

1 **The architecture of cell differentiation in choanoflagellates** 2 **and sponge choanocytes**

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26 SUMMARY

27 Collar cells are ancient animal cell types which are conserved across the animal
28 kingdom [1] and their closest relatives, the choanoflagellates [2]. However, little is
29 known about their ancestry, their subcellular architecture, or how they differentiate.
30 The choanoflagellate *Salpingoeca rosetta* [3] expresses genes necessary for animal
31 multicellularity and development [4] and can alternate between unicellular and
32 multicellular states [3,5], making it a powerful model to investigate the origin of
33 animal multicellularity and mechanisms underlying cell differentiation [6,7]. To
34 compare the subcellular architecture of solitary collar cells in *S. rosetta* with that of
35 multicellular “rosettes” and collar cells in sponges, we reconstructed entire cells in
36 3D through transmission electron microscopy on serial ultrathin sections. Structural
37 analysis of our 3D reconstructions revealed important differences between single
38 and colonial choanoflagellate cells, with colonial cells exhibiting a more amoeboid
39 morphology consistent with relatively high levels of macropinocytotic activity.
40 Comparison of multiple reconstructed rosette colonies highlighted the variable nature
41 of cell sizes, cell-cell contact networks and colony arrangement. Importantly, we
42 uncovered the presence of elongated cells in some rosette colonies that likely
43 represent a distinct and differentiated cell type. Intercellular bridges within
44 choanoflagellate colonies displayed a variety of morphologies and connected some,
45 but not all, neighbouring cells. Reconstruction of sponge choanocytes revealed both
46 ultrastructural commonalities and differences in comparison to choanoflagellates.
47 Choanocytes and colonial choanoflagellates are typified by high amoeboid cell
48 activity. In both, the number of microvilli and volumetric proportion of the Golgi
49 apparatus are comparable, whereas choanocytes devote less of their cell volume to
50 the nucleus and mitochondria than choanoflagellates and more of their volume to
51 food vacuoles. Together, our comparative reconstructions uncover the architecture
52 of cell differentiation in choanoflagellates and sponge choanocytes and constitute an
53 important step in reconstructing the cell biology of the last common ancestor of the
54 animal kingdom.

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58 RESULTS AND DISCUSSION

59 Three-dimensional cellular architecture of choanoflagellates

60 Collar cells were likely one of the first animal cell types [1,8,9] and persist in most
61 animal phyla (Figure 1A). Therefore, characterising the microanatomy of
62 choanoflagellates and sponge choanocytes has important implications for the origin
63 and evolution of animal cell types. To fully characterise and reconstruct both single
64 and colonial *S. rosetta* cells, we used high-pressure freezing and 3D serial ultrathin
65 TEM sectioning (3D ssTEM), in addition to fluorescent microscopy. Three randomly
66 selected single cells and three randomly selected colonial cells from a single colony
67 were chosen for the reconstruction of entire choanoflagellate cells and subcellular
68 structures (Figures 1 and S1-2, Videos S1-6). Both single and colonial *S. rosetta*
69 cells exhibited a prominent, central nucleus enveloped by a mitochondrial reticulum
70 and basal food vacuoles – as well as intracellular glycogen reserves - consistent with
71 the coarse choanoflagellate cellular architecture reported in previous studies [10,11]
72 (reviewed in [7,12]) (Figures 1 and S1-2, Videos S1-6). However, with the increased
73 resolution of electron microscopy we detected three morphologically distinct
74 populations of intracellular vesicles with distinct subcellular localizations (Figure 1G
75 and S1): 1) Large vesicles (extremely electron-lucent, 226 ± 53 nm in diameter), 2)
76 Golgi-associated vesicles (electron-dense inclusions, 50 ± 10 nm in diameter), and
77 3) Apical vesicles (electron-lucent, 103 ± 21 nm in diameter). Extracellular vesicles
78 were also observed associated with two of the single cells (electron-lucent, 173 ± 36
79 nm in diameter) and appeared to bud from the microvillar membrane (Fig S1L).
80 Choanoflagellate cells subjected to fluorescent labelling were congruent with 3D
81 ssTEM reconstructions in terms of organelle localization (Figure 1B-C), providing
82 evidence that the 3D models presented herein are biologically representative.

83 Ultrastructural commonalities and differences between single and colonial 84 choanoflagellate cells

85 Our 3D ssTEM reconstructions allowed for detailed volumetric and numerical
86 comparisons among single and colonial *S. rosetta* cells (Figures 2 and S2, Table S1
87 and S2). Overall, the general deposition of organelles was unchanged in both cell
88 types (Figures 2A, B and S2A-C). In addition, single and colonial cells devote a
89 similar proportion of cell volume to most of their major organelles (nucleus: single

90 cells $12.92 \pm 0.58\%$ vs colonial cells $11.56 \pm 0.27\%$; nucleolus: $1.85 \pm 0.33\%$ vs 2.2
91 $\pm 0.22\%$; mitochondria: $5.08 \pm 1.14\%$ vs $6.63 \pm 0.42\%$; food vacuoles: $9.22 \pm 2.75\%$
92 vs $6.85 \pm 0.87\%$ and glycogen storage: $8.71 \pm 2.36\%$ vs $7.50 \pm 1.12\%$) (Figures 2
93 and S2, Table S1 and S2).

94 We also uncovered some ultrastructural differences between single and colonial
95 cells (Figure 2C). Colonial cells devoted a higher proportion of cell volume to
96 endoplasmic reticulum (ER) (single: $3.27 \pm 0.35\%$ vs colonial: $6.86 \pm 0.39\%$). This
97 contrast was coupled to a differential ER morphology across cell types. The ER of
98 colonial cells frequently displayed wide, flat sheets (Figure 3E), which were not
99 observed in the reconstructed single cells. Single cells exhibited a higher number of
100 Golgi-associated vesicles (single: 166.3 ± 32.7 vs colonial: 72.3 ± 26.5) and
101 individual mitochondria than colonial cells (single: 25.3 ± 5.8 vs colonial: 4.3 ± 4.2)
102 (Figure 2C, Table S2), despite lacking volumetric differences between cell types. The
103 ultrastructural differences in ER, Golgi-associated vesicles and mitochondria suggest
104 differences in endomembrane trafficking and energetic physiology between single
105 and colonial cells. ER and mitochondrial morphology change dynamically, and stark
106 changes have been observed in other eukaryotic cells due to changes in cell cycle
107 [13] and cytoskeletal activity [14,15]. Mitochondria and the ER too show an intimate
108 association [16], and the contrast in the number of individual mitochondria in different
109 cell types was particularly striking (Table S2).

110 In animal cell types, fusion/fission dynamics have been previously associated with
111 cellular stress [17] and substrate availability [18], but it is of most interest for
112 choanoflagellates in the context of aerobic metabolism. For example, the fresh water
113 choanoflagellate *Desmarella moniliformis* exhibits a shift in mitochondrial profile prior
114 to encystment and metabolic dormancy [19] and choanoflagellates have been
115 uncovered from hypoxic waters [20]. The role of oxygen in the origin and evolution of
116 animals has long been discussed [21] and is currently met with controversy [22,23].
117 Coupled to a previous report of positive aerotaxis in *S. rosetta* rosette colonies [24],
118 this finding places even more emphasis on understanding variation in aerobic
119 metabolism between single and colonial choanoflagellates.

120 Finally, we found that colonial cells are characterised by a more amoeboid
121 morphology than single cells (Figure 3A). Colonial cells exhibited a higher relative

122 proportion of endocytotic vacuoles by volume (single: 0.07 ± 0.07 vs colonial: $0.32 \pm$
123 0.12) - a phenomenon coupled to a higher overall number of endocytotic vacuoles
124 (single: 1 ± 1 vs colonial: 5 ± 2) and pseudopodial projections per cell (single: 1 ± 1
125 vs colonial 8 ± 2) (Figure 2C and Tables S1 and S2). Many of the pseudopodial
126 projections and endocytotic vacuoles bore the morphology of lamellipod ruffles and
127 macropinosomes (Figure 3A), suggesting that colonial cells are typified by high
128 macropinocytotic activity. Macropinocytosis – defined as the formation of phase-
129 lucent vacuoles $>0.2 \mu\text{m}$ in diameter from wave-like, plasma membrane ruffles [25] –
130 is conserved from the Amoebozoa [26] to animal cell types [27]. It is therefore
131 parsimonious to infer that the macropinocytotic activity of *S. rosetta* colonial cells
132 represents a trophic adaptation, particularly considering that previous biophysical
133 studies have reported more favourable feeding hydrodynamics in rosette colonies
134 [28]. Even in macropinosomes with no observable cargo, dissolved proteins [29] and
135 ATP [27] from extracellular fluid have been previously reported to be metabolically
136 exploited by animal macropinocytotic cell types. This non-selectivity, coupled to the
137 large volume of engulfed fluid, makes macropinocytosis an efficient cellular process
138 to sample and process the extracellular milieu.

139 Our comparison between single and colonial cells provides new insights into
140 ultrastructural commonalities and differences associated with the conversion from
141 solitary to colonial cells and shows that colonial cells might represent a distinct and
142 differentiated cell type.

143 **Reconstruction of multiple rosettes reveals colony-wide cell arrangement,** 144 **different cell shapes and complete cell-cell contact network**

145 While high magnification 3D ssTEM enabled the high-resolution reconstruction of
146 individual colonial cells, their context and interactions with neighbouring cells were
147 lost. To address this, we reconstructed the subcellular structures of a seven-cell
148 rosette colony (complete rosette, RC1) from 80 nm sections taken at lower
149 magnification (Figure 3A-D, Video S7), as well as the gross morphology of four
150 larger rosettes (RC2-5) from 150 nm sections to provide a more representative
151 survey (Figure 3E-P).

152 We found that individual cells in rosette colonies vary widely in volume (Figure 3M,
153 N), although no pattern was detected in the volumetric cellular arrangement along

154 the rosette z-axis (Figure 3M). In addition, mean cell size was comparable among
155 different rosettes, including those that contained different numbers of cells (Figure
156 S4B). However, we did find a positive correlation between cell number and the
157 number of intercellular bridges per cell across rosette colonies (Figure S4B).

158 Importantly, we uncovered the presence of unusually shaped cells in two of the five
159 *S. rosetta* rosette colonies (Carrot-shaped cell 5 in RC3 and chili-shaped cell 5 in
160 RC4, both labelled orange with an asterisk) (Figure 3M). These unusual cells were
161 both found at the same location along the rosette z-axis, exhibited an elongated
162 morphology distinct from other colonial cells (Figure 3O, P and Videos S8 and S9),
163 and were small in volume. Cells 5 from RC3 and RC4 were 9.87 and $13.35 \mu\text{m}^3$
164 respectively (Figure 3N) - the mean volume of the cells in RC3 and RC4 was 27.38
165 and $27.25 \mu\text{m}^3$ respectively (Figure 3N). While each of these unusual cells
166 possessed a flagellum, a collar, connections to neighbouring cells via intercellular
167 bridges and had a similar proportion of cell volume dedicated to most of their major
168 organelles as observed in other colonial cells, these cells devoted a larger volumetric
169 percentage of the cell body to the nucleus (29.8% and 30.78% respectively versus
170 the mean colonial proportion of $13.76 \pm 0.49\%$). These data hint that cell
171 differentiation within colonies may be more complex than previously realized.

172 Our 3D ssTEM reconstructions of rosette colonies also revealed the distribution of
173 intercellular bridges, and the connections formed between individual cells (Figure
174 3M). We found intercellular bridges in all analysed rosette colonies (RC1-5), totalling
175 36 bridges. There was no detectable pattern regarding bridge networking across
176 rosette colonies. Bridges were distributed from the cell equator to either of the poles
177 along the cellular z-axis and the average bridge was $0.75 \pm 0.38 \mu\text{m}$ in length (Figure
178 S4D). Prior studies [3,4] of *S. rosetta* bridges suggested that bridges are typically
179 short ($0.15 \mu\text{m}$), connecting two adjacent cells and containing parallel plates of
180 electron-dense material. In contrast, the bridges detected in this study exhibited
181 striking morphological diversity (Figure 3M, Q-U), with lengths ranging from 0.21 –
182 $1.72 \mu\text{m}$. The majority of bridges consisted of a protracted cytoplasmic connection
183 between two cells, and in many cases, the septum was localized asymmetrically
184 along the bridge (Figure S4C). Most surprisingly, some bridges were not connected
185 to any neighbouring cells at all, but rather the septum was situated on the end of a
186 thin, elongated cellular protrusion (Figure 3S). In addition, we observed asymmetric

187 bridge width and degraded electron dense structures proximal to bridge remnants
188 being incorporated into the cell body of a contiguous cell (Figure 3T, U). These data
189 suggest that intercellular bridges could be disconnected from neighbouring cells and
190 that the electron-dense septum may be inherited.

191 The asymmetric and disconnected morphology of intercellular bridges provides
192 important clues to choanoflagellates colony formation and potentially the evolution of
193 animal multicellularity. Bridges, displaying electron-dense septa reminiscent of those
194 found in *S. rosetta*, have been previously identified in other colony-forming
195 choanoflagellate species [30,31] and it has been hypothesized that these structures
196 represent stable channels for intercellular communication [4]. Our data suggest that
197 bridges can be disconnected, and that the electron-dense septum may be
198 asymmetrically inherited. In this way, choanoflagellate bridges may resemble the
199 mitotic midbody in animal cells [32]. It may still be that *S. rosetta* bridges play a role
200 in cell-cell communication, albeit transiently. However, the exit of colonial cells from
201 the rosette (as previously reported [3]) must involve bridge disconnection, and a
202 proper understanding of the fate of the septum could augment our understanding of
203 choanoflagellate cell differentiation and destiny in colony development.

204 **Three-dimensional cellular architecture of sponge choanocytes**

205 To place our choanoflagellate reconstructions into the context of collar cells from an
206 early-branching animal, we reconstructed a section of a sponge choanocyte
207 chamber (Figure 4A) from the homoscleromorph sponge *Oscarella carmela* [33].
208 Both choanoflagellates and sponge collar cells influence local hydrodynamics by
209 beating their single flagellum to draw in bacteria that are captured by the apical collar
210 complex [34], however sponge choanocytes are part of an obligately multicellular
211 organism. Our 3D ssTEM reconstructions allowed for the reconstruction of five
212 choanocytes and for the volumetric and numerical comparison of choanocyte and
213 choanoflagellate subcellular structures (Figure 4B-E, Figures S5, S6 and Video S10).
214 We detected little ultrastructural variability within the five choanocytes (Figure S5,
215 Table S3 and S4). All five cells exhibited a prominent basal nucleus, small and
216 unreticulated mitochondria, food vacuoles scattered around the entire cell, and an
217 apical Golgi apparatus (Figure 4B-D and Figure S5, S6) - consistent with the coarse

218 choanocyte cellular architecture reported in previous studies [32,34,36] (reviewed in
219 [1,37]).

220 Furthermore, our data showed many ultrastructural commonalities between
221 choanocytes and choanoflagellates. For example, the number of microvilli that
222 surround the apical flagellum in single and colonial choanoflagellates is comparable
223 to the number of microvilli in sponge choanocytes (single: 32 ± 2 vs colonial: $35.3 \pm$
224 4.9 vs choanocytes: 30.6 ± 4.1) (Figure S6A). We also found that the number of food
225 vacuoles and the number and volumetric proportion of the Golgi apparatus are
226 similar in all three cell types (Figure S6A). Although, choanocytes did not appear to
227 exhibit the same macropinocytotic activity as colonial choanoflagellates throughout
228 the cell (some micropinocytotic inclusions are present towards the cell apex (Figure
229 S6 D-E)), basal sections of choanocytes were heavily amoeboid (Figure S6 B-C).
230 These amoeboid protrusions may not only be for mechanical anchorage into the
231 mesohyl, but may play a role in phagocytosis as we observed bacteria in the
232 mesohyl to be engulfed by basal pseudopodia (Figure S6 F-G). Thus, both
233 choanocytes and colonial choanoflagellates are typified by high amoeboid cell
234 activity.

235 Not unexpectedly, we also observed some ultrastructural differences between
236 choanocytes and choanoflagellates. In contrast with cells from choanoflagellate
237 rosettes, sponge choanocytes lack filopodia and intercellular bridges. Choanocytes
238 also do not possess glycogen reserves and devote significantly less of their cell
239 volume ($9.25 \pm 0.39\%$) than choanoflagellates (single: $12.92 \pm 0.58\%$ and colonial:
240 $11.56 \pm 0.27\%$) to the nucleus, and less to mitochondria ($2.5\% \pm 0.3\%$ versus single:
241 $5.08 \pm 1.14\%$ and colonial: $6.63 \pm 0.42\%$) (Figure S6A). However, choanocytes
242 devote significantly more of their volume to food vacuoles ($20.7 \pm 1.01\%$) than
243 choanoflagellates (single: $9.22 \pm 2.75\%$ and colonial: $6.85 \pm 0.87\%$) (Figure 4E).
244 High-resolution reconstructions of the choanocyte and choanoflagellate apical pole
245 (Figure 4 F-G and Videos S11, S12) showed differences in terms of vesicle type and
246 localisation, Golgi positioning and collar arrangement (conical in choanoflagellates
247 while cylindrical in choanocytes, as previously noted [34]). The flagellar basal body
248 has previously been meticulously characterised in both choanocytes and
249 choanoflagellates and some differences have been reported between the two by

250 other authors [38–43] These findings are reiterated by our reconstructions and
251 observations (Figure 4F, G).

252 **Concluding Remarks**

253 The comparative 3D reconstruction of collar cells from two different phyla,
254 choanoflagellates and sponges, allowed for an unbiased view of their cellular
255 architecture and for the reconstruction of key properties of the enigmatic ancestral
256 collar cell. Our data reveal distinct ultrastructural features in single and colonial
257 choanoflagellates and demonstrate that cells within rosette colonies vary significantly
258 in their cell size and shape. The newly identified ‘carrot’ and ‘chili cells’ reveal that
259 cells within choanoflagellate colonies do not simply consist of an assemblage of
260 equivalent single cells but some may represent a distinctly differentiated cell type
261 displaying ultrastructural modifications. Likewise, our data suggest that sponge
262 choanocytes are not simply an incremental variation of the choanoflagellate cell, but
263 are specialised feeding cells as indicated by their high volumetric proportion of food
264 vacuoles. Together, our data show a remarkable variety of collar cell architecture
265 and suggest cell type differentiation was present in the stem lineage leading to
266 animals.

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288

289 **AUTHOR CONTRIBUTIONS**

290 DL, KM, PB designed the study; DL, BL, KM, PB performed experiments; DL, KM,
291 PB analysed data; DL, NK, PB wrote the paper and all authors reviewed,
292 commented on, and edited the manuscript.

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294 **DECLARATION OF INTRESTS**

295 The authors declare no competing financial interest

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455 **FIGURE LEGENDS**

456 **Figure 1. Three-dimensional cellular architecture of choanoflagellates and**

457 **collar cells across the Choanozoa.** (A) Phylogenetic distribution of collar cells

458 across the Choanozoa (Choanoflagellata + Animals [1][44]) showing the presence

459 (black circle), absence (white circle) and putative losses (brown cross) of collar cells

460 across lineages. The origin of collar cells is marked by the orange circle. Adapted

461 from [1]. *Some lineages within the Bilateria have secondarily lost collar cells. (B-C)

462 Characterisation of major organelles in *S. rosetta* labelled with fluorescent vital dyes

463 (B) and by immunofluorescence (C). Arrowhead indicates nucleus of

464 choanoflagellates cell, asterisks indicate the stained nucleoids of engulfed prey

465 bacteria. Scalebar = 1 μm . (D-G) 3D ssTEM reconstruction of a single *S. rosetta* cell

466 (S3) exterior (D). The plasma membrane was made transparent (E) and glycogen

467 and ER were removed to allow better visualisation of subcellular structures (F) and

468 vesicle populations (G). Shown are apical vesicles (pink), food vacuoles (green),

469 endocytotic vacuoles (fuschia), endoplasmic reticulum (yellow), extracellular vesicles

470 (grey), filopodia (external – purple), flagellar basal body (light blue), flagellum (dark

471 green), glycogen storage (white), Golgi apparatus and vesicles (purple), intercellular

472 bridges (external – yellow; septa - red), large vesicles (brown), microvillar collar (light

473 orange), mitochondria (red), non-flagellar basal body (dark orange) and nuclei (dark

474 blue). Scale bar = $\sim 1 \mu\text{m}$ (depending on position of structure along the z-axis).

475 **Figure 2. 3D ssTEM reconstructions allow for volumetric and numerical**

476 **comparison of high-resolution single and colonial *S. rosetta* cells.** Shown are

477 the mean volumetric breakdowns of three single (A) and three colonial (B) *S. rosetta*

478 cells (left) and a generalised diagram of cell-type ultrastructure (right). Colours are as

479 in Figure 1. (C) Volumetric (%) (\pm SEM) (endoplasmic reticulum and endocytotic

480 vacuoles) and numerical (μm^{-3}) (\pm SEM) (endocytotic vacuoles, pseudopodia, Golgi-

481 associated vesicles and mitochondria) differences were found between single and

482 colonial ($n = 3$) *S. rosetta* cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

483 **Figure 3. Reconstructions of complete choanoflagellate rosette colonies**

484 **places colonial cells into context, unveils ultrastructural features involved in**

485 **rosette formation and a novel cell type.** (A-D) 3D ssTEM reconstruction of a

486 complete rosette colony 1 (RC1). The plasma membrane was made transparent (B)

487 to allow better visualisation of subcellular structures. Highlighted are contacting
488 filopodia (C) and intercellular bridges (D). Cellular structures coloured as in Figure 1.
489 Scalebar = $\sim 1 \mu\text{m}$. (E-L) 2D TEM and 3D ssTEM reconstructions of structures (*)
490 differentially exhibited by colonial cells or involved in colony formation. Shown are
491 the endoplasmic reticulum (ER) (E&F), intercellular bridges (IB)(G&H), endocytotic
492 vacuoles (EV) (I&J) and filopodia (FP) (K&L). Scalebars = 200 nm. (M-P)
493 Reconstruction of multiple *S. rosetta* colonies shows no strong pattern of volumetric
494 distribution and bridge networks, but reveal the presence of highly derived cell
495 morphologies. (M) 3D ssTEM reconstructions of five complete rosettes (RC1-5)
496 coloured by cell number (above) and 2D projections of bridge connections in 3D
497 ssTEM reconstructions of rosette colonies (below). Disconnected intercellular
498 bridges marked by white arrowheads and lines. Asterisks mark the presence of
499 highly derived cell morphologies in RC3 and RC4. Cells in rosette colonies are
500 numbered in order of their appearance along the z-axis. (N) Volumetric distribution of
501 mean cell volumes (RC1-5) in rosette colonies reveals no apparent pattern of cell
502 distribution across the z-axis. (O, P). Two highly derived cell types, the 'carrot cell'
503 (O) from RC3 and the 'chili cell' (P) from RC4 were identified in rosette colonies.
504 Colours as in Figure 1. Scalebar = $\sim 1 \mu\text{m}$. (Q-U) Intercellular bridges in colonial *S.*
505 *rosetta* exhibit a high diversity of morphologies, suggestive of disconnection. In
506 addition to prior descriptions of intercellular bridges (arrowheads) and electron-dense
507 septa (asterisks), bridges in colonial *S. rosetta* often display an asymmetrically
508 distributed septum (Q), protracted and elongated morphology (R), disconnection
509 from one of the contiguous cells (S) and evidence of abscission (T) and putative
510 inheritance of the septum (U). Scalebar = 200 nm.

511 **Figure 4. Three-dimensional cellular architecture of sponge choanocytes.** (A)
512 Choanocytes line interconnected chambers in members of the Porifera and serve as
513 feeding cells. (B) Mean volumetric breakdown of five sponge choanocytes. Colours
514 are as in Figure 1. (C-E) 3D ssTEM of a section of choanocyte chamber containing
515 five complete cells (B). The plasma membrane was rendered transparent (D) and
516 food vacuoles and ER were removed to allow better visualisation of subcellular
517 structures (E). Colours are as in Figure 1. Scalebar = $\sim 1 \mu\text{m}$. (F-G) Reconstruction
518 and comparison of the sponge choanocyte (F) and choanoflagellate (G) apical poles
519 shows distinct differences between the two cell types. Shown in the choanocyte

520 reconstruction are the basal foot (red, associated with basal body), food vacuole
521 (light green), endoplasmic reticulum (yellow) flagellar basal body (light blue),
522 flagellum (dark green), Golgi apparatus and Golgi-associated vesicles (purple),
523 microtubules (grey), mitochondria (red) non-flagellar basal body (dark orange),
524 Type 1 vesicles (light orange) and Type 2 vesicles. Shown in the choanoflagellate
525 reconstruction are the apical vesicles (pink), food vacuole (light green), endoplasmic
526 reticulum (yellow) flagellar basal body (light blue), flagellum (dark green), Golgi
527 apparatus and Golgi-associated vesicles (purple), glycogen (white), large vesicles
528 (brown), microtubules (grey), microtubular ring (red) and non-flagellar basal body
529 (dark orange). Scalebars = 200 nm. Diagrams of the choanocyte fine kinetid (F) and
530 choanoflagellate fine kinetid (G) structure highlight the distinct differences.

531 **Figure S1. High magnification TEM panel of the *S. rosetta* (A-L) and *O. carmela***
532 **(M-T) subcellular components discussed herein.** (A) *S. rosetta* nucleus showing
533 endoplasmic reticulum (er), euchromatin (eu), heterochromatin (he), nuclear
534 membrane (nm), nuclear pore complex (npc) and nucleolus (n). (B) Mitochondrion
535 (m) showing flattened, non-discoidal cristae (cr). (C) Apical pole showing flagellum
536 (f), flagellar basal body (fbb), non-flagellar basal body (nfbb), tubulin filaments (tf)
537 and transversal plate (tp). (D) Area of high glycogen storage (gly). (E) Food vacuole
538 (dv). (F) Posterior filopodia (fp) projecting from the basal plasma membrane (pm).
539 (G) Golgi apparatus (ga). (H) microvillus (mv) from the apical collar displaying actin
540 filaments (af). (I) Large, extremely electron-lucent vesicles. (J) Golgi-associated,
541 electron dense vesicles. (K) Apical, electron-lucent vesicles. (L) Extracellular
542 vesicles were observed in two of the single cells and appeared to bud from the
543 microvillar membrane. (M) *O. carmela* nucleus showing euchromatin (eu),
544 heterochromatin (he) and nuclear pore complex (npc). (N) Mitochondria (m)
545 displaying cristae (cr). Also visible are cell-cell contacts between two adjacent
546 choanocytes (cc). (O) Collar microvillus (mv). (P) Apical pole and Golgi apparatus
547 showing flagellum (f), flagellar basal body (fbb), non-flagellar basal body (nfbb),
548 tubulin filaments (tf) and basal foot (bf). (Q) Food vacuole (dv). (R) Rough (rer) and
549 smooth (ser) endoplasmic reticulum. (S) Basal pole of *O. carmela* shows bacteria
550 located in the mesohyl (b), basal pseudopodia (ps) and endocytotic invagination (ev).
551 (T) Vesicles type 1 (V1) and type 2 (V2) are located throughout the choanocyte
552 cytoplasm. Scale bars = 200 nm.

553 **Figure S2. 3D ssTEM reconstructions of high resolution single and colonial *S.***
554 ***rosetta* cells.** (A) Gross external morphologies of reconstructions of both single (S1-
555 3) and colonial (C1-3) *S. rosetta* cells. (B-C) Structomic reconstructions of single (B)
556 and colonial (C) *S. rosetta* cells, with the plasma membrane removed to reveal
557 subcellular ultrastructure. Colours are as in Figure 1. Asterisks indicate engulfed
558 prey bacteria. Cells are labelled with their corresponding cell ID number and
559 volumetric breakdown for each cell is shown below reconstructions. Scalebar = ~1
560 μm .

561 **Figure S3. Methodological overview of 3D ssTEM reconstruction of *S. rosetta***
562 **and *O. carmela* cells.** (A) ssTEM stacks are imported into the Fiji plugin TrakEM2,
563 aligned, and scaled. Subcellular structures are then manually segmented. (B) 3D
564 ssTEM reconstructions are conducted in TrakEM2 by merging traced structures
565 along the z-axis, initially smoothed and imported into Blender (C). In Blender, final
566 reconstruction artefacts are smoothed using the F Smooth Sculpt Tool and final
567 materials are added for the ultimate render (D). (E) The aforementioned
568 methodology applied to single cells (S1-3), colonial cells (C1-3), a complete rosette
569 colony (CR) and a section of an *O. carmela* choanocyte chamber

570 **Figure S4. Mean cell volume per colony cell number, intercellular bridges per**
571 **colony cell number and bridge length** (A) No correlation was found between cell
572 volume and colony cell number. (B) A positive correlation was found between
573 bridges per cell and colony cell number ($p < 0.05$). (C) No apparent pattern was
574 observed between the length of an intercellular bridge and its position along the
575 colony z-axis.

576 **Figure S5. 3D reconstructions and volumetric breakdown of five sponge**
577 **choanocytes.** (A-B) 3D ssTEM reconstructions of five *O. carmela* choanocytes and
578 their volumetric breakdown is shown below. Scalebar = ~1 μm .

579 **Figure S6. Volumetric and numerical comparison of choanocyte and**
580 **choanoflagellate major subcellular structures.** (A) Choanocytes from *O. carmela*
581 are significantly larger by volume (μm^3) than the single and colonial choanoflagellate
582 *S. rosetta* cells. Volumetric (%) (\pm SEM) (nucleus, nucleolus, mitochondria,
583 endoplasmatic reticulum, food vacuoles and glycogen storage) and numerical (μm^{-3})
584 (\pm SEM) (mitochondria) differences were found between sponge choanocytes ($n = 5$)

585 and single (n = 3) and colonial (n = 3) choanoflagellates. * $p < 0.05$, ** $p < 0.01$, *** p
586 < 0.001 . (B-G) TEM and 3D ssTEM reconstructions of amoeboid cell behaviour in
587 sponge choanocytes. Shown are the highly invaginated (inv) and pseudopodiated
588 (ps) basal pole of the choanocyte (B&C), macropinocytotic activity (*) at the apical
589 pole (D&E) and a mesohyl-associated bacterium being engulfed by a pseudopodium
590 (ps) at the basal pole (F&G).

591 **Table S1. Volumetric measurements of *S. rosetta* cells and components**

592 **Table S2. Numbers of various organelles and components in *S. rosetta* cells**

593 **Table S3. Volumetric measurements of *O. carmela* choanocytes and**
594 **components**

595 **Table S4. Numbers of various organelles and components in *O. carmela***
596 **choanocytes**

597

598 **SUPPLEMENTARY EXPERIMENTAL PROCEDURES**

599 **Cell Culture**

600 Colony-free *S. rosetta* cultures (ATCC 50818) were grown with co-isolated prey
601 bacteria in 0.22 μm -filtered choanoflagellate growth medium [45] diluted at a ratio of
602 1:4 with autoclaved seawater. Cultures were maintained at 18°C and split 1.5:10
603 once a week. Colony-enriched *S. rosetta* cultures (Px1) were likewise maintained,
604 but monoxenically cultured with the prey bacterium *Algoriphagus machipongonensis*
605 [46] to induce rosette formation.

606 **Fluorescent Labelling of Organelles**

607 To support the annotation of organelles from ssTEM sections, the microanatomy of
608 *S. rosetta* cells was chemically characterized by fluorescent vital staining. Cells were
609 pelleted by gentle centrifugation (500x g for 10 min at 4°C) in a Heraeus™
610 Megafuge™ 40R (ThermoFisher Scientific) and resuspended in a small volume of
611 culture medium. Concentrated cell suspension (500 μl) was applied to glass-bottom
612 dishes coated with poly-L-lysine solution (P8920, Sigma-Aldrich) and left for 10-30
613 min until cells were sufficiently adhered. Px1 cultures were concentrated into 100 μl
614 of culture medium to promote the adherence of rosette colonies.

615 Adhered cells were incubated in 500 μ l of fluorescent vital dye diluted in 0.22 μ m-
616 filtered seawater. Cells were incubated with 4.9 μ M Hoechst 33342 Dye for 30 min
617 (to label nuclei); 1 μ M LysoTracker® Yellow HCK-123 for 1.5 h (to label food
618 vacuoles); 3 μ M FM® 1-43 Dye for 1 min (to label the plasma membrane); and 250
619 nM MitoTracker® Red CM-H2Xros for 30 min (to label mitochondria). All vital dyes
620 were from ThermoFisher Scientific (H3570, L12491, T35356 and M7513
621 respectively). Fluorescent-DIC microscopy was conducted under a 100 x oil-
622 immersion objective lens using a Leica DMI8 epifluorescent microscope (Leica,
623 Germany). Vital dyes were viewed by excitation at 395 nm and emission at 435-485
624 nm (Hoechst 33342 Dye); 470 nm and emission at 500-550 nm (LysoTracker®
625 Yellow HCK-123 & FM® 1-43 Dye); and 575 nm and 575-615 nm (MitoTracker® Red
626 CM-H2Xros). Micrographs were recorded with an ORCA-Flash4.0 digital camera
627 (Hamamatsu Photonics, Japan). All cells were imaged live. No-dye controls using
628 only the dye solvent dimethyl sulfoxide (DMSO) (D4540, Sigma-Aldrich) were run for
629 each wavelength to identify and control for levels of background fluorescence.
630 Chemical fixation during vital staining and TEM sectioning was avoided where
631 possible in this study to reduce fixation artefacts.

632 To visualize cell bodies, flagella, filopodia and collars adherent cells were fixed for 5
633 min with 1 ml 6% acetone, for 15 min with 1 ml 4% formaldehyde. Acetone and
634 formaldehyde were diluted in artificial seawater, pH 8.0. Cells were washed gently
635 four times with 1 ml washing buffer (100 mM PIPES at pH 6.9, 1 mM EGTA, and 0.1
636 mM MgSO₄) and incubated for 30 min in 1 ml blocking buffer (washing buffer with
637 1% BSA, 0.3 % Triton X-100). Cells were incubated with primary antibodies against
638 tubulin (E7, 1:400; Developmental Studies Hybridoma Bank) diluted in 0.15 ml
639 blocking buffer for 1 h, washed four times with 1 ml of blocking buffer, and incubated
640 for 1 h in the dark with fluorescent secondary antibodies (1:100 in blocking buffer,
641 Alexa Fluor 488 goat anti mouse). Coverslips were washed three times with washing
642 buffer, incubated with Alexa Fluor 568 Phalloidin for 15 min and washed again three
643 times with washing buffer. Coverslips were mounted onto slides with Fluorescent
644 Mounting Media (4 ml; Prolong Gold Antifade with DAPI, Invitrogen). Images were
645 taken with a 100x oil immersion objective on a Leica DMI6000 B inverted compound
646 microscope and Leica DFC350 FX camera. Images presented as z-stack maximum
647 intensity projections.

648 **Electron Microscopy**

649 *High Pressure Freezing.* Cultured *S. rosetta* single and colonial cells were
650 concentrated by gentle centrifugation (500x g for 10 min), resuspended in 20% BSA
651 (Bovine Serum Albumin, Sigma) made up in artificial seawater medium and
652 concentrated again. Most of the supernatant was removed and the concentrated
653 cells transferred to high pressure freezing planchettes varying in depth between 50
654 and 200 μm (Wohlwend Engineering). For sponges, tiny pieces of *O. carmela* were
655 excised and mixed with 20% BSA made up in seawater before transferring to 200
656 μm -deep high pressure freezing planchettes. Freezing of both the choanoflagellate
657 and sponge samples was done in a Bal-Tec HPM-010 high pressure freezer (Bal-
658 Tec AG).

659 *Freeze Substitution.* High pressure frozen cells stored in liquid nitrogen were
660 transferred to cryovials containing 1.5 ml of fixative consisting of 1% osmium
661 tetroxide plus 0.1% uranyl acetate in acetone at liquid nitrogen temperature (-195°C)
662 and processed for freeze substitution according to the method of McDonald and
663 Webb [47,48]. Briefly, the cryovials containing fixative and cells were transferred to a
664 cooled metal block at -195°C ; the cold block was put into an insulated container such
665 that the vials were horizontally oriented, and shaken on an orbital shaker operating
666 at 125 rpm. After 3 hours the block/cells had warmed to 20°C and were ready for
667 resin infiltration.

668 *Resin Infiltration and Embedding.* Resin infiltration was accomplished according to
669 the method of McDonald [48]. Briefly, cells were rinsed 3X in pure acetone and
670 infiltrated with Epon-Araldite resin in increasing increments of 25% over 30 min plus
671 3 changes of pure resin at 10 min each. Cells were removed from the planchettes at
672 the beginning of the infiltration series, and spun down at 6,000 X g for 1 min between
673 solution changes. The cells in pure resin were placed in between 2 PTFE-coated
674 microscope slides and polymerized over 2 h in an oven set to 100°C .

675 *Serial Sectioning.* Cells/tissues were cut out from the thin layer of polymerized resin
676 and remounted on blank resin blocks for sectioning. Serial sections of varying
677 thicknesses between 70 - 150 nm were cut on a Reichert-Jung Ultracut E microtome
678 picked up on 1 X 2 mm slot grids covered with a 0.6% Formvar film. Sections were
679 post-stained with 1% aqueous uranyl acetate for 7 min and lead citrate [49] for 4 min.

680 *Imaging.* Images of cells on serial sections were taken on an FEI Tecnai 12 electron
681 camera.

682 **3D Reconstruction & Analysis**

683 ssTEM sections were imported as z-stacks into the Fiji [50] plugin TrakEM2 [51] and
684 automatically aligned using default parameters, except for increasing steps per
685 octave scale to 5 and reducing maximal alignment error to 50 px. Alignments were
686 manually curated and adjusted if deemed unsatisfactory. Organelles and subcellular
687 compartments were manually segmented and 3D reconstructed by automatically
688 merging traced features along the z-axis. Meshes were then preliminarily smoothed
689 in TrakEM2 and exported into the open-source 3D software Blender 2.77 [52]. Heavy
690 smoothing of the cell body in TrakEM2 sacrifices fine structures associated with
691 cellular projections or does not remove all distinct z-layers, which exist as
692 reconstruction artefacts. Therefore, cell bodies were manually smoothed using the F
693 Smooth Sculpt Tool in Blender of final distinct z-layers for presentation purposes
694 only (Figure S3). All organelles were subjected to the same smoothing parameters
695 across individual cells. All analysis was conducted using unsmoothed, unprocessed
696 meshes. Organelle volumes were automatically quantified by the TrakEM2 software
697 and enumerated in Blender 2.77 by separating meshes in their total loose parts.

698 The microvillar collar and flagellum were excluded from volumetric analysis as their
699 total, representative length could not be imaged at this magnification. Cytosolic
700 volume was calculated by subtracting total organelle volume from cell body volume,
701 and is inclusive of cytosol, ribosomes and unresolved smaller structures excluded
702 from 3D reconstruction. Endocytotic vacuoles were distinguished from food vacuoles
703 by connection to the extracellular medium in ssTEM's or by localisation to a cell
704 protrusion. Cells in rosette colonies are numbered in order of their appearance along
705 the image stack z-axis. Rosette colony diameters were calculated by measuring the
706 largest distance of the z-axis midsection. Bridge length was measured in one
707 dimension along the bridge midsection. Mean vesicle diameters were calculated
708 from 20 measurements (or as many as possible if the vesicle type was rare) from
709 single cells.

710

711

712 **Data Analysis**

713 Univariate differences in the volume and number of subcellular structures between
714 the two cell types were evaluated using Two-Sample t-tests. Shapiro-Wilk and
715 Levene's tests were used to assess normality and homogeneity of variance
716 respectively. Statistical comparisons were conducted using data scaled against total
717 cell volume. Correlations between colony cell number, cell volume and bridges per
718 cell were assessed using Pearson correlation tests. All statistical analyses were
719 conducted using R v 3.3.1 [53] implemented in RStudio v 0.99.903 [54].

Figure 1

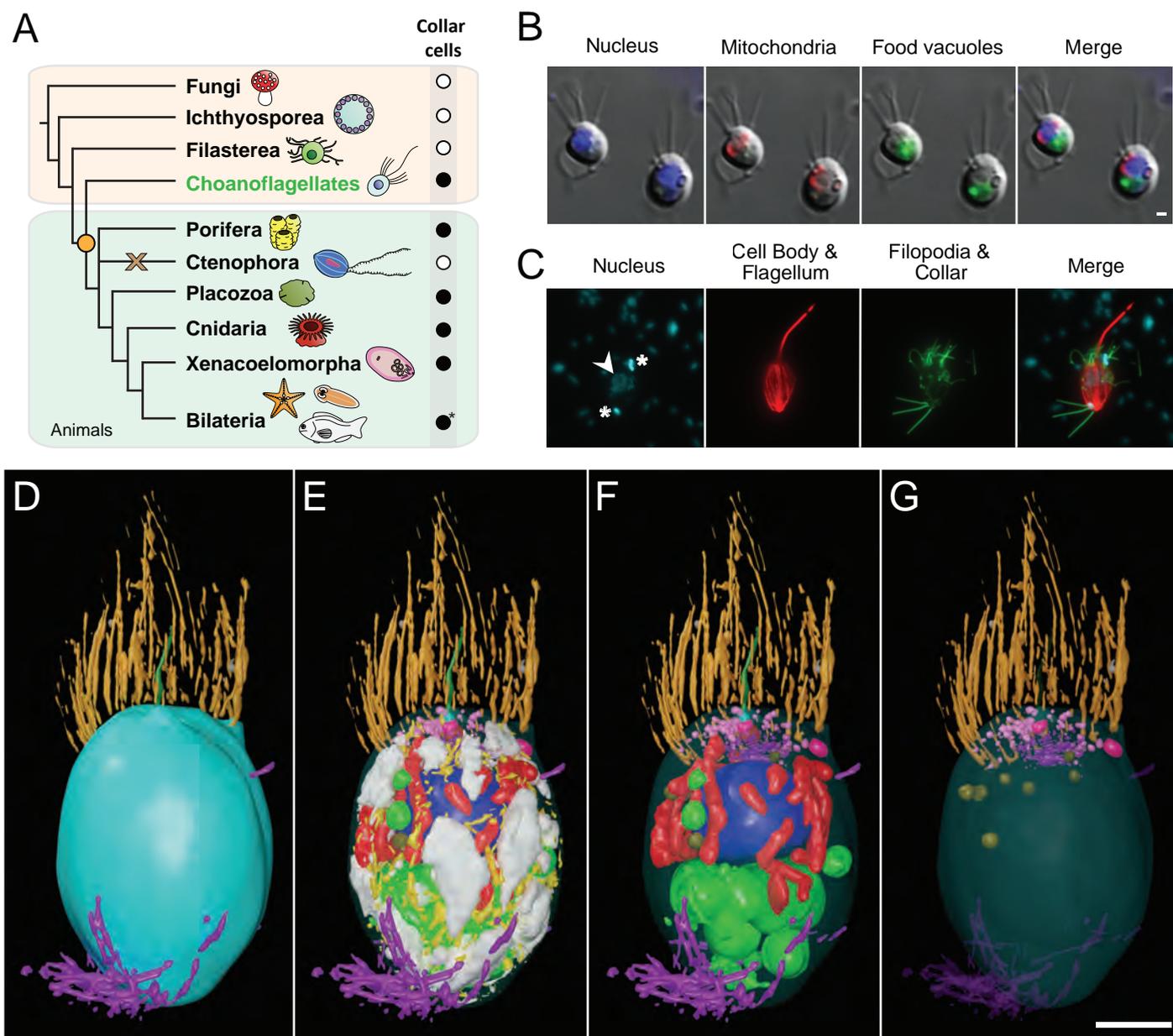


Figure 2

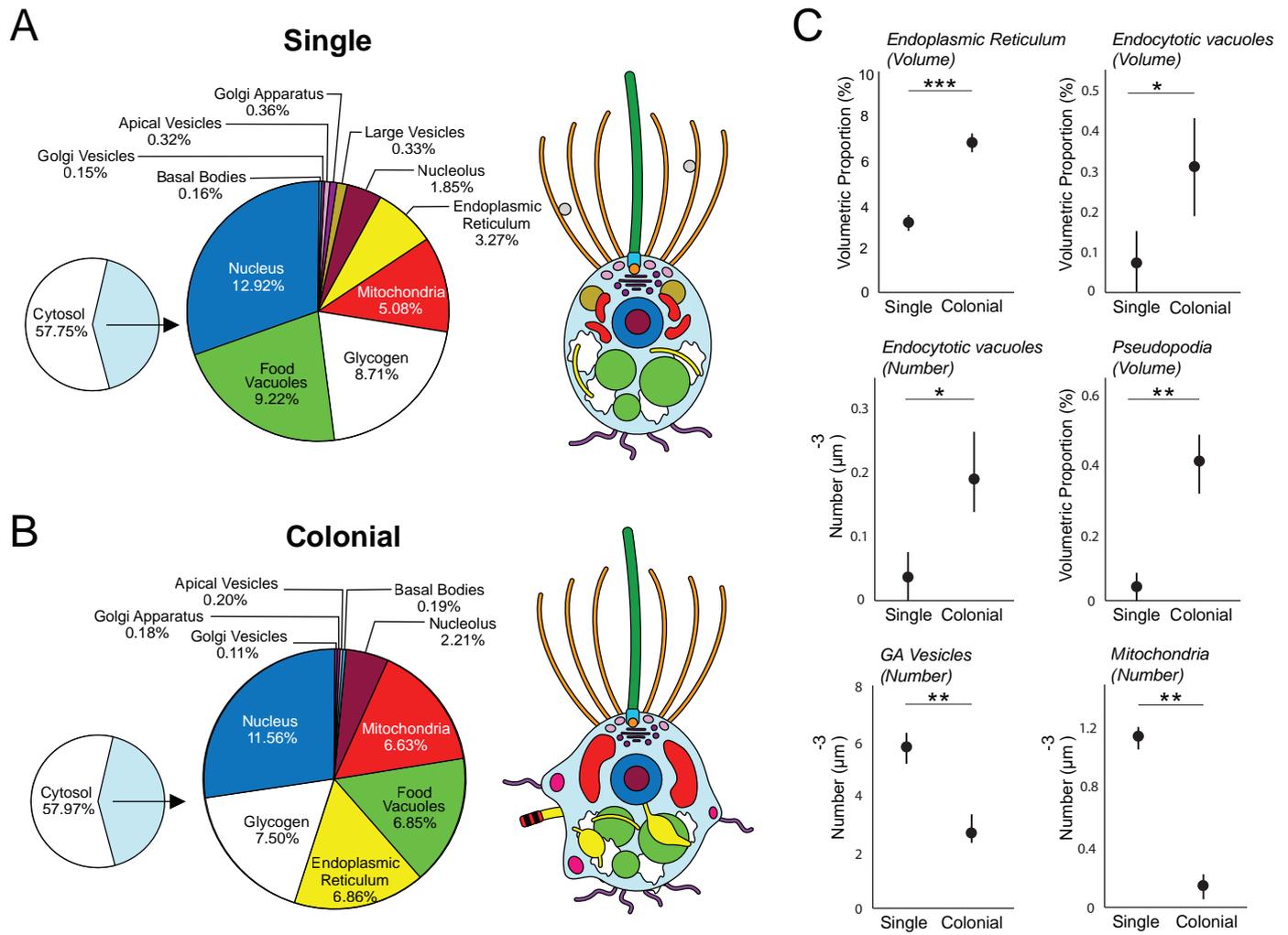


Figure 3

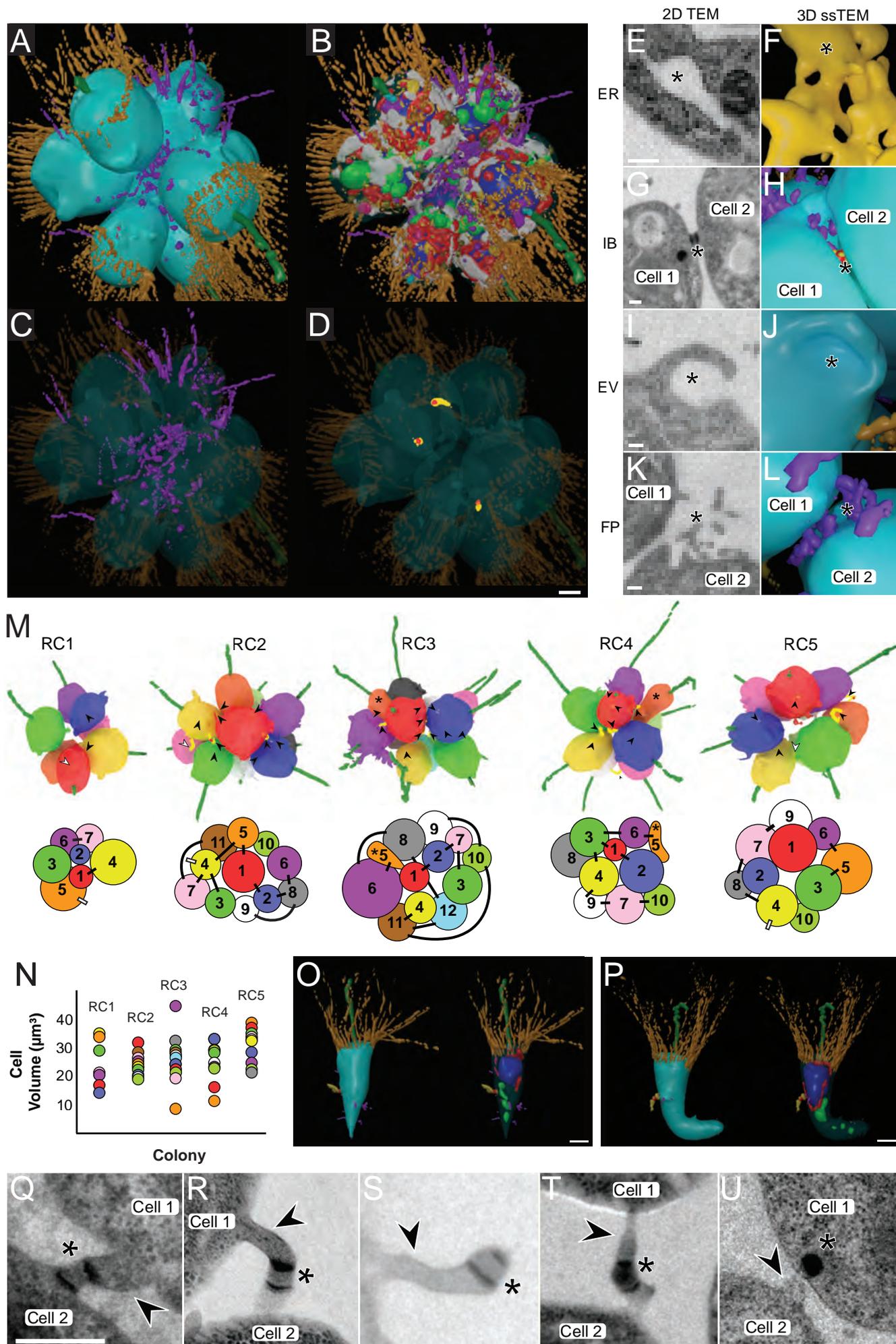
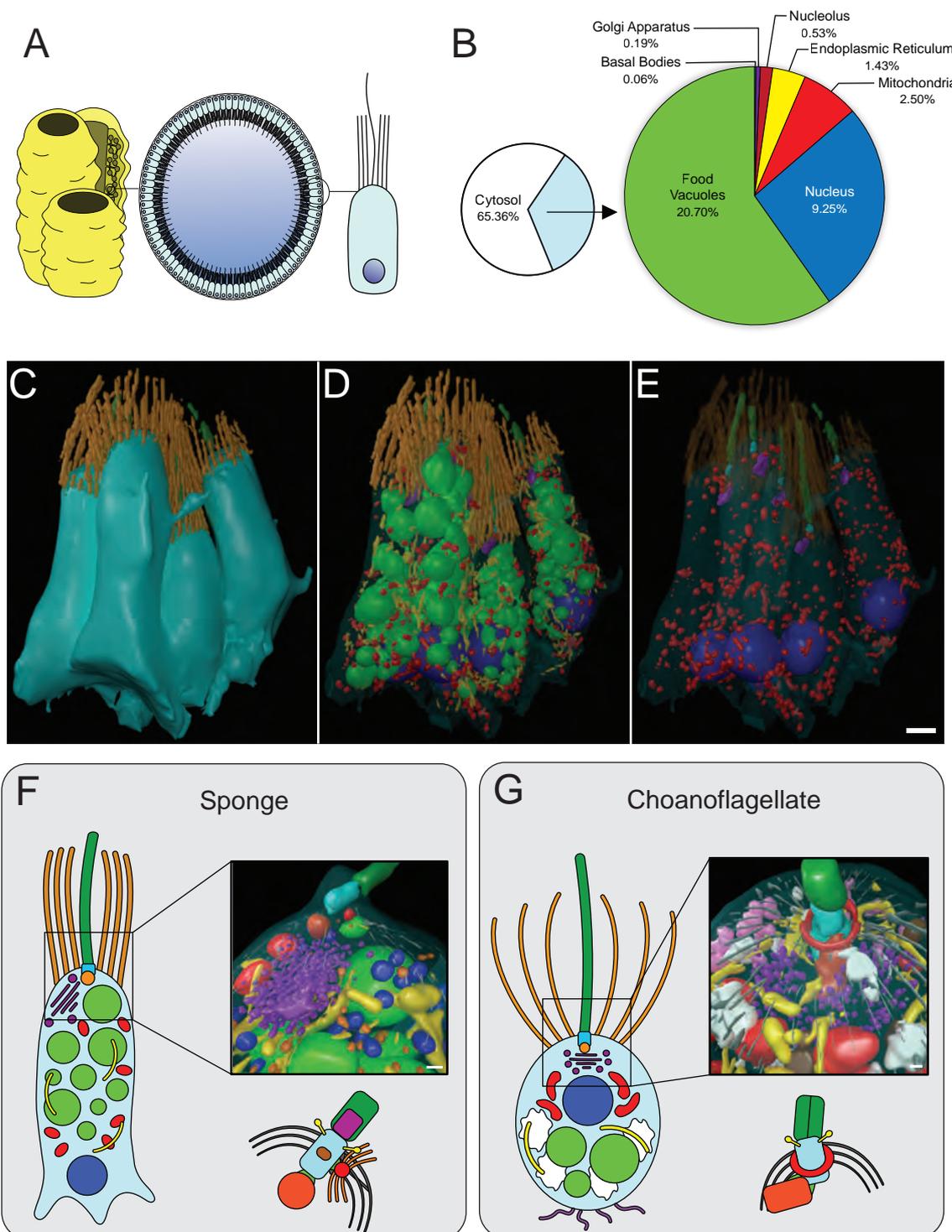
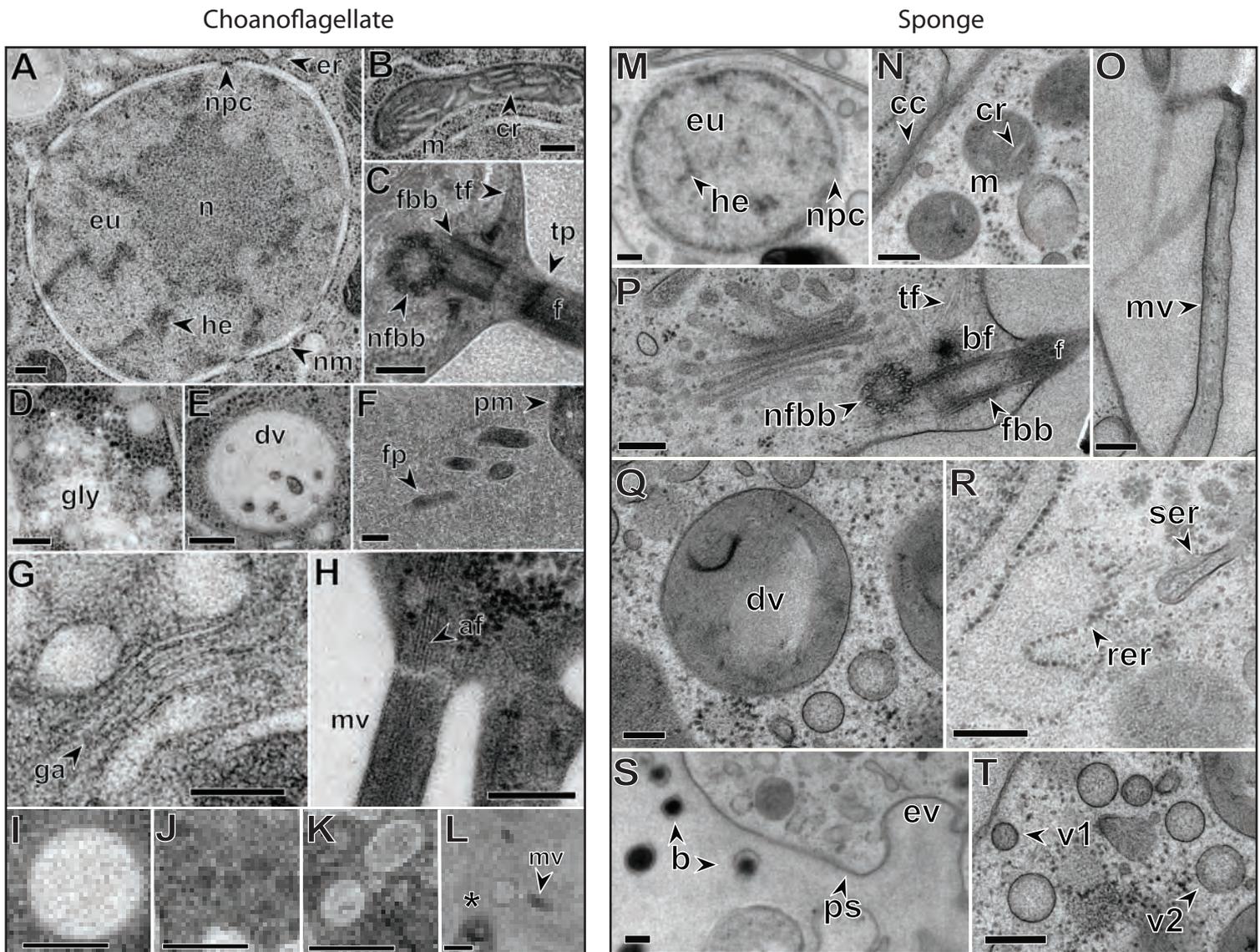


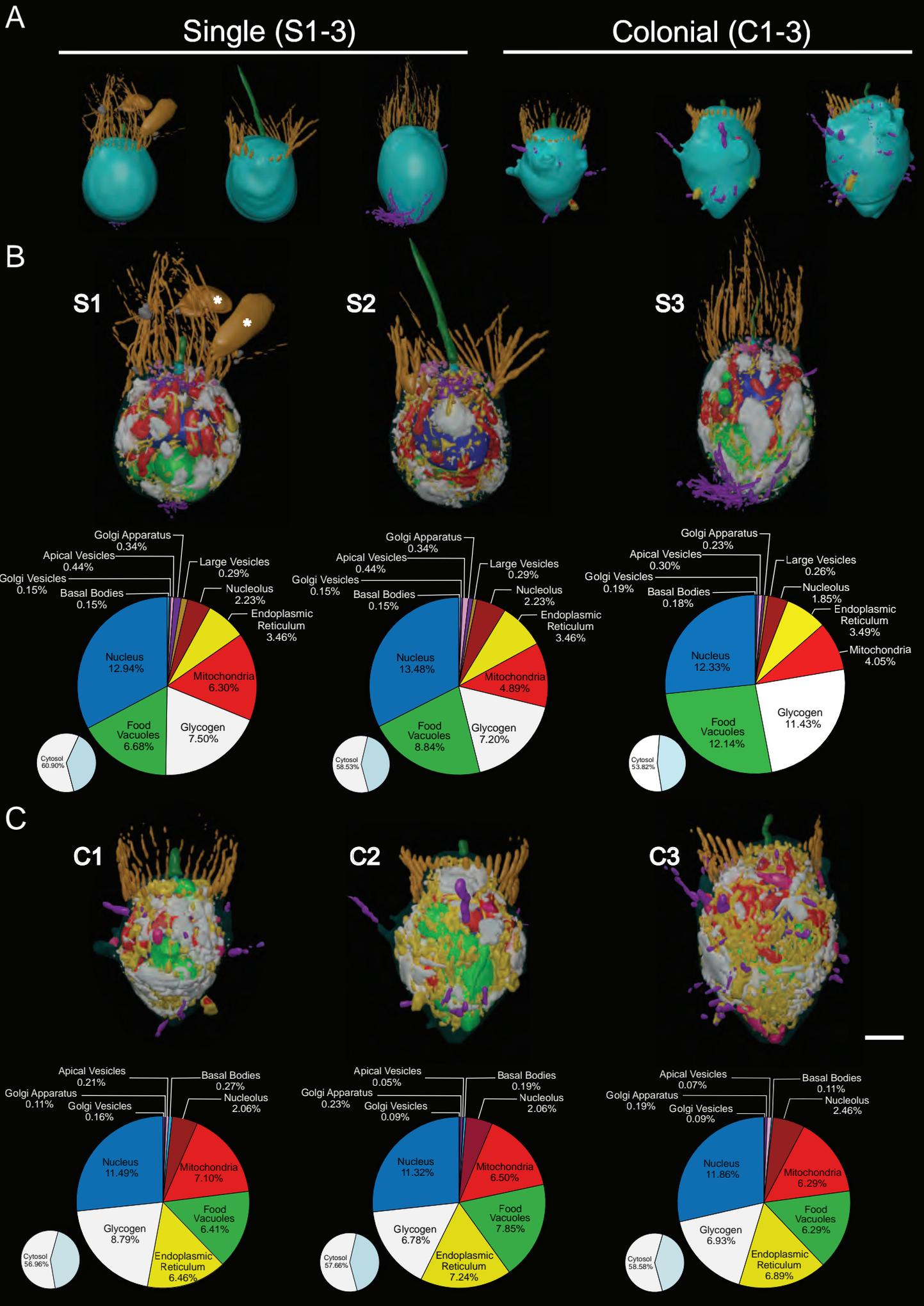
Figure 4



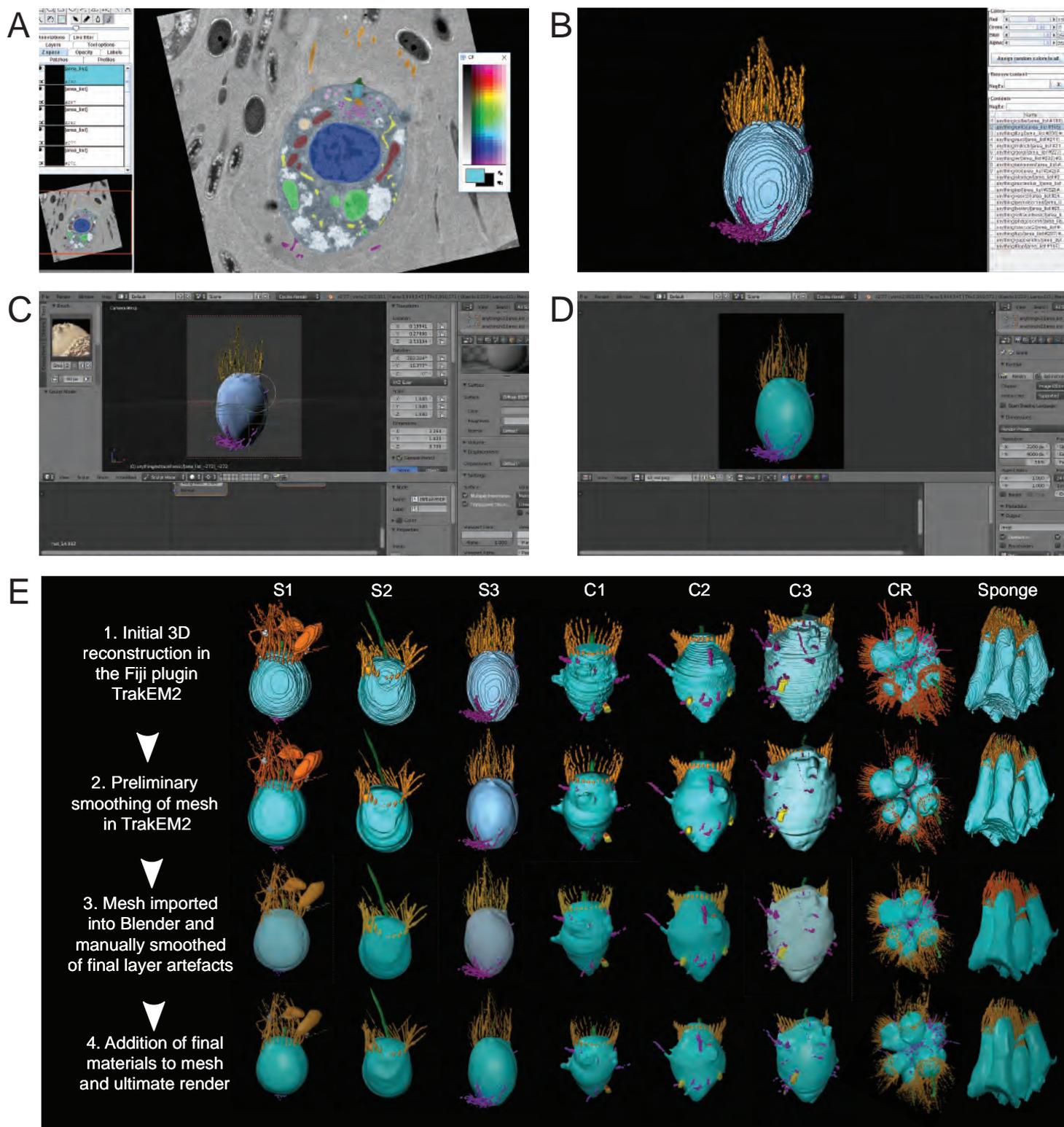
Suppl. Figure 1



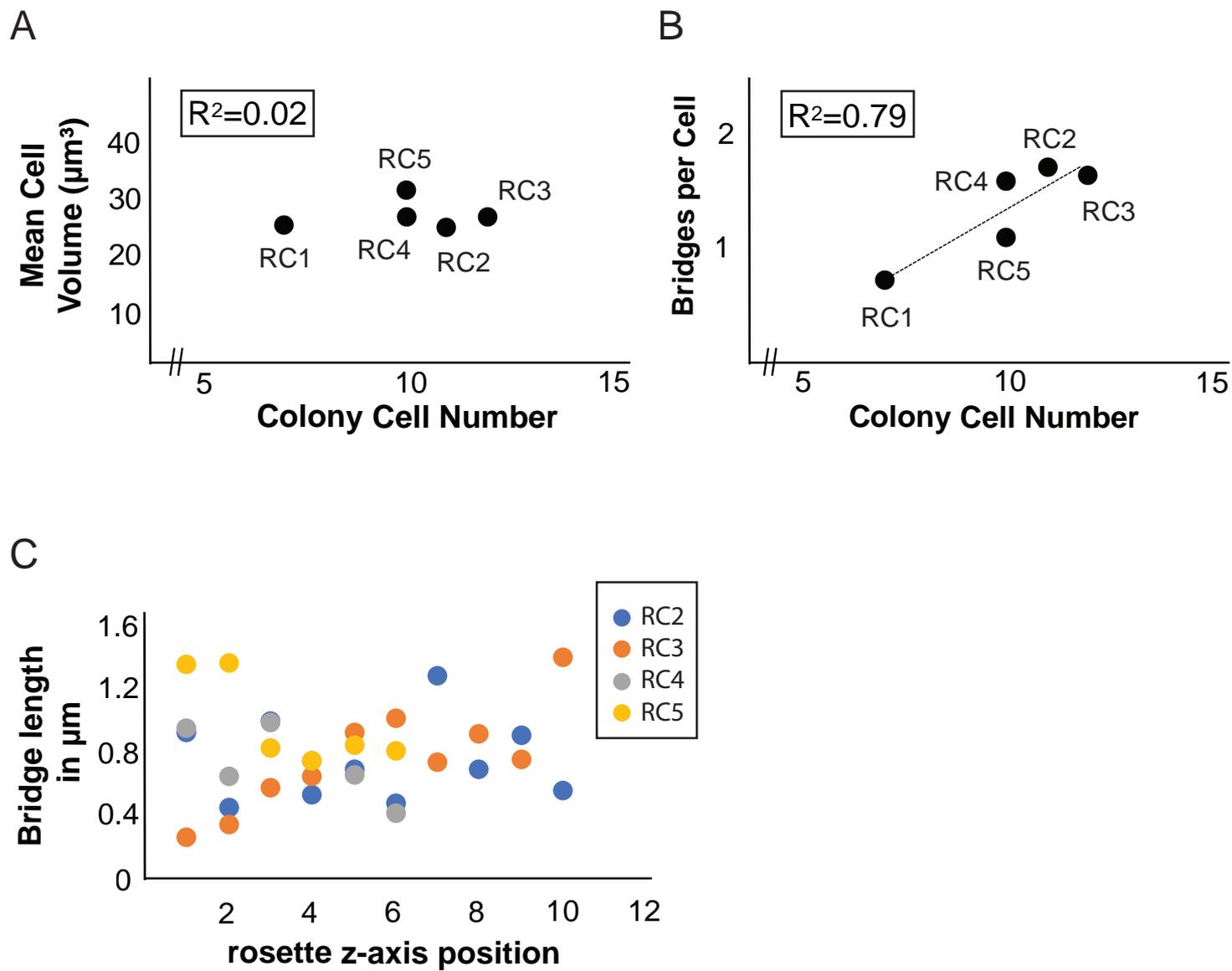
Suppl. Figure 2



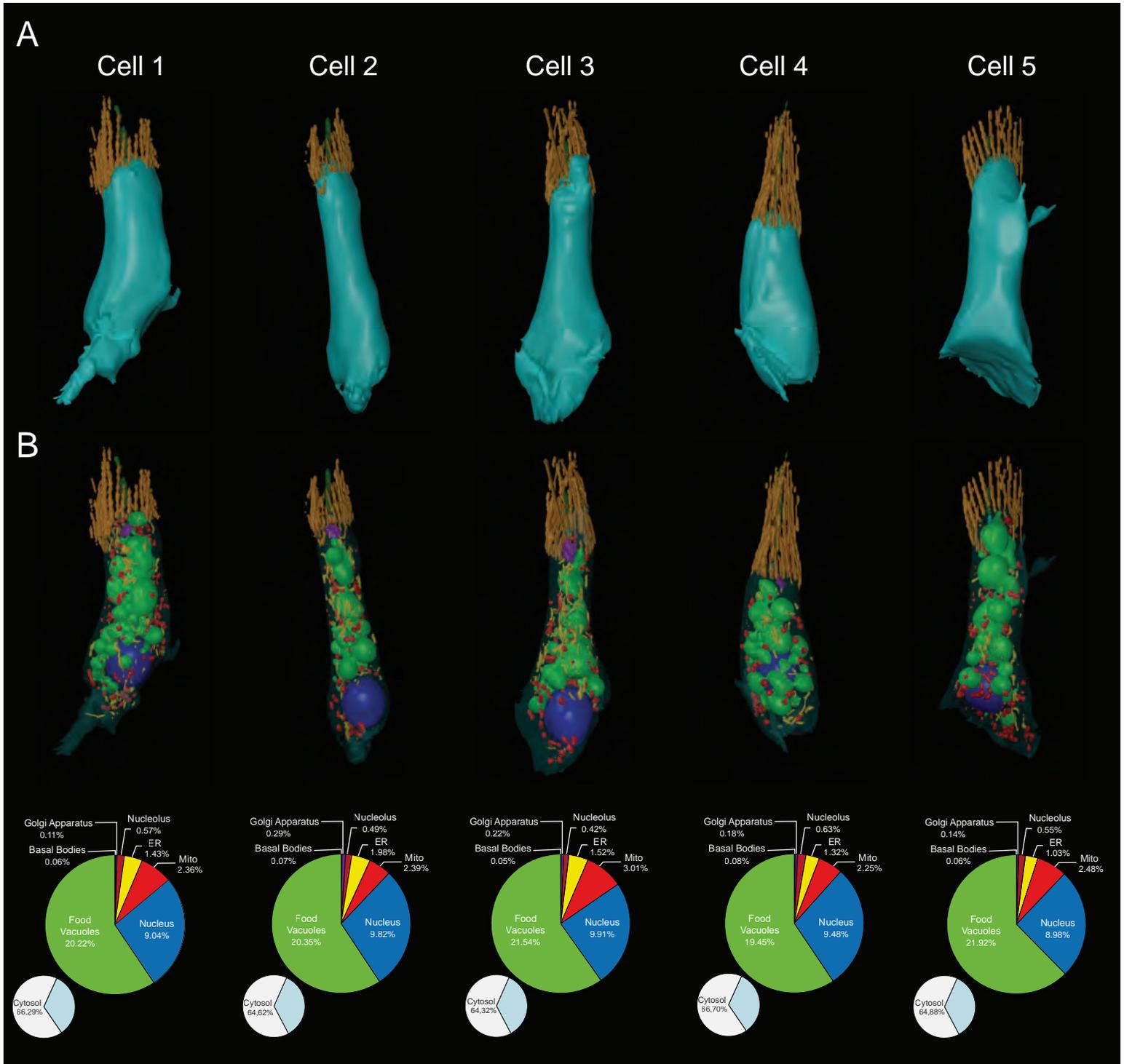
Suppl. Figure 3



Suppl. Figure 4



Suppl. Figure 5



Suppl. Figure 6

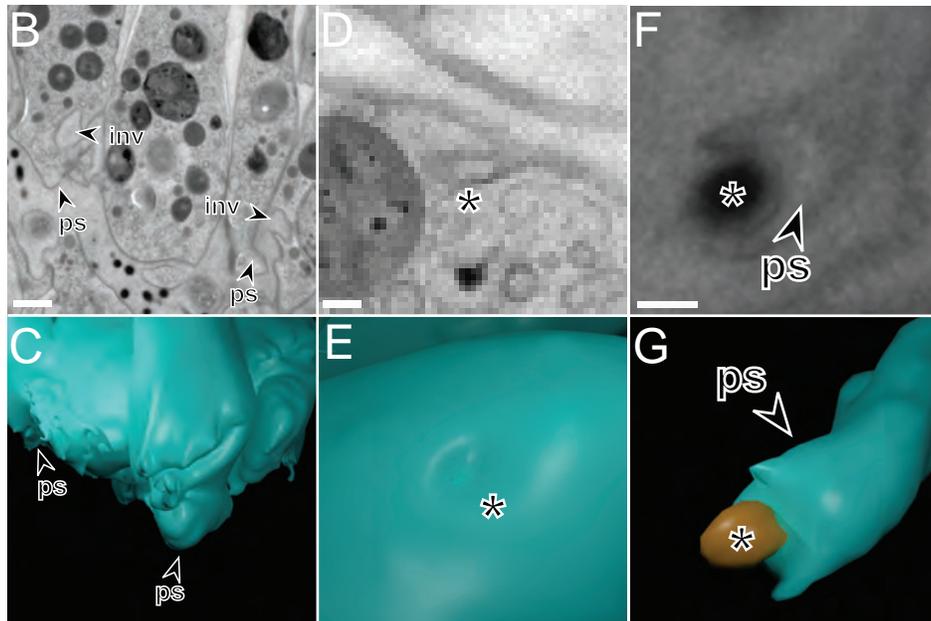
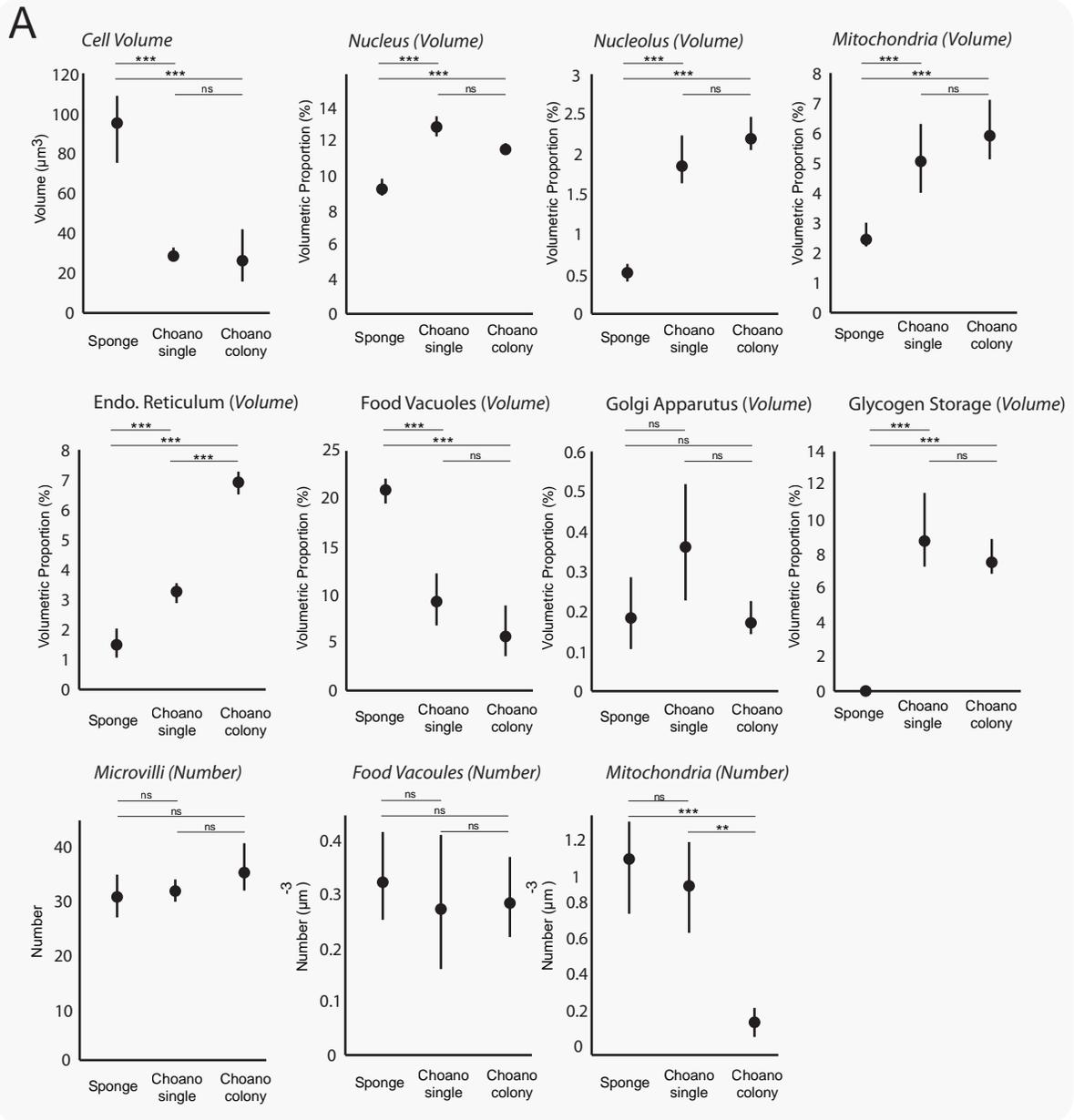


Table S1. Volumetric measurements of *S. rosetta* cells and components

<u>Organelle</u>	Single cells				Colonial cells			
	S1	S2	S3	Mean +/- SD	C1	C2	C3	Mean +/- SD
Cell Body	26.81 (100)	32.34 (100)	26.68 (100)	28.61 ± 3.23 (100 ± 0)	18.89 (100)	21.54 (100)	41.82 (100)	27.41 ± 12.54 (100 ± 0)
Nucleus	3.92 (12.94)	5.08 (13.48)	3.73 (12.33)	4.24 ± 0.73 (12.92 ± 0.58)	2.56 (11.49)	2.89 (11.33)	5.99 (11.86)	3.81 ± 1.89 (11.56 ± 0.27)
Nucleolus	0.45 (1.68)	0.72 (2.23)	0.44 (1.65)	0.54 ± 0.16 (1.85 ± 0.33)	0.39 (2.06)	0.45 (2.09)	1.03 (2.46)	0.62 ± 0.35 (2.2 ± 0.22)
Mitochondria	1.69 (6.30)	1.58 (4.89)	1.08 (4.05)	1.45 ± 0.33 (5.08 ± 1.14)	1.34 (7.10)	1.4 (6.50)	2.63 (6.29)	1.79 ± 0.73 (6.63 ± 0.42)
Endoplasmic Reticulum	0.77 (2.87)	1.12 (3.46)	0.93 (3.49)	0.94 ± 0.18 (3.27 ± 0.35)	1.22 (6.46)	1.56 (7.24)	2.88 (6.89)	1.89 ± 0.88 (6.86 ± 0.39)
Food Vacuoles	1.79 (6.68)	2.86 (8.84)	3.24 (12.14)	2.63 ± 0.75 (9.22 ± 2.75)	1.21 (6.41)	1.69 (7.85)	2.63 (6.29)	1.84 ± 0.72 (6.85 ± 0.87)
Glycogen Storage	2.01 (7.50)	2.33 (7.20)	3.05 (11.43)	2.46 ± 0.53 (8.71 ± 2.36)	1.66 (8.79)	1.46 (6.78)	2.9 (6.93)	2.01 ± 0.78 (7.50 ± 1.12)
Flagellar Basal Body	0.02 (0.07)	0.03 (0.09)	0.01 (0.10)	0.02 ± 0.01 (0.09 ± 0.02)	0.03 (0.16)	0.03 (0.14)	0.02 (0.05)	0.03 ± 0.01 (0.12 ± 0.06)
Non-Flagellar Basal Body	0.02 (0.07)	0.02 (0.06)	0.02 (0.08)	0.02 ± 0 (0.07 ± 0.01)	0.02 (0.11)	0.01 (0.05)	0.02 (0.05)	0.02 ± 0.01 (0.07 ± 0.03)
Golgi Apparatus	0.14 (0.52)	0.11 (0.34)	0.06 (0.23)	0.10 ± 0.04 (0.36 ± 0.15)	0.02 (0.11)	0.05 (0.23)	0.08 (0.19)	0.05 ± 0.03 (0.18 ± 0.06)
Golgi Associated Vesicles	0.03 (0.12)	0.05 (0.15)	0.05 (0.19)	0.04 ± 0.01 (0.15 ± 0.04)	0.03 (0.16)	0.02 (0.09)	0.03 (0.07)	0.03 ± 0.01 (0.11 ± 0.05)
Apical Vesicles	0.06 (0.22)	0.14 (0.44)	0.08 (0.30)	0.09 ± 0.04 (0.32 ± 0.11)	0.04 (0.21)	0.01 (0.05)	0.14 (0.33)	0.06 ± 0.07 (0.20 ± 0.14)
Large Vesicles	0.03 (0.45)	0.09 (0.29)	0.07 (0.26)	0.06 ± 0.03 (0.33 ± 0.10)	0 (0)	0 (0)	0 (0)	0 (0)
Extracellular Vesicles	0.06 (0.22)	0 (0)	0.01 (0.04)	0.02 ± 0.03 (0.09 ± 0.12)	0 (0)	0 (0)	0 (0)	0 (0)
Endocytotic Vacuoles	0.04 (0.15)	0 (0)	0.02 (0.07)	0.02 ± 0.02 (0.07 ± 0.07)	0.06 (0.32)	0.04 (0.19)	0.18 (0.43)	0.09 ± 0.08 (0.32 ± 0.12)
Filopodia	0.07 (0.26)	0 (0)	0.28 (1.05)	0.12 ± 0.15 (0.44 ± 0.55)	0.07 (0.37)	0.08 (0.37)	0.18 (0.43)	0.11 ± 0.06 (0.39 ± 0.03)
Cytosol	16.33 (60.9)	18.93 (58.53)	14.36 (53.82)	16.54 ± 2.29 (57.75 ± 3.6)	10.76 (56.96)	12.42 (57.66)	24.50 (58.58)	15.89 ± 7.49 (57.97 ± 0.81)

Volumes were measured in μm^3 .

Values between parentheses are percentages of cell volume.

Table S2. Numbers of various organelles and components in *S. rosetta* cells

<u>Organelle</u>	Single cells				Colonial cells			
	S1	S2	S3	Mean +/- SD	C1	C2	C3	Mean +/- SD
Nucleus	1	1	1	1 ± 0	1	1	1	1 ± 0
Nucleolus	1	1	1	1 ± 0	1	1	1	1 ± 0
Flagellum	1	1	1	1 ± 0	1	1	1	1 ± 0
Flagellar Basal Body	1	1	1	1 ± 0	1	1	1	1 ± 0
Non-Flagellar Basal Body	1	1	1	1 ± 0	1	1	1	1 ± 0
Microvilli	30	34	32	32 ± 2	32	33	41	35.3 ± 4.9
Golgi Apparatus	1	1	1	1 ± 0	1	1	1	1 ± 0
Golgi Associated Vesicles	140	203	156	166.3 ± 32.7	64	51	102	72.3 ± 26.5
Food Vacuoles	6	8	11	8.3 ± 2.5	5	9	9	7.7 ± 2.3
Mitochondria	32	22	22	25.3 ± 5.8	1	3	9	4.3 ± 4.2
Apical Vesicles	41	53	68	54 ± 13.5	23	8	64	31.6 ± 29.0
Large Vesicles	11	12	7	10 ± 2.7	0	0	0	0
Extracellular Vesicles	12	0	4	5.3 ± 6.1	0	0	0	0
Endocytotic Vacuoles	1	0	2	1 ± 1	5	3	7	5 ± 2
Pseudopodia	0	1	2	1 ± 1	6	8	10	8 ± 2
Intercellular Bridges	0	0	0	0	2	2	2	2 ± 0

Table S3. Volumetric measurements of *O. carmela* choanocytes and components

<u>Organelle</u>	Five sponge choanocytes					
	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Mean +/- SD
Cell Body	108.56 (100)	75.35 (100)	107.37 (100)	83.95 (100)	100.29 (100)	95.1 ± 14.8 (100 ± 0)
Nucleus	10.47 (9.07)	7.77 (9.82)	10.02 (8.91)	8.49 (9.48)	9.54 (8.98)	9.26 ± 1.11 (9.25 ± 0.39)
Nucleolus	0.62 (0.57)	0.37 (0.49)	0.45 (0.42)	0.53 (0.63)	0.55 (0.55)	0.51 ± 0.10 (0.53 ± 0.08)
Mitochondria	2.56 (2.36)	1.8 (2.39)	3.23 (3.01)	1.89 (2.25)	2.49 (2.48)	2.39 ± 0.58 (2.50 ± 0.30)
Endoplasmic Reticulum	1.43 (1.32)	1.49 (1.98)	1.63 (1.52)	1.03 (1.32)	1.03 (1.03)	1.32 ± 0.28 (1.43 ± 0.35)
Food Vacuoles	21.95 (20.22)	15.33 (20.35)	23.13 (21.54)	16.33 (19.45)	21.98 (21.92)	19.74 ± 3.62 (20.70 ± 1.01)
Glycogen Storage	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Flagellar Basal Body	0.03 (0.03)	0.02 (0.03)	0.03 (0.03)	0.03 (0.04)	0.02 (0.02)	0.03 ± 0.01 (0.03 ± 0.02)
Non-Flagellar Basal Body	0.03 (0.03)	0.03 (0.04)	0.02 (0.02)	0.03 (0.04)	0.02 (0.02)	0.03 ± 0.01 (0.03 ± 0.01)
Golgi Apparatus	0.12 (0.11)	0.22 (0.29)	0.24 (0.22)	0.15 (0.18)	0.14 (0.14)	0.17 ± 0.05 (0.19 ± 0.07)
Filopodia	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cytosol	71.97 (66.29)	48.69 (64.62)	69.07 (64.32)	56 (66.70)	65.07 (64.88)	62.16 ± 9.64 (65.36 ± 1.06)

Volumes were measured in μm^3 .

Values between parentheses are percentages of cell volume.

Table S4. Numbers of various organelles and components in *O. carmela* choanocytes

	Five sponge choanocytes					
Organelle	Cell 1	Cell 2	Cell 3	Cell 4	Cell 5	Mean +/- SD
Nucleus	1	1	1	1	1	1 ± 0
Nucleolus	1	1	1	1	1	1 ± 0
Flagellum	1	1	1	1	1	1 ± 0
Flagellar Basal Body	1	1	1	1	1	1 ± 0
Non-Flagellar Basal Body	1	1	1	1	1	1 ± 0
Microvilli	35	27	35	27	29	30.6 ± 4.1
Golgi Apparatus	1	1	1	1	1	1 ± 0
Food Vacuoles	42	22	27	35	27	30.6 ± 7.9
Mitochondria	125	81	140	66	117	105.8 ± 31.1
Pseudopodia	0	0	0	0	0	0
Intercellular Bridges	0	0	0	0	0	0