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Investigating pH Banding Kinetics of Chara corallina in Alternating Light Conditions with Rectangular Pulse Voltammetry

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Investigating pH Banding Kinetics of *Chara corallina* in Alternating Light
Conditions with Rectangular Pulse Voltammetry

Matthew C. Zandee

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

in

Partial Fulfillment of the Requirements

for the Degree of

Master of Science

Cell and Molecular Biology

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Dedication

This work is dedicated to my Grandma and Grandpa, Carolyn and Ed Chandler. Their constant interest in my studies and their never-ending generosity and encouragement have made this work possible. Without them, I would not be able to pursue my love of science at this level. Thank you for your love and support.

Acknowledgements

I would like to thank first and foremost my advisor, Dr. Eric Ramsson, for teaching me how to think before I speak. For guiding me, with “hands off,” and letting me learn and even struggle in the process. Many thanks to him for offering insight along the way, providing career advice, and making my graduate research experience a very positive one. I want to thank my awesome committee for being fun to work with, organized, and challenging me to be a better scientist, presenter, and student. I thank my wife, Estee, for working a “normal” job tirelessly and making it possible for me to be able to be a full time student and complete my degree. Thanks for being my thesis guinea pig and for nodding your head just the right amount as I explain my research. Thank you to my parents for their constant support and investment these last couple of years. I especially appreciated all the genuine interest and extra-curricular help I received from the many undergraduates in the Ramsson lab. Albert, thanks for your moments of brilliance, solving the inexorable questions that vexed me but made perfect sense to you. To Nick and Rhiannon for the countless electrodes I stole or borrow from you, thank you. Thank you Alex, Sami, Tyler, Drew, and Nolan, for showing such interest in my project and helping in any and every way you could. A special thanks to Dr. Zeitler, my statistics professor, as well as Keith and Ben at the Statistical Consulting Center for helping me display and analyze my data and properly describe the statistics. To the many other professors and resources that I sent emails to about plant biology or bounced my ideas off, thank you for your patience.

Abstract

Chara corallina is an important biological model due to its large internodal cells, the simple shape, and the wide range of techniques that can be used to study it. The alternating acidic and basic banding pattern of *Chara corallina* upon illumination has been studied and well described over the past several decades. However, much of this complex mechanism is not fully understood. Few studies have shown how the acidic and basic regions have responded in real time as lighting conditions change. Utilizing rectangular pulse voltammetry (RPV) and pH-sensitive carbon microelectrodes along the cell wall allows for a real time profile of the banding activity upon removal and subsequent reintroduction of an illumination source. The live current profile obtained reveals aspects of the kinetics and upstream mechanism of each banding region. The acidic bands are shown to be active at a relatively constant level, regardless of light. However, the basic bands appear to be strictly controlled by photosynthesis and, far beyond that of the acidic bands, account for the majority of the pH change in the banding phenomenon. In addition, this data reveals much about the kinetics of the basic region, namely that the time to restart banding takes significantly longer than that to stop. Understanding these rates will lead to a better understanding of the overall mechanism and the unique characteristics of each pH zone.

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List of Abbreviations

AP—Action potential

APW—Artificial pond water

APWpr—Artificial pond water with phenol red indicator dye

ATP—Adenosine triphosphate

DNS—Data not shown

LED—Light-emitting diode

NPQ—Non-photochemical quenching

RPV—Rectangular pulse voltammetry

V—Volts

nA—Nanoamps

Chapter 1

Literature Review

Overview of the Characean Banding Phenomenon

Introduction

Characean algae have for several decades been an important model in biological research due primarily to the large size of its internodal cells - up to several inches in length (Lucas and Smith, 1973a; Walker, 1960). These organisms grow in fresh water and undergo unique biochemistry, namely the formation of alternating acidic and basic bands along their cell walls. *Chara* have also been identified as carbon sinks as they absorb large quantities of environmental carbon via this banding mechanism, either through gaseous carbon dioxide or storing it in the form of calcium carbonate for proper metabolism (Lucas and Nuccitelli, 1980a). The study of these important cells has shed light on aspects of many biochemical cellular mechanisms in plants including cytoplasmic streaming, membrane conductance, photosynthesis, carbon uptake, signaling, pH-homeostasis, and others (Wayne, 1994).

Physiology

Characean algae have been one of the benchmark species used in electrochemical studies. The membrane potential can be easily measured using

microelectrodes (Beilby and Bisson, 1992; Fisahn et al., 1992; Lucas, 1979; Walker, 1960), the thin layer of cytoplasm lies along the large vacuole and allows for streaming to be easily visualized (Bulychev and Kamzolkina, 2006; Eremin et al., 2013), and these cells exhibit the classic alternating pH banding phenomenon that has been studied for decades (Beilby and Bisson, 2012; Bulychev et al., 2001).

Chara have been categorized as either corticate or ecorticate species. Corticates have a thick “bark-like” thallus, or shoot-stem region. Ecorticates, like *Chara corallina* often used in these banding studies, lack this thick layer and require more of an actively mediated boundary between the cell and its environment. Calcified encrustations on ecorticates are not seen in corticoids; however, calcification may occur on the inner layer of the internodal cell wall, beneath this thick thallus. pH banding aids in formation of calcium carbonate in the basic bands. Furthermore, calcification along the cell can influence the banding array due to their fixed position in long-acting basic bands (Kawahata et al., 2013).

Banding

pH banding is the unique alternation of acidic and basic regions along the internodal cells that occur during photosynthesis. The banding phenomenon in *Chara* evolved to maintain the pH balance across the cell membrane with the environment and create micro-environments that aid in cellular metabolism (Beilby and Bisson, 2012; Bulychev et al., 2001; Lucas and Smith, 1973; Rubin

and Riznichenko, 2014; Walker and Smith, 1977). This pH regulation was probably the precursor to many H^+ or OH^- ion transport systems. The regulation of cytoplasmic pH is crucial in maintaining a proper internal environment for metabolic processes, like proper enzymatic activity (Smith and Raven, 1979).

One of the key homeostatic aspects of banding, other than simply regulating membrane potential in changing environments, is for carbon fixation (Mimura and Shimmen, 1994). Because the natural environment of *Chara* is usually rather basic, acidifying local regions along the cell has been shown to aid in the absorption of carbon. A more acidic environment favors the presence of CO_2 , which is easily absorbed by cells and utilized by the chloroplasts during photosynthesis (Lucas and Smith, 1973a; Schmölzer et al., 2011). In the basic regions, environmental calcium reacts with carbonate to form calcium carbonate on the outside of the cell wall. This precipitate builds up in the basic bands where these ions are in higher concentrations. Therefore, older cells often have more of a crusty precipitation layer in these regions (Kawahata et al., 2013).

Although these alternating acidic and basic bands are repeatable and often uniform, there is much unknown regarding the many factors that control each region (Beilby and Bisson, 2012; Lucas, 1979). The exact mechanism of how photosynthesis controls banding is not fully understood. For instance, the acidic bands are thought to be active even in the dark, but the basic bands require light (Mimura and Tazawa, 1986). Several studies have shown a clear connection between the banding phenomenon, photosynthesis and cytoplasmic streaming, adding to the larger picture of the banding mechanism (Bulychev et

al., 2004, 2005; Dodonova and Bulychev, 2012). Banding is often observed in *Chara* kept on a 12 hour/12 hour light/dark cycle in artificial pond water (APW) or in natural light. The cells are recorded in APW solution along with an indicator dye to be able to visually discern the bands. The red bands that form reveal basic regions and the yellow bands, acidic regions (**Figure 1**). Quantification of these bands can be obtained by pH electrode readings of current (Lucas and Smith, 1973; Bulychev et al., 2001; Kawahata et al., 2013; Lucas and Nuccitelli, 1980b; Lucas and Smith, 1973; Shimmen and Wakabayashi, 2008).

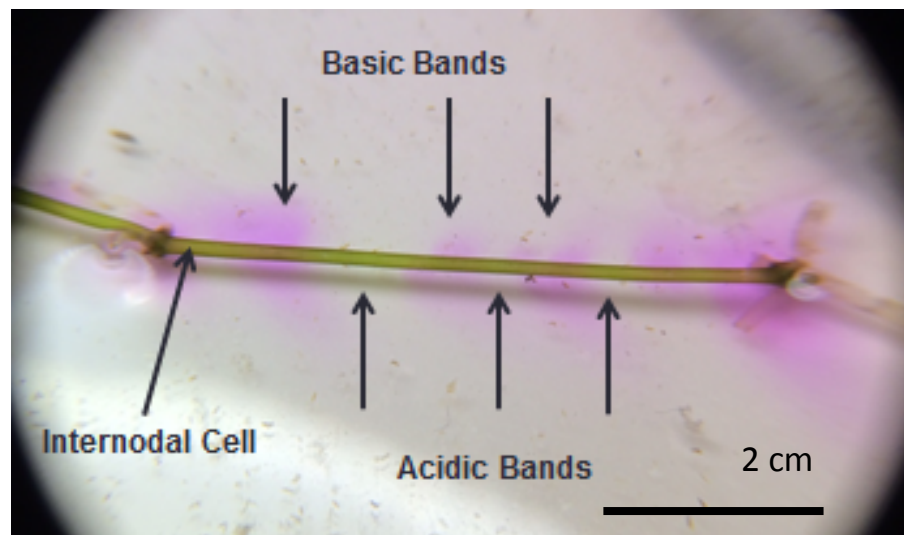


Figure 1: Alternating acidic-basic bands. Banding regions along *Chara corallina* internodal cells are visualized with 0.25 μ M phenol red indicator dye in artificial pond water (APW), pH 5.6. Bands can be seen clearly with the naked eye as full banding occurs after only several minutes in lighted conditions.

Banding Distribution

Only recently has the three-dimensional band distribution been studied in *Chara*. Bulychev et al. (2001) utilized pH microelectrodes to analyze the exact pH variation around these bands to deduce more about their three dimensional structure, function, and mechanism (**Figure 2**). Scanning along the cell (longitudinal scan) at a fixed distance of 50-100 micrometers shows the large fluctuations of pH as you go from acidic to basic bands, showing the change in pH between bands to be upwards of 2 pH units (**Figure 2a**). Scanning away from the cell (transverse scan), the pH is also drastically different (**Figure 2b**). This indicates that where you record pH banding, the distance to the cell, can significantly alter the measurement. As the electrode moves farther away from the cell wall, the signal weakens (Bulychev et al., 2001). Furthermore, upon scanning the longitudinal profile of the cell on three different sides, the authors found that these “bands” do not form closed rings of low pH (**Figure 2c**). The scans of each surface were different for the acidic regions which revealed that the region responsible for the acidic “band” was localized in a single region of the cell wall – not a constant band of low pH around this region (Bulychev et al., 2001).

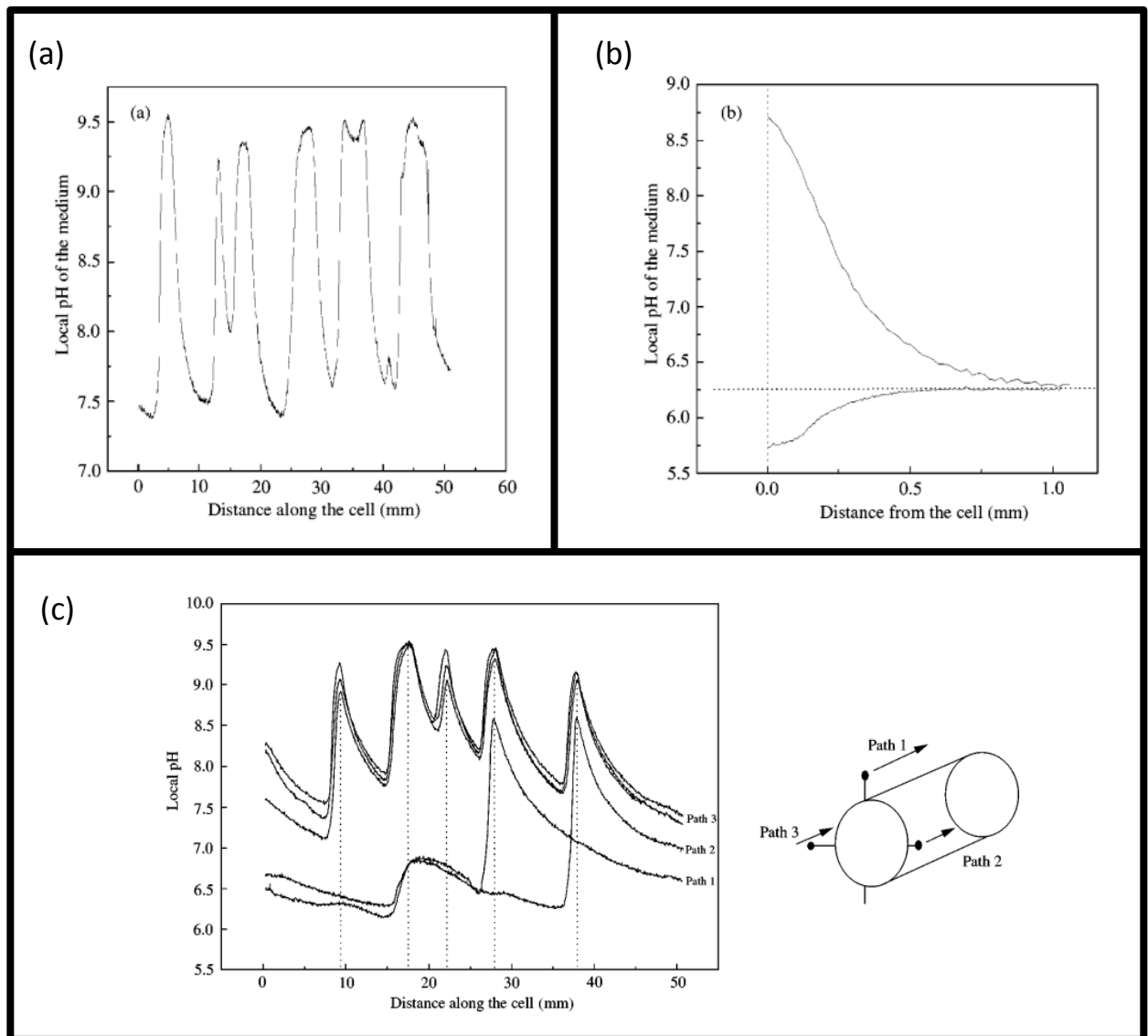


Figure 2: Banding distribution revealed by current profiles. Using antimony pH microelectrodes, pH profiles were obtained by scanning (a) along the cell (longitudinal scan) and (b) away from the cell (transverse scan). (c) The pH profiles of different paths along the cell reveal heterogenous banding regions about the cylindrical area of the cell (Bulychev et al., 2001).

Mechanisms of Banding

The many models of banding mechanisms in the literature share common players, which include bicarbonate, hydroxyl groups, and protons. They often vary in the type of transport, whether active or passive, symport or antiport, as well as the various roles solutes play in banding (Bulychev et al., 2001). The acidic region is the area in high concentration of H^+ due to an active process from the well-characterized H^+ pump driven by ATP. This region is more straightforward and the proton pump has been shown to be active even in low light and dark conditions (Mimura and Tazawa, 1986). The proton pump responsible for acidic bands has been documented and verified using rate studies and various pump-specific inhibitors (Bisson, 1986; Bulychev et al., 2001). Due to the much more consistent nature of the acidic band, the primary focus has been given to the formation of the alkaline regions.

The basic region is often referred to as an area of H^+ intrusion or OH^- extrusion and remains an area of contention in the literature (Fisahn et al., 1992; Kawahata et al., 2013; Lucas and Nuccitelli, 1980b; Lucas and Smith, 1973a; Shimmen and Wakabayashi, 2008). Much of the classical literature calls for the active pumping of both the H^+ in acidic regions and OH^- in basic regions. Lucas and Nuccitelli (1980) proposed a HCO_3^-/OH^- exchange symport in the alkaline zone, of which the carbonate reacts with the extracellular calcium to form the calcium carbonate precipitate observed in *Chara*. While acidic banding has been previously described, the mechanism of banding at these alkaline regions is

more elusive. The measurements obtained from various kinds of pH-sensitive electrodes have shown that the basic regions have a higher relative concentration of hydroxide ions, as expected. As to whether this is from the extrusion of hydroxide from inside the cell (OH^- efflux) or from the influx of hydrogen ions inward from the environment in these regions has been debated (Bulychev et al., 2001; Lucas and Nuccitelli, 1980b; Shimmen and Wakabayashi, 2008).

In the classic review on the maintenance of intracellular pH, Smith and Raven (1979) lay out the evolutionary importance of the conservation of homeostatic “pumps.” The dogma is that the H^+ fluxes, to speak generally, are due to an actively extruding H^+ zone, and a passive H^+ inward flowing zone. Chemically speaking, this active H^+ extrusion could be active OH^- intrusion as the two are functionally indistinguishable. They go on to say that convention lends itself to speaking in terms of H^+ flux, as protonated reactions are simpler. It should be noted that the authors also speak to the necessity for cells to actively control the influx of H^+ (OH^- efflux) when the cell is in an extremely alkaline environment.

Lucas et al. (1980) have long pointed to bands forming in order for the absorption of necessary solutes like HCO_3^- ; it being absorbed in tandem with hydrogen ion extrusion ($\text{H}^+/\text{HCO}_3^-$ symport). The channels were referred to as OH^- extruding and HCO_3^- extruding channels, as the authors focused on what was being transported across the membrane (Lucas and Nuccitelli, 1980b). When it comes to the mechanism of banding for a biochemical purpose such as

assimilation of extracellular solutes like calcium, one would think this would be easily distinguished by simply monitoring the solute activity or “silencing” banding by removing the regulating solute, if that were the case.

Bulychev et al. (2009, 2013) proposed a mechanism of banding other than the $\text{H}^+/\text{HCO}_3^-$ symport or $\text{OH}^-/\text{HCO}_3^-$ antiport that monitors membrane conductance and extracellular pH changes and reports on the proton pump in acidic regions. They found that an induced action potential directly inhibited the proton pump, raising pH in the acidic banding regions. However, the basic regions were also less basic with the AP, but this local change was not due directly to the pump inhibition. Rather, it was due to the change in membrane conductance with the AP. This slowed the kinetics of the proposed passive H^+ influx in basic banding regions, indirectly affected by the pump activity and directly affected by the change in membrane conductance. The authors propose that these basic regions arise primarily from the passive diffusion of H^+ ions inward, and that the “ OH^- efflux” condition is due to the influx of H^+ ions from the external medium, driven by the active diffusion in the acidic regions, that give the local region a higher concentration of OH^- relative to H^+ (Bulychev and Krupenina, 2009; Eremin et al., 2013). An overview of this movement of solutes is depicted in **Figure 3**.

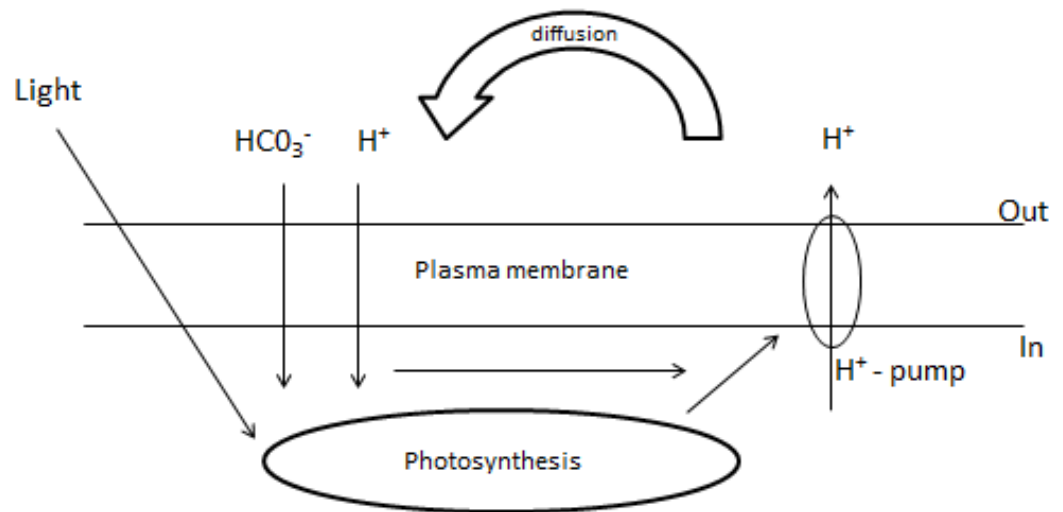


Figure 3: An overview of the solute movement during banding. Protons are absorbed in basic zones and actively pumped outward at acidic bands, which can be recycled and absorbed again in basic zones, creating circular currents along the cell. Redrawn from Bulychev et al. (2001).

Photosynthesis and Banding

Photosynthesis has been shown essential for banding as it is a dynamic process that changes the intracellular environment due to the light reaction metabolites (Foissner et al., 2014; Krupenina et al., 2008). Due to the necessity of photosynthesis for *Chara*, it makes sense these cells positively regulate the ability to utilize energy from light at all costs. Given the relationship between light and banding, the intensity of an artificial light source was scanned until the energy threshold was obtained (Bulychev et al., 2001). Rather than banding decreasing as light intensity decreases, a threshold effect was seen in that

banding remained relatively constant over two orders of magnitude of intensity. At a lower limit of intensity, banding was inhibited entirely (Bulychev and Krupenina, 2009; Bulychev et al., 2001). This seems to tie together photosynthesis at the level of the chloroplast with the extracellular banding profiles.

These results bring up the question of whether intracellular biochemistry regulates the amount of available ATP for these active proton pumps in low pH regions. To answer this, Shimmen and Yamamoto (2002) removed chloroplasts from several acidic regions of *Chara* and basic bands were observed in all the chloroplast-removed regions. The authors observed “random” switches from acidic to basic regions in younger cells, presumably due to this more dynamic state, and were confirmed to be in regions without chloroplasts. The authors, therefore, found they could remove bands as well as induce new banding physiology, further connecting banding with the direct action of the chloroplasts, or perhaps a photosynthetic byproduct, during illumination (Shimmen and Yamamoto, 2002).

In efforts to connect membrane composition with each banding region, Schmolzer et al. (2001) found protein clusters, referred to as “charasomes,” in the membrane co-localized with acidic bands. These large structures contain H⁺ ATPase pumps, which are visualized by staining and confirmed by Western blot analysis, which are responsible for the proton pumping activity. These charasome regions correlate positively with the acidic banding and are scarce in basic regions. Furthermore, there was a strong correlation between the acidic

banding location (charasome pumps) and the chloroplasts (**Figure 4b**). There was a much higher density of chloroplasts in the acidic regions when compared to the extracellular basic regions **Figure 4b and c**). This confirms that these alternating pH regions are involved with a self-organization phenomenon where CO₂ can be absorbed easier in these acidic bands to be utilized for photosynthesis by the nearby chloroplasts. Increased photosynthetic activity was confirmed by higher chlorophyll fluorescence and Photosystem II activity in the acidic region. It must be noted that, while the “charasomes” strongly co-localized in the acidic regions near chloroplasts, the banding phenomenon in *Chara corallina* and other *Chara* species is not limited by the presence of these structures and display banding but do not seem to contain these protein complexes. Schmolzer et al. (2011) suggest that these proteins cannot be ignored in how they help banding, and they may be integral in initiating the alternating regions or in aiding membrane potential changes necessary for diffusion.

In contrast, Foissner et al. (2014) found little correlation between chloroplast location and charasome-containing regions in *Chara*. They found charasomes able to form in any vegetative part of the plant, even nodal cells, and this was irrespective to chloroplast location. They found that these ‘chlorophyll-free windows’ described by Schmolzer et al. (2001) were not specific to one banding morphology but rather could exhibit either pH band. Many of these discrepancies may be due to the sensitivity of the *Chara* cells to various stimuli

that alter the membrane conductance (and streaming necessary to transport solutes).

Not only are metabolic solutes involved in the banding mechanism, electrical activity, like induction of an action potential (AP) also effects banding (Babourina et al., 2004; Bulychev and Kamzolkina, 2006; Bulychev and Krupenina, 2011). This shows that membrane excitation has a profound input on photosynthetic metabolism and reveals the strong correlation between banding, metabolism, and membrane conductance. Studies utilizing uni-directional light along the long *Chara* cells have shown that regions of “irradiated cytoplasm” flow towards the area that is to form an alkaline band and aid in channel opening (Bulychev and Kamzolkina, 2006). This cytoplasm is transported via cyclosis from a region dense in actively photosynthesizing chloroplasts (**Figure 5**).

Dodonova et al. (2011) found that the shaded regions on either side of these locally illuminated regions varied distinctly in pH, solute composition, chloroplast activity, and extracellular banding due to the direction of streaming in relation to the illuminated region. The importance of this directional flow of solutes for banding relates the classic banding phenomenon with another highly regulated aspect of these long cells—cytoplasmic streaming.

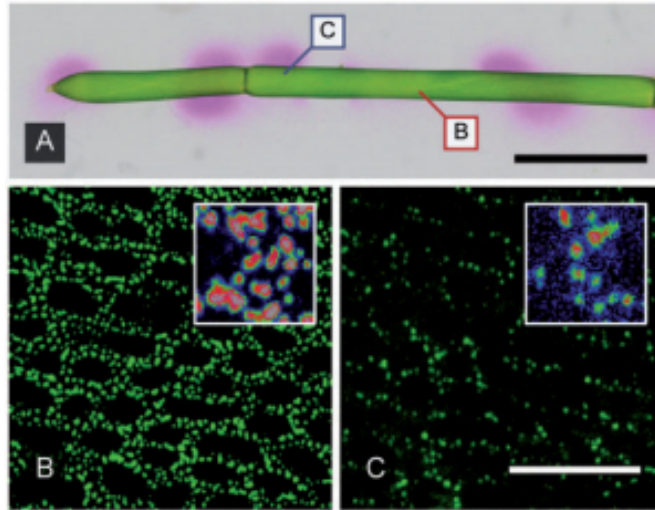


Figure 4: Cellular composition of banding zones. *Chara corallina* internodal cell banding visualized by phenol red indicator dye (A). Panel B shows chloroplast fluorescence in the acidic region. Panel C shows chloroplast fluorescence from the basic region. This seems to show a higher composition of chloroplasts in the acidic bands (Schmölzer et al., 2011).

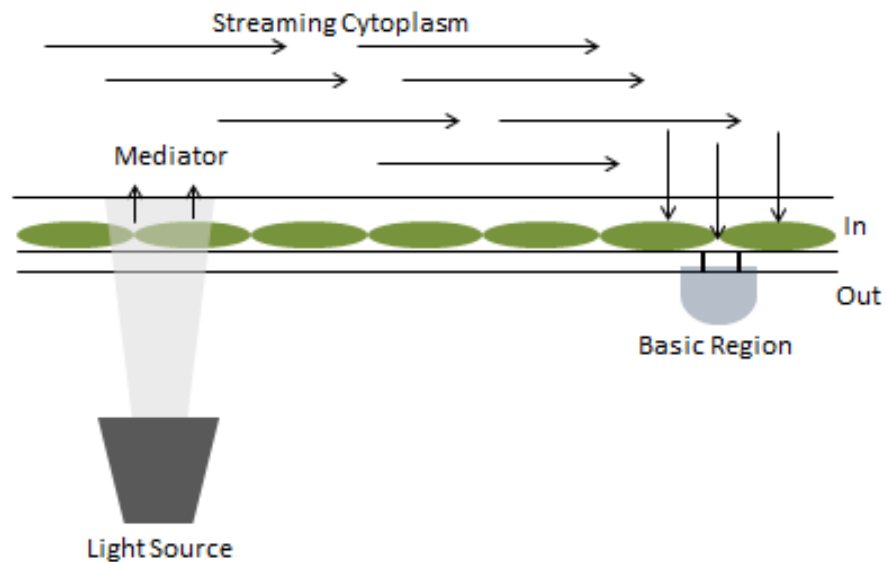


Figure 5: Uni-directional light experiments. Light activates photosynthesis in illuminated chloroplasts. A photosynthetic byproduct, labelled “mediator” is carried in the direction of cytoplasmic streaming to downstream basic channels to illicit channel opening. Figure redrawn from Dodonova and Bulychev, 2011.

Cytoplasmic Streaming and Banding

Due to the size of the internodal *Chara* cells, they rely heavily on cytoplasmic streaming to carry the many necessary solutes from one region of the cell to the other. Simple diffusion rates alone are not sufficient due to this large distance. Cytoplasmic streaming, or cyclosis, in *Chara* involves ATP-dependent actin-myosin systems that move up to 100µm/s. Streaming ceases upon the generation of an action potential, as the influx of Ca^{2+} inhibits the

functional interaction of the myosin and actin (Shimmen and Yokota, 2004). It makes sense, then, that cyclosis is intimately involved in the banding phenomenon.

Upon induction of an action potential and cessation of streaming, Shimmen and Wakabayashi (2008) found both the acidic and alkaline regions to be affected. Given this ability of a single action potential to inhibit banding, we see the direct relationship between band formation and membrane conductance. Bulychev et al. (2004) have shown that this single change in the membrane conductance during an action potential inhibits banding by somehow interfering with the thylakoid membrane of the chloroplasts. In a more recent paper, the chloroplasts of cells undergoing whole-cell and local illumination were examined. Chloroplasts of cells which were exposed wholly to the light were heavily taxed and showed Non-Photochemical Quenching (NPQ). When streaming was inhibited by cytochalasin B, the locally illuminated cells did not receive necessary solutes and did not display NPQ (Dodonova and Bulychev, 2012).

In order to resolve between the effect that streaming and the actively pumping regions have on banding, Bisson (1986) utilized inhibitors that targeted the proton pumps or passive regions in a different time course than how they inhibited streaming. Their results were inconclusive and unable to completely separate these two processes and their control on pH homeostasis (Bisson, 1986). Eremin et al. (2013) looked at the photosynthetic byproduct hydrogen peroxide. H_2O_2 may act as a signaling molecule when released during photosynthesis and transported throughout the cell by cyclosis. The redox effect

of H_2O_2 may be involved in changing the diffusion rates in the basic banding regions, possibly by directly affecting a protein like an H^+ channel and allowing an influx of hydrogen ions. This may be part of a complex, long-distance chloroplast-plasma membrane communication system. This suggests the role banding plays in this self-organization of charge across the membrane and the strong tie to photosynthesis and carbon fixation (Eremin et al., 2013).

Chloroplasts are known to alkalize the cytoplasm during photosynthesis from local chloroplast activity, which also releases hydrogen peroxide. H_2O_2 could act downstream on the passive regions to allow H^+ intrusion—the areas responsible for high extracellular pH bands. These regions could also be acted on to leak the overflow OH^- ions in these basic regions, which could reconcile the long withstanding dilemma of “ H^+ influx or OH^- efflux?”

Precipitation Chemistry

The aqueous environment of *Chara* reacts with environmental carbon dioxide that causes, over time, the acidification of the medium. The acidic solution levels off at a pH of 4.5 as the gaseous CO_2 is released into the atmosphere. If the solution becomes more basic, the CO_2 reacts with water to form bicarbonate, which disassociates to form carbonate (and a proton) and precipitates out of solution with the environmental calcium ions as calcium carbonate. While the formation of this encrustation in the alkaline zones is well characterized, several models for the exact reactions in these regions are proposed (**Figure 7**) (Brownlee and Taylor, 2001; McConnaughey, 1991).

Kawahata et al.(2013) suggest that the basic banding regions always aid in CO₂ uptake for the cell by decreasing the amount of free CO₂. This is accomplished by increasing the concentration of HCO₃⁻ or CO₃²⁻ which can form the precipitate. This, they suggest, may activate carbonic anhydrase which increases the CO₂ use. Therefore, CO₂ utilization is enhanced in alkaline areas as well as the acidic areas that are known to absorb CO₂ efficiently (Kawahata et al., 2013). The reactions involved in precipitation, occurring during banding, are described more in **Figure 6**.

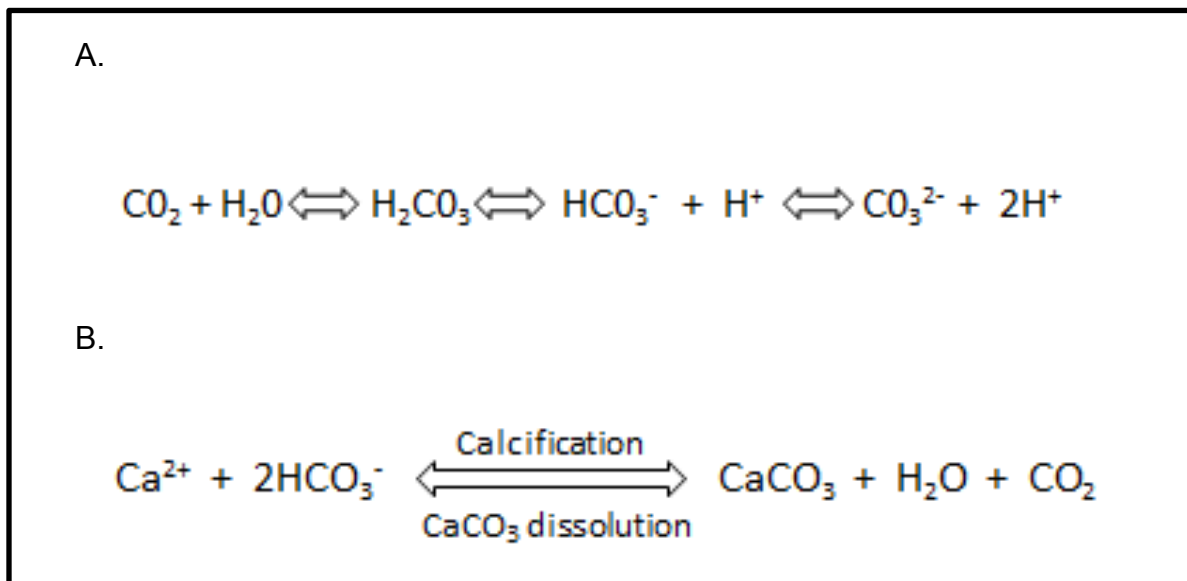


Figure 6: *Chara* chemistry. The overall reaction at play in the aqueous environment (A) that results in the calcification reaction precipitation on the older internodal cells as CaCO₃ (B). The free CO₃²⁻ may react with environmental

calcium ions to form the CaCO_3 precipitate found on the outside of the basic zones on older *Chara* cells.

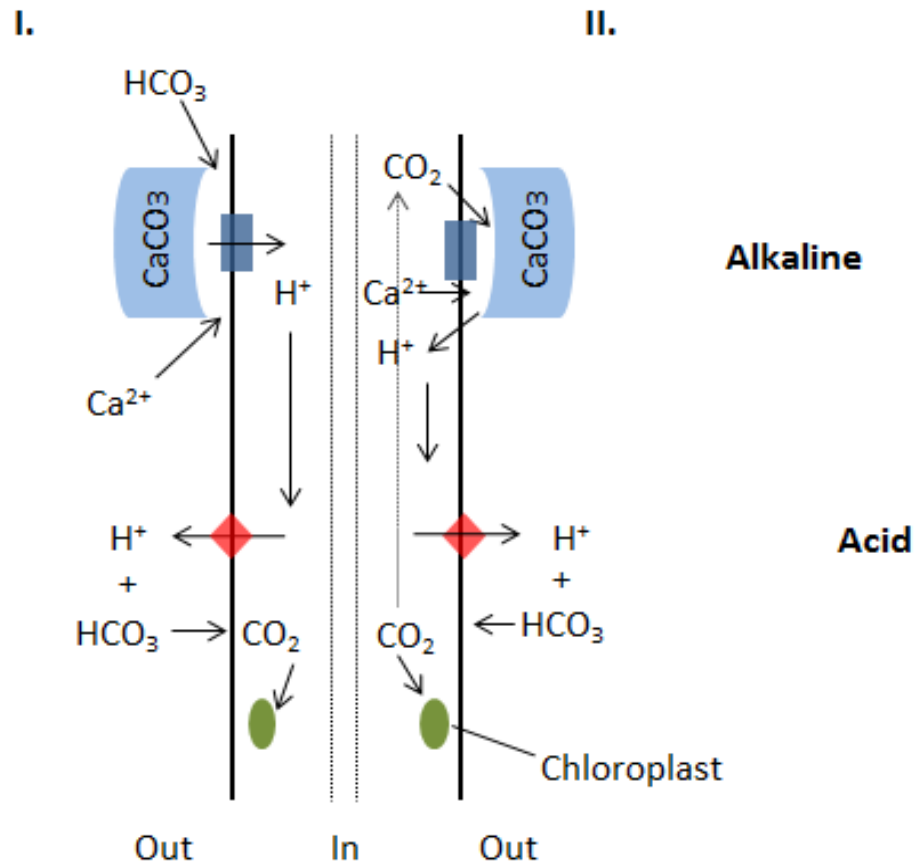


Figure 7: Two proposed models of calcification in the basic regions of internodal cells. The first model (I.) relies upon environmental calcium ions in the formation of the precipitate. The second (II.) involves intracellular calcium ions. Calcium carbonate is formed in both but various mechanisms responsible for the local chemistry are in question. Figure redrawn from Brownlee and Taylor, 2001.

Buffer Chemistry

With the many facets of banding in mind, there is an obvious regulation of the cellular pH in various environments, presumably for proper cellular function and photosynthetic metabolism. Several key papers have pointed the buffering effect of several metabolites and chemical species throughout the many reactions. Carbonic acid, in particular, actively regulates the intracellular and extracellular pH to an extent (Bulychev et al., 2001; Fisahn et al., 1992; Lucas, 1979). With a pKa of 6.4, an accurate recording of the pH profiles along the *Chara* can be influenced due to the buffering effects of this acid, keeping the solution in the range of pH 5.4 to 7.4. This buffering effect, and others like it, must be considered when recording pH profiles in various solutions. Characean cells have been shown to alkalize local environments up to a pH of 10 while actively photosynthesizing (Shimmen and Wakabayashi, 2008). In this case, the local environment is above the buffering range of carbonic acid.

The buffering effect is visualized by changes in the current profiles—they are gradual as you scan along the cell if the buffer does not have an effect, and they are instantaneous if in the buffering range of 5.4 to 7.4. The characteristics of this current profile, obtained by pH microelectrode, can give insight into the kinetics of these regions. For example, the current profile reveals whether a region is controlled by a pump or not, is a symport or antiport, or if it is buffered or un-buffered (Fisahn et al., 1992). Certain pH behavior with pH microelectrodes can even reveal specific compounds being transported. For example, OH⁻

extrusion has a far different current signature than an influx of protons, even though both can be responsible for local alkalization.

Inversion of Banding

Figure 8 shows the inversion of the pH profiles along *Chara* cells when an initial solution of pH 8 was exchanged for a pH 4 solution (Fisahn and Lucas, 1990; Fisahn et al., 1992). This seems to indicate and further support the idea of a self-organizing, spontaneous formation of currents in response to both the internal and external pH. Because the inside of the cell is alkalized during photosynthesis, it must allow a recovery of H^+ from the environment in order to maintain a relatively constant balance of charge across the membrane, as well as the correct concentration of metabolites necessary to the cell. This could include simply the distribution of ions such as protons which contribute to the polarity across the cell or metabolites more specific to photosynthesis regulation like sulfuric acid (Eremin et al., 2013).

When the local environment is significantly changed so that the H^+ concentration increases (strongly acidic), the H^+ recovery is not as difficult in the presence of so many free hydrogen ions. Likewise, the pumping out of H^+ may be in demand, and so there is an inversion of the previous banding profiles in the more alkaline solution. The difference in pH environments across membranes is further complicated taking into account the effect photosynthesis has, both on intracellular and extracellular pH. In a local environment pH 5-6, the intracellular pH was 7.7 in the light (photosynthetically active) and 7.3 in the dark. When the

extracellular environment was as basic as pH 10, the cytosol had a pH of around 8 (Smith and Raven, 1979). Knowing these values gives a glimpse as to what pH the cell must maintain for optimal function as well as the membrane potential across the cell across a range of environments. This further supports and complicates the overall paradigm involving the interplay between banding regions to promote homeostasis.

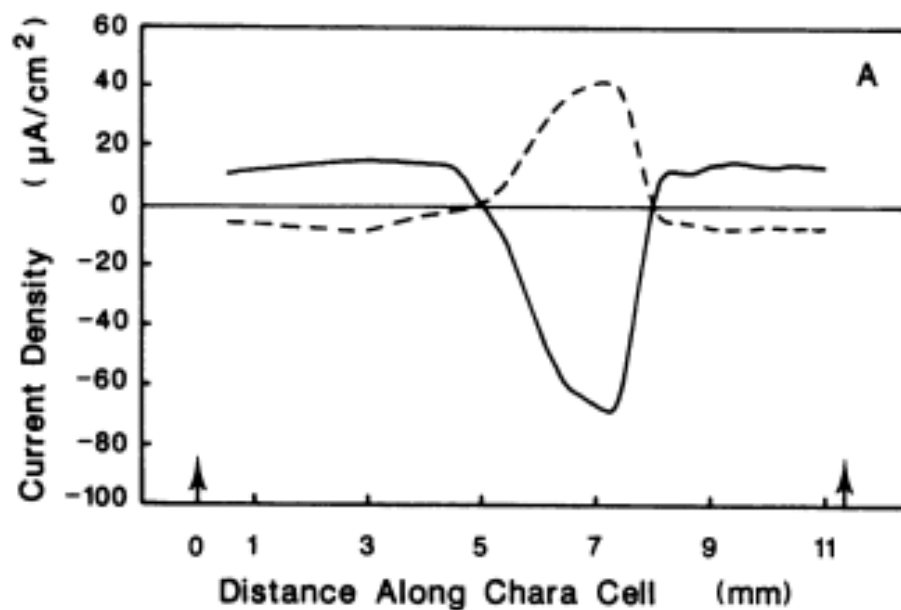


Figure 8: Inversion of banding. This figure shows the banding inversion phenomenon when switching from an extracellular solution of pH 8.2 (solid line) to a solution of pH 5 (dotted line) (Fisahn et al., 1992).

Discussion

Chara cells are an excellent research model due to their large size and ease of observation of biological processes such as cytoplasmic streaming, photosynthesis activity, membrane potential, and the pH banding phenomenon. The alternating acidic and basic banding observed in Characean cells has been established as a means of self-organization of charges across the membrane and for solute uptake, dependent on photosynthesis and cytoplasmic streaming, to allow these cells to grow in a wide range of environments. The regulation of intracellular pH and membrane conductance is crucial in optimizing the integrity of the intracellular processes like enzymatic activity, signaling pathways, correct buffer chemistry, and especially monitoring metabolites for and byproducts of photosynthesis.

Over the last several decades, the mechanism of banding has been a serious focus in *Chara* research, yet the complexities of these algae have allowed this exact mechanism to elude our understanding. Much has been revealed about the many purposes of banding, its distribution, inversions, timeframe, and other phenomenological effects on various aspects of the mechanism. Banding is observed as the alternating regions of high and low pH found along the internodal cells when exposed to light past a certain threshold. These bands may invert upon changing their extracellular environment from a slightly acidic pH to very alkaline. The acidic regions are dependent on a proton ATPase pump, regions of H^+ efflux, and the alkaline regions are passive regions, dependent on the membrane conductance, noted for H^+ influx (OH^- -efflux).

Banding is furthermore regulated by cytoplasmic streaming, as banding is promptly stopped when an action potential is generated or when streaming mechanisms are halted directly. This seems to point to the necessity of cyclosis to bring proper solutes to these regions across the very long cell with a very low surface area to volume ratio. Whether these solutes directly or indirectly affect banding at the acidic or basic zones is to be determined. Several mechanistic models point to the need for these processes for proper absorption or reaction of different solutes in the intracellular and extracellular environment. An acidic region drives the reaction one way, while a basic region drives it in the other, each creating a microenvironment favorable for the respective reaction. An all-inclusive examination of the many factors at play in the banding mechanism is required in order to reach a comprehensive understanding of this complex phenomenon.

Chapter 2

Introduction

Chara corallina algae has been a central biological model for studying a wide range of concepts such as photosynthesis, cytoplasmic streaming, cellular metabolism, solute transport and uptake, membrane potential, and the pH banding phenomenon (Beilby and Bisson, 2012; Lucas and Smith, 1973a). Studying the long internodal cells of this freshwater alga has furthered the overall understanding of many of these complicated biochemical concepts as well improved our understanding of *Chara* itself.

The pH banding phenomenon in particular is a most interesting aspect of *Chara* as the mechanism involves many of the above concepts working in accord. Banding can be easily initiated and visualized in a small dish of artificial pond water with phenol red indicator in artificial or natural light (Lucas and Smith, 1973b). Understanding the mechanism of banding has been an active area of research for several decades and the model has been fine-tuned over the years (Lucas, 1975; Lucas and Smith, 1973a; Walker and Smith, 1975, 1977). While the purpose of pH banding is well-characterized, there is still not a clear, overarching understanding of the mechanism—specifically in the alkaline zones. More recently, Bulychev et al. (2001) have elegantly characterized the banding profiles of *Chara* in various light conditions. Schmoltzer et al. (2011) looked at the cellular composition along the membrane as well as the chloroplast location in relation to pumping regions. Cytoplasmic streaming has been shown to play a significant role in banding induction (Dodonova and Bulychev, 2012; Eremin et

al., 2013; Shimmen and Wakabayashi, 2008; Shimmen and Yokota, 2004). In addition, even small changes in temperature have been shown to affect streaming rates, which introduces further variability in any direct connection between streaming and banding (Shimmen and Yokota, 2004). Beilby and Bisson (2012) have compiled much of this understanding into a simple model, but a more detailed model is lacking. **Figure 9** combines several of these concepts into a mechanism for channel opening during banding. It is thought that the photosynthetic intermediate responsible for the alkaline channel opening during banding could be a signaling molecule binding to a channel protein, a “voxel” (compositionally unique three dimensional space) of water of a more severe pH, or possibly another charged intermediate that opens the channel by some other means (Beilby and Bisson, 2012; Bulychev and Komarova, 2014; Dodonova and Bulychev, 2012; Eremin et al., 2013).

Understanding the nature of these banding regions and their activity upon stimulation gives insight into how each region works in an effort to clarify the overall mechanism. Much of the current literature describes these pH bands by both the hydroxyl and proton transport. The alkaline bands are proton sinks, either releasing hydroxyl ions or simply creating a more basic (hydroxyl-rich) environment. Discussions as to the exact composition of the solutes involved in each banding region have been considered in many previous studies (Bisson, 1986; Brownlee and Taylor, 2001; Lucas, 1979; Lucas and Nuccitelli, 1980a; Lucas and Smith, 1973a; Walker and Smith, 1977). Understanding banding rates upon induction and removal of visible light can ideally unite rate-dependent

banding concepts such as cytoplasmic streaming, channel opening, and photosynthesis. The acidic region has been shown to be active even in dark conditions, as the ATP-dependent proton pump has been well-characterized, but that would mean that the basic region is the primary switch for full banding (Mimura and Tazawa, 1986; Mimura et al., 1993; Schmölzer et al., 2011). In some cases, by drastically altering the pH of the cell's environmental solution, an inversion of banding current can be induced (Fisahn and Lucas, 1990). This unique flip of relative proton currents between bands could provide more understanding of the nature of each region by knowing what sort of solutes can pass both in and out of the cell during such conditions.

The present study found that the alkaline bands are tightly regulated by visible light, and it is these regions that are primarily responsible for the pH banding phenomenon. The acidic bands were confirmed to be relatively constant in both lighted and dark conditions. Upon illumination, the alkaline bands were activated and these regions became several pH units more basic than the adjacent acidic zones. Banding rate experiments in the alkaline regions revealed the time of activation to be significantly longer than that of banding cessation. Lastly, banding inversion was accomplished by exposing internodal cells to drastically different basic and acidic environments to induce a relative flip in the pH of the banding region from the initial basic bands. This implicates alkaline regions in the role of environmental homeostasis and provides a deeper understanding of these passive channels and their activation mechanism.

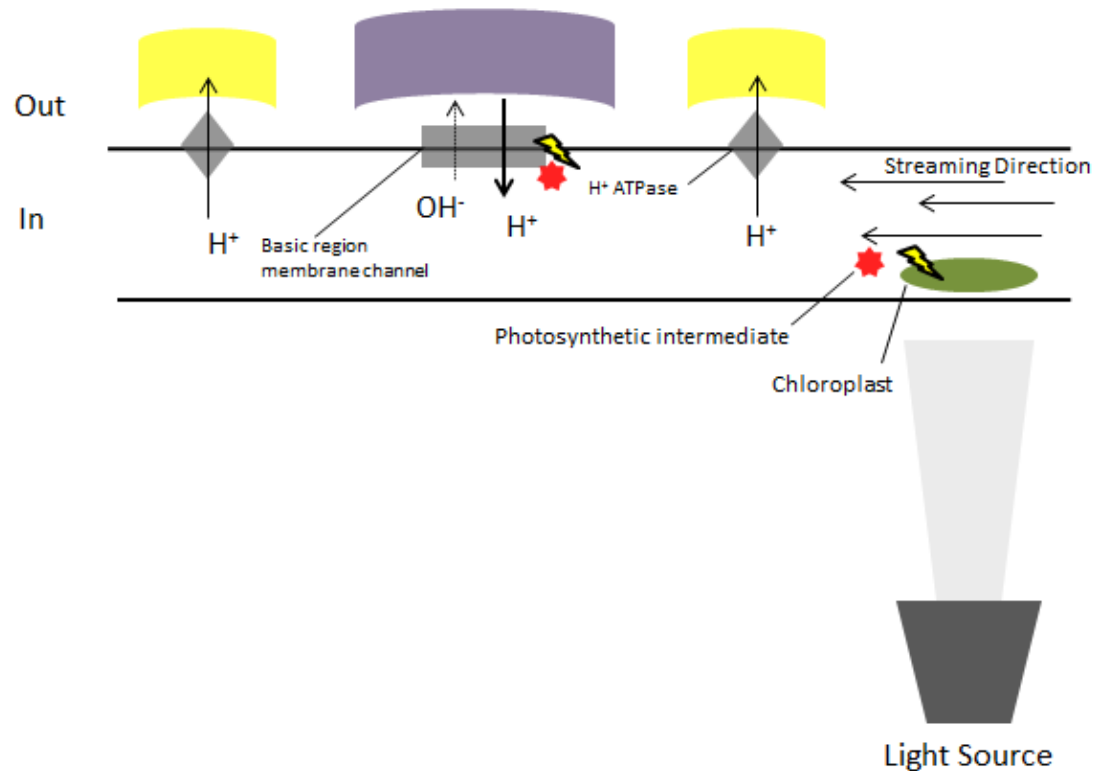


Figure 9: Overview illustrating the various aspects of the pH banding

phenomenon. Diagram of the internodal cell and the key players involved in light-induced banding including the light source, acidic and basic channel regions (yellow and purple, respectively), ion transport, cytoplasmic streaming, and the resulting bands formed along the cell exterior. Chloroplast activation by light creates a photosynthetic intermediate that travels the length of the cell due to streaming and interacts with basic membrane channels.

Chapter 3

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Plant Material

C. corallina Klein ex Willd. were grown in an aquarium exposed to scattered daylight at room temperature. Young, non-elongated internodal cells with little calcification were cut and used for all experiments. Cells were kept in artificial pond water (APW) containing 0.1 mM CaCl_2 , 1.0 mM NaCl, and 0.1 mM KCl before experimentation and a 16/8h light cycle was provided by a LED lamp at room temperature.

Banding

Cells were incubated in banding buffer (BB - 2.0 mM NaHCO_3 , 0.3 mM CaCl_2 , 1.0 mM NaCl, 0.1 mM KCl) for several minutes prior to recording to help stimulate banding and inhibit a stress response (Schmölzer et al., 2011). The light-induced banding pattern was visualized in an APW solution with 0.25 μM phenol red indicator solution (APWpr). Cells were isolated in a small volume of APWpr solution in a petri dish and immobilized by a small channel of petroleum jelly. The cells were illuminated by an LED bulb (3.4-4.3 Lumen) at a distance of about 3 inches from the cell. This light source was suitable for this study because

it introduces little electrical noise to the system and meets the energetic threshold requirements for photosynthesis that have previously been described in detail (Bulychev and Vredenberg, 2003; Bulychev et al., 2001; Lucas, 1975).

Images

The photos of electrode placement and *Chara* intermodal cell banding were taken with an iPhone 5 (8 Megapixel HD camera) staged on a Roachscope (Backyard Brains, Ann Arbor, MI) platform. Images were improved with both digital zoom and auto-focus on the iPhone as well as mechanical zoom provided by the staging unit.

Electrode Construction

pH-sensitive carbon fiber glass microelectrodes were fabricated by the methods described previously (Ramsson et al. 2015). In brief, a carbon fiber filament was drawn in capillary tube with a vacuum and loaded into a horizontal pipette puller (P-77 Sutter Flaming-Brown Micropipette Puller, Sutter Instruments, Novato, CA). The fiber was cut to the appropriate length, and sealed in paraffin wax (Ramsson et al., 2015).

pH Recordings

Glass-pulled carbon microelectrodes from 100-300 micrometers in size were used to measure the current of the proton movement responsible for the large variation of pH of the bands. Rectangle pulse voltammetry (RPV) allowed

for a repeatable, quantifiable platform from which to observe the current profile in real time. The carbon surface was held at 0 volts (V) and switched to 0.2 V and back every 100 ms (10 Hz). The 0.2 V excursion lasted 30 milliseconds. This waveform monitors changes in pH as capacitive changes in the electrode upon voltage switching (Yoshimi and Weitemier, 2014). Recordings were done in a grounded Faraday cage (built in house) to eliminate any potential electrical noise that is easily picked up by the sensitive electrodes. The electrode was fixed to an arm of a small animal stereotaxics (Stoelting, Wood Dale, IL) for precision placement and security once positioned. For scanning experiments, the electrode was manually moved uni-directionally by the adjusting wheel of the stereotaxics at a relatively constant rate. The electrode location is crucial to the accuracy of the banding measurements. Three directional factors were involved in electrode tip location: first, the depth of the electrode as it is lowered into the solution with the tip nearest to the active banding region, second the “transverse” distance away from the cell, and third, the “longitudinal” location, or the orientation along the large cell, from one pole to the other. All of these factors were achieved by referring to both the visual confirmation of the electrode in the phenol red, with the aid of a headband magnifier, and the from the current profile viewed in real-time, all while scanning the electrode in one of three movements (Bulychev et al., 2001). A visual representation of electrode placement is shown in **Figure 10**. The location was considered optimal when the electrode was placed in the region that produced the largest change in current for each given region without touching the cell and fully immersed in solution.

Light-response banding kinetics

Internodal cells were allowed to band in APWpr, pH 5.6. Although natural conditions are far more basic (pH ~8), these conditions allowed the phenol red indicator to be more effective in visually distinguishing between banding zones for proper electrode placement. Changing the pH of the recording environment to match experimental requirements is consistent with electrophysiology protocols with Chara (Bulychev et al., 2001; Lucas, 1979; Schmölzer et al., 2011). Phenol red indicator dye has long been used in detecting pH changes in the medium of banding cells (Spear et al., 1969). Once the cell was banding in this lighted condition, the electrode was scanned along the cell to verify the region as either basic or acidic for each recording. This only confirmed the banding profile seen easily with the indicator dye. Bands were verified by scanning with the electrodes for optimal placement. The area with the greatest current disturbance was recognized as the bands' center. The electrode was placed and subsequently given 5 minutes to equilibrate to this region. This also gave the cell and the medium time to recover from any minute changes to its banding profile to reduce any possibility of error introduced from the electrode placement. After this time, the current was recorded with only the light condition changing. The room was sufficiently dark during the entire experiment with only the LED bulb responsible for altering the light condition. This reduced any external noise to the system as well as any potential disturbances to the cell during the dark control time. There was no change to the electrode placement relative to the cell. After the 5 minute

equilibration time in light, the cell was recorded in light for 1 minute, followed by 9 minutes in the dark, and 10 minutes with the light restored. These times were chosen to show the full banding activity initially in light, to give the cell ample time to stop full banding in the dark, and to restart banding fully. Even a few minutes in the dark has been shown to sufficiently stop banding (Bulychev et al., 2001). Refer to **Figure 11** for an overview of the light-response experimental model. The output in nanoamps was converted to pH units by establishing a calibration factor. The recordings in the alternating light conditions described were compared to dark control recordings. We placed the electrode in the same manner for both the dark control and the alternating light recordings, but the controls were allowed to equilibrate for 10 minutes in the dark before a 20 minute recording. This provided a baseline activity level in each region to properly understand the effect of light on banding.

Electrochemistry restrictions

The magnitude reached in a given region is a function of electrode distance to the cell and proximity to the “actual” pump or channel opening. This banding epicenter has been shown to be heterogeneous as the membrane composition differs around the cylindrical cell (Bulychev et al., 2001; Schmölzer et al., 2011). Although these factors contribute to some inaccuracy, recordings were repeated on multiple internodal cells using the same method of electrode placement and results were averaged to obtain an accurate current profile in these regions.

Calibrations

By recording the pH with a probe and the current using RPV, we added acid (HCl) or base (NaOH) to APWpr and noted the change in pH and the accompanying change in current. This provides for a conversion from nanoamp change to a change pH.

Inversion test

Internodal cells were allowed to band in APWpr in full light conditions. The high pH group was recorded in alkaline APWpr, pH 10. The low pH group was recorded in acidic APWpr, pH 4. The basic and acidic solutions were altered by the addition of NaOH and HCl, respectively, dropwise until the solutions reached the desired pH. This change was recorded to the nearest 0.01 pH unit and could be verified visually by noting the obvious color change of the indicator dye. Rather than fixing the electrode in one banding region, as in the previous study, pH inversion was monitored by scanning from the basic region (the region that is basic in APWpr pH 5.6), through the adjacent acidic region, and into the next closest basic region in approximately 30 seconds. This was repeated in both the high (10) and low (4) pH environments comparing the very same banding zones of the same internodal cell. The 30-second banding profile described was recorded in the acidic condition several times before the solution was extracted with a bulb pipette. The basic APWpr solution was introduced and the cell was allowed to band fully before recording, while the electrode remained in the

“original” basic region from the previous recording. Banding profiles were obtained from the high pH condition by scanning the same region of the cell as the previous scans. **Figure 12** provides an overview of the relative proton concentrations and pH of the internal and external environments during the inversion experiments. Internal pH values are approximate and were inferred from past works where the intracellular *Chara* pH was recorded in changing external conditions (Smith and Raven, 1979; Walker and Smith, 1977).

Statistics

In comparing the acidic (n=11) and basic recordings (n=15) in the alternating light and dark conditions, the values reported were the max disturbance from the initial banding in nanoamps. Each region was compared against its dark control recordings in that same region, acidic n=5 and basic n=4, that show banding activity in the dark. When comparing the stop and start times of basic banding, the stop time was the time (minutes) it took for banding to fully stop when light was removed. Restart time was the time the basic region took to begin banding again after reintroduction of light after the 9 minutes in the dark. Due to small sample size of recordings, we performed a non-parametric Wilcoxon rank sum analysis.

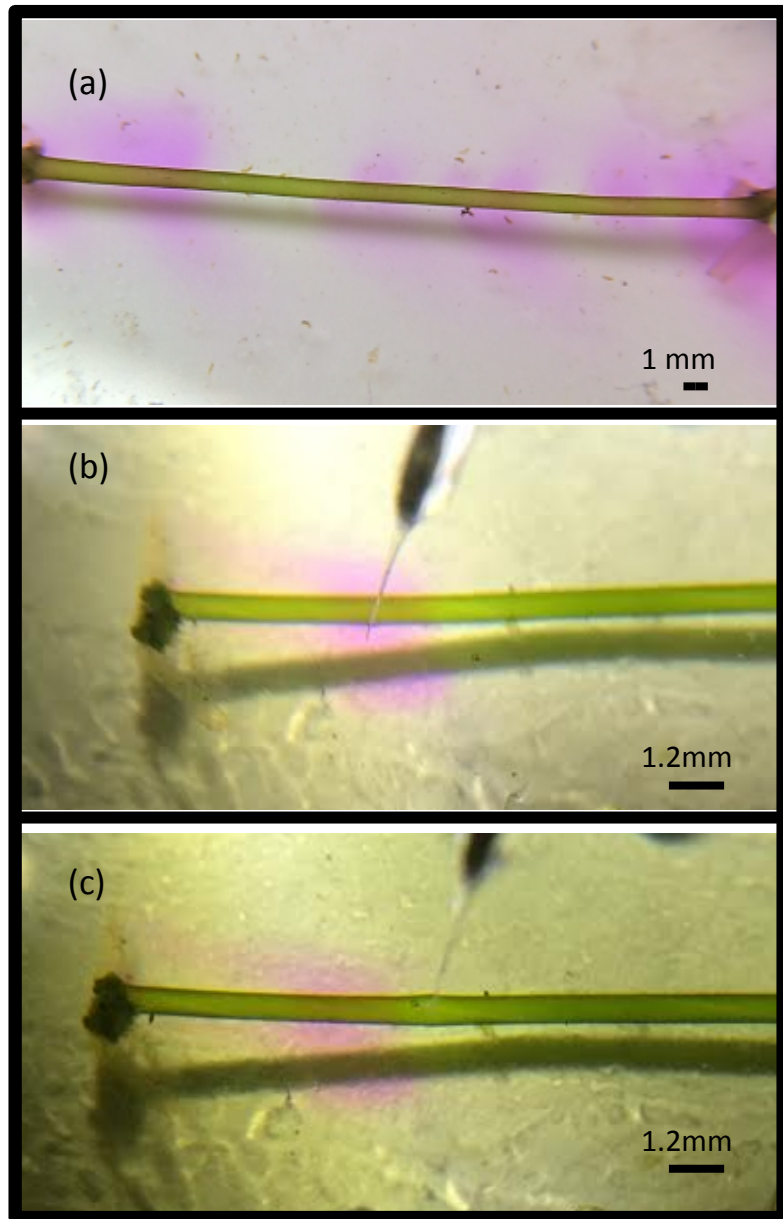


Figure 10: Proper electrode placement along the internodal cell. Placement was obtained by recording current while scanning along the cell and adjusting in three dimensions to reveal the most active region of each banding zone, also confirmed by indicator dye. (a) Cell fully banding in APWpr before electrode placement. (b) Electrode placed in basic region. (c) Electrode placed in acidic region.

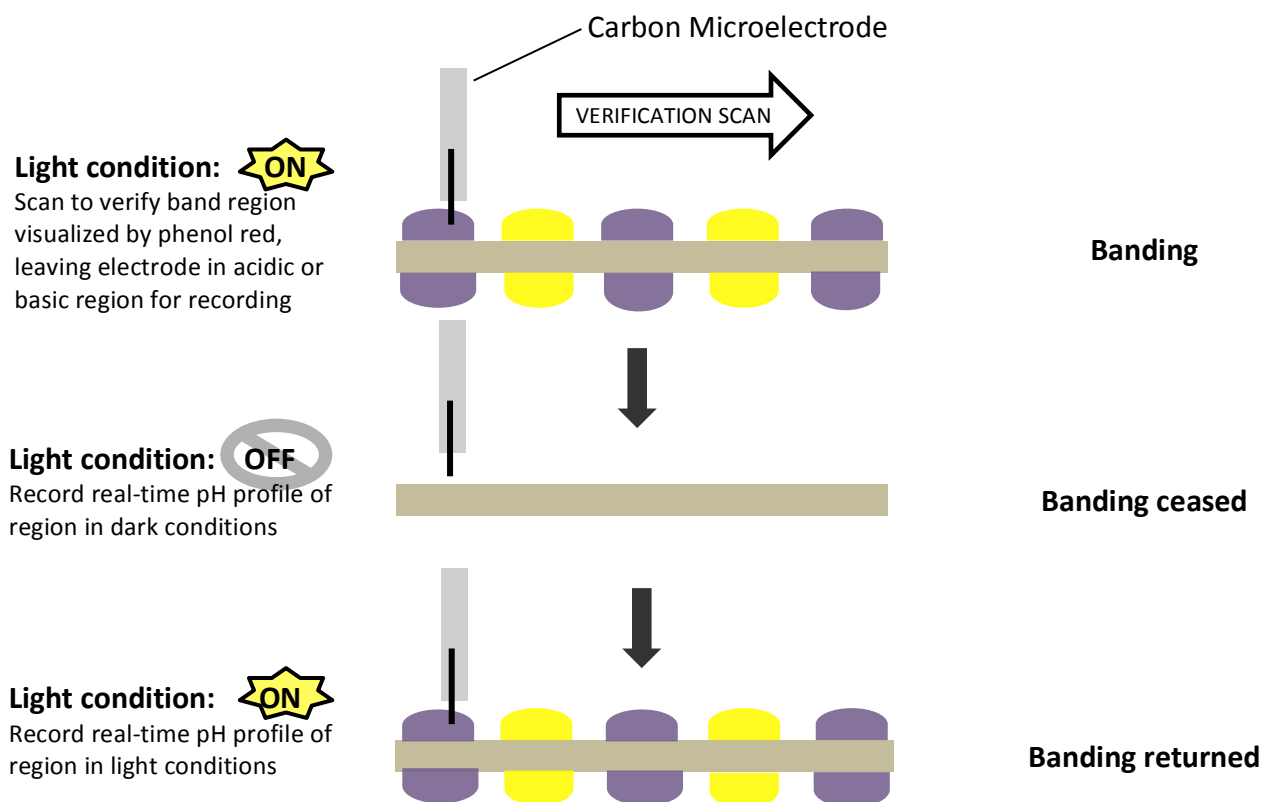


Figure 11: Overview of the light-response banding kinetics experimental design. The electrode is placed along the cell as described in the methods and records the pH , as a function of current, of each banding region in the lighted, dark, and then lighted conditions to show the real time kinetics and compare each region's response.

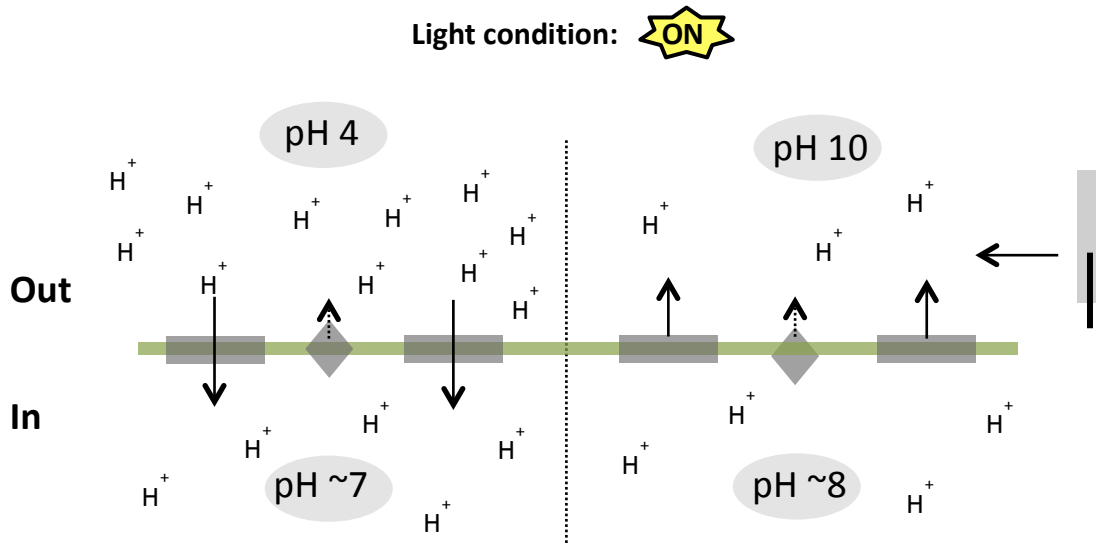


Figure 12: An overview of the two environments responsible for pH banding inversion. Internal and external cellular pH is represented by proton concentrations. The diamonds represent proton pumps responsible for acidification of the media, and blocks represent the basic channel regions along the membrane that allow for proton diffusion. Arrow direction and size indicate current direction and relative magnitude.

Chapter 4

Results and Discussion

Comparing banding zones

To determine the light response rates of banding, control measurements were recorded for 20 minutes in the dark in both acidic and basic regions with no photosynthetic activation. The cells were also recorded in alternating light conditions to compare to the dark control recordings. In response to the darkness after the first minute when the light was removed, the basic region increases in current, indicating an acidic swing and diminishing banding. When light is reintroduced, the current drops. This indicates a lower proton composition, a more alkaline pH, and the return of full banding. In most cases, the initial pH in the basic region returned after the reintroduction of light for 10 minutes. The acidic recordings seemed more random in their proton flux. Several recordings seemed to respond to changes in light condition, while others remained relatively constant (data not shown). However, any apparent response to the changing light condition was much less pronounced when compared to the large swings in current of the basic bands. **Figure 13** shows a representative recording of the behavior of both regions in alternating light conditions. This file, showing one basic and one acidic recording, reflects the trend seen in the average of all the recordings. In order to test the effect of light on banding, we quantified any significant differences between the dark control and alternating light condition recordings from each banding region. The values used for this comparison were

generated from the change in nanoamps from the initial current baseline (time = 0) to the greatest disturbance in current. This large swing was often seen after the cells responded to the absence of light. This is an appropriate marker for comparing banding activity in response to light as it succinctly summarizes the change in current as a single value – the maximum disturbance from the baseline. Due to small sample size of recordings (n=11 acidic, n=15 basic), we performed a non-parametric Wilcoxon rank sum analysis. A Wilcoxon two-sample p-value of 0.6612 provided no evidence of a significant difference between the light and dark recordings in the acidic region. However, a significant difference was seen in the basic region with a p-value of 0.0005 (**Appendix A, Table 1**). **Figure 14** shows this difference. When compared to the 20 minute dark control recording, the acidic region does not respond to the light stimulus, while there is a significant increase in current during the alternating light condition. Using RPV allows for current measurements in nanoamps, but this can be easily converted to pH units as in **Figure 15**. These results are consistent with the drastic differences in pH between the two regions previously reported of around 2 pH units (Beilby and Bisson, 2012; Bulychev et al., 2001; Lucas, 1979; Schmölzer et al., 2011). The acidic banding regions were at a pH of 5-6, while the alkaline regions were a pH of 6-8 during full banding in a lighted condition when the regions were most distinct.

Basic regions key regulators of banding

Due to the semi-random nature of the acidic zones' pump rates and

smaller contribution to full banding in response to light (**Figure 13-14**), our focus was directed towards the role the alkaline zones play in banding control. These findings in the acidic bands also confirm the notion that the proton ATPase pumps in these regions continue in either light condition – acidic bands are always on, assuming both protons and ATP are available (Mimura and Tazawa, 1986). The acidic pump properties could be due to the changing internal concentration of protons and ATP during the recording. While the acidic alternating light files were not significantly greater than the dark control acidic files, we noticed a small alkalization trend in the acidic bands in the dark, as this region remained acidic but became less active (data not shown). This could be explained by the cessation of proton intrusion with the basic band channel closing and a higher concentration of environmental protons outside the cell that may have diffused into the acidic region during the 9 minutes of darkness.

While the acidic region persistently pumps, the basic region is under strict regulation (**Figures 13 – 15**). With this established, our focus shifted to the basic region, given that this appears to have a more regulatory effect on the large difference between the regions and control by a light source. The same light-dark-light data were analyzed for the time it took the alkaline zones to stop banding after removing the light and the time to restart banding after reintroduction of light. To properly compare the time in minutes it took to stop banding (when the light was removed) to the time it took to restart banding, again we performed a non-parametric analysis. The p-value of 0.0158 indicates the two time frames are significantly different (**Appendix A, Table 2**). **Figure 16**

compares the time to stop and restart banding, showing a significantly longer time cells take to restart banding. This sheds light onto the molecular mechanism controlling the opening and closing of the basic channel. Closing of the channel took longer than channel opening (or restarting of banding) which appears to be the rate-limiting step. We propose that this could be due to the action of a signaling molecule that may interact with the channel to open and close it and directly control the current in alkaline zones, in line with previous assertions (Bulychev and Komarova, 2014; Dodonova and Bulychev, 2012; Eremin et al., 2013). If this were the case, disassociation from the channel, and banding cessation, would theoretically be faster than restarting banding, which would necessitate the transport of this signaling molecule downstream to the alkaline channels. We propose that this rate is strongly tied to the rate of cytoplasmic streaming and photosynthetic activation of the chloroplasts upon the introduction of light. A photosynthetic byproduct may be responsible for directly or indirectly affecting the basic region downstream to re-open the channel in the recorded time, about 6 minutes (Dodonova and Bulychev, 2012).

Band inversions induced by changing environmental pH

Our observed band inversion confirmed the phenomenon described by Fisahn et al. (1992) in which acidic and basic bands seem to invert proton current direction when introduced to a drastically different environmental pH (**Figure 17**). This phenomenon confirms the homeostatic regulation capabilities of *Chara* internodal cells to regulate internal ion compositions in changing environments.

These capabilities speak to the aforementioned ambiguous nature of the solutes being transported (proton intrusion or hydroxyl extrusion), as well as the flow direction along the cell in basic bands. Assuming the ATP-controlled acidic proton pumps are uni-directional and solely a function of internal conditions, the basic regions must act as bi-directional regulators of solute homeostasis. **Figure 17** shows this inversion of current direction clearly. The low pH (4) recording in panel b reveals a spike in the positively charged protons as it enters the acidic (yellow) environment and returns to a lower current, higher pH region in the adjacent basic zone (purple). These two regions are interdependent as the acidic region pumps out internal protons, reducing the levels inside the cell, and increasing the relative concentration of hydroxyl ions. In this sense, one region is dependent on the other, creating circular currents of solute motion from one region to the next until homeostasis is met (Beilby and Bisson, 2012; Walker and Smith, 1977). Although this environment is more acidic than ordinary freshwater conditions, it mirrors the banding profile observed in previous experiments (pH 5.6). This process never fully stops as photosynthesis and other metabolic factors are always changing the composition of internal pH. This self-organization of charges also creates favorable micro-environments for carbon assimilation from CO₂ and in the acidic region and precipitation of CaCO₃ in the alkaline bands. Banding is halted if cytoplasmic streaming is ceased due to an action potential or other shock responses (Bulychev and Kamzolkina, 2006; Mimura et al., 1993; Shimmen and Wakabayashi, 2008; Shimmen and Yokota, 2004).

A unique inversion is seen in the high pH (10) scan in panel c of **Figure**

17. Here entering the initial “acidic” region (purple) reveals a drop in current, an increase in pH, and a more basic region in relation to the proper basic bands on either side, which are now more acidic (yellow). This theoretically means that the basic bands (those without proton pumps) have reversed their uptake of protons and now passively allow protons out into the media, seeming to show the bi-directional transport of protons following the diffusion gradient. Both regions are “pumping” outward, but the proper basic zones are responsible for a greater current in that direction, relative to the proper acidic region. **Figure 12** is an overview of the proposed environments and this inversion of current in the changing pH solution. This understanding also reveals key aspects of the banding mechanism, agreeing with the alternating light findings described previously. In the inversion experiments, the cells are in fully lighted conditions for both conditions. Assuming a photosynthetic intermediate is responsible for basic channel opening, the channel is open during both high and low pH scans. If so, this agrees with the inversion of current in the alkaline environment as the internal protons both could and would favorably diffuse from the cell into the H^+ -deficient environment.

Summary

Utilizing rectangular pulse voltammetry (RPV) to detect the smallest changes of pH in the *Chara corallina* banding regions in real time has offered new insights of the overall mechanism of the pH banding phenomenon. Investigations of the kinetics of each banding region in response to light and

changing environmental pH has confirmed much of the current model of *Chara corallina* banding. The alternating light study has clearly shown that the basic bands are controlled by light most profoundly, while the acidic regions are much more constant and contribute far less than the alkaline region to the large pH swings. This data also confirms a photo-induced mechanism responsible for alkaline channel activation. Furthermore, comparing the stop and restart times of the basic region in the alternating light study revealed it took significantly longer for cells to restart banding. The inversion study revealed a bidirectional nature of the membrane in the basic bands that allows cells to maintain a favorable membrane potential with external media (Shimmen and Wakabayashi, 2008). The acidic bands seem to be unidirectional and pump at a relatively persistent rate.

Future Direction

Due to the dynamic nature of cytoplasmic streaming, which is controlled by temperature, membrane potential, and many other factors, an accurate understanding of banding rates upon light stimulation is difficult. A molecular understanding of this proposed mechanism of a photo-induced intermediate opening the basic channel must be further investigated. Because of the spontaneous, yet still highly regulated nature of the pH banding phenomenon, and the many functions it plays for the organism, the overall mechanism is difficult to describe without a large-scale examination and understanding of these many factors. A more robust comparison of these many aspects of banding is

required. Comparing photosynthetic activation time and streaming rates over the distances from the activated chloroplast to a given alkaline channel-opening would further confirm the role of a photosynthetic intermediate in basic banding. Photosynthetic intermediates, such as hydrogen peroxide (Eremin et al., 2013), as well as large changes in internal pH (Beilby and Bisson, 2012) have been implicated in channel activation. Resolution of the exact trigger for the alkaline channel interaction and subsequent opening is required.

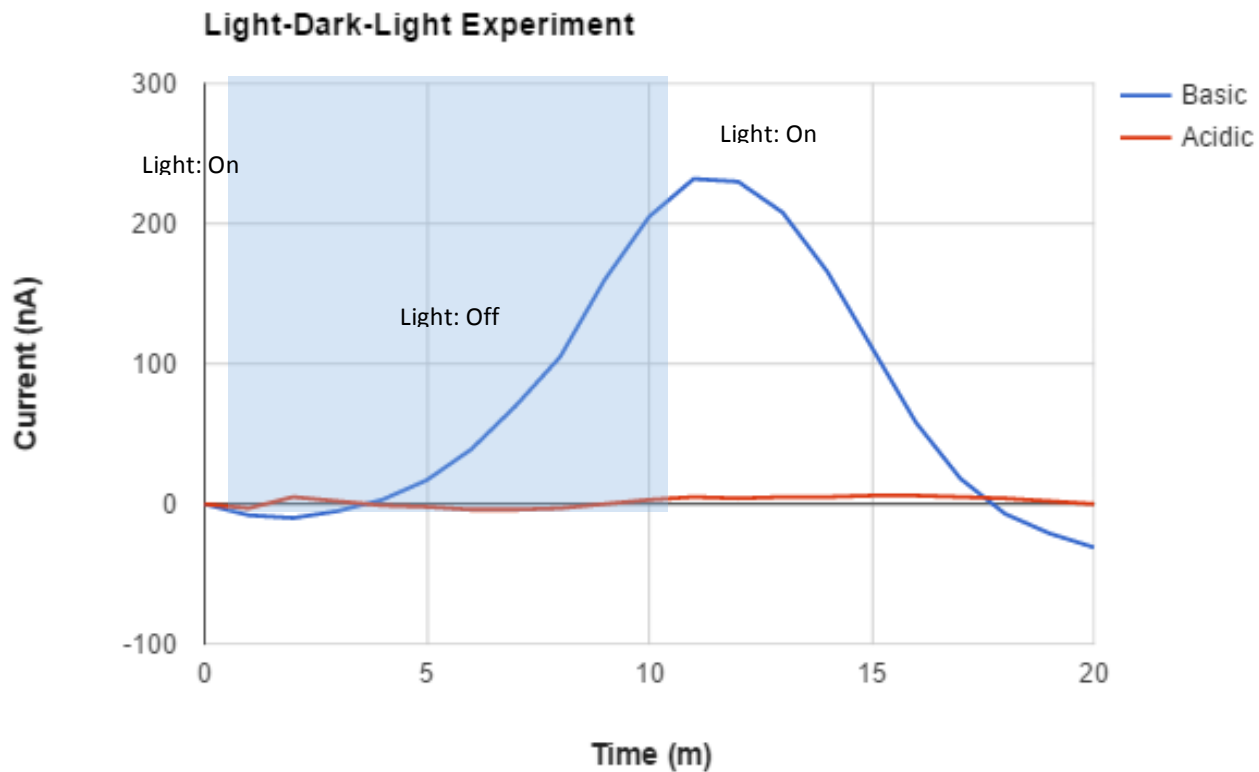


Figure 13: The representative recording of the basic and acidic regions during the light-dark-light experiment. Starting with cells sufficiently banding, recordings started in 1 minute lighted conditions, darkness for 9 minutes, and 10 minutes light. The basic region appears to be a function of the lighted environment, as it gets more acidic in darkness, and returns to its full basicity when the light is reintroduced. The acidic region remains relatively constant in changing light environments.

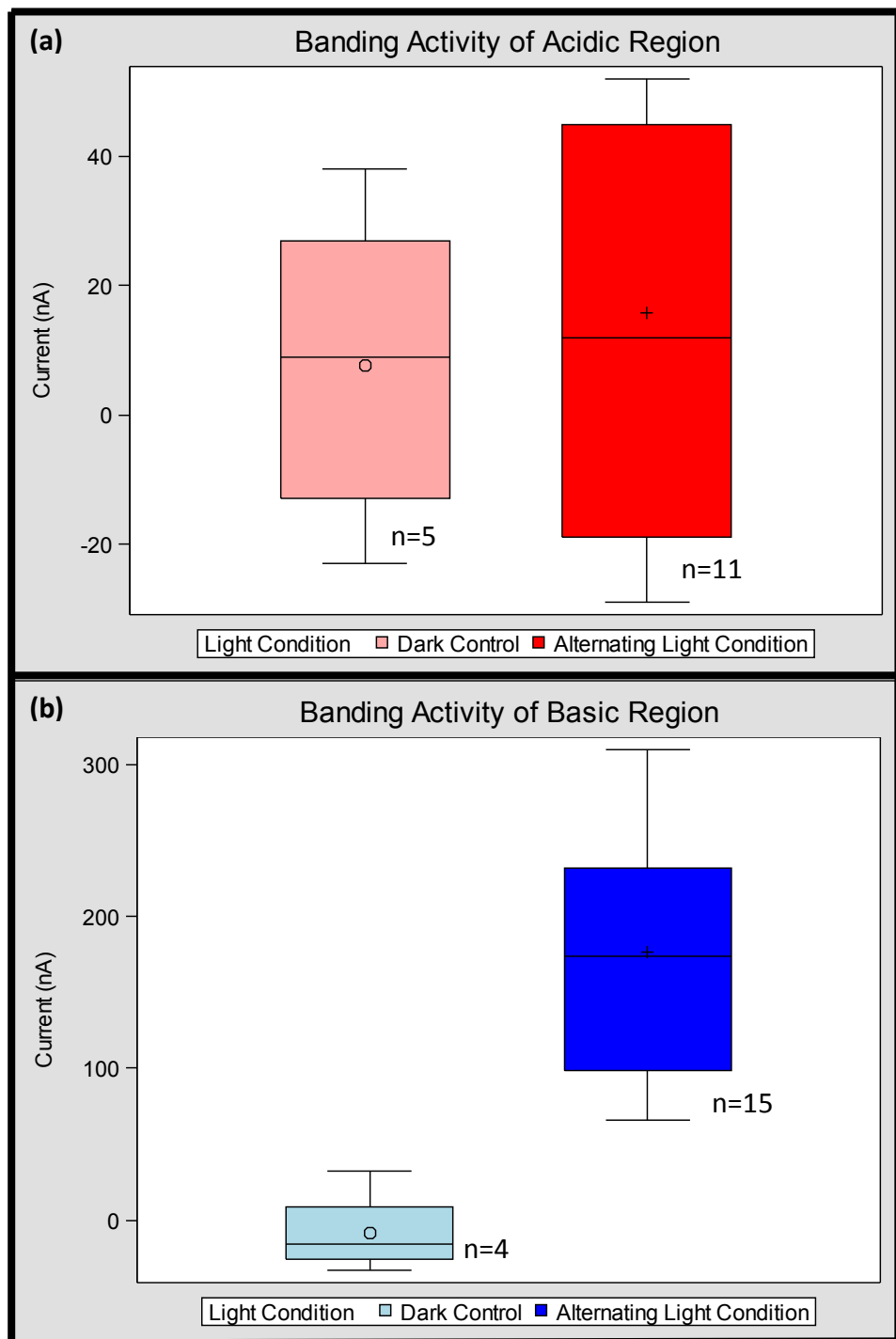


Figure 14: Comparing banding activity between the basic and acidic regions in alternating light conditions. Recorded in APWpr pH 5.6, current profiles of the dark controls and the alternating light condition are compared. The average of the max current disturbance during the 20 minute recording (a) Banding activity of acidic region. (b) Banding activity of basic region.

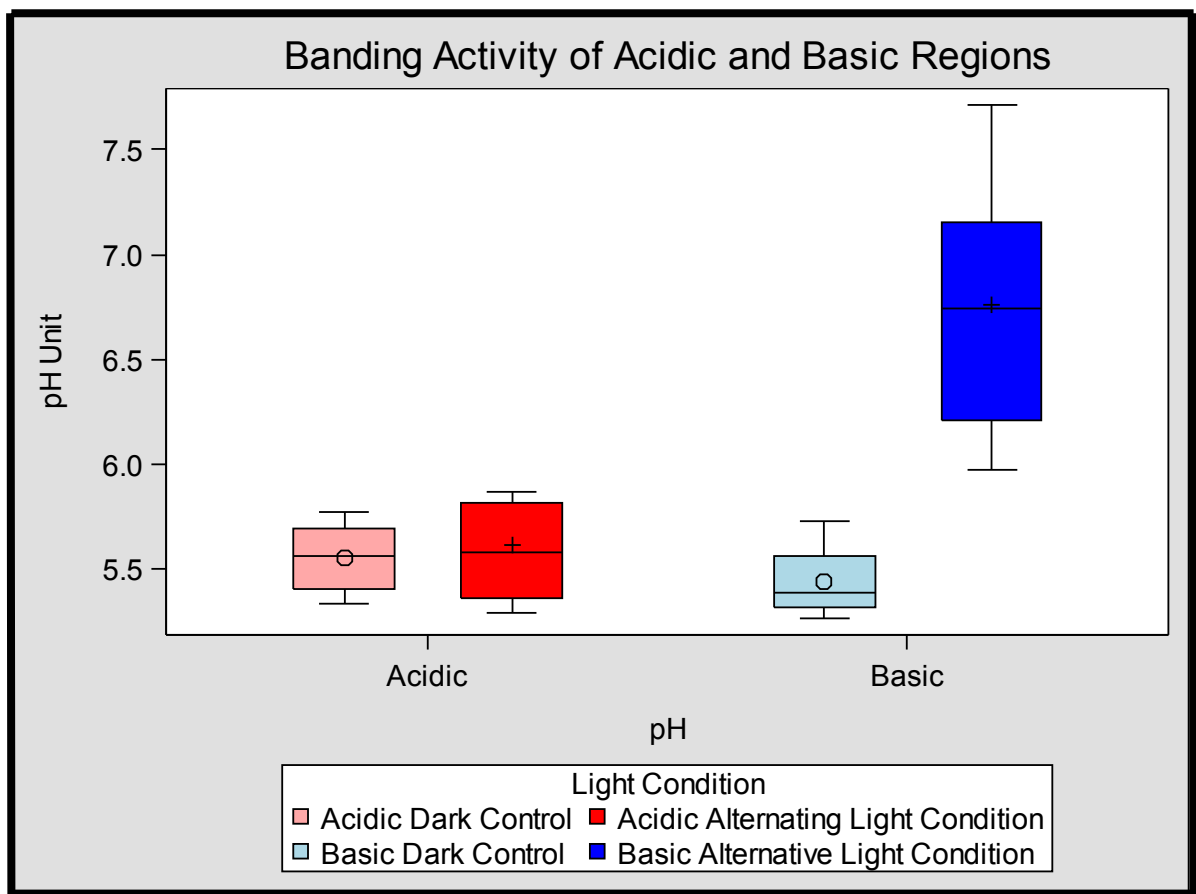


Figure 15: Comparing banding activity in pH units. Banding measured in nanoamps (**Figure 14**) was converted to pH units and the acidic and basic regions are compared. The effect of the light was only significant in the basic regions while acidic banding remains relatively unaffected by light as compared to the dark control.

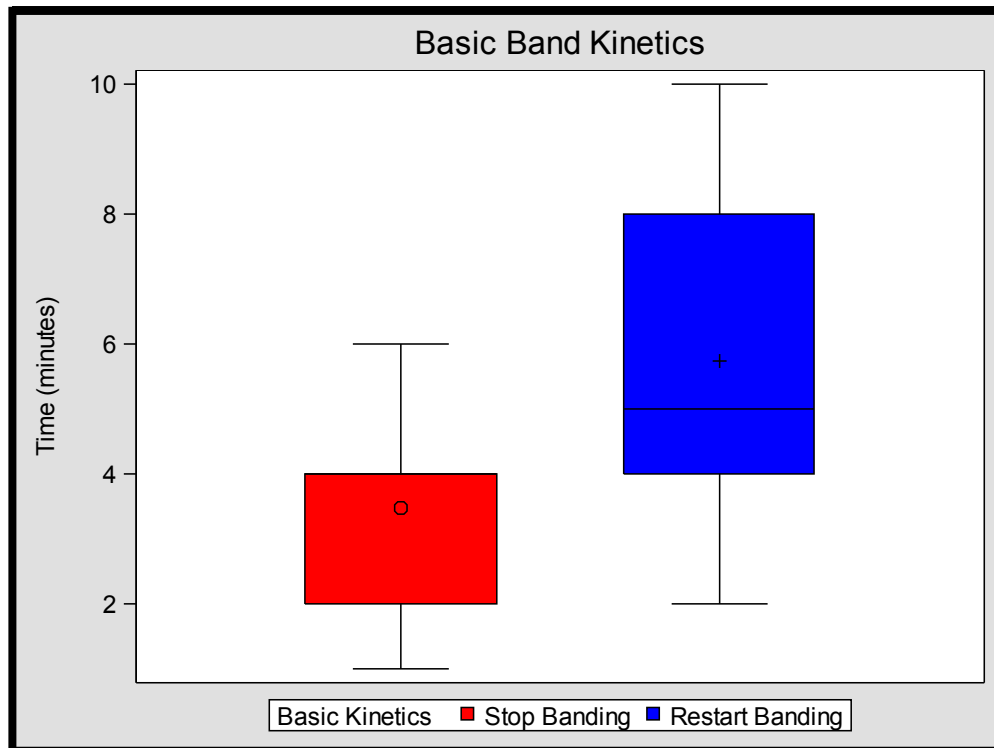


Figure 16: Basic band kinetics. Comparing the time it takes for the basic band to stop banding upon the removal of the light source (red) and the time it takes for banding to be restarted after the introduction of light (blue). Measurements were taken from the time the light was removed (stop banding) and from the time the light was reintroduced (restart banding).

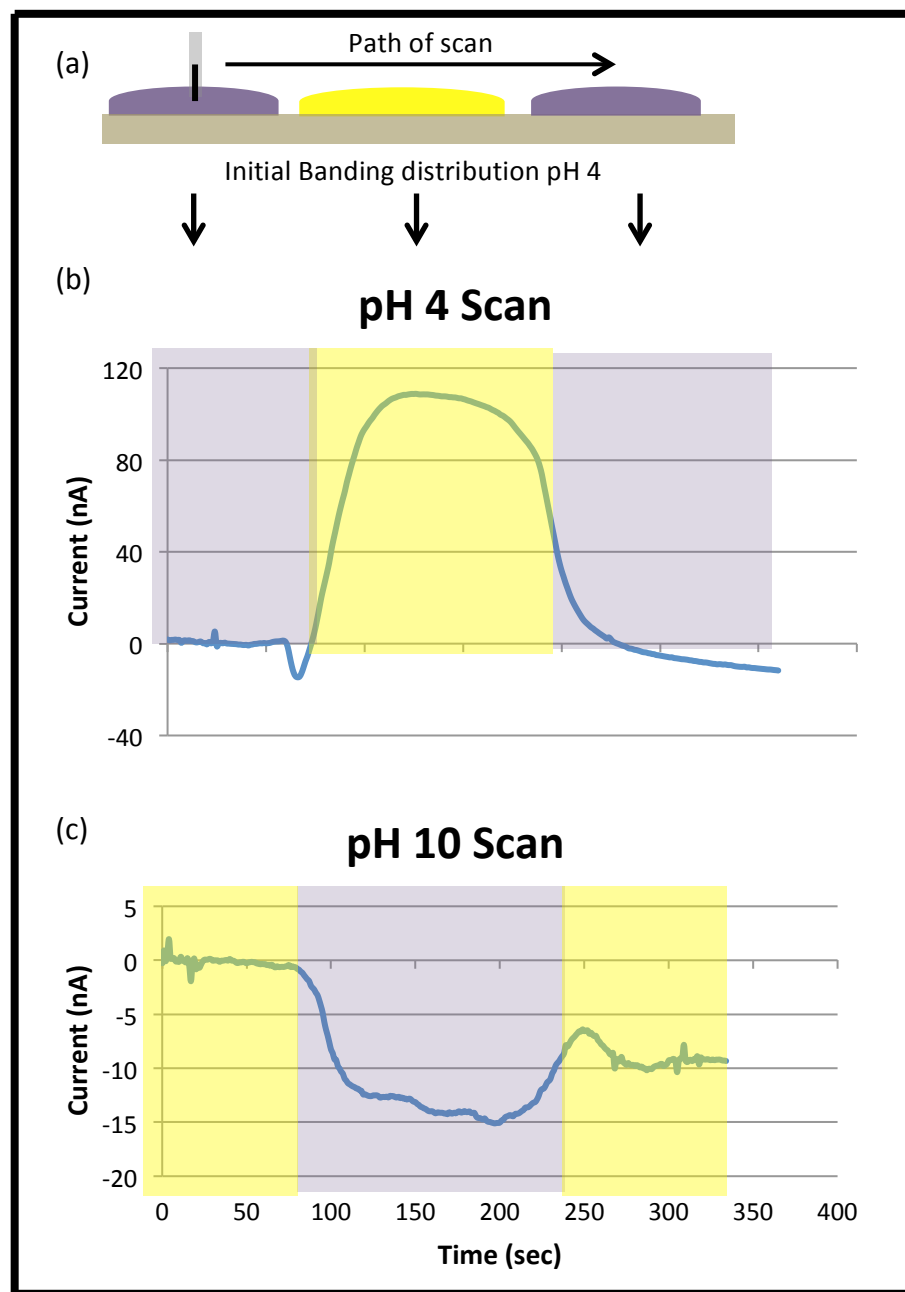


Figure 17: Repeated longitudinal scans showing the effects of high and low pH on the banding profile. (a) Path of the electrode along the cell from alkaline band (purple), through acidic band (yellow), into the far alkaline band in pH 4. (b) Banding profile of acidic environment scan, pH 4 and (c) the profile in an alkaline environment, pH 10. Colored regions highlight the pH inversion.

Appendix A

Statistical Tables

Region	Light Condition	N	Min	Q1	Med	Q3	Max
Basic	Alternating	15	66	99	174	232	310
Basic	Dark control	4	-33	-25.5	-16	9	32
Acidic	Alternating	11	-29	-19	12	45	52
Acidic	Dark control	5	-23	-13	9	27	38

Table 1: Descriptive statistics comparing basic and acidic regions in alternating light experiments. The values reported are a measure of the max disturbance from the initial banding in nanoamps. This measures the banding activity of each region of the alternating light recordings and the dark control recordings.

Region	Condition	N	Min	Q1	Med	Q3	Max
Basic	Restart	11	2	4	5	8	10
Basic	Stop	15	1	2	4	4	6

Table 2: Descriptive statistics comparing the restart and stop times of the basic region. Stop time is the time in minutes it took for banding to fully stop when light was removed. Restart time was the time in minutes the basic region took to band again from when the light was re-introduced after 9 minutes dark.

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