Supporting Online Material for

Cellular and Subcellular Structure of Neoproterozoic Animal Embryos


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Materials and Methods

Bulk rock samples were collected from the upper Doushantuo Formation at Weng’an, Guizhou Province, South China. To obtain the studied embryos, several kg of grey dolomitic phosphorite were immersed in 10% acetic acid to dissolve the dolomitic cements and free phosphatized microfossils. Freed microfossils were repeatedly extracted every 3–5 days, and the undissolved rock fragments were subjected to repeated maceration. Fossils were examined using a dissecting microscope and a scanning electron microscope, and a suite of unbroken, uncollapsed spheroidal fossils were chosen for further analyses. The spheroidal shape was used as a rough indicator of their relative preservation – but a number of specimens were subsequently discarded after only partial preservation was discovered on their interiors. 102 well-preserved specimens representing a range of possible ontogenetic stages, from 1- to ~1000 cells, were scanned using microfocus and synchrotron X-ray computed tomography. Three 4-celled specimens, some bearing reniform-spheroidal subcellular structures, were embedded in EPON epoxy for ultra-thin sectioning (70 nm thick) at controlled orientations in order to observe their internal structures and assess compositional and diagenetic features using TEM. For petrography, 40 specimens were embedded and standard 30 μm thin sections were made. In the process of polishing embedded samples for petrography, one of the best specimens was accidentally overpolished (see NW hemisphere of embryo in Fig. 1F).

TEM was performed on a JEM 1230 at Nanjing Institute of Geology and Paleontology. The voltage was 80-120 kV. Field emission scanning electron microscopy (FE-SEM) was performed on a LEO 1550 FE-SEM (Virginia Tech) and a LEO 1530 VP (Nanjing Institute of Geology and Paleontology). The voltage was 5-10 kV.

Microfocus X-ray computed tomography (microCT) was performed on Skyscan 1072 microtomographs at NASA-JPL and Amherst College (S1-2). Samples were scanned unwedged and were exposed for 1.6-2 seconds at 75 keV and 132 μA, with 200 equiangularly spaced projections across 180°. Replicate scans were made of some samples, after wedging in apatite powder, to reduce beam hardening effects resulting from usage of polychromatic X-rays [sensu (S3)]. Three frames were averaged for each image to improve the signal-to-noise ratio, and greyscale spectra of shadow images were homogeneously contrast stretched such that X-ray attenuation values for air were zero. X-ray attenuation slices were generated using modified filtered backprojection of shadow images (NRecon; www.skyscan.be) into an
image stack, and resulting voxels are 1.6 \mu m \times 1.6 \mu m \times 1.6 \mu m. Volume rendering and isosurface modeling was performed using contrast thresholding and manual and auto-segmentation using ANT (www.skyscan.be) and AMIRA (www.tgs.com).

Synchrotron-radiation X-ray tomographic microscopy (SRXTM) was performed at the tomography station of the Materials Science beamline of the Swiss Light Source at the Paul Scherrer Institute (S4). Samples were scanned unwedged and were exposed for three seconds at 17.5 keV, with 501 or 1001 equiangularly spaced projections across 180°. Two frames were binned for each image, and greyscale spectra of shadow images were homogeneously contrast stretched to maximize X-ray attenuation contrast within embryos. X-ray attenuation slices were generated using optimized filtered back-projection, and resulting voxels are 1.4 \mu m \times 1.4 \mu m \times 1.4 \mu m. Volume rendering and isosurface modeling was performed using manual and auto-segmentation in AMIRA.

Downsized animations of selected image stacks are in Supporting Online Materials. Complete computed tomography datasets and raw SEM, TEM, and tomography parameters for all samples are available from authors on request.

Unequal Cell Size and Asynchronous Cleavage in Marine Embryos

Unequal embryo cell sizes and asynchronous cell division within in marine embryos can result from several processes. The first three in the following list are normal events that result in different cell sizes as a normal part of embryo cell cleavage in particular taxa.

1) Unequal timing as a normal part of cleavage of distinct cell lineages. In marine embryos, the canonical 2ⁿ division pattern is followed, but because not all cells divide at the same rate, the numbers of cells at any slice of time do not necessarily match this count sequence. For example, one gastropod illustrated by Lindberg and Guralnick (S5) shows a pattern of 2-cell, 3, 5, 7, 11…cells. They show that heterochronic variations in cell numbers occur across gastropod taxa, due to differing rates of cleavage of particular cell lineages. Non-canonical cell counts have been documented for other mollusks (S6), annelids, ascarid nematodes, nemertines (S7), echinoids (S8), and sponges (S9). In many cases these cell lineage events produce regular and reproducible patterns of cells.
2) Unequal cell sizes are often produced, even with equal rates of division, by unequal division to yield different cell sizes, e. g. macromeres and micromeres in sea urchins or in spiralians. This does not depend on differences in rate, but in cell division machinery that produces a large and a small daughter cell rather than two equal sized ones. Cell patterning can be strikingly and characteristically regular or it may be irregular, depending on the taxon involved.

3) A gradient of slower cleavages from animal to vegetal pole can produce a distribution of cell sizes, generally distributed from one pole to the other. Cells at one end will be smaller than at the other end. This is typical of some large eggs, and seen in various taxa, e. g. some cnidarians (S10) and barnacles (S7).

4) Abnormal production of differing sized cells or patches of cells can result from anoxia, toxicity, and other pathological causes. The patterns are irregular and likely variable among individuals of the same species (S11).

References
Supplemental Figure and Movie Captions

**Fig. S1.** Distribution of embryo cleavage states and occurrence of subcellular structures among the studied population. This population only includes unbroken embryos that are bound by envelopes and/or are spheroidal. Note the general $2^n$ pattern, but also the presence of specimens that contain aberrant numbers of cells. Subcellular structures occur in approximately 25% of the 3-16 celled embryos; this frequency is consistent with our own observations of such features in thin section, and their size and shape is similar to previously figured specimens (12).

**Fig. S2.** 4-celled embryo (specimen DOU-23). (A) Reflected light photomicrograph. One of the cells is partially deflated. (B) Sketch of embryos, with cells labeled to correspond to their models. (C) Exterior isosurface model. (D-G) Digitally extracted cell models. Cell (F) rendered transparent to show subcellular structure (dark green) in embryo center. Scale bar for A, C: 300 µm.

**Fig. S3.** 8-celled embryo (specimen DOU-30). (A) Reflected light photomicrograph. (B) Exterior isosurface model. (C) A X-ray section that captures only four of the eight cells. Image has been placed on a black background to match (A). (D) A X-ray section that shows subcellular structure (arrowhead) with slightly greater attenuation. Image has been placed on a black background to match (A, C). (E) Schematic drawing with cells labeled to correspond to their models. (F-M) Cell models. Three of the models are rendered transparent to show subcellular structures. The other five do not have discernable subcellular structures and are opaque. Relative scale bar size: A, B, D: 200 µm; C: 100 µm.

**Fig. S4.** (A) Scanning electron micrograph of a *Megaclonophycus onustus* embryo. (B) Volume rendering of 754 discrete cells from this embryo; additional cells are visible in the orthoslice projections, but because their attenuation values are at the extremes of the greyscale spectrum, they were not rendered. We estimate there are probably ~1024 cells within this embryo, representing the 10$^{th}$ cleavage stage. (C) Cells are evenly distributed throughout the embryo interior, visible in the $\frac{1}{4}$ section. Together with thin section images (D-F) of *Megaclonophycus onustus*, this suggests that embryos progress through many stages of cell division with no evidence of gastrulation. (D-F) Thin section photomicrographs of *Megaclonophycus onustus*, showing the arrangement of cells.
(E) and (F) are modified from Xiao and Knoll (2000). Scale bar in A: 200 µm for (A), 169 µm for (D), 189 µm for (E), 85 µm for (F).

**Movie S1.** Animation of serial X-ray attenuation images through 7-celled embryo in Fig. 2D (specimen MPLa). Image sizes and contrast spectra have been reduced in all movies to facilitate rapid download. Lighter greyscale values represent greater X-ray attenuation.

**Movie S2.** Animation of serial X-ray attenuation images through 2-celled embryo in Fig. 3A (specimen MPKxiv). Lighter greyscale values represent greater X-ray attenuation.

**Movie S3.** Animation of serial X-ray attenuation images through 4-celled embryo in Fig. 3E (specimen DOU-8). Darker greyscale values represent greater X-ray attenuation.

**Movie S4.** Animation of volume rendered cells and reniform subcellular structures in Fig 3E (specimen DOU-8). Two of the cells are rendered transparent and dislocated from the embryo to facilitate visualization.

**Movie S5.** Animation of serial X-ray attenuation images through 16-celled embryo in Fig. 1B (specimen DOU-10). Darker greyscale values represent greater X-ray attenuation. Specimen is ~820 µm at its maximum diameter.

**Movie S6.** Animation sequence of X-ray attenuation images through 32-celled embryo (specimen DOU-32). Darker greyscale values represent greater X-ray attenuation. Specimen is ~610 µm at its maximum diameter.

**Movie S7.** Animation sequence of X-ray attenuation through a many-celled (~250 < n <1250 cells) *Megaclonophycus* embryo (specimen PhilDii). Grey region in left-center of embryo reflects ovoidal cells where cell boundaries are sutured together and/or where isopachous cements lining cell boundaries are compositionally less distinct from cell interiors. Lighter greyscale values represent greater X-ray attenuation. Specimen is 590 µm at its maximum diameter.