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

Introduction

34

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 shot 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 ± 2.08° (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° 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 6th or 7th '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 (α=119.01 ± 6.11°, 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 we
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

A potential link between auxin and brown algal phyllotaxis is unlikely

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

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

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

Elongating organs are predicted to have softer walls and the apical cell to have 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

Discussion

Phyllotaxis is a phenomena found in evolutionary distant photosynthetic lineages

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

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

The apical cell is the centre of the brown algal meristem; its sequential face divisions create a pool of cells which build the body of the adult alga. It has been hypothesised that the division pattern of the algal apical cell directly relates to phyllotactic patterning (Klemm and Hallam, 1987). In the spiral meristem of *S. muticum* this does not appear to be the case. Firstly, the difference in the divergence angles between the two patterns does not support a causative relationship – in apical cell divisions, the angle of the newly produced daughter cell to the previous is 120°, whereas the observed phyllotactic angle centres on the golden angle of 137.5°. In the moss *Atrichum undulatum*, the triangular apical cell exhibits sequential face divisions but these occur at ~137.5°, and angle which is reflected directly in the phyllotactic angle (Gola and Banasiak, 2016). In *Physcomitrella patens*, spiral apical cell divisions 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 ~137.5° 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-immunlocalisations. 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 aslso
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 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 meistoderm physical buckling may result through compression
512 of the outer tissue (reviewed in Dumais, 2007). This possibility is worthy of further
513 investigation.

Future directions

514

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.

Materials and Methods

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 4^0C. 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

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

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

Apex ablation

Sargassum apices were dissected and handled as described above. They were then precisely stabbed using a fine needle in the middle of the meristem, where the apical cell is located. The images of the stabbed meristems were taken using a VHX 5000 microscope (Keyence (UK) Ltd, UK). The samples were kept in culture under 16^{0}C, 12:12 hour day night cycle, 60 μmol m^{-2} s^{-1}. After 20 days, they were dissected again to remove the newly grown leaves and imaged again. They were fixed, dehydrated, embedded in resin as previously described. The embedded apices were then cut into serial 1 μm sections (every 5 μm throughout the meristem), stained with TBO as above and imaged under Zeiss Axio Imager M2 (Zeiss, Germany).

Alginate immunolocalisation

The apices were fixed, dehydrated and embedded in resin as described above. The sections were placed on Vectabond coated multitest 8-well slides, 2 sections per well (Vector Laboratories, USA). They were then incubated in a blocking solution of 5% milk for 2 hours. The sections were rinsed with phosphate buffered saline (PBS; 2.7 mM KCl, 6.1 mM Na_2HPO_4, and 3.5 mM KH_2PO_4) and incubated in the 60 μl of 1/5 (in 5% milk) monoclonal primary antibody for 1.5 hours. After the incubation, the slides were washed with PBS 3 times for 5 minutes each, followed by the incubation in the 60 μl of 1/100 (in 5% milk) IgG-FITC secondary antibody (F1763, Sigma-Aldrich). The sections were then washed 5 times for 5 minutes in PBS, mounted in Citifluor (Agar Scientific, UK), covered with a coverslip, sealed and imaged under a Leica SP8 confocal microscope (Leica Microsystems, Germany). The antibodies used were gifts from Prof. Paul Knox (University of Leeds).

Auxin immunolocalisation

The protocol was adapted from Le Bail et al. (2010). The *Sargassum* and *Arabidopsis* apices were dissected and prefixed in 3% of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC, Sigma-Aldrich, USA) followed by an overnight fixation in FAA ((47.5% ethanol, 5%acetic acid, and 10% formaldehyde in ASW). Samples were then dehydrated and embedded in resin as described above. 1μm sections were cut using the Leica ultramicrotome and placed on Vectabond coated slides. The slides were placed into PBS for 5 minutes and then incubated in a blocking solution (0.1% [v/v] Tween 20, 1.5% [w/v] Glycine, and 5% [w/v] bovine serum albumin (BSA) in dH_2O) 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 µl 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_2O) 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 µl of 1:100 (v/v) dilution of the $1mgmL^{-1}$ 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 was 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

Competing Interests

619   The authors have no competing interests to declare.

620

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625

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 µm, 50 µm (B, D), 20 µm (apical cell inset in D).

825

826 **Figure 3. IAA and mannuronate immunolcalisation 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 µm (A, B,

839 C, F, G), 50 µm (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 µm, 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
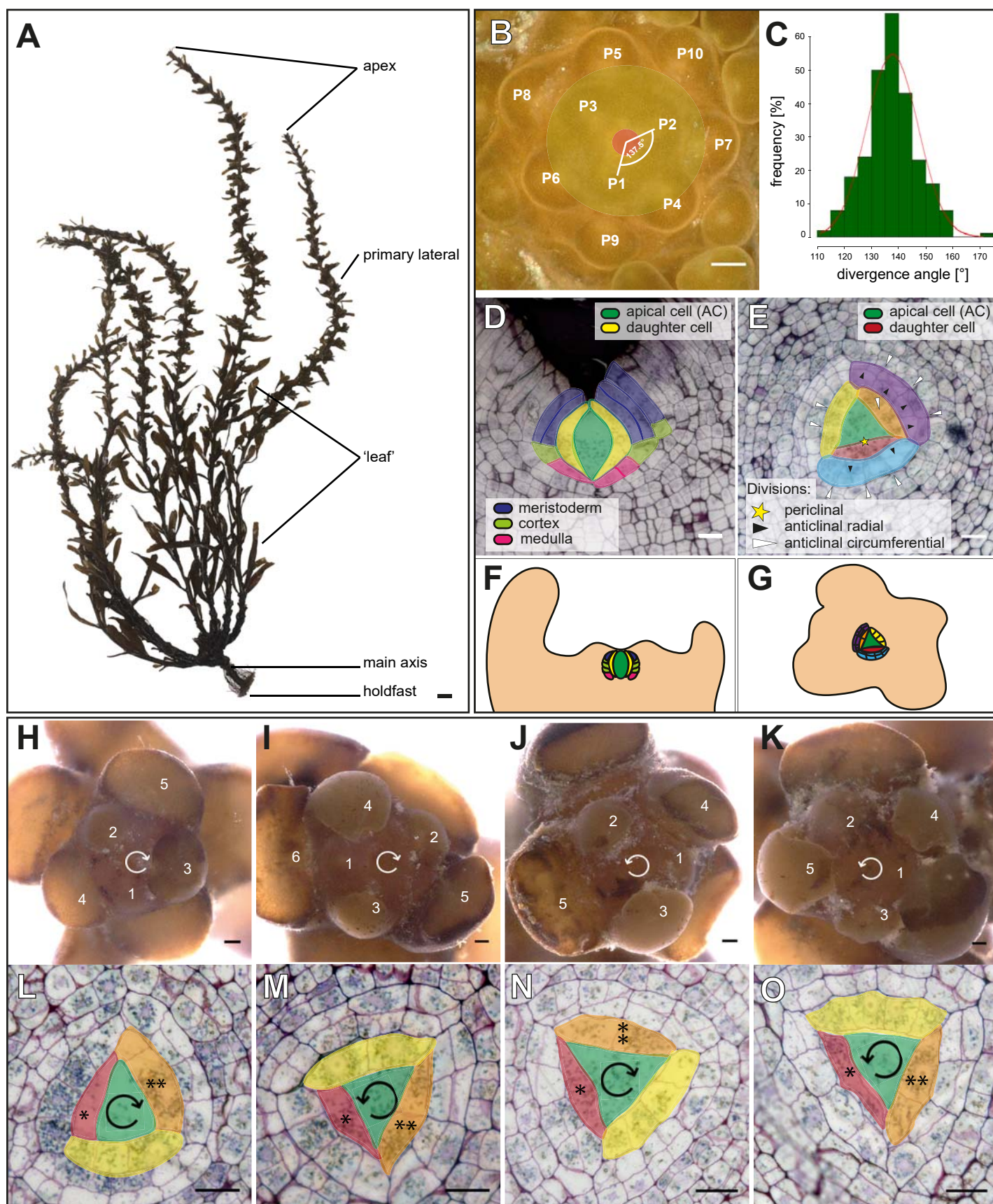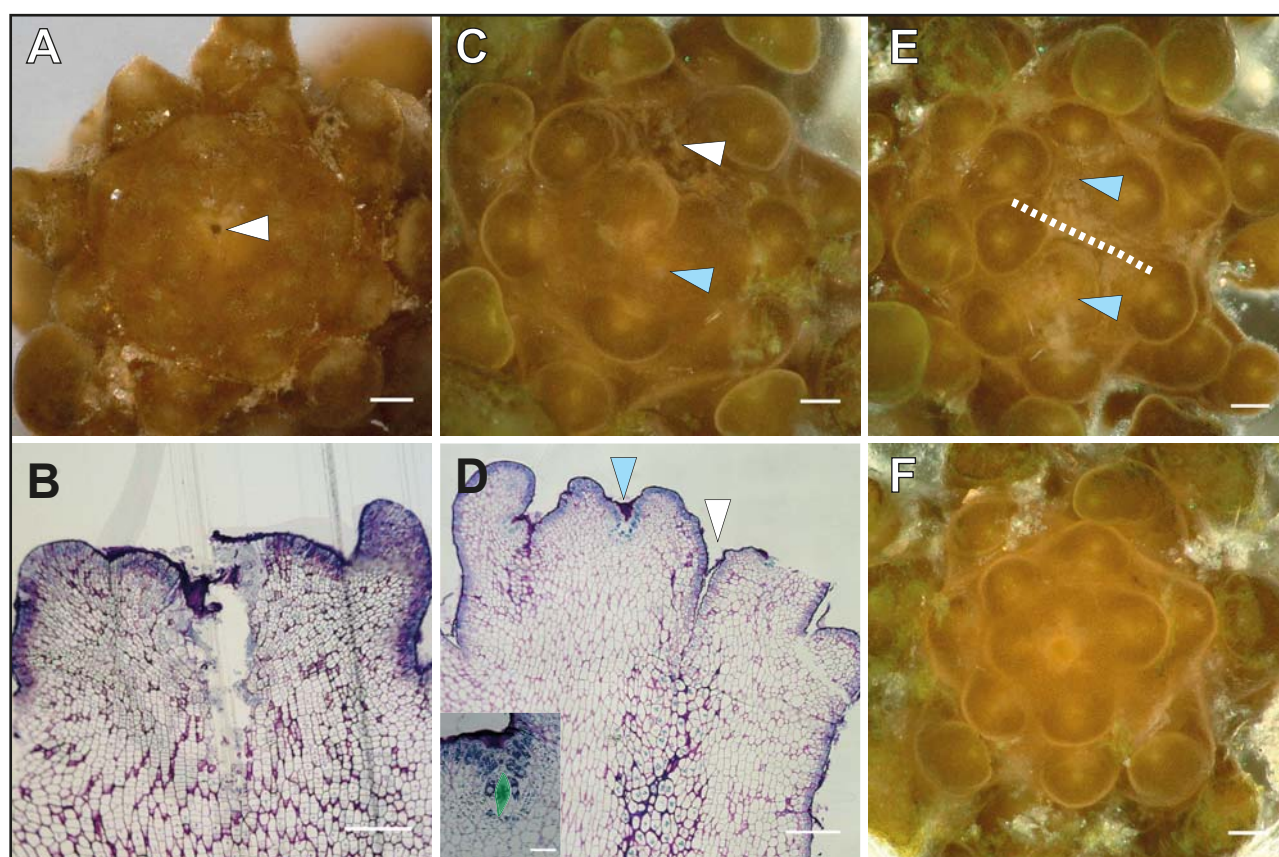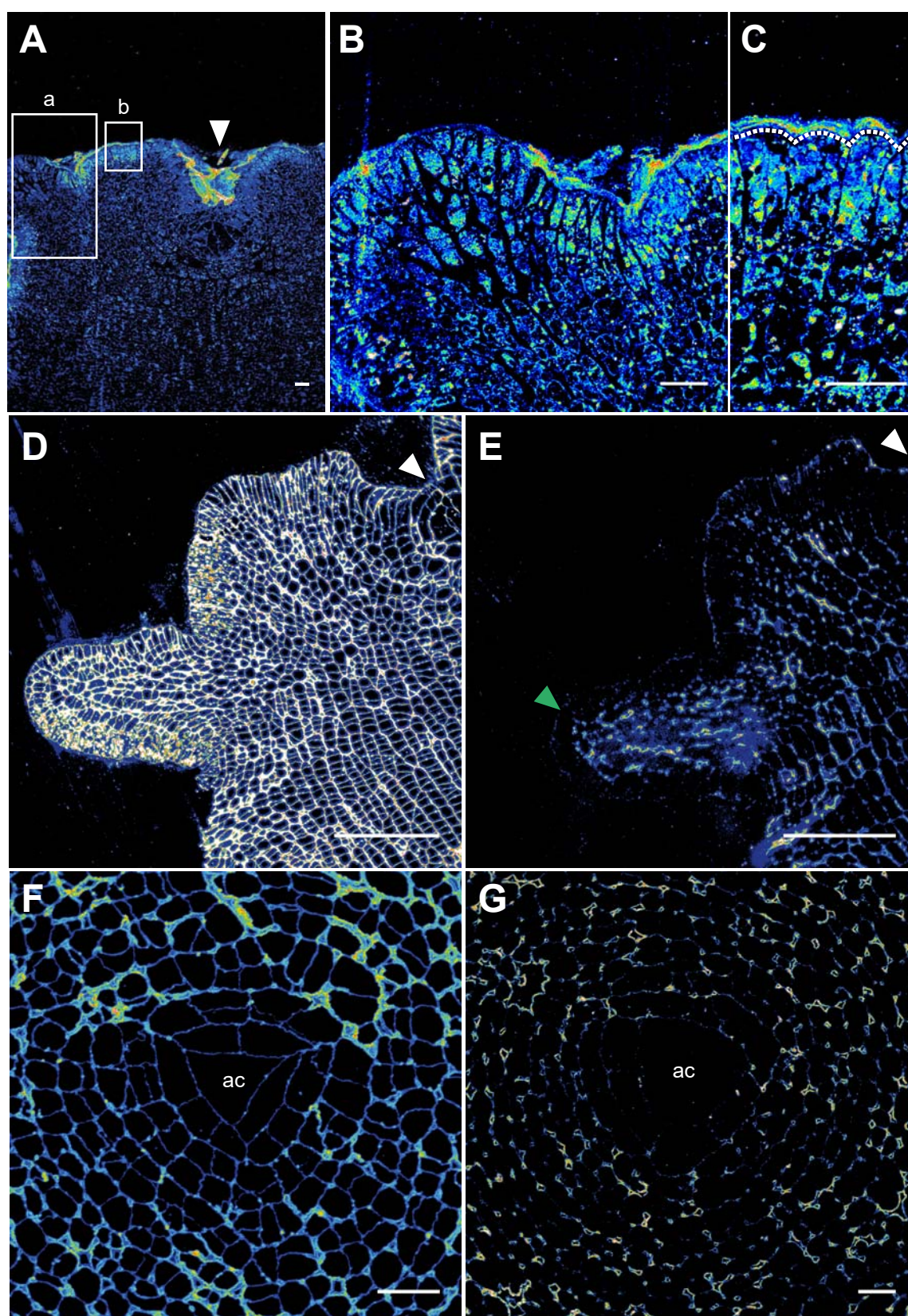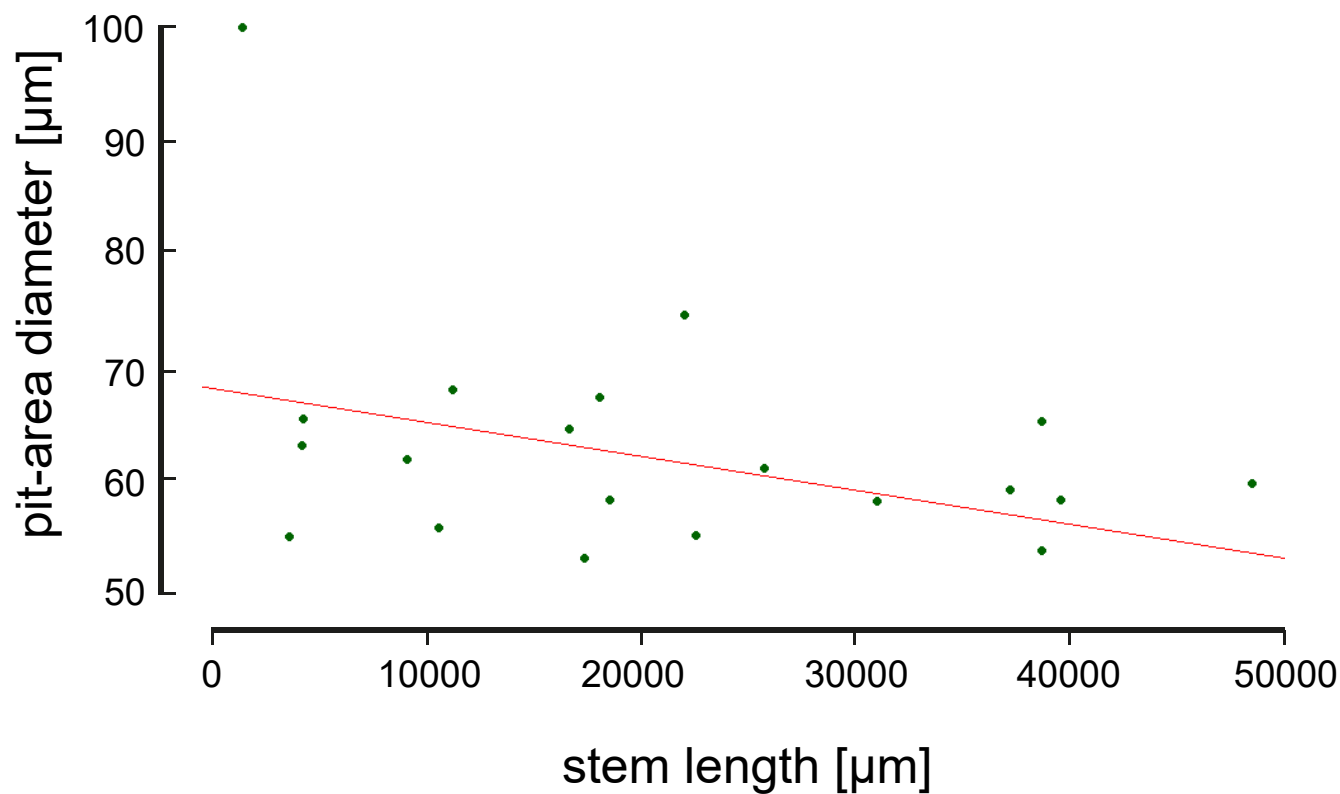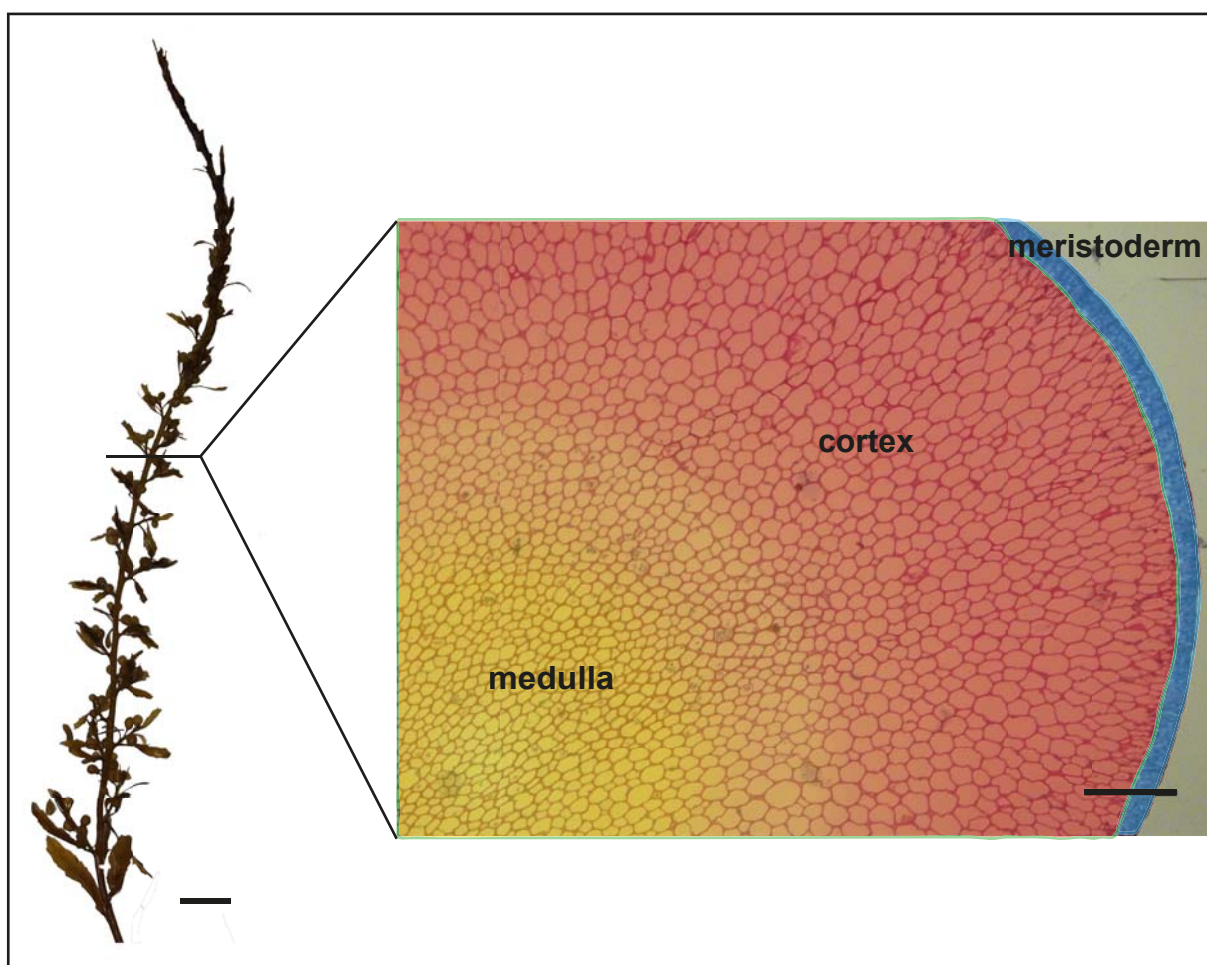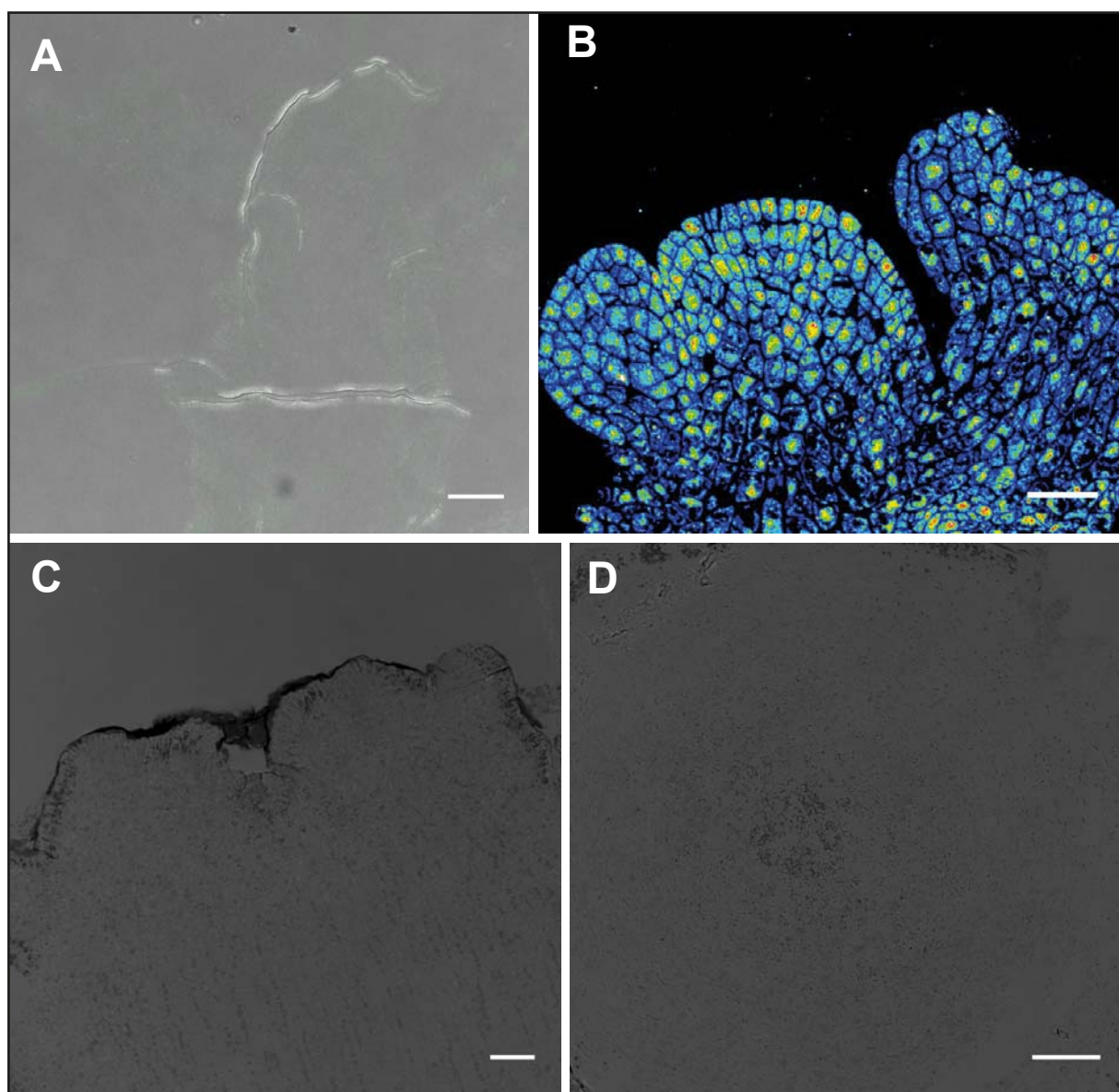
855 µm.

Figure 1.

Figure 2.

Figure 3.

Supplementary Figure 1.

Supplementary Figure 2.

Supplementary Figure 3.