The application of confocal microscopy and 3D imaging software in Functional, Evolutionary, and Developmental Zoology: reconstructing myo- and neurogenesis in space and time

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Although Marvin Minsky invented the basic principles as early as in 1955, it took several decades until confocal laserscanning microscopy (CLSM) finally entered the centre stage of zoological research. However, while CLSM datasets *per se* contain three-dimensional information, merged projection images resulting from confocal stacks provide a representation of the respective signal in 2 dimensions only. With the dramatic increase of computational capacity and the availability of advanced imaging software, it is nowadays possible to fully exploit the intrinsic 3D information of CLSM datasets and produce high-resolution, spatial depictions of labelled structures of metazoan bodyplans. In this chapter, I describe the three-dimensional reconstruction of neural and muscular systems in larvae and adults of minute invertebrate animals, based on confocal datasets computed by isosurface algorithms of 3D reconstruction software. In addition, I will outline the potential of these tools for reconstructing metazoan organogenesis events over time and highlight the significance of these applications for comparative functional, evolutionary, and developmental analyses in zoological research.

Keywords confocal laserscanning microscopy; 3D reconstruction; bioimaging; fluorescence; organogenesis; immunocytochemistry; neurobiology; evolution; invertebrate; segmentation; phylogeny

1. Introduction

Accurate reconstruction of metazoan bodyplan features – and especially of nervous and muscle systems - has traditionally been a major aim in zoological research, both because they hold a wealth of characters potentially important to infer animal interrelationships, and in order to clarify functional aspects of organ systems. While macroscopic dissection techniques may be applied to animals above the centimetre-range, depictions of the often highly complex, three-dimensionally arranged muscular and neural meshworks in microscopic specimens have for a long time been very time consuming, if not impossible altogether, due to methodological restrictions to serial sectioning and light or electron microscopical means of analysis.

In order to overcome this dilemma, Marvin Minsky constructed the first confocal scanning microscope in 1955. This technical breakthrough rendered it possible to not only record sharp images in various focal planes by scanning through the entire study specimen, but also to eliminate scattered light from out-of-focus regions, which otherwise results in blurred images [1]. However, having been invented prior to the age of routine availability of laser excitation and digitized signal processing and storage media, this genius idea was almost entirely forgotten until its renaissance some 30 years later. By that time, integration of technical innovations such as laser excitation, enhanced computational power, and refined microelectronic technology had rendered routine use of confocal laserscanning microscopy (CLSM) principally possible.

Indeed, with steadily increasing means of data processing and scan speed in combination with advanced software tools and improved fluorochrome stability, it is nowadays possible to perform high-throughput CLSM analyses of *whole-mount* fluorescence preparations by generating optical sections

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through an entire specimen without physical sectioning. This is not only possible for individual specimens, but also for entire developmental sequences of a species and allows precise 3D reconstruction of ontogenetic patterns of, e.g., myo- and neurogenetic pathways in minute invertebrate larvae in the size range from below 50μm to a few millimetres. It should be noted, however, that CLSM applications in *whole-mount* preparations are limited by the dimensions of the study organism. Accordingly, specimens with a thickness of more than 300μm will be difficult to be scanned in their entity. In practice, tissue conditions, the mode of fixation, and the fluorochromes used may limit signal yield to the upper 100μm or even less of a sample.

While, by definition, a series of confocal images ("stack") contains the entire information needed to perform three-dimensional reconstructions of the labelled organ systems, it were again the limitations in data processing power that for a long time prevented high resolution 3D depictions of CLSM datasets, although "pseudo-3D" images could be produced, which gave a quite realistic three dimensional impression of these data if viewed through appropriate goggles (Fig. 1).

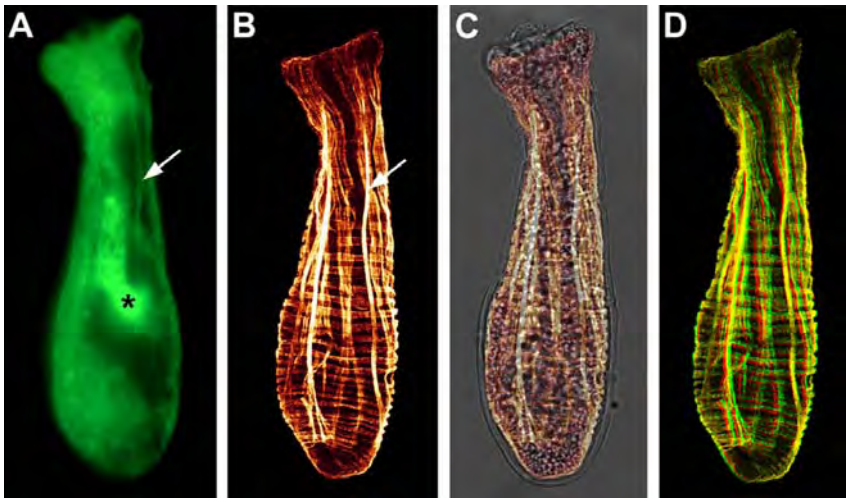

Fig. 1 A juvenile sipunculan worm stained for F-actin to visualize the body musculature. Identical specimen in all images.
A is an epifluorescence micrograph from a single focal plane, while B-D are CLSM recordings of a merged stack of optical sections through the entire specimen. The arrow in A and B depicts an identical longitudinal muscle fibre, the asterisk in A marks the high level of autofluorescence of the stomach from scattered signal from outside the range of the excitation wavelength of the fluorochrome. The significantly higher signal yield by CLSM and much better signal-to-noise ratio is evident. C is an overlay of the CLSM image shown in B and a light micrograph scan, while D is a red-green stereo image composed by the CLSM stack used to produce the projection image shown in B. The specimen is about 150μm in length.

It was only during the past few years that data processing and computer-based storage means as well as user-friendly 3D reconstruction software packages became inexpensive enough for research laboratories that focus on non-applied, basic zoological research. One of the milestones of these software tools is the possibility to depict the reconstructions not only as still images but also as animated video sequences that may be created and viewed on commercially available desktop computer systems and may easily be integrated in scientific presentations or lectures. The easy-to-use, full exploitability of CLSM-based 3D datasets, including the possibility to acquire animated video sequences, is one of the most exciting recent innovations in Evolutionary Zoology and spearheads new developments by linking bioimaging tools to microscopy-based basic organismic research, thereby also opening new educational horizons for universities and other research and teaching institutions.
In the following, I will demonstrate the scientific power of accurate three dimensional reconstructions of muscular and neural bodyplans of microscopic invertebrates based on immunofluorescence labelling and CLSM in combination with 3D reconstruction software, and I will highlight the use of these data for a number of research areas under the umbrella of "Evolutionary Developmental Zoology".

2. Fluorescence labelling, confocal microscopy, and 3D reconstruction of nervous and muscle systems in microscopic invertebrates

The motivation to reveal and understand animal bodyplan features can be manifold, but may generally be ascribed to one of the following three research areas: *functional* (or *constructional*) *morphology*, *evolutionary and phylogenetic systematics*, or *developmental zoology*.

2.1 Functional (or constructional) approach: "How does the animal function?"

This approach tries to answer questions dealing with issues as to what kind of mechanisms and morphological features animals have developed during the course of evolution to meet environmental, physical, or other selective pressures and constraints imposed on them. Some mud-burrowing worm-shaped animals, for example, use a complex three-dimensional meshwork of ring- and longitudinal muscles in order to create peristaltic movements. These are generated by selective adjustment of the pressure of the body fluid in certain body regions by increasing or reducing the diameter of the respective body areas through contraction or relaxation of individual muscles. Accordingly, the body wall functions as a muscular hydrostat against the inner body pressure. These processes can only be fully understood with detailed knowledge of the relative arrangement of the muscles to each other, which, in microscopic animals, can only be revealed in sufficient resolution by fluorescence labeling of muscular F-actin in combination with CLSM (Fig. 2).

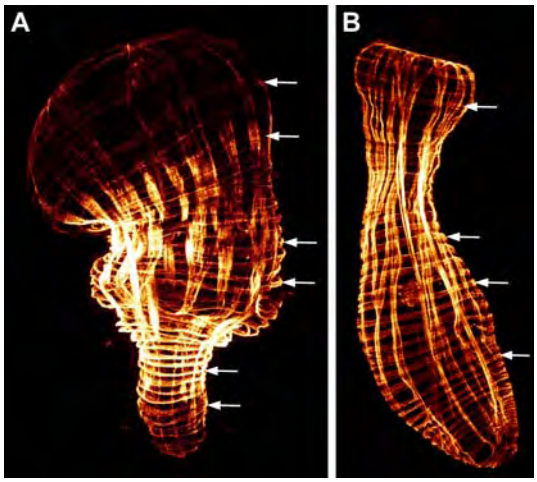

Fig. 2 Juvenile sipunculan worms. The specimen in A was fixed during burrowing movements and shows ring muscles in various stages of contraction (arrows), while the specimen in B is in a much more relaxed state. Relaxation of ring- and simultaneous contraction of longitudinal muscles results in enlargement of the body diameter and in reduction of body fluid pressure in the respective body region. Both specimens are about 150μm in length.

In contrast to mud-dwelling, cylindrical worm-shaped animals, some microscopic sessile invertebrates with a body size below the millimetre range are filter-feeding organisms that depend on relatively strong water currents in order to assure a steady supply with food particles (e.g., the tentacle-bearing kamptozoans or entoprocts, see Figs. 3, 4B). However, due to the lack of skeletal hardparts, one wonders how these animals maintain their upright body position. A closer look at their anatomy reveals a very dense and interwoven meshwork of longitudinal and oblique muscle fibres in the trunk region and in their disk-shaped attachment organ (Fig. 3) [2]. This muscular scaffold functionally replaces the endoskeleton of vertebrates or the calcareous exoskeleton of other invertebrates such as snails, thus avoiding collapsing of the body and allowing them to withstand currents and to orient their body in the water column.

Recognizing the distinct, fundamentally different functional roles of body wall muscles through detailed three-dimensional reconstructions, zoologists are able to understand the evolutionary response of animals to the different bodyplan requirements caused by the various life history strategies and ecological niches these species inhabit.

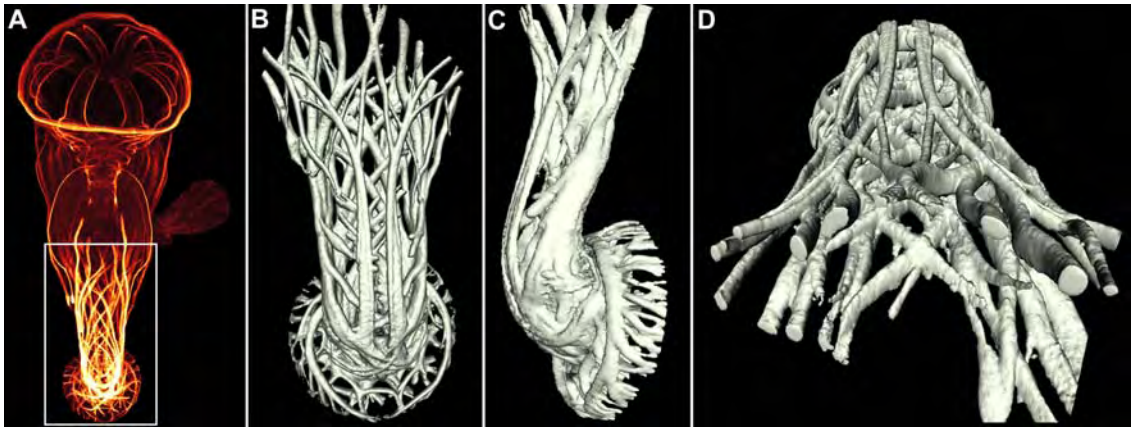

Fig. 3 Muscular meshwork of the trunk and the adhesive disk of an adult entoproct (kamptozoan). A. Musculature of the entire specimen as revealed by F-actin staining and CLSM. B-D. 3D reconstructions of the trunk and adhesive disk musculature from the boxed area in A using 3D imaging software. B. frontal view, C. lateral view, D. top view. The length of the entire specimen is approx. 250μm.

2.2 Evolutionary and phylogenetic approach: "Who is closest related to whom?"

Inferring phylogenetic relationships between animal taxa is a constant search for shared character states that allow alignment of a certain taxon with another one. Since all life cycle stages of an organism are exposed to selection pressures, it appears consequent to look for such traits not only in the adult stage, but during the entire life span of a species. Thus, a closer look at embryological and larval stages seems particularly promising if the adult phenotypes of the respective taxa do not yield enough data for solid phylogenetic inferences. In such cases, detailed reconstruction of larval body plan features on the one hand and developmental mechanisms that lead to the establishment of larval and adult phenotypes on the other (see below), may significantly add to the database in order to infer metazoan sistergroup relationships.

A drastic example comes from recent investigations dedicated to the identification of the direct sistergroup of Mollusca, a phylum that comprises groups with body plans as diverse as mussles, squids, snails, or basal worm-shaped taxa (aplacophoran molluscs). Accordingly, it was believed for a long time that molluscs are closely related to other worm-shaped forms such as annelids or sipunculans [3]. However, morphological analyses of larval stages employing electron microscopy and, in particular, CLSM in combination with immunocytochemistry, revealed that molluscs appear to be closest related to the enigmatic group of minute, tentacle-bearing invertebrates named Kamptozoa (or Entoprocta) mentioned above [4], a phylum that had previously been aligned with another tentacle-bearing group, Bryozoa (or Ectoprocta) (Fig. 4) [5]. However, the nervous system (NS) of the larvae of entoprocts and basal molluscs shows a number of homologous structures and, in its entity, exhibits a complexity unmatched by any other potentially closely related invertebrate taxon (Fig. 4D-G). This example emphasizes the high potential of combined CLSM and 3D reconstruction software applications on all life cycle stages of the target species and thus allows exploration of the full range of morphological characters present, making them available for phylogenetic and evolutionary analyses.

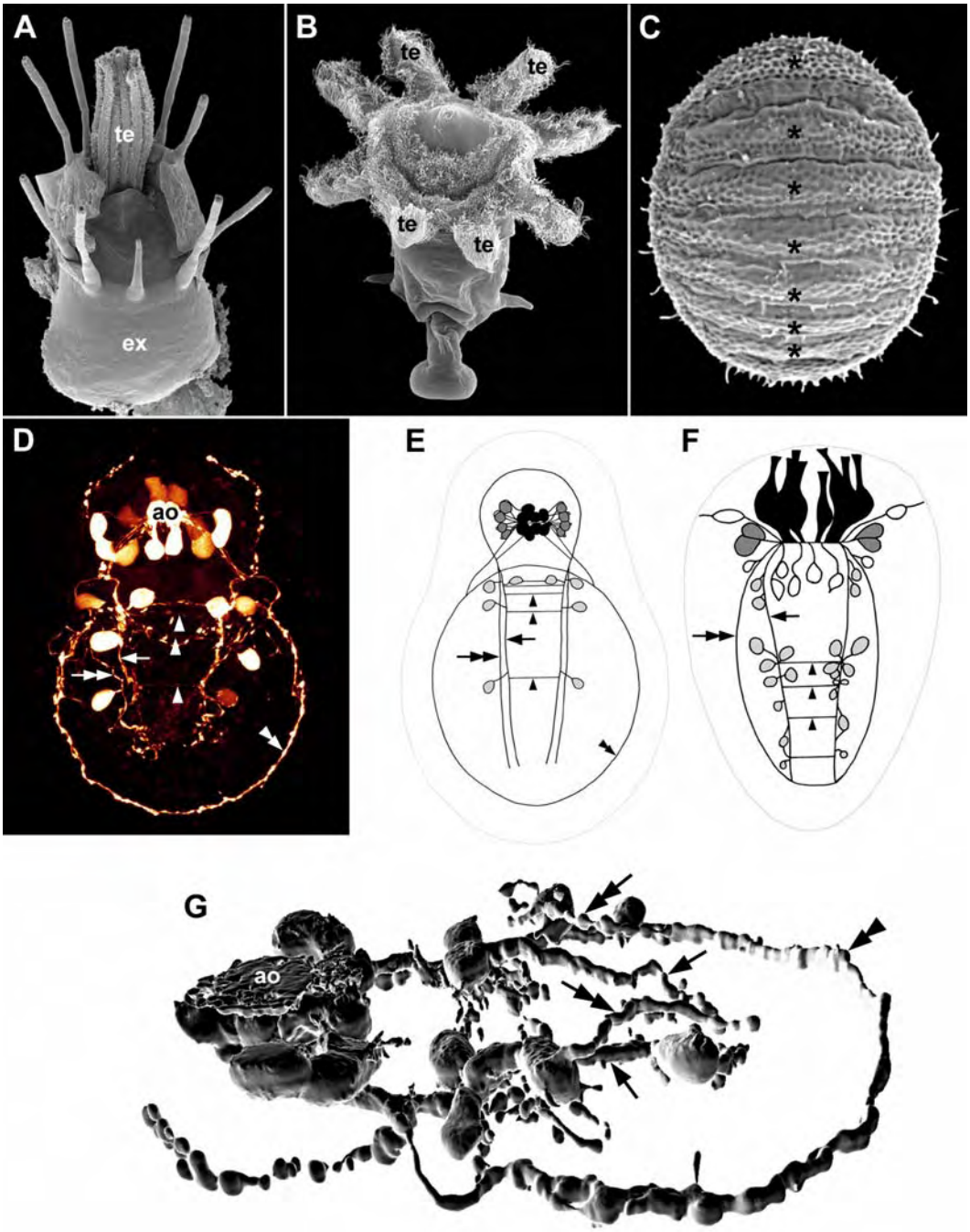

Fig. 4 The exoskeleton- (ex) and tentacle- (te) bearing Ectoprocta (Bryozoa; A) were previously thought to be the sister taxon of Entoprocta (Kamptozoa; B). However, larval neuroanatomy of Entoprocta and polyplacophoran molluscs (C; shell plates marked with asterisks) suggests an entoproct-mollusc clade. D. CLSM micrograph of the larval entoproct NS with complex apical organ (ao), outer visceral (double arrow) and inner pedal nerve cords (arrow) with commissures (arrowheads) and prototroch nerve (double arrowhead). E-F. Graphic representations of larval entoproct (E) and polyplacophoran (F) NS with homologous structures indicated. Proposed homologous immunoreactive cell types are depicted in respective shades of grey. G 3D reconstruction of the larval entoproct NS based on the CLSM stack shown in D. All specimens are approx. 250μm in length.

2.3 Evolutionary developmental approach: "How do animals and their organs form?"

A shown above, not only adult, but also embryological and larval life cycle stages may yield characters useful for evolutionary and phylogenetic inferences. As I will demonstrate in the following, it is, however, not only the stages alone, but also the developmental mechanisms leading to these phenotypes, that may hold important information about the evolution of animal form and metazoan relationships.

The mollusc scenario proposed that – based on larval characters – this phylum is closest related to the tentacle-bearing entoprocts rather than to the worm-shaped sipunculans or annelids. Analysis of the ontogenetic formation process of the ventral nervous sytem of sipunculans provides further (indirect) evidence for this hypothesis: While adult sipunculans exhibit a single nerve cord running along the midline of the animal in anterior-posterior direction, young sipunculan larvae clearly show a paired ventral nerve (Fig. 5A, B). Moreover, in later stages, labelling with an antibody against the neuropeptide FMRFamide reveals that these two nerve cords are bridged by 3 commissures – a situation resembling a typical "ladder-like" nervous system as is typical for annelids (Fig. 5C). It is only at the beginning of the benthic life phase, after the onset of metamorphosis, that this dual ventral nerve cord begins to fuse, eventually resulting in the single ventral nerve cord of the adult [6]. These findings indicate that sipunculans and annelids may stem from a common worm-like ancestor that possessed a paired ventral nerve cord and serially arranged commissures, while molluscs and entoprocts form a second evolutionary pathway with a tetraneurous nervous system consisting of one pair of ventral and an additional pair of visceral longitudinal nerve cords.

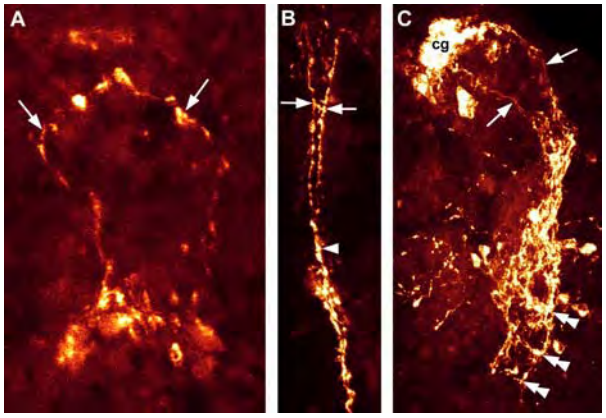

Fig. 5 Neurogenesis in sipunculan worms reveals the dual origin of the ventral nerve cord by serotonin immunostaining (A; arrows). Fusion starts at the onset of metamorphosis (B). From the initial merging point (arrowhead), the single ventral nerve cord grows continuously in posterior direction. Late stage larvae stained for FMRFamide (C) show commissures (double arrowheads) bridging both strands of the still paired ventral nerve cord. cg – cerebral ganglion. Total length of the specimens is approx. 150μm.

Concerning the evolution of animal bodyplans one key issue that has traditionally received considerable attention deals with the origin of annelid segmentation. While some authors have argued that segmentation may be a primitive trait that was already present in the last common ancestor (LCA) of lophotrochozoan or even protostome animals, others have proposed that it might have evolved independently in annelids and arthropods, respectively (see [7] for review). The former hypothesis implies that animals such as annelids, sipunculans, molluscs, entoprocts, etc. all emerged from a segmented stem species, and indeed, several taxa (e.g., annelids, basal molluscs, sipunculans) show serially repeated structures such as ventral commissures or body wall ring muscles. A comparison of the ontogenetic processes that lead to the serial arrangement of these organs in the various taxa, however, reveals striking differences (Fig. 6) [6, 8-10]. In annelids, segments (i.e., metamerically arranged, paired body cavities, so-called coelomic pouches) are formed along the anterior-posterior body axis. In the most basal species, each of these coelomic cavities houses a number of organs such as excretory systems, gonads, ganglia, ventral commissures, and ring muscles. Since these segments are formed from a posterior growth zone, development occurs in a strict anterior-posterior pattern, where anterior segments are always older than posterior ones. This is also reflected in the subsequent development of the organ systems associated with each segment. Accordingly, in developing stages, segmental muscles show an anterior-posterior gradient in their degree of differentiation, with anterior sets of muscles being further developed than more posterior ones (Fig. 6A). In sharp contrast to this condition, myogenesis of the body

wall ring muscles of the basal mollusc *Chaetoderma* occurs randomly, resulting in myocytes in different stages of differentiation in any given region along the body axis (Fig. 6B). A third way of ring muscle formation occurs in sipunculan worms, where the early rudiments of the musculature are formed synchronously (Fig. 6C). During longitudinal growth of the animal, multiplication of myocytes occurs by fission of existing muscle filaments along the entire anterior-posterior axis (Fig. 6D). Interestingly, in polyplacophoran molluscs, which exhibit 8 paired dorsoventral muscle units in the adult bodyplan, the early *anlagen* likewise appear synchronously (Fig. 6E, F). However, it is due to gradual postmetamorphic fusion processes of individual myocytes, that the adult 8-seriality eventually is established (Fig. 6G, H).

These differences in the ontogenetic mechanisms of muscle formation illustrate the importance of data on the dynamics of developmental processes for reconstructing evolutionary scenarios. Based on these data on myogenesis, the most parsimonious conclusion is that annelids are the only lophotrochozoans that express a "segmented" bodyplan emerging from a posterior growth zone, thus rendering the hypothesis of a segmented LCA of all lophotrochozoans highly unlikely [6].

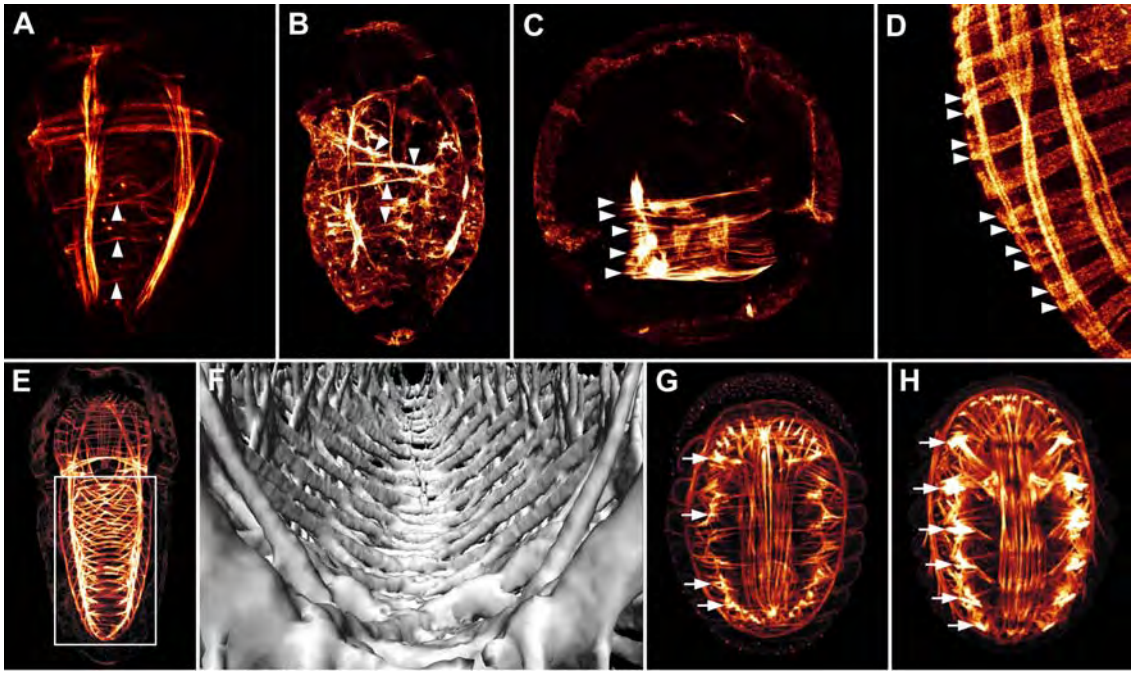

Fig. 6 Myogenesis of invertebrates shows various different ontogenetic mechanisms. All images are CLSM micrographs, except F, which is a 3D reconstruction of the boxed body region in E. A. Segmented annelid worms form their body wall muscles in strict anterior-posterior (a-p) direction, hence the signal decreases from anterior to posterior (arrowheads). B. Worm-like molluscs form their muscles in a random-like manner with no direction of formation discernable along the a-p axis. C. Sipunculan worms form the first muscle rudiments simultaneously. During growth of the animal, subsequent muscles are formed by fission from existing ones along the entire a-p axis (D). E. Polyplacophoran molluscs form their dorso-ventral muscles simultaneously, resulting in homogeneous muscle arrangement along the a-p axis (F), while concentration into the 8 units of the adult animal is a secondary condition that occurs subsequently after metamorphosis (G,H; arrows). All specimens are approx. 200μm in length.

3. Documentation of neuro- and myogenesis: adding the 4th dimension

The usefulness of developmental processes for evolutionary inferences has been outlined above. The three-dimensional information included in each confocal scan, however, allows an even greater broadening of data presentation and thus a gain of information: three-dimensional reconstruction of the dynamics of muscle and nervous system formation through time. Starting out with an originally two-

dimensional microscopic image, through exploitation of CLSM intrinsic 3D information by sophisticated reconstruction software, it is thus possible – provided that all significant stages in the ontogeny of the study specimen are available – to add time as the fourth dimension in microscopy. This adds significantly to our understanding as to how complex, three-dimensional networks of muscles and nerves are formed during ontogeny of small invertebrate animals. Hence, comparisons of character-states at each ontogenetic stage, as well as comparative analysis of the very process that shape larval and adult bodyplans, are possible (see above). Applying 3D reconstruction software to CLSM stacks of sipunculan neurogenesis, for example, enables 3D depiction of the neural anatomy of each individual developmental stage (Fig. 7). 3D representation of crucial developmental stages thus allows "4D" reconstruction of this process and facilitates understanding of the developmental mechanisms underlying neurogenesis in these animals (Fig. 7).

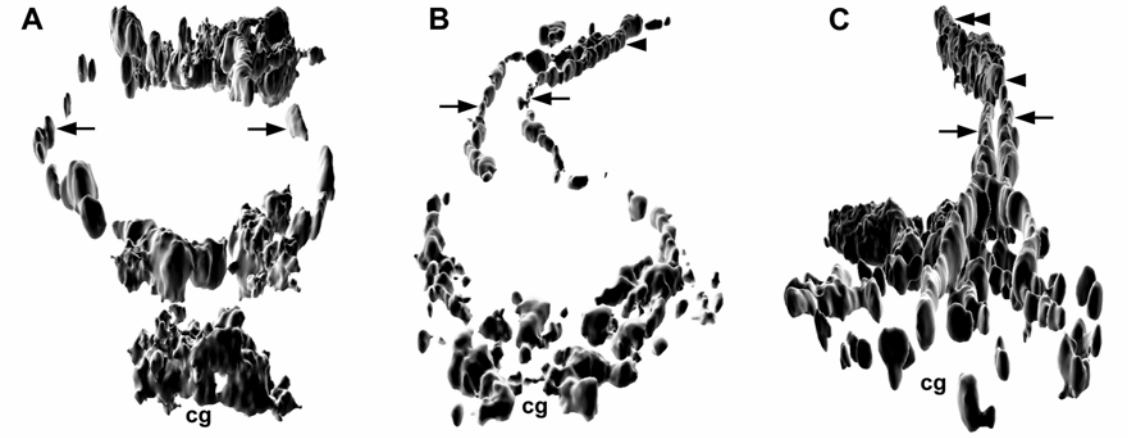

Fig. 7 4D reconstruction of neurogenesis in space and time in a sipunculan worm. Images were created with 3D reconstruction software on the basis of CLSM stacks. All aspects are from anterior with the cerebral ganglion (cg) in the foreground. In early larvae (A) the ventral nerve cord (arrows) is a clearly paired structure that fuses during subsequent development (B, C; arrowheads), and eventually continues to grow as a single nerve cord in posterior direction (C; double arrowhead). Note the decreasing space between the nerve cords from stage A to C.

4. Outlook

Digitized microscopic techniques such as confocal laser scanning microscopy in combination with advanced, commercially available 3D reconstruction software packages render it possible to perform microanatomical analyses of fixed samples in 4 dimensions, if complete developmental sequences of the life cycle of the study species are available. In addition, animated movie clips can easily be produced with these software tools, and since most publishers now offer the possibility of storing online supplementary material linked to the respective articles, these clips become available to the scientific community and may also be used as teaching material at universities. These novel advances are due to increased digital data processing power, which in recent years has become inexpensive and thus readily available to organismic zoological research. The enhanced scientific power of these 4D recordings does not only lie in the detailed reconstruction of animal bodyplans at any given stage of development, but also in the possibility of reconstructing the dynamics and morphogenetic mechanisms underlying these ontogenetic processes. Reconstructing animal morphogenesis in time and space opens a whole new field of research for developmental zoologists, as it is now possible to compare not only the character states as such, but also the very processes that shape the phenotypes of microscopic species, thus allowing insights into the evolutionary pathways that have led to the diversity of animal form.

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