 0.5, the median section sectioning trough and canal concentrically. Bottom: lateral view of trough and canal as low-density structures in the lateral sections (light green and pink) and as unstained areas (white) in the median section. Note that only situations given in D and E present the chance of observing a patent canal in longitudinal sections. F Principal building block representation of part of a 10µm paraffin section (section no. 34-1-1 of embryo 93.2.2 containing a patent blind-ending canal, applying the principal section situation shown in C. The canal appears as a deep drawn-out trough, its cellular lining partially indicated by black outlines. The yellow asterisk marks unpolarized cells forming the ventral wall of the canal in contradistinction to the polarized cells in the dorsal wall of the canal. The position of the focus planes of the three photomicrographs (G, H, I) taken from this section is represented by the black lines labeled G, H, and I, respectively, in F. The dorsal opening of the canal (black arrow in I) is either lined by a clear (G) or a blurred (H and I) representation of the honey-comb structure of the cells’ apicolateral membranes (black arrow head in G and I). The sequential appearance of the same apicolateral cell membranes in neighboring sections illustrate that the cells’ apices face an empty space, i.e. the lumen of the canal. Scale bar of I also valid for G and H.

Supplementary figure 2: ‘Narrow’ vs. ‘wide’ 3-dimensional reconstruction mode. A Hematoxylin-Eosin stained paraffin section no. 34.1.1 of embryo 93.2.2 used for ‘narrow’ (B, D) or ‘wide’ (C, E) segmentation (B, C) and reconstruction (D, E). Colour coding: neuroectoderm blue, notochordal plate purple, mesoderm red, basement membrane green. B Completely translucent intercellular areas only are segmented which results in three uncoded intercellular spaces (lumina) without connection to each other or to the dorsal embryonic disc surface. C Semi-translucent areas next to completely translucent intercellular areas are excluded from segmentation which results in a continuously patent canal with a dorsal opening. D Lateral view of a ‘narrow’ 3D-reconstruction using 24 sections and ‘narrow’ segmentation as shown in B: a continuously patent notochordal canal with a dorsal opening is not visible. E Lateral view of a ‘wide’ reconstruction using 4 sections and ‘wide’ segmentation as shown in C: a continuously patent notochordal canal with a dorsal opening (black arrow) is seen and bordered dorsally by the involuting ectodermal epithelium in the HINC. Scale bar of C also valid for B, D and E.

Supplementary figure 3: Left-right asymmetries in 3-dimensional reconstructions as a function of section seriality. A Schematic representation of five consecutive transverse sections with morphologically defined dorsal vs. ventral surfaces, a ventral black base plate, and a dorsal red pillar-like structure of interest (SOI); the SOI appears in a central position in the most cranial section (no. 1) and lies in a more lateral position in the most caudal section (no. 5). B Dorsal view of a 3D-reconstruction of the sections shown in A when aligned by starting with the cranial-most section and connecting the sections 1 to 5 serially into one image plane (‘away from the reader’): the SOI appears as curved structure located on the left side of the black rectangular base plate and leaning from caudal left to cranial right. C Dorsal view of a 3D-reconstruction of the sections shown in A when aligned by starting with the cranial-most section and connecting the sections 1 to 5 serially out of one image plane (‘towards the reader’). D Dorsal view of B inverted by 180 degrees (cranial to the top) for easier comparison with B: The SOI appears as a curved structure located on the right side of the black rectangular base plate and leaning from caudal right to cranial left (inverse to B).

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