

Short communication

## **The morphology of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) spores released from *Fredericella sultana* (Bryozoa: Phylactolaemata)**

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Proliferative kidney disease (PKD) is recognized as an economically important disease of salmonid culture in Europe and North America (Hedrick, MacConnell & de Kinkelin 1993). The life cycle of *Tetracapsuloides bryosalmonae*, the myxozoan parasite that causes the disease, has been partially elucidated and requires a bryozoan host to produce spores that infect fish (Feist, Longshaw, Canning & Okamura 2001). While myxosporean spores are encompassed with hard valves formed from valvogenic cells that subsequently degenerate, the outer surface of the spore of *T. bryosalmonae* and its sister taxon *Buddenbrockia* comprises valve cells that retain their cellular integrity and lack hardened valve coats (Canning, Curry, Feist, Longshaw & Okamura 2000). Hence, the class Malacosporea (from the Greek for 'soft spores') was established to accommodate these organisms. While light- and electron-microscopical studies have revealed many developmental and mature features of these organisms, the precise morphology of the fully formed spores has remained elusive, with only basic features being discernable. Diagrams based on light microscopical examinations have added little to our understanding of how the cells are arranged to form spores.

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The development of confocal laser scanning microscopy (CLSM) techniques has facilitated the production of three-dimensional (3D) reconstructions of biological specimens with a minimum of artefacts and with little or no processing of tissue (Kubinova, Janacek, Karen, Radochova, Difato & Krekule 2004). CLSM has proved especially useful in the imaging of microorganisms (Martin-Cereceda, Alvarez, Serrano & Guinea 2001) and this short communication describes the morphology of *T. bryosalmonae* spores as interpreted from CLSM data.

In September 2003 and February 2004, colonies of the bryozoan *Fredericella sultana* were collected from the inlet of a trout farm in southern England known to be endemic for PKD. They were placed in aerated plastic containers filled with water from the site and transported to the University of Stirling. The colonies were maintained at 18 °C in laboratory aquaria filled with culture medium comprising a diet of protozoa and algae in artificial fresh water (Morris, Morris & Adams 2002). Using an inverted microscope, several colonies of *F. sultana* were observed to contain characteristic malacosporean tetracapsulid spores. Spores were observed in the bryozoans 28 and 15 days following collection in September and February, respectively, confirming previous findings of *T. bryosalmonae* being present in *F. sultana* throughout the year (Gay, Okamura & de Kinkelin 2001). The identity of these spores was confirmed to be *T. bryosalmonae* by experimentally exposing 12 naïve rainbow trout, *Oncorhynchus mykiss* (Walbaum), to the infective spores. Fifty days after exposure, the fish were killed

and examined for evidence of PKD. All the fish showed characteristic signs of development of clinical PKD as confirmed by established immunohistochemistry and polymerase chain reaction analyses including positive and negative control samples (Morris, Adams, Feist, McGeorge & Richards 2000).

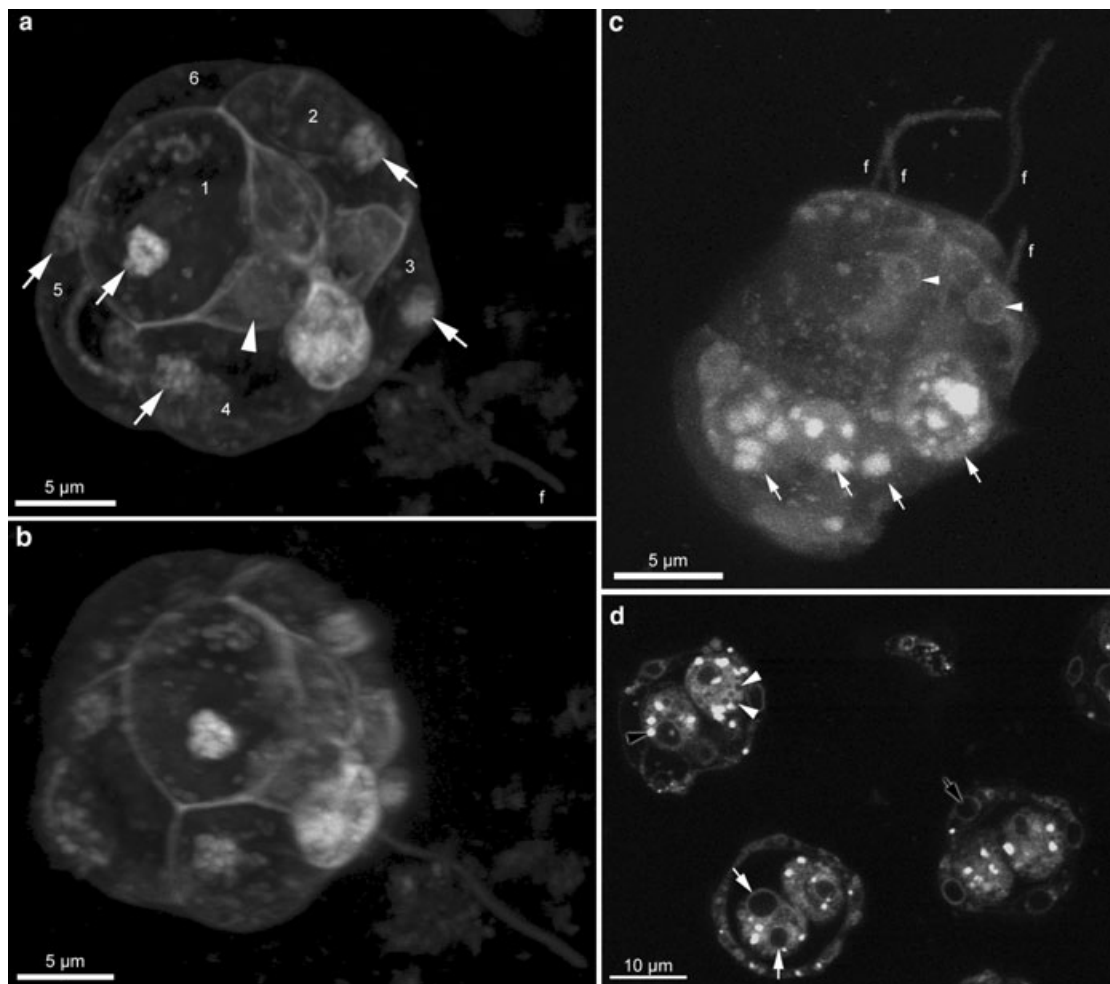
Portions of the infected bryozoans were carefully teased apart on microscope slides under a dissecting microscope and the released spores stained with one of the following fluorophores: Blankophor (4,4'-bis [(4-anilino-6-substituted 1,3,5-triazine-2-yl)amino] stilbene-2,2'-disulphonic acid; ICN Biomedicals, Irvine, CA, USA), BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Molecular Probes, Eugene, OR, USA) or DAPI (4,6-diamidino-2-phenylindole, dilactate; Molecular Probes). They were diluted respectively to working dilutions of 0.1% v/v, 80 µm and 300 nm in dechlorinated tap water and then added to the slide with the spores. Cover slips were placed over the samples and sealed with nail varnish to prevent evaporation of the medium. The slides were stored in the dark for between 30 min and 2 h until fluorescent staining could be observed and were then examined by CLSM.

Spores were observed using a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems AG, Wetzlar, Germany) coupled to a Leica DM IRE2 inverted microscope employing a ×63 oil-/glycerol-immersion lens. In addition to laser excitation of fluorochromes, transmitted light was also used to visualize some of the samples. Sequences of images were taken along the optical Z-axis using the confocal optical sectioning facility. The images were acquired on a PC running Windows XP using Leica Confocal Software (version 2.5), with 3D reconstructions generated for a selection of the images using the same application. All the spores examined were positioned in a similar orientation, with the capsulogenic cells facing down towards the inverted objective lens. No morphological differences were noted between the spores resulting from the two collection visits.

The optical brightener Blankophor has been widely used in the staining of microorganisms, showing a high affinity for β-glycosidically linked polysaccharides (Ruchel & Schaffrinski 1999). Its use allowed the structural details of released *T. bryosalmonae* spores to be visualized. The mean spore diameter was 19.16 µm (SD = 1.15,  $n = 18$ ). Each spore possessed four spherical polar

capsules of mean diameter 1.73 µm (SD = 0.03,  $n = 4$ ), each of which contained a coiled polar filament as observed by Canning *et al.* (2000). The measurements proved consistent with previous descriptions of *T. bryosalmonae* (Canning, Curry, Feist, Longshaw & Okamura 1999; Canning *et al.* 2000). In some of the specimens, up to four polar filaments of approximate length 12.5 µm and thickness 800 nm were seen to be everted from the polar capsules of an individual spore. In one spore, the capsulogenic cell from which a polar filament had extruded exhibited more fluorescence than the other three capsulogenic cells (Fig. 1a). Presumably, upon firing of the polar filament, the Blankophor dye was able to penetrate the interior of the capsulogenic cell, demonstrating an intimate connection between the capsulogenic cell and the sealing plug of the polar capsule. The polar capsules were located within four capsulogenic cells arranged in a cruciate pattern. Four valve cells could be seen beneath the capsulogenic cells rotated at an angle of 45° to the latter. The Z stack reconstructions of the spores indicated the presence of a further four cells below these, similarly rotated at 45° with respect to the upper valve cells, making a total of eight valve cells. Some of the valve cells contained eccentrically located structures interpreted to be nuclei (Fig. 1b). Figure 1c shows a spore which had extruded its complement of four polar filaments, leading to apparent morphological changes in the structure of the spore. Fluorescent dye was able to enter this spore, allowing visualization of the internal sporoplasmic constituents. Prior to examination, the spore had been freshly released from the bryozoan, suggesting that the morphological alterations were due to extrusion of the polar filaments as opposed to degenerative changes.

The lipophilic qualities of non-polar BODIPY fluorophores make them highly suitable for investigating membrane structures (Yamada, Toyota, Takakura, Ishimaru & Sugawara 2001). From the scans conducted, internal components including the germinative sporoplasms of the spores could easily be visualized. Internally, each spore was seen to possess twin ellipsoid sporoplasms of mean length 9.01 µm (SD = 0.47,  $n = 6$ ) and width 6.39 µm (SD = 0.43,  $n = 6$ ), and each of these included two distinct non-fluorescent regular areas of contrasting dimensions, believed to represent primary and secondary cell nuclei (Fig. 1d). Within the sporoplasms, non-fluorescent spheres of approximate diameter 500 nm were observed, while highly fluor-

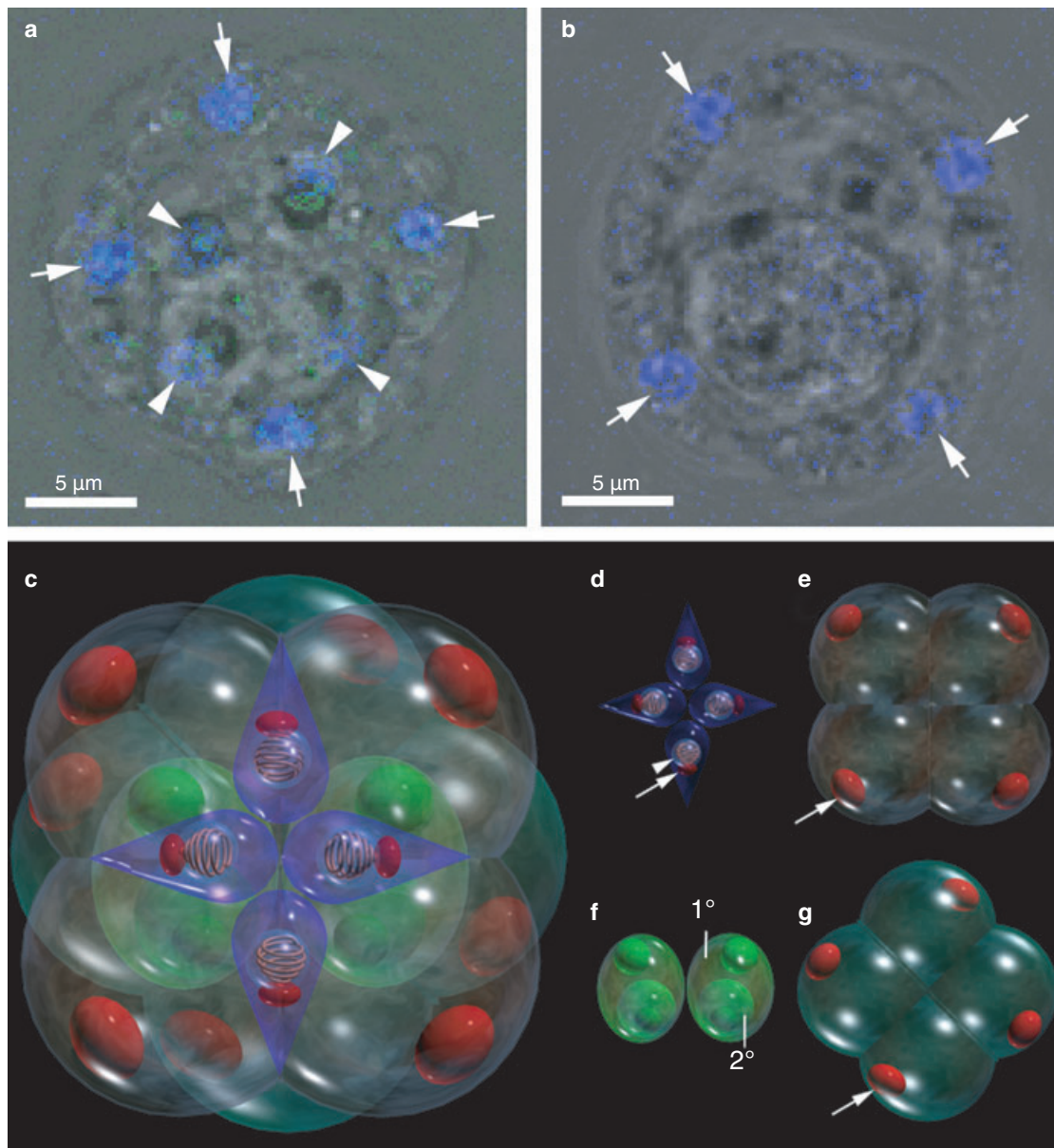


**Figure 1** (a–c) CLSM 3D reconstructions of spores of *Tetracapsuloides bryosalmonae* stained with Blankophor. (a) A spherical polar capsule (arrowhead) can be seen within one of four central capsulogenic cells, while a polar filament 'f' has been extruded from another. Six valve cells '1–6' can be seen including nuclei (arrows). (b) Further rotation of the spore shown in (a), demonstrating the eccentric position of the nucleus of cell 5. (c) Showing extrusion of four polar filaments 'f' from spherical polar capsules (arrowheads); four sporoplasmic constituents (arrows) can also be visualized. (d) CLSM section of three *T. bryosalmonae* spores stained with BODIPY. Each spore possesses two sporoplasms within which twin nuclei (white arrows) of contrasting size and non-fluorescent bodies (white arrowheads) were detected. Lipid vesicles (black arrowhead) can be seen throughout the spores, while valve cell nuclei (black arrow) were observed in the outer membranes.

escent bodies of a similar size were also seen throughout the cytoplasm of cells forming the spore, presumably representing lipid vesicles as previously described by Canning *et al.* (2000).

DAPI has previously been found to be effective in staining nuclear accumulations of DNA in myxozoans (El-Matbouli, Holstein & Hoffmann 1998), and in the current study demonstrated the presence of capsulogenic and valve cell nuclei in the *T. bryosalmonae* spores. However, no sporoplasmic nuclei were imaged convincingly by this technique, presumably due to insufficient penetration of the

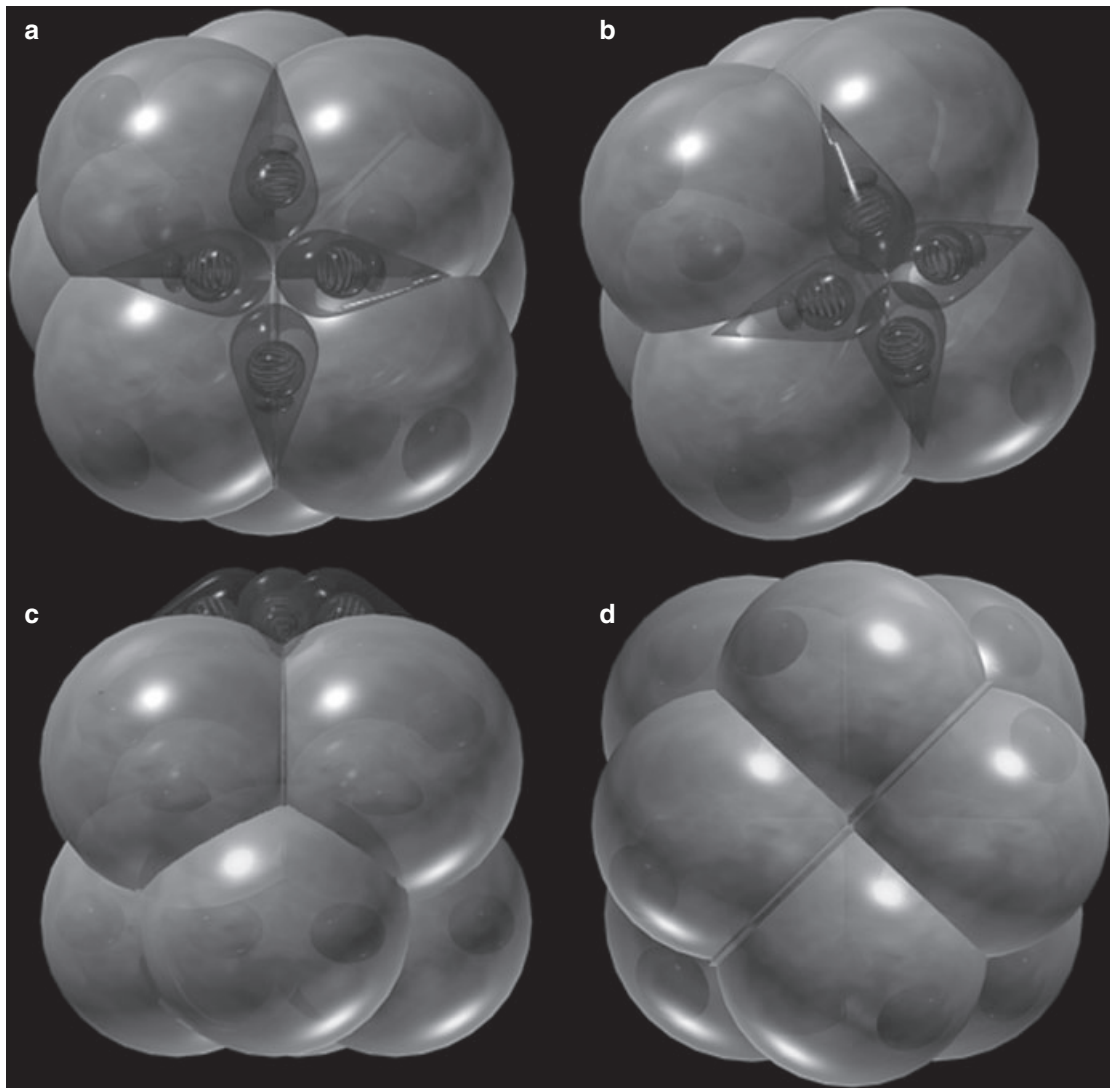
fluorophore into the spore interior. At a depth of 6.9 µm within the spore, four peripheral valve cell nuclei and four capsulogenic cell nuclei could be detected (Fig. 2a), while at a depth of 11.5 µm, four further valve cell nuclei were observed (Fig. 2b). This, in concert with observations from the Blankophor study, confirmed that a total of eight valve cells form the outer structure of the spore. This observation contrasts with those of ultrastructural studies of *T. bryosalmonae* which suggested the presence of up to four valve cells in each spore (Canning *et al.* 1999, 2000). In each section where valve cell nuclei were



**Figure 2** (a) CLSM section of *Tetracapsuloides bryosalmonae* spore stained with DAPI, showing four capsulogenic cell nuclei (arrowheads) and four valve cell nuclei (arrows). (b) Deeper section showing four further valve cell nuclei (arrows). (c–g) Schematic 3D model of *T. bryosalmonae* spore showing internal components. (c) Composite image showing all layers *in situ*. (d) Representation of nearest layer to view, comprising four capsulogenic cells, each containing a spherical polar capsule (arrowhead) with internal coiled polar filament and a nucleus (arrow). (e) Deeper layer of four valve cells, each with one nucleus (arrow). (f) Deeper layer of two sporoplasms, each comprising nucleated primary '1°' and secondary cells '2°'. (g) Deepest layer from view, comprising four nucleated (arrow) valve cells.

imaged, the four nuclei were seen to be positioned approximately equidistantly around the circumference. The orientation of the nuclei in the lower layer of valve cells was seen to roughly correspond to the position of the upper layer rotated through 25° anticlockwise. The mean diameter of the valve cell

nuclei was 2.46  $\mu\text{m}$  (SD = 0.09,  $n = 8$ ), while that of the capsulogenic cell nuclei was 2.37  $\mu\text{m}$  (SD = 0.18,  $n = 4$ ). The relative positions of the cells are represented in schematic models of the spore produced using 3DS Max 7 (Discreet Software, Discreet, Montreal, QC, Canada) (Figs 2c–g & 3).



**Figure 3** Schematic 3D model of *Tetracapsuloides bryosalmonae* spore. (a) Front view showing four spherical polar capsules within capsulogenic cells forming a cruciate pattern, surrounded by valve cells. (b) Oblique view comparable with Fig. 1a. (c) Side view. (d) Interpretation of rear view showing four valve cells.

CLSM has been demonstrated as a powerful tool in the study of myxozoan parasites. Due to the minimal processing of material required, few artefacts developed to alter the morphology of the spores before examination. Thus, structures alluded to in previous electron microscopical studies can now be viewed *in situ*. The use of a complementary range of fluorescent dyes allows detailed visualization of morphological features. Three-dimensional reconstructions of the image slices obtained from CLSM allowed the proposition of a hypothetical conformational architecture of the spore of *T. bryosalmonae* released from Bryozoa.

Previous light microscopical interpretations of malacosporean spores have depicted them as spherical with the arrangement of their valve cells not being observed (Canning, Okamura & Curry 1996; Canning, Tops, Curry, Wood & Okamura 2002; Morris *et al.* 2002). The use of CLSM in the current study represents the first documented 3D visualization of the cellular components of malacosporean spores. The *T. bryosalmonae* spore has been shown to be highly organized in structure, with consistency in architecture noted between the examined spores. Future examination of other malacosporean spores such as *Buddenbrockia*

*plumatellae* and fish stages of *T. bryosalmonae* utilizing similar CLSM techniques would represent a powerful method to compare species of the class Malacosporea.

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