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► **To cite this version:**

Garry Farnham, Martina Strittmatter, Susana Coelho, J. Mark Cock, Colin Brownlee.
GENE SILENCING IN FUCUS EMBRYOS: DEVELOPMENTAL CONSEQUENCES OF RNAi-
MEDIATED CYTOSKELETAL DISRUPTION. *Journal of Phycology*, 2013, 49 (5), pp.819-829.
10.1111/jpy.12096 . hal-01806438

HAL Id: hal-01806438

<https://hal.science/hal-01806438>

Submitted on 6 Jun 2018

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**GENE SILENCING IN FUCUS EMBRYOS: DEVELOPMENTAL
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Journal:	<i>Journal of Phycology</i>
Manuscript ID:	JPY-12-279-ART.R1
Manuscript Type:	Regular Article
Date Submitted by the Author:	11-May-2013
Complete List of Authors:	Brownlee, Colin; Marine Biological Association, ; Farnham, Garry; Marine Biological Association, Strittmatter, Martina; UPMC University Paris 06, Coelho, Susana ; CNRS, UMR 7139, Station Biologique de Roscoff, Cock, J. Mark; Station Biologique de Roscoff, UMR 7139
Keywords:	gene silencing, brown algae, Fucus, RNAi, cytoskeleton, tubulin, actin

1 **GENE SILENCING IN *FUCUS* EMBRYOS: DEVELOPMENTAL**
2 **CONSEQUENCES OF RNAi-MEDIATED CYTOSKELETAL**
3 **DISRUPTION.**

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1 **Abstract**

2 **Brown algae (Phaeophyta) are an important algal class that play a range of key**
3 **ecological roles. They are often important components of rocky shore communities. A**
4 **number of members of the Fucales and Ectocapales have provided models for the study**
5 **of multicellular evolution, reproductive biology and polarised development. Indeed the**
6 **furoid algae exhibit the unusual feature of inducible embryo polarisation, allowing many**
7 **classical studies of polarity induction. The potential of further studies of brown algae in**
8 **these important areas has been increasingly hindered by the absence of tools for**
9 **manipulation of gene expression that would facilitate further mechanistic analysis and**
10 **gene function studies at a molecular level. The aim of this study was to establish a**
11 **method that would allow the analysis of gene function through RNAi-mediated gene**
12 **knockdown. We show that injection of dsRNA corresponding to an α -tubulin gene into**
13 ***Fucus serratus* zygotes induces the loss of a large proportion of the microtubule**
14 **cytoskeleton, leading to growth arrest and disruption of cell division. Injection of dsRNA**
15 **targeting β -actin led to reduced rhizoid growth, enlarged cells and the failure to develop**
16 **apical hair cells. The silencing effect on actin expression was maintained for three**
17 **months. These results indicate that the *Fucus* embryo possesses a functional RNA**
18 **interference system that can be exploited to investigate gene function during**
19 **embryogenesis.**

20

1

2

3 **Introduction**

4 The brown algae are an important group of organisms that dominate the flora of many rocky
5 shore environments. These organisms have attracted interest for a number of reasons,
6 including their capacity to synthesise novel biomolecules (McHugh 2003; Klarzynski et al.
7 2003), as models for the study of early embryogenesis (Brownlee et al. 2001) and as one of
8 the few major eukaryotic groups that has evolved complex multicellularity (Cock et al.
9 2010a). Brown algae belong to the stramenopile group, which also includes the diatoms and
10 the oomycetes, and are hence only very distantly related to classical laboratory model
11 organisms such as *Drosophila* and *Arabidopsis*. There is a pressing need to adapt tools that
12 have been developed for such classical model organisms to brown algal systems in order to
13 investigate the unusual biological features of these seaweeds. However, to date no reports of
14 gene knock down or manipulation of gene expression have been reported for any brown algal
15 species. This represents a major bottleneck in further development of brown algal models.

16 Small, 18-30 nucleotide RNA molecules derived by nuclease digestion of larger double-
17 stranded or hairpin RNA molecules play important regulatory roles in cells including
18 defending the genome against mobile elements, chromatin regulation and modulation of gene
19 expression {Carthew and Sontheimer 2009; Vionnet 2009; Chapman and Carrington 2007}.

20 The RNA interference (RNAi) system, which is mediated by small interfering RNAs
21 (siRNAs), has been exploited in many different model organisms in order to develop gene
22 knockdown systems and has proven to be a powerful tool to investigate gene function.

23 RNAi was initially characterised in green plants and animals (Carthew and Sontheimer 2009;
24 Voinnet 2009) but it is becoming increasingly clear that this is a very ancient cellular system

1 that is present in a broad range of eukaryotic groups. For example, proteins resembling key
2 components of the RNAi machinery, such as dicer, argonaute and RNA-dependent RNA
3 polymerase (RdRP) have been identified in almost all of the major eukaryotic lineages
4 (Cerutti et al. 2011) and small RNA molecules have been characterised from a broad range of
5 species, including stramenopiles (Cock et al. 2010a; Norden-Krichmar et al. 2011; Huang and
6 Wang 2011). The genome of the brown alga *Ectocarpus*, for example, appears to encode all
7 the above components of the RNAi machinery together with a broad range of small RNAs
8 including microRNAs (Cock et al. 2010a). Moreover, the potential of RNAi as a tool for
9 investigating gene function in stramenopiles was recently demonstrated in two studies that
10 used this approach to investigate light signalling pathways in the yellow-green alga *Vaucheria*
11 *frigida* (Takahashi et al. 2007) and the diatom *Phaeodactylum tricornutum* (De Riso et al.
12 2009).

13 The aim of the work described here was to investigate whether RNAi could be used to knock
14 down gene function in a brown alga. Experiments were carried out using a furoid species,
15 *Fucus seratus*, for several reasons. First, this group of brown algae produce large zygotes
16 allowing double stranded RNA (dsRNA), the substrate for the production of siRNAs, to be
17 introduced directly by microinjection. Second, the cellular events associated with early
18 embryogenesis have been investigated in detail in this group of brown algae and a number of
19 molecules involved in this process have been identified (Corellou et al. 2005; Fowler et al.
20 2004; Brownlee et al. 1998, 2001). These previous studies, therefore, both provide potential
21 targets for RNAi knockdown and a reference framework that allows identification and
22 characterisation of deviations from the normal process of embryogenesis. The roles of
23 cytoskeletal components in determining polarity and division pattern during early
24 embryogenesis has been particularly well characterised in furoid zygotes and embryos
25 (reviewed in Bisgrove, 2007; Brownlee et al. 2001). Fixation of the polar axis depends on the

1 localization of F-actin at the presumptive rhizoid apex and rhizoid germination is dependent
2 on a functional actin cytoskeleton but is independent of the microtubule polymerization
3 (Corellou et al, 1995; Hable et al, 2003). Microtubule connections between the growing
4 rhizoid apex and the mitotic apparatus ensure appropriate alignment of the division axis
5 (Peters and Kropf, 2010; Bisgrove et al. 2003). Mitotic divisions are associated with
6 substantial dynamics of the microtubule cytoskeleton (e.g. Corellou et al. 2005; Hable et al.
7 2003). We show that microinjection of *Fucus serratus* zygotes with dsRNA corresponding to
8 α -tubulin leads to growth inhibition and disruption of cell division in a manner consistent
9 with that observed following treatment with the microtubule depolymerising molecule
10 nocodazole. Moreover, immunocytochemistry revealed that injection with the α -tubulin
11 dsRNA led to a dramatic reduction in the cellular levels of α -tubulin and disruption of
12 microtubule cytoskeletal dynamics. Injection of dsRNA targeting β -actin produced
13 substantially different long-lasting morphological responses that included inhibited rhizoid
14 growth, multicellular embryos with enlarged cells and the failure to develop apical hair cells.

15

16 MATERIALS AND METHODS

17 *Biological material.* Collection of fertile *Fucus serratus* individuals, gamete production,
18 fertilization and embryo culture were carried out as described previously (Corellou et al.
19 2005). Briefly, gamete release was triggered by immersing receptacles in fresh water for
20 several minutes before transferring to filtered seawater (FSW) at 15-18°C and a light intensity
21 of 70 $\mu\text{mol s}^{-1}$. Released gametes (eggs and spermatozooids) were filtered through a 100 μm
22 nylon mesh. Eggs were washed gently with FSW before addition of sperm, washed with FSW
23 to remove excess sperm after 30 min. and incubated at 18°C at a light intensity of 70 $\mu\text{mol m}^{-2}$
24 s^{-1} for 1 h. Following fertilization, zygotes were transferred to microinjection dishes in FSW

1 and kept on ice in the dark until microinjection, which was either carried out on the same or
2 the following day.

3 *Plasmid constructs.* ORF Esi0053_0070 of the *Ectocarpus* genome (add REF Cock et al.,
4 2010), which is predicted to encode α -tubulin, was used in a nucleotide BLAST search to
5 interrogate a *F. serratus* EST data set (Pearson et al. 2010). The best hits were to a contig
6 designated TC_00471 that aligned with 750 bp of the Esi0053_0070 coding sequence (90%
7 identity). PCR primers MB1 (TGCGGTATCAACTACCAGCC) and MB2
8 (ATACAGCTTCTCATAGCATAGG) were designed to amplify a region consisting of 306
9 bp of the predicted α -tubulin protein coding sequence and 236 bp of the 3'UTR in TC_00471.
10 A second contig, designated TC_00244 was also identified. In the 306 bp region spanning
11 the MB1 primer binding site in TC_00471 to the end of the predicted α -tubulin coding
12 sequence there are 15 variant nucleotides between the two sequences. In contrast, the 239 bp
13 region spanning the 3'UTR of TC_00471 to the primer binding site MB2 has no identical
14 regions greater than 5 bp and MB2 does not match any sequences in this region of TC_00244
15 (Fig. S1). Thus the *F. serratus* genome contains at least 2 tubulin genes, only one of which
16 (TC_00471) was investigated in this study. Total RNA was extracted from desiccated 24h *F.*
17 *serratus* embryos using the method of Pearson (Pearson et al. 2010). First strand cDNA
18 synthesis was performed using 1 μ g of *F. serratus* RNA, an oligo dT(18) primer and
19 Invitrogen Superscript III reverse transcriptase as per manufacturers recommendations. A 542
20 bp fragment corresponding to the *F. serratus* α -tubulin mRNA sequence was amplified with
21 TAQ DNA polymerase using primers MB1 and MB2 and 1 μ l of first strand cDNA. The
22 complete *F. serratus* β -actin mRNA sequence including 69 bp of the 5'UTR and 157bp of the
23 3'UTR was determined by RACE PCR (Fig. S2). Primers ACTFS1 (5'-
24 ATGGCGGACGAGGACGTG-3') and ACTFS2 (5'-CGCCCGGCGAGGTCGAG-3') were
25 used to generate a 551bp product starting at the ATG start codon. Additionally, primers MB3

1 (CACTCTTGTACTCCACCTTC) and MB4 (TTAGAAGCACTTGCGGTG) were designed
2 to amplify a region spanning 69 bp of the 5' untranslated region and the entire 1131bp coding
3 sequence including the stop codon in order to generate the 1200bp fragment. All products
4 were cloned into pCR2.1 TOPO and the nucleotide sequence and orientation relative to the T7
5 promoter was confirmed by sequencing individual clones in both directions. Plasmid
6 constructs used in subsequent procedures, designated pCR2.1 TOPO:Tubulin1 (sense to T7
7 promoter), pCR2.1 TOPO:Tubulin 4 (antisense), pTOPO:GF actin (sense; 1200 bp fragment)
8 and pTOPO:GF (antisense; 1200 bp fragment), pTOPO:LD actin (sense; 551 bp fragment)
9 and pTOPO:LD (antisense; 551 bp fragment) were harvested from *E. coli* Top10 cells
10 (Invitrogen) using Qiagen miniprep columns, linearized with *Bam*HI and subjected to a
11 proteinase K/SDS treatment followed by phenol chloroform extraction (Sambrook and
12 Russel) to reduce RNase contamination.

13
14 *Synthesis of double-stranded (ds) RNA.* Synthesis of dsRNA by T7 polymerase transcription
15 was carried out using the MEGAscript® RNA kit (Ambion, Life Technologies) according to
16 the manufacturer's instructions. For the transcription reaction, 1µg of each plasmid (sense and
17 antisense) was used in a single 20 µl reaction. The reaction was incubated for 4 h at 37°C and
18 this was followed by an annealing step in which the sample was heated to 90°C for 5 min
19 followed by gradual cooling to 20°C at a rate of 0.2°C s⁻¹. The quality of the dsRNA was
20 checked by agarose gel electrophoresis and the concentration was determined on a NanoDrop
21 1000 spectrophotometer (Thermo Scientific, Cramlington, UK). The 551 bp and 1200 bp
22 Actin dsRNA constructs gave the same phenotype but we found that the Megascript derived
23 dsRNA from the 1200 bp template was more abundant than dsRNA deriving from the 551bp
24 template. Therefore the former was used in the β-actin silencing experiments we describe
25 here.

1 *Microinjection of dsRNA.* *F. serratus* zygotes were secured in glass wedges formed from a 1
2 cm length piece of glass capillary (microdispenser 250 μ L replacement tubes; Drummond
3 Scientific, Broomall, PA, USA) glued onto a glass coverslip. The zygotes were then immersed
4 in FSW containing 0.4 M sorbitol in order to decrease turgor pressure. The 0.4 M sorbitol was
5 then replaced by 0.8 M sorbitol and zygotes were microinjected at 18°C using a pressure
6 system (Corellou et al. 2005). The microinjection pipettes were made from 1.2 mm filamented
7 borosilicate glass (Clarke Electromedical, Reading, UK), dry-beveled and back filled with 10
8 μ L of artificial intracellular solution (200 mM KCl, 10 mM HEPES, 550 mM mannitol, pH 7)
9 containing 0.1 mM fluorescein isothiocyanate (FITC) and 10 ng μ L⁻¹ dsRNA (dsRNA:tubulin,
10 dsRNA:actin or dsRNA:control). The microinjection process was monitored by observing
11 FITC fluorescence (450 nm excitation, 510 nm emission) so that the volume microinjected
12 corresponded to about 5-10% of the zygote cell volume. Following microinjection the zygotes
13 were kept in the dark at 18°C and the sorbitol solution was gradually replaced with FSW. The
14 dishes were then transferred to and placed in unidirectional light at 18°C. The following day
15 (19-24 h after microinjection), the development of injected zygotes was scored and images
16 obtained using a CCD camera (DCM A series; ScopTek, Kelowna, BC, Canada) on an
17 Axiovert microscope (Zeiss, Jena, Germany). Both dsRNA:control (positive non-coding
18 sequence control supplied with the Ambion Megascript kit) and dsRNA:tubulin or
19 dsRNA:actin injections were carried out during each microinjection session.

20 *Immunolabeling and confocal microscopy.* Immunolabeling of α -tubulin was carried out
21 between 19 and 24h after the microinjection of dsRNA. The protocol was as described by
22 Bisgrove and Kropf (2001), with the following modifications. The initial step of freezing the
23 material in liquid nitrogen was omitted since this step usually led to considerable loss of
24 injected *F. serratus* zygotes. The material was incubated in freshly prepared fixation solution
25 (FSW containing 0.5% v/v glutaraldehyde and 3.2% v/v formaldehyde) for 1 h and then

1 rinsed three times for 5 min in modified PBS before incubating in 5% v/v Triton X-100
2 overnight. The following day, the material was washed again in mPBS and incubated for 4h in
3 0.1 M NaBH₄ in mPBS. After washing in mPBS the material was rinsed in solution C, then
4 incubated in solution C (100 mM NaCl, 20 mM MgCl₂, 2 mM KCl, 0.2% BSA, 10 mM MES,
5 0.85 M sorbitol, 1 mM EGTA, with pH adjusted to 5.8 with Tris base), containing
6 hemicellulase (40 mg/mL; Sigma H2125) and cellulase (7 mg/mL; Sigma C8546) for 90 min
7 and washed in mPBS. Anti- α -tubulin (Sigma, T9026) was used at a dilution of 1/50 in 250-
8 500 μ L mPBS and the secondary antibody (rhodamine-conjugated goat anti-mouse IgG;
9 Pierce Thermo Scientific) was used at a dilution of 1/100 in mPBS. All incubation steps were
10 performed at room temperature. Confocal sections of rhodamine fluorescence were obtained
11 with a Zeiss LM510 confocal laser scanning microscope with a x40, 1.3 n.a. objective using
12 four line averaging (image size 512 x 512 pixels, 8 bit). Z stacks were viewed as planar
13 projections using the Zeiss LSM software.

14 *Quantification of fluorescent signal and microtubules.* In order to estimate the levels of α -
15 tubulin, we measured the fluorescence of immunolabeled *Fucus* zygotes from planar
16 projections of confocal sections of dsRNA:tubulin-injected, dsRNA:control-injected and
17 uninjected samples. Quantification was performed using ImageJ software
18 (<http://rsbweb.nih.gov/ij/>). Two quantification methods were employed. First the mean gray
19 value (equivalent to the sum of pixel intensities divided by the number of pixels) of the
20 complete embryo was measured. We also determined the relative area of microtubules. A
21 standard threshold look up table was applied to each image to maximize the contrast of
22 microtubule fluorescence. Binary images of pixels above and below the threshold value were
23 used to determine the relative area occupied by microtubules. The data were analyzed using
24 GraphPad Prism version 5.03 for Windows and significance ($p < 0.05$) was determined by

1 applying Kruskal Wallis and Dunn's multiple comparison post test. In total, images of three
2 independent experiments were analyzed in this way.

3 *Length measurements.* Lengths of developing *F. serratus* zygotes and embryos from the
4 thallus apex to the rhizoid tip were measured as a proxy of growth using a measuring
5 microscope ocular. Measurements of dsRNA:control, dsRNA:tubulin and uninjected zygotes
6 were made 1 d (19-24 h), 2 d (40-43 h) and 3 d (64-67 h) after injection in two independent
7 experiments.

8 *Nocodazole treatment.* Nocodazole was dissolved in DMSO to a concentration of 5 mg ml⁻¹
9 and further diluted in FSW to a final concentration of 1 µg ml⁻¹. Zygotes were incubated in
10 nocodazole for 3 d and the medium containing the inhibitor was changed daily. Zygotes
11 incubated in 0.02 % (v/v) DMSO served as controls and were treated in the same manner.

12

13 **Results**

14 *Effects of tubulin dsRNA injection on rhizoid growth and embryonic cell divisions.* Disruption
15 of the microtubule cytoskeleton in *Fucus* zygotes with pharmacological agents such as
16 nocodazole or colchine leads to characteristic abnormal developmental phenotypes including
17 inhibition of rhizoid growth and aberrant cell divisions (Corellou et al. 2005, Katsaros et al.
18 2006; Quatrano, 1973). We tested whether introduction of tubulin dsRNA into *Fucus* zygotes
19 could induce similar effects. *F. serratus* zygotes were microinjected with either *Fucus*
20 dsRNA:tubulin or with dsRNA:control (dsRNA corresponding to a non-coding sequence
21 supplied with the Ambion Megascript kit). The length of the developing zygotes and embryos
22 (from the apical thallus region to the rhizoid tip) was measured daily as a proxy for tip growth
23 (Figure 1). No differences in zygote length were observed between the different treatments
24 prior to 24 h post-injection (PI) (Fig. 1a). However, the dsRNA:tubulin injected embryos,

1 grew more slowly over the following 2 d and in most cases no increase in length was detected
2 after 3 d PI. In contrast, the dsRNA:control-injected zygotes grew continuously, in a manner
3 comparable to uninjected zygotes. Three d after injection of dsRNA:tubulin, embryos showed
4 loss of pigmentation and increased vacuolation, indicating that they were becoming senescent
5 (Fig. 1b). Moreover, the rhizoids of the dsRNA:tubulin-injected zygotes appeared swollen and
6 misshapen (Figs. 1b and 2f-h). Similar rhizoid phenotypes were observed when *Fucus* zygotes
7 were incubated with nocodazole, an inhibitor of microtubule polymerization (Fig. 2b-d).

8 *Cell division and cell plate formation in dsRNA injected zygotes.* Cell plate formation was
9 monitored in developing embryos using normal light field microscopy 19-24h after injection
10 with dsRNA. The observed phenotypes could be classified into four different categories (Fig.
11 3): a) Normal cell plate formation with regard to the developmental stage of the zygote as
12 described in the literature (e.g. Goodner and Quatrano 1993; Bouget et al. 1998), b) absence
13 of cell plate formation, c) aberrant, but symmetrical cell plate formation and d) aberrant,
14 asymmetrical cell plate formation (atypical division patterns and/or incomplete cell plate
15 formation). Only 25.5% of the dsRNA:tubulin injected zygotes exhibited normal cell plate
16 formation, compared with 90.2% of the uninjected controls (Fig. 3e). The zygotes injected
17 with dsRNA:control showed a slightly lower frequency of normal cell plate formation
18 (73.1%) than the uninjected controls but the effect was much less marked than in the
19 dsRNA:tubulin injected sample. Both the dsRNA:tubulin and dsRNA:control injected zygotes
20 included a proportion of individuals with aberrant cell plate formation. However, in contrast
21 to the dsRNA:tubulin injected zygotes, the aberrant cell plates observed in dsRNA:control-
22 injected zygotes were always symmetrical, whereas 41.8% of the dsRNA:tubulin-injected
23 *Fucus* zygotes showed cell plates that were not only aberrant, but also asymmetrical and
24 incomplete. A small percentage of *Fucus* egg cells may be fertilised by more than one male
25 gamete (polyspermy) (Brawley 1987). The presence of more than one pair of paternal

1 centrioles in these zygotes causes aberrant positioning of the cell division planes (Bisgrove et
2 al. 2003; Motomura and Nagasato 2004). This may explain the small percentage of embryos
3 with aberrant, but symmetrical, cell division patterns observed following dsRNA:control
4 microinjection (Fig. 3 c, e), assuming that polyspermy levels were high in this sample due to
5 the presence of a large number of male gametes during fertilisation. The proportion of
6 embryos that completely failed to form a cell plate was markedly higher in dsRNA:tubulin-
7 injected zygotes (32.7%) compared to control-injected (11.5%) and uninjected (4.8%)
8 embryos.

9 *Reduced microtubule abundance and aberrant microtubule organisation in zygotes injected*
10 *with tubulin dsRNA.* To determine whether the phenotypic changes observed after injection of
11 dsRNA:tubulin were due to inhibition of tubulin gene expression, immunolabeling was
12 carried out 19-24h post injection (Fig. 4). At this stage, dsRNA:control-injected cells had
13 usually passed the two-cell embryo stage. Immunolabelling patterns of α -tubulin typical of
14 those found in other studies (e.g. Bisgrove and Kropf 2001; Corellou et al. 2005) were
15 detected in all of the dsRNA:control-injected individuals, regardless of the developmental
16 stage. Microtubules were observed in abundance in both the thallus and the rhizoid area in the
17 perinuclear region, with perinuclear microtubules orientated perpendicular to the position of
18 the future cell division plane (Fig. 4c, d). The same pattern of microtubule organisation was
19 observed in uninjected zygotes .

20 The α -tubulin immunolabeling patterns of dsRNA:tubulin-injected zygotes differed
21 dramatically from those of dsRNA:control injected and uninjected zygotes. Microtubules
22 were much less abundant in dsRNA:tubulin treated individuals than in dsRNA:control and
23 uninjected *Fucus* zygotes. The extensive microtubule arrays seen in the apical area of control
24 samples were absent in dsRNA:tubulin-injected zygotes and only highly localised condensed
25 nuclear accumulations were apparent (Fig. 4a, b). Occasionally, microtubule labelling could

1 still be observed in the rhizoid region of the zygote but was less pronounced compared to the
2 controls. Reduced tubulin immunostaining was not due to displacement of microtubules to
3 another part of the cell since the entire volume of the cell was analysed. We measured tubulin
4 immunofluorescence and microtubule area as an proxy for α -tubulin abundance since more
5 direct immunoblot quantification of α -tubulin protein or qPCR measurement of α -tubulin
6 transcript abundance were not feasible on the limited amount of injected material available in
7 this study. Fluorescence levels, represented as mean grey values of immunolabeled zygotes
8 injected with dsRNA:tubulin, were significantly lower compared to dsRNA:control injected
9 individuals and uninjected zygotes (Fig. 5a). The relative area of microtubules in
10 dsRNA:tubulin treated individuals was also significantly smaller than in dsRNA:control
11 treated and uninjected embryos (Fig. 5b).

12 In the putative polyspermic zygotes, which showed aberrant but symmetrical cell division
13 planes, normal abundances of microtubules with characteristic perinuclear organisation, were
14 detected following immunostaining (Fig. S3), rather than the reduced levels of microtubules
15 characteristic of dsRNA:tubulin-injected embryos (see Fig. 4).

16 *Silencing of β -actin is maintained in *F. serratus* embryos over a period of at least three*
17 *months.* The rapid senescence of embryos injected with dsRNA α -tubulin did not allow an
18 assessment of the persistence of RNAi knock down in the *Fucus* embryo. However in addition
19 to targeting α -tubulin in our RNAi experiments we also targeted the highly abundant mRNA
20 of the single copy β -actin gene. In 75.9% of the dsRNA:actin injected embryos the basal
21 rhizoid had thickened by 48h and there was little or no rhizoid growth after 72h (Fig. 6). This
22 response was never observed in dsRNA:control injected embryos. The severity of this
23 phenotype increased over 12 weeks and was typified by distension and enlargement of cells
24 which often protruded from the embryo surface (Fig. 7). The dsRNA:actin injected embryos

1 showed no signs of senescence during the experiment; they maintained their pigmentation and
2 there was no visible breakdown in cellular structure. The progressive nature of the observed
3 phenotype and the lack of senescence suggests that actin silencing was maintained during the
4 12 weeks that the embryos were followed.

5

6 **Discussion**

7

8 In this study we show that injection of dsRNA is a viable method to knock down gene
9 expression in zygotes and embryos of the brown alga *F. serratus*. Injection of dsRNA
10 corresponding to an α -tubulin gene resulted in growth inhibition and disruption of cell
11 division during subsequent embryo development. The rhizoid failed to elongate and exhibited
12 swelling. Cell division was either prevented completely or showed dramatic disruption of the
13 normal regular division pattern. Immuno-staining showed that these effects on growth and cell
14 division were correlated with disruption of the microtubule cytoskeleton and reduced levels of
15 α -tubulin in the cell. The phenotypes observed following injection of dsRNA:tubulin were
16 very similar to those observed following treatment with the inhibitor nocodazole, which
17 interferes with microtubule polymerisation. Previous studies have also reported growth
18 inhibition and swelling of the rhizoid tip following continuous treatment with nocodazole
19 (Corellou et al. 2005; Bisgrove and Kropf 1998). Moreover, a pulse treatment with
20 nocodazole, applied 12 h after fertilization, has been shown to increase the number of
21 embryos that have a misaligned first division plane (i.e. an aberrant division plane) and this
22 was also associated with inhibition of elongation and a reduced growth rate (Bisgrove and
23 Kropf 1998). Aberrant division planes were also observed when *Sylvetia compressa* zygotes
24 were treated with monastrol 6 h after fertilization (Peters and Kropf 2006). Monastrol is an
25 inhibitor of kinesin 5 motor proteins, which are involved in microtubule organisation (Miki et

1 al. 2005). Other microtubule inhibitors such as colchicine have been shown to have similar
2 effects on cell division without affecting zygote polarization (e.g. Quatrano, 1973; Brawley
3 and Quatrano, 1979).

4 Significant growth inhibition, compared to control embryos was only manifest after 2 d in
5 embryos injected with dsRNA:tubulin (see Fig. 1a). Effects on cell division were, however,
6 detected earlier, at 19-24 h (i.e. at the first cell division) and reduced levels of α -tubulin were
7 also detected by this time (see Figs. 3,4). The lag period between injection and growth
8 inhibition may have corresponded to the time necessary for the RNAi system to reduce α -
9 tubulin levels below a threshold necessary for growth, whereas cell division is likely to
10 require higher tubulin levels and microtubule dynamics. This is consistent with observations
11 that nocodazole treatment does not inhibit zygote polarization but does inhibit the first cell
12 division (Corellou et al. 2005). The mRNAs encoding structural cytoskeletal proteins
13 including α -tubulin are maternally inherited and are amongst the most abundant mRNAs in
14 the *Fucus* embryo as evidenced by representation in EST data sets (Masters et al. 1992;
15 Pearson et al. 2010). Moreover, while there is no specific data available on the stability and
16 turnover rates of tubulin and actin protein in *Fucus* embryos is very likely that low turnover
17 and high stability contribute significantly to the observed lag between microinjection of
18 dsRNA and phenotypic response. RNAi has been used successfully to silence maternal
19 mRNAs in unfertilised animal oocytes and to study the role of developmental genes in
20 *Xenopus* oocyte maturation and early embryogenesis (Svoboda et al. 2000). In this work we
21 introduced dsRNA constructs into fertilised embryos of *F. serratus* because injection of
22 unfertilised eggs is technically challenging (e.g. Roberts et al. 1994). Developing
23 methodologies such as biolistic loading (Bothwell et al. 2008) of dsRNAs into unfertilised
24 oocytes may lead to earlier silencing of maternally inherited and abundant mRNAs further
25 increasing the potential of this approach in the *Fucus* system.

1 The presence of substantially reduced, but detectable levels of α -tubulin, rather than complete
2 absence, in embryos injected with dsRNA:tubulin indicates a degree of incomplete silencing.
3 Two contigs with homology to *E. siliculosus* α -tubulin genes, designated TC_00471 and
4 TC_00244, were identified in a *F. serratus* EST data set (see Fig. S1). The presence of at least
5 two α -tubulin genes in *F. serratus* has two main implications. First, long regions of identical
6 sequence shared between genes, as seen between the 306 bp region spanning MB1 and the
7 predicted protein coding sequence in both TC_00471 and TC_00244 would likely cause co-
8 silencing of gene family members (Miki et al. 2005). This, combined with the high
9 abundance of α -tubulin mRNAs, could potentially saturate the silencing system leading to a
10 delay in the onset of and incomplete penetration of the RNAi phenotype (Arvey et al. 2010).
11 *Xenopus* embryos are especially prone to saturation of the RNAi system (Lund et al. 2011)
12 and further experiments are needed to understand this phenomenon in *Fucus* embryos.
13 Alternatively, the function of the targeted gene may be complemented by redundant functions
14 of other gene family members that are not targeted by the specific RNAi, which could again
15 result in delayed onset and attenuation of the phenotype. Future studies that utilise the
16 observed long untranslated regions (3'UTRs) in many *Fucus* genes (Pearson et al. 2010) may
17 offer a solution to the co-silencing problem. Targeting of silencing constructs to more
18 variable 3' UTRs of *OSrac* mRNAs in rice resulted in silencing of specific gene family
19 members whereas targeting to homologous of coding regions silenced multiple isoforms
20 (Miki et al. 2005). Differentiating between these possibilities; co-silencing, saturation and
21 redundancy will require detailed information concerning the complexity of the α -tubulin
22 complement of *Fucus* embryos and parallel quantification of multiple mRNAs.

23 The responses of individual embryos to injection of α -tubulin dsRNA were quite variable,
24 33% exhibited no cell division, 42% exhibited aberrant cell divisions and in 25% normal cell
25 division was observed. Our preliminary observations suggest that this variability in RNAi

1 response is most likely the variable amount of dsRNA loaded into individual zygotes, as
2 judged by the co-loading of fluorescent marker dyes with the dsRNA, since only poorly-
3 loaded zygotes underwent normal development (results not shown). However, variability
4 may be an inherent feature of RNAi in some systems. For example in the stramenopile
5 oomycete, *Phytophthora infestans*, activation of RNAi by lipofection of dsRNA into
6 protoplasts, which presumably leads to a more uniform loading than the microinjection
7 system employed here for *Fucus* embryos, results in variable silencing both between
8 individual organisms in a population and within different cells of a single organism (Whisson
9 et al. 2005). Further RNAi studies in the *Fucus* embryo will benefit from accurate
10 quantification of the introduced silenced dsRNA species.

11 In contrast to the eventual senescence over several days of dsRNA:tubulin-treated embryos,
12 the phenotype induced after the introduction of dsRNA:actin into the *F. serratus* embryos
13 persisted for 12 weeks even though the dsRNA:actin trigger molecule was introduced only
14 transiently. Silencing of the aureochrome receptor in the stramenopile alga *Vaucheria frigida*
15 was maintained for over 6 months after the microinjection of a transient dsRNA trigger
16 molecule (Takahashi et al. 2007). The recently sequenced genome of the brown alga *E.*
17 *siliculosus* provides evidence for a mechanism for the prolonged maintenance of the RNAi
18 phenotype. *E. siliculosus* contains components of the RNAi machinery including homologs of
19 *Dicer*, *Argonaute* and two RNA-dependent RNA polymerases (RDRP). In the green plant
20 *Arabidopsis thaliana*, the RDRP SDE1 is required for amplification and maintenance of gene
21 silencing mediated by a transgene (Dalmay et al. 2000), RDRP may play a similar role in
22 maintenance of RNAi gene silencing in brown algae.

23 A caveat to interpreting our actin RNAi experiments is that the levels of the β -actin protein
24 were not determined in individual embryos. The phenotypes of *F. serratus* embryos injected
25 with dsRNA-actin however are wholly consistent with current models of β -actin function in

1 fucoid algae. Moreover, the different phenotypes observed in response to dsRNA:tubulin and
2 dsRNA:tubulin microinjection strongly imply that the observed effects are due to specific
3 gene knock down rather than non-specific effects on gene expression.

4

5 In dsRNA:actin-treated *F. serratus* embryos compromised cell wall deposition is consistent
6 with the distended appearance of the cells in these embryos. Low concentrations of the actin
7 depolymerising compound latrunculin B have been shown to block cell division and
8 polarisation in *F. serratus* embryos (Bothwell et al. 2008). RNAi-mediated reduction of β -
9 actin protein below a critical level would be expected to similarly compromise cell division,
10 and is also consistent with reduced rhizoid length after 72 h and the appearance of enlarged
11 cells throughout the embryo.

12 The demonstration that RNAi is functional and maintained for long periods in the *F. serratus*
13 embryo is the first example of targeted gene silencing in a brown macroalga and paves the
14 way for studies into the molecular basis of the phenomenon. Using microinjection, it was
15 possible to inject up to 60 *F. serratus* zygotes in a single session of approximately 4 h,
16 sufficient to allow the reproducibility of a response to be accessed and statistical analyses of
17 phenotyping data to be carried out. The protocol described here opens up the possibility of
18 analysing the functions of specific *Fucus* genes during early development. Moreover, the
19 demonstration that injection of dsRNA can be used to knock down gene expression in a
20 brown alga suggests that RNAi may be a viable technique for gene function analysis in other
21 brown algae. It will be particularly productive, for example, to adapt the method described
22 here for the filamentous brown alga *Ectocarpus*, for which a complete genome sequence is
23 available (Cock et al. 2010a,b) together with a broad range of genetic, transcriptomic,

1 proteomic and metabolomic tools (Heesch et al. 2010; Coelho et al. 2011; Peters et al. 2008;
2 Dittami et al. 2009; Ritter et al. 2010; Dittami et al. 2011).

3

4 **Acknowledgements**

5 This work was supported by the Interreg IV Program France (Channel)-England (project
6 Marinexus), the Centre National de Recherche Scientifique, the University Pierre and Marie
7 Curie and the *Marine Genomics Europe* Network of Excellence.

8

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- 19

1 **Figure Legends**

2 **Figure 1.** Growth and morphology of *F. serratus* embryos following microinjection of
3 zygotes with dsRNA:tubulin. (a) Embryo lengths 1, 2 and 3 days post-injection (PI). Data
4 represent geometric means of two independent experiments. Error bars show standard
5 deviations. dsRNA:tub: *Fucus* zygotes injected with dsRNA:tubulin (n=15); dsRNA:cont:
6 *Fucus* zygotes injected with dsRNA:control (n=23); uninj: uninjected *Fucus* zygotes that were
7 in the same dish as injected zygotes (n=18). (b-d) representative images of *F. serratus*
8 embryos at 3 days PI. dsRNA:tubulin-injected embryos showed no complete cell divisions
9 and growth was arrested 1-2 days PI (b) whereas dsRNA:control-injected (c) and uninjected
10 zygotes (d) exhibited multiple cell divisions and elongated rhizoids.

11

12 **Figure 2.** Phenotypes of nocodazole-treated or dsRNA:tubulin-injected *F. serratus* embryos.
13 (a) Control incubation in 0.02% DMSO. (b-d) Bright field images of representative embryos 3
14 days after treatment with 1 $\mu\text{g mL}^{-1}$ nocodazole. (e-h) representative embryos 3 days after
15 injection with dsRNA:control (e) or dsRNA:tubulin (f-h). Scale bars 25 μm

16

17 **Figure 3.** Representative images of the four categories of cell plate phenotypes observed in
18 dsRNA:tubulin-injected *Fucus serratus* embryos. (a) Normal division pattern. (b) Absence of
19 cell plate formation. (c) Aberrant symmetrical division pattern. (d) Aberrant asymmetrical
20 division patterns. Arrowheads in (a, c and d) indicate cell plates. (e) Proportions of embryos
21 in each phenotype category following microinjection of either dsRNA:tubulin (n=55),
22 dsRNA:control (n=26) or uninjected (n=21). Data derive from 5 independent microinjection
23 experiments. Embryos were observed 24 h after injection.

24

25

1 **Figure 4.** Planar z-stack projections of confocal sections through α -tubulin immunolabelled
2 *Fucus serratus* embryos 24h after microinjection of double stranded RNA. Two
3 representative embryos are shown for each treatment. (a, b) dsRNA:tubulin-injected embryos.
4 Highly localized and condensed microtubules are visible. Note the aberrant cell divisions in
5 (a). (c, d) dsRNA:control-injected and (e, f) uninjected embryos show extensive microtubule
6 arrays in perinuclear and rhizoid regions. Scale bars 50 μ m

7
8 **Figure 5.** Quantification of microtubule fluorescence as a proxy of α -tubulin content in
9 immunolabelled *Fucus serratus* zygotes injected with either dsRNA: control (n=8),
10 dsRNA:tubulin (n=32) or uninjected (n=9). Data represent mean values and standard
11 deviation of three independent experiments. a) Mean gray value of the zygotes and b) relative
12 microtubule areas. a*** indicates highly significant difference (p=0.01) between
13 dsRNA:tubulin and uninjected zygotes and b** indicates significant difference between
14 dsRNA;tubulin and dsRNA: control injected zygotes (p < 0.05; tests: Kruskal Wallis and
15 Dunn's multiple comparison post test).

16 **Figure 6.** Progression of the dsRNA:actin phenotype in *F. serratus* over 12 weeks. Embryo
17 development post injection (PI) of zygotes with dsRNA:control (a, b, c) or dsRNA:actin (d, e,
18 f, g) 48h PI (a, d), 21 d PI (b, e), 6 weeks PI (c, f) and 12 weeks PI (g). The first evidence of
19 altered development in dsRNA:actin injected embryos was a reduction in the length and
20 thickening of the rhizoid (indicated by arrows in (b)) by 48 h PI. At 21 d dsRNA:actin
21 injected embryos contained many enlarged cells and shortened rhizoids (e) compared to
22 dsRNA:control embryos (b). At 6 weeks embryos were still viable, producing occasional
23 adventitious rhizoids (arrow in (f)). Apical hairs, apparent in dsRNA:control injected embryos
24 (arrows in (c)) were always absent in dsRNA:actin injected embryos (f). Distended cells are
25 apparent in dsRNA:actin injected embryos. By 12 weeks several of these distended cells

1 visibly bulged out from the periphery of the dsRNA:actin injected embryos (g). At 12 weeks
2 dsRNA:actin injected embryos showed little further rhizoid elongation compared with 21 d or
3 42 d embryos.

4

5 **Figure 7.** Relative numbers of phenotypes of *F. serratus* embryos at 14 days after
6 microinjection with either dsRNA:control or dsRNA:actin. Embryos were scored according to
7 the following criteria. Normal development compared to uninjected embryos of the same age
8 (normal), failure to develop beyond initial polarisation and second cell division (stall), failure
9 to polarise and undergo the first cell division (dead), thickening of the basal rhizoid at 48
10 hours, failure to elongate after 72 hours and development of enlarged distended cells, referred
11 to as bloated cells, by 14 days (stunt/ bloated phenotype). The results are shown as a
12 percentage of embryos exhibiting each phenotype using data derived from 4 (dsRNA:actin)
13 and 5 (dsRNA:control) independent experiments, respectively ($n=106$ embryos).

14

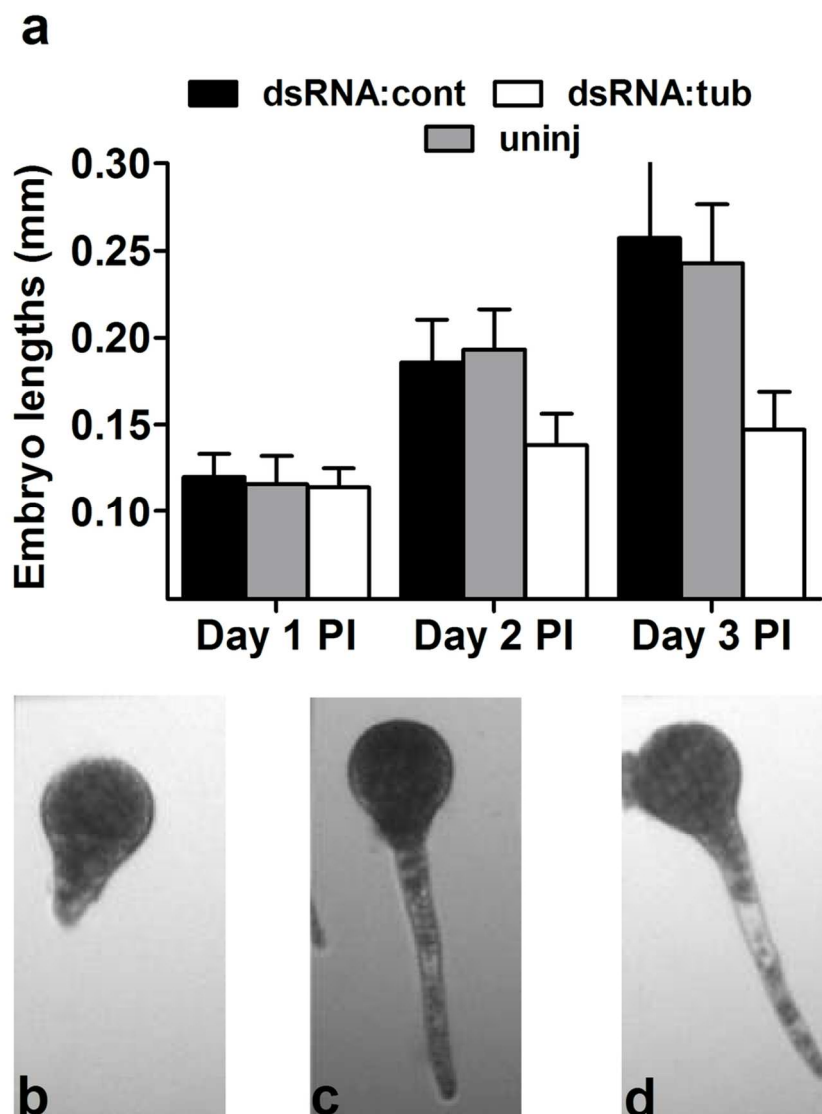


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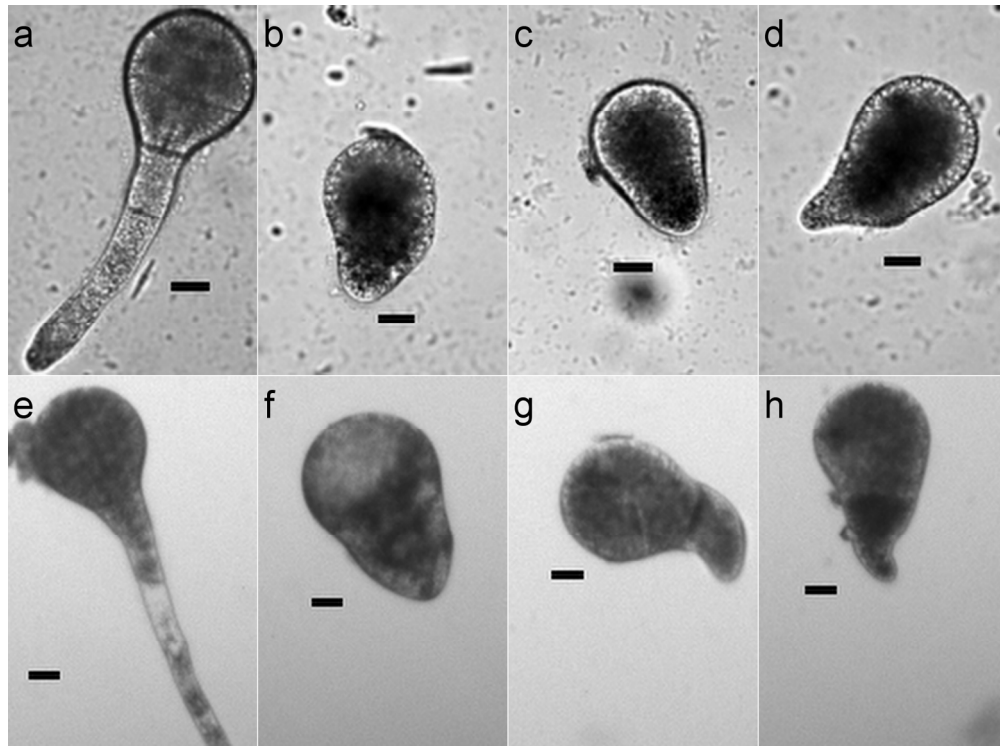


Figure 2. Phenotypes of nocodazole-treated or dsRNA:tubulin-injected *F. serratus* embryos. (a) Control incubation in 0.02% DMSO. (b-d) Bright field images of representative embryos 3 days after treatment with $1 \mu\text{g mL}^{-1}$ nocodazole. (e-h) representative embryos 3 days after injection with dsRNA:control (e) or dsRNA:tubulin (f-h). Scale bars $25 \mu\text{m}$
674x499mm (72 x 72 DPI)

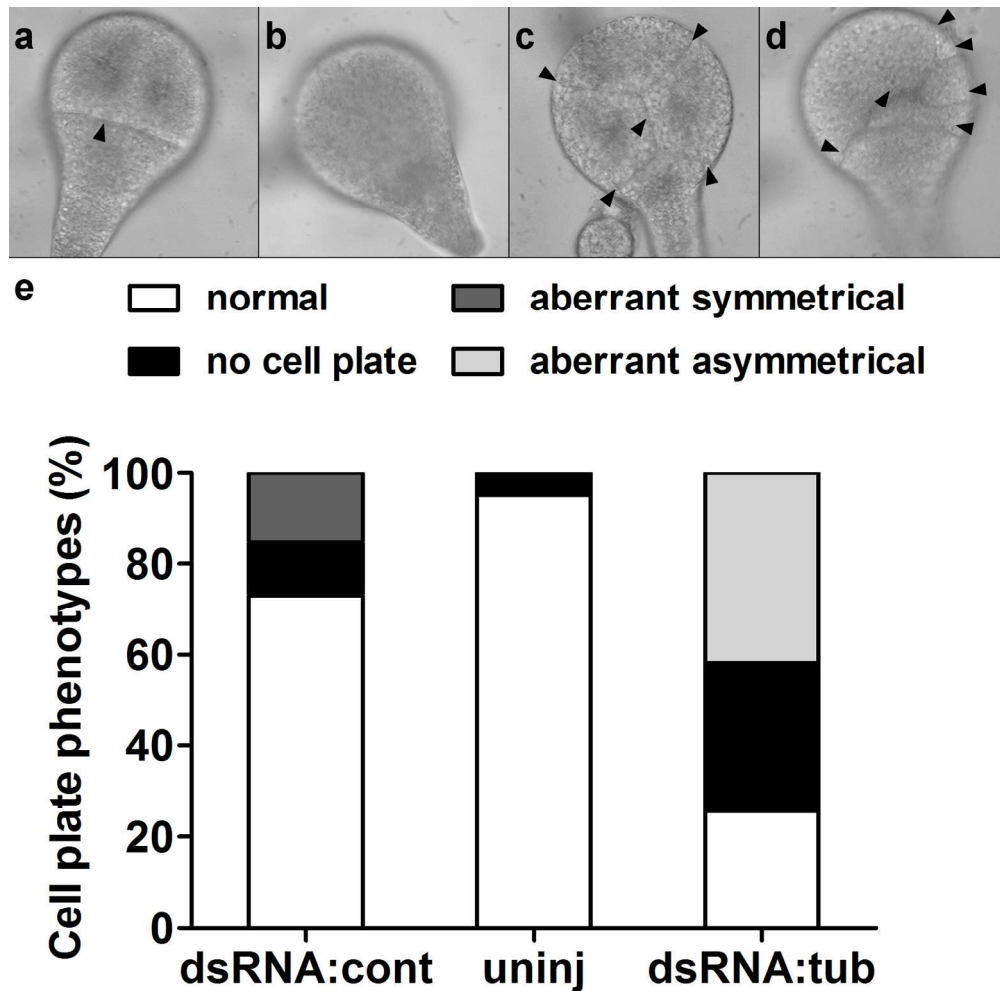


Figure 3. Representative images of the four categories of cell plate phenotypes observed in dsRNA:tubulin-injected *Fucus serratus* embryos. (a) Normal division pattern. (b) Absence of cell plate formation. (c) Aberrant symmetrical division pattern. (d) Aberrant asymmetrical division patterns. Arrowheads in (a, c and d) indicate cell plates. (e) Proportions of embryos in each phenotype category following microinjection of either dsRNA:tubulin (n=55), dsRNA:control (n=26) or uninjected (n=21). Data derive from 5 independent microinjection experiments. Embryos were observed 24 h after injection.

674x674mm (72 x 72 DPI)

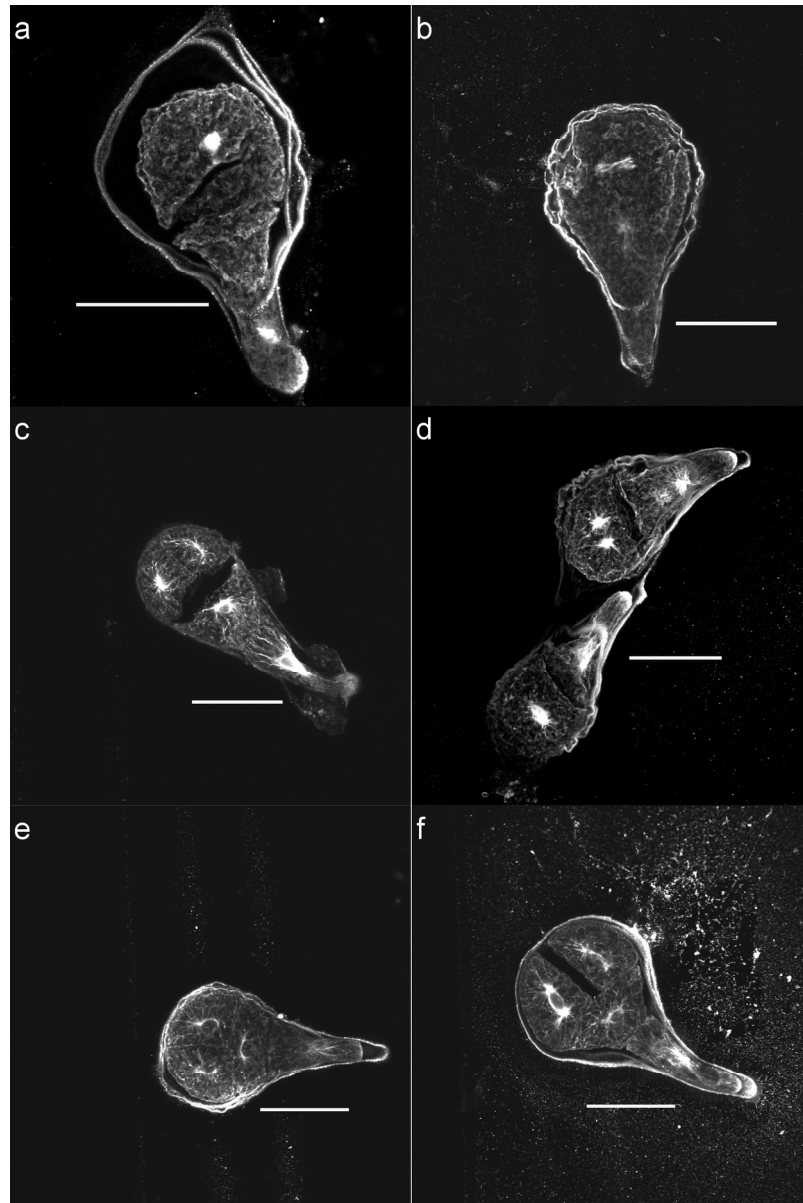


Figure 4. Planar z-stack projections of confocal sections through α -tubulin immunolabelled *Fucus serratus* embryos 24h after microinjection of double stranded RNA. Two representative embryos are shown for each treatment. (a, b) dsRNA:tubulin-injected embryos. Highly localized and condensed microtubules are visible. Note the aberrant cell divisions in (a). (c, d) dsRNA:control-injected and (e, f) uninjected embryos show extensive microtubule arrays in perinuclear and rhizoid regions. Scale bars 50 μ m
674x1008mm (72 x 72 DPI)

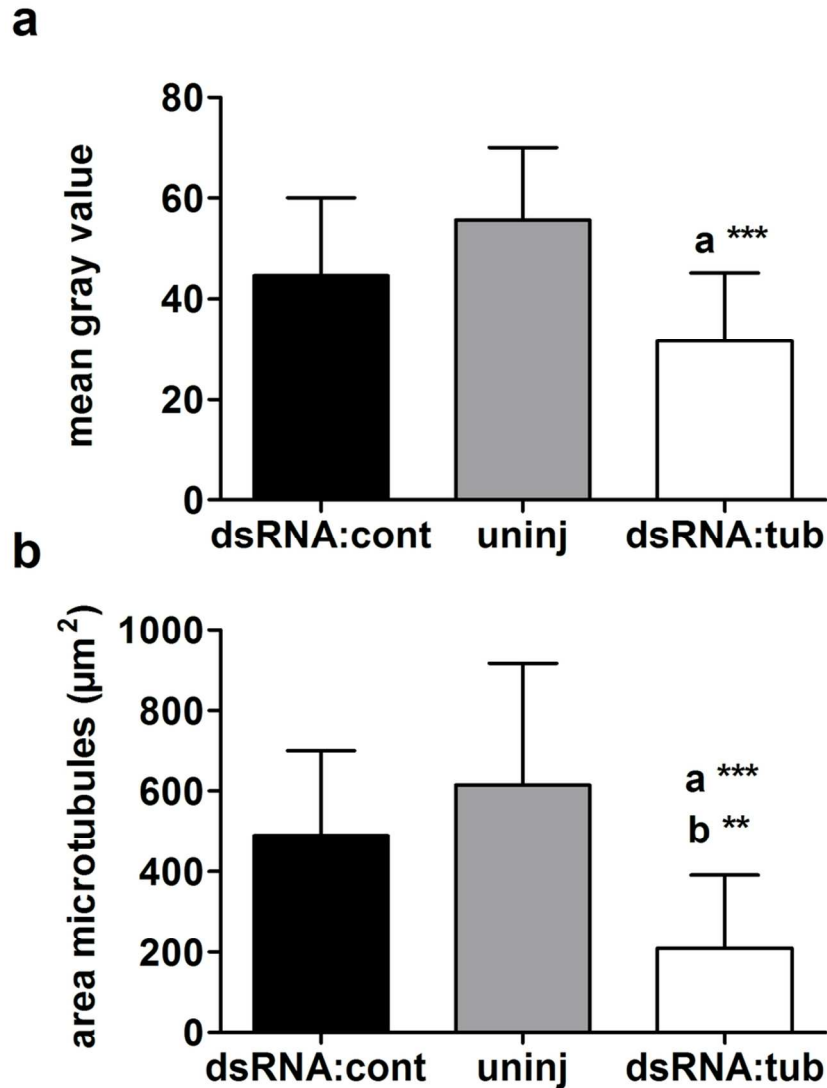


Figure 5. Quantification of microtubule fluorescence as a proxy of α -tubulin content in immunolabelled *Fucus serratus* zygotes injected with either dsRNA: control (n=8), dsRNA:tubulin (n=32) or uninjected (n=9). Data represent mean values and standard deviation of three independent experiments. a) Mean gray value of the zygotes and b) relative microtubule areas. a*** indicates highly significant difference ($p=0.01$) between dsRNA:tubulin and uninjected zygotes and b*** indicates significant difference between dsRNA;tubulin and dsRNA: control injected zygotes ($p < 0.05$; tests: Kruskal Wallis and Dunn's multiple comparison post test). 96x115mm (300 x 300 DPI)

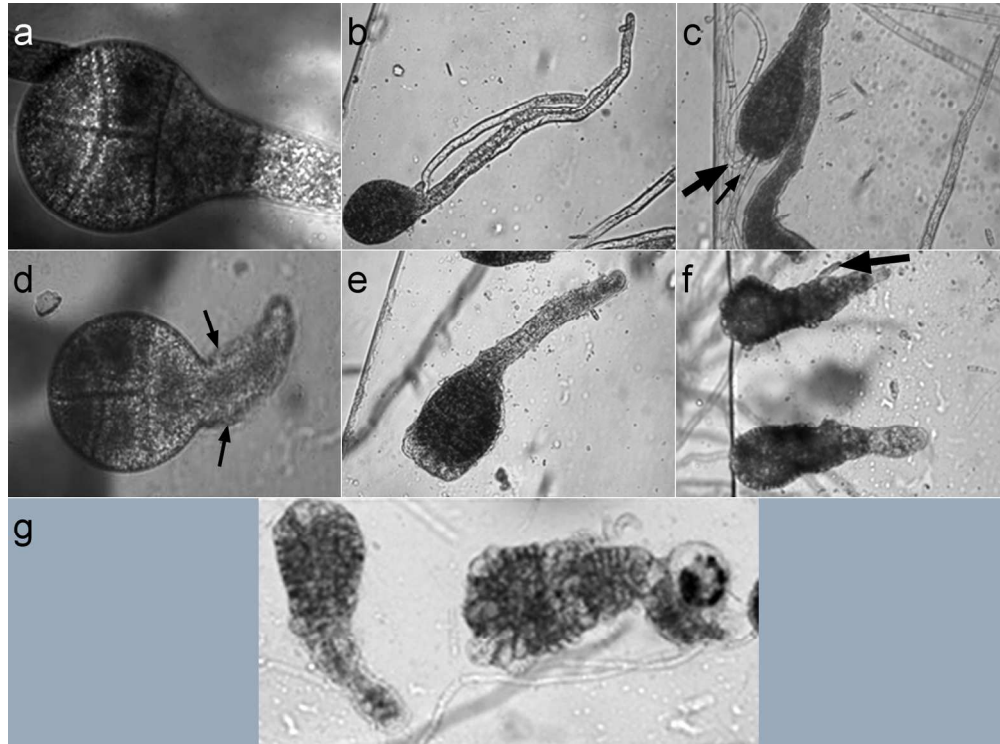


Figure 6. Progression of the dsRNA:actin phenotype in *F. serratus* over 12 weeks. Embryo development post injection (PI) of zygotes with dsRNA:control (a, b, c) or dsRNA:actin (d, e, f, g) 48h PI (a, d), 21 d PI (b, e), 6 weeks PI (c, f) and 12 weeks PI (g). The first evidence of altered development in dsRNA:actin injected embryos was a reduction in the length and thickening of the rhizoid (indicated by arrows in (b)) by 48 h PI. At 21 d dsRNA:actin injected embryos contained many enlarged cells and shortened rhizoids (e) compared to dsRNA:control embryos (b). At 6 weeks embryos were still viable, producing occasional adventitious rhizoids (arrow in (f)). Apical hairs, apparent in dsRNA:control injected embryos (arrows in (c)) were always absent in dsRNA:actin injected embryos (f). Distended cells are apparent in dsRNA:actin injected embryos. By 12 weeks several of these distended cells visibly bulged out from the periphery of the dsRNA:actin injected embryos (g). At 12 weeks dsRNA:actin injected embryos showed little further rhizoid elongation compared with 21 d or 42 d embryos.

674x499mm (72 x 72 DPI)

ript

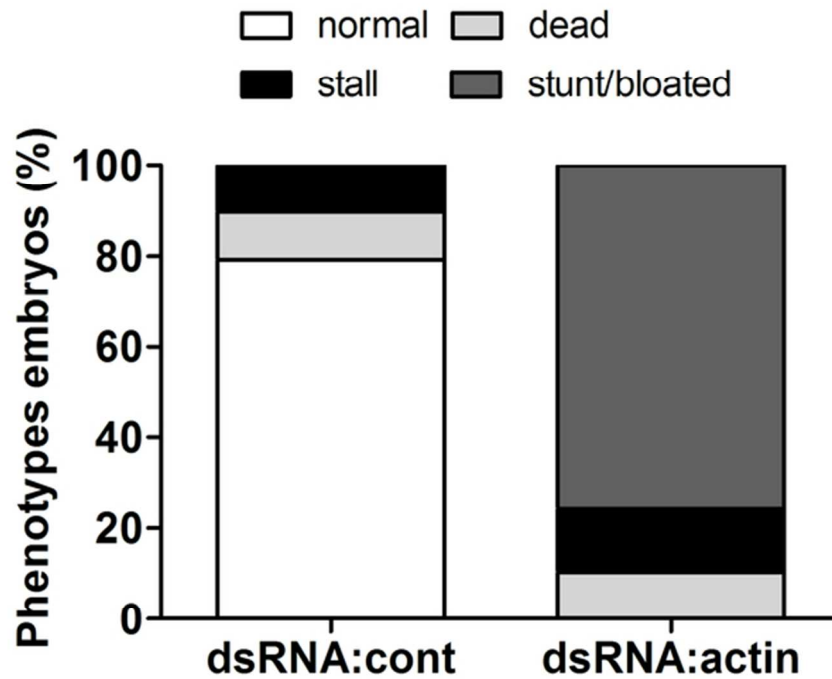


Figure 7. Relative numbers of phenotypes of *F. serratus* embryos at 14 days after microinjection with either dsRNA:control or dsRNA:actin. Embryos were scored according to the following criteria. Normal development compared to uninjected embryos of the same age (normal), failure to develop beyond initial polarisation and second cell division (stall), failure to polarise and undergo the first cell division (dead), thickening of the basal rhizoid at 48 hours, failure to elongate after 72 hours and development of enlarged distended cells, referred to as bloated cells, by 14 days (stunt/ bloated phenotype). The results are shown as a percentage of embryos exhibiting each phenotype using data derived from 4 (dsRNA:actin) and 5 (dsRNA:control) independent experiments, respectively (n=106 embryos).
59x44mm (300 x 300 DPI)

script

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+++++ (MB1)
TC_00471   TCGGGTATCAACTACCAGCCGCCACGGCCGTTCCCGGGTGACCTTGCCCGAGTTCAG 60
TC_00024   TCGGGTATCAACTACCAGCCGCCACCGTCGTGCCTGGCGGTGACCTTGCCCGAGTTCAG 60
*****

TC_00471   CGCGCCGTGTGCATGGTGGCCAACACCACGGCCATCGCCGAGGCTCTCTCGCGCATCGAC 120
TC_00024   CGCGCCGTGTGCATGGTGGCCAACACCACGGCCATCGCCGAGGCTCTCTCGCGCATCGAC 120
*****

TC_00471   CACAAGTTCGACCTCATGTACGCCAAGCGTGCCTTCGTGCACTGGTACGTGCGGTGAGGGC 180
TC_00024   CACAAGTTCGATCTCATGTACGCCAAGCGTGCCTTCGTGCACTGGTACGTGCGGTGAGGGC 180
*****

TC_00471   ATGGAAGAGGGCGAGTTCTCGGAGGCTCGTGAGGATCTTGCTGCGCTGAAAAGGACTAC 240
TC_00024   ATGGAAGAGGGTGTGAGTTCTCGGAGGCTCGTGAGGATCTTGCCGCTCTGAAAAGGACTAC 240
*****

TC_00471   GAAGAGTTCGGAGCCGAGACCGCTGATGGTGACGGCGAGGAGGAGGACTTCGGAGAGGAG 300
TC_00024   GAAGAGTTCGGAGCAGAAACCGCTGAGGGCGAGGGCGAGGAGGAGGACTTCGGAGAGGAG 300
*****

      XXX
TC_00471   TACTAAGCT----GCAACAAATGCCGTGGCTGATTATAT-ATGGCAGTGAAA---- 349
TC_00024   TACTGATCATATCAGCATCCAAC-TCGTGAAGTTGTCCCTGACGGCAAACCTTT 359
*****

TC_00471   TGCTGCCGGTTGAG--AGCAACGTTGTGCTCGTATTACGGATGATGTACACCGATG--T 405
TC_00024   TGTTTCTGTTGGGTTAGGAGTAAATGCC---TTTCGAAAGGTGTTTGTCCGACGCCT 416
*****

TC_00471   AGAGGATGCCCCAATACTTGGCGTTTC----CCCGT--CATAC-----GCGA-TGTG 450
TC_00024   ACACAGTGCATGCAAAATTTGTAGTGTTCGAGTCTTGTGGCATACAAAATCACGGCGAATGTG 476
*****

TC_00471   GAAGT-----GTCAGAACA-----TGATTCGGG 473
TC_00024   CAGGATTTTGGTAAAAACAGAAACGGGATGGGAGGAAGCGGAAGCGGTGCTGTTTTGGC 536
*****

TC_00471   ---TAATTGTC-----TGGAAGCCT-----AGG-----CAACGC 499
TC_00024   CTGTAATTATTAATAAATAAATAAATAAATAGATGTTTTCAGTAGGAGTCTCACCACGT 596
*****

      +   +++  ++++++ (MB2)
TC_00471   GCGGGGATTTGCTT---GCGCATC--CTA--TGCTATGAGAAGCTGTA 540
TC_00024   GGCATCGTTTCGCTTCTGACAAATCGATCAGTTGCTGGGAAGTGGTTCG 644
*****

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Figure S1: CLUSTAL 2.1 multiple sequence alignment of α -tubulin sequences from a *F. serripes* EST data set (Pearson et al. 2010). The alignment shows the region of TC_00471 and TC_00024 between the MB1 and MB2 primer binding sites (indicated by '+' above the sequence in the 5' and 3' regions of TC_00471). In the predicted protein coding sequence of TC_00471 and TC_00024 there are 15 nucleotide substitutions in 333 bases from the MB1 primer binding site to the predicted stop codon (Indicated by 'XXX'). The region spanning the stop codon to the MB2 primer binding site in TC_00471 is less well conserved and identity is low at the MB2 primer binding site.

MB3 (f)

cactcttgactccaccttcccccaagaaccctccagctctcttttagaacaaaaactttatca

ACTFS1 (f)

gagaaca**ATGgcgacgaggacgtgcaa**gctttggtggtggacaacgggtccggcatgtgcaaggccg
gcttcgccggtgacgacgcgcgcgtgcccgttttccctccatcgtgggtcgccccaaagcaccgccgga
atcatggtcggcatggaccagaaggatgcctacgtgggacgagggcccagtcacaagcgtggtgttct
tactctcaaataccatcgagcacggcatcgtgaccaactgggacgacatggagaagatttggcacc
acaccttctacaacgagctccgtgtagccccgaggagcaccctgtttgctcacggaggccccctc
aaccacaaggccaacaaggagcgcgatgactcagatcatgttcgagacctcaacgtgcccgctatgta
cgtcaacatccaggccgttctctccctctacgcctccggctcgtaccaccgggtgtgtgctcgattcgg
gtgacggagtgtcccacacg

ACTFS2 (r)

gtgcccatacagaggggtacgctctacccccagcaatcaaccgc**ctcgacctcgccgggctg**acct
gaccgataacctcatgaagggttttgaccgagcgtggttactccttcacgaccaccgcggagcgcgaga
tcgttcgcgacatcaaggagaagctcacctacgtggcgctggacttcgaccaggagatgaagacggcc
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ctatcctcgcgctcgtgtccactttccagcaaatgtggatttccaaggcagagt

MB4 (r)

acgatgagctctggcccgtccatcgtt**caccgcaagtgtctcTAA**aagccgaattccagcacactggcg
gccgttactagtggtaccgagctcggtagccaagcttggcgtaatcatggatcatagctgtgtgctagtt
ggtttgctaaagaacccccctccagggggggggggggtattgtcaccttaagactttttgata

Figure S2: Sequence of the *F. serratus* β -actin mRNA that was used to construct templates for the generation of dsRNA in this work. The sequence includes the 69 nucleotide 5'UTR region (underlined), the complete coding sequence delineated by ATG (Start) and TAA (Stop) codons shown in capitalised text and the 157 nucleotide 3'UTR region (underlined). The binding sites of forward (f) and reverse (r) primers used to generate the template sequences for the 551bp (Actsf1 and Actfs2) and 1200bp (MB3 and MB4) dsRNA actin constructs are shown in bold text.

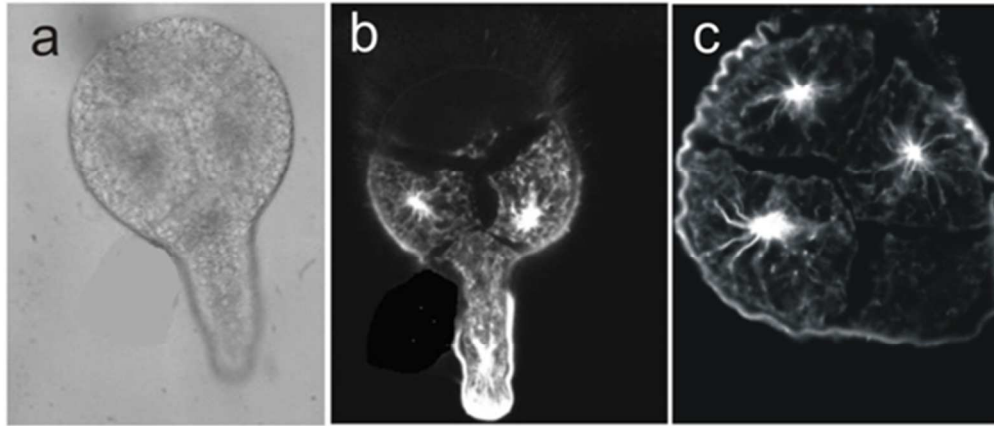


Figure S3. Labelling of α -tubulin in putative polyspermic *F. serratus* zygotes. (a) Brightfield image of a polyspermic embryo. (b, c) Representative α -tubulin labelling in similar zygotes.