

Locating The Sites of Active Plastids in Coralline Algae

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Abstract

Coralline algae (Corallinales) are ecologically important producers found amongst a variety of marine habitats across the globe. Their secreted calcium carbonate cell walls and the distribution of some families at deep water depths calls for a closer examination into coralline anatomy and physiology. This study located plastids across crustose and articulated morphologies using laser scanning confocal microscopy and scanning electron microscopy. Phycocyanobilin and chlorophyll a fluorescence was highest in cortical and meristematic regions in both crusts and fronds, sometimes in two fluorescent bands. These findings challenged conventional definitions for *Calliarthron* medulla and cortex layers and were re-defined accordingly. Extending this work across the taxon of Corallinales may provide a unifying definition for medulla and cortex, and plastid roles in medullary cells may reveal more efficient photosynthesis adaptations than previously thought.

Abbreviations

Chl a = Chlorophyll a

FB = Fluorescent band

HB = Hypofluorescent band

IFB = Inner fluorescent band

LSCM = Laser scanning confocal microscope

OFB = Outer fluorescent band

SEM = Scanning electron microscope

SFB = Solitary fluorescent band

PAR = Photosynthetically active radiation

PC = Phycocyanobilin

PE = Phycoerythrobilin

PSI = Photosystem I

PSII = Photosystem II

UC = Unidentified crust

Introduction

Coralline algae (phylum Rhodophyta, order Corallinales; also referred to as corallines) are benthic primary producers that secrete calcium carbonate (CaCO_3) skeletons from their cell walls.¹ Corallines are important habitat engineers, serving as a nursery habitat for several animal taxa (e.g. gastropods² and bivalves³), and greatly contribute to the biodiversity of coral-rich habitats.⁴ Coralline algae's ability to form a calcareous layer also makes them valuable when studying paleoclimates, as their internal Mg/Ca ratios correlate to surrounding ocean Mg/Ca ratios.⁵

The wide distribution of corallines has inspired concern over their potential to dissolve as ocean acidification increases^{6,7,8} and sea temperatures rise.^{8,9} Recent research has begun

exploring coralline algae's ecological roles and physiology,⁸ though less is known about what adaptations allow for their unique ability to grow in deeper and darker environments compared to other red algae taxa. Corallines have been found to grow at irradiance levels less than 0.001% irradiance levels,¹⁰ an especially intriguing phenomenon considering the calcium carbonate covering secreted by coralline cell walls. The success in colonizing these unique niches is generally attributed to slow growth rates and red algae's more efficient light-capturing phycobilisomes, light-capturing protein complexes unique to red algae and cyanobacteria.¹¹ However, researchers have only just begun to investigate their light-capturing physiology.¹² Specifically, anatomical locations of photosynthesizing plastids within coralline algae has yet to be fully identified past epithallial cells,¹³ (non-calcareous cuticle cells produced from an intercalary meristem)¹⁴ and vegetative cells in emerging reproductive organs¹⁴ despite the presence of red pigments in perithallial regions as well. Since photosynthetic pigments such as phycoerythrin (PE), phycocyanin (PC), and chlorophyll a (chl a)^{12,15} exhibit autofluorescence,^{12,15} analytical methods capable of detecting these pigments may be used to locate photosynthetically active plastids.

The aim of this study is to identify the anatomical locations of photosynthesizing plastids in coralline algae via autofluorescence of phycobilisome pigments PE, PC, and chl a, especially in upright vs crustose morphologies. Understanding coralline algae's light-capturing physiology leads to a better understanding of life in extreme environments, as low light exposure for any autotroph is a challenging and unique niche to occupy. This knowledge not only expands our general understanding of the order Corallinales but challenges the field of biology's boundaries on what we consider habitable for phototrophs. As with previous research, exploring the diversity of light-capturing adaptations across phototrophs lays down a framework for future technological innovations.^{16,17,18}

Methods

I. Specimens

Seven specimens were collected across several taxa within the order Corallinales within San Juan County, WA. Two crustose-only algae were collected from a -10 m MLLW dredge at Mosquito Pass (48.3538°N, -123.1033°W). Five articulated algae were collected, two of which were from Eagle Cove (48.4609°N, 123.0310°W; +0.13 m MLLW), two from Deadman's Bay (48.5131°N, 123.1464°W; -0.08 m MLLW), and one from Argyle Creek (48.5216°N, 123.0135°W; -0.33 m MLLW) Upon this study's completion, specimens were preserved with aerosol epoxy and catalogued for future DNA analysis.

II. Preparing for Microscopy

Specimens were air-dried for one day minimum on lab benches in ambient, uncontrolled air and temperature conditions immediately upon return to the laboratory. Six specimens were decalcified with 0.5 M EDTA solution by full submersion for four days maximum, 6 hours minimum (depending on time available), and three were left calcified. Decalcified specimens were subsequently rinsed and air-dried in the same fashion as the pre-decalcification air-drying for half a day minimum.

Upon drying, specimens were cut using a thin razor both transversely and longitudinally along thalli (see *Fig. 1*). Images of still calcareous sections were taken under a dissection scope

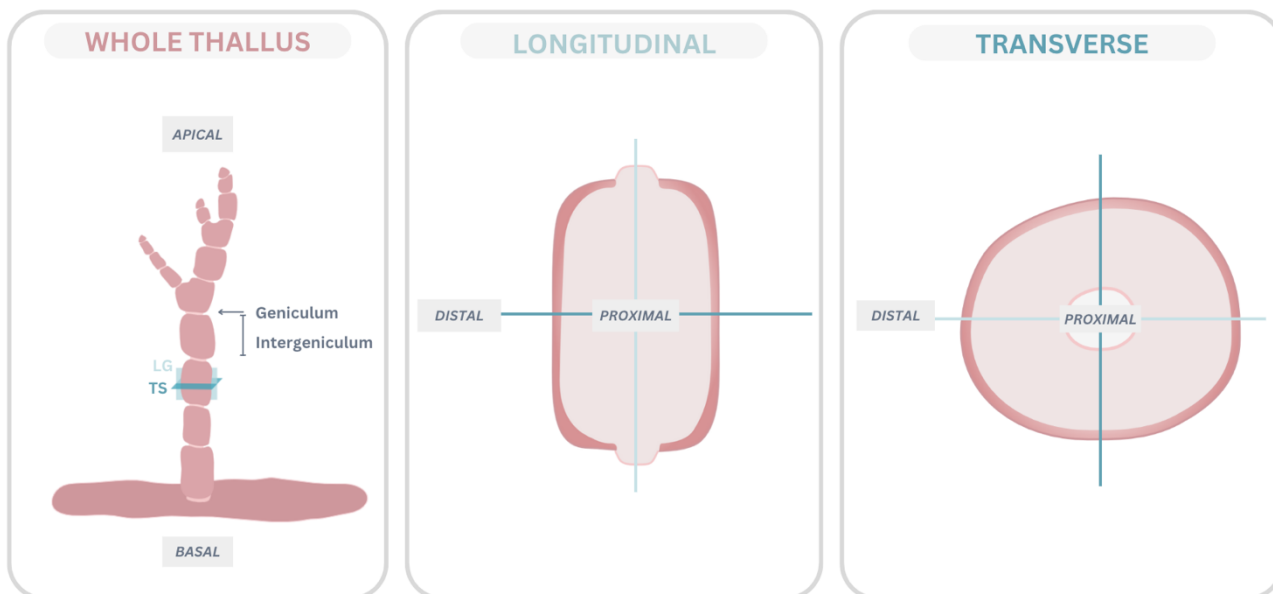


Figure 1. Thallus diagram of a generic articulated coralline alga (order Corallinales) with a crustose holdfast. Longitudinal plane (LG) is depicted in light blue, and transverse plane (TS) in darker blue. **1A.** Whole thallus of an articulated coralline, showing the genicula and intergenicula. The longitudinal plane (LG) is depicted in light blue, and transverse plane (TS) in darker blue. **1B.** Longitudinal cross section (of LG plane). The longitudinal axis is depicted in light blue, and the transverse axis in darker blue. **1C.** Transverse cross section (of TG plane). The longitudinal axis is depicted in light blue, and the transverse axis in darker blue.

LG = longitudinal plane; TS = transverse plane.

using an Apple iPhone 15 Pro. Both cross sections mounts and whole mounts of these planes were mounted on carbon disks and coated in gold with a Cressington 108 Sputter Coater (model 6002-8) for scanning electron microscopy (SEM). Separately, both types of specimen mounts were also prepared onto a microscope slide using 50% Karo® solution for laser scanning confocal microscopy (LSCM).

III. Microscopy

SEM images of mounts were taken using a JEOL Neoscope (JCM-5000), at high vacuum and a 5 kV accelerating voltage. JEOL proprietary software was used to capture images, where scale bars were provided by default. Fiji (ImageJ) was used to measure lengths of regions of interest for SEM, LSCM, and dissection scope images.

LSCM images were taken using a Nikon D-Eclipse C1Si laser scanning confocal system connected to a Nikon Eclipse C800 confocal microscope. Laser channel wavelengths used for excitation were 488 nm, 561 nm, and 640 nm.

NIS-Elements AR software was used to capture fluorescence LSCM images. Fiji (ImageJ) software was used to add scale bars, stitch confocal image stacks, and combine the three laser channels into a single composite image. Autofluorescence observed in images were assigned to photosynthetic pigments at the wavelength ranges below:

- PE (560-600 nm)¹²
- PC (620-660 nm)¹²
- Chl a (peak at 680 nm, ranges from 660-740 nm)¹²

Results

Crustose Fluorescence

In an unidentified exclusively crustose coralline (UC) showed two distinct bands of high fluorescence (FB; fluorescent band) in a decalcified longitudinal section. FBs were on the apical and basal sides, with diffused fluorescence in between (*Fig. 2*). The apical FB showed higher fluorescence compared to the basal FB.

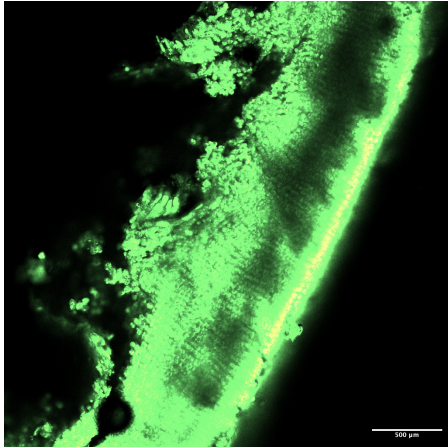


Figure 2. Unidentified crust (UC). Composite LSCM micrograph of all laser channels (PE, PC, and chl a fluorescence shown) of a longitudinal cross section, fully decalcified. The left side is basal, attaching the alga to rock substrate.

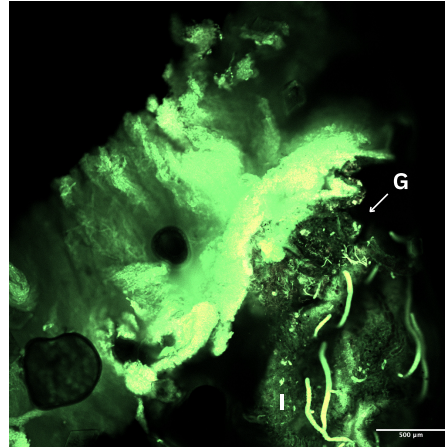


Figure 3. *Bossiella*. Composite LSCM micrograph of all laser channels (PE, PC, and chl a fluorescence shown) of a longitudinal cross section of crust with a young upright branch, decalcified. The upper left side is basal, attaching the alga to rock substrate.

G = geniculum; I = intergeniculum.

In a *Bosiella* sp. decalcified longitudinal section, the same two FBs are observed, though the basal band is much less fluorescent relative to the apical band (*Fig. 3*). The apical band in the *Bossiella* sp. also showed far higher fluorescence than the first geniculum and intergeniculum.

Articulated Fronds Fluorescence

Fluorescence was consistently greater in intergenicula than genicula in a calcified *Calliarthron tuberosum* (*Figs 4.A, 5.A*), though genicula do minimally fluoresce (*Fig. 4.A*). Growth lines are visible in LSCM micrographs of a calcified *C. tuberosum*, as fluorescence is confined to each growth line but partially connected within one line in both longitudinal (*Fig. 4.A, 5.A*) and transverse planes (both sections calcified).

Within the intergeniculum, a singular distal band of high intensity fluorescence (SFB; solitary fluorescent band) measured to be 102 μm thick (*Fig. 4.A*). The cortex of the same intergeniculum measured 272 μm thick in the SEM micrograph (*Fig. 4.C*).

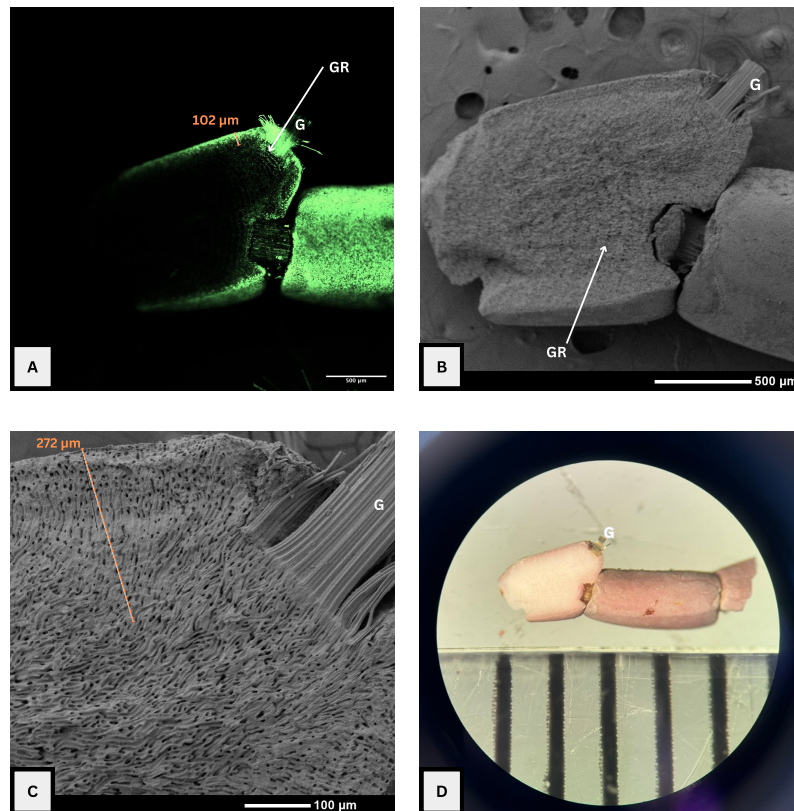


Figure 4. *Calliartheron tuberculosisum*. Longitudinal cross section of a branch intergeniculum, fully calcified. All subfigures show the same intergeniculum. **G** labels the same geniculum in all four subfigures. The labeled geniculum has an unidentified epiphytic red alga.

4.A. Composite LCSM micrograph of all laser channels (PE, PC, and chl a fluorescence shown). A growth ring is labeled as **GR**. The orange bar runs the length of the band of high intensity fluorescence, measuring 102 µm.

4.B. SEM micrograph of the same intergeniculum at a comparable scale to *Subfig. 4.A*. A growth ring is labeled as **GR** (different ring from *subfigure 4.A*).

4.C. SEM micrograph at a smaller scale, zoomed in on the cortex (note the labeled geniculum **G** for reference). The orange bar runs the length of the cortex, measuring 272 µm.

4.D Photograph of the intergeniculum under a dissection microscope. Tick marks on ruler are spaced 1 mm apart.

G = geniculum (same in all subfigures); **GR** = growth ring.

PC and chl a strongly out-fluoresced PE in all LCSM micrographs, but PE was strongest in a *C. tuberculosisum* transverse section (see *Fig. 5.A-B*). Of the minimal PE fluorescence was visible, PE was most concentrated in the outermost layers of the intergenicular cortex. Two bands of relative maximum fluorescence were observed in the geniculum, termed the outer fluorescent band (OFB) and inner fluorescent band (IFB). The OFB measured 123 µm thick, and the sum of the OFB, hypofluorescent band (HB; between OFB and IFB), and IFB measured 212 µm thick (*Fig. 5.A*).

SEM micrographs of the same intergeniculum reveal two layers with distinct morphology distal to perpendicular cortical filaments. The outermost layer was smoother and had a 78 µm maximum thickness, while the innermost was 136 µm thick at the maximum and more porous,

adding to a combined 214 μm thick region constituting the most distal region of the cortex (*Figs. 5.C-D*). Thicknesses varied around the circumference of the intergeniculum, though both maxima occurred at the same position.

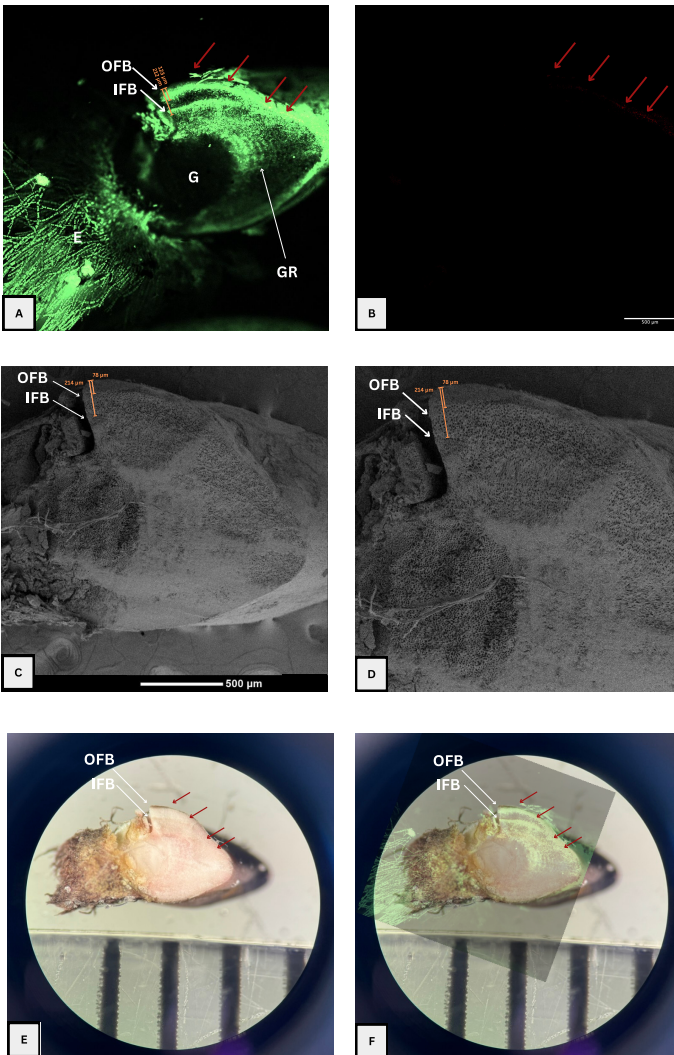


Figure 5. *Calliarthron tuberculosum*. Transverse cross section looking top down of a branch intergeniculum, fully calcified (same branch from the same individual as in *Fig. 4*). All subfigures show the same intergeniculum. **G** labels the base of the next geniculum (not fully shown). The labeled geniculum has an unidentified epiphytic red alga labeled as **E** (same sp. as in *Fig. 4*).

5.A. Composite LSCM micrograph of all laser channels (PE, PC, and chl a fluorescence shown). Two bands of intense fluorescence are labeled as **OFB** (outer fluorescent band) and **IFB** (inner fluorescent band). The OFB is 123 μm wide, while the distal end of the OFB to the proximal end of the IFB is 212 μm (shown in orange bars). Red arrows point to PE fluorescence (see *Subfig. 5.B*). A growth ring is labeled **GR**.

5.B. LSCM micrograph from *Subfig. 5.A*, but isolated for the red laser channel showing only PE fluorescence. Red arrows point to fluorescence regions for visibility.

5.C-D. Low and high magnification of an SEM micrograph of the same section. Positions of the **OFB** and **IFB** from *Subfig. 5.A* are labeled in white. The shorter orange bar spans the smoother band of cells, and is 78 μm wide. The longer orange bar spans both the smooth band and the next band of cells running in and out of the plane of the image, measuring 214 μm .

5.E. Photograph of the intergeniculum under a dissection microscope. Tick marks on ruler are spaced 1 mm apart.

5.F. Photograph from *Subfig. 5.E*, overlaid with LSCM micrograph from *Subfig. 5.A*.

G = base of geniculum (same in all subfigures); **E** = epiphytic red alga; **OFB** = outer fluorescent band; **IFB** = inner fluorescent band; **GR** = growth ring.

Due to the limited time and resources during this study, three of the specimens initially dried, sectioned, and mounted produced quality micrographs that are included here. These specimens were an unidentified crust likely from the family Haplidiaceae collected from Mosquito Pass (UC) and decalcified for four days (*Fig. 2*), *Bossiella* sp. from Argyle Cove that also decalcified for four days (*Fig. 3*), and *Calliarthron tuberculosum* from Deadman's Bay (*Figs. 4-5*).^{19,20,21}

Discussion

The Need to Redefine The Medulla and Cortex

In coralline algae, the cortex is defined by at least two primary features: (1) filaments perpendicular to the longitudinal axis (contrasted to medullary filaments in parallel with the axis), and (2) the presence of plastids (epithallium), especially within *Calliarthron*.^{13,24} These will henceforth be referred to as Type I and Type II definitions respectively, as this study demonstrates how the two regions are not synonymous. This reveals a need to re-define the cortex and medulla in the fronds of articulated coralline algae.

This author proposes the medulla to be defined by the presence of (1) growth rings and (2) filaments parallel to the longitudinal axis (Type I), within *Calliarthron* (see *Table 1* and *Fig. 6*). The medulla is easiest visualized by the region with the growth rings in both LSCM and SEM micrographs, (*Figs 4.A-B, 5.A*), and is met distally by an intercalary, dividing in both directions inward and outward directions.^{1,14,22,23}

Table 1. Proposed Definitions for Medulla and Cortex Tissue Layers for *Calliarthron*.

Summary of medulla and cortex characteristics observed, with associated figures where characteristics are observable. Non *Calliarthron* micrographs were included if characteristics were still present.

Term	Defining Characteristics	Figures for Reference
Medulla	Filaments that run parallel to the longitudinal axis.	Fig. 4.B, C.
	Growth rings.	Fig. 5.
Cortex	Filaments that curve from off of longitudinal axis, eventually running perpendicular to the axis more apically.	Fig. 2. Fig. 3.
	Fluorescent band (local maximum) at most proximal boundary; begins at IFB if multiple fluorescence bands present.	Fig. 4.B, C.
		Fig. 5.A, F.

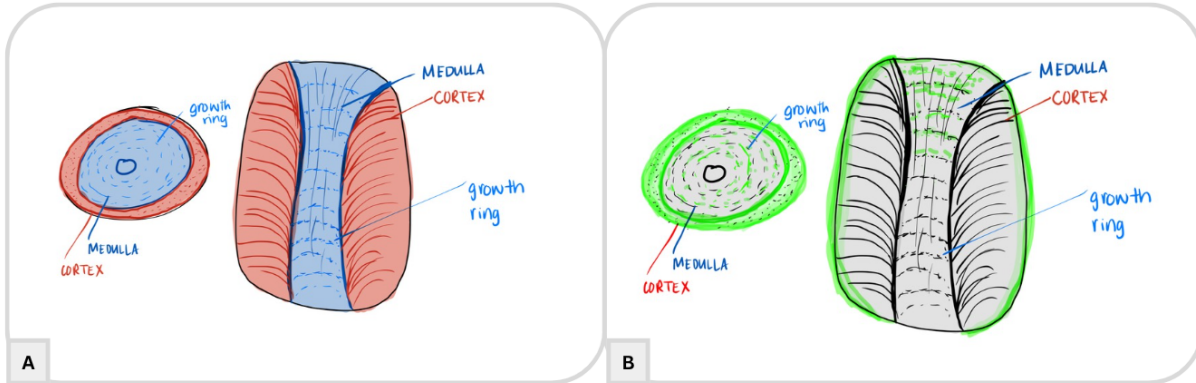


Figure 6. Illustration of proposed definitions for medulla and cortex. In both subfigures, the transverse cross section is featured on the left, and the longitudinal cross section on the right. See *Table 1* for accompanying summary.

6.A. Anatomy of cross sections both cross sections highlighting filament directions, consistent with the Type I definition. The blue highlighted region represents the medulla, and the red highlighted region the cortex. Both filaments and growth rings are shown.

6.B. Proposed fluorescence definitions, inconsistent with the Type II definition. Regions of fluorescence are indicated by green, imposed *Subfig. 6.A*. Note the fluorescence gradient in the cortex, the bright fluorescent band meristem separating the medulla and cortex, as well as the dispersed fluorescence along growth lines.

Growth Rings

By this scheme, the inner fluorescent band (IFB) of *Fig. 5* would correspond to a series of close-together growth rings and/or the cortical meristem. SEM micrographs rule out the former, as no growth rings are apparent in the IFB region in *Fig.s 5.C-D*. Therefore, the most proximal cortical meristem may be identified IFB, meaning it is capable of high photosynthetic activity.

Bands of High Fluorescence: Two vs. One

The outer fluorescent band (OFB) intensity and location aligns with the high plastid density¹⁴ and distal location^{13,14} of the epithallium, however, epithallial cells were likely missing from the micrographs as they tend to erode off during both drying and SEM preparation.²³

Not all regions of intergenicula have two FBs as in *Fig. 4*, however. In *Fig. 5*, both one and two FBs occur, visible from the same transverse cross section. Considering that in the latter case, (1) the two FBs taper off into a singular band, (2) the IFB+HB+OFB (HB = hypofluorescent band) region spans about the width of *Calliarthron* genicula, (3) the IFB+HB+OFB region was located opposite to the winged end, and (4) intercalary meristems have been known to split off and create an apical meristem when about to initiate a new branch,^{14,23} the OFB likely corresponds to an apical meristem. Though the OFB-labeled band would likely include several tiers of cells produced by the apical meristem as well as the meristem itself, the OFB to measure 123 μm thick.^{14,23}

Both the OFB in *Fig. 5* and the SFB in *Fig. 4* are comparable in size, with 123 μm and 102 μm thickness respectively. Some variation is expected between intergenicula, and a 21 μm difference is more than reasonable for a natural cortex thickness variation as each cortical cell varies by 25 μm .²⁴ The OFB+HB+IFB collective band thickness was about the same as the two cortical layers described in *Fig.s 5.C-D*, differing by 2 μm . This difference is likely due to low

precision measurements, and therefore the OFB+HB+IFB collective band likely constitutes the cortex.

Regions of Low Fluorescence

Though the cortex is thought to have plastids, literature does posit that “the number of plastids per cell decreases from the periphery toward the interior cell, and they become smaller.”²⁴ When applying this study’s definitions for cortex and medulla, this claim is supported, but misleading. Fluorescence does indeed taper proximally but may be found to increase again in both crustose and upright morphologies (tapers basally for crustose; *Figs* 2, 5.A). To add another layer of complexity, fluorescence is still observed in medullary cells, though minimally (*Fig. 4.A*).

Surprisingly, there is an abrupt fluorescence drop off in the more light exposed HB than in the more light-shielded medulla in *Fig 5.A*. The HB therefore likely relies on translocation than endogenous photosynthesis, which is supported by previous research noting a higher density of starch bodies in the medulla than the starch bodies in the epithallium.^{14,24} Further investigation into the translocation within the intergenicular tissue layers would be welcomed.

Future Directions

The presence of the HB suggests two fascinating physiological implications: (1) more light-shielded medullary meristem cells likely rely primarily on their own photosynthesis for sugars, rather than on translocation²⁵ from more outward-facing (and thus more light-exposed) cells; and (2) photosynthetic pigments, and likely the plastids themselves, are either being recycled, assimilated to distal cells, or both.

Future investigations benefit from uncovering (1) whether these HB cells lose entire plastids as they grow, or if plastids just become inert/lose functional pigments but remain in cells; (2) if translocation sources and sinks differ significantly in crustose vs upright morphologies; and (3) how much photosynthesis is occurring in medullary cells within growth rings, and (4) whether medullary cell pigments are functional or vestigial. These findings offer a hopeful outlook for parameterizing the different tissue layers in Corallinales by fluorescence, as ubiquitous definitions for corallines are a challenge of the field. The presence of medullary cell fluorescence also opens possibilities to undiscovered adaptations for photosynthesis.

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