

1 **Title:** Towards an understanding of spiral patterning in the *Sargassum muticum* shoot  
2 apex

3 **Running title:** Phyllotaxis in *Sargassum muticum*

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8

9 **Keywords:**

10 *Sargassum muticum*, brown algae, phyllotaxis, apical cell, meristem

11

12 **Summary**

13 The brown alga *Sargassum muticum* displays spiral phyllotaxis developed from a  
14 position-dependent self-organising mechanism, different from that understood in  
15 plants.

16

17 **Abstract**

18 In plants and parenchymatous brown algae the body arises through the activity of an  
19 apical meristem (a niche of cells or a single cell). The meristem produces lateral  
20 organs in specific patterns, referred to as phyllotaxis. In plants, two different control  
21 mechanisms have been proposed – one is position-dependent and relies on  
22 morphogen accumulation at future organ sites whereas the other is a lineage-based  
23 system which links phyllotaxis to the apical cell division pattern. Here we examine the  
24 apical patterning of the brown alga, *Sargassum muticum*, which exhibits spiral  
25 phyllotaxis (137.5° angle) and an unlinked apical cell division pattern. The *Sargassum*  
26 apex presents characteristics of a self-organising system, similar to plant meristems.  
27 We were unable to correlate the plant morphogen auxin with bud positioning in  
28 *Sargassum*, nor could we predict cell wall softening at new bud sites. Our data  
29 suggests that in *Sargassum muticum* there is no connection between phyllotaxis and  
30 the apical cell division pattern indicating a position-dependent patterning mechanism  
31 may be in place. The underlying mechanisms behind the phyllotactic patterning appear  
32 to be distinct from those seen in plants.

33

## 34 **Introduction**

35           When discussing spiral patterns in nature, D'Arcy Thompson (1917) wrote:  
36 'When the bricklayer builds a factory chimney, he lays his bricks in a certain steady,  
37 orderly way, with no thought of the spiral patterns to which this orderly sequence  
38 inevitably leads, and which spiral patterns are by no means "subjective".' This  
39 proposition, now 100 years old, implies that spiral patterns are an emergent property  
40 of local decision making processes; the underlying mechanisms may be various while  
41 the result remains the same. In developmental biology, fate decisions (such as where  
42 to place a brick, or new organ) often exhibit characteristics of emergent phenomenon.  
43 Such decisions are often made based on a position-dependent patterning system  
44 where the position of a cell within a tissue or organ specifies its fate and a signal (or  
45 morphogen) acts as an instructive agent (Scheres, 2001). An alternative mechanism  
46 depends on cell lineage, although this seems less prevalent in walled organisms such  
47 as plants (Scheres, 2001). When one examines the processes behind areal organ  
48 positioning in plants, phyllotaxis, two major theories emerge: in some early diverging  
49 land plants, phyllotactic patterning is attributed to patterned divisions at the  
50 meristematic apical cell; in spermatophytes (seed plants), they are attributed to a  
51 morphogen-based mechanism. The latter is position-dependent patterning and the  
52 former lineage-dependent.

53           Early diverging land plants, such as mosses and ferns, maintain a single apical  
54 cell which acts as a stem cell for the apex (Nägeli, 1845a, 1845b; Schüepp, 1926 via  
55 Korn, 1993). In mosses, the pattern of leaf production may be seen as lineage-  
56 dependent as it follows the apical cell patterning directly (Renzaglia et al., 2000;  
57 Harrison et al., 2009). In horsetails, the whorled arrangement of the leaves is  
58 independent of the division pattern in the apical cell (Golub, 1948). In fern apices, the  
59 division of the apical cell occurs successively from each face of a three-sided apical  
60 cell producing packets of daughter cells at 120° angles (Bierhorst, 1977); the spiral  
61 phyllotactic pattern observed later likely occurs through another imposed patterning  
62 signal. These two examples hint at a position-dependent patterning mechanism which  
63 takes place post-apical cell divisions; position-dependent mechanisms tend to be  
64 robust and self-organising - just what Thompson might have alluded to above.  
65 Evidence for a self-organising and robust patterning mechanism comes from  
66 experiments where apical cell ablation does not lead to growth arrest, but instead to a

67 new apical cell establishment and subsequent spiral phyllotaxis about the new centre  
68 (Wardlaw, 1949; Cutter, 1965). Work from Wardlaw (1949) and Snow & Snow (1935)  
69 explored positional patterning mechanisms which were both physical (tissue tension)  
70 and morphogen (the phytohormone auxin) based (reviewed in Philipson, 1990);  
71 however, no further modern explorations have been conducted in these species to our  
72 knowledge.

73 In Spermatophytes (seed plants) the meristematic activity in the shoot apex is  
74 attributed to an organised group of cells. This niche serves as a reservoir for  
75 production of cells which then give rise to the lateral organs (Steeves and Sussex,  
76 1989; Meyerowitz, 1997). Phyllotactic patterning occurs independent from division  
77 patterns within the meristematic niche and evidence exists for a position/morphogen-  
78 based patterning mechanism: organs emerge due to local softening of tissues at  
79 specific positions at the shoot apex (Braybrook and Peaucelle, 2013; Milani et al.,  
80 2014); local maxima of the phytohormone auxin dictate the position of new organs  
81 (Reinhart et al., 2003); auxin maxima are positioned by the cell-cell dynamics of polar  
82 auxin transporters whose direction relates to auxin concentration of neighbouring cells  
83 (Reinhart et al., 2003); stochastic fluctuations in auxin concentration can lead to  
84 coordinated polarisation of auxin transporters and result in a self-organising pattern of  
85 organs (Jönsson et al., 2006). Here again, ablation of the meristematic niche leads to  
86 re-establishment of a new niche and organised phyllotaxis about the new centre  
87 lending weight to a robust self-organising mechanism rooted in the morphogen auxin  
88 (Steeves and Sussex, 1989; Reinhardt et al., 2003).

89 Plants are not the only organisms to display spiral organ arrangement: two  
90 genera of parenchymatous multicellular brown algae, in the order Fucales, arrange  
91 their organs in spirals: *Sargassum* and *Cystoseira* (Church, 1920). Other members of  
92 the order tend to display dichotomous branching (e.g. *Fucus*). The body of  
93 parenchymatous brown algae is built through the meristematic activity of an apical cell  
94 (Fritsch, 1945; Katsaros, 2000). Most complex brown algal species belong to the order  
95 Fucales and have only one apical cell per thallus tip (Yoshida 1983; exceptions  
96 detailed in Nizamuddin, 1967; Clayton, 1985). In the Fucales, the apical cell presents  
97 as three or four sided in transverse view and divides from these faces (Nizamuddin,  
98 1963; Moss, 1967, 1969; Yoshida, 1983; Klemm and Hallam, 1987; Kaur, 1999). In  
99 some cases, the apical cell is thought not to divide but rather stimulate the cells around

100 do so (Moss, 1967). In *Cystophora*, it has been proposed that the division pattern of  
101 the apical cell drives the observed branching pattern of the thallus, similar to the theory  
102 for moss (Klemm and Hallam, 1987). In *Fucus*, if the apical cell is removed growth of  
103 the branch ceases (Moss, 1967) indicating a less robust patterning mechanism than  
104 seen in spermatophytes, ferns and lycophytes. As such, the literature indicates that  
105 phyllotactic patterns in parenchymatous brown algae may be lineage-dependant.

106 In *Sargassum muticum*, while a clear apical cell is present (Yoshida, 1983) the  
107 shoot apex itself is similar in organisation to that seen in ferns and spermatophytes: a  
108 large central area is surrounded by emerging organs in a spiral pattern (Simons,  
109 1906). At the apex a leaf bud is formed when a new apical cell differentiates from the  
110 epidermal tissue; subsequently it is proposed that other cells are differentiated into  
111 apical cells, between the main apical cell and the leaf apical cell, and these go on to  
112 form air bladders and other organs (Oltmann, 1889 via Critchley, 1983). As such the  
113 main bud identity observed at the apex is that of a leaf but subsequently branch  
114 development occurs, including specification of new lateral branch apices. Since the  
115 brown algae have evolved completely independently from plants, it is fascinating to  
116 see similar spiral leaf patterns emerging in the shoots of *Sargassum* as are seen in  
117 that distant kingdom. Here we explore the apical organisation and spiral phyllotaxis  
118 observed in *Sargassum muticum*, and begin to investigate its robustness and  
119 underlying mechanisms with comparisons to those proposed in plants.

120

## 121 **Results**

### 122 **The arrangement of leaf buds in the *Sargassum muticum* meristem follows the** 123 **golden angle**

124 The *S. muticum* apex has a striking 'phyllotactic' pattern, where subsequent  
125 branches are spirally organised with respect to each other (Church, 1920). At the apex,  
126 these branches begin as leaf buds (Critchley, 1983). In order to characterise the spiral  
127 pattern more fully, we performed detailed analysis of *S. muticum* apices collected in  
128 the field.

129 *S. muticum* apices were qualitatively divided into two zones: the apical pit-  
130 region, where pro-meristem cells were produced (Fig. 1B, pink), and the peripheral  
131 region where new leaf buds formed (Fig. 1B, yellow). In primary lateral apices the  
132 meristem size (proxied by presented area of the pit-region, Fig. 1B, pink) was not

133 correlated with stipe length which is representative of apex age (n (individuals) = 7, n  
134 (branches) = 22, Fig. S1). Within the peripheral region, the phyllotactic pattern was  
135 spiral and presented an average divergence angle (angle between two sequentially-  
136 aged buds) of  $137.53 \pm 2.08^\circ$  (Fig. 1B, C; n (meristems) = 57). The organisation and  
137 phyllotactic pattern observed in the apices of *S. muticum* was highly regular and  
138 resembled that seen in complex multicellular plant apices.

139

### 140 **The *Sargassum muticum* apical cell area suggests a highly organised division** 141 **pattern**

142 As the literature seemed to indicate that brown algae phyllotaxis might be  
143 lineage-dependent, we next examined the division patterns of the *Sargassum* apical  
144 cell to see if its pattern exhibited a golden angle, as in moss. The apical cell (AC) of  
145 *Sargassum* species has been described as a three-sided lenticel (Yoshida, 1983). In  
146 order to investigate the possible patterning of cell divisions in the promeristem, and  
147 any connection to the phyllotactic pattern, we examined transverse and longitudinal  
148 sections of *S. muticum* apices.

149 In sections, the apical cell of *S. muticum* presented as bi-convex and lenticular  
150 (longitudinally; Fig. 1D) and as three-sided (transversely; Fig. 1E) consistent with other  
151 species in the order Fucales (Nizamuddin, 1963; Moss, 1969; Yoshida, 1983; Kaur,  
152 1999). Unlike Fucales apical cells, which are reported to stimulate their neighbours to  
153 divide but refrain themselves (Moss, 1967), evidence of apical cell division was  
154 observed. In the longitudinal view, divisions appeared to give rise to three tissues –  
155 outer layer (meristoderm) and two inner layers (cortex and medulla). The upper cell of  
156 the first anticlinal division likely gave rise to the meristodermal and medullar cells  
157 whereas the lower cell likely created the medullar and cortex cells (Fig. 1D, F, S2).

158 In the transverse direction, the first division always appeared to be an  
159 asymmetric periclinal division which followed a sequential face division pattern of the  
160 apical cell producing promeristem daughters at  $119^\circ$  angles to each other (Fig. 1E,  
161 red). Following from this division, the subsequent anticlinal divisions from the initial  
162 daughter cell could be followed up to the 6<sup>th</sup> or 7<sup>th</sup> 'division round' (a division round  
163 was defined as a pseudo-time progression that each daughter cell would undergo as  
164 it moved away from the apical cell). In the first three rounds of division, the daughter  
165 cell then underwent one or more anticlinal radial divisions (Fig. 1E, orange/yellow/blue;  
166 white arrowhead); the rounds of radial division never produced more than four cells

167 (Fig. 1E, blue). The further divisions were anticlinal circumferential and created 8 cells  
168 in total (Fig. 1E, blue, purple; black arrowhead). These cells then underwent another  
169 round of anticlinal radial and circumferential divisions; however, at this point it became  
170 difficult to discern lineages in histological sections. The pattern described here was  
171 highly conserved although occasionally an anticlinal circumferential division was  
172 observed before the 4-cell stage (n=1/30). From these data, it was concluded that the  
173 *S. muticum* apical cell divided asymmetrically from sequential faces, producing  
174 daughter cells at 120° angles, and that these promeristematic daughter cells further  
175 underwent a regimented division pattern.

176

177

### 178 **The phyllotaxis pattern and the apical cell division pattern are not linked**

179 In *Cystophora*, the apical cell division pattern (bifacial divisions) has been  
180 correlated with the apical branching pattern (sympodial branching giving rise to an  
181 alternate phyllotactic presentation; Klemm and Hallam, 1987). Our observations in *S.*  
182 *muticum* suggest that the apical cell divides from all three faces to produce  
183 promeristem daughter cells at an approximate 120° angle ( $\alpha=119.01 \pm 6.11^\circ$ , n=74),  
184 while the phyllotactic pattern follows at ~137.5° spiral pattern. In order to examine  
185 whether these two patterns in *S. muticum* were linked, we examined the chirality in  
186 both the apical and phyllotactic patterns in the same meristems.

187 The spiral phyllotaxis in *Sargassum muticum* had either a clockwise or a  
188 counter-clockwise direction with a ratio of ~1:1 (58/118 clockwise, 60/118 counter-  
189 clockwise). In the clockwise orientation the older buds were located to the left side of  
190 the younger bud forming a right-handed spiral (Fig. 1H, I). Likewise, in the counter  
191 clockwise orientation, the spiral produced was left-handed (Fig. 1J, K). This 1:1 ratio  
192 is observed in plants as well (e.g. Thompson, 1917). With respect to apical cell division  
193 patterning, two patterns were observed: moving out from the apical cell, daughter cells  
194 were produced to the left or the right yielding both counter- and clockwise patterns in  
195 a 1:1 ratio (Fig. 1L, M, N, O; 28/56 clockwise, 28/56 counter-clockwise).

196 In order to examine if a connection in chirality was observed, individuals were  
197 imaged under a light microscope and subsequently sectioned to check the orientation  
198 of the apical cell division. In either the counter or clockwise phyllotactic groups, the  
199 apical cells presented as ~1:1 counter- and clockwise (Fig. 1H-O, clockwise  
200 phyllotaxis – 8/16 clockwise, 8/16 counter-clockwise apical cell divisions; counter-

201 clockwise phyllotaxis – 7/11 clockwise, 4/11 counter-clockwise apical cell divisions).  
202 This data strongly suggests that these two patterning mechanisms are unlinked and  
203 may be under separate control. This is highly similar to the patterning mechanisms  
204 seen in multicellular plant apices where the phyllotactic pattern is defined in the  
205 peripheral zone by the morphogen auxin and the stem cell niche is positioned by  
206 another phytohormone, cytokinin (Reinhardt et al., 2003; Chickarmane et al., 2012).

207

### 208 **Ablation of the apical cell leads to formation of a new apical centre indicating** 209 **pattern self-organisation**

210 Given the observed similarities to multicellular plant meristems, we next  
211 examined whether the apical cell and phyllotaxis could re-establish after ablation of  
212 the apical cell. In plants, the stem cell region can re-establish in this way pointing to a  
213 robust self-organising patterning system (Reinhardt et al., 2003). In *Fucus*, such  
214 manipulations led to growth arrest and termination (Moss, 1967).

215 Apical cell ablations were performed on partially dissected apices using a thin  
216 needle aimed at the centre of the pit-area (Fig. 2A, B; white arrowhead). Apices were  
217 grown in culture, and re-dissected after a 3-week recovery period before a second  
218 imaging. Two scenarios were observed – in 30% (7/23) of the apices the growth of the  
219 central zone had stopped or they were dead (6/7 dead, 1/7 no new meristem formed,  
220 but the existing buds continued to grow). In 30% of the samples growth continued from  
221 what appeared to be a new pit-region (Fig. 2C, D; blue arrowhead, n= 7/23); in these  
222 apices, the phyllotactic pattern after recovery exhibited a spiral pattern. In another 20%  
223 apices, the meristems seemed to split in two (Fig. 2E; n= 5/23) but again appeared to  
224 present spiral patterning. In the remaining 20% of the samples, the results were  
225 inconclusive as the imaging methods did not always produce sufficient quality data for  
226 pit-area positioning. Culturing itself did not alter the pattern of buds (Fig. 2F).

227 In the samples where a new pit-area appeared to establish, the wound had  
228 moved to the side of the meristem and the new pit-area was roughly centrally  
229 positioned (Fig. 2C, D). These data suggest that the meristematic region of the  
230 *Sargassum* meristem could re-establish itself indicating a self-organising system  
231 similar in nature to that in plant meristems. The data also imply that when a new apical  
232 cell is established, the spiral phyllotactic pattern can also re-establish.

233

234

## 235 **A potential link between auxin and brown algal phyllotaxis is unlikely**

236 In plants, auxin distribution within the peripheral zone dictates the phyllotactic  
237 pattern (Reinhardt et al., 2003). Auxin maxima in the peripheral zone lead to cell wall  
238 softening and organ outgrowth (Braybrook and Peaucelle, 2013). The brown algae  
239 *Fucus vesiculosus* and *Ectocarpus siliculosus* have both exhibited auxin-triggered  
240 morphogenetic changes: aberrant embryo rhizoid branching in *Fucus* and filamentous  
241 adult branching changes in *Ectocarpus* (Basu et al., 2002; Le Bail et al., 2010). In  
242 addition, auxin has been detected using gas chromatography mass spectroscopy and  
243 an anti-indole acetic acid (IAA) anti-body in *Ectocarpus* (Le Bail et al., 2010). Since  
244 the phyllotaxis in *Sargassum muticum* is spiral and highly resembles the one observed  
245 in higher plants and given the potential for auxin response in brown algae, we next  
246 examined whether auxin could alter, or be correlated with, the phyllotactic pattern.

247 We applied auxin externally in the artificial sea water cultivation medium in  
248 order to see if phyllotaxis could be altered. In our conditions and experiments, this  
249 treatment had no effect on growth or the phyllotactic pattern (50  $\mu$ M; data not shown).  
250 Due to the aqueous nature of the culture system it was not possible to apply auxin  
251 locally as has been performed in tomato and *Arabidopsis* (Reinhardt et al., 2000;  
252 Braybrook and Peaucelle, 2013). These experiments were therefore inconclusive but  
253 lightly suggest that external auxin could not alter phyllotaxis in *Sargassum*, in these  
254 conditions.

255 In order to determine if auxin showed patterned distribution within the apex, we  
256 performed immunolocalisations on sectioned apices using the anti-IAA antibody. The  
257 anti-IAA signal was strongest in the meristoderm and mucilage external to the  
258 meristoderm with accumulation at apical pits and the bases of buds (Fig. 3A, white  
259 arrowhead). Internally, there were regions of high signal within the apex in the  
260 meristoderm although these did not correlate with bud size or position (Fig. 3A, B).  
261 Upon close examination, a large amount of signal originated from the mucilage  
262 external to the meristoderm (Fig. 3C). These data suggest that auxin may be  
263 accumulating in the meristoderm and mucilage, although its source is undetermined  
264 (see Discussion), and there was little correlation with bud position.

265

266

267 **Elongating organs are predicted to have softer walls and the apical cell to have**  
268 **stiffer cell walls**



269 In plants, new organs form in the peripheral zone after auxin maxima lead to  
270 wall softening (Braybrook and Peaucelle, 2013). The central zone, containing the  
271 meristematic stem cells, exhibits stiffer cell walls than the peripheral zone or new  
272 primordia (Milani et al., 2014). In most walled organisms, it is assumed that cell growth  
273 is limited by the cell wall and its mechanical properties, in turn linked to its biochemical  
274 composition (Peaucelle et al., 2013; Braybrook and Jönsson, 2016). Since *Sargassum*  
275 apices were so similar in pattern to the *Arabidopsis* apex, we checked whether they  
276 might follow similar mechanical rules. Alginate biochemistry was examined *in muro*  
277 using antibodies raised against different biochemical epitopes: the BAM10 antibody  
278 preferentially recognises guluronic acid within alginate (Torode et al., 2016), which  
279 should be mechanistically rigid through calcium cross-linking (Grant et al., 1973);  
280 conversely, the BAM6 antibody preferentially binds to mannuronic acid residues within  
281 alginate, which should be mechanically less rigid as they are unable to form calcium  
282 cross-links (Torode et al., 2016). Control immunolocalisations may be found in the  
283 Supplement (Fig. S3).

284 In longitudinal sections, BAM10 showed a wide distribution of signal across the  
285 apex (Fig. 3D). BAM6 signal was found at the junctions of cells and on the outer  
286 periclinal walls of the meristoderm cells (Fig. 3E). There was no obvious pattern of  
287 mannuronic/soft alginate associated with young buds, however signal did appear  
288 higher in slightly older elongating buds (BAM6; Fig. 3E; green arrowhead). Using these  
289 antibodies we were unable to determine if alginate softening could be predicted at the  
290 sites of new bud formation; however, it appears that elongating buds have more  
291 mannuronic/soft alginate than other areas.

292 Since the outer wall was thick, and the apical cell covered by a large plug of  
293 alginate mucilage (Kaur, 1999; Fig. 1D, dark stained area above the AC), we next  
294 looked at the apical cell alginate biochemistry using immunolocalisations on  
295 transverse sections. The BAM10 signal was equally distributed across the apical cell,  
296 promeristem cells and the surrounding cells (Fig. 3F) in the transverse section. The  
297 BAM6 signal was excluded from the apical cell and promeristem cells (Fig. 3G). BAM6  
298 signal was detected in more mature tissues at the junctions between cells (Fig. 3G).  
299 These data suggest that the apical cell, young promeristem cells and the peripheral  
300 area in the apex have more guluronic acid residues than mannuronic, which may lead  
301 to stiffer cell walls.

302

## 303 **Discussion**

### 304 **Phyllotaxis is a phenomena found in evolutionary distant photosynthetic** 305 **lineages**

306 Here we report that the spiral phyllotactic pattern in *S. muticum* follows the  
307 Golden Angle ( $\sim 137.5^\circ$ ) in a pattern almost identical to that found in many multicellular  
308 plants. Developmentally, this may not be so surprising: both organisms display  
309 indeterminate growth and produce new organs from an apical meristem-like region;  
310 both utilise their shoots and branches for light interception; and both utilise branches  
311 to produce numerous reproductive structures. However, it is interesting to see a similar  
312 pattern when the building blocks of multicellularity are completely independent: while  
313 both organisms have cell walls and cell adhesion, the polysaccharides which make up  
314 the wall matrix (pectin and alginate) are distinct. These organisms have evolved  
315 multicellularity independently (Baldauf, 2008) and therefore may have different  
316 communication strategies (morphogen identity): they may be seen as examples of  
317 D’Arcy Thompson’s bricklayers whose material is different but results in the same  
318 pattern. The data presented here indicate that complex parenchymatous brown algae  
319 have position-dependent patterning mechanism which results in spiral phyllotaxis,  
320 similar to that observed in plants.

### 321 322 **AC-based patterning does not underlie phyllotactic patterning in *S. muticum***

323  
324 The apical cell is the centre of the brown algal meristem; its sequential face  
325 divisions create a pool of cells which build the body of the adult alga. It has been  
326 hypothesised that the division pattern of the algal apical cell directly relates to  
327 phyllotactic patterning (Klemm and Hallam, 1987). In the spiral meristem of *S. muticum*  
328 this does not appear to be the case. Firstly, the difference in the divergence angles  
329 between the two patterns does not support a causative relationship – in apical cell  
330 divisions, the angle of the newly produced daughter cell to the previous is  $120^\circ$ ,  
331 whereas the observed phyllotactic angle centres on the golden angle of  $137.5^\circ$ . In the  
332 moss *Atrichum undulatum*, the triangular apical cell exhibits sequential face divisions  
333 but these occur at  $\sim 137.5^\circ$ , and angle which is reflected directly in the phyllotactic  
334 angle (Gola and Banasiak, 2016). In *Physcomitrella patens*, spiral apical cell divisions  
335 lead to spiral leaf arrangement (Harrison et al., 2009). As this correlation in pattern is

336 not seen in *S. muticum*, apical cell division pattern and phyllotaxis appear to be  
337 unrelated. While growth distortion post-apical-cell cannot be discounted, we believe it  
338 is unlikely given the highly organised nature of divisions seen in the apex. In fact, the  
339 *Sargassum* apex seems more closely aligned with that of the ferns, which also present  
340 a three-sided, sequentially-diving, apical cell and robust spiral phyllotaxis (Wardlaw,  
341 1949; Bierhorst, 1977).

342 A second piece of evidence comes from the observation that both patterns  
343 could follow either a left- or a right-handed rotation but the two could be disconnected:  
344 a counter-clockwise AC pattern could pair equally with a left- or right-handed  
345 phyllotactic spiral. Our experiments suggest that apical cell division pattern and  
346 phyllotactic pattern are not correlated. We hypothesise that an apical-cell-independent  
347 patterning mechanism exists in *Sargassum*, that position is more instructive than  
348 lineage.

349

### 350 **Apical robustness in *S. muticum***

351 When a stem-cell niche or apical cell are destroyed in plants, there are two  
352 outcomes reported in the literature: in ferns the apical growth can cease, or the apex  
353 will develop a new stem-cell centre and restart growth (Wardlaw, 1949; Cutter, 1965;  
354 Steeves and Sussex, 1989). More recently, in *Solanum lycopersicum cv esculentum*  
355 a new meristem centre was re-established after laser removal of the original one and  
356 the new organs formed in a spiral manner with a  $\sim 137.5^\circ$  divergence angle (Reinhardt  
357 et al., 2003). In brown algae, destruction of the apical cell in leads to the termination  
358 of apical growth (Moss, 1967; Clayton and Shankly, 1987); no re-establishment of  
359 patterning has been observed to our knowledge.

360 In our experiments, *S. muticum* apices exhibited both outcomes upon apical-cell  
361 destruction: 30% of the apices ceased growing, while another 50% showed continued  
362 growth after re-organisation. Our surviving apices fell into two categories: those where  
363 a single new apical cell was established or double-meristems where it is likely that two  
364 new apical cells were established. Similar to plants, our data showed that the  
365 phyllotactic pattern in the *Sargassum* meristem was also re-established (or  
366 maintained) upon apical cell destruction. These data indicate that the *S. muticum* apex  
367 is capable of re-organisation after apical cell destruction, in a similar way to that seen

368 in plants. This further supports a morphogen-based position-dependent patterning  
369 mechanism.

370 The spiral apices observed in *Sargassum* and *Cystoseira* represent the most  
371 complex apices found in the brown algal lineage. This complexity, and its similarity to  
372 those of plants, may represent a more robust system when it comes to development:  
373 spiral phyllotaxis may allow the algal body a more thorough exploration of space in  
374 comparison to the dichotomous thallus found in other Fucales. The ability to re-  
375 establish its apical cell and continue patterned growth could also hint at a more robust  
376 patterning mechanism in this alga, again as compared to other Fucales.

377

### 378 **Auxin is an unlikely candidate for the phyllotactic morphogen**

379 Hormones are one good candidate for morphogen-based positioning  
380 mechanisms in systems with cell walls and fixed position. Auxin's potential as a  
381 morphogen has been established in plants (Reinhardt, 2003) and it has been found to  
382 have an effect in the development of Bryophytes (Bennett et al., 2014; Coudert et al.,  
383 2015) and brown algae (Basu et al., 2002; Sun et al., 2004; Le Bail et al., 2010).  
384 Furthermore, it has been shown in plants that the localisation of auxin at the sites of  
385 future primordia changes the cell wall mechanical properties allowing outgrowth to  
386 take place (Peaucelle et al., 2011; Braybrook and Peaucelle, 2013). As such, auxin  
387 was a prime candidate for morphogen-like behaviour in the *Sargassum* apex.

388 We were unable to detect an effect of exogenous auxin on phyllotactic  
389 patterning in *S. muticum*. In our hands, using a relatively high (but effective in other  
390 brown algal cultures; Basu et al., 2002; Le Bail et al., 2010) concentration of auxin we  
391 were unable to disturb patterning. It is possible that the auxins used (NAA and 2,4-D)  
392 were not effective or that their concentrations require further optimisation. It is also  
393 possible that local applications might have been more effective: local applications  
394 could alter phyllotaxis in the plants *Lupinus* and *Epilobium* (Snow and Snow, 1937)  
395 but when more broadly applied in *Tropaeolum* a lesser effect was seen (Ball, 1944).

396 In order to gain a more spatial view of possible endogenous auxin, we then  
397 switched to IAA-immunolocalisations. Our data suggest that there is no particular  
398 localisation of auxin to newly growing buds observed in sections of *S. muticum* apices.  
399 The localisation seems to be spread over the whole section with a higher signal  
400 localized in the meristoderm cells and mucilage attached to the surface of the alga.

401 We cannot completely rule out a stickiness effect with the mucilage and non-specific  
402 antibody reactions; however, the no primary antibody controls were negative (Fig. S3)  
403 and the cell wall antibodies did not show such signals (Fig. 3C, D). There are also  
404 limitations in using an immunolocalisation approach to detect auxin since as it is small  
405 and highly dynamic and may be difficult to fix in place; however, using a specific pre-  
406 fixation step auxin could be fixed and detected using this approach in plants and brown  
407 algae (Avsian-Kretchmer et al. 2002, Le Bail et al. 2010). It is possible that IAA is not  
408 the functional auxin in *Sargassum*, although it has been detected by GCMS in the  
409 brown alga *Ectocarpus* (Le Bail et al., 2010). The data presented here suggest auxin  
410 is an unlikely morphogen for brown algal phyllotaxis, but without a tool such as the  
411 molecular reporter constructs in plants we cannot be certain.

412 The transport of auxin is key to establishing local maxima in plants, while in our  
413 algae we did not see evidence of strong maxima. There is no evidence to date that  
414 brown algae have homologues of the auxin transporters found in plants (Le Bail et al.,  
415 2010; Yue et al., 2014), although the algae may have completely different transport  
416 mechanisms. It remains unknown how and if auxins are being transported through the  
417 algae. It is always possible that in less complex organisms such as the filamentous  
418 *Ectocarpus*, mentioned above, auxin could move by diffusion. Classical phyllotactic  
419 patterning mechanisms have relied on reaction-diffusion equations in the past  
420 (Bernasconi, 1994; Swinton, 2004), and as such it is possible that auxin diffusion may  
421 be instructive in brown algae.

422 Another question that has been a topic of discussion is whether the auxin detected  
423 is being produced by the alga itself rather than being provided by auxin-producing  
424 associated bacteria on the surface of their thallus (Evans et al., 1991). Bacteria have  
425 been found to mineralize organic substrates giving the algae nutrients and growth  
426 factors (Matsuo et al., 2005). They have also been described living in association with  
427 brown algae (Hengst et al., 2010; Lachnit et al., 2011), as well as having an effect on  
428 their development (Tapia et al., 2016). The marine bacteria *Sulfitobacter* has recently  
429 been shown to produce IAA, which is then used by co-incident diatoms to regulate  
430 growth (Amin et al., 2015); diatoms are in a sister class to the Phaeophyceae (brown  
431 algae). It still remains uncertain how bacteria might be influencing brown algal  
432 development, especially when considering about the more complex parenchymous  
433 species such as those found in the Fucales. The recent interest in exploring and  
434 understanding the algal-bacterial interactions could lead to a better understanding if

435 and how the bacteria might be affecting the development in a more “advanced” brown  
436 algal species such as *Sargassum muticum*.

437

### 438 **Cell wall softening and algal bud outgrowth**

439 The cell walls of brown algae are composed mostly out of matrix polysaccharides  
440 called alginates and sulphated fucans and a small amount of cellulose (Deniaud-Bouët  
441 et al., 2014). It is believed that these are the functional analogues of pectin,  
442 hemicelluloses and cellulose in land plants, respectively. In plants, the role of the cell  
443 wall and its relationship to auxin in organ formation has been previously explored  
444 (Fleming et al., 1997; Reinhardt et al., 1998; Peaucelle et al., 2008, 2011; Braybrook  
445 and Peaucelle, 2013), but no information of such is available for the brown algae.  
446 Given the recent evidence for a role of pectin in organ emergence, and the  
447 predominant nature of alginate in the algal cell wall it seems plausible that alginate  
448 may be involved in bud formation.

449 Alginate is formed of two residues, mannuronic and guluronic acid, the latter being  
450 able to cross-link with  $\text{Ca}^{2+}$  ions (Grant et al., 1973), similarly to pectin in plants, and  
451 thus change its mechanical properties (Mancini et al., 1999). New alginate is added to  
452 the wall in the softer, mannuronic acid, form and may then be selectively epimerised  
453 into the guluronic acid form which can calcium cross-link (Grant et al., 1973). New  
454 techniques have been recently developed to look into the brown algal cell wall  
455 biochemistry by using immunolocalisations targeted towards specific epitopes in  
456 alginate chains (Torode et al., 2016). We have used two antibodies which bind to two  
457 different alginate epitopes, one rich in guluronic acid (BAM10) and the other rich in  
458 mannuronic acid residues (BAM6). Our data suggests that the guluronic acid rich  
459 areas are more abundant throughout the apex of *S. muticum*, but there is no clear  
460 distinction between growing and non-growing parts. The mannuronic acid (BAM6)  
461 signal tended to be higher in rapidly growing older buds but did not obviously mark  
462 young buds. These observations differ from the ones seen in *A. thaliana* meristems  
463 where pectin biochemical changes did mark new organ sites (Peaucelle et al., 2011).  
464 In slightly older, elongating buds, a stronger signal from BAM6 was detected,  
465 indicating a possible role for alginate biochemistry in elongation but not initiation of  
466 buds.

467 When looking into the apical cell and the cells around it in transverse sections, we  
468 observed that the guluronic acid signal (BAM10) was high whereas that for  
469 mannuronic acid (BAM6) was barely detected. Based on these data, we could suggest  
470 that the area around the apical cell and the apical cell itself have stiffer walls. This is  
471 similar to the situation observed in plants, where the stem cells have been shown to  
472 be stiffer than the surrounding peripheral cells (Milani et al., 2014). It may be  
473 interesting to explore whether this increased stiffness regulates cell division rates, in  
474 both plants and algae.

475 Taken together, it seems that the apical cell in *Sargassum* may behave  
476 mechanically similar to the stem-cell niche in spermatophyte plants and that softer  
477 alginate may be present in rapidly elongating new buds. However, it does not appear  
478 that new bud positions display biochemical markers of softer alginate and as such it is  
479 possible that alginate biochemistry is not related to bud positioning in *Sargassum*, only  
480 in outgrowth.

#### 481 **Possible mechanisms of phyllotaxis in *S. muticum***

482 In the *Sargassum* apex, a new leaf will develop its own apical cell, and further  
483 cells along the meristoderm between this and the primary apical cell will follow suit,  
484 each giving rise to another organ on each branch (Oltmann 1889 via Critchley 1983).  
485 All meristodermal cells have a meristematic ability which could indicate that any cell  
486 from this cell layer could “switch on” and become an apical cell and start producing its  
487 own bud (Moss, 1967). This is not dissimilar to the specification seen in ferns for the  
488 production of new leaves from the epidermis (Bierhorst, 1977; Mueller, 1982). Based  
489 on the robust, self-organising, nature of the *Sargassum* apex and the lack of  
490 correlation between the apical cell division pattern and that of new buds, it seems likely  
491 that a positional mechanism is in place for phyllotactic patterning in *Sargassum*. It is  
492 then plausible that an unknown morphogen instructs the conversion of a  
493 meristodermatic cell into a new apical one, in a positioned manner. The secondary  
494 specification of further apical cells between the leaf and main apical cell also hints at  
495 a position-dependent specification of meristodermal conversion into apical cells.

496 It has been observed that the apical cell can divide to potentially create a  
497 daughter apical cell which then continues to create a new branch (Kaur, 1999). In our  
498 experiments, we never observed an equal division of the apical cell which could  
499 explain the previously described situation. This is similar to the case in *Cystophora*

500 where division to produce a second apical cell was rarely observed (Klemm and  
501 Hallam, 1987). If we assume this hypothesis might be true and that we simply missed  
502 such special divisions, based on the observed patterns of longitudinal division it seems  
503 unlikely that this could produce a golden-angled spiral and would more likely produced  
504 a 120° spiral.

505 The absence of cell wall biochemical marks associated with alginate softening  
506 (mannuronic vs. guluronic acid) correlated to new bud positions indicates that the  
507 physical events of initial bud outgrowth may be different than that in plants. This does  
508 not rule out a physically-based positioning system for the brown algal apex; physical  
509 buckling may give rise to phyllotactic patterns. While the mechanical properties of the  
510 meristoderm remain homogeneous, if the underlying cortex tissue is growing at a  
511 differential rate to the meristoderm physical buckling may result through compression  
512 of the outer tissue (reviewed in Dumais, 2007). This possibility is worthy of further  
513 investigation.

#### 514 **Future directions**

515 The similarity between the *Sargassum* apex and that of complex multicellular plant  
516 meristems is striking: the presence of a golden-angled phyllotactic spiral; the robust  
517 reorganisation after meristem ablation; the presence of equal clockwise and counter-  
518 clockwise patterns; the apparent independence of phyllotactic patterning to meristem  
519 divisions. However, there are many obvious differences as well: we do not currently  
520 have strong evidence for auxin as a patterning morphogen; we also cannot detect  
521 softening of the algal cell walls coincident with new bud outgrowth.

522 While the experiments presented here make a case for the *Sargassum* apex as  
523 being more plant-like in its patterning and organisation principals, we have many new  
524 questions. Is there a re-specification of a meristodermal cell into a new apical cell, and  
525 how is this regulated? If auxin is an unlikely morphogen, what might be the identity of  
526 the algal functional analogue (if it exists at all)? If auxin can in fact be instructive, is it  
527 produced by the algae or by associated bacteria (implying a more communal evolution  
528 of patterning in the brown algae)?

529 The answers to many of these questions undoubtedly require advances in  
530 molecular techniques and genetics within the brown algae. These techniques are  
531 beginning to be developed in *Ectocarpus* and hopefully can be translated into other



532 interesting algae (Le Bail et al., 2011). Another hurdle is the inability to culture many  
533 brown algae for their full life cycle *in vitro*, thus limiting when questions might be asked  
534 (seasonally). Currently methods exist for *Ectocarpus* and *Dictyota* (Le Bail et al., 2011;  
535 Bogaert et al., 2016). In addition, it remains unclear how axenic growth conditions can  
536 become in this cultures while still supporting growth and development.

## 537 **Materials and Methods**

### 538 **Sample collection and *in vitro* culture**

539 The samples were collected in Rottingdean (East Sussex, United Kingdom) between  
540 November 2015 and February 2017. After collection, they were transported in  
541 seawater to the laboratory and kept at 4°C. Processing was done shortly after  
542 returning back to the laboratory – the specimens were dissected using fine tweezers  
543 and used for further experiments. The medium used in the experiments was filter  
544 sterilised artificial seawater (ASW, Tropic Marin Sea Salt; Tropic Marin, Germany).

545

### 546 **Imaging of the apices for divergence angle measurements**

547 *Sargassum* apices were dissected using fine tweezers by removing all the leaves from  
548 their base, until the central region of the apex was clearly visible. The apices were  
549 then cut to a 1 cm length and anchored by insertion into Petri dishes containing 1%  
550 agarose melted in ASW and flooded with ASW to cover. Images were taken using a  
551 VHX 5000 microscope (Keyence (UK) Ltd, UK). Measurements for the divergence  
552 angle were done using the VHX 5000 Keyence software; centres of each organ were  
553 used in reference to the centre of the apex.

554

### 555 **Histology**

556 The apices were fixed in a fixative containing 2.5% glutaraldehyde and 2%  
557 formaldehyde in artificial seawater. They were then dehydrated through 10% ethanol  
558 steps and embedded in resin (LR White resin, Agar Scientific Ltd, UK). Samples were  
559 then cut in 1 µm slices using a Leica EM UC7 ultramicrotome (Leica Microsystems,  
560 Germany). Sections were placed onto Superfrost Ultra Plus slides (Thermo Scientific,  
561 USA) and left to dry at room temperature. Sections were then stained with 0.05%  
562 Toluidine blue O solution for 5 minutes, washed, covered with a cover slip and imaged  
563 under the Zeiss Axio Imager M2 (Zeiss, Germany).

564

565 **Apex ablation**

566 *Sargassum* apices were dissected and handled as described above. They were then  
567 precisely stabbed using a fine needle in the middle of the meristem, where the apical  
568 cell is located. The images of the stabbed meristems were taken using a VHX 5000  
569 microscope (Keyence (UK) Ltd, UK). The samples were kept in culture under 16°C,  
570 12:12 hour day night cycle, 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After 20 days, they were dissected again  
571 to remove the newly grown leaves and imaged again. They were fixed, dehydrated,  
572 embedded in resin as previously described. The embedded apices were then cut into  
573 serial 1  $\mu\text{m}$  sections (every 5  $\mu\text{m}$  throughout the meristem), stained with TBO as above  
574 and imaged under Zeiss Axio Imager M2 (Zeiss, Germany).

575

576 **Alginate immunolocalisation**

577 The apices were fixed, dehydrated and embedded in resin as described above. The  
578 sections were placed on Vectabond coated multitest 8-well slides, 2 sections per well  
579 (Vector Laboratories, USA). They were then incubated in a blocking solution of 5%  
580 milk for 2 hours. The sections were rinsed with phosphate buffered saline (PBS; 2.7  
581 mM KCl, 6.1 mM  $\text{Na}_2\text{HPO}_4$ , and 3.5 mM  $\text{KH}_2\text{PO}_4$ ) and incubated in the 60  $\mu\text{l}$  of 1/5 (in  
582 5% milk) monoclonal primary antibody for 1.5 hours. After the incubation, the slides  
583 were washed with PBS 3 times for 5 minutes each, followed by the incubation in the  
584 60  $\mu\text{l}$  of 1/100 (in 5% milk) IgG-FITC secondary antibody (F1763, Sigma-Aldrich). The  
585 sections were then washed 5 times for 5 minutes in PBS, mounted in Citifluor (Agar  
586 Scientific, UK), covered with a coverslip, sealed and imaged under a Leica SP8  
587 confocal microscope (Leica Microsystems, Germany). The antibodies used were gifts  
588 from Prof. Paul Knox (University of Leeds).

589

590 **Auxin immunolocalisation**

591 The protocol was adapted from Le Bail et al. (2010). The *Sargassum* and *Arabidopsis*  
592 apices were dissected and prefixed in 3% of 1-ethyl-3-(3-dimethylaminopropyl)-  
593 carbodiimide (EDAC, Sigma-Aldrich, USA) followed by an overnight fixation in FAA  
594 ((47.5% ethanol, 5% acetic acid, and 10% formaldehyde in ASW). Samples were then  
595 dehydrated and embedded in resin as described above. 1  $\mu\text{m}$  sections were cut using  
596 the Leica ultramicrotome and placed on Vectabond coated slides. The slides were  
597 placed into PBS for 5 minutes and then incubated in a blocking solution (0.1% [v/v]  
598 Tween 20, 1.5% [w/v] Glycine, and 5% [w/v] bovine serum albumin (BSA) in dH<sub>2</sub>O) for

599 45 minutes. The sections were rinsed in a salt rinse solution for 5 minutes, a quick  
600 wash with 0.8% (w/v) BSA in PBS and incubated in 60  $\mu$ l of 1:100 monoclonal anti-  
601 IAA antibody (Sigma Aldrich, USA) overnight at 4°C. The slides were vigorously  
602 washed three times for 10 minutes with a high salt rinse solution (2.9% [w/v] NaCl,  
603 0.1% [v/v] Tween 20, and 0.1% [w/v] BSA in dH<sub>2</sub>O) and then washed for an additional  
604 10 minutes in a salt rinse solution and a rinse with 0.8% (w/v) BSA and then in PBS.  
605 60  $\mu$ l of 1:100 (v/v) dilution of the 1mgmL<sup>-1</sup> goat anti-mouse IgG antibody Alexa Fluor®  
606 488 (Invitrogen, USA) was added to each well and incubated for 4 h at room  
607 temperature. The slides were washed 5 times for 10 minutes in the salt rinse solution  
608 followed by a brief wash in PBS, mounted in Citifluor (Agar Scientific, UK), covered,  
609 sealed and imaged under a Leica SP8 confocal microscope (Leica Microsystems,  
610 Germany).

611

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617

## 618 **Competing Interests**

619 The authors have no competing interests to declare.

620

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625

## 626 **Data availability**

627 All raw data and images are available from the corresponding author upon request.

628

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796

## 797 **Figure Legends**

798 **Figure 1. The *Sargassum muticum* apex displays distinct patterns which are**  
799 **independent of each other.** (A) The morphology of an adult *S. muticum* alga. (B)  
800 Newly forming buds numbered by increasing age (P1 -> P10) with a representative  
801 divergence angle illustrated between the two consecutive buds. (C) Divergence angles  
802 distribution of measured apices (mean=137.53 ± 2.08°; n=260). (D) Division pattern  
803 in a longitudinal section of a *Sargassum* apex; AC divides to give rise to three tissues  
804 (meristoderm, cortex, medulla). (E) Apical cell division pattern in a transverse section  
805 of a *Sargassum* apex; first periclinal apical cell division (red; yellow star) followed by  
806 radial (orange, yellow; white arrowhead) and circumferential (blue; black arrowhead)  
807 anticlinal divisions. Schematic representation of the division in the longitudinal  
808 direction (F) and the transverse direction (G). (H, I) Clockwise phyllotaxis with a (L)  
809 clockwise or (M) counter-clockwise apical cell division orientation. (J, K) Counter-  
810 clockwise phyllotaxis with a (N) clockwise or (O) counter-clockwise apical cell division  
811 orientation (n=27). \*youngest daughter cell, \*\*next-to-youngest daughter cell. Scale  
812 bars 1 mm (A), 100 µm (B, H, I, J, K), 20 µm (D, E, L, M, N, O).

813

814 **Figure 2. Removal of the apical cell can induce formation of a new central**  
815 **meristem.** *Sargassum* meristem stabbed (white arrowhead) in the region of the apical  
816 cell in top view (A) and in subsequent longitudinal section (B). (C) Formation of a new  
817 central meristem; white arrowhead marks the spot of the stab, blue arrowhead shows  
818 the location of the new meristem. (D) Longitudinal section of a newly formed meristem  
819 (as in C) showing a new apical cell in the new meristem centre (blue arrowhead) and  
820 stabbed area (white arrowhead). Inset: magnified view of the new apical cell. (E) Apex  
821 presenting a split meristem; dashed line shows the separation of the two new

822 meristems, centers indicated by blue arrowheads. (F) Control apex, not stabbed.  
823 Sample numbers: n=23 stabbed (12 recovered, 7 ceased growth, 4 unclassified).  
824 Scale bar 100  $\mu\text{m}$ , 50  $\mu\text{m}$  (B, D), 20  $\mu\text{m}$  (apical cell inset in D).

825

826 **Figure 3. IAA and mannuronate immunolocalisation signals do not appear to**  
827 **correlate with new bud formation in the *S. muticum* apex.** (A) Anti-IAA antibody  
828 localising to the buds, surface of the meristoderm and the apical pit (white arrowhead).  
829 Higher magnification view of the bud (B) and of the meristoderm cells (C). Dashed line  
830 in (C) delineates the meristoderm outer cell wall. (D) BAM10 antibody binds to the  
831 guluronic acid rich areas in the cell wall with a homogeneous distribution throughout  
832 the apex. (E) BAM6 antibody binds to mannuronic acid rich areas in the cell wall,  
833 localised at the surface and on cell-cell junctions in the inner tissues and with a slightly  
834 higher abundance in a young bud (green arrowhead). (F) BAM10 antibody signal is  
835 distributed throughout the apical cell and promeristem region in transverse sections.  
836 (G) BAM6 antibody signal is not detected in the apical cell and immediate neighbors.  
837 BAM6 localises mainly in the cell junctions around the apical cell. White arrowheads  
838 mark the pit-area (with an apical cell). ac= apical cell location. Scale bar 20  $\mu\text{m}$  (A, B,  
839 C, F, G), 50  $\mu\text{m}$  (D, E).

840

841 **Supplementary Figure 1.** The age of the stipe and the meristem area. Scatter plot  
842 showing a lack of correlation between the length (proxy for age) of an individual stipe  
843 with the diameter of its pit-area (proxy for meristem size; apical cell and the  
844 promeristem cells around it) (n=22, p-value=0.07, r=-0.39; two-sample t-test).

845

846 **Supplementary Figure 2.** Types of tissues found in *S. muticum* as illustrated on a  
847 stipe section. Outer layer (meristoderm), middle layer (cortex) and inner layer  
848 (medulla). Scale bar 150  $\mu\text{m}$ , 1 mm (whole algal body).

849

850 **Supplementary Figure 3.** Control confocal images for the auxin and alginate  
851 immunolocalisations. *Arabidopsis thaliana* longitudinal section with no primary  
852 antibody control (A) and anti-IAA (B). (C) No primary antibody controls of *S. muticum*  
853 apex sections for alginate immunolocalisation (C) longitudinal section and (D)  
854 transverse. All controls merged with a brightfield image for visualisation. Scale bar 20  
855  $\mu\text{m}$ .

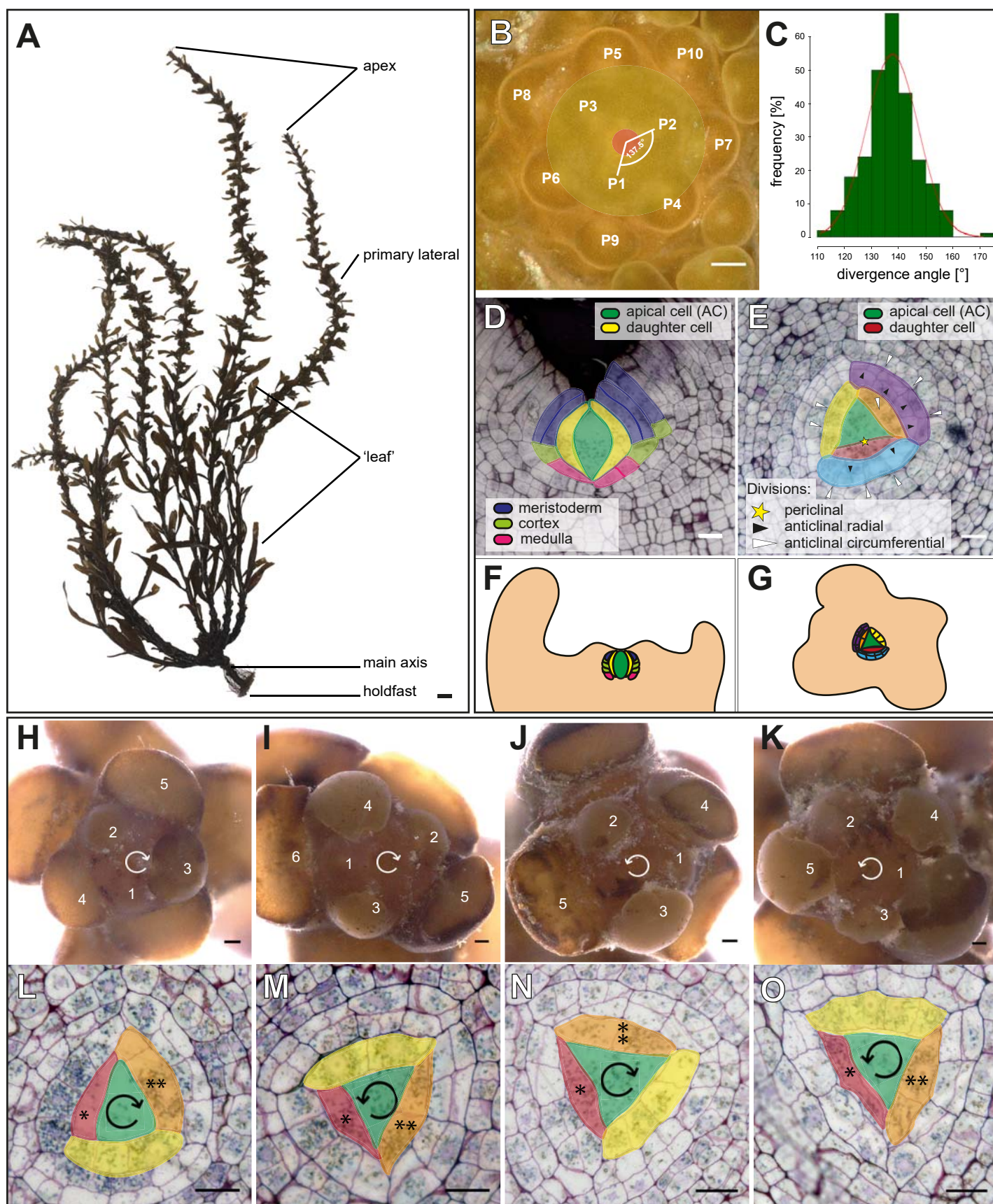


Figure 1.

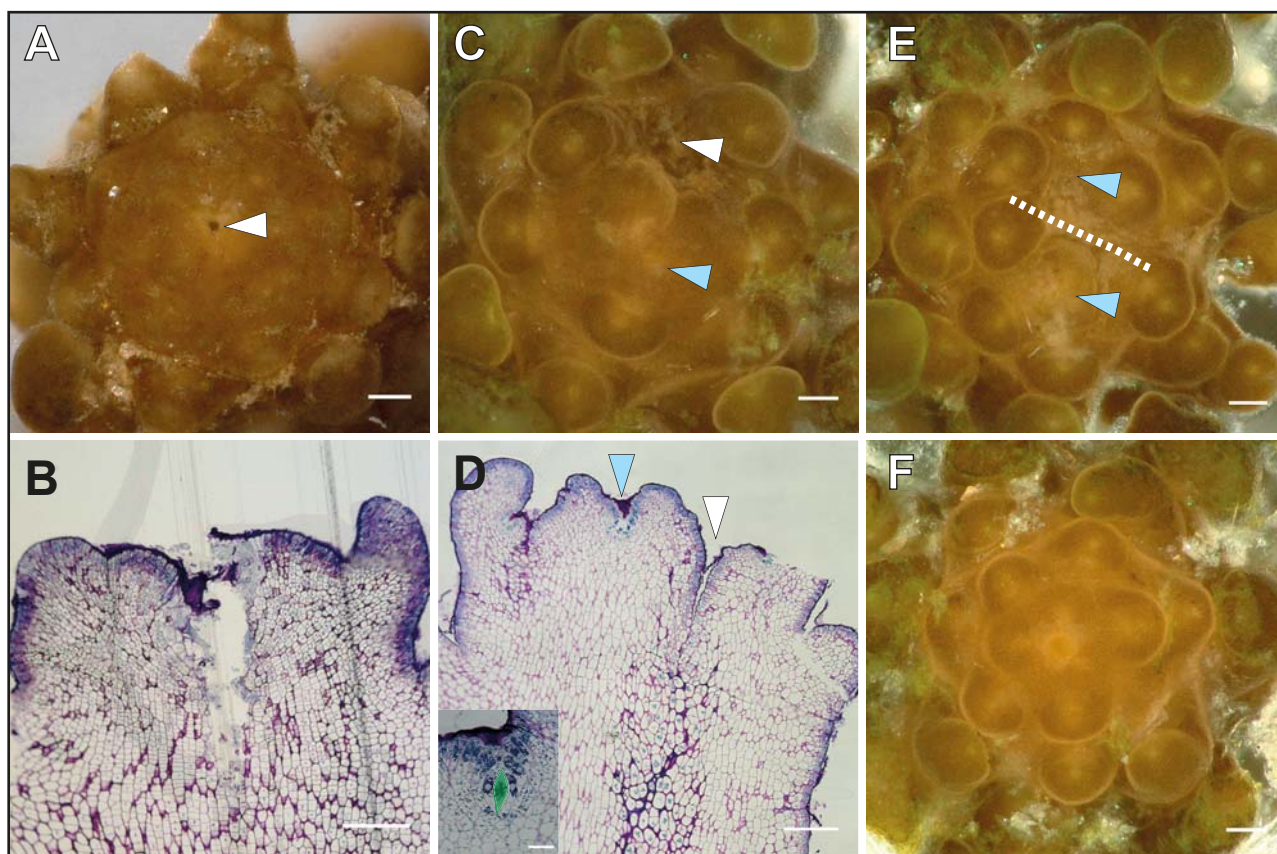


Figure 2.

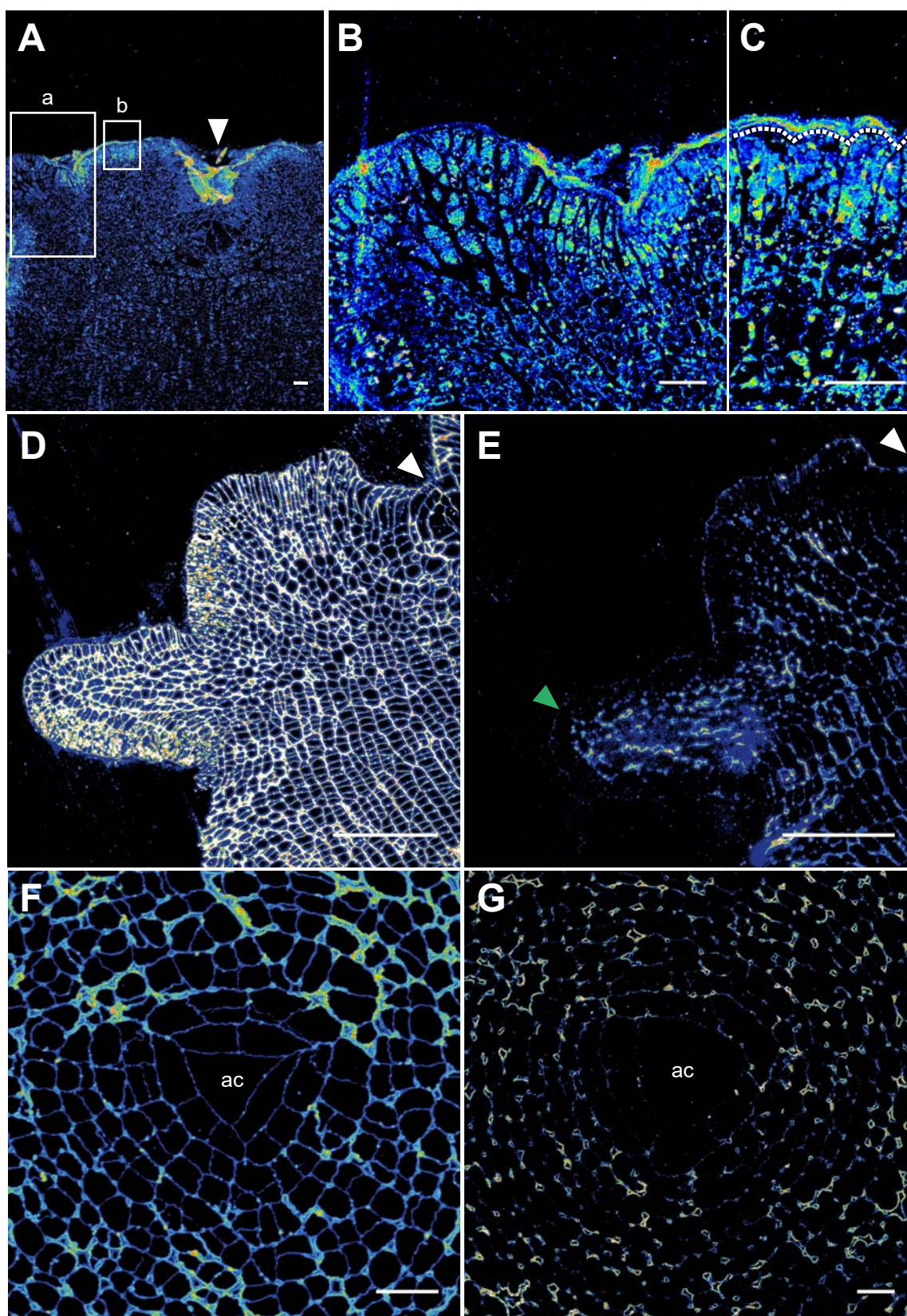
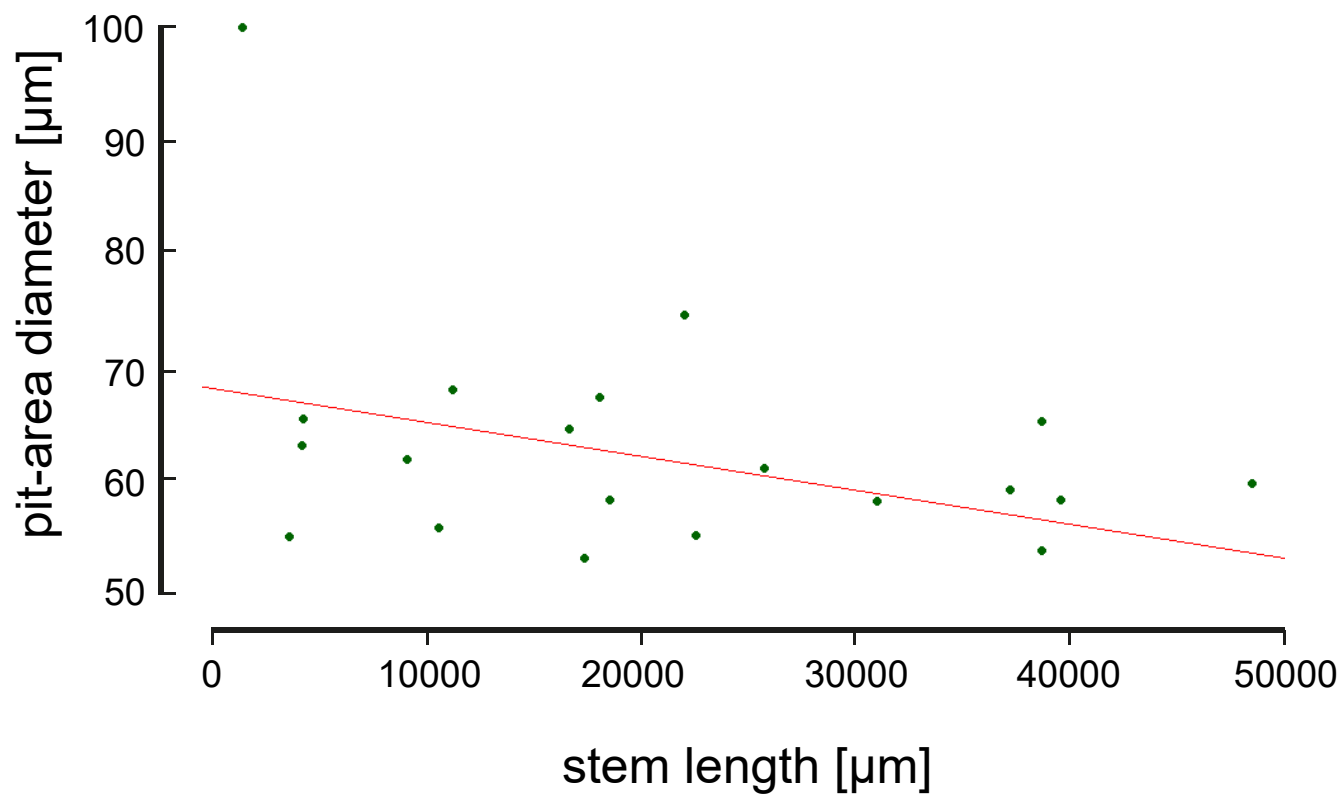
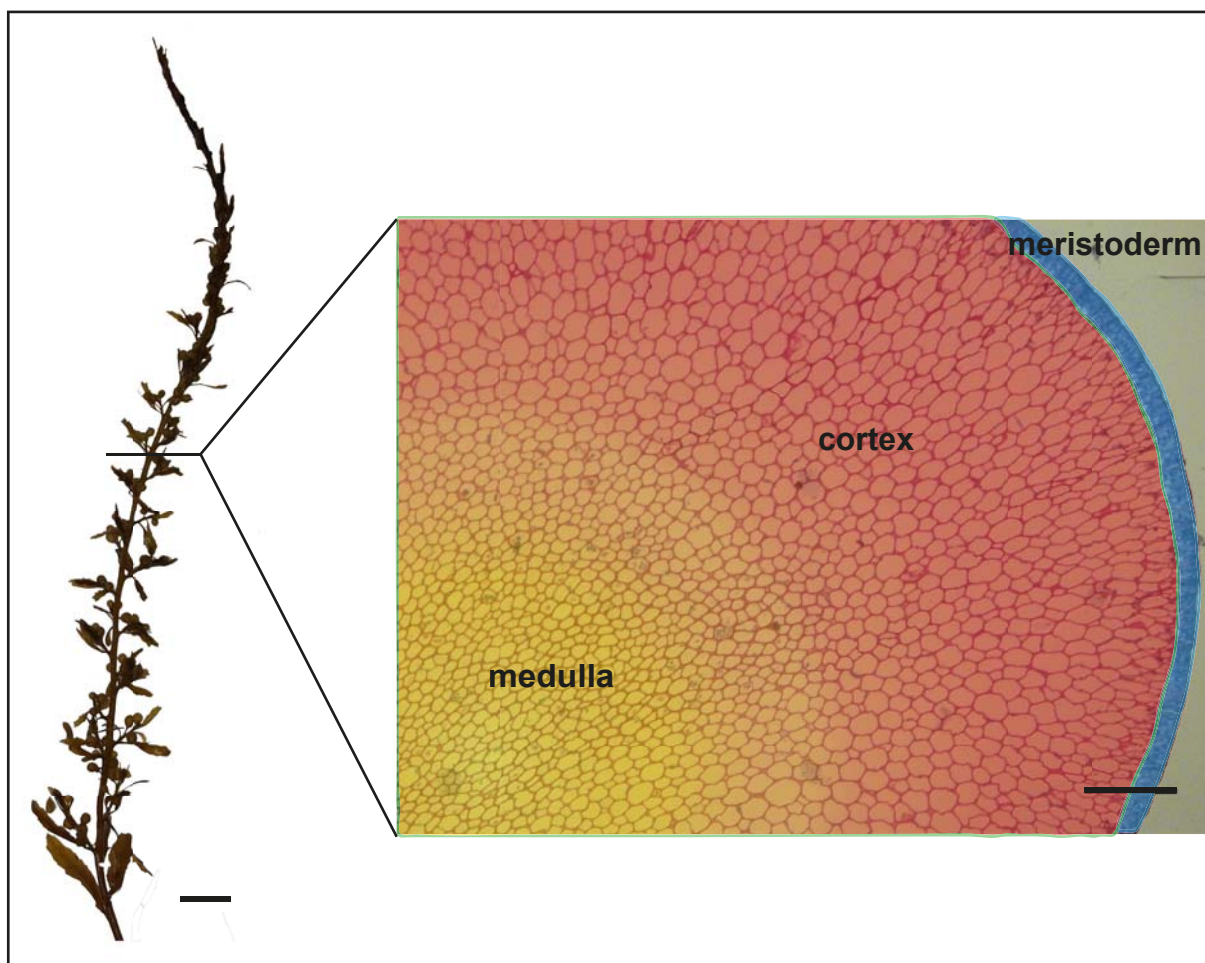


Figure 3.

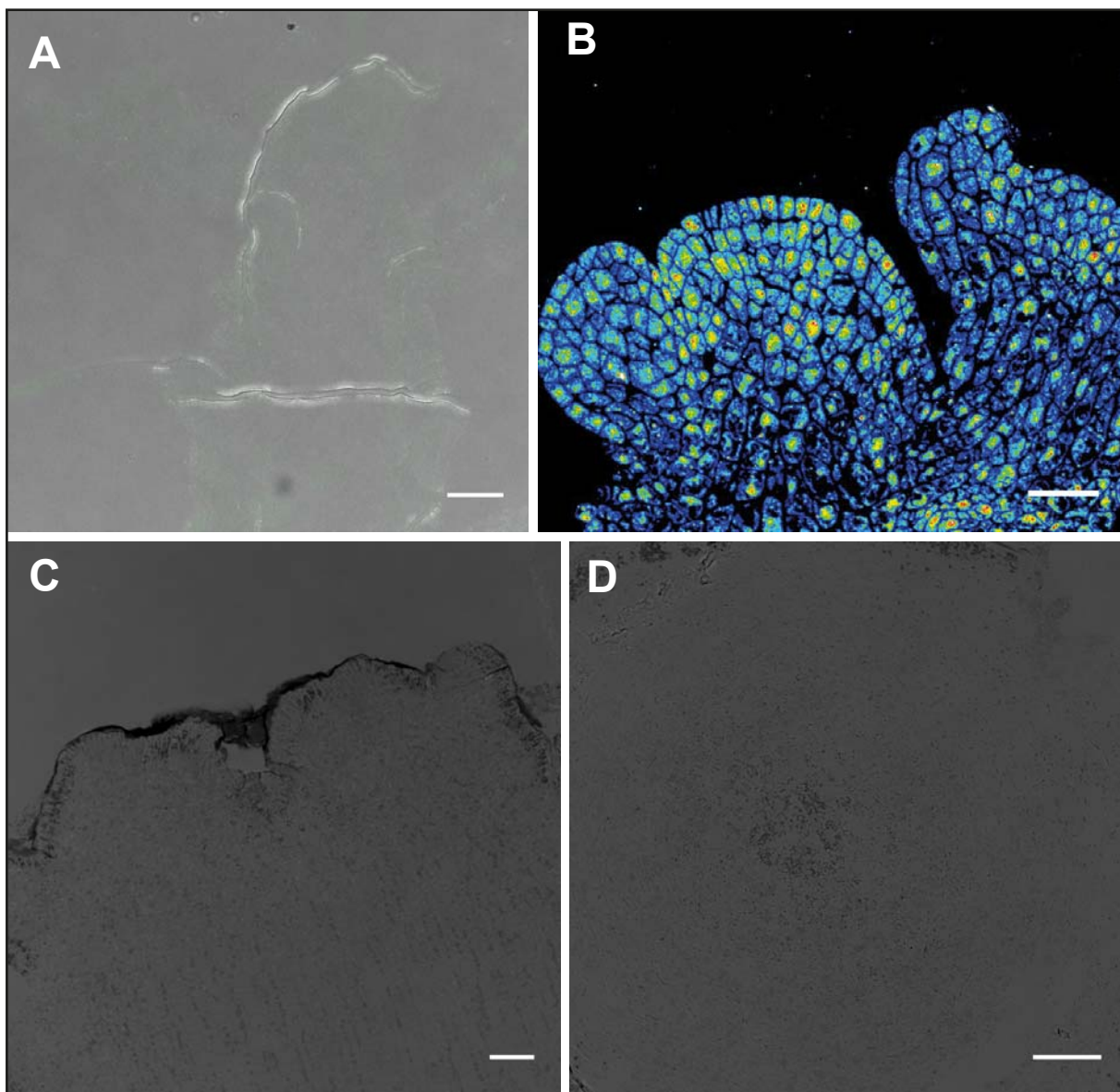


Supplementary Figure 1.



Supplementary Figure 2.





Supplementary Figure 3.