

LETTERS

Synchrotron X-ray tomographic microscopy of fossil embryos

Philip C. J. Donoghue¹, Stefan Bengtson², Xi-ping Dong³, Neil J. Gostling¹, Therese Hultgren², John A. Cunningham^{1,4}, Chongyu Yin⁵, Zhao Yue^{2,5}, Fan Peng³ & Marco Stampanoni⁶

Fossilized embryos from the late Neoproterozoic and earliest Phanerozoic have caused much excitement because they preserve the earliest stages of embryology of animals that represent the initial diversification of metazoans^{1–4}. However, the potential of this material has not been fully realized because of reliance on traditional, non-destructive methods that allow analysis of exposed surfaces only^{1–4}, and destructive methods that preserve only a single two-dimensional view of the interior of the specimen^{5,6}. Here, we have applied synchrotron-radiation X-ray tomographic microscopy (SRXTM)⁷, obtaining complete three-dimensional recordings at submicrometre resolution. The embryos are preserved by early diagenetic impregnation and encrustation with calcium phosphate, and differences in X-ray attenuation provide information about the distribution of these two diagenetic phases. Three-dimensional visualization of blastomere arrangement and diagenetic cement in cleavage embryos resolves outstanding questions about their nature, including the identity of the columnar blastomeres. The anterior and posterior anatomy of embryos of the bilaterian worm-like *Markuelia* confirms its position as a scalidophoran, providing new insights into body-plan assembly among constituent phyla. The structure of the developing germ band in another bilaterian, *Pseudoooides*, indicates a unique mode of germ-band development. SRXTM provides a method of non-invasive analysis that rivals the resolution achieved even by destructive methods, probing the very limits of fossilization and providing insight into embryology during the emergence of metazoan phyla.

The study involves various developmental stages from cleavage to pre-hatching. Fossil cleavage embryos are notoriously difficult to study because of their tiny size and precarious preservation, but

tomography data provide complete information about the size, shape, and distribution of preserved blastomeres. Thus we have been able to test the recent claim that large columnar structures in some earliest Cambrian embryos, originally interpreted as an outer

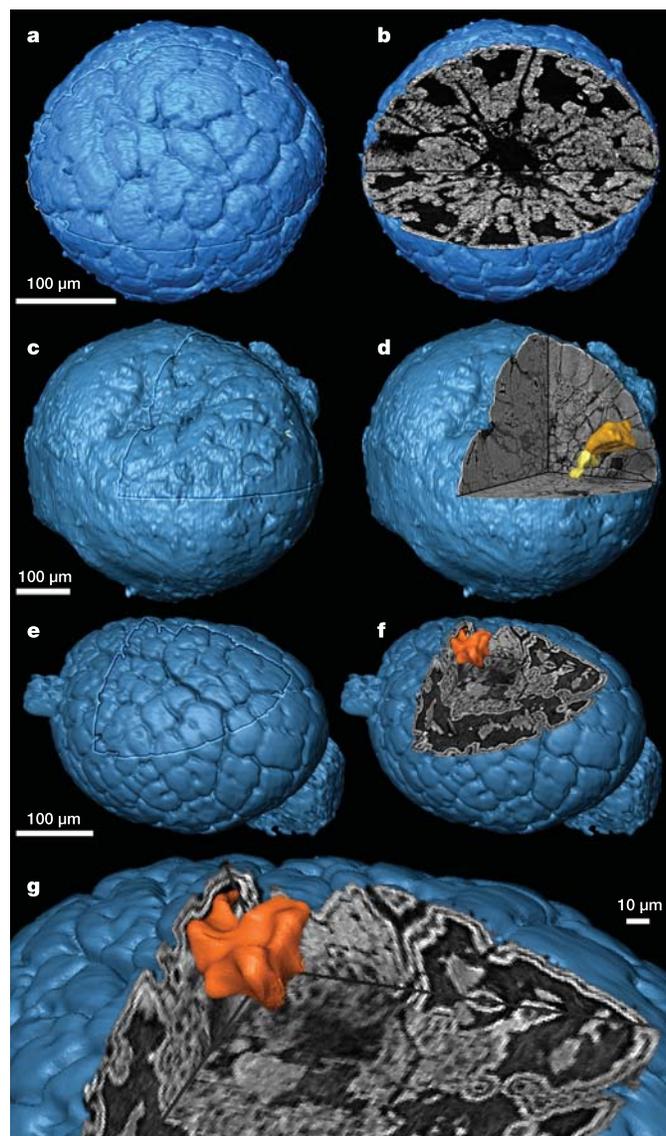


Figure 1 | Tomographic reconstructions of cleavage-stage embryos from the Lower Cambrian Kuanchuanpu Formation, Shizhonggou section, Kuanchuanpu, Ningqiang County, Shaanxi Province, Southern China. Divisions between adjacent blastomeres variably preserved on the surface and within. **a, b**, Museum of Earth Science, Institute of Geology, Chinese Academy of Geological Sciences (MESIG) 20061. Divisions between some, but not all, blastomeres are preserved internally. **c, d**, MESIG 20062. Divisions between all, or nearly all, blastomeres are preserved to their full extent; the orange and yellow structures are renderings of the morphology of a column of blastomeres. **e–g**, Geological Museum of Peking University (GMPKU) 2204. Divisions between blastomeres are generally not preserved, and instead the core of the embryo is characterized by the centrifugal addition of diagenetic crust layers (easily distinguished from edge artefacts through their absence from some of the objects seen in the slices); orange structure represents a rendering of one of the cavities within the diagenetic infilling.

¹Department of Earth Sciences, University of Bristol, Bristol BS8 1RJ, UK. ²Department of Palaeozoology, Swedish Museum of Natural History, Stockholm, Sweden. ³School of Earth and Space Sciences, Peking University, Beijing, China. ⁴Department of Earth and Ocean Sciences, 4 Brownlow Street, Liverpool L69 3GP, UK. ⁵Institute of Geology, Chinese Academy of Geological Sciences, Beijing, China. ⁶Swiss Light Source, Paul Scherrer Institut, CH-5232 Villigen, Switzerland.

layer of blastomeres⁸, instead represent yolk pyramids of a kind known from modern arthropods, with the implication that arthropod evolutionary history is thereby pushed back in time⁹.

Our SRXTM scans of embryos from the same Cambrian deposits show that they are fully subdivided into blastomere-like structures, among which the narrow columnar ones form the outer layer (Fig. 1a–d) (tomographic slice data available as Supplementary Information for all specimens we describe). Arthropod yolk pyramids do not have such arrangement¹⁰, and would also be expected to preserve evidence (not seen here) of the constituent yolk particles. The evidence for complete cleavage in these embryos removes one of the central arguments for associating them with the later-stage embryos of the enigmatic *Pseudoooides*³ rather than other co-occurring taxa, including the putative cnidarian *Olivoooides*^{1,8}. This also removes support for a projected prehistory of the arthropod phylum.

The SRXTM scans also illustrate the layered diagenetic crusts of apatite that often line the walls of cavities in fossilized embryos (Fig. 1g). Such layers have been interpreted^{5,6,11–14} as original cellular layers, but can now be imaged to their full three-dimensional extent (see, for example, the orange object in Fig. 1f, g), making them clearly distinguishable from cellular tissue layers. Thus, together with evidence from comparative taphonomy^{15–18}, the case is now very strong for rejecting claims of anthozoan planula larvae, hydrozoan and bilaterian gastrulae^{5,6,12}, as well as minute adult bilaterians^{12–14} from the Ediacaran Doushantuo phosphorites.

A similar mode of infilling is encountered in specimens of *Markuelia secunda*, a putative scalidophoran from the Early Cambrian of Siberia¹ (Fig. 2j). The SRXTM analysis demonstrates layered infilling, but this is clearly secondary to the replication of the

anatomy of the embryo: the two phases are distinguished by different levels of X-ray attenuation.

SRXTM has also facilitated analysis of embryonic morphological features that are not exposed at the surface. For instance, the two best-characterized species of *Markuelia* differ ostensibly in the number of pairs of posterior spines: two in *M. secunda*¹ and three in *M. hunanensis*^{4,19}. However, tomographic sections through the embryos of *M. secunda* reveal a third, unexposed pair of spines (Fig. 2j), confirming its similarity to *M. hunanensis*.

Although these data are crucial to understanding the disparity of embryo body forms, they are not germane to understanding the affinities of *Markuelia*. *Markuelia* has been proposed to fall into the Scalidophora (phyla Kinorhyncha, Loricifera, Priapulida)^{4,19}, although this conclusion is hostage to missing data, with reassignments possible within Scalidophora, or to Nematodea (phyla Nematoda, Nematomorpha)¹⁹. In particular, the organizational symmetry of the circummoral and pharyngeal spines is crucial to the systematics of these two clades, with nematoids exhibiting hexaradial symmetry and scalidophorans exhibiting 20- and 25-fold (pentaradial) symmetry²⁰. The symmetry of the spines in *Markuelia* was unknown because of the difficulty in interpreting even the very best specimens^{4,19}, but now SRXTM has made it possible to dissect such specimens virtually and resolve the scalid arrangement.

Figure 2a–f shows an incomplete embryo of *M. hunanensis* in which spines are preserved around the margins of the mouth. The exact arrangement cannot be seen in surface view, but virtual sections reveal that the spines continue internally. Their orientation towards—rather than away from—the orifice is consistent with their proposed nature as scalids situated on an introvert capable of

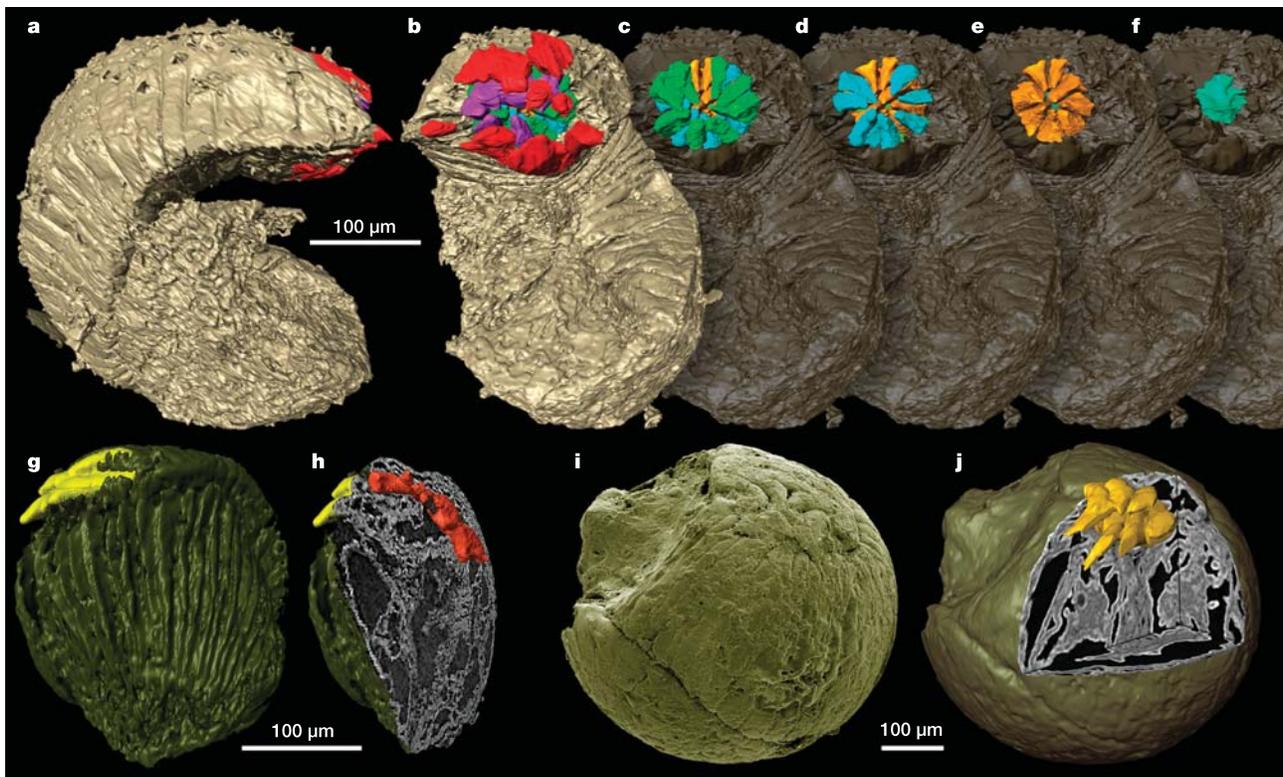


Figure 2 | The scalidophoran *Markuelia* from the Cambrian of China and Siberia. **a–f**, GMPKU 2205. Whole-mount tomographic reconstruction of *Markuelia hunanensis* from the Upper Cambrian of Wangcun, Hunan, Southern China. **b–f** are rotated 90° from **a**. **a** and **b** show the whole reconstruction, with all scalids. **c–f**, 25 circumpharyngeal scalids arranged in rings surrounding the mouth cone (**f**). 25 scalids are shown in the first three rings (**c**), 16 scalids are shown in the first two rings (**d**), and 8 scalids are

shown in the inner first circummoral ring (**e**). **g, h**, GMPKU2011.

Tomographic reconstruction of posterior spines and a section revealing the terminal end of the digestive tract of *M. hunanensis*. **i, j**, Swedish Museum of Natural History (SMNH) X2240. *Markuelia secunda* from the basal part of the Pestrotsvet Formation at Dvortsy, Aldan River, Siberia. Scanning electron micrograph (**i**) shows four terminal spines arranged in two pairs, while the tomographic reconstruction (**j**) shows a third, obscured, pair.

inversion. All but the most distal rows of scalids are located within an expansive and externally open lumen, separated from the closed pharynx by a mouth cone (Fig. 2f). Approximately forty scalids are present (including broken external scalids), arranged in five rings. The first three rings (counted away from the mouth cone) are composed of eight (Fig. 2e), eight (Fig. 2d) and nine (Fig. 2c) scalids, respectively, and are arranged such that each individual scalid intercalates with others in the preceding ring. Thus, the first three rings define 25 radii—as in living priapulids in which there are three initial rows of eight, nine and eight scalids²¹.

This overall pentameric arrangement in *Markuelia* and priapulids is also shared by loriciferans and kinorhynchs, but not nematodes and nematomorphs²¹. The position of the scalids, restricted to a pre-pharyngeal lumen in the best preserved specimens, is the same in embryos and larvae of nematomorphs, kinorhynchs and priapulids²², in which the introvert occurs naturally in an inverted position. The introvert normally assumes an everted position in later developmental stages and the adult^{22,23}, and it is likely that embryos of *Markuelia* in which the mouth cone is everted^{4,19,24} represent the latest-stage embryos known. We conclude, therefore, that the introvert of the *Markuelia* larva was capable of complete inversion. Together with the evidence of structure and scalid shape, it is likely that the introvert was used in peristaltic locomotion, as in the priapulid adults²³.

Data from SRXTM also reveal that the lumen at the posterior terminus, from which the three pairs of terminal spines emerge, extends internally to form an axial tube that can be traced adorally for over 200 μm (Fig. 2h). The most likely interpretation of this is a digestive tract opening to a terminal anus, a feature shared with nematodes, loriciferans, kinorhynchs and basal panarthropods, but not nematomorphs or most living priapulids^{4,19}.

The inclusion of these new data on the anatomy of *Markuelia* into the phylogenetic analysis presented by Dong *et al.*^{4,19} (see Supplementary Information) adds further weight to the existing hypothesis of affinity in which *Markuelia* is resolved as a member of the scalidophoran stem, corroborating unfolding evidence that similarities to priapulids are superficial and represent shared-primitive characters of scalidophorans. Additional support is also found for the view that pentameric organization of the introvert and pharyngeal armature is a scalidophoran character²⁰, and that despite differences in adult and larval body form among scalidophorans, the primitive function of the introvert is peristaltic locomotion²⁵.

As a final example, we also examined the enigmatic fossil embryo taxon *Pseudoooides prima* (Fig. 3), which has been interpreted to exhibit a long germ-band pattern of development³. The putative germ band has been claimed to exhibit secondarily imposed metamorphism³ and is delineated from the surrounding extra-embryonic material by a marginal furrow. However, there is no evidence of progressive development, and the germ-band feature exhibits a peculiar transverse pinching at mid-length³. The SRXTM analysis reveals that in most specimens the furrows separating the presumed metameres extend internally to some depth but do not fully enclose them. The marginal furrow delineating the germ band exhibits a highly wrinkled micromorphology (Fig. 3f and Supplementary Fig. 1) but extends to only a shallow depth (Fig. 3f and the stereo Supplementary Fig. 2). Where a pinch occurs in the centre of the germ band, the marginal furrow shallows to the surface, possibly indicating the point of segment addition (Supplementary Figs 1, 2). The latter interpretation is consistent with the pattern of folding of the surface at both ends of the germ band (for example, Fig. 3a), which indicates outward movement along the band emanating from the pinched centre. A further structure, superficially like a surface scratch, is situated between the pointed ends of the germ band, at the pole opposite to its pinched centre, and oriented perpendicular to it (lower left quadrant in Fig. 3b, d; compare also Fig. 2N in ref. 3 and Fig. 4.4A in ref. 26). Tomographic slices (Fig. 3c, e) show that it is connected to internal features and is thus not merely superficial. It is

clearly of biological origin, although its nature is uncertain. We interpret it as the remains of a blastopore formed before the wrinkled surface membrane. The lower, presumably ventral, surface of the germ band is never seen intact, though it has been observed to extend to more than a radius depth within. There is no evidence to support the original interpretation of a long germ-band pattern of development³, nor of terminal segment addition, characteristic of short germ-band development. Instead, the new data suggest a unique mode of establishing metamerism in which segments were added to the germ band from a centrally located generative zone.

It is clear from this study that scanning electron microscopy and light-microscopy of thin sections are insufficient to reveal fossil embryo structure. Analyses of internal and external structure in concert by means of SRXTM have allowed us to clarify the nature of diagenetic infills, to decide between opposing interpretations of cleavage modes, and to resolve the anatomy of the later-stage embryos of *Pseudoooides* and *Markuelia*, helping to constrain their affinity and evolutionary significance. Perhaps more importantly, it

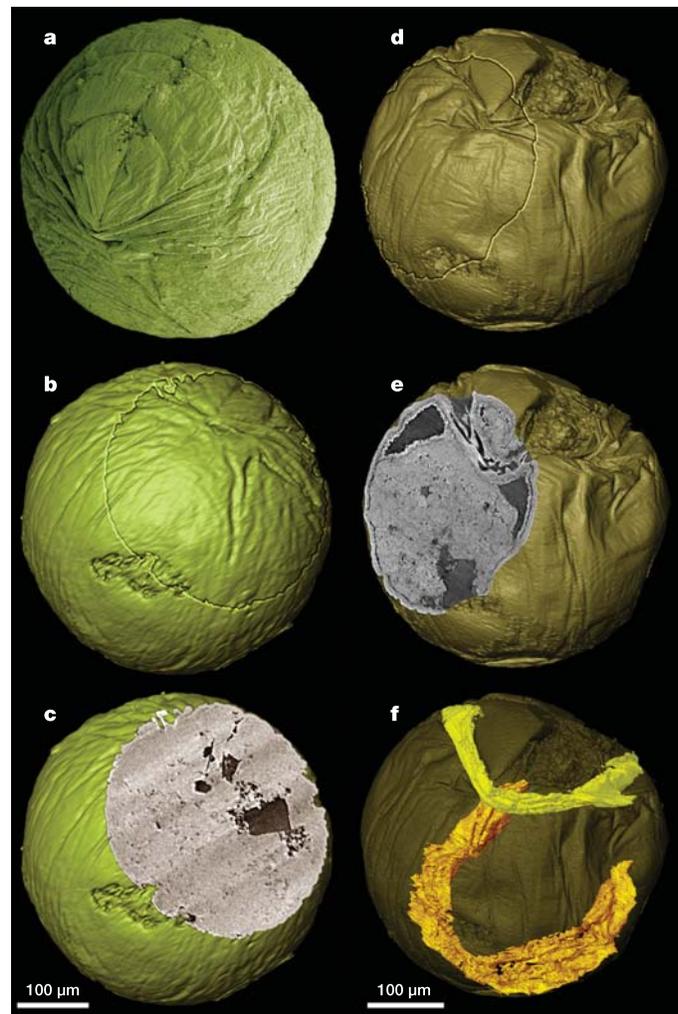


Figure 3 | Germ-band embryos of *Pseudoooides prima*, same locality as for Fig. 1. a–c, MESIG 20063. a, Scanning electron micrograph. b, c, Tomographic reconstruction with virtual histological section (c) showing diagenetic cuboidal crystals and possible blastopore (below and left of centre) at opposite pole from the germ band. d–f, MESIG 20065. Tomographic reconstruction, oriented with germ band uppermost. d shows position of virtual section in e, which shows that the terminal tip has an extension of the marginal furrow below it and that the possible blastopore is connected to a lacuna. f, rendered cast of the marginal furrow revealed as broadly horseshoe-shaped, enclosing the pointed ends of the germ band.

has revealed aspects of the anatomy of these organisms that would never have otherwise been resolved.

While authigenic phosphatisation of microscopic organisms has been widely recognized as an exceptional mode of fossilization²⁷, it has been held that preservation is limited to surface structure²⁸. SRXTM confirms that at least in instances of embryo fossilization, preservation can be much more extensive. More likely, the extent of anatomical preservation in fossils such as those from the Orsten and Orsten-like deposits²⁸ has been significantly underestimated and warrants further investigation with SRXTM technology.

SRXTM provides a non-invasive method of analysis of small and microscopic fossil materials that rivals even destructive approaches in terms of the resolution of data recovered, unlocking the finest details of preserved anatomy from fossilized remains. The present study demonstrates the feasibility of the method for a variety of questions concerning developmental processes in early fossil animals. The method has wide applicability in the study of microscopic structures in originally mineralized and exceptionally preserved fossil materials and may thus bring about a revolution in palaeontology on a par with that once brought about by the scanning electron microscope.

Received 21 February; accepted 10 May 2006.

- Bengtson, S. & Yue, Z. Fossilized metazoan embryos from the earliest Cambrian. *Science* **277**, 1645–1648 (1997).
- Xiao, S., Zhang, Y. & Knoll, A. H. Three-dimensional preservation of algae and animal embryos in a Neoproterozoic phosphate. *Nature* **391**, 553–558 (1998).
- Steiner, M., Zhu, M., Li, G., Qian, Y. & Erdtmann, B.-D. New early Cambrian bilaterian embryos and larvae from China. *Geology* **32**, 833–836 (2004).
- Dong, X., Donoghue, P. C. J., Cheng, H. & Liu, J. Fossil embryos from the Middle and Late Cambrian period of Hunan, south China. *Nature* **427**, 237–240 (2004).
- Chen, J. *et al.* Precambrian animal diversity: putative phosphatised embryos from the Doushantuo Formation of China. *Proc. Natl Acad. Sci. USA* **97**, 4457–4462 (2000).
- Chen, J. *et al.* Precambrian animal life: Probable developmental and adult cnidarian forms from Southwest China. *Dev. Biol.* **248**, 182–196 (2002).
- Stampanoni, M. *et al.* High resolution X-ray detector for synchrotron-based microtomography. *Nucl. Instrum. Methods Phys. Res. A* **491**, 291–301 (2002).
- Yue, Z. & Bengtson, S. Embryonic and post-embryonic development of the Early Cambrian cnidarian *Olivoides*. *Lethaia* **32**, 181–195 (1999).
- Chen, J., Braun, A., Waloszek, D., Peng, Q.-Q. & Maas, A. Lower Cambrian yolk-pyramid embryos from southern Shaanxi, China. *Prog. Nat. Sci.* **14**, 167–172 (2004).
- Chipman, A. D., Arthur, W. & Akam, M. Early development and segment formation in the centipede, *Strigamia maritima* (Geophilomorpha). *Evol. Dev.* **6**, 78–89 (2004).
- Chen, J. Y. & Chi, H. M. Precambrian phosphatised embryos and larvae from the Doushantuo Formation and their affinities, Guizhou (SW China). *Chin. Sci. Bull.* **50**, 2193–2200 (2005).
- Chen, J. Y. *The Dawn Of Animal World* 1–366 (Jiangsu Science and Technology Publishing House, Nanjing, 2004).
- Chen, J. *et al.* Small bilaterian fossils from 40 to 55 million years before the Cambrian. *Science* **305**, 218–222 (2004).
- Chen, J.-Y., Oliveri, P., Davidson, E. & Bottjer, D. J. Response to ‘Comment on “Small bilaterian fossils from 40 to 55 million years before the Cambrian”’. *Science* **306**, 1291b (2004).
- Bengtson, S. & Budd, G. Comment on “Small bilaterian fossils from 40 to 55 million years before the Cambrian”. *Science* **306**, 1291a (2004).
- Bengtson, S. in *The New Panorama Of Animal Evolution* (eds Legakis, A., Sfenthourakis, S., Polymeni, R. & Thessalou-Legaki, M.) 289–300 (Pensoft, Moscow, 2003).
- Xiao, S., Yuan, X. & Knoll, A. H. Eumetazoan fossils in terminal Proterozoic phosphorites? *Proc. Natl Acad. Sci. USA* **97**, 13684–13689 (2000).
- Raff, E. C., Vilinski, J. T., Turner, F. R., Donoghue, P. C. J. & Raff, R. A. Experimental taphonomy shows the feasibility of fossil embryos. *Proc. Natl Acad. Sci. USA* **103**, 5846–5851 (2006).
- Dong, X., Donoghue, P. C. J., Cunningham, J., Liu, J. & Cheng, H. The anatomy, affinity and phylogenetic significance of *Markuelia*. *Evol. Dev.* **7**, 468–482 (2005).
- Schmidt-Rhaesa, A. Phylogenetic relationships of the Nematomorpha—a discussion of current hypotheses. *Zool. Anz.* **236**, 203–216 (1998).
- Lemburg, C. *Ultrastrukturelle Untersuchungen an den Larven von Halicyptus spinulosus und Priapulid caudatus: Hypothesen zur Phylogenie der Priapulida und deren Bedeutung für die Evolution der Nematelminthes* 1–393 (Cuvillier, Göttingen, 1999).
- Adrianov, A. V. & Malakhov, V. V. *Kinorhyncha: Structure, Development, Phylogeny and Taxonomy* 1–260 (Nauka Publishing, Moscow, 1994).
- Hammond, R. A. The burrowing of *Priapulid caudatus*. *J. Zool.* **162**, 469–480 (1970).
- Donoghue, P. C. J. *et al.* Fossilized embryos are widespread but the record is temporally and taxonomically biased. *Evol. Dev.* **8**, 232–238 (2006).
- Dzik, J. & Krumbiegel, G. The oldest ‘onychophoran’ *Xenusion*: a link connecting phyla? *Lethaia* **22**, 169–182 (1989).
- Steiner, M., Li, G. & Zhu, M. Lower Cambrian small shelly fossils of northern Sichuan and southern Shaanxi (China), and their biostratigraphic significance. *Geobios* **37**, 259–275 (2004).
- Briggs, D. E. G. The role of decay and mineralization in the preservation of soft-bodied fossils. *Annu. Rev. Earth Planet. Sci.* **31**, 275–301 (2003).
- Waloszek, D. The ‘Orsten’ window—a three-dimensionally preserved Upper Cambrian meiofauna and its contribution to our understanding of the evolution of Arthropoda. *Paleontol. Res.* **7**, 71–88 (2003).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank W. Guo for field assistance, and A. Groso for her support at the beamline, and K. Robson-Brown for her assistance in running the experiments. This work was funded by the Swiss Light Source (P.C.J.D. and S.B.), European Union FP6, as well as grants from the Natural Environment Research Council (P.C.J.D.), the Swedish Research Council (S.B.), Bristol University and the National Natural Science Foundation of China (X.-p.D.).

Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.C.J.D. (phil.donoghue@bristol.ac.uk) or M.S. (marco.stampanoni@psi.ch).