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A possible billion-year-old holozoan with differentiated multicellularity

Graphical abstract

Differentiation in a Billion-year-old Protist
new genus *Bicellum* demonstrates cell-cell adhesion

- naked stage
- cyst stage

Highlights

- The multicellular microfossil *Bicellum brasieri* possesses two distinct cell types
- 3D preservation in phosphate preserved different life cycle stages
- Differential adhesion may have contributed to cell segregation during morphogenesis
- This billion-year-old freshwater protist shows evidence of holozoan affinity

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In brief

Strother et al. describe life cycle morphogenesis in a new billion-year-old microfossil, which may provide clues to the evolutionary roots of embryonic development in animals.
A possible billion-year-old holozoan with differentiated multicellularity

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SUMMARY

Sediments of the Torridonian sequence of the Northwest Scottish Highlands contain a wide array of microfossils, documenting life in a non-marine setting a billion years ago (1 Ga). 1–4 Phosphate nodules from the Diabaig Formation at Loch Torridon preserve microorganisms with cellular-level fidelity, 5, 6 allowing for partial reconstruction of the developmental stages of a new organism, Bicellum brasieri gen. et sp. nov. The mature form of Bicellum consists of a solid, spherical ball of tightly packed cells (a stereoblast) of isodiametric cells enclosed in a monolayer of elongated, sausage-shaped cells. However, two populations of naked stereoblasts show mixed cell shapes, which we infer to indicate incipient development of elongated cells that were migrating to the periphery of the cell mass. These simple morphogenetic movements could be explained by differential cell-cell adhesion. 7, 8 In fact, the basic morphology of Bicellum is topologically similar to that of experimentally produced cell masses that were shown to spontaneously segregate into two distinct domains based on differential cadherin-based cell adhesion. 9 The lack of rigid cell walls in the stereoblast renders an algal affinity for Bicellum unlikely: its overall morphology is more consistent with a holozoan origin. Unicellular holozoans are known today to form multicellular stages within complex life cycles, 10–13 so the occurrence of such simple levels of transient multicellularity seen here is consistent with a holozoan affinity. Regardless of precise phylogenetic placement, these fossils demonstrate simple cell differentiation and morphogenetic processes that are similar to those seen in some metazoans today.

RESULTS

Butterfield 14 has pointed out that multicellular organisms in pre-Ediacaran age deposits were likely to have left behind ontogenetic stages in the fossil record. We have examined about 50 petrographic thin sections of phosphatic lenses in the Diabaig Formation (ca. 1 Ga) that preserve populations of benthic and planktic organisms trapped in former lake bottom sediments (Figures 1A–1D). These include unicells and cell clusters of various kinds, some of which have been documented previously. 1, 2, 6, 15 In several thin sections, we observed cell clusters that are composed of aggregations of two distinct cell types, indicating a condition that constitutes a step toward complex multicellularity sensu Knoll. 16 Further investigation revealed a second set of cell clusters that appeared very similar in size and form but that lacked the fully differentiated second cell type. Here, we describe these interesting fossils and show intermediate morphologies that are consistent with an ontogenetic series driven by a differential cell-adhesion model.

The morphology of the new multicellular organism consists of a spheroidal mass of mutually adpressed cells enclosed by a peripheral layer of elongate, sausage-shaped cells. The interior cell mass forms a stereoblast (Figures 2A–2C, 2F, 2H, 2J, and S1–S3) of roughly isodiametric cells that average ~2.5 µm in diameter (Table S1). Exceptional preservation in calcium phosphate (francolite) and authigenic clay minerals 5 (Figure S3) retains intracellular biological features that, in this case, consist of a single dense, organic “spot” (Figures 2A and 2C, arrows). In well-preserved specimens, such inclusions occur in about half of the interior cells. These might represent preserved nuclei, but we consider that, more likely, they are the condensed remains of the entirety of the cytoplasmic cell content. 1 The cells of the stereoblast retain mutually compressed walls, so that the original multicellular topology, including Y-shaped junctions 17 (Figures 2B and 2C, circles), is retained. There is no evidence that these interior cells possessed rigid cell walls, because the shape of each cell is established by mutual compression with adjacent cells. This indicates the likelihood that individual cells were bounded by just a cell membrane or a thin, non-rigid cell wall. A carbon map of a specimen from an ultrathin section (Figures 2J and S3) also shows very thin interior walls as compared with the exterior cell layer. Although it is not
possible to completely rule out that each of the interior cells possessed a thin, flexible cell wall, we found no examples of cells that possessed interior membranes that might have pulled away from any such cell wall. This is not the case for various other isolated cells of different organisms found throughout the Diabaig phosphates in which multiple concentric layers are apparent and in which true cell walls are quite evident.\(^6\)

The outermost cell layer consists of thicker walled, sausage-shaped (elongate) cells, which form an enclosing layer that is unmistakably distinct from the isodiametric cells found in the interior stereoblast (Figures 2A, 2B, 2D–2K, and S1–S3). The elongate cells that form the peripheral layer are around 1.5 to 2 μm in diameter and generally about 3 to 4 times that in length, although, in some cases, they can be much longer (e.g., Figure 2K). The average width-to-length ratio for a set of 6 specimens was 0.28 (Table S1). The elongate shape is best demonstrated in surficial focus, as seen in Figures 2E–2G, 2I, and 2K (see also Figures S1–S3). Here, these cells crowd together to form what appears to be a rigid, outer spherical shell. The peripheral cells often occur in sets of 4 or more adjacent cells that are positioned parallel to each other (Figures 2F, 2G, 2I, 2K, and S1–S3), creating a tiled arrangement of sets of parallel cells, or, in some cases, covering the entire surface of the stereoblast in parallel-aligned, elongate cells (Figure 2K).
Figure 2. *Bicellum Brasieri* in mature form
All specimens were preserved in petrographic thin sections from the Diabaig Formation stratotype, Lower Diabaig, Scotland, UK. Scale bars in (A)–(J), 5 μm; scale bar in (K), 10 μm.
(A) *Bicellum brasieri* n. g. n. sp. holotype specimen. Arrows indicate condensed, intracellular organic "spots" Sample TS09-1.
(B) *Bicellum brasieri* n. g. n. sp. paratype. Larger ellipsoidal specimen with incomplete preservation in the interior. Circle indicates an example of a Y-shaped junction. Sample TS09-1.
(C) Enlargement of holotype specimen showing typical "Y" junctions (circle) and a condensed intra-cellular "spot." Total field of view is 10 μm.
(D) Specimen in equatorial view in which the interior cells are only very faintly preserved. Sample TS09-2.
(E) Surface view of specimen in (D), showing the tiled sets of parallel-aligned elongate cells.
(F) Specimen in sub-equatorial optical section showing elongate exterior cells in surface view and the thinner walled isodiametric cells of the interior stereoblast. Sample TS09-2.
(G) Surface view of specimen in (F), showing the tiled pattern of sets of elongate cells.
(H) Scanning electron microscopy (SEM) image of an *in situ* milled medial section through *B. brasieri*. Sample TOR11-108.
(I) SEM image of a milled tangential section of same specimen as in (H). Note that, here, the cells of the surface layer are quite elongate.

(legend continued on next page)
In medial cross-section, the peripheral cell layer is shown to be one cell in thickness (Figures 2A, 2B, 2D, 2H, 2J, and S1–S3). In light microscopy (LM), the walls of these outer cells appear darker than those of the stereoblast, indicating a thicker cell wall, distinct from those of the interior cells. This is seen in the holotype (Figure 2A) and in many of the other illustrated specimens (e.g., Figures 2D, 2HJ, S1, and S2). This is also somewhat evident in the carbon map in Figure 2J, although in this specimen, the carbon signal from the cells of the epidermal layer is masked somewhat by the carbon signal from the enclosing francolite (see also Figures S3E and S3F). The cells of the peripheral layer never show an interior “spot” like many cells of the stereoblast, indicating a persistent taphonomic difference in the two cell types, or perhaps loss of the protoplast at maturity in the epidermal cells, as, under LM, their interiors are more transparent than those of the interior stereoblast. Many specimens show some degree of cell loss within the stereoblast as a whole. This can be seen in medial sections of Figures 2B and 2D, where parts of the interior are missing well-preserved cells (see also Figure S1). Overall, this arrangement of two distinct cell types forming a spherical organism has not been previously described in the fossil record and is formalized here as Bicellum brasieri Strother & Wellman gen. et sp. nov.

The structural data used to describe Bicellum exist because of the unique qualities of cellular and sub-cellular preservation provided by phosphate and authigenic clay mineralization.5,6 The taxonomic richness that characterizes the Torridonian lake deposits, however, is recorded primarily in palynological preparations of fine-grained siliciclastic rocks that yield organic-walled microfossils (OWMs).1 An OWM comparable to Bicellum was also recovered in palynological strew mounts, but, as documented in Figure 3, its appearance as a flattened, dispersed OWM is somewhat different than its 3D form. Here, the wall is characterized by marginal circular structures (arrows in Figures 3A, 3C, and 3D), which correspond to cross-sectional views of the elongated cell peripheral layer. The average diameter (28.5 μm; Table S2) and ovoid (Figures 3D and 3E) to circular (Figures 3A–3C) shape are similar to the 3-dimensional form, but the interior stereoblast is not structurally preserved in the dispersed form, nor is the cellular nature of the peripheral layer readily apparent. In spite of these preservational differences, Bicellum, in its dispersed form, has now been recognized from 11 sample localities found throughout the Torridonian sequence (Table S2).

Tightly bound, spherical cell clusters found in association with B. brasieri fall outside the prescribed complex morphology for the species as presented here. These multicellular cell clusters consist of naked stereoblasts without an enclosing wall or cell layer. They are most commonly quite spherical (Figures 4A–4E), although some larger, ellipsoidal specimens have also been found (Figure 4F). The cells that compose each mass are tightly adpressed without intervening spaces; indeed, many exhibit straight lines of contact and clear 120° (Y-shaped) junctions where three cells meet. They are generally isodiametric, with diameters of 2 to 3 μm. The closeness of the cells indicates the originally cohesive nature of these cell masses; they appear as if they were tightly pressed together in life. The lack of intervening space also indicates that, in life, these cells did not possess rigid cell walls; if present, the cell walls clearly had a

Figure 3. Examples of the distributed (palynological) form
All figured specimens are from sample TOR11-9, Eilean Fladday, Scotland, UK. Scale bar, 10 μm for all images.
(A) Spherical form characterized by circular cross sections in an epidermal layer (arrows).
(B) Typical specimen without clearly demarked cell outlines.
(C) Spherical specimen showing circular cross-sections in the epidermal layer (arrows).
(D) Somewhat larger ellipsoidal specimen. Here, arrows indicate more elongated cells of the epidermal layer. Note that the right-hand margin indicates epidermal cells in circular cross-section.
(E) Oblate specimen without clear cellular structural interior.
See also related size data in Table S2.
degree of plasticity. The adjoining cell walls are thin, never thickened like those in the peripheral cell layer of *B. brasieri*.

These free, spherical cell masses (Figures 4A and 4B) are indistinguishable from the stereoblasts that characterize the interior cells of *B. brasieri*. After examination, it became apparent that some of these masses occasionally included elongate cells, unlike the enclosed stereoblasts described earlier. The incomplete cell mass in Figure 4C contains only one such elongate cell (indicated by an arrow) in the midst of what are generally isodiametric cells. Figure 4D illustrates another example photographed in median optical section. Here, embedded among generally isodiametric cells, is a single row of adjacent elongate cells (between arrows) in the midst of the cell mass, but cells at the margin are isodiametric in shape.

Figure 4E, Naked stereoblasts
Except where noted, all specimens are from sample TOR11-108, Diabaig Formation stratotype, Lower Diabaig, Scotland, UK. Scale bar, 10 μm.
(A) Naked stereoblast of mutually adpressed isodiametric cells. Note the lack of a discrete outer layer of cells.
(B) Another example of a simple ball of isodiametric cells amassed together to form a naked stereoblast. Sample TS09-1, Diabaig Formation stratotype, Lower Diabaig, Scotland, UK.
(C) This somewhat smaller specimen shows a single elongate cell (arrow) in the midst of a solid mass of isodiametric cells. Sample TS09-2, Diabaig Formation stratotype, Lower Diabaig, Scotland, UK.
(D) Naked stereoblast with an admixture of elongate and isodiametric cells. There is a row of adjacent elongate cells (between arrows) in the middle of the cell mass, but cells at the margin are isodiametric in shape.
(E) Here, some elongate cells (arrows) now appear at the periphery of the cell mass, although a clearly distinct epidermal layer has yet to become established. Note that there is no apparent distinction between the cell walls of either cell type.
(F) This ellipsoidal specimen, which is cut in a sub-tangential section, shows substantial alignment of sausage-shaped cell types (arrows).

DISCUSSION

In spite of its simple morphology, the characterization of the *Bicellum* stereoblast, combined with an understanding of its
dynamic assembly into a differentiated condition of two distinct cell aggregations, provides clues as to its systematic placement. It seems reasonable to assume that Bicellum falls within one of the lineages leading to one of the six clades that possess complex multicellularity today: animals, plants, florideophyte algae, brown algae, ascomycete fungi, and basidiomycete fungi. The precise dynamics of how Bicellum attained its initial multicellular state has yet to be determined. In broad terms, this would have been through palintomy, aggregative assembly, or cellularization of a coenobium (syncytium). If the initial multicellular condition of Bicellum occurred through successive mitotic divisions, then we would expect to find cell clusters of similar overall size, exhibiting combinations of 2n cells, as is the case, for example, in the embryo-like fossils of the Doushantu fossils. Although it is the case that many different kinds of cell clusters have been found in the same thin sections that contain Bicellum, no such palintomic sequence has yet been recognized. This indicates that the multicellular condition in Bicellum more likely occurred either through cell aggregation or through the cellularization of either a syncytium or a coenobium. Since these non-palintomic forms of multicellularity are also somewhat limited in their distribution within extant protist groups, they provide a means of limiting the potential placement of Bicellum. Other than in the opisthokonts, aggregative multicellularity occurs in the amoebozan, Dictyostelium, three SAR supergroup genera (Sorogena, Sorodiplophys, and Guttulinopsis), and also some labyrinthulids and the Excavate Acrasia, none of which are a morphological match with Bicellum. Formation of syncytia and/or coenobia occurs in the Archaeplastida and in all holozoan groups, with the exception of the choanoflagellates and the Filastera, which do show aggregative multicellularity. 

However, the multicellular condition, as exhibited in the Bicellum stereoblast—that is, Y-shaped cell junctions and lack of fixed, or determinate, cell placement—indicates that these cells probably lacked rigid cell walls. This eliminates both cyanobacteria and the eukaryotic chlorophyte algae as likely homologues, because multicellular form in these taxa is strongly influenced by their possession of rigid cell walls. This is also the case for comparison with the florideophyte red algae, which possess cellular and cell walls and are fundamentally of filamentous or pseudoparenchymatous thallus organization. In addition, the red algae are predominantly marine in their habitat distribution, as are the laminarian brown algae. Floulike and Steinberg manipulated cadherin levels in cultured cells, showing that changing the expression level of that single protein was able to produce structures with an inner cell wall of strongly adhering cells surrounded by a cortex of more weakly adhering ones. However, even without a change in protein expression, an increase in surface area of the elongate cells, compared with that of the isodiametric cells, would reduce the surface density of adhesion molecules, leading to weaker adhesion between elongate and isodiametric cells. Whatever the mechanism, Bicellum does show that differentiation and morphogenesis occurred in the life cycles of freshwater protists as long as a billion years ago. This early example of complex multicellularity adds to a nascent body of evidence indicating the importance of selection in terrestrial settings during late Mesoproterozoic to early Neoproterozoic time. Indeed, if Bicellum does belong to the clade Holozoa, as we suspect, it would provide support for recent models proposing Mesoproterozoic eukaryotic crown group origins, and it could prove to be a key fossil clue in an ongoing

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debate on the importance of oxygen in the origin and rise of animals.50

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.03.051.

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AUTHOR CONTRIBUTIONS

Original conceptualization was by P.K.S. and M.D.B., with input from C.H.W.; fieldwork was conducted by P.K.S., C.H.W., and M.D.B.; data acquisition and analysis were performed by D.W., M.S., and L.T.; light photomicrography was conducted by P.K.S.; and P.K.S. and C.H.W. wrote the paper, with contributions from D.W. and L.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE                     | SOURCE     | IDENTIFIER |
|-----------------------------------------|------------|------------|
| Biological Samples                      |            |            |
| Bicellum brasieri holotype and syntype specimens | This paper | TS09-1     |
| other B. brasieri figured specimens     | This paper | TS09-2, TOR11-108 |
| dispersed variants of B. brasieri       | This paper | TOR11-9    |
| milled specimens of B. brasieri         | This paper | TOR11-108  |
| Software and Algorithms                 |            |            |
| Adobe Photoshop                         | Adobe Systems | CS6       |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for access to prepared slides and thin sections should be directed to and will be fulfilled by the Lead Contact, Charles Wellman (c.wellman@sheffield.ac.uk).

Materials availability
This study did not generate new unique reagents. Thin sections, including the holotype, along with prepared strew slides, are curated in the palynology collections at the University of Sheffield.

Data and code availability
Data associated with Figures 2H–2J and S3B–S3F are stored in the UWA research repository archive. No codes were generated by this research.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Information about the specimens
All the specimens of Bicellum brasieri mature form (Figure 2) and the variants (Figure 4) illustrated in this report are found embedded in rock thin sections of phosphatic nodules collected by PKS and CHW in 2009 (TS09-1, TS09-2) and in 2011 (TOR11-108) from the Diabaig Formation at the stratotype section at Lower Diabaig, Scotland, UK (Figures 1A–1D). These thin sections are housed in the Palynological Collections at the University of Sheffield, Department of Animal and Plant Sciences, The University of Sheffield, UK. The palynomorphs illustrated in Figure 3 were collected by PKS, CHW and Christopher T. Baldwin from a section of the Diabaig Formation that is exposed on Eilean Fladday immediately opposite the short causeway that when exposed at low tide, gives access to the island from the Isle of Raasay. The sample, TOR11-09 is located 11 m above the base of the section exposure. This material is curated in the Palynological Collections at the University of Sheffield, Department of Animal and Plant Sciences, Sheffield, UK.

METHOD DETAILS

Systematics
A formal systematic description of the taxon, Bicellum brasieri Strother & Wellman, gen. et sp. nov. can be found in Data S1, Systematics.

Electron image and elemental map acquisition
Images in Figures 2H, 2I, S3B, and S3C were acquired using a FEI Helios NanoLab G3 CX dual beam FIB-SEM instrument using an in lens back-scattered electron detector. The image in Figure S3D was acquired using a FEI Titan G2 80-200 TEM/STEM in high angle annular dark field (HAADF) mode. Minor image corrections (brightness/contrast) were performed using the open source ImageJ software.

The elemental maps presented in Figures 2J, S3E, and S3F were acquired using a FEI Titan G2 80-200 TEM/STEM with ChemiSTEM Technology (Super-X EDX system) operating at 200 kV. Data were acquired from sample wafers of 100-150 nm that were prepared using a FEI Helios NanoLab G3 CX dual beam instrument and attached to Omniprobe copper TEM holders using platinum connector strips. Multicolored image overlays were performed using the Gatan Digital Micrograph software.
Cell size measurements

Cell size measurements in Tables S1 and S2 were made using the Ruler tool in Photoshop using a scale calibration based on an optical micrometer slide ruled in 10 μm intervals.

QUANTIFICATION AND STATISTICAL ANALYSIS

Descriptive statistical data presented in Tables S1 and S2 were calculated in Microsoft Excel based on the size data extracted in Photoshop.