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The HV1 proton channel of Lingulodinium polyedrum localizes to the bioluminescent scintillon

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A Thesis Presented in Partial Fulfillment of Requirements for the Master of Science in Integrative Biology for the Department of Molecular and Cellular Biology, May 2016

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Abstract

In 1972, J. Woodland Hastings and colleagues predicted the existence of a proton selective channel that opens in response to depolarizing voltage (H_V1) across the vacuole membrane of bioluminescent dinoflagellates and conducts protons into specialized luminescence compartments (scintillons), thus causing the pH drop that triggers the light flash. RNA-Seq data from several luminescent dinoflagellate species provided candidate H_V1 genes. When expressed in mammalian cells, the predicted H_V1 from *Lingulodinium polyedrum* displays the hallmark properties of bona fide proton channels, including time-dependent opening with depolarization, perfect proton selectivity, and characteristic pH dependent gating. RT-PCR and Western blotting confirm expression of H_V1 in *L. polyedrum* and isolated scintillons. Fluorescence confocal microscopy of *L. polyedrum* cells stained with antibodies to luminescence proteins luciferase (LCF), luciferin binding protein (LBP) and to H_V1 (LpH_V1) reveal structures consistent with H_V1's proposed function in bioluminescence. Isolated scintillons immunostained with antibody to LpH_V1 displayed LpH_V1 expression, showing that LpH_V1 is present in this organelle. In addition, proteomics analysis demonstrated that isolated scintillon preparations contain peptides that map to LpH_V1, including a portion of the epitope used to raise the antibody. These results indicate that LpH_V1 is the voltage gated proton channel that triggers bioluminescence in *L. polyedrum*.

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Introduction

Dinoflagellates are single celled organisms, primarily marine but also found in fresh water. Dinoflagellates can be found in coastal waters of South America, South Africa, western Mediterranean Sea, Australia, Tasmania, Japan, and New Zealand (Zonneveld *et al.,* 2013).The dinoflagellates are very diverse in morphology, food sources, and size which range from 15 microns in size up to two millimeter in diameter (World of Microbiology and Immunology *et al.,* 2003). Dinoflagellates are surrounded by a complex covering called the amphiesma, which consists of outer and inner continuous membranes, between which lie a series of flattened vesicles (Evitt, *et al.,* 1961). In armored forms, these vesicles contain thecal plates composed of cellulose that provide "armor" in the form of a strong cell wall (Evitt, *et al.,* 1961). Dinoflagellates species differ on their primarily energy sources; some feed on other organisms, while others produce their energy using photosynthesis (World of Microbiology and Immunology *et al.,* 2003). Many dinoflagellates are photosynthetic and can form vital endosymbiotic associations with invertebrates such the zooxanthellae dinoflagellates of corals, sea anemone and other marine invertebrates. Whereas zooxanthellae are beneficial to their invertebrate host, other dinoflagellates are endoparasites of animal and protist hosts. However, a large fraction of dinoflagellates are in fact mixotrophic, combining photosynthesis with ingestion of prey. (World of Microbiology and Immunology *et al.,* 2003).

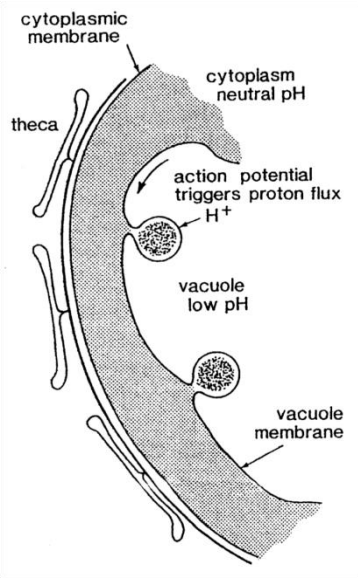


Figure 1. Cross section of *L. polyedrum.* (Fritz and Hastings, *et al.,* 1990)

A few dinoflagellate species have the interesting characteristic of bioluminescence, the production of light by living organisms. Several proposals have been offered for dinoflagellates' use of bioluminescence, the most widely accepted being defense (burglar alarm), offense (illuminate the prey) and mate attraction (Moline *et al.,* 2010).

Lingoludinium polyedrum (L. polyedrum) is a well-studied bioluminescent dinoflagellate species. *L. polyedrum* is an armored structure, marine, bioluminescent dinoflagellate species. This warm-water species is a red tide former that has been associated with fish and shellfish mortality events (Steidinger, *et al.,* 1997). *L. polyedrum* bioluminescence is primarily produced in response to mechanical stimulation in the form of shear stress; for example, upon contact by breaking waves or agitation by swirling in a flask (Fogel and Hastings et al., 1972). Shear stress exerts force on the cell membrane and most likely stimulates a mechanoreceptor. Supporting this model, luminescence was strongly inhibited in presence of calcium chelator and calcium intracellular blocker (Dassow *et al.,* 2002), suggesting that calcium mediates the response to mechanoreceptor in *L. polyedrum.* The presumed mechanoreceptor probably activates G-protein coupled receptors leading to a change from the inactive state of G-proteins (GDP) to the active state of G-proteins (Frangos *et al.,* 2007). Mechanical stimulation leads to an action potential propagated along the vacuole membrane (Eckert *et al.,* 1965).

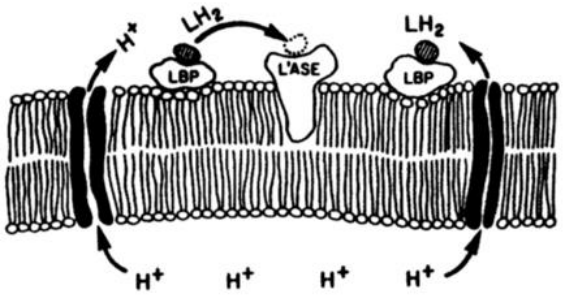


Figure 2. Proposed mechanism of bioluminescence. Voltage-gated proton channels within the scintillon membrane trigger the bioluminescent light flash in dinoflagellates. LBP: luciferin binding protein, LCF: luciferase, LH_2: luciferin, H^+: hydrogen ion/proton (Herring, *et al.,* 1978)

Bioluminescence in *L. polyedrum* appears to be mediated by small organelles within the dinoflagellates called scintillons. Scintillons are small organelles that emit the light in *L. polyedrum (*Nicolas *et al.,* 1987*).* These organelles are individual cytoplasmic bodies (0.5 µm in diameter, by electron microscopy) distributed mainly in the cortical region of the cells. In addition, the scintillons are inside or surrounded by the vacuole membrane (Nicolas *et al.,* 1987).

Scintillons (Figure 1 and 2) contain the biochemical components necessary for light production (Fogel and Hastings et al., 1972): the small molecule luciferin (LH_2) and two proteins, luciferin binding protein (LBP) and the oxidative enzyme luciferase (LCF). The bioluminescence of *L. polyedrum* is controlled by circadian rhythm, with the phase set by light-dark cycles (Morse *et al.,* 1990). The Morse group showed that the flashing response to mechanical stimulation was greater during the night than during the day; they further demonstrated that the cells exposed to constant light display a persistent circadian rhythm, which was maintained for 20 days (1990). The biochemical components that result in luminescence are also under circadian control; in *L. polyedrum* these proteins are produced and destroyed on a 24 hour cycle (Morse *et al.,* 1990).

In the scintillons, pH plays a central role in producing the light. The reaction occurs in acidic conditions when the pH is lowered from 8 to 5.7 (Fogel and Hastings et al., 1972). When this drop in pH occurs, luciferin is released from its binding protein (LBP) and LCF conformation shifts from inactive to active form to catalyze the reaction with luciferin, emitting a photon (Figure 2) (Herring *et al.,* 1978). Based on the correlation of the action potential known to propagate along the vacuole membrane (DeSa & Hastings *et al*., 1968) and a change in pH, Fogel and Hastings (1972) proposed the existence of a voltage dependent proton channel that conducts protons from the acidic vacuole into the scintillon lumen in response to voltage change in the vacuole membrane; this pH drop would trigger the light flash from the scintillons. The signaling cascade signaling from mechanical stimulation to bioluminescence is illustrated in Figure 3 (Iglesias *et al*., 2013).

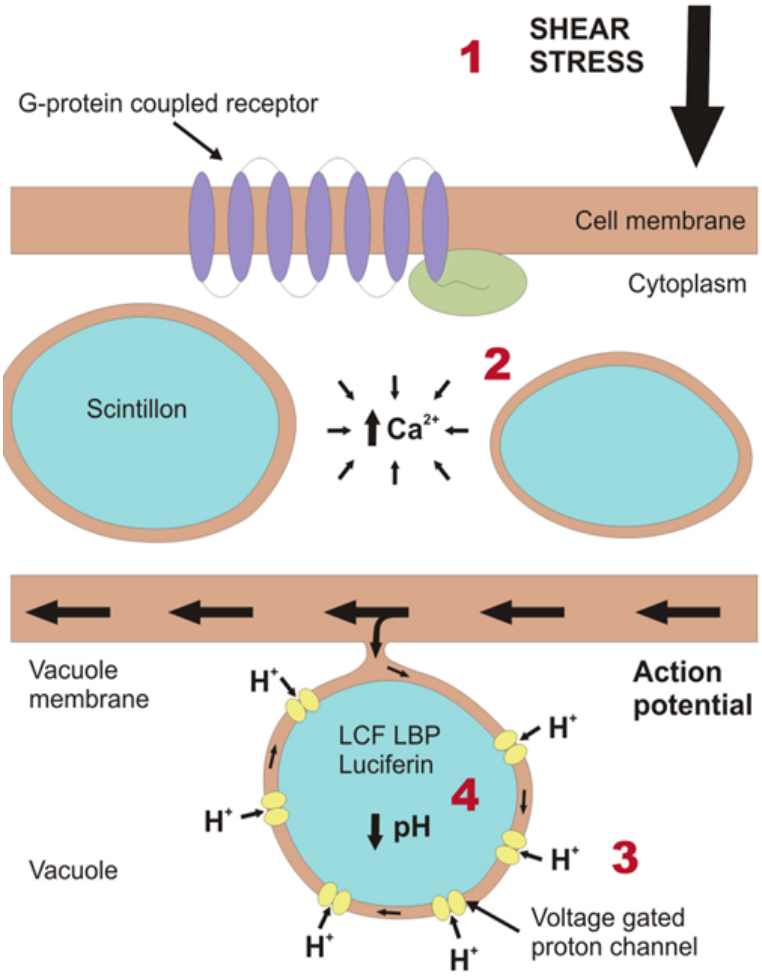


Figure3. Schematic representation of part of a dinoflagellate cell, depicting the cellular processes that take place to generate a bioluminescence flash. (1) Mechanical action on the cell membrane provides the signal which activates the receptor. (2) Cytoplasmic calcium levels increase. (3) Differences in ion concentrations create an action potential. (4) The voltage proton channel is activated, allowing proton movement into the scintillon thereby dropping the pH and promoting the chemical reaction resulting in bioluminescence (Images from Iglesias *et al*., 2013).

The H_V1 voltage gated proton channel is a transmembrane protein, which transports protons out of many types of cells (Sasaki *et al*., 2006). The gating of H_V1 is controlled by voltage and modulated by the pH gradient (ΔpH) (DeCoursey *et al.,* 2012). H_v1 displays, as far as can be measured, perfect proton selectivity (DeCoursey *et al.,* 2012). The best known function of the voltage gated proton channel is its role in phagocytes, where H_V1 participates in regulating production of the reactive oxygen species (ROS) by which phagocytes kill bacterial pathogens (DeCoursey *et al.,* 2012). Sequence and structural analysis showed that H_V1 is a homolog of the voltage sensor domains (VSD) of the long-studied voltage gated ion channels and of the more recently discovered voltage sensitive enzymes. Proposed 'signature sequence' elements were used to discover a bona fide H_V1 in the non-bioluminescent dinoflagellate *Karlodinium veneficum* (Smith *et al*., 2011)

The first objective of this work was to use the same signature elements to search for homologous sequences within genomic units generated by RNA seq experiments on dinoflagellates (Place *et al*., unpublished results) including the RNA-seq library of *L. polyedrum* generated by the Beauchemin group (Beauchemin *et al.*, 2012). From the RNA seq data, a putative H_V1 was identified in the bioluminescent dinoflagellate *L. polyedrum,* which is referred to as LpH_V1 (***L****ingoludinium* ***p****olyedrum* **H_V1**). Based on the predicted sequence, the next objective was to construct a synthetic gene for expression in a cell line. The resulting expression in mammalian cells showed LpH_V1 was a *bona fide* H_V1 with hallmark perfect proton selectivity (DeCoursey *et al*, unpublished results). It was hypothesized that if LpH_V1 conducts protons through the scintillon membrane, LpH_V1 should be detectable in isolated scintillons. The final objective was to determine that LpH_V1 localizes to the scintillon using immunostaining techniques and confocal laser scanning microscopy. Bioluminescence proteins LBP and LCF were used as positive markers of the scintillon (Desjardins and Morse *et al.,* 1993). The results presented here demonstrate that LpH_V1 does localize to the scintillons, strongly supporting the prediction that a voltage gated proton channel triggers the bioluminescent light of *L. polyedrum*.

Materials and Methods

Dinoflagellate culture. Cultures of *L. polyedrum* (CCMP 1932, obtained from National Center for Marine Algae and Microbiota-Bigelow Laboratory for Ocean Sciences) were established by inoculation of the dinoflagellate into L1-Silicate (Si) (L1 minus silica; Guillard & Hargraves, 1993) or f/2-Si (f/2 minus silica; Guillard & Ryther, 1962) algal growth medium prepared in artificial seawater (Instant Ocean, Blacksburg) and maintained in 12hr:12hr or 14hr:10hr light:dark cycle (photon flux 100µmoles/m^2/s) at 18-20°C. Experiments were performed when the cultures reached a cell density of 4,000-10,000 cells/ml. At this density cells were collected at two different phases: at mid-dark and mid-day time points.

Preparation of purified recombinant proteins. The LBP clone in pGEX4T plasmid was obtained from David Morse. LCF and LpH_V1 genes were synthesized (Genscript, Piscataway NJ) based on the published or the RNA-Seq data, respectively; both were codon optimized for human expression and cloned into pQE30 (LCF) or pGEX-6P (LpH_V1) . The recombinant LBP and LpH_V1 proteins tagged with glutathione S-transferase (GST) were expressed in *Escherichia coli* (*E. coli)* after induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 23°C (LBP) or 30°C (LpH_V1) for 8-24 hours. Recombinant LCF protein tagged with 6-His was expressed in *E. coli* by induction with IPTG at 23°C for 16-24 hours. Proteins were purified from *E. coli* according to the manufacturer's instructions (GE-Healthcare Life Sciences, Buckinghamshire, UK for LBP and LpH_V1; Qiagen, Valencia, CA for LCF). PreScission protease (GE-Healthcare Life Sciences, Pittsburgh, PA) was used to remove the GST tag from the LBP-GST and LpH_V1-gst proteins while bound to a glutathione affinity column, according to the manufacturer's instructions. For the LBP-GST fusion protein, cleavage of the GST resulted in formation of a protein precipitate.

Antibody preparation. Polyclonal antibodies to LCF and LpH_V1 were produced against synthetic peptides by GenScript Antibody Services (Piscataway, NJ). The

peptide used to raise LCF antibody is CLDYPKKRDGWLEKN, which is in the D3 domain of LCF.. For LpH_V1 the peptide used was CDAGRQLSSDGDQ, which is in a loop between two LpH_V1 helices. Primary antibodies were raised and affinity purified from rabbit (LpH_V1, final protein concentration 1.1 mg/ml) or chicken (LCF, final protein concentration 0.88 mg/ml). Primary antibody to LBP (rabbit) was a gift from David Morse; we do not know the concentration. (Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal).

***L. polyedrum w*hole cell lysate and protein extraction**. One liter of *L. polyedrum* cells at a density of 10,000 cells/ml at the mid-dark point was collected by filtering through Hardened Ashless 150mm diameter filter paper (GE-Healthcare Life Sciences, Buckinghamshire, UK). Cells were rinsed off the filter paper surface and into 50ml conical tubes with artificial seawater, followed by centrifugation at 3200 x *g* for 5 minutes. The supernatant was discarded and the cells were resuspended in Zymo lysis buffer (Zymo RNA isolation kit, Zymoresearch, Irvine CA). Cells were mechanically disrupted by bead-mediated lysis at 4°C for 1 minute. The bead-lysate mixture was centrifuged at 12,000 x *g* for 1 minute at 4°C followed by transfer of the supernatant to a 1.5ml tube. Beads were rinsed three times with 300µL Zymo lysis buffer and the rinses were added to the tube holding the supernatant. Two hundred microliters of chloroform was added to the supernatant/rinse, vortexed and incubated for 5 minutes at room temperature, followed by centrifugation at 12,000 x *g* for 15 minutes at 4^oC. The supernatant layer was carefully removed and the pellet was resuspended in 300µL of 95% ethanol, and centrifuged 2,000 x *g* for 5 minutes at 4°C. The supernatant was transferred to a new tube, to which an equal volume (1:1) of 100% cold isopropanol was added and incubated at -20°C for ~1hour to precipitate proteins. After incubation, the suspension was centrifuged at 14,000 x *g* for 30 minutes at 4°C. The supernatant was removed, followed by resuspension of the pellet in 1ml of 95% ethanol, and then centrifuged at 14,000 x *g* for 30 minutes at 4°C. The supernatant was removed and the pellet was

allowed to air dry. Finally, the dry pellet was dissolved in 120µL SDS-PAGE buffer.

Scintillon isolation. *L. polyedrum* cells were collected by filtration at mid-dark point as described above. Scintillons were isolated by density gradient according to established methods (Desjardins & Morse, 1993). Briefly, a 90% Percoll gradient in pH 8 phosphate buffer was created whereby scintillons should be at the bottom of the gradient. Cell fractions were collected and the presence of scintillons in fractions was detected by using the luminescence assay (Desjardins & Morse, 1993). One microliter of the fraction was placed in a luminometer cuvette to which 300µL of acidic luminescence assay buffer (0.2M phosphate (pH 6), 0.25mM EDTA and 0.1mg/ml BSA) was injected. Light was detected by the FB-12 single tube luminometer (Berthold, Bad Wildbad, Germany). Fractions containing > 25% of the peak light production were collected. Percoll was removed by repeated dilution with ice-cold extraction buffer (100mM Tris pH8, 10mM EDTA, 0.25M sucrose and 14mM β-ME) (Desjardins & Morse, 1993) and centrifuged at 30,000 x *g* until less than 5% of Percoll remained. After final centrifugation all supernatant (liquid) was removed and the pellet was resuspend in 100µL extraction buffer per 10g initial wet weight of cells.

Sample concentration assay. Protein concentrations in whole cell lysates, purified proteins and isolated scintillons were determined using the Pierce 660nm protein reagent protocol (ThermoFisher Scientific, Waltham, MA). To set up the standards, serial dilutions of bovine serum albumin (BSA) were made representing protein concentrations from 25 µg/ml to 2000 µg/ml. The samples were loaded in a 96 wells plate, absorbance was read at 660 nm on a BioTek (Vermont) plate reader, and protein quantification was determined by the Gen 5 program.

Western blot analysis. Total cell lysate, purified scintillon proteins, and/or preparations of purified recombinant proteins were analyzed by Western blot analysis using the polyclonal antibodies previously described. In each case, sample was denatured by heating for 10 min at 95°C in 4X SDS-PAGE sample buffer with 5% βeta-mercaptoethanol (β-me). Proteins were separated by gradient SDS-PAGE, blotted onto a polyvinylidene fluoride (PVDF) transfer membrane for 30minutes at 25 mV (Bio-Rad, California USA) and blocked for 1 hour at room temperature with Odyssey Licor blocking buffer (Licor, Lincoln, NE). After blocking, membranes were immersed in solutions containing diluted primary antibodies to LCF (1:1000), LBP (1:10000), and LpH_V1 (1:3000) and incubated overnight at 4°C (cold room). Primary antibody binding to proteins was detected with secondary antibodies to the appropriate animal primary immunoglobulin conjugated with Licor fluorescent tags. Secondary antibody for LBP and LpH_V1 (anti-rabbit) was diluted at 1:20000 and for LCF (anti-chicken) was diluted 1:5000; both were diluted in Odyssey Licor blocking buffer and incubated at room temperature for 1 hour. Reactions on the PVDF membrane were visualized using the program Image Studio from the Licor Odyssey system (Nebraska, USA); LBP and LpH_V1 were detected with secondary goat anti-rabbit IgG IRDye 680LT (Nebraska, USA) and LCF was detected with secondary donkey-anti chicken IgG tagged with IRDye 800CW (Nebraska, USA). To determine the molecular weight of our proteins interest, PageRuler prestained standard protein marker was used (Fisher Scientific, Pittsburgh USA).

Fixation and immunostaining of whole cells and isolated scintillons. Whole cells were cultured and collected at mid-dark point as previously described. The following fixation and staining procedure is a modification of the procedure described by Brunelle, Hazard, Sotka, & Dolah (2007). Preliminary experiments indicated that low speed centrifugation was optimal to preserve cell integrity; therefore, all centrifugation steps for this procedure were performed at 300 x *g*.

Cells were pelleted from 50-100 mL of culture by a 5 minute centrifugation, resuspended in 1mL seawater, concentrated again by centrifugation for 1 minute, followed by careful removal of the supernatant. Cells were fixed by incubating in 4% paraformaldehyde in seawater at room temperature for 10 minutes, pelleted by centrifugation, and washed 3 times with 1mL of phosphate buffered saline (PBS). Cells were incubated in 1.5mL of 100% methanol at 4°C for 1 hour, washed 3x at room temperature with PBS supplemented with 0.1% Tween-20 and 1 % BSA (PBST-BSA), and then blocked by incubation in PBST-BSA at room temperature for 1 hour. Primary antibodies were added at the following dilutions: α-LBP 1:300 dilution, α-LCF 1:100 dilution and α- LpH_V1 1:300 dilution. The serum negative controls were diluted to match the dilutions of the primary antibodies. Primaries experimental and negative control were diluted onto PBST-BSA (0.1%tween and 1%BSA) and incubated overnight rotating at 4°C.

Following overnight incubation, cells were pelleted and washed 3 times by incubation at room temperature with PBST-BSA. For detection, Alexa555-Fluor-conjugated goat anti-rabbit IgG (H+L) and/or Alexa555-Fluor-conjugated goat anti-chicken IgG (ThermoFisher, Waltham MA) were added to the appropriate primary antibody treatment and incubated in the dark for 1 hour at room temperature. Cells were washed 3 times for 10 minutes with PBST-BSA at room temperature, incubated with 4', 6-diamidino-2-phenylindole (DAPI) 5µg/mL in PBS for 5 minutes, and washed again with PBST-BSA. Cells were centrifuged for 30 seconds at 300 x *g*, and almost all the supernatant was removed. Cells were incubated in 2 drops of Vectashield overnight at 4°C in the dark and 15 to 50 µL were mounted on slides.

Fixation and immunostaining of isolated scintillons were performed as described for whole cells, but without the methanol permeabilization step. The native luciferin fluorescence excitation (DAPI filter) were used to identify the scintillons. In addition, the confocal microscopy was used to score for the presence or absence of secondary antibody fluorescence. Scintillons treated with primary antibodies to both LBP and LCF were scored for fluorescence from secondary antibodies to both the primary antibodies, and scintillons treated with

primary antibodies to both LCF and LpH_V1 were also scored for fluorescence from fluorochrome-conjugated secondary antibodies to both primary antibodies. The negative controls groups (No 1^{st} ab, LCF pre-serum and LpH_V1 pre-serum) were also scored the same way. Cells and scintillons were visualized using a Zeiss LSM 700 confocal microscope, equipped with a 20x (whole cells) and 40x (scintillons) 1.2 NA C-Apochromat water objective For immunofluorescent localization, all channel pinholes were set to 1 Airy Unit. Confocal slices or maximum intensity projections of the Z-stack were rendered using Zeiss Zen software (ZEISS Microscopy, Pleasanton, CA), and processed using Adobe Photoshop.

Scintillon Immunoprecipitation by LpH_V1 antibody. Immunoprecipitation (IP) experiments with the isolated scintillons were performed. About ~40µg of scintillons was combined with 10µg of LpH_V1 antibody; then the volume was brought up to 1mL with Pierce IP lysis/wash buffer from the Pierce Co-Immunoprecipitation kit (Thermo Scientific, Rockford USA); 1% octylphenoxypolyethenoxyethanol (IGEPAL) (Sigma life sciences, St. Louis USA) was added and the mixture was incubated overnight at 4°C. IGEPAL was added to release LpH_V1 from the membrane. Pierce protein G magnetic beads (Thermo Scientific, Rockford USA) for the IP targets (LpH_V1) were used. After the beads were prepared, the combined sample (scintillons + α-LpH_V1) was added to the beads, then washed and eluted with 100µL SDS-PAGE buffer. Also a 25µL of 4x LDS-β-me was added and heated at 95 °C for 10minutes.

Image analysis. Images were analyzed using the Zeiss Zen software image analysis tools. Whole cells were identified by the DAPI stained nuclei. Zen blue software Image analysis functions were used to select the cell shape and to measure the fluorescent intensity per area. We selected and measured 30 cells area fluorescence intensity and reported the average cell area of each cell. Scintillons were identified by native luciferin fluorescence and outlined. Because of the small size of the scintillons, we selected the region of interest by drawing a

circle to “encapsulate” the scintillon. Then the fluorescence intensities in that selected area for luciferin and each fluorochrome conjugated antibody were measured. The diameter of the scintillons was also measured, using the line feature from the same software program. In addition, scintillons from antibody and negative control treatments were identified by luciferin fluorescence, and the proportion of scintillons displaying secondary antibody fluorescence was determined. Graphs for whole cells and scintillons immunostaining analysis were prepared using the Graphpad Prism program.

RNA extraction and cDNA synthesis. Fifty milliliter aliquots (7,000cells/mL) from mid-dark cultures were collected and centrifuged at 500 x *g* for 20 minutes at 4°C. RNA was extracted as described in the Sigma-Aldrich TRI reagent protocol (Montana, USA) with modifications. Supernatant was removed and cells were resuspended in 1mL TRI-reagent (Zymoresearch, Irvine CA).). Resuspended cells were mechanically disrupted by bead-mediated lysis at 4°C for 1.5 minutes, with lysis confirmed by observing broken cells using a light microscope 10x magnification. Lysed cells were transferred to a 1.5mL Eppendorf tube, incubated at room temperature for 15 minutes, then centrifuged at 12,000 x *g* for 10 minutes at 4°C. After centrifugation, 200µL of chloroform was added followed by mixing the sample by repeated inversion until the sample appeared homogenous appearance. The preparation was incubated at room temperature for 10 minutes, and then centrifuged for 15 minutes at 12,000 x *g* at 4°C. After this centrifugation, three layers were formed. RNA (colorless layer) was transferred to a new tube and an equal volume of cold isopropanol was added, and then centrifuged at 12,000 x *g* for 15 minutes at 4°C. The supernatant was removed and 1 mL of 75% cold ethanol was added onto the pellet. The sample was centrifuged at 7,500 x *g* at 4°C for 5 minutes. Supernatant was removed and the pellet (RNA) was allowed to air dry at room temperature. The pellet was resuspended with 25µL DNA/RNASE free water.

RNA concentration was quantified via 260nm absorbance using a BioTek microplate reader (Vermont, USA). RNA quality was assessed by the ratio of 260/280 nm absorbance with an acceptable quality ratio of >1.65 and by agarose bleach gel analysis (Aranda *et al*., 2012). One microgram of RNA was loaded into a 1% agarose gel supplemented with 1% Clorox bleach and ~0.5µg/ml of ethidium bromide. RNA sample integrity was verified by examining for the presence of ribosomal RNA bands (28S, 18S or 5S) in "bleach gel".

RNA was reverse transcribed using Superscript III reverse transcriptase kit (Invitrogen by Life Technologies, ThermoFisher Scientific, Waltham, MA) with random hexamer primers according to the manufacturer's protocol. The cDNA concentration was quantified via 260nm absorbance using the same microplate reader and then used as template for polymerase chain reaction (PCR), using primers designed against LpH_V1 (Table 1). PCR products were visualized by loading 12µL of each PCR product (contains 2µL of 6X loading dye) into a 1% of agarose gel.

Statistical Analyses. For the immunostaining of the whole cells, an ANOVA was used to test the differences in secondaries antibodies fluorescent intensity between the experimental and the negative controls. The ANOVA analysis was followed by unpaired Tukey test for significant differences. For the immunostaining of the scintillons different analytical measurements were performed. To analyze the secondaries antibodies detection on the scintillons a statistics for difference between two proportions was performed (Figure 10 graph). The null hypothesis was that both compared proportions are the same (i.e. LBP vs No 1^{st} ab), meaning that secondaries antibodies detection would be the same between the experimental and the negatives controls antibodies. If the calculated difference between two proportions (z) is greater than 2.33, the null hypothesis would be rejected. To compare the native luciferin (LH2) across all treatments an ordinary one-way ANOVA multiple comparisons was performed (Figure 11 LH2). To analyze the differences between the experimental antibodies versus the negative controls; an ANOVA followed by t test was performed (Figure 11 Ab).

RESULTS

H_V1 sequence identification. A sequence search within RNA-seq assemblies (Beauchemin *et al*. 2012; Keeling *et al*., 2014) from the bioluminescent dinoflagellate *L. polyedrum* revealed a sequence with the signature sequence (Smith *et al.*, 2011) diagnostic of H_V1. cDNA libraries prepared from *L. polyedrum* populations sampled at mid-light and mid-dark and probed with PCR primers designed using the RNA-seq data produced PCR products matching predicted sizes. Additionally, qRT-PCR showed that the RNA for the putative H_V1 was expressed in *L. polyedrum.*

LpH_V1 is a bona fide voltage-gated proton channel. Synthesis of a mammalian codon-optimized gene corresponding to the predicted *L. polyedrum* H_V1 gene was constructed by Genscript (Picataway, NJ). When the gene was expressed in the human cell line HEK-293 the gene product produced voltage- and time-dependent currents in voltage-clamped cells (Fig. 1). The current during depolarizing voltage pulses turned on much more rapidly at more positive voltages. Measurement of the reversal potential (V_{rev}) at a variety of pH_o and pH_i confirmed that these currents were proton selective, because V_{rev} was close to the Nernst potential for H^+ (E_H) (Fig. 2). Deviations of V_{rev} from E_H most likely reflect the difficulty of controlling the pH perfectly. The gene product is clearly a highly proton-selective voltage-gated channel, so it was named LpH_V1 (*L. polyedrum* H_V1).

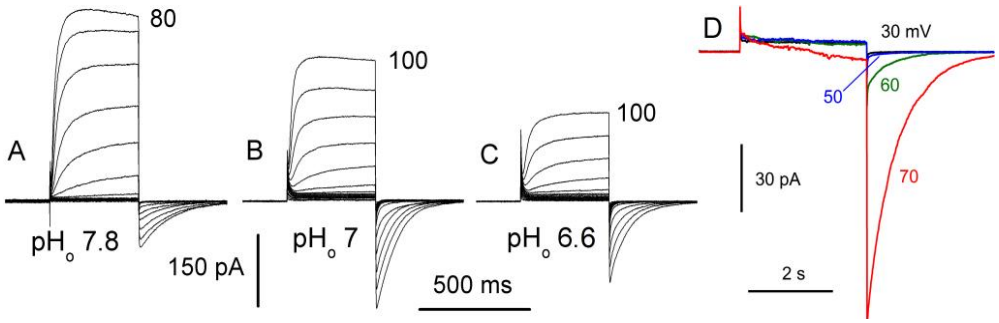


Figure 1. LpH_V1 is a voltage gated proton channel. A-C, Families of whole-cell proton currents at different pH_o in a cell transfected with LpH_V1, with pH_i 7.0. Pulses were applied from a holding potential of -60 mV (A, B), or -40 mV (C), in 10 mV increments up to the voltage indicated. D. Inward H^+ currents can be seen with inward pH gradients. Currents are shown during pulses to 30, 50, 60, and 70 mV as indicated, in an inside-out patch with pH_o 7 (pipette) and pH_i 9 (bath). From the tail currents upon repolarization to the holding potential of -40 mV, it is clear that the g_H was activated detectably by the pulse to 50 mV, with small inward current evident during the pulse to 60 mV, and larger inward current at 70 mV.

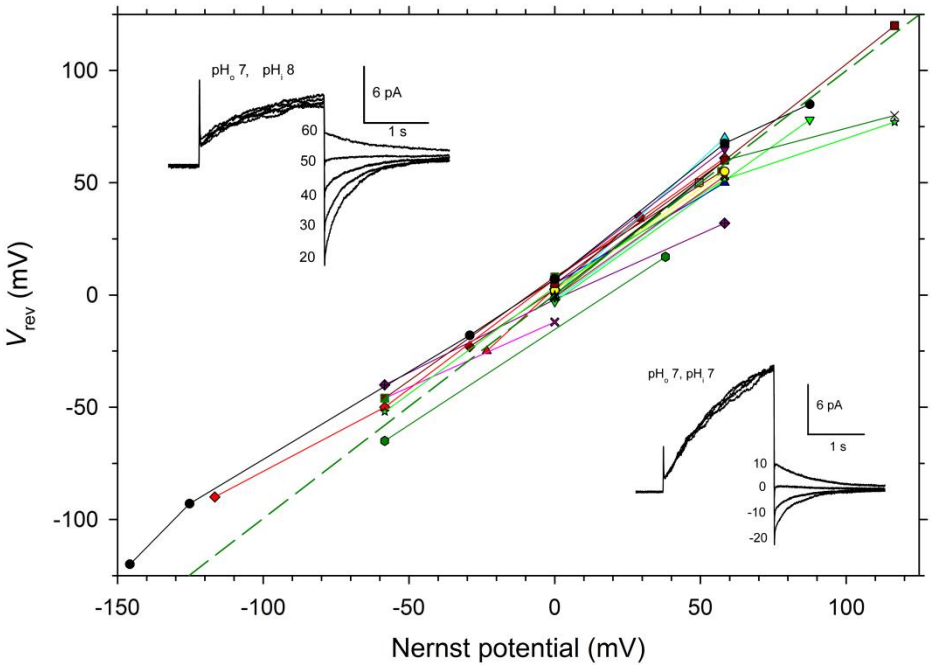


Figure 2. LpH$_V$1 is a proton selective channel. The reversal potential (V_{rev}) was measured, usually by tail currents, in both whole-cell and excised-patch configurations over a wide range of pH (pH$_o$ 4.5-9.0; pH$_i$ 4.5-10.0). Measurements from individual cells or patches are connected by lines. The dashed green line indicates $V_{rev} = E_H$. Insets are tail current measurements from an inside-out patch, with pH$_o$ 7 in the pipette, and pH$_i$ 6 or 7, as indicated, in the bath. V_{rev} shifts from -2 mV at pH$_i$ 7 to 53 mV at pH$_i$ 6, a change of 55 mV, near the Nernst expectation of 58 mV.

LpH$_V$1 protein is found in *L. polyedrum* whole cell lysate. SDS-PAGE followed by Western blotting was performed to confirm specificity of commercially raised antibodies to recombinant LCF and LpH$_V$1 proteins, which were expressed and purified from *E. coli.* As a positive control, LBP reactive antibody provided by Dr. David Morse was included (Roy and Morse, 2014). LBP and LpH$_V$1 with GST affinity tags were purified, and had expected sizes (protein + tag) of 100KDa and 60KDa, respectively. LCF recombinant protein with a histidine tag was purified and had an expected weight near its native size of 134KDa. As shown in Figure 3, all three antibodies detected the respective recombinant protein at the expected sizes. Furthermore, recombinant proteins were detected at their expected sizes when probed with antibodies against the tag component including anti-GST antibody for LBP-GST and LpH$_V$1-GST, anti-6His antibody for LCF-6xHis (data not shown). When the GST-tag was cleaved from LBP, the protein precipitated. GST-free LBP precipitate boiled in SDS-PAGE sample buffer and

probed with anti-LBP did show a prominent band at about 75 kDa (data not shown), but this preparation was difficult to solubilize so the remaining work utilized GST-LBP, which was soluble.

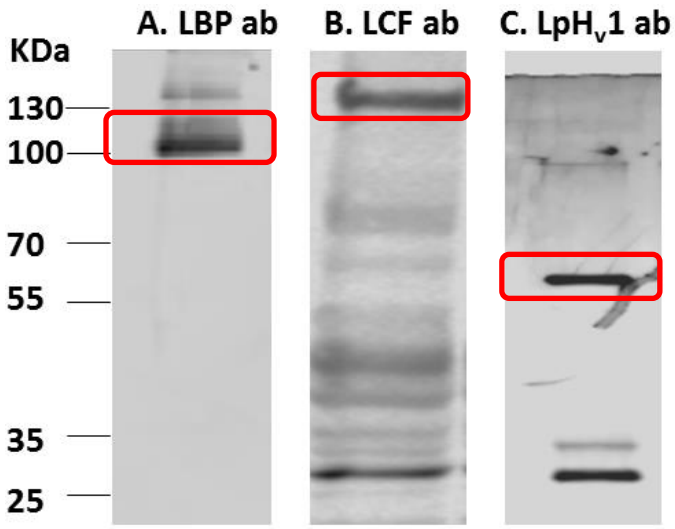


Figure 3. SDS-PAGE of purified recombinant proteins, followed by Western blot. A. Purified LBP-GST protein, probed with anti-LBP. B. LCF-His protein probed with LCF antibody. C. LpH_V1-GST protein probed with LpH_V1 antibody. Primary antibodies to LBP and LpH_V1 raised in rabbit were detected with secondary anti-rabbit IgG tagged with IRDye 680LT. Primary antibody to LCF raised in chicken was detected with secondary anti-chicken IgG tagged with

To test the presence of native LpH_V1 in *L. polyedrum* the protein was purified from a whole-cell lysate preparation followed by SDS-PAGE and Western blotting. Cells for the lysate were collected at mid-dark point, which was previously reported as the time of highest scintillon protein expression (Fogel and Hastings *et al*., 1972). Antibodies to LBP and LCF, positive controls for the Western blot analysis, detected proteins at the expected size (Figure 4, A and B). Some additional bands were observed and likely represent degradation products as seen in earlier studies (Fogel and Hastings *et al*., 1971), such as the lower molecular weight bands at approximately 35-55 KDa in the LCF Western blot results. The antibody to LpH_V1 detected several prominent bands at ~60 kDa and ~55 kDa, and at 30 kDa, and several other minor bands (Figure 4C). Western

blots of scintillon preparations isolated in another laboratory and probed with our antibody detected a protein at 30 kDa. Because of this, and evidence presented below, in the case of LpH_V1, we believe that the band at ~60 KDa may represent an oligomeric state, while the band at ~30 kDa represents the native LpH_V1 protein (Figure4C).

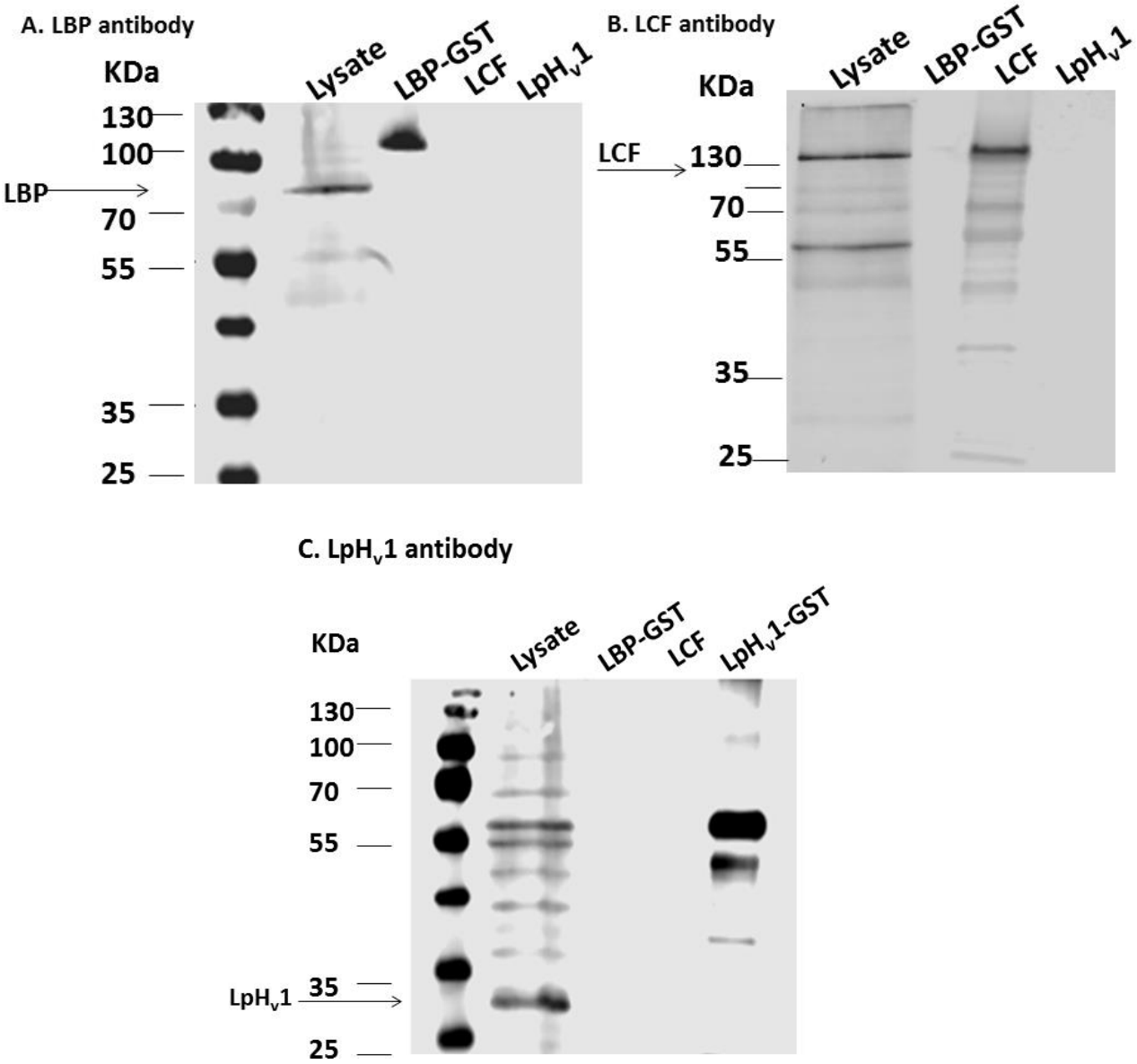


Figure 4. SDS-PAGE separation of protein from *L. polyedrum* whole cell lysate and purified proteins, followed by Western blot. Figure 4 shows native expression of LBP 75kDa (A), LCF 134kDa (B) and LpH_V1 ~30kDa (C). Cell lysate and purified proteins were probed with same primary and secondary antibodies as in figure 3.

LpH_V1 stains multiple membranes in whole cells. The presence and location of LpH_V1 in *L. polyedrum* cells was detected by immunostaining assay. If the function of LpH_V1 is to allow protons to transport across the vacuole into the scintillons as predicted, LpH_V1 should be localized to the scintillon membranes. As a positive control bioluminescence proteins LCF and LBP were used, which were previously demonstrated to localize to scintillons (Desjardins & Morse, 1993; Hastings & Dunlap, 1986; M. T. Nicolas, Sweeney, & Hastings, 1987). PFA-fixed, methanol-dehydrated *L. polyedrum* cells were probed with primary antibodies (chicken and rabbit polyclonal antibodies as described in materials and methods) to LCF, LBP, and/or LpH_V1. Localization of primary antibodies was detected using α-chicken IgG or α-rabbit IgG secondary antibodies, each labeled with a different fluorophore (Figure 5 A, C, E). Staining with each antibody (to LBP, LCF or LpH_V1) showed a "punctate" distribution, indicative of scintillons as previously reported from dark harvested *L. polyedrum cells* (Johnson *et al.*, 1985). Total fluorescence intensity per area of antibody labeled cells was significantly different than both pre-serum- and no-primary-antibody negative controls (Figure 5 B, D, and F).

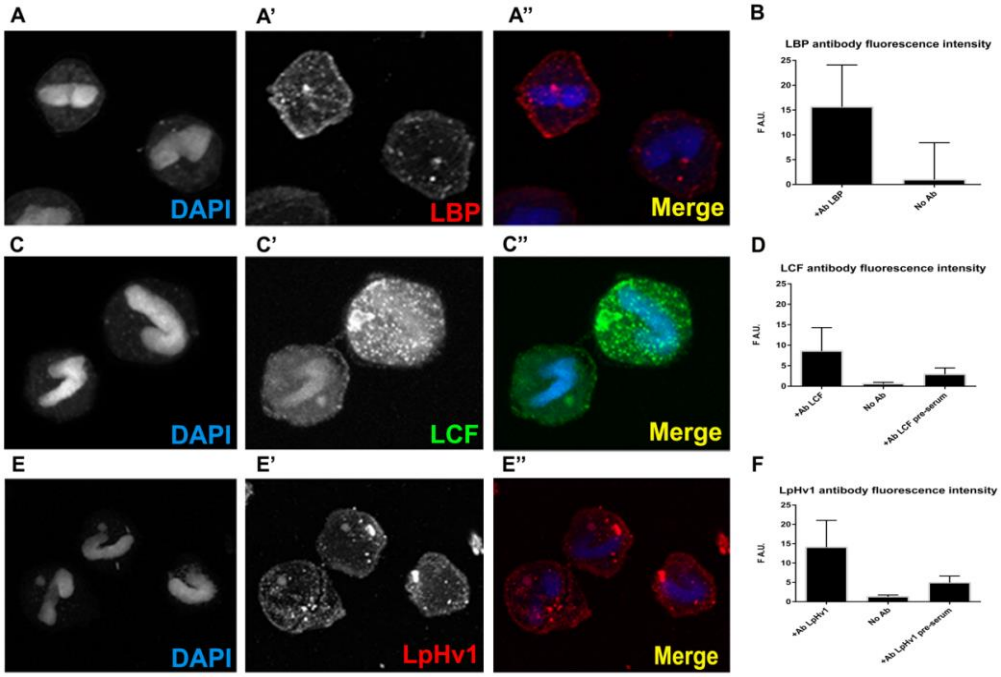


Figure 5. Immunostaining in whole cells: Expression of LBP (Panel A), LCF (Panel C) and LpH_V1 (Panel E) proteins were detected in paraformaldehyde fixed and methanol dehydrated *L. polyedrum* cells using primary antibodies to LBP, LCF and LpH_V1, secondary antibodies to appropriate IgG tagged with different fluorescent tags, and visualized using confocal microscopy. Secondary antibodies used were: Alexa555 Fluor goat anti-rabbit IgG (H+L), ex555/em580 to detect LBP; Alexa488 Fluor goat anti-chicken IgG ex 490/em525 to detect LCF; and Alexa555 Fluor goat anti-rabbit IgG ex555/ em580 to detect LpH_V1. Images were analyzed for per area fluorescence using Zen software analysis tools. Panel B, D and F; Bars represent means +/- s.d. of fluorescence from 20-30 cells from 2-5 separate preps.

LpH$_V$1 is present in isolated scintillons. Since a punctate pattern of immunostaining in whole cells was observed, isolated scintillons obtained by density gradient according to established methods (Desjardins & Morse, 1993; Fogel *et al*., 1972) were immunostained. Isolation of scintillons from *L. polyedrum* was confirmed by two different methods. First, bioluminescence measurements were performed by dropping the pH (Desjardins & Morse, 1993) of the collected fractions, and those fractions that flashed were retained. We also observed the final scintillon preparation, both fixed and unfixed, via fluorescent confocal microscopy; scintillons could readily be identified by native luciferin fluorescence (Johnson *et al.*, 1985). Western blots of proteins extracted from isolated scintillons probed with antibodies to LBP and LCF demonstrated the presence of these known scintillon markers (Figure 6). As previously described, presumed proteolysis products for LBP and LCF were visible in these Western blots (Morse and Hastings *et al*., 1989). Recombinant GST-LBP and LCF purified from *E. coli* served as positive controls.

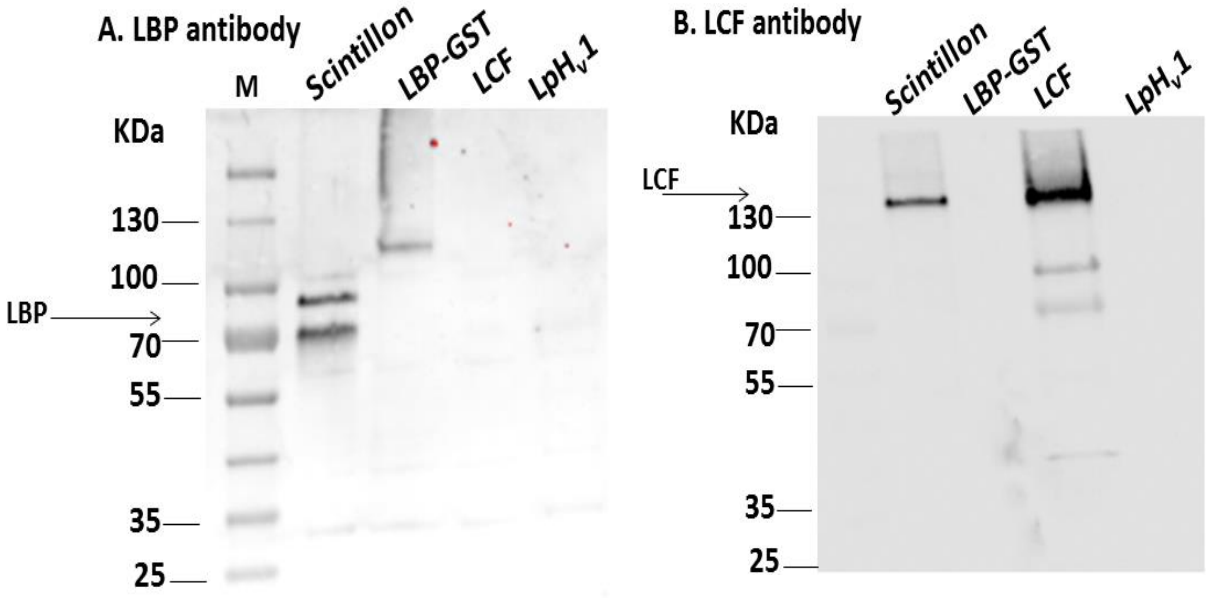


Figure 6. Western blot shows native expression of LBP 75kDa, LCF 134kDa Scintillon proteins and purified recombinant LCF and LBP were separated by SDS-PAGE, blotted, and probed with rabbit anti-LBP (A) or chicken anti-LCF (B). Primary antibody to LBP was detected with secondary anti-rabbit IgG tagged with IRDye 680LT. Primary antibody to LCF was detected with secondary anti-chicken IgG tagged with IRDye 800CW.

Figure 7 shows that antibody to LpH_V1 detects both a purified recombinant LpH_V1 protein and the native protein from purified scintillons. Note that this antibody does not cross react to either LBP or LCF, while Figure 6 demonstrates that LBP and LCF antibodies do not cross react with purified recombinant LpH_V1. In purified recombinant protein preparations from which the GST tag has been removed, and in preparations of native scintillon protein, antibody to LpH_V1 consistently detects proteins at ~60 KDa, consistent with the size of an LpH_V1 dimer. LpH_V1 has a strongly predicted coiled-coil region in its C-terminus, so it may dimerize like H_V1 from other species (Koch *et al*., 2008; Lee *et al*., 2008; Smith *et al*., 2013; Tombola *et al*., 2008).

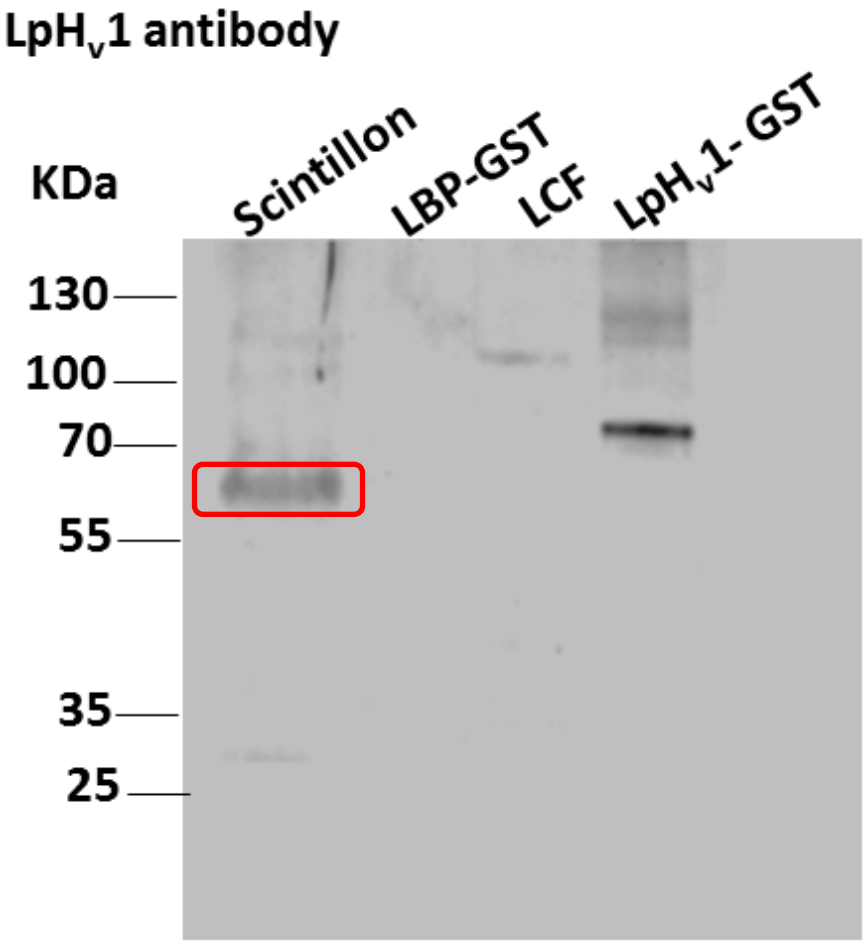


Figure 7. SDS-PAGE separation of protein from isolated scintillons, followed by western blotting, showed expression of LpH_V1 ~60kDa. Recombinant versions of these proteins were expressed and purified from *E. coli.* Primary antibodies to LpH_V1 (this case has the GST tag) raised in rabbit were detected with secondary anti-rabbit IgG tagged with IRDye 680LT.

Immunoprecipitation of LpH_V1 from scintillon preparations. To confirm the smaller apparent size of native LpH_V1 (~30KDa) immunoprecipitations (IP) with both total lysate (not shown) and with isolated scintillons from *L. polyedrum* were performed. Total lysate and scintillon preparations treated with antibody to LpH_V1 immunoprecipitated a protein of ~30KDa, whereas precipitation of LpH_V1 recombinant protein (GST-free) was ~37 KDa (Figure 8A). Antibody heavy (H-chain) and light (L-chain) chains were also detected. Figure 8B confirmed the GST-free recombinant protein size and lack of antibody cross-reactivity.

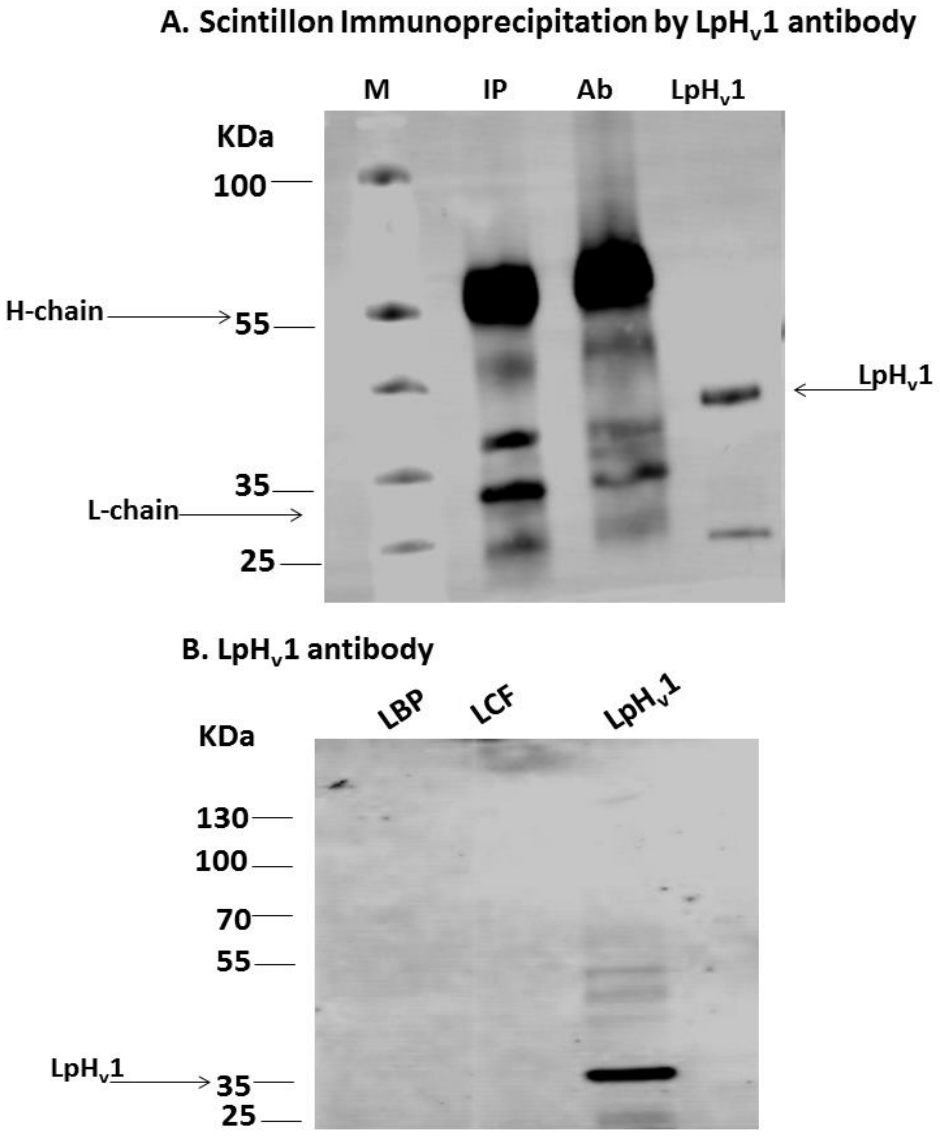


Figure 8. Immunoprecipitated scintillons by LpH_V1 antibody and antibody cross reactivity. IP and recombinant proteins were separated by SDS-PAGE gel followed by Western blotting, using LpH_V1 (1:3000dilution) antibody. In Panel A, a prominent band approximately 30 KDa in the immunoprecipitant corresponds to native LpH_V1. Primary antibody reactivity was detected by the same method as in Figure 6. The panel B showed reactivity with LpH_V1 recombinant protein, but not with LBP or LCF.

Scintillon immunostaining demonstrated the presence of LpH_V1 protein. To test the hypothesis that LpH_V1 is in scintillon membranes, isolated scintillons were fixed in 4% PFA and subjected to immunostaining using the same antibodies to LBP, LCF and LpH_V1 as with whole cells. Native luciferin fluorescence was used as a marker to positively identify scintillons (Dunlap and Hastings *et al*., 1981). As previously described by Johnson *et al*. (1985) a low level of contaminating chlorophyll fluorescence was observed; however, in these preparations the chlorophyll fluorescence rarely overlapped with luciferin fluorescence. Further, detection of structures with luciferin-specific fluorescence occurred without interference from nearby chlorophyll fluorescence. To determine the size range of the scintillons we measured the diameter dimension of 64 scintillons from two different experiments. As shown in Figure 9, the diameters were in the expected range 0.5µM ±1µM (Johnson *et al*., 1985).

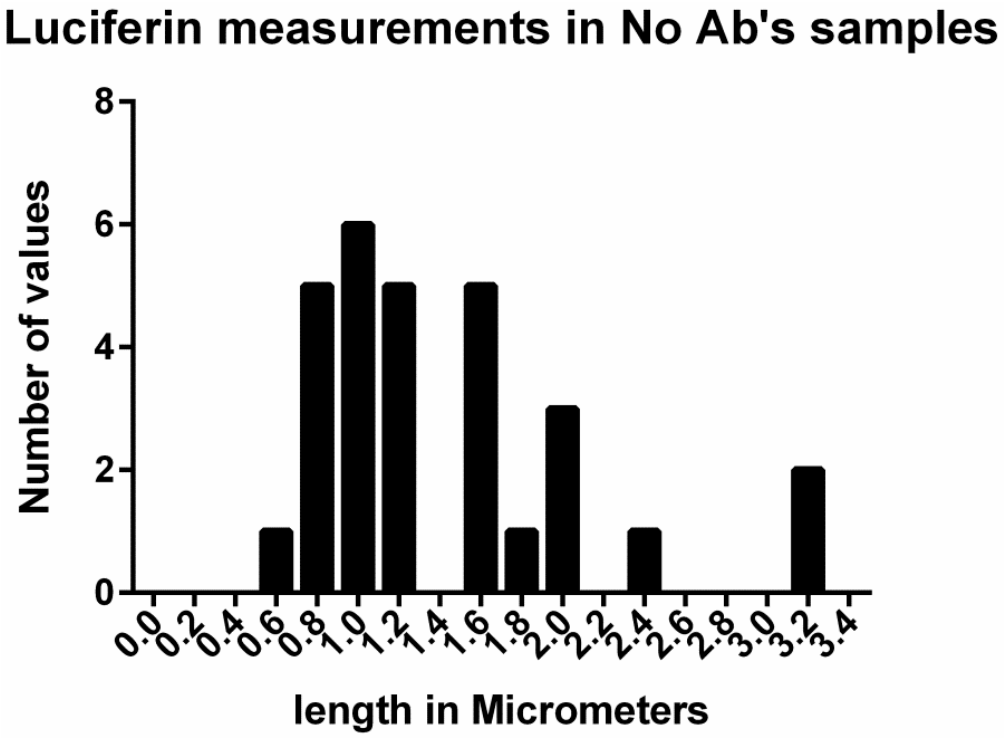
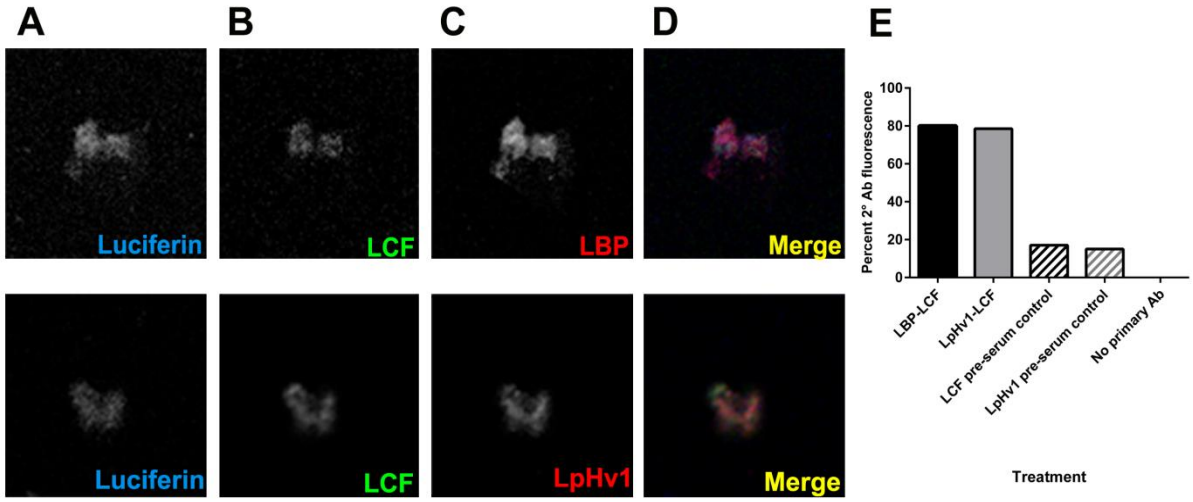


Figure 9 Distribution of scintillon longest dimension. The longest dimension of 64 scintillons identified by native luciferin fluorescence was measured using Zen software. Histogram was prepared using Graphpad Prism program

Immunostaining of fixed scintillon preparations confirmed the presence of LBP and LCF in isolated scintillons (Figure 10 A-D upper). Immunostaining of fixed scintillon preparations showed that LpH_V1 localizes to luciferin-containing structures that also contain LCF (Figure 10A-D lower). The proportion of scintillons (identified by native luciferin fluorescence) that showed fluorescence was determined using fluorochrome-labelled secondary antibodies to primary antibodies, as mentioned in the material and methods. The proportion of scintillons that showed fluorescence from secondary antibodies to their controls was also determined: The proportion of scintillons showing fluorescence from secondary antibodies was significantly higher in scintillons treated with primary antibodies compared to the corresponding pre-serum or vehicle-only controls (Figure 10 E, F; $p < 0.05$ for the following comparisons: LBP-LCF vs. no antibody; LCF- LpH_V1 vs. LCF pre-serum; and LCF- LpH_V1 vs. LpH_V1 pre-serum).

In one preparation, scintillons were identified by native luciferin fluorescence and the mean fluorescence intensity per area of native luciferin (LH_2) was measured using approximately 30 scintillons for each of the primary antibody or control treatments. The mean fluorescence intensity per area of secondary antibody fluorescence for each treatment was for the same treatments or controls was also measured (Figure 11). As expected, native luciferin intensity was similar across treatments. The one-way ANOVA followed by Tukey multiple comparisons showed no significant differences between any treatments; $p > 0.05$. Secondary antibody fluorescence per area was markedly higher in the primary antibody-treated samples compared to the pre-serum and vehicle-only controls ($p < 0.0001$ for the following comparisons determined by t-test: LBP vs. no antibody, LCF vs LCF pre-serum, and LpH_V1 vs LpH_V1 pre-serum). Together these data provide strong evidence that LpH_V1 localizes to scintillons.



F. Negative controls

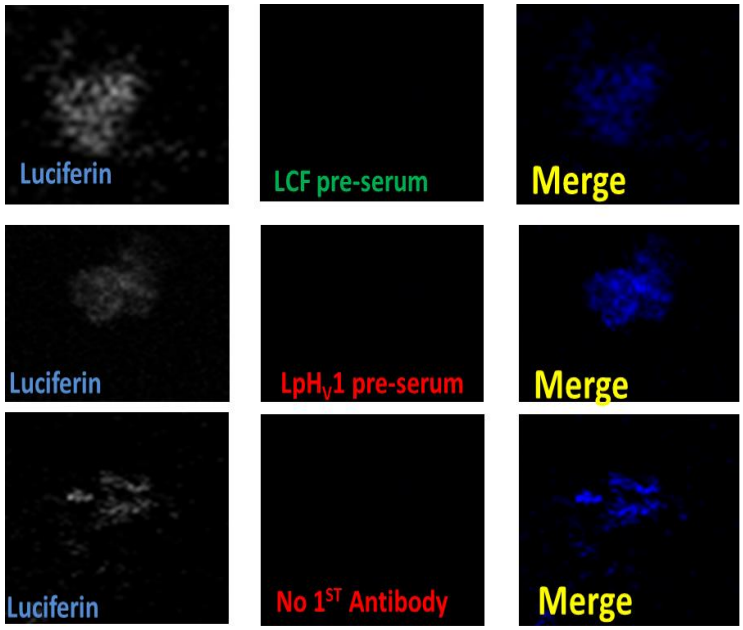


Figure 10. Immunostaining from isolated scintillon detects of LBP-LCF and LpH$_V$1-LCF. Isolated scintillons were fixed and immunostained with the same antibodies as in Fig.3. Scintillons were identified using the native fluorescence of luciferin (Column A). Top row shows scintillons probed with antibodies to LCF (Column B) and LBP (Column C). Bottom row shows scintillons probed with antibodies to LCF (Column B) and LpH$_V$1 (Column C,). Column D shows the merged image for all fluorescence channels. Percentage of scintillons in which both 2nd antibody .fluorescence was detected is shown in Panel E. Number of scintillons scored for each treatment: LBP-LCF=55, LpH$_V$1-LCF =55, LCF pre-serum=41, LpH$_V$1 pre-serum=15, No 1st ab =35. Column F shows scintillons probes with the antibodies controls, LCF pre-serum, LpH$_V$1 pre-serum and No 1st antibody

The crucial point for the proposed H_V1 function in bioluminescence is that it be localized in scintillon membranes; nonetheless, *a priori*, no cellular membrane could be excluded as a potential site for H_V1 localization. As seen in Figure 5, a significant fraction of the LpH_V1 localizes around the periphery of the organism, consistent with a plasma membrane location. The results shown in Figure 10 do not directly demonstrate that LpH_V1 is in the scintillon membranes, because we cannot clearly see the scintillon membrane. As seen in Figure 10, most scintillons imaged showed a staining pattern suggestive that LCF is surrounded by LpH_V1.

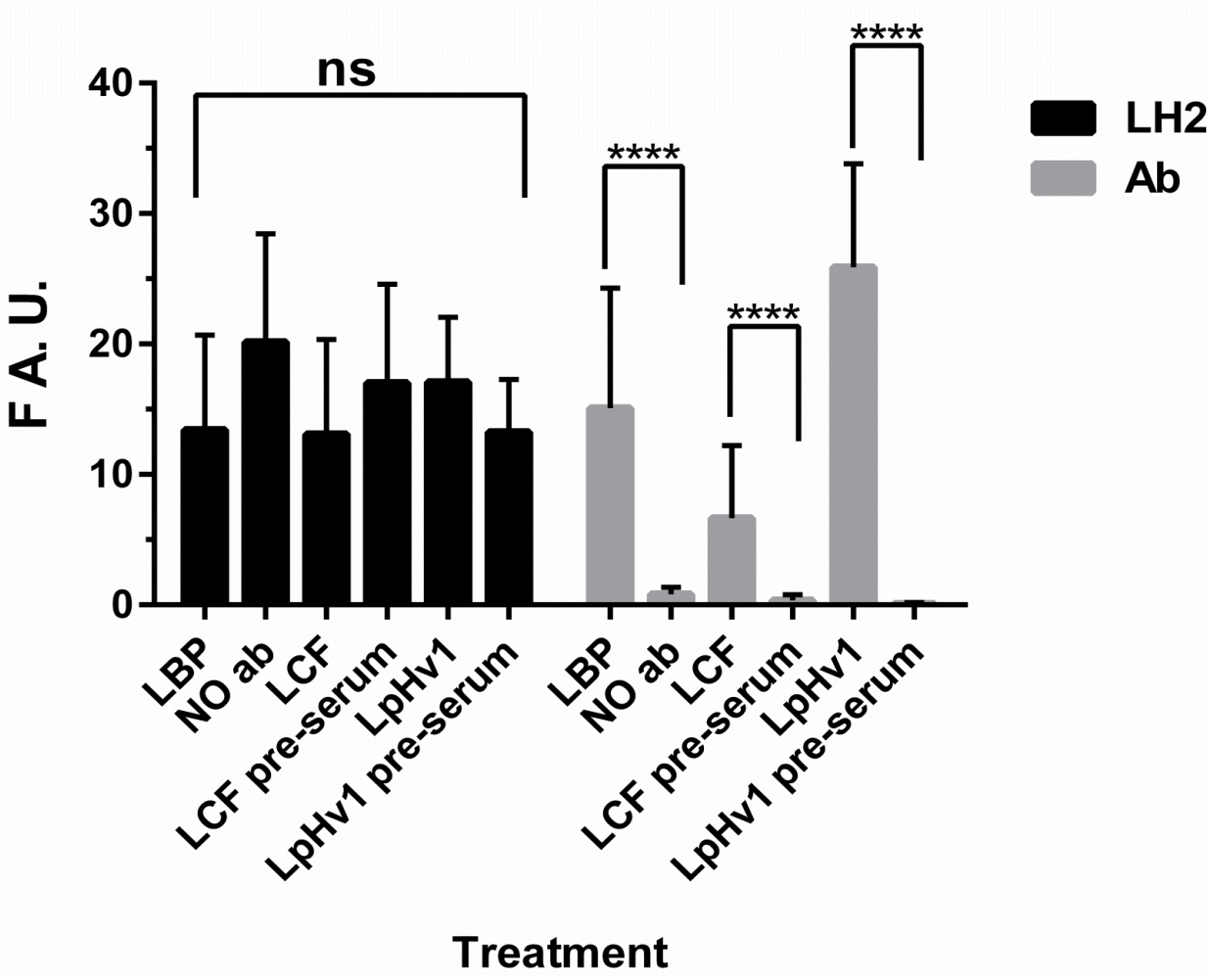


Figure 11. Fluorescence intensity of native luciferin and of secondary antibodies in experimental and control scintillon treatments. Mean fluorescence per area of scintillons identified by luciferin fluorescence was quantified using Zen analysis tools. Scintillons were identified by native luciferin fluorescence. Paired primary antibody treatments were scored if fluorescence from both secondary antibodies was visible. Scintillons quantified for each treatment; LBP=25, LCF=25, LpH_V1=21, LCF pre-serum=27, LpH_V1 pre-serum=7, No 1st ab=13. The Luciferin (LH2) fluorescence levels shown in black and antibody (Ab) fluorescence in gray.

PCR products from RNA extraction and cDNA synthesis of the *L. polyedrum.* As a first step to establishing the native sequence of LpH_V1 RNA extraction from *L. polyedrum* followed by cDNA synthesis was performed. This required optimizing the RNA extraction conditions. Two different methods were used to break the cells, bashings beads (Zymo research, Irvine USA) and homogenizer. The quality of the RNA obtained was determined using an agarose gel supplemented with bleach. Figure 12 shows the expected 28S, 18S RNA bands from the bead-basher method, but not in the sample produced by the homogenizer method. Yield of RNA from the bead-basher method was also higher (data not shown). From these results it was decided to use the bead-mediated lysis method for RNA extraction.

PCR primer sets (Table 1) were obtained that previously were shown to result in products of the expected size from *L. polyedrum*, using cDNA template (Place *et al*. unpublished results). The portion of the predicted coding region that contains the epitope for the primary antibody to LpH_V1 lies between primer sets 2 and 3. Figure 12 show that all of the primer sets used resulted in products of the expected size. These results support the authenticity of the sequence of LpH_V1 predicted by RNA seq, and therefore the fidelity of the epitope used for our antibodies. However, additional bands were observed, optimization for these experiments is ongoing.

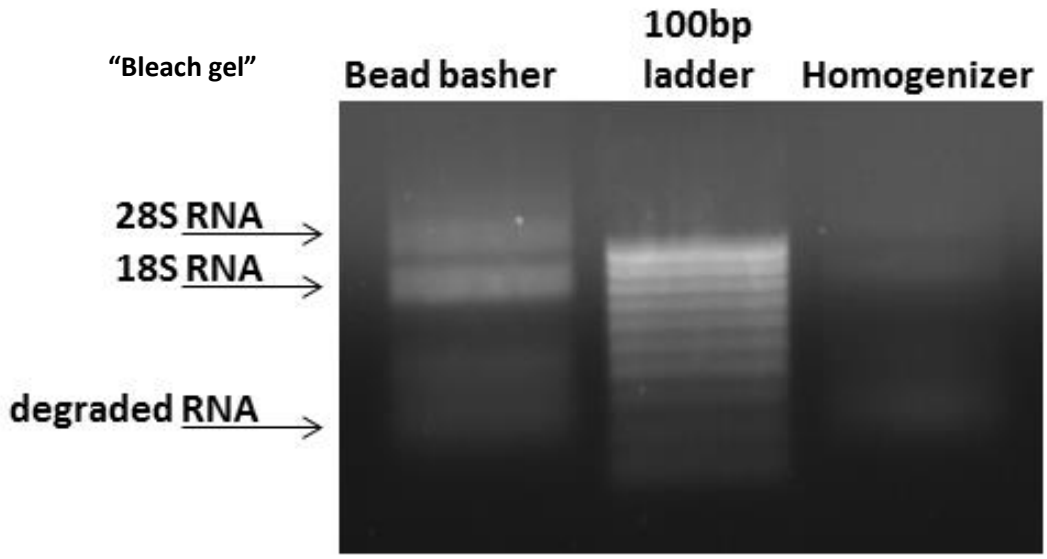


Figure 12. RNA protective effect from bleach as a measure of RNA quality. A 1% agarose gel with 1% of bleach and 0.5µg of ethidium bromide. A 100bp ladder was used for RNA localization. Stained RNA bands from the bead-bash method (left) and homogenizer method (right).

Table1. Primers used for the amplification of 3 different regions within the cDNA of LpH_V1.			
Gene	**Primer name (F- forward; R-reverse)**	**Primer Sequence, 5´ - 3´**	**Length size**
LpH_V1	HV1_1_F HV1_1_R	CTTCAAAGCACGAGGAGCAT AGGTAGTGCGTCTCCAGGAC	157bp
LpH_V1	HV1_2_F HV1_2_R	ACTGCAAGGCCTACGTGGA GTGCCCAGCTAGGAAGAGG	185bp
LpH_V1	HV1_3_F HV1_3_R	GGCATCCTCGTGATCTTCAT CAGGTTCGTCACCAGGATCT	141bp

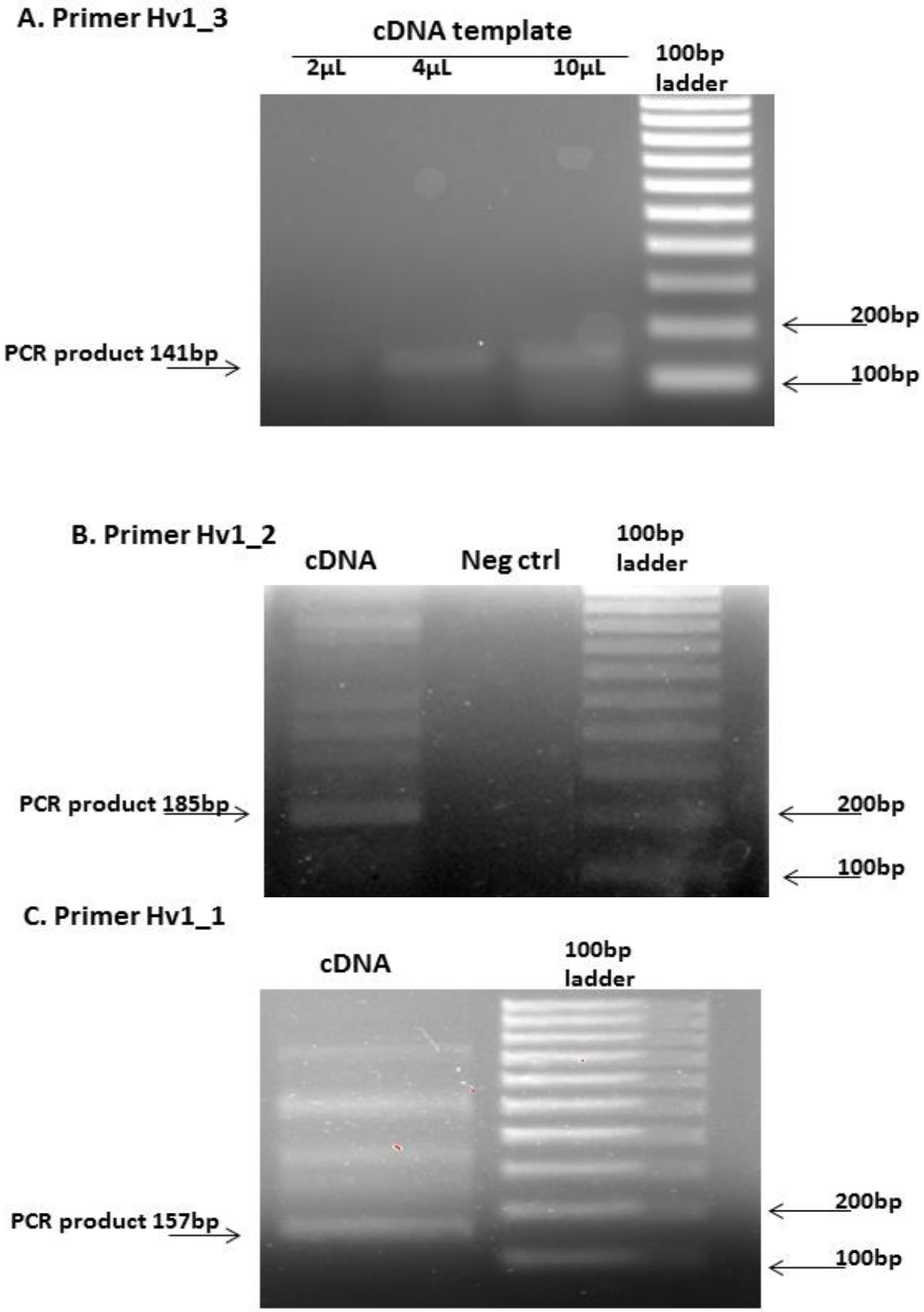


Figure 13. PCR products using Hv1_3, Hv1_2 and Hv1_1 primers. A 1% of agarose gel and 100bp ladder was used to visualize the PCR product and also to compare the different cDNA template amount (A). The PRC product expected size for primer Hv1_3 was 141bp (A), for Hv1_2 was 185bp (B) and for Hv1_1 was 157bp indicated by the arrows.

Discussion

The electrophysiological properties of LpH_V1 are consistent with their proposed function. J. Woodland Hastings predicted the existence of a voltage gated proton channel in bioluminescent dinoflagellates (Fogel and Hastings, *et al.,* 1972). This proposed idea came from two lines of evidence: electrophysiological measurements showed an action potential in the vacuole membranes of the bioluminescent dinoflagellate *Noctiluca* (Eckert & Sibaoka, 1968); and a drop in pH is required to trigger the catalysis of the light reaction of the bioluminescent dinoflagellate *L. polyedrum* (Fogel and Hastings, 1971). Our evidence indicates that LpH_V1 is the predicted voltage gated proton channel from *L. polyedrum*. The electrophysiology data of exogenously expressed LpH_V1 clearly shows that LpH_V1 has all the characteristics of a bona fide voltage gated proton channel: it is activated by depolarization (Figure 1), displays the ΔpH dependence (Figure 1 A-C), and displays perfect proton selectivity (Figure 2).

LpH_V1 is found in *L. polyedrum* whole cell lysate. Western blotting data using antibodies against LBP, LCF and LpH_V1 indicated that these antibodies specifically detected the correct protein to which they were raised without cross reaction with other proteins (Figures 3 and 4). Western blotting results from the whole cell lysate shown in figure 4 A and B indicated the predicted sizes of 75kDa and 134kDa for LBP and LCF, respectively. Antibody to LpH_V1 detected several different bands including a dominant protein band around 30kDa. A clear protein band at the expected size of 36kDa was not detected. Nonetheless we are confident that the band at 30kDa corresponds to LpH_V1 because our collaborators used the same antibody and detected a band of this size from another source of *L. polyedrum* (Place *et al*., unpublished results). We believe that the 30kDa band represents the mobility of the native protein, which could happen because of compact folding or from partial proteolysis. Interestingly, a band around 60kD was consistently detected, which could represent a dimerized form of LpH_V1 and is a typical characteristic for other Hv1 proton channels (Koch *et al.,* 2008).

LpH_V1 stains multiple membranes in whole cells. Immunostaining of whole cells showed that LBP, LCF and LpH_V1 were mostly expressed around the peripheral membranes (Figure 5). Previous work established that LBP and LCF were found nearly exclusively in the scintillons and that immunostaining of LBP and LCF produces the punctate pattern observed in Figure 5 (Johnson *et al.,* 1985). Positive LpH_V1 immunostaining (Figure 5) provides additional evidence that LpH_V1 is present in *L. polyedrum* and its punctate pattern strongly suggests that LpH_V1 localizes in the scintillons.

LpH_V1 is present in isolated scintillons showed by Western blotting and immunostaining experiments. The isolated scintillons were validated by probing Western blots of scintillon preparations with antibodies to scintillon markers LBP and LCF. Consistent with previous results, Figure 6 shows that LBP and LCF are present in the isolated scintillons (Desjardins & Morse, *et al.,* 1993). In addition, scintillons Western blots were probed with antibody reactive to LpH_V1. From the LpH_V1predicted amino acid sequence it was expected that LpH_V1 would be around 36kDa, we consistently observed scintillons protein bands of 30 and/or 60kDa. The larger band could indicate that LpH_V1 is dimerized *in vivo* (Figure 7). In attempt to resolve the molecular weight discrepancy, an immunoprecipitation of LpH_V1 from the isolated scintillons was performed. Figure 8 shows three lanes, the immunoprecipitation (IP), antibody negative control (Ab) and the positive control of purified recombinant LpH_V1. As expected, in both the IP and Ab samples we see the heavy and light chain of the primary antibody, 55kDa and 25kDa. In the recombinant protein lane we see the expected band at 37 kDa. A band around 35 kDa is prominent in the IP sample, consistent with Figure 8, but this band also is faintly visible in the negative control. We did not see any band at 60 kDa in the IP sample. Thus the IP experiments did not resolve the size discrepancy. As additional controls, antibodies against His and GST tags were used to probe Western blots of scintillon preparations; the recombinant GST-LBP and His-LCF proteins were

used as positive controls. The antibodies detected the positive controls but no bands were observed in the scintillons (data not shown).

Next, immunostaining of isolated scintillons was performed omitting the methanol dehydration step to avoid removing luciferin. As with other reported work, when this preparation was observed under the microscope without any antibody stain we could distinguish scintillons from chloroplast contaminants by endogenous fluorescence of luciferin or chlorophyll (Johnson *et al.,* 1985). Thus in all subsequent manipulations scintillons were positively identified by their native luciferin fluorescence. Figure 9 shows the size range of 64 scintillons, which is consistent with the size estimated in previous studies (Johnson *et al.,* 1985). When isolated scintillons were immunostained with antibodies to LBP and LCF, detection or colocalization of these proteins in the scintillons was observed as predicted (Figure 10 upper A-D). Immunostaining with antibodies to LpH_V1 and LCF also showed colocalization of these two proteins in scintillons (Figure 10 lower A-D). The proportion of negative controls (no 1st antibody, LCF-pre-serum, LpH_V1 pre-serum) that displayed antibody fluorescence was significantly lower than that of scintillons in the experimental treatments (figure 10F). In addition, the native luciferin fluorescence intensity per area in the several treatments to the secondary antibody fluorescence intensity per area was compared. As expected, there was no significant difference in native luciferin fluorescence among the treatments or between any two treatments (Figure 11). This contrasts sharply with the secondary antibody fluorescence, each of which was significantly higher than its negative control. As a control for nonspecific antibody binding, the isolated scintillons were immunostained with antibody to the GST tag, and no significant signal was observed (data not shown). Importantly, these results indicate not only that direct immunostaining of fixed scintillons was successful, but that LpH_V1 was present in the scintillons. The main point with these experiments was to show that LpH_V1 was present in scintillons and associated with the scintillon membranes. Our data suggest that LpH_V1 enfolds LCF (figure 10 A-D lower). To definitively confirm that LpH_V1 is in the scintillon

membranes, further experiments using vacuole membrane markers to confirm colocalization of LpH_V1 with the markers will be necessary.

PCR products of LpH_V1 from RNA extraction and cDNA synthesis. To demonstrate conclusively that LpH_V1 is present in *L. polyedrum* we sought for the nucleotide sequences corresponding to the amino acid of the epitope that we used to raise the antibody to LpH_V1. Primers were designed to amplify this sequences of ~532bp to confirm the LpH_V1 presence and expression in *L. polyedrum*. Our collaborators used primers listed in Table 1 to perform qRT-PCR and showed that the LpH_V1 is expressed in *L. polyedrum* (Place *et al.,* unpublished). In a parallel effort, we extracted RNA from *L. polyedrum* and analyzed the resulting cDNA.

First, optimization of the RNA extraction protocols were needed; Figure 12 shows that the bead basher method produced better RNA quality than the homogenizer method. Additionally, the RNA and cDNA yield from the bead basher methods was higher.

PCR products from primers designed against the sequence predicted from the RNA seq results were analyzed (Table 1). These primers were designed by our collaborators for qPCR and produced predicted amplicon sizes (Place et al, unpublished results). Figure 12 shows that our *L. polyedrum* cDNA also produced PCR products of expected sizes from three different primer sets. These results provide convincing evidence for LpH_V1 mRNA expression. The PCR primers were reverse designed off the epitope amino acid sequence. Therefore, positive amplification using *L. polyedrum* cDNA from extracted RNA can only occur if the primers annealed to the correct target sites. This could occur only if the predicted nucleotide sequence was correctly derived, which the evidence supports. Experiments to confirm this epitope in *L. polyedrum* cDNA are ongoing.

Conclusion

It was hypothesized that LpH_V1 is the voltage gated proton channel predicted by Hastings and colleagues, and that its activity triggers the bioluminescent light in *L. polyedrum* (Fogel and Hastings, *et al.,* 1972). Confirming this hypothesis required demonstration of two important characteristics: function and localization. What was known is that light emission resulting from a biochemical reaction occurs in the scintillon of the bioluminescent dinoflagellate, requires a drop in pH (Fogel and Hastings, *et al.,* 1971), and matches the time course of an action potential along the vacuole membrane (Eckert *et al.,* 1965). Therefore, LpH_V1 should be localized in the scintillon membrane, be voltage dependent, and conduct protons (H^+). The evidence presented in this work strongly supports this location and function. In addition, preliminary data from a proteomic analysis (collaborator's unpublished data) showed that LpH_V1 was present in the isolated scintillons. These analyses showed the expected presence of LBP and LCF. For LpH_V1 the peptides found overlap to LpH_V1 predicted amino acid sequence. More importantly is that these peptides included the portion of the epitope used to raise the LpH_V1 antibody.

Electrophysiology experiments (Figure 2) clearly show that LpH_V1 is voltage dependent and conducts protons. The localization of LpH_V1 in the scintillon was conclusively demonstrated via Western blotting and immunostaining of isolated scintillons (Figures 7, 10, 11). Although not conclusive, immunostaining of isolated scintillons suggested scintillon membrane localization (Figure 10). Because H_V1 is known to be a transmembrane protein, cytoplasmic localization was not expected (Sasaki *et al*., 2006). TEM experiments using immunogold labeling and the antibody to LpH_V1 described in this work are ongoing in the laboratory of Dr. David Morse; these experiments should provide the necessary evidence for localization of LpH_V1 to the scintillon membrane. Further experiments using inhibition of LpH_V1 by zinc (Zn) in concert with bioluminescent measurements would reinforce this proposed idea. Zinc inhibition experiments would work if LpH_V1 is more sensitive to inhibition by this

metal compared to LCF. Recent bioluminescent measurements using the illuminometer showed that LCF is inhibited at Zn concentration of ~100 µM. Recent data from our collaborators indicate that H_V1 can be inhibited by Zn at much smaller concentrations ranging from 0.5 µM to 2 µM (DeCoursey *et al.,* unpublished results). To test the Zn effect in the bioluminescent light, an assay that involves synthetic scintillons in the liposomes will be used. The synthetic scintillons will have all the components of the endogenous scintillons; the luciferin binding protein (LBP), luciferase (LCF), luciferin (LH2) and the voltage gated proton channel (LpH_V1). Because the synthetic scintillons mimic the endogenous (native) *L. polyedrum* scintillons, the light measurements could be the same or very similar to the native scintillons. If low or no bioluminescent light is obtained in the present of the LpH_V1 inhibitor (Zn), this will confirm that LpH_V1 triggers the bioluminescence light.

Integration of the thesis research

The result of this study strongly supports the hypothesis that that LpH_V1 is the voltage gated channel that triggers the bioluminescence in *L. polyedrum.* During this study I was able to address this hypothesis by integrating aspects of several different disciplines.

The main organism in this study is the aquatic dinoflagellate *Lingulodinium polyedrum*, which is in *euglenophyta phylum* group**.** One ecological role of dinoflagellates is their symbiotic relationship with corals (Muscatine *et al.,* 1977). Most corals capture and consume prey; however, this form of feeding does not satisfy the complete energy needs of the animal. Many corals rely on photosynthetic endosymbionts for energy and in return provide structural protection to the endosymbionts (Muscatine *et al.,* 1977). The majority of these endosymbionts, called zooxanthellae, are dinoflagellates of the genus *Symbiodinium*. Zooxanthellae reside within the cells of a coral's gastrodermis where up to 90 percent of the organic material produced through photosynthesis is transferred to the host coral tissue. This study provides a foundation for investigation into the role H_V1 across several dinoflagellates species which could provide new information regarding the mechanisms important to stabile endosymbiosis with corals, which are experiencing a rapid global decline.

One of the main points of this study is to confirm the function of LpH_V1, the proposed voltage gated channel that triggers the light in the bioluminescence. Therefore, this study also incorporated several biochemical, biophysical, and cell biological techniques to confirm the location and function of LpH_V1: the proposed voltage gated channel that triggers the light in the bioluminescence. These techniques included electrophysiology (performed by collaborators), organelle purification by density gradient centrifugation, various immunostaining techniques using fluorescently labeled antibodies, confocal laser scanning microscopy, RNA purification and cDNA generation, bioinformatics, polymerase chain reaction and agarose gel electrophoresis. Through application of multiple techniques, it was

shown that LpH_V1 has all the characteristics of a voltage gated proton channel, had structural characteristics as predicted using sequence information and was localized in the scintillon. These results are in agreement with data from a proteomic assay on isolated scintillons performed by collaborators. Their preliminary data showed that predicted peptides of LpH_V1 were detected in the scintillons. Finally, biostatistics analyses were used to measure the significance of all the confocal microscopy experiments. The data obtained from the confocal microscope was statically analyzed and each analyzed experiments was statistically significant.

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