

Characterization of the Bioluminescent Organelles in *Gonyaulax polyedra* (Dinoflagellates) after Fast-freeze Fixation and Antiluciferase Immunogold Staining

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Abstract. To characterize the microsources of bioluminescent activity in the dinoflagellate *Gonyaulax polyedra*, an immunogold labeling method using a polyclonal antiluciferase was combined with fast-freeze fixation and freeze substitution. The quality of the preservation and the specificity of the labeling were greatly improved compared to earlier results with chemical fixation. Two organelles were specifically labeled: cytoplasmic dense bodies with a finely vermiculate texture, and mature trichocysts, labeled in the space between the shaft and the membrane. The available evidence indicates that the dense bodies are the light-emitting microsources observed in vivo. The dense bodies appear to originate in the Golgi area as cytoplasmic densifications and, while migrating peripherally, come into contact with the vacuolar membrane. Mature organelles protrude and hang like drops in the vacuolar space, linked by narrow necks to the

cytoplasm. These structural relationships, not previously apparent with glutaraldehyde fixation, suggest how bioluminescent flashes can be elicited by a proton influx from a triggering action potential propagated along the vacuolar membrane. Similar dense bodies were labeled in the active particulate biochemical fraction (the scintillons), where they were completely membrane bound, as expected if their necks were broken and resealed during extraction. The significance of the trichocyst reactivity remains enigmatic. Both organelles were labeled with affinity-purified antibody, which makes it unlikely that the trichocyst labeling is due to a second antibody of different specificity. But trichocysts are not bioluminescent; the cross-reacting material could be luciferase present in this compartment for some other reason, or a different protein carrying similar antigenic epitopes.

THE unicellular marine dinoflagellate, *Gonyaulax polyedra*, emits brief (100 ms) bright ($\sim 10^8$ photons) flashes upon stimulation, primarily during the night (Krasnow et al., 1981). Such flashes are triggered by an action potential along the vacuolar membrane (Eckert, 1965; Eckert and Sibaoka, 1968). Although the flashes were known to originate from subcellular fluorescent sites in *Gonyaulax* (Johnson et al., 1985), the identity of the presumptive organelle had not been established (Fogel et al., 1972; Sweeney, 1980).

In extracts of *Gonyaulax* cells, bioluminescence occurs in both soluble and particulate fractions, and in both cases, luminescence may be triggered by a pH change. In the soluble fraction extracted at pH 8, the enzyme (*Gonyaulax* luciferase) and a substrate-binding protein occur together with the bound substrate (dinoflagellate luciferin, an open chain tetrapyrrole; see Dunlap et al., 1981). Activity lasting for many minutes is triggered simply by lowering the pH to 6.5; the

substrate is released and enzymatically oxidized with concomitant light emission (Fogel and Hastings, 1971).

The sedimentable particles, which contain luciferase, luciferin, and the binding protein (Henry and Hastings, 1974), emit a flash that mimics closely the in vivo flash upon the rapid shift of the pH from 8 to 5.7 (Fogel et al., 1972). These particles, or "scintillons", have a buoyant density of ~ 1.23 gm/cc and a sedimentation constant of $\sim 10,000$ S. Discharged scintillons can be recharged by incubation with free luciferin at pH 8; a subsequent reacidification elicits a new flash.

In previous studies we used an antibody raised against *Gonyaulax* luciferase and the immunogold technique of De Mey (1983) on sections of cells which had been fixed with glutaraldehyde and osmium (Nicolas et al., 1985). A significant labeling occurred over dense vesicles, found generally within the vacuolar space. Their size and cortical position corresponded well to that of the microsources ob-

served *in vivo*, but their location within the vacuole made it difficult to account for the excitation–bioluminescence coupling. In addition, the sheath areas of trichocysts were also labeled. The trichocysts connect with the plasma membrane, which might also be involved in the control of the flash response, but in living cells no bioluminescence or fluorescence emanates from structures of the size and shape of trichocysts (Johnson et al., 1985). Moreover, trichocysts occur rather commonly in a variety of nonluminescent unicellular organisms, and are also labeled there (Nicolas et al., 1987).

In the study reported here we used fast-freeze fixation (Heuser et al., 1979), which improved the quality of the preservation and the precision of the labeling. We used affinity-purified antiluciferase and found that both organelles were labeled as before. But the improved fixation allowed us to visualize for the first time the association of the dense bodies with the vacuolar membrane and to see that they protrude into the vacuole while retaining cytoplasmic connections. These relationships support the candidacy of the dense bodies as the light-emitting organelles and allow us to suggest a plausible model for the cellular control of bioluminescence flashing.

Materials and Methods

Fast-freeze Fixation/Freeze Substitution (FFFS)¹

G. polyedra (strain 70) cells were grown at $21^{\circ} \pm 3^{\circ}\text{C}$ under a light–dark cycle (12 h light, 12 h dark) in a modified sea water medium (F/2; Guillard and Ryther, 1962), harvested by gentle centrifugation (200 g for 30 s), and transferred onto filter paper for fast-freeze fixation. The filter paper was placed on a foam rubber block covered with a layer of mica on the specimen mount of a Reichert–Jung cryoblock (Escaig, 1982). Excess water was removed, and the sample was promptly “slammed” onto a polished copper block cooled under vacuum with liquid helium to -260°C . The frozen sample was then quickly transferred to and stored in liquid nitrogen.

For freeze substitution, the sample was transferred to acetone, or to acetone containing 5% osmium tetroxide at -85°C , in the presence of a molecular sieve (Merck, 0.4 nm) to absorb water. These conditions were maintained for 3 d at -85°C , followed by 2 h at -30°C and 30 min at room temperature. The specimen was then washed in pure acetone and embedded in Epon. Freeze drying was carried out at -80°C under vacuum for 3 h. The samples were then embedded directly in Epon. Thin sections were cut with a diamond knife and mounted directly on gold or nickel grids (300 mesh).

Luciferase and Antiluciferase Antibody

Luciferase was purified ($\sim 90\%$ pure) from *Gonyaulax* cells harvested and extracted in the middle of the night phase by the procedure of Hastings and Dunlap (1985) and stored at -80°C . The antiluciferase antibody used was prepared by Dunlap and Hastings (1981); the IgG fraction (7 mg/ml final concentration) of rabbit antisera was purified by Na_2SO_4 precipitation and DEAE–cellulose chromatography. This polyclonal antibody, hereinafter referred to as “partially purified” antibody, inhibits luciferase activity *in vitro* and recognizes a single major polypeptide of 135,000 D in immunoblots (Johnson et al., 1984).

Affinity Purification of Antiluciferase

Luciferase-containing portions of nitrocellulose blots of SDS–polyacrylamide gels were used to obtain affinity-purified antibodies (Olmsted, 1981). An extract of *Gonyaulax* cells was subjected to SDS–PAGE and then transferred to nitrocellulose (Towbin et al., 1979; Johnson et al., 1984). This blot was washed twice for 5 min in Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris, pH 7.3) plus 0.05% Tween 20, washed twice in TBS for 5 min,

1. *Abbreviations used in this paper:* FFFS, fast-freeze fixation/freeze substitution; IGS, immunogold staining; NRS, normal rabbit serum.

stained for 10 min in Ponceau S (Sigma Chemical Co., St. Louis, MO; 0.2% in 3% TCA), and destained briefly in water until the protein bands were clearly visible. The luciferase area was then excised with a razor blade, washed twice in TBS plus 0.05% Tween 20 and incubated for 1 h in the same solution with 3% BSA added. After overnight incubation at 4°C in a 1/25 dilution of the polyclonal antibody diluted into TBS–BSA, the nitrocellulose strip was washed five times for 10 min each in TBS–Tween, loaded in a filter syringe and washed again by forcing two 5-ml aliquots of TBS–Tween through the filter. Then 1 ml of 0.2 M glycine (pH 2.8), 0.5% Tween 20, and 3% BSA was drawn up into the syringe, incubated for 5 min in order to elute the antibody and expelled into 200 μl of 1 M Trizma base. This affinity-purified antibody was dialyzed overnight against 1 liter of TBS buffer (pH 8.2) with 0.1 mM EDTA plus 0.02% azide and used directly for immunoblots and for labeling EM sections.

Immunocytochemistry

We used a two-step immunogold staining (IGS) method (De Mey, 1983). The first antibody, antiluciferase, was raised in a rabbit; the second, goat anti-rabbit IgG, was labeled with 10-nm colloidal gold particles. Grids with mounted sections were washed with TBS buffer with 0.1 mM EGTA and 3% BSA at pH 8.2, and incubated in the first antibody (with the partially purified IgG fraction, a 1/100 dilution) overnight at 4°C in humid atmosphere (Nicolas et al., 1985). After washing in the same buffer (five 15-min washes) at room temperature, the grids were incubated for 2 h at room temperature with the goat anti-rabbit serum labeled with colloidal gold particles (Janssen Pharmaceutica, Brussels), diluted 1/50 with buffer. After five washes with buffer and two with distilled water, the grids were dried on filter paper, stained with uranyl acetate and lead citrate. Controls were carried out either by using normal rabbit serum (NRS) diluted 1/100 with buffer or with buffer alone in place of the antiluciferase, or with purified antibody adsorbed with an excess of pure luciferase and diluted in buffer.

Electron microscope observations were made on a Phillips EM 300 or EM 301.

Isolation and Assay of Scintillons

To isolate scintillons, cells from two liters of unialgal culture were collected at the end of the light period by filtration on Whatman paper 541 and resuspended in 3 ml mannitol extraction buffer (MEB; 300 mM mannitol, 50 mM Tris, 10 mM EDTA, 5 mM 2-mercaptoethanol, 0.2% BSA, pH 8.3) with 10 μm glass beads (0.5 mm diameter; Sigma Chemical Co.). Cells were broken by vortexing at the highest setting for 20 s in a conical centrifuge tube (Samuelsson et al., 1983). The extract was then filtered through one layer of Miracloth with three washings of 5 ml mannitol extraction buffer. This extract (~ 20 ml) was centrifuged for 10 min at 1,000 g; the pellet was resuspended in 5 ml mannitol extraction buffer and centrifuged again. The supernatants were combined, assayed for activity, and then centrifuged at 27,000 g for 20 min. The pellet containing the scintillon activity was resuspended in 4 ml MEB and assayed; 2 ml were layered on each of two 50-ml tubes prepared with discontinuous gradients of Percoll and propane-1,2-diol (propylene glycol; Schwitzguebel and Siegenthaler, 1984). These gradients were prepared by layering respectively from the bottom to the top of the tube: 60 (6 ml) and 45% (14 ml) Percoll diluted in gradient buffer (250 mM sucrose, 50 mM Tris, 10 mM EDTA, 5 mM 2-mercaptoethanol, 0.2% BSA, pH 8.0), then 27 (8 ml) and 21% (8 ml) Percoll diluted into gradient buffer containing also 100 mM propanediol. The tubes were centrifuged at 13,000 g in the Sorvall centrifuge (SS-34 rotor) of 1 h at 0°C . Fractions (1.8 ml) were collected and assayed for light-emitting activity by injection of 1 ml of 0.01 M acetic acid into a 10- μl aliquot of the fraction suspended in 1 ml buffer (50 mM Tris, 10 mM EDTA, pH 8) (Fogel and Hastings, 1972).

Gradient fractions containing the highest activities were pooled and vortexed while adding 25% glutaraldehyde to a final concentration of 3%, and fixed for 1.5 h at 4°C . The solution was then filtered through a 0.67- μm millipore filter (0.22- and 0.45- μm filters clogged rapidly). The filters were postfixed with 0.5% OsO_4 in 0.1 M phosphate buffer for 1.5 h at 4°C and embedded in agarose before Epon infiltration.

Results

Gonyaulax Ultrastructure after Fast-freeze Fixation/Freeze Substitution (FFFS)

Although the dimensions of *G. polyedra* cells ($25 \times 40 \mu\text{m}$)

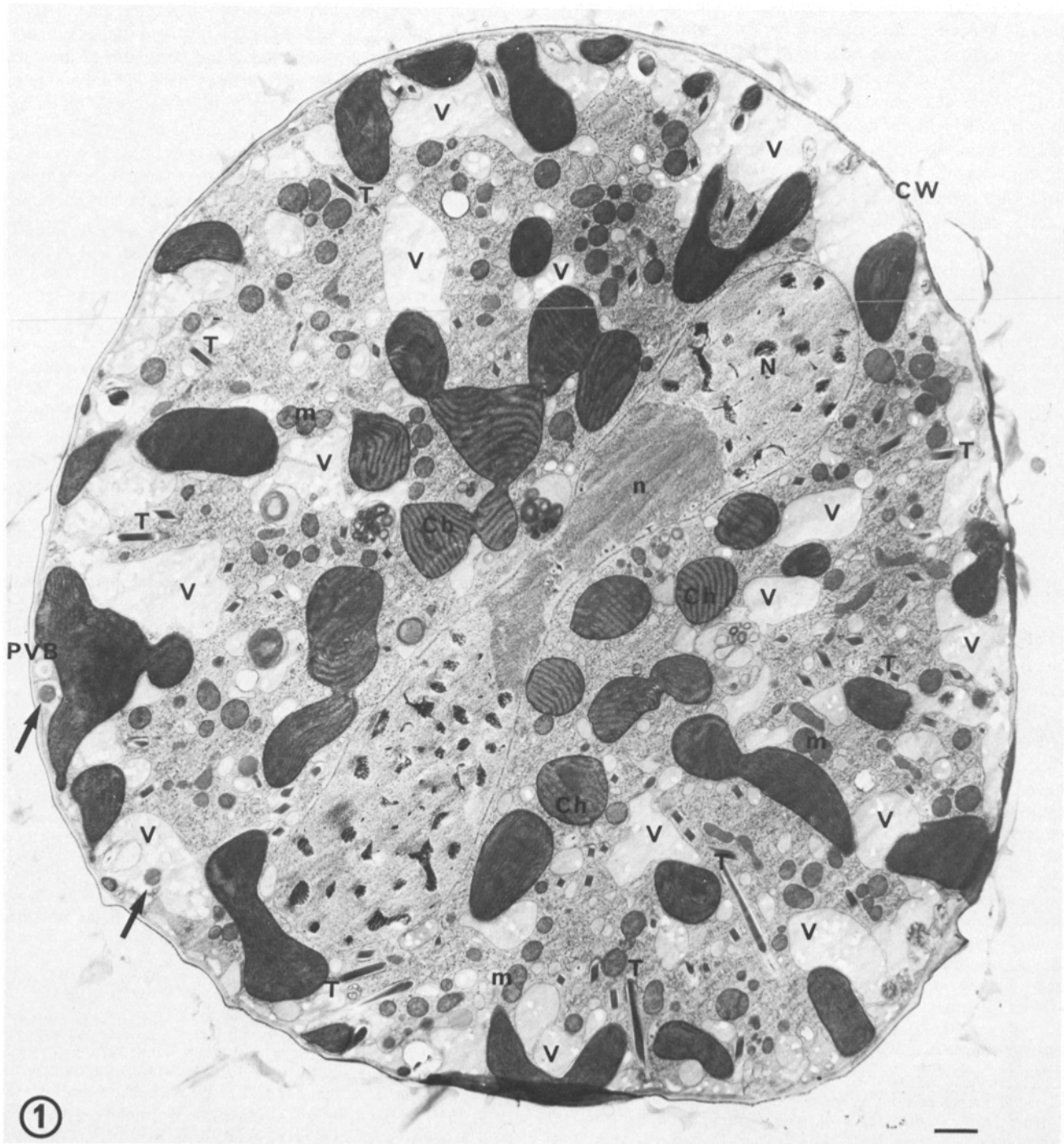


Figure 1. General view of a cell of *Gonyaulax* after FFFS in OsO₄ and acetone. The cell, bounded by the cell wall (CW) composed of thecal plates, is well preserved throughout its entire section (~40 μm in diameter). The nucleus (N) contains a prominent nucleolus (n) and typical peridinin chromosomes. The cytoplasmic organelles comprise numerous chloroplasts (Ch), mitochondria (m), and ergastoplasm; trichocysts (T) are oriented perpendicular to the cell surface; their shafts appear as rods in longitudinal sections and as squares in cross sections. The vacuole (V), which may form a continuous internal compartment (tonoplasm), is mostly peripheral, but its ramifications extend to the center of the cell. Its content is characterized by filamentous material punctuated by white holes, which correspond to the abundant guanine crystals which were extracted during the preparation procedures. Polyvesicular bodies (PVB) and dense bodies or scintillons (arrows) are localized in the cortical cytoplasm. Bar, 1 μm.

are greater than those usually considered to be the limit for optimal fixation by this method (10–15 μm; Gilkey and Staehelin, 1986), the preservation was generally excellent throughout the cell, with very few ice crystal artifacts, allowing ob-

servations in the center of the cell as well as at its periphery.

Figs. 1–3 illustrate the preservation and structural complexity of the cell. Around the large C-shaped nucleus, with its distinctive chromosomes (Haapala and Soyer, 1973; Soyer

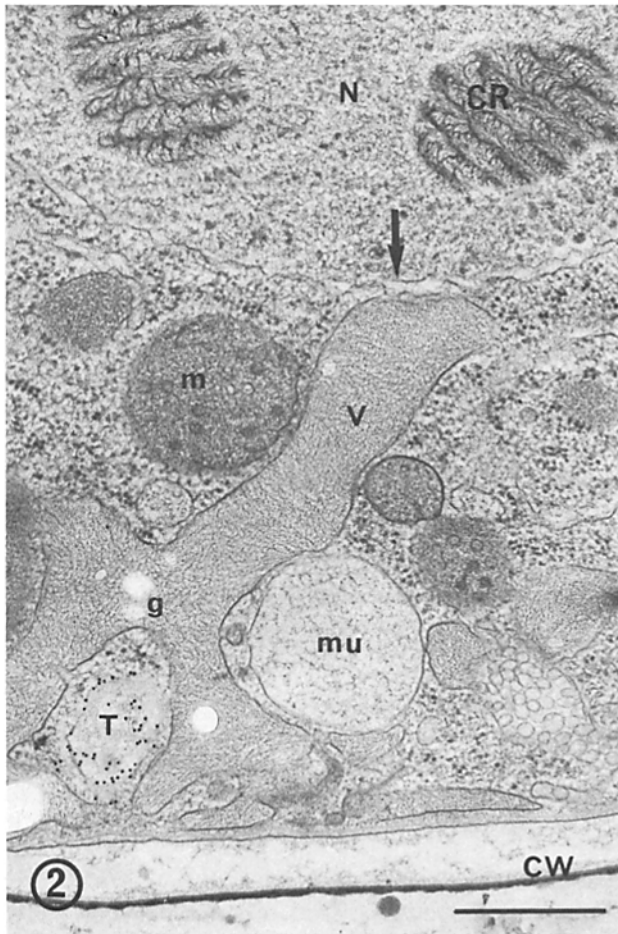


Figure 2. FFFS (OsO₄-acetone substitution) and IGS, first with affinity-purified antibody against *Gonyaulax* luciferase followed by a second antibody with 10-nm gold particles attached. The vacuolar space (*V*) is characterized by its texture and by white spaces (*g*) which are the ghosts of guanine crystals dissolved during the alkaline staining procedure. In this field there is only one trichocyst (*T*), labeled at its tip. No gold labeling occurs over the cell wall (*CW*), a mucus vesicle (*mu*), the mitochondria (*m*), the nucleus (*N*) with its typical chromosomes (*CR*), the polyvesicular body (lower right), with small vesicles in a larger one, or the ground cytoplasm with its numerous ribosomes, often associated as polysomes. The ribosomes are the dark bodies which are slightly larger and somewhat less dense than the gold particles. A possible connection or continuity between the vacuolar membrane (tonoplast) and the outer membrane of the nuclear envelope is indicated (*arrow*). Bar, 0.5 μ m.

and Haapala, 1974; Livolant, 1984; Spector, 1984), the numerous cytoplasmic organelles include the large chloroplasts disposed more or less radially. There are numerous mitochondria dispersed throughout, and mature trichocysts are oriented perpendicularly to the cell surface. There are many different membrane-bound structures, including lysosomes, mucus vesicles, and others of various sizes and different contents. The endoplasmic reticulum includes some large cisternae filled with a dense material that may be involved in the formation of the cell wall, as described in other plant cells (Heath et al., 1985). There are many ribosomes, both attached and free, the latter being arranged sometimes in double rows of polysomes. Packages of filaments were oc-

asionally observed. Polyvesicular bodies, first described by Schmitter (1971), and which may be involved in theca formation (Durr, 1979), are localized at the periphery of the cell. Dense bodies, which we will consider in detail later and refer to also as "scintillons" (flashing units), were found either associated with the vacuolar membrane or as free dense cytoplasmic condensations in the Golgi area.

FFFS preserved remarkably well the vacuolar topography, which is seen to be more complex than after usual liquid chemical fixation, where the vacuole was seen as a "peripheral vesicle" (Sweeney, 1976). The vacuolar sap appears finely reticulated, and guanine crystals, earlier noted as abundant in this cell (De Sa et al., 1963), occur exclusively in vacuolar spaces, appearing as white spaces because they are extracted during the preparation procedure. The vacuole, though mostly peripheral, possesses a very tortuous topography, with digitations penetrating deeply in every part of the cytoplasm, creating numerous channels separated by thin cytoplasmic strands (Figs. 1 and 3). In some sections, the vacuolar membrane appeared to make contact with the outer membrane of the nuclear envelope; the two might be continuous (Fig. 2).

In the Golgi region (Herman and Sweeney, 1975), many Golgi apparatuses abut one another, forming a crown which delimits a central cytoplasmic area that is strikingly different from the rest of the cytoplasm (Fig. 4). The Golgi apparatuses are evidently involved in secretory processes of several kinds, two which were of particular interest since they lead to the differentiation of trichocysts and dense bodies.

Pretrichocysts first appear in the Golgi area as relatively large spherical vesicles ($\sim 0.8 \mu$ m) with a characteristic amorphous content (Fig. 4). They then migrate radially, become ovoid, and a crystalline shaft appears on one side (Fig. 3). The vesicle elongates, and the shaft condenses to give the mature trichocyst, which ultimately contacts the outer membrane of the cell (Fig. 5), ready to eject its content (Bouck and Sweeney, 1966; Hausman, 1978). As will be detailed later, the scintillons also appear to differentiate along radial paths which start in the Golgi area and reach the cortical cytoplasm.

Luciferase Localization by Immunogold Staining (IGS)

After FFFS, the IGS localization of luciferase was, as with chemical fixation, specific for two different organelles: dense bodies (scintillons and prescintillons) and the space between the crystalline shaft and the sheaths of mature trichocysts (Fig. 3). The labeling was similar in specimens prepared by FFFS (not shown). Adsorption of antibody with purified luciferase antigen before immunolabeling led to complete absence of labeling of both the scintillons and the trichocysts (Figs. 8 and 9), indicating that the labeling is specific for luciferase.

Spotty and light labeling occurred on the cell wall, polyvesicular bodies, and occasionally on packages of filaments enclosed in vesicles. Controls showed that these labelings were not specific, since they occurred as well when NRS was used alone (Fig. 7) or disappeared when affinity-purified antiluciferase was used (not illustrated). A faint labeling of some lysosome-like vesicles was observed in one instance in an area close to the flagellum and the pusule system (see Cauchon et al., 1970). Some of the vesicles around the Golgi area

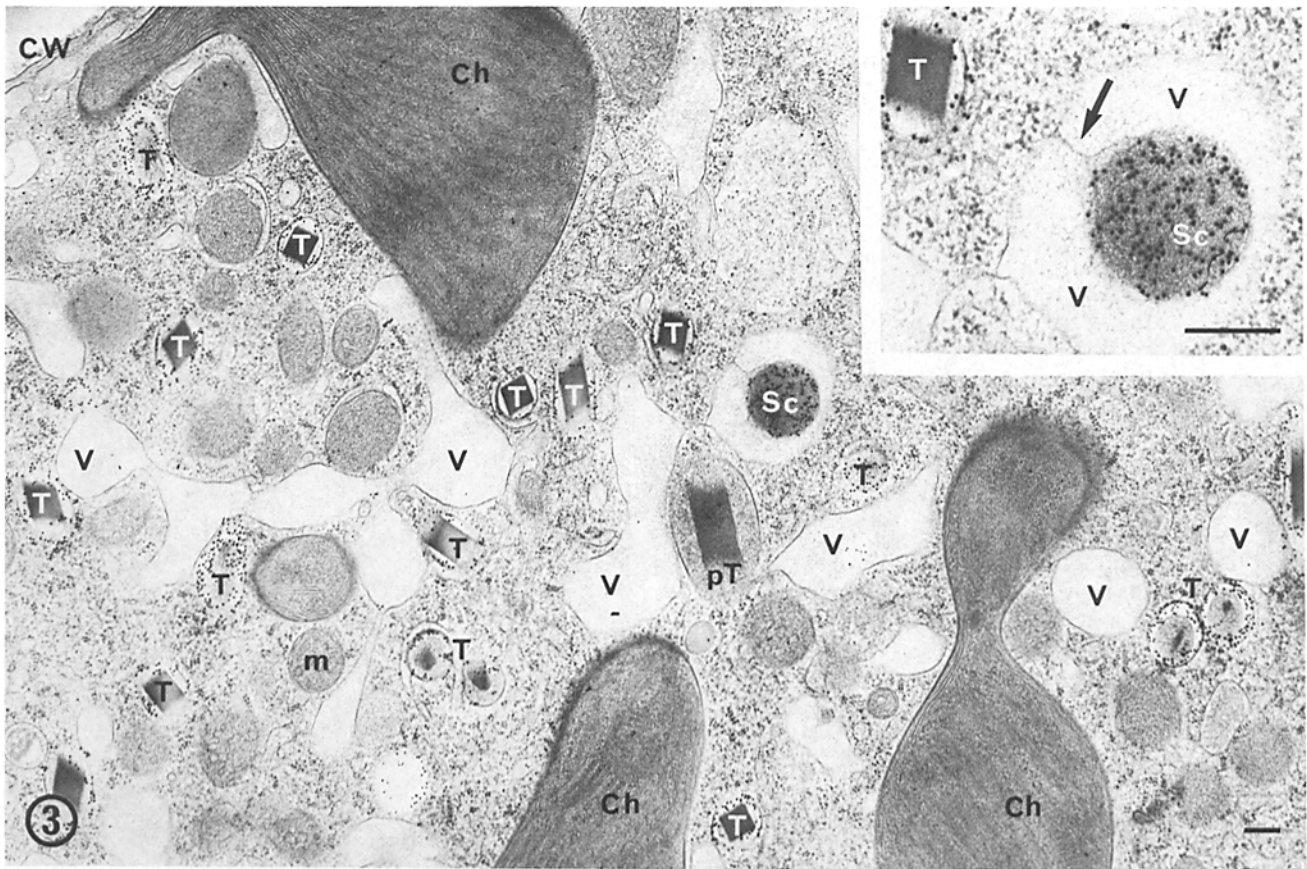


Figure 3. A scintillon (*Sc*), heavily labeled, hanging as a drop in the vacuolar space (enlarged in *inset*, with the narrow neck indicated by an arrow). FFFS-IGS technique with partially purified antiluciferase. The many ribosomes are similar in size to but slightly less dense than the gold particles. In this field, which is cortical, the vacuole (*V*) interdigitates extensively. Labeling also occurs on mature trichocysts (*T*). Most are seen here in cross section, showing the crystalline shaft, which is absent at their tips (for example two such sections are at the right part of the picture). Gold particles are seen in the space between the shaft and the trichocyst membrane. A pretrichocyst (*pT*) is not labeled at all, and the background labeling is very low. *Ch*, chloroplast; *CW*, cell wall; *m*, mitochondria. Bars, 0.2 μm .

were also occasionally labeled with affinity-purified antibody (Fig. 4). They might correspond to a precursor stage in the luciferase synthesis.

Only mature trichocysts in the cortical region of the cell were labeled, and not pretrichocysts, and exclusively on the space between the membrane of the organelle and the crystalline shaft (Figs. 3 and 5). The labeling was the same with affinity-purified antibody (Fig. 2). Gold particles were occasionally seen aligned over dense but fuzzy structures parallel to and on the membrane of the organelle (Fig. 5). Labeling was especially heavy over the tip of the trichocyst, which contained similarly fuzzy condensations.

Labeling with Affinity-purified Antiluciferase

The specific labeling of two organelles is difficult to explain. To investigate the possibility that this could be due to the presence of a second (different) antibody in our preparation, we prepared affinity-purified antiluciferase antibody which should lack the putative second antibody. As is evident in the sections treated with this affinity-purified antibody (see Figs. 2, 4, 10, and 11), the specificity of the labeling for the two organelles persists. This indicates that the antigenically reactive protein in the trichocysts is luciferase or a protein that shares antigenic epitopes with it.

The antibodies before and after affinity purification resulted in similar immunoblots (Fig. 6, *A* and *B*). As previously reported, the partially purified polyclonal antibody recognizes only a single major polypeptide from crude SDS extracts (made at pH 8) of *Gonyaulax* cells (Johnson et al., 1984). This experiment was repeated (Fig. 6 *A*), with over-exposure in order to visualize minor bands. With the affinity-purified antibody the staining of the luciferase, and also the minor bands, were essentially the same (Fig. 6 *B*), thus giving no indication for the presence of a second protein.

The possibility remains that the 135-kD protein band in the SDS gel comprises two proteins having the same molecular weights and other properties in common, resulting in their copurification. However, immunoblots of 2-D gels showed only a single protein spot with a pI of 6.8–7.0 at 135 kD (data not shown). This means that if a second cross-reacting 135-kD protein exists, it must also have the same pI as luciferase, which seems unlikely.

A further observation which rules out a cross-reacting 135-kD protein comes from immunoblots of proteolytic fragments of luciferase. This relies upon a special property of luciferase that would not be expected of a second and different protein, namely its sensitivity to a specific endogenous protease. In extracts made at pH 6, this protease converts luciferase



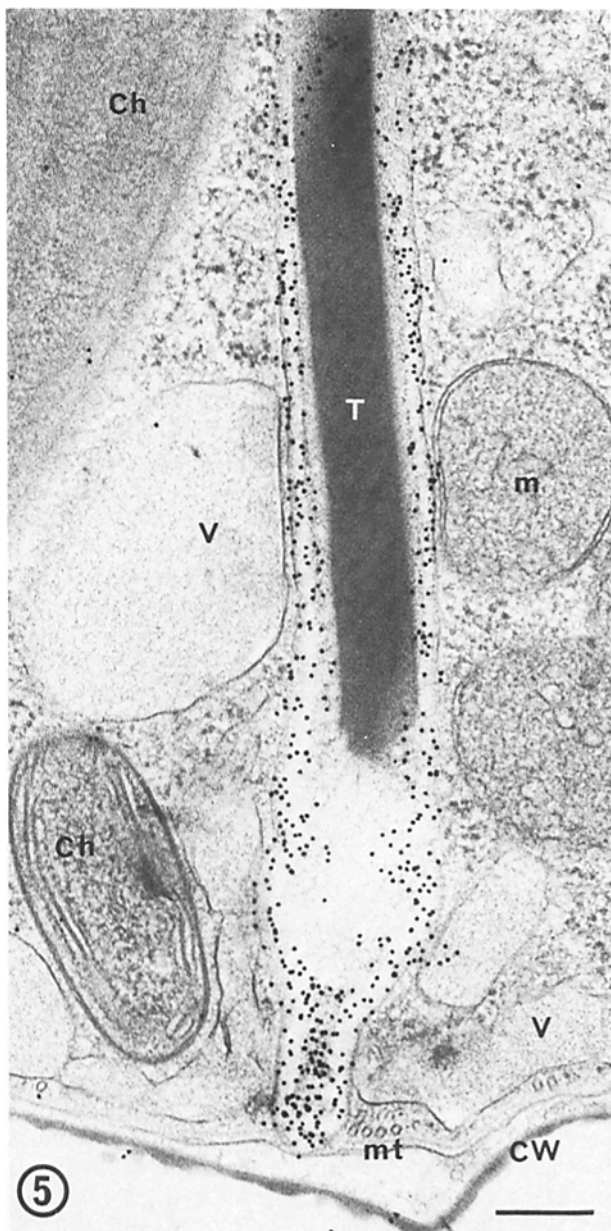


Figure 5. A mature trichocyst (*T*) in longitudinal section. FFFS-IGS technique, with partially purified antiluciferase antibody. Labeling occurs in the internal space of the trichocyst, between the limiting membrane and the crystalline shaft. Gold particles are apparently aligned along the length of the shaft; they are also numerous at the tip of the trichocyst in contact with the outer membrane. Note the adjacent microtubules (*mt*). Other unlabeled structures include chloroplasts (*Ch*), the cell wall (*CW*), mitochondria (*m*), and vacuoles (*V*). Bar, 0.2 μm .

Figure 4. Prescintillons in the Golgi area. FFFS-IGS technique, with affinity-purified antiluciferase antibody. A crown of Golgi apparatuses (*G*) delimits a central cytoplasmic area (right part of the picture) extremely rich in Golgi-related vesicles, coated vesicles (*cv*), and ribosomes, but devoid of chloroplasts and mitochondria (*m*). Branches of the vacuolar system (*V*) penetrate between adjacent Golgi apparatuses in this central area. The largest vesicles are filled with a product similar in appearance to that of trichocysts, and are considered pretrichocysts (*pT*). They are not labeled. Second, local condensations (*dark arrows*), occur free in the cytoplasm without a limiting membrane; these are considered prescintillons and are heavily labeled. Some also occur outside of the Golgi-enclosed area (left part of the picture), some in contact with the vacuolar membrane (*arrow, lower left*). A group of vesicles (*center at left, open arrow*) is also lightly labeled (see text). Artifacts of the freezing technique are occasionally observed to disrupt the ultrastructure (*asterisk, lower left*). *N*, nucleus; *CR*, chromosome. Bar, 1 μm .

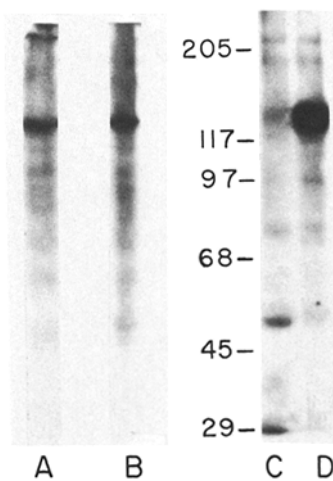


Figure 6. Immunoblots (greatly overexposed in order to visualize minor bands) of SDS crude extracts, made at pH 8 (extraction buffer: 100 mM Tris, 10 mM EDTA, 5 mM mercaptoethanol) with partially purified antibody (*A*) and with affinity-purified antibody (*B*). In both cases, there is a major band at 135 kD, corresponding to luciferase, and minor bands which are essentially the same in *A* and *B*. *C* and *D* show immunoblots from crude SDS extracts made at pH 8.3 (*D*) or at pH 6 (*C*; extraction buffer). In *C*, the 135-kD luciferase band has been

largely lost, and new bands of lower molecular masses appear (30 kD, 50 kD), corresponding to the proteolysed fragments of luciferase. Lanes *C* and *D* were overexposed in order to intensify the 50,000-D band in *C*. When the autoradiograms were exposed for shorter times, the 135,000-D band in *D* appeared as a single discrete band.

ase to smaller molecular mass fragments, which are active but which exhibit a different pH profile for enzyme activity (Krieger et al., 1974). In Fig. 6 *D*, an immunoblot is shown with an extract made at pH 8 (as in Fig. 6 *A*, but even more overexposed); with an extract made at pH 6 (Fig. 6 *C*), the material in the area of the luciferase band is largely lost, and new bands corresponding to the proteolysed fragments appear at lower molecular masses (30 and 50 kD). Thus, there is no indication of a second putative (trichocyst-related) protein resistant to the endogenous protease.

The Scintillons

In contrast to the trichocysts, which were labeled only at the mature stage, dense bodies were labeled at early stages of development. We use the term "prescintillons" to refer to the dense bodies found within or near the central Golgi area, before their envelopment by the vacuolar membrane. They occur as small (0.05–0.3 μm) subspherical electron-dense cytoplasmic condensations, completely free in the ground cytoplasm, apparently not associated with any other special cellular structure such as microtubules (Figs. 4, 8, and 10). They might have remained unnoticed without antiluciferase labeling and there was no indication of even earlier stages in their formation, except for the possibility that occasional groups of lightly labeled vesicles near the Golgi region might be involved.

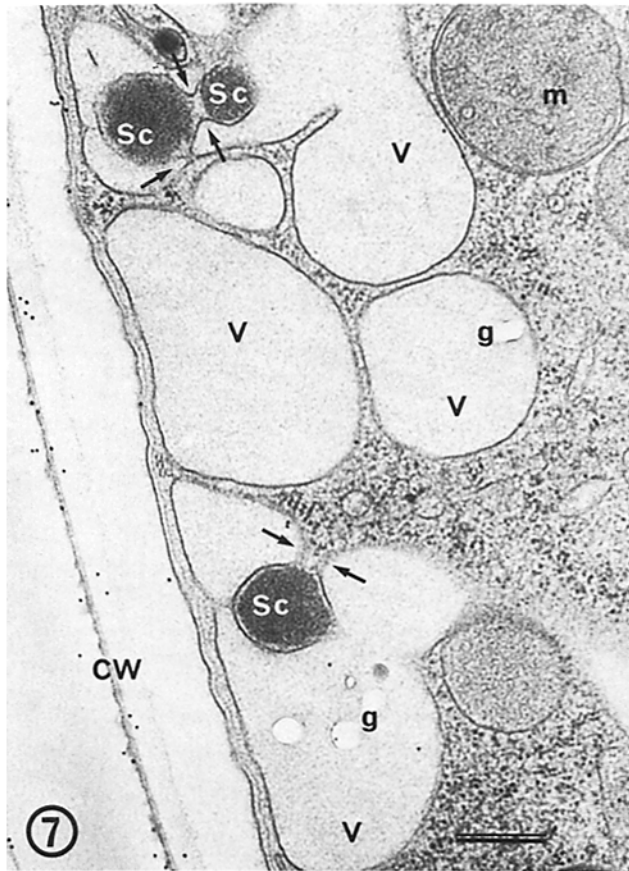


Figure 7. Control treated with NRS in place of the antiluciferase. The scintillons (Sc) are not labeled, whereas some (nonspecific) label occurs over the cell wall (CW). The scintillons, which here have a close association with the vacuolar membrane, protrude significantly in the vacuolar (V) space, but remain connected to the cytoplasm by one or more narrow bridges (arrows). *m*, mitochondria; *g*, "ghosts" of guanine crystals. Bar, 0.3 μm .

During development, it may be postulated that prescintillons migrate radially, establishing an association with a region of the vacuolar membrane (Fig. 11) and ultimately protruding into the vacuole (Fig. 7). This association appears to begin in the Golgi area into which, as previously noted, vacuolar digitations penetrate (Fig. 4). In the peripheral part of the cell the mature scintillons, which have sizes ranging from 0.3 to 0.5 μm , are seen protruding in the vacuolar space in a variety of ways (Figs. 7 and 12). However, they appear to remain in communication with the bulk of the cytoplasm by one or more narrow bridges, sometimes hanging like drops with just one narrow neck. At high magnification (Fig.

13), the dense part of the scintillons appears to be composed of an irregularly aggregated threadlike vermiform material with which the labeling appears to be associated.

Isolated Scintillons

Sedimentation in a Percoll/propanediol gradient resulted in a good resolution of the active bioluminescent fractions from the chloroplasts, with a major peak of scintillon activity within the gradient (Fig. 14). The peak fractions (12–15) were filtered on Millipore filters for microscopic study. The amount of biological material which could be deposited on the filters was limited; filters were quickly clogged by Percoll particles, which were abundant in the sections. In addition to dense bodies (scintillons) which were 0.3–0.5 μm in diameter, the active fractions examined contained miscellaneous membranes, a few mitochondria, and some discharged trichocyst shafts in the expanded state with their characteristic periodic structure. Although the dense bodies were few in number (2 or 3 per frame of a 300-mesh grid), they were the only labeled structures (Fig. 15). Discharged trichocysts found in these samples did not label. When NRS was used in place of antiluciferase antibody, the dense bodies did not show IGS labeling. The presence of dense bodies in peak fractions of sucrose density gradients, and their absence elsewhere, had previously been observed (Schmitter, 1971; Fogel et al., 1972).

Discussion

Identity of the Luminous Organelle

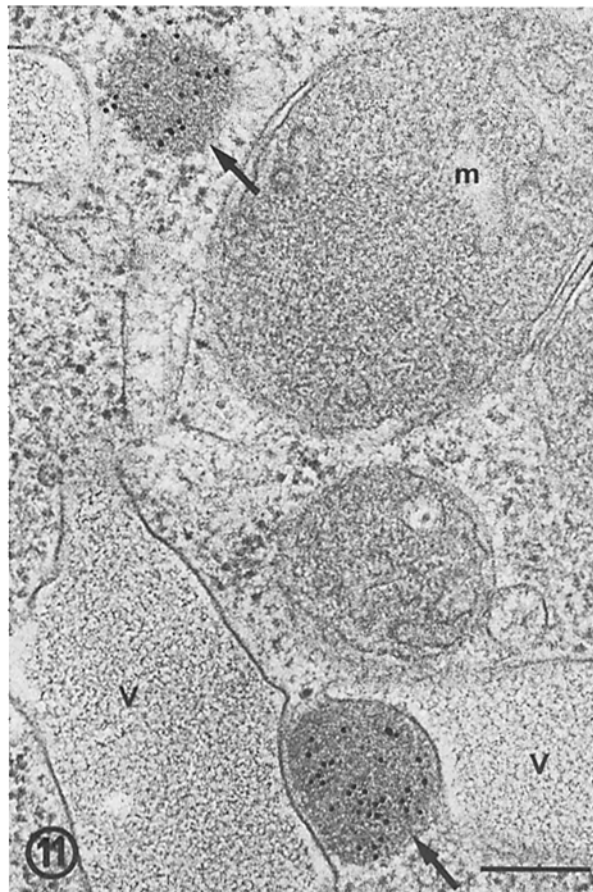
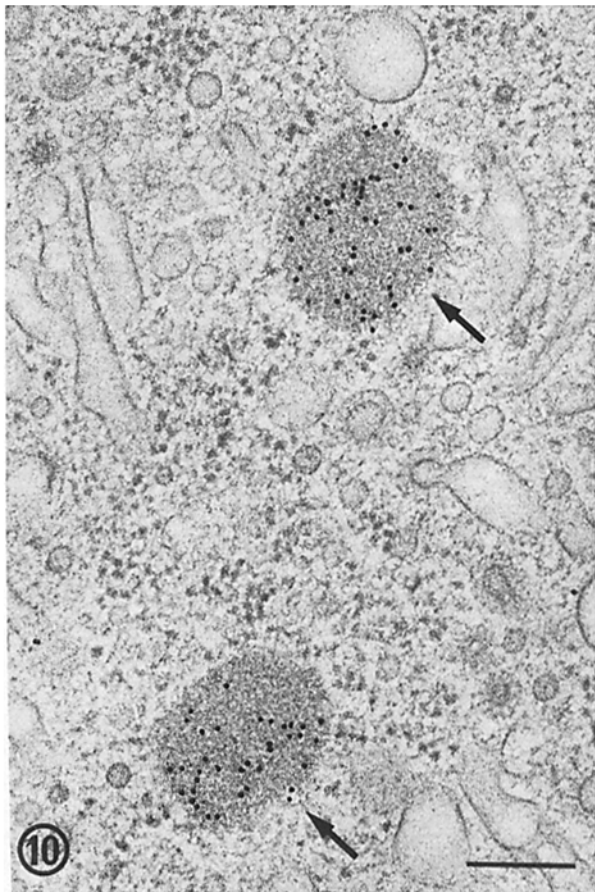
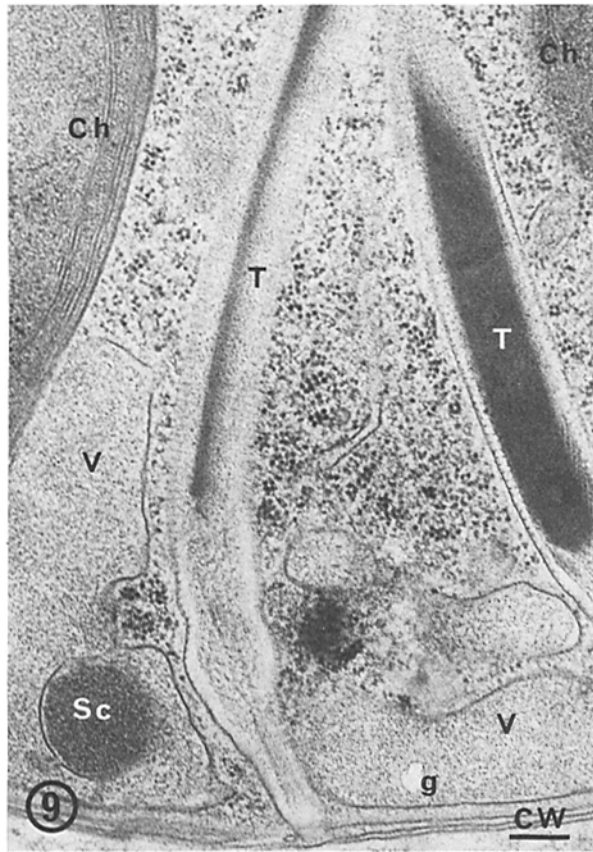
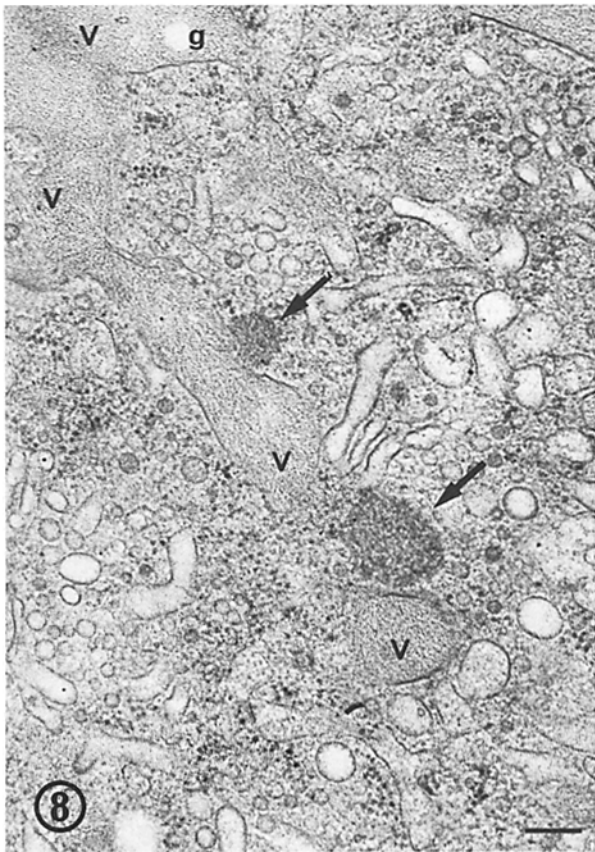
Luminescent systems in phylogenetically disparate groups are very different (Hastings, 1983). But the identification of a luminous organelle by immunocytochemistry has been previously reported only in the firefly, where it appears to be identifiable as a peroxisome (Neuwirth, 1981). In *Gonyaulax* the labeling with antiluciferase was found to be specific for two organelles, trichocysts and dense bodies, the latter being identified as the bioluminescent organelles, or scintillons. Although scintillons were originally believed to be associated with guanine crystals (De Sa et al., 1963), it was later shown that this was not so (Fogel et al., 1972). Rather than creating a neologism, we retain the term scintillon, defining it functionally as the flashing unit and now morphologically as the cytoplasmic dense bodies which are partially (in vivo) or completely (in vitro) surrounded by vacuolar membrane.

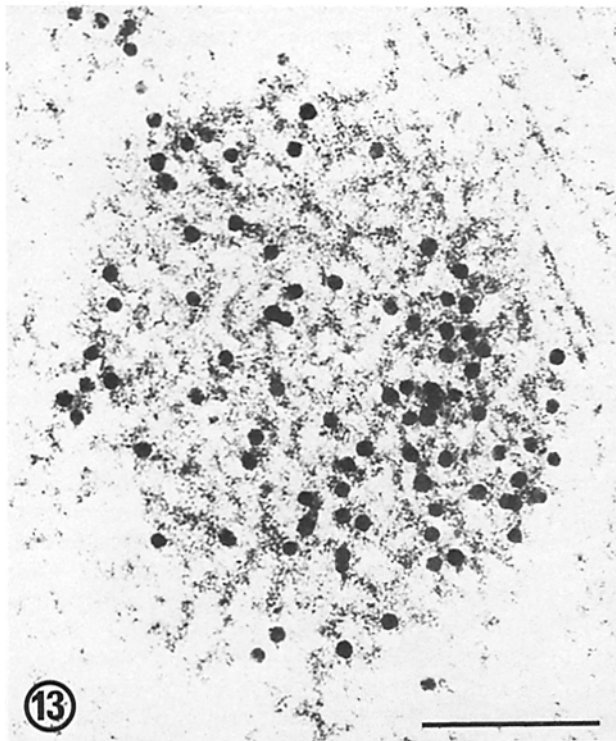
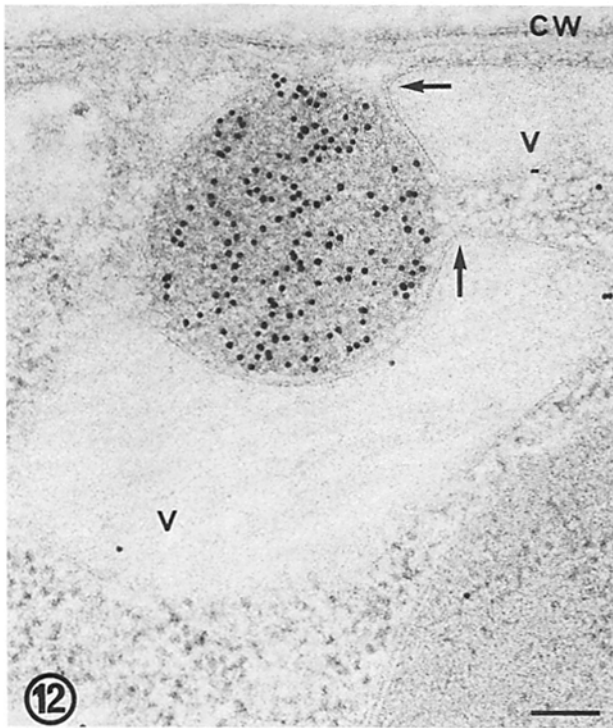
Specificity and Resolution of the Labeling

In the present study, the FFFS procedure was successfully coupled with the IGS technique, as also recently reported by

Figures 8 and 9. Controls, in which the affinity-purified antiluciferase was incubated with purified luciferase before treatment of sections. (Fig. 8) Prescintillons (arrows) in contact with the membrane of the vacuole (V) in the Golgi area remain unlabeled. (Fig. 9) At the periphery of the cell, neither the mature trichocyst (T) nor the mature scintillon (Sc) are labeled. CW, cell wall; Ch, chloroplast; g, "ghosts" of guanine crystals. Bars, 0.2 μm .

Figures 10 and 11. Enlargements of Fig. 4. Prescintillons (arrows) and the vacuolar membrane. FFFS-IGS technique, with affinity-purified antiluciferase antibody. At their early stages of formation the prescintillons (arrows) are found completely free in the cytoplasm among ribosomes, polysomes, and vesicles. Then, usually outside the central area, prescintillons are seen to contact the vacuolar membrane (V) (Fig. 11). A thin clear border separates the dense body from the vacuolar membrane, which is nevertheless depressed, partially wrapping around the scintillon. Note the coated vesicles abutting Golgi vesicles (upper left, lower right, Fig. 10). Bars, 0.2 μm .





Figures 12 and 13. Labeling of fine structure of the scintillons. FFFS-IGS technique, substitution in acetone alone (Fig. 12) or OsO_4 -acetone (Fig. 13) with partially purified antibody. In Fig. 12, a mature scintillon located near the cell periphery is hanging in the vacuolar space (V) by cytoplasmic bridges (arrows). Only the dense matrix is labeled. In Fig. 13, a prescintillon, free in the cytoplasm, shows a vermiculate structure, with which the 10-nm gold particles seem to be associated. CW, cell wall. Bar, 0.1 μm .

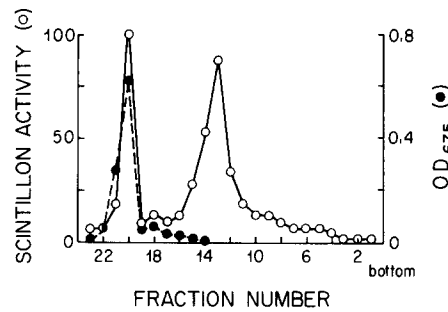


Figure 14. Isolation of the active bioluminescent fraction by percoll/propanediol density gradient centrifugation. One peak of bioluminescence scintillon activity is associated with chloroplasts (OD-675) at the surface of the gradient; the other one is clearly separate from the chloroplasts and was used for EM observations and labeling. In the fractions selected (12–15), fluorescent particles, previously shown to correlate with bioluminescence both in vivo and in vitro (Johnson et al., 1985), were confirmed to be present.

Hisano et al. (1985) and Dudek and Boyne (1986). As noted by Dudek et al. (1982, 1984), the specificity of immunolabeling is improved; in our studies, using affinity-purified antibodies, there was practically no background labeling. The high quality of the preservation allowed us to visualize for the first time the ultrastructural features of the scintillons, including their unusual association with the vacuolar membrane. By virtue of these structural features and also their biochemical properties (e.g., luminescence), scintillons must be considered as a new type of organelle. But, as noted above, homologous organelles should not be assumed to be present in luminous organisms from other phyla, since most are believed to have had evolutionarily independent origins (Hastings, 1983).

With 10-nm gold particles, the resolution is sufficient to localize the antigen to the space between the sheath and the core of the trichocysts (Fig. 5), and to the matrix of the dense bodies but not the surrounding membrane (Fig. 12). However, when labeling is light, the interpretation becomes more difficult. For example, there were some isolated gold particles associated with lysosome-like structures, others scattered over regions rich in free ribosomes, and some within vesicles or the endoplasmic reticulum cisternae (Fig. 4). In these cases, it could not be ascertained whether this reflected the existence of some small amount of the antigen or was just background labeling.

Why Are Trichocysts Labeled?

The labeling of the trichocysts by IGS is perplexing. The trichocysts do not show luciferin fluorescence nor do cells emit bioluminescence from trichocysts upon stimulation (Johnson et al., 1985). Therefore, although the trichocyst sheath may contain luciferase, it does not contain luciferin nor does it contribute to the bioluminescent flash. On the other hand, IGS labeling of trichocysts does not appear to be a simple artifact of nonspecific absorption because (a) trichocysts are labeled in spite of differences in fixation and processing techniques, (b) trichocysts are labeled by affinity-purified antiluciferase antibody, and (c) no trichocyst labeling occurs after antiluciferase is incubated with purified luciferase protein.

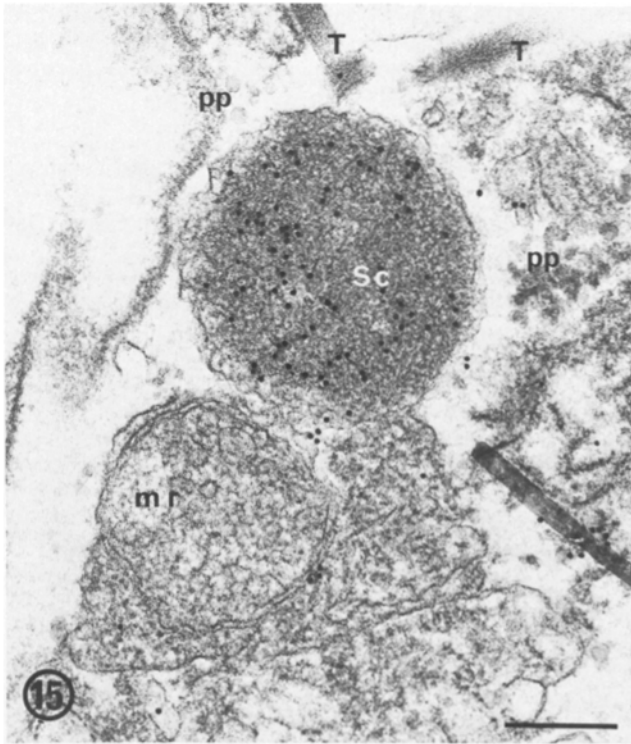


Figure 15. An isolated scintillon (*Sc*) from an active peak fraction of a Percoll gradient was fixed with glutaraldehyde and OsO_4 and embedded in Epon and treated with partially purified antiluciferase and IGS staining. The fraction contains many different cell components, including mitochondria (*mi*), diverse membranes and vesicles, expanded shafts of ejected trichocysts (*T*); the scintillon (*Sc*) is the only structure labeled. Its dense matrix has a structure similar to that seen in scintillons in situ, but with a limiting membrane completely enclosing the dense body. *pp*, Percoll particles. Bar, $0.2 \mu\text{m}$.

Why might an antiluciferase-reactive protein be located in the trichocyst sheath? It is possible that some processing or storage steps for luciferase involve trichocysts. Another possibility is that the antiluciferase-reactive trichocyst protein shares some antigenic epitopes with luciferase but is not itself luciferase. This last possibility is supported by the fact that our antiluciferase antibody labels the trichocysts (but no other organelle) of nonluminescent species of dinoflagellates (Nicolas et al., 1987). However, on immunoblots of these nonluminescent species, no polypeptide is labeled by our antibody (Johnson, C. H., unpublished observation). This result indicates that the luciferase protein which does label on immunoblots of *G. polyedra* is specific to scintillons. Thus, although trichocyst labeling is enigmatic, it does not appear to be relevant to the intracellular organization of bioluminescence because it appears in nonluminescent species as well.

Soluble vs. Particulate Luciferase and the Origin of In Vitro Activity

Cytoplasmic labeling was too low to suggest that soluble luciferase occurs uniformly in the cytosol. Such a putative soluble material could have been extracted and lost during liquid chemical fixation and the subsequent washing and dehydration, but it should have been preserved with the FFFS procedure. The distinction between the soluble and the particulate fractions (Schmitter et al., 1976; Hastings, 1978,

1986) could well be an artifact of extraction; some dense bodies might be disrupted and their soluble contents released, while in others the necks might be pinched off and resealed to form vesicles—the active in vitro scintillons. A similar pinching off and resealing during liquid chemical fixation, but not during the fast-freeze procedures, might account for the fact that the dense bodies appear as fully membrane-bound vesicles in sections prepared by the first method, whereas the cytoplasmic connections are seen with FFFS.

In the active particulate fractions (Fig. 15), only the dense vesicles are labeled. A comparison between these in vitro dense vesicles and those observed in situ shows that they are similar in structure and size ($0.3\text{--}0.5 \mu\text{m}$), and in both cases only the matrix is labeled. All these observations lead us to conclude that the dense vesicles in the gradient correspond to the dense bodies in situ whose neck has been disrupted during isolation procedure, and that they can exhibit in vitro flashing activity.

It is significant that only the matrix (Fig. 12), and not the membrane or the clear space between, is labeled. This suggests that luciferase is not an integral membrane protein, as had been proposed by Henry and Hastings (1974). They based their conclusion on the fact that luciferase could be extracted from isolated active particles by detergents. Considering the in vitro labeling, those results could be explained as a permeabilization by the detergent of the vesicle membrane, allowing luciferase to be released.

Association of the Scintillons with the Vacuole: The Proton Trigger Model

The labeling pattern of scintillons in different parts of the cell suggests that scintillons originate near ribosomes in the Golgi area, then form a special association with vacuolar membrane and move to the periphery of the cell. No cytoskeletal structure which might be involved in this migration was observed; the postulated displacement mechanism is thus unknown. The membrane association with the dense body appears to occur secondarily, and true vesicles are not formed. Instead, the dense material evaginates and hangs like a drop in the vacuolar lumen, forming the scintillon, with virtually all of the cytoplasm other than the dense material excluded. These features are represented in Fig. 16. Scintillons are thus unambiguously in the cytoplasmic compartment but nevertheless isolated and intimately associated with the vacuole, which, as in other plant cells (Matile, 1978; Marty et al., 1980), is acidic. In *Noctiluca*, the pH within the vacuole is ~ 3.5 (Nawata and Siboaka, 1979), and in *Gonyaulax* it is $\sim \text{pH } 4.5$, as estimated in vivo by weak base partitioning (Johnson, C. H., unpublished observations).

The involvement of the vacuolar membrane in the structure of the scintillon supports a specific model concerning the mechanism of the coupling between the conducted action potential, which can be elicited by mechanical stimulation, and the bioluminescent flash. An action potential, propagated along the vacuolar membrane, is postulated to allow protons to flow from the vacuole to the scintillon, which lowers the pH within the scintillon matrix, triggering the acid-sensitive light emission. The membrane around the scintillon could be specialized for this process; it might also contain a proton translocating ATPase whose function would be to restore the pH within the scintillon to a value greater

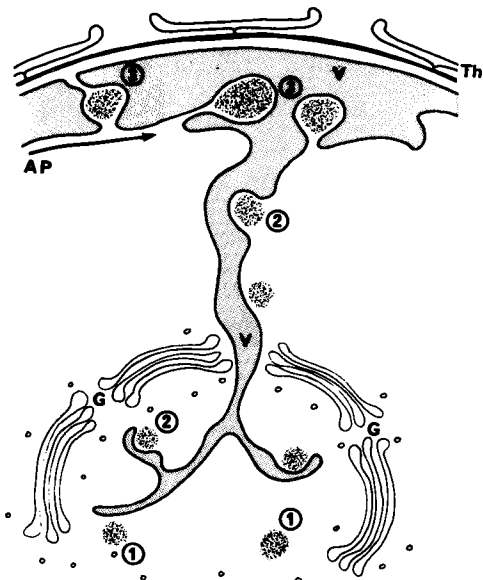


Figure 16. Schematic representation of the postulated steps in the formation and differentiation of the scintillons and of the excitation-bioluminescence coupling. Prescintillons appear as free cytoplasmic condensations in the Golgi area (1), contact the vacuolar membrane (2), and migrate towards the periphery of the cell. In the final stage of development, mature scintillons (3) protrude into the vacuolar space (V). In such a situation, a tonoplast action potential (AP), induced by a stimulation, spreads along the vacuolar membrane and could allow a proton influx from the acidic vacuole to the dense body of the scintillon, thus triggering the flash. Th, thecal plates.

than 7 by transporting protons from the scintillons back to the vacuole. The cytoplasmic bridges could allow for recharging by the transfer of cytoplasmic components to the scintillon (e.g., luciferin for the binding protein and ATP needed for the proton-translocating ATPase).

In addition to biochemical (Fogel and Hastings, 1972; Hastings, 1986) and cytological data, this model draws from electrophysiological studies in *Noctiluca miliaris*, where it was shown that flashing is correlated with an action potential propagated along the vacuolar membrane (Eckert, 1965; Eckert and Sibaoka, 1968). In addition, Nawata and Sibaoka (1979) showed that neutralizing the pH of the vacuole of *Noctiluca* with a buffer results in a reversible loss of action potential propagation and flashing capability. These data indicate both that the source of bioluminescence-stimulating protons is the vacuole and that the charge-carrying ions involved in the action potential are protons.

All the scintillons of a cell are thus connected by the vacuolar membrane; transvacuolar strands, where they occur, greatly increase the interface vacuole-cytoplasm (Wagner, 1982). Some recent studies (Hilling and Amelunxen, 1985; Schnepf, 1984) propose the vacuole of plant cells to be a part of the endoplasmic reticulum, and the possible connection in *Gonyaulax* cells between the vacuolar membrane and the outer membrane of the nuclear envelope (Fig. 2) is consistent with this idea.

The vacuole, therefore, in spite of its complex topography, constitutes a continuous internal compartment, and the propagation of the action potential along its excitable mem-

brane would result in a concerted firing of the individual microsomes, as has been observed in *Gonyaulax* (Johnson et al., 1985) and other dinoflagellates (Eckert, 1966; Sweeney, 1982; Widder and Case, 1982a, b).

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