

# Chlorarachniophytes form light- and Arp2/3 complex-dependent extensions that are involved in motility and predation

Long protrusions from several microalgal species appear to help cells move, capture prey, transport mitochondria and chloroplasts, and more. Are they filopodia that evolved abilities more like other actin- or microtubule-based structures, or are they something new?

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## Purpose

Many algal species have divergent cytoskeletons, lacking key components found in other eukaryotes. We are looking at a class of amoeboid algae called chlorarachniophytes (Greek for “green spider”) to explore how they carry out actin-based functions with divergent actin-binding proteins.

We observed intricate, arm-like extensions in *Bigeloviella longifila* and several other chlorarachniophyte species. We sought to understand the function and underlying cytoskeletal structure of these extensions, and found that they seem to be performing a slew of roles for which cells would typically rely on multiple different structures. They seem involved in motility, like actin-based filopodia, and require actin-interacting proteins for their formation, but their structures are much more elaborate than typical filopodia. Further, we see tubulin in the extensions, but have not yet been able to visualize actin. The extensions also carry out bidirectional molecular transport, so in this respect they seem closer to microtubule-based structures or possibly actin-based tunneling nanotubes. Finally, they also appear to sense light, capture prey, and may be involved in cell division.

Our observations may be useful to anyone who thinks about novel cell structures and functions. We'd appreciate any feedback on what you think these extensions are, and how we might further tease apart their roles.

- This pub is part of the **project**, "[Understanding the evolution of actin-binding proteins across diverse species](#)." Visit the project narrative for more background and context.

# We've put this effort on ice! ☒

## #HardToScale

While these results were intriguing, we've shifted as a company from exploring unique organisms in the lab to first developing broad-scale computational tools for prediction and *then* following up on key leads at the bench. We therefore decided to pause this particular effort.

[Learn more](#) about the Icebox and the different reasons we ice projects.

# Background and goals

Algae tend to have divergent actin-binding proteins (ABPs), but some species still carry out actin-dependent processes like motility, cell division, and predation. We are generally interested in how unique sets of divergent ABPs can accomplish these classic functions.

In reading about chlorarachniophytes, a taxonomic class of amoeboid algal cells believed to have evolved through a secondary endosymbiotic event where an amoeboid cell engulfed a green algal cell [1], we came across a species called *Bigelowiella longifila*. *B. longifila* is the founding member of the “beast” group of chlorarachniophytes, which switch between amoeboid (“crawling”) and flagellate (“swimming”) morphotypes [2][3]. Since many well-studied green microalgae swim, the fact that *B. longifila* can also crawl intrigued us to learn more about its cytoskeleton and regulators. Once we sourced the organism and did some preliminary imaging under the scope, we noticed prominent, arm-like extensions projecting off from the cells.

These projections have been described previously. Multiple chlorarachniophytes in this class develop web-like cytoplasmic networks, lending the nickname “green spider” (*chlor-arachnio*) algae. The amoeboid cells are reported to form filopodia up to 500 µm in length [2]. Filopodia are thin, actin-based protrusions involved in cell motility [4]. However, to our knowledge, the only published evidence that the structures observed in *B. longifila* are actin-based comes from immunofluorescence of similar pseudopodial structures in the related species *Cryptochlora perforans* [5].

As we continued watching *B. longifila* and other chlorarachniophytes under the scope, we saw the extensions participating in a variety of unexpected behaviors. They seem involved in motility, prey capture, molecular transport, and possibly environmental sensing and cell division. We are actively exploring these roles and trying to tease apart whether these extensions are a novel type of protrusion or perhaps filopodia or another known structure that has gained additional functions. Here, we describe our initial observations and experimental insights into the extensions of several chlorarachniophyte species.

# The approach

Not interested in methodological details? Jump straight to our [Observations](#) or [Experimental results](#).

We imaged *B. longifila* and other chlorarachniophyte cells at high spatial and temporal resolution and stained for a handful of subcellular structures to get a better sense of what functions their extensions carry out. We also performed experiments to explore the light-dependence and cytoskeletal basis of extension formation and dynamics.

## Materials and methods

### Strain maintenance

We obtained *Bigelowiella longifila*, *Amorphochlora amoebiformis*, and *Gymnochlora* sp. cultures from the National Center for Marine Algae and Microbiota (NCMA) at Bigelow Laboratory (East Boothbay, Maine, USA) ([Table 1](#)). We obtained *Lotharella* cultures from the Canadian Centre for the Culture of Microorganisms (CCCM) at the University of British Columbia (Vancouver, British Columbia, Canada). We maintained cells in static liquid cultures of either modified Erdschreiber's Medium (UTEX), L1-Silica Media (NCMA, Bigelow Laboratories), K Medium (NCMA, Bigelow Laboratories), or Prov50 Medium (Provasoli & Guillard, unpublished) + short-grain rice (NCMA, Bigelow Laboratories) at ambient temperature in 12:12 h light:dark cycles, unless otherwise mentioned.

Species	Strain	Source	Media	Temperature
<i>Bigelowiella longifila</i>	CCMP242	NCMA	PROV-50 + shortgrain rice	16 °C
<i>Amorphochlora amoebiformis</i>	CCMP2058	NCMA	L1-Si	20 °C
<i>Gymnochlora</i> sp.	CCMP2014	NCMA	K	20 °C
<i>Lotharella globosa</i>	CCCM0811	CCCM	Erdschreiber's	20 °C
<i>Lotharella</i> sp. (LEX01)	CCCM0920	CCCM	Erdschreiber's	20 °C

**Table 1**

**Sources and growth conditions for all organisms studied in this pub.**

NCMA: National Center for Marine Algae and Microbiota, CCCM: Canadian Centre for the Culture of Microorganisms.

## Staining cells

We fixed cells according to methods optimized to observe F-actin networks in the green algae *Chlamydomonas reinhardtii* [6]. Briefly, we took ~200 µL of cells from culture and placed them on poly-L-lysine coated coverslips, allowing them to adhere for 5 min prior to washing off excess cells in 1× PBS. We then fixed cells in 4% PFA in 1× HEPES for 15 min before washing in 1× PBS. We then permeabilized cells in 80% acetone at –20 °C for 5 min, followed by a second incubation in 100% acetone at –20 °C for 5 min. We then allowed coverslips to dry out at room temperature. Next, we rehydrated coverslips in 1× PBS and then incubated in 100 µL of 0.5 nM Phalloidin Atto-488 (Sigma-Aldrich; cat. # 49409) and 100 nM MitoTracker Orange CMTMRos (Invitrogen; cat. # M7510) for 15 min prior to washing in 1× PBS. We then stained cells in 2.9 µM 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich; cat. # D9542) for 5 min. We washed the cells again in 1× PBS and then mounted on microscope slides with Fluoromount-G (Southern Biotech; cat. # 0100-01) mounting media. We performed all incubation steps at room temperature in the dark.

## Live cell imaging

We took cells from culture in their respective media and plated in microchamber slides from Ibidi or 96-well plates with #1.5 glass coverslips. We observed DNA using 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich; cat. # D9542). We attempted to observe the actin cytoskeleton using SiR-Actin (Spirochrome), SPY650-FastAct (Spirochrome), or SPY555-FastAct (Spirochrome), but were unable to observe any successful staining. We visualized mitochondria using 100 nM MitoTracker Orange CMTMRos (ThermoFisher Scientific; cat. # M7510). We attempted to observe microtubules using SiR-Actin (Spirochrome) or SPY555-Tubulin (Spirochrome) but were unsuccessful. Tubulin Tracker Green (ThermoFisher Scientific; cat. # T34075) was more successful when we used it according to the manufacturer's protocol. Briefly, we washed cells in fresh media, treated with 1× Tubulin Tracker Green with Pluronic F-127 for 30 min, washed again in fresh media, and immediately imaged. We imaged chloroplasts by taking advantage of their autofluorescence when excited with a 640 nm laser.

## Drug treatments

We cultured *B. longifila* cells in PROV50 + rice and collected them at 1150 × g for 5 min. We washed the cells three times in fresh Erdschreiber's medium, resuspended in 200 µL of Erdschreiber's medium, and transferred to poly-L-lysine-coated 96-well plates. There, we treated them at a final concentration of 1–2.5% DMSO, 10–25 µM latrunculin B, 100–250 µM cytochalasin D, 100–250 µM SMIFH2, 100–250 µM blebbistatin, 100–250 µM CK-666, or 100–250 µM CK-689. Cells were allowed to rest for 3 h prior to adding paraformaldehyde to a final concentration of 4%.

## Light dependence

We took cells from culture and collected them at 1150 × g for 5 min before washing in fresh medium. We then resuspended cells in fresh medium and plated in either 8-well microchamber slides or 96-well plates. We grew cells under 12:12 h light:dark, 24:0 h light:dark, or 0:24 h light:dark (in a closed cabinet) conditions. We allowed cells to grow for 30–48 h prior to imaging.

## Microscopy

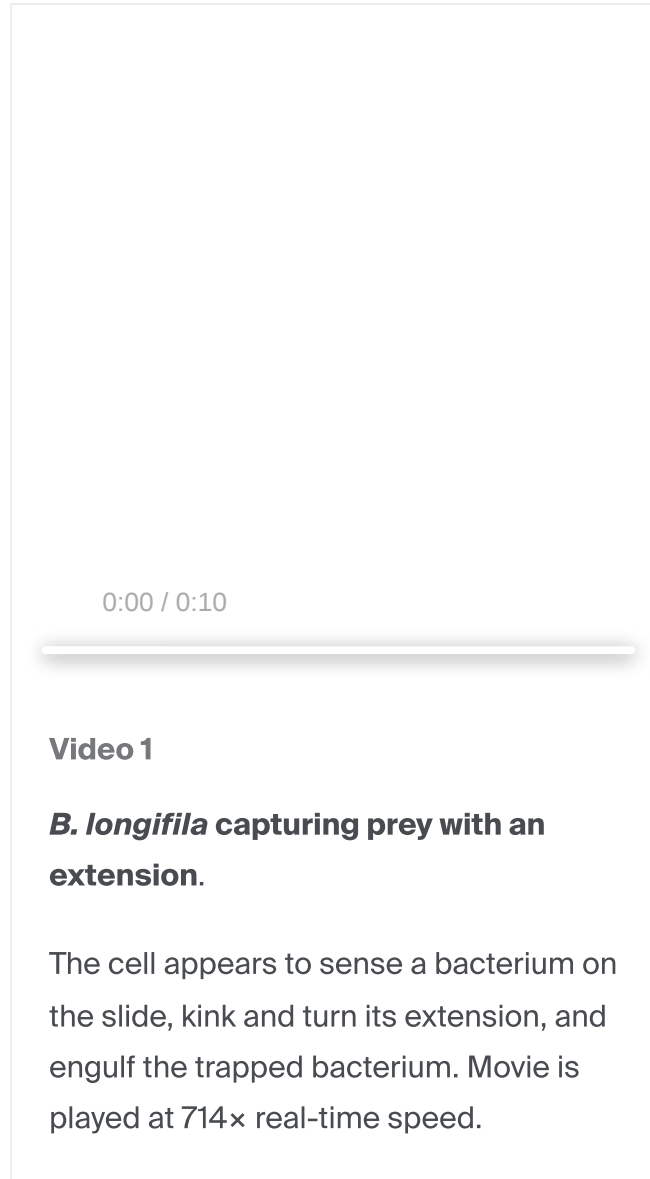
We acquired micrographs with a Yokogawa CSU W1-SoRa scanner unit attached to a Nikon Ti2-E confocal microscope. We acquired fluorescent images using a 100× 1.35 Plan Apo Silicone objective and acquired DIC and brightfield images using either a 100× 1.35 Plan Apo Silicone objective or a 40× 0.95 Plan Apo Air objective. We acquired Z-stacks in 0.2  $\mu\text{m}$  slices using either the Ti2-zDrive or a Piezo Nano-ZDrive. The microscope was equipped with an ORCA-Fusion BT digital camera that we used to acquire all fluorescent data and monochrome brightfield or DIC images. We acquired the DIC images shown in RGB color on the same system equipped with a Nikon Digital Sight 10-Color CMOS camera.

## Image analysis and statistics

We used Fiji software for all quantification [7]. Since these cultures are non-axenic, we counted cellular extensions manually using the Fiji Cell Counter tool to avoid bacterial artifacts being counted as extensions. We determined statistical significance using ordinary one-way ANOVA for multiple comparisons with GraphPad Prism software version 9.4.1 (458) for Mac.

# The results

## Observations



0:00 / 0:10

**Video 1**

***B. longifila* capturing prey with an extension.**

The cell appears to sense a bacterium on the slide, kink and turn its extension, and engulf the trapped bacterium. Movie is played at 714x real-time speed.

Initial imaging of non-axenic *Bigelowiella longifila* CCMP242 revealed rapid elongation of thin, cellular protrusions extending outward. Interestingly, these filopodia-like structures appeared to carry out chemotaxis towards bacterial prey. Upon encountering the prey, the extensions fanned out and engulfed the prey, revealing a unique process ([Video 1](#)). Filopodia are known to contribute to phagocytosis by reaching, grabbing, and pulling prey to the cortical phagosome [8]. Surprisingly, we



also observed these cells preying on the plastids of lysed *Phaeodactylum tricornutum* cells ([Video 2](#)) and even smaller cells of their own species ([Video 3](#))!

0:00 / 0:11



### Video 2

***B. longifila* with an already-formed protrusion degrading the plastids of a lysed *Phaeodactylum tricornutum* diatom.**

You can see membranous ruffles at the end of the extension. Movie is played at 404× real-time speed.

0:00 / 0:16

### Video 3

#### ***B. longifila* preying on other cells of its own species.**

The *B. longifila* cell reaches its extension past the nearby *P. tricornutum* diatoms to a smaller *B. longifila* cell and “drinks” up the cell. Movie is played at 2302× real-time speed.

Many chlorarachniophyte members do not take on an amoeboid life stage. For instance, *Lotharella globosa* lacks an amoeboid life cycle stage, but an isolated strain of the same species is able to crawl, which is amoeboid-type cell motility [9]. While we were able to observe amoeboid cells in our *Lotharella* LEX01 cultures (Video 4), amoeboid cells were much more frequent in other chlorarachniophytes, including *Amorphochlora amoebiformis* and *Gymnochlora* sp. (CCMP2014) (Video 5 and Video 6, respectively).

0:00 / 0:11



#### Video 4

***L. globosa* using its extensions to move between a cluster of cells.**

Movie is played at 98× real-time speed.

0:00 / 0:10



#### Video 5

***A. amoebiformis* using its extensions to move along a slide.**

As the cell body passes through another *A. amoebiformis* cell's extension, you can observe a fraction of the extension snapping back. Movie is played at 978× real-time speed.



0:00 / 0:10

#### **Video 6**

***Gymnochloa* sp. using its extensions  
to move back and forth along a slide.**

Movie is played at 30× real-time speed.

These cells all appear to depend on their thin, protruding extensions to perform this type of motility. Interestingly, these structures may also aid in cell division, since short protrusions appear around the body of dividing cells, possibly anchoring the cells to the surface ([Video 7](#)).

0:00 / 0:10



### **Video 7**

#### ***A. amoebiformis* undergoing cell division.**

The dividing cell appears to use shorter extensions as possible anchors to aid in this process. Movie is played at 594× real-time speed.

Frequently, we observed large clusters of *B. longifila* cells with multiple protrusions extending outward. While these structures branched out into an intricate root-like system, we observed trunks of these networks as thick as 3  $\mu\text{m}$  wide! Interestingly, using DIC microscopy, we observed rapid transport of cytoplasmic molecules throughout this network of tubes ([Video 8](#) and [Video 9](#)).

0:00 / 0:10



### Video 8

**A cluster of *B. longifila* cells form a large, wide extension with rapid cytoplasmic transport visible throughout.**

This is a mixed culture of microalgae. The wide extension measures  $\sim 3\ \mu\text{m}$  in diameter and several extensions branch out of this central protrusion. Movie is played at 6× real-time speed.



0:00 / 1:01

### **Video 9**

**Zoomed-in version of the cytoplasmic trafficking in Video 8, played at real-time speed.**

To better understand the purpose of these extensions, we stained the cells with multiple live-cell dyes. While many dyes did not work under the recommended conditions, we observed strong signal from the mitochondria stain MitoTracker Orange CMTMRos and the DNA stain DAPI. Additionally, the chloroplasts in these cells autofluoresce brightly when excited at far-red wavelengths, allowing us to observe endogenous chloroplast dynamics.

0:00 / 0:05



### Video 10

#### **A single *B. longifila* cell with stained substructures.**

DNA is stained with DAPI (cyan) and mitochondria with MitoTracker Orange (yellow). We've pseudo-colored chloroplast autofluorescence in magenta. Movie is played at 7× real-time speed.

Using these stains, we observed mitochondria and DNA (likely mitochondrial DNA) being trafficked through *B. longifila* extensions ([Video 10](#)). Interestingly, the mitochondria that remained in the cell body were localized to the base of the extension. Similarly, we observed mitochondria and chloroplasts being trafficked through *A. amoebiformis* extensions ([Video 11](#)). Within the first few frames, the chloroplasts retract back into the cell body, likely because of the high light intensity, which may explain why we don't always observe chloroplasts in these extensions.



0:00 / 0:08



### Video 11

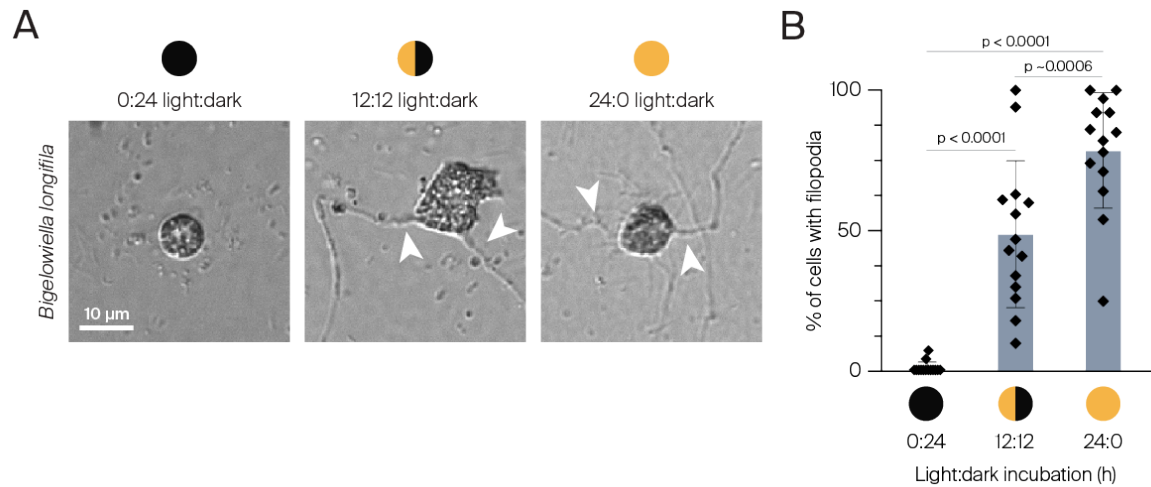
#### **An *A. amoebiformis* cell with stained substructures.**

Mitochondria are stained with MitoTracker Orange (green), and we've pseudo-colored chloroplast autofluorescence in magenta. Movie is played at 7× real-time speed.

## Experimental results

### Light dependence

Cytoplasmic streaming of chloroplasts in the macroalgal species *Chara corallina* enhances photosynthetic activity [10]. Since we observed chloroplasts in these extensions ([Video 11](#)), we wondered how light would impact their dynamics. Under standard physiological 12:12 light:dark cycles, nearly half of *Bigeloviella* cells have obvious protrusions extending from the cell body. However, when grown in complete darkness, almost all of the cells we observed lacked protrusions altogether. Conversely, over 75% of cells grown under constant light had extensions ([Figure 1](#)).



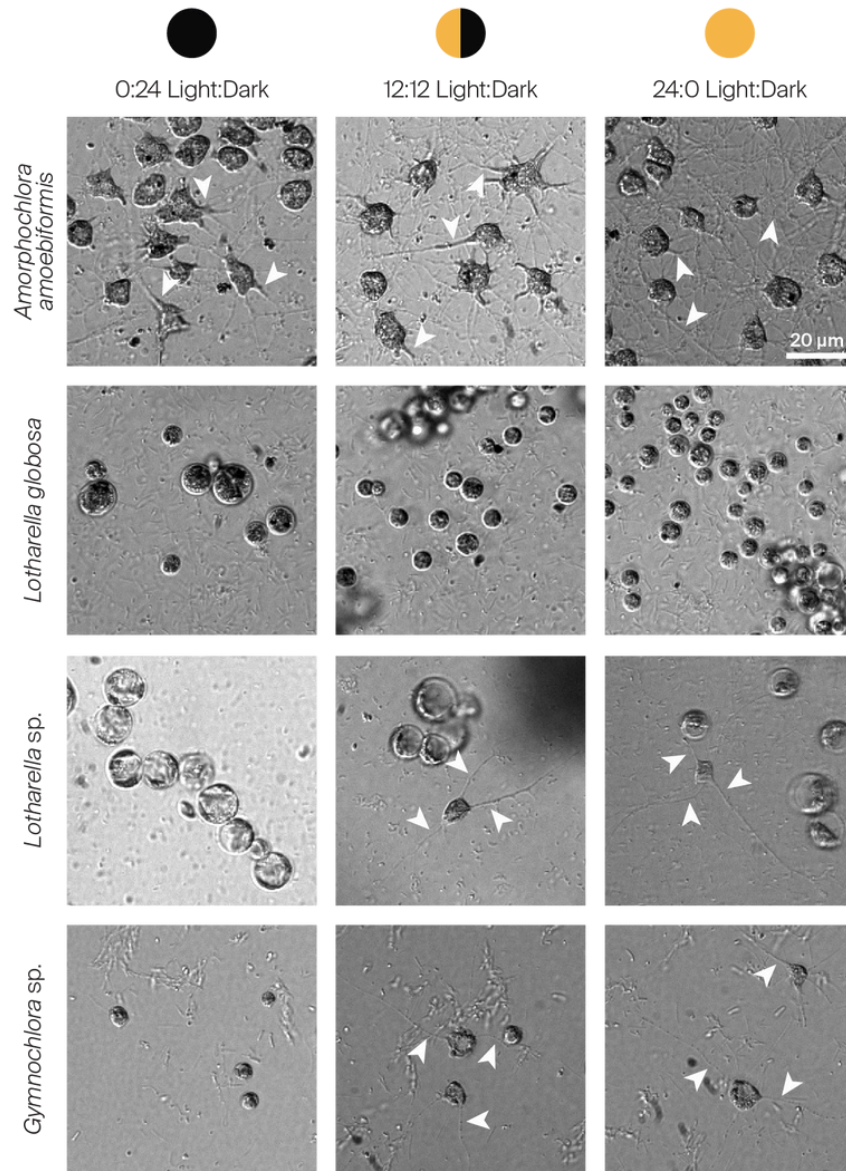
**Figure 1**

**The arm-like extensions projecting off from *Bigelowiella longifila* cells are light-dependent.**

(A) Representative images of *B. longifila* cells grown under constant darkness (0:24 light:dark), environmentally mimetic illumination (12:12: light:dark), or constant light (24:0 light:dark) for 30 h prior to imaging. White arrows indicate the presence of extensions.

(B) Quantification of the percentage of *B.* cells with one or more extensions protruding from the cell body. We collected data from 14 independent samples on two separate days. We performed statistics on the raw count values and overlaid them on the graph. Total cells counted: 349 (24:0), 505 (12:12), 609 (0:24).

Interestingly, this dependence on light appears to be conserved in the species *Lotharella* sp. (CCCM0920) and *Gymnochlora* sp. (CCMP2014); however, *Lotharella globosa* did not have extensions under any of these conditions and *Amorphochlora amoebiformis* continued to form intricate structures independent of light exposure (Figure 2).



**Figure 2**

**Multiple chlorarachniophyte species form extensions.**

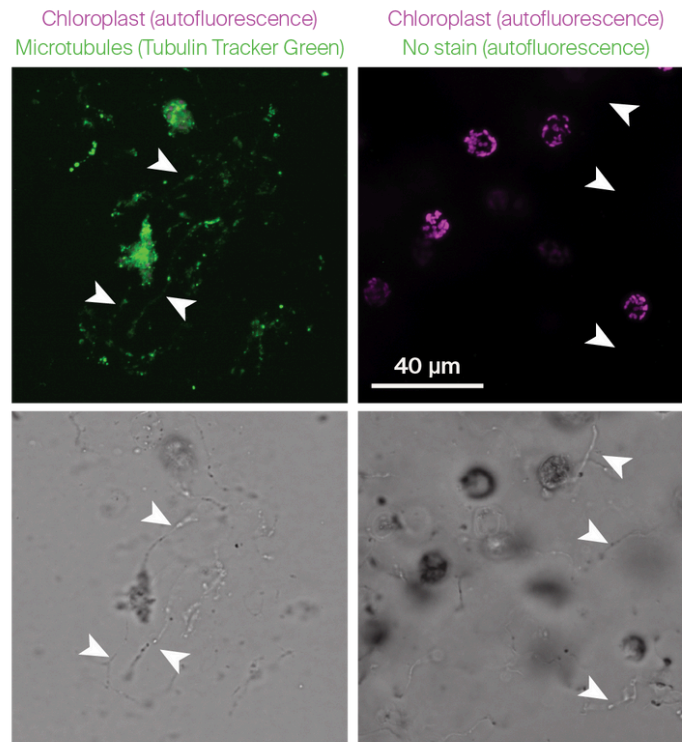
*A. amoebiformis* appears to form extensions under all light conditions. *Lotharella* sp. LEX01 CCCM0920, and *Gymnochlora* sp. CCMP2014 form extensions in light-dependent manner. *L. globosa* CCCM0811 does not form extensions.

We grew all species under the same conditions as in Fig. 1. White arrows indicate the presence of extensions.

## Cytoskeletal dependence

To determine whether these extensions are truly actin-based filopodia, as reported for *B. longifila* [2], we attempted to visualize the cytoskeleton in these structures.

Unfortunately, we have not yet been able to observe actin in these structures using standard protocols for phalloidin in fixed cells or live cell dyes such as SPY555-FastAct, SPY650-FastAct, or SiR-Actin; however, we are actively working to optimize these protocols. Similarly, we were unable to visualize the microtubule cytoskeleton with SPY555-Tubulin or SiR-Tubulin dyes, but did have some success using Tubulin Tracker Green, which researchers have used to visualize microtubule networks in other amoeboid species [11]. We observed tubulin stain in the cell bodies and by web-like networks of these extensions ([Figure 3](#)), more in line with microtubule-based structures like cilia [12][13] and axons [14] or actin-based tunneling nanotubes [15][16] than standard filopodia. We will soon test the specificity of this dye using tubulin inhibitors. In this experiment, chloroplasts were *not* visible within the extensions like they were at other times ([Video 11](#)).



**Figure 3**

**Tubulin is present in both the cell bodies and their extensions.**

Images of *B. longifila* extensions coming from a cluster of cells.

(Top row) Fluorescence images excited with 488 nm and 640 nm wavelengths.

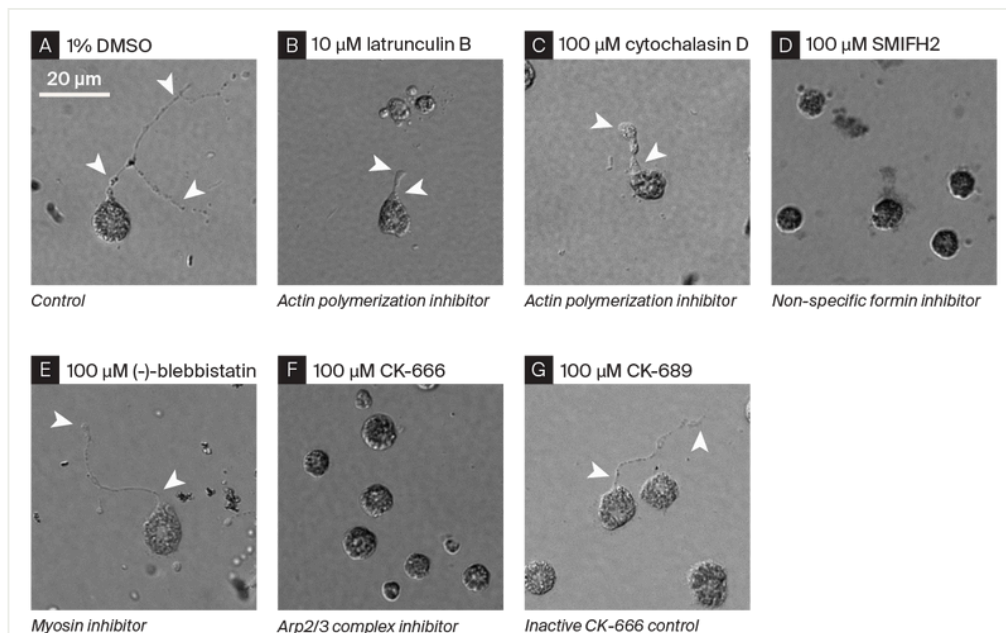
(Bottom row) Brightfield image of the same xyz plot from above.

(Left column) Cells treated with 1× Tubulin Tracker Green.

(Right column) Untreated cells.

Since we were unable to observe actin in these structures, we opted to measure the effects of actin cytoskeleton inhibitors on the initiation of extensions in *B. longifila* cells. Interestingly, treatment with actin polymerization inhibitors latrunculin B and cytochalasin D did not impact the percentage of cells that formed extensions, but did

result in shorter, stubbier extensions (compare control conditions in [Figure 4](#), A to drug treatments in B and C). Surprisingly, the only drugs that reduced the frequency of extensions were actin nucleation inhibitors SMIFH2 and CK-666 (representative images in [Figure 4](#), overall quantifications in [Figure 5](#), D and F). CK-689, the inactive CK-666 control, had no effect ([Figure 4](#) and [Figure 5](#), G). SMIFH2 targets linear microfilament-nucleating formins and CK-666 inhibits the branched-actin-nucleating Arp2/3 complex; however, recent work has shown SMIFH2 has multiple off-target effects [17], making it difficult to clearly interpret those results. Finally, the myosin II motor protein inhibitor (-)-blebbistatin did not impact the formation of these structures ([Figure 4](#) and [Figure 5](#), E), but we have not yet determined whether the rate of cytoplasmic streaming is reduced under these conditions. Although we have not observed actin in these structures, these data clearly suggest that actin, or more specifically actin nucleators, are important for extension formation.

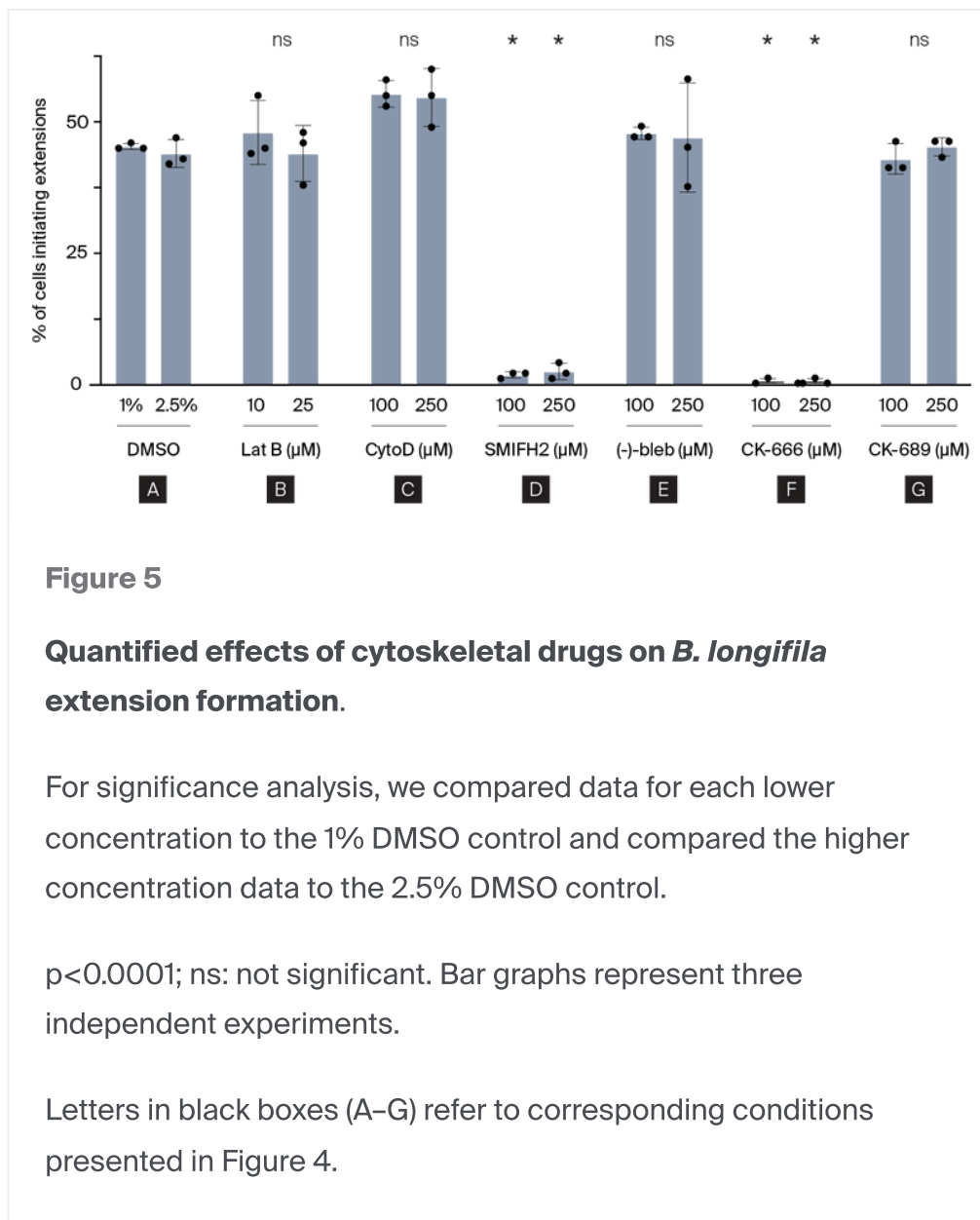


**Figure 4**

**Actin-inhibiting drugs impact extension length and formation in *B. longifila*.**

Representative images of *B. longifila* cells adhered to coverslips for 3 h in the presence of the indicated cytoskeletal inhibitors.

Letters in black boxes (A–G) refer to corresponding quantification data in Figure 5.



# Summary and conclusions

## Key findings

- *Chlorarachniophyte* cells have extensions that protrude outward and appear to participate in predation ([Video 1](#), [Video 2](#), and [Video 3](#)), motility ([Video 4](#), [Video 5](#), and [Video 6](#)), and possibly cell division ([Video 7](#)).
- These extensions also perform cytoplasmic streaming of organelles, including mitochondria and chloroplasts ([Video 8](#), [Video 9](#), [Video 10](#), and [Video 11](#))



- *B. longifila*, *Lotharella* sp. LEX01, and *Gymnochlora* sp. form extensions in a light-dependent manner. *A. amoebiformis* appears to form extensions under all light conditions and *L. globosa* CCCC0811 species does not seem to form extensions at all ([Figure 1](#) and [Figure 2](#)).
- Tubulin appears to be present in the extensions ([Figure 3](#)).
- Actin polymerization inhibitors make extensions short and stubby ([Figure 4](#), B and C).
- Formin and Arp2/3 inhibitors appear to block extension formation altogether ([Figure 4](#) and [Figure 5](#), D and F).

## Conclusions

Amoeboid algal members of the chlorarachniophyte taxonomic group form long, intricate extensions. We've observed these structures aiding in cell motility, predation, and possibly cell division. We investigated how these extensions form and discovered two major requirements for their formation: light and actin nucleators. Although we were unable to observe actin filaments in these extensions, we did observe tubulin. We also saw that chloroplasts and mitochondria are bidirectionally transported through the extensions.

Together, these observations and results suggest that cellular protrusions may perform more versatile functions than previously recognized. We're excited to delve deeper into the purposes they serve and further illuminate the underlying mechanisms.

## Reflections and outstanding questions

Tubulin seems to be in both the cell body and in the extensions of *B. longifila*, but so far, we haven't been able to visualize the actin cytoskeleton. One of the most surprising results in this study was the subtle effect of actin polymerization inhibitors latrunculin B and cytochalasin D compared to the actin nucleator inhibitors SMIFH2 and CK-666. While latrunculin B concentrations were 10-fold lower than the other inhibitors, we used cytochalasin D at the same concentration as SMIFH2 and CK-666. It could be



that *B. longifila* actin is partially resistant to treatment with these actin polymerization inhibitors if it contains a partially compensating divergent actin that doesn't bind the inhibitor, similar to the divergent *Chlamydomonas reinhardtii* actin protein NAP1, which is resistant to latrunculin B [18]. We are, in parallel, probing the presence and classification of additional actin genes across taxa, which may provide insight [19]. Given these results so far, we'd appreciate any additional ideas for probing the role of actin in the extensions.

Further, amoeba have long been known to respond to light [20]. We were intrigued to see chloroplasts trafficked within the extensions, and hypothesize that these localized chloroplasts may directly control the extensions' response to light. Maybe the extensions serve as light-harvesting organs that elongate to capture more energy when light is present, and are unable to grow when light is absent and there is reduced ATP available for actin polymerization. Or perhaps in the cell's natural environment, light correlates generally with favorable growth conditions, so it triggers cells to produce more extensions to carry out functions like prey capture or cell division. Reduced light could also spur a transition into a more dormant state, preventing the initiation of these structures.

We'd love any insights into what we should call these extensions and whether others have observed anything similar. Are they filopodia that have taken on new functions? Something new altogether? Please leave a comment if you have thoughts to share!

## Next steps

Chlorarachniophytes have fascinating biology and we will continue to use them to answer questions, but their long life cycle is a major hurdle. However, recent work uncovered that co-culturing the chlorarachniophyte *Bigelowiella natans* with the cyanobacterium *Synechococcus* sp. strain CC9311 doubles the growth rate of *B. natans* [21]. We plan to test whether co-culturing with CC9311 increases the growth rate of additional chlorarachniophytes, which would make this species much more conducive to research in a laboratory setting.

Further, the Marine Microbe Eukaryote Transcriptomic Sequencing Project (MMETSP) provided transcriptomes for all known chlorarachniophytes [22]. Recent work has re-assembled and annotated these transcriptomes into more useful versions [23]. We

are actively using these data sets to map out potential cytoskeletal regulation within these cells.

Additionally, a major roadblock for this work so far is our inability to observe actin networks *in vivo*. Another group recently developed a protocol to successfully transform fluorescent proteins into *Amorphochlora amoebiformis* cells [24]. We will adopt these tools to visualize endogenous actin-binding proteins *in vivo* to better understand actin's contributions to these structures.

Finally, in the absence of additional tools, another approach we plan to use is comparing genomic and/or transcriptomic information across multiple chlorarachniophytes and imaging in high-throughput to generate phenotypic insights. Our hope is that this effort will help us make hypotheses and uncover broad mechanisms of filopodia formation across the tree of life.

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