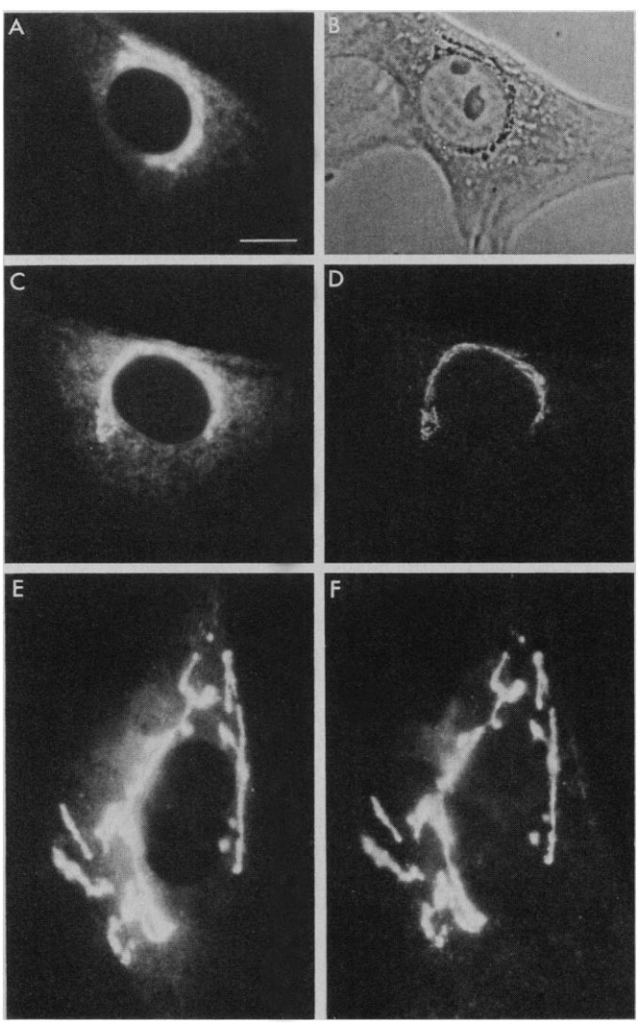
A Vital Stain for the Golgi Apparatus

Abstract. *The Golgi complex, a membranous organelle with important functions in membrane traffic and macromolecular synthesis, has been stained in living cells with a fluorescent sphingolipid. Cells were first incubated with liposomes containing* N-*[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl sphingosine (C_6-NBD-ceramide), or with a bovine serum albumin complex of the fluorescent lipid, and then examined by fluorescence microscopy. An intensely fluorescent perinuclear structure was identified as the Golgi apparatus by its colocalization with known Golgi markers in fixed cells. C_6-NBD-ceramide was used to observe the morphology of the Golgi apparatus in living cells in the presence or absence of monensin or Colcemid, and during mitosis. In all cases, C_6-NBD-ceramide revealed a Golgi apparatus in the living cell that was identical to that obtained with conventional procedures that require fixation.*

"Students of the Golgi element have tended to work towards a desired sharp image, without questioning whether in fact the structure is sharply defined in life. . . . Nevertheless, the sharpness and beauty of the Golgi element in impregnated preparations are irrelevant, unless it can be shown to be sharp and beautiful in the living cell" (*1*). In the 40 years since Baker made this criticism, a great deal has been learned about the functions and characteristics of the Golgi apparatus (*2*), but the nature of this organelle in the living cell remains to be established (*3*). Although sophisticated Golgi stains have been developed, including the stain for thiamine pyrophosphatase activity (*4*), and those involving fluorescently labeled lectins (*5*) or antibodies to the Golgi apparatus (*6–8*), these methods are time-consuming and utilize fixed cells. In this report, we describe a simple and rapid procedure for vital staining of the Golgi apparatus in cultured cells.

When cultured Chinese hamster lung fibroblasts are incubated at 2°C with liposomes containing the fluorescent lipid *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl sphingosine (C_6-NBD-ceramide) the mitochondria, nuclear envelope, and endoplasmic reticulum become fluorescently labeled (*9*). When these cells are washed and warmed to 37°C, the Golgi apparatus and, later, the plasma membrane become intensely fluorescent. During this time, the C_6-NBD-ceramide is metabolized to fluorescent cerebroside and sphingomyelin. In the present study we (i) developed general procedures for the administration of C_6-NBD-ceramide to cells; (ii) rigorously established the staining of the Golgi apparatus by C_6-NBD-ceramide; and (iii) examined the morphology of the Golgi apparatus in the living cell in the presence of several drugs and during mitosis.

C_6-NBD-ceramide (*10*) can be administered to cells from liposomes (*11*), or as a complex with bovine serum albumin (C_6-NBD-ceramide–BSA) (*12*). Cells were washed free of culture medium and incubated with liposomes (for 30 minutes



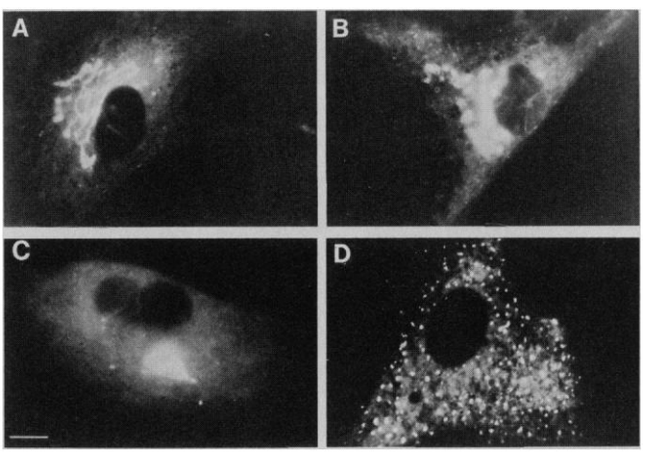


Fig. 1 (left). Colocalization of C_6-NBD-ceramide fluorescence with traditional markers of the Golgi apparatus. (A and C) Fluorescence micrographs of BHK cells after incubation with C_6-NBD-ceramide–BSA. (B) The same cell as in (A) after fixation and staining for thiamine pyrophosphatase activity (*4*), as viewed by phase-contrast microscopy. (D) The same cell as in (C) after fixation and staining with a mouse immunoglobulin G specific for the Golgi apparatus (*6*) followed by a rhodamine-labeled goat antibody to mouse immunoglobulin G. (E) Human skin fibroblast vitally stained with C_6-NBD-ceramide as in (A) and (B). (F) The same cell as in (E) after fixation and labeling with rhodamine-conjugated wheat germ agglutinin. Scale bar, 10 μm. Fig. 2 (right). Effect of monensin and Colcemid on the morphology of the Golgi apparatus in living cells. Cells were stained with C_6-NBD-ceramide–BSA in the presence or absence of inhibitors and viewed in the fluorescence microscope. Human skin fibroblasts in the absence (A), or presence (B) of 10 μ*M* monensin. Mouse 3T3 cells in the absence (C), or presence (D) of 10 μ*M* Colcemid. Scale bar, 10 μm.

at 2°C), or with C_6-NBD-ceramide–BSA (for 10 minutes at 37°C) (*13*). The cells were then washed, incubated in 18 m*M* Hepes-buffered minimum essential medium (HMEM) for 30 minutes at 37°C, and observed in the fluorescence microscope (*14*). Figure 1A shows a representative fluorescence micrograph of a BHK cell labeled with C_6-NBD-ceramide in which the nucleus is partially surrounded by a bright threadlike structure. The cell was then fixed and the Golgi apparatus visualized by means of the histochemical stain for thiamine pyrophosphatase activity developed by Novikoff *et al.* (*15*). A comparison of the different staining protocols on the same cell shows an excellent correspondence between C_6-NBD-ceramide fluorescence in the living cell (Fig. 1A) and the characteristic black reaction product resulting from the histochemical stain (Fig. 1B). Subtle discrepancies between these two techniques may be due to the difficulty in recapturing the same focal plane in the fixed cell as in the living cell.

Another colocalization study was carried out in BHK cells with a rhodamine-labeled monoclonal antibody specific for the Golgi apparatus (*16*). Again, the C_6-NBD-ceramide fluorescence in the living cell corresponded to the pattern of rhodamine fluorescence in the fixed specimen (Fig. 1, C and D). In a human skin fibroblast from a preparation labeled with C_6-NBD-ceramide, the fluorescent region was highly reticular and perinuclear (Fig. 1E). The cell preparation was subsequently fixed, rendered permeable, and stained (*9*) with rhodamine-conjugated wheat germ agglutinin, a lectin with high affinity for the Golgi apparatus (*5*) (Fig. 1F). The lectin-stained Golgi apparatus coincided with the fluorescence pattern obtained in the living cell after incubation with C_6-NBD-ceramide. Based on these three colocalization experiments, we conclude that C_6-NBD-ceramide reliably labels the Golgi apparatus in living cells.

Labeling of other cytoplasmic membranes also occurs during incubation with C_6-NBD-ceramide (*9*), resulting in a faint "background" of NBD-fluorescence against which the more brightly labeled Golgi apparatus can be seen. This background can be minimized for each cell type by varying the concentration of C_6-NBD-ceramide and the incubation times during and after treatment with this fluorescent lipid. In addition, the intensity of the fluorescently stained Golgi apparatus decreased with time after the initial incubation with C_6-NBD-ceramide, presumably reflecting the metabolism of the fluorescent ceramide to fluorescent sphingomyelin and cerebroside and its translocation to the plasma membrane (*9*). Reincubation with C_6-NBD-ceramide again produced prominent labeling of the Golgi apparatus.

The staining pattern obtained after incubation with C_6-NBD-ceramide was also examined in other established cell lines, in primary cultures of chick sympathetic ganglia, chick myotubes, and rat hepatocytes, and in a newt intestinal epithelial cell (*17*). In each case, a bright region of fluorescence, often reticular and usually perinuclear, appeared in the cytoplasm of the treated cells. Although we did not carry out systematic colocalization studies of the type shown in Fig. 1 for all these cell types, we believe that the prominent labeling in each case was most likely the Golgi apparatus.

The morphology of the Golgi apparatus in the presence of metabolic inhibitors has been described for fixed, processed tissues and is characteristic for different inhibitors. Monensin, for example, causes vacuolation and distension of the Golgi apparatus without affecting the perinuclear distribution (*18*), whereas Colcemid causes the Golgi apparatus to fragment throughout the cytoplasm (*19*). We examined the effect of these agents on the morphology of the Golgi apparatus in living cells by staining with C_6-NBD-ceramide. In the presence of 10 μ*M* monensin, the Golgi apparatus of a human skin fibroblast became vesiculated and less distinct (Fig. 2B) as compared to the characteristic reticular structure of the control (Fig. 2A). This confirms the observations made with monensin-treated fixed cells. The altered structure was identified as the Golgi apparatus by colocalization with rhodamine-conjugated wheat germ agglutinin in monensin-treated fibroblasts (*9*). The Golgi apparatus in a 3T3 cell appeared as a compact, perinuclear structure (Fig. 2C), and at other planes of focus its

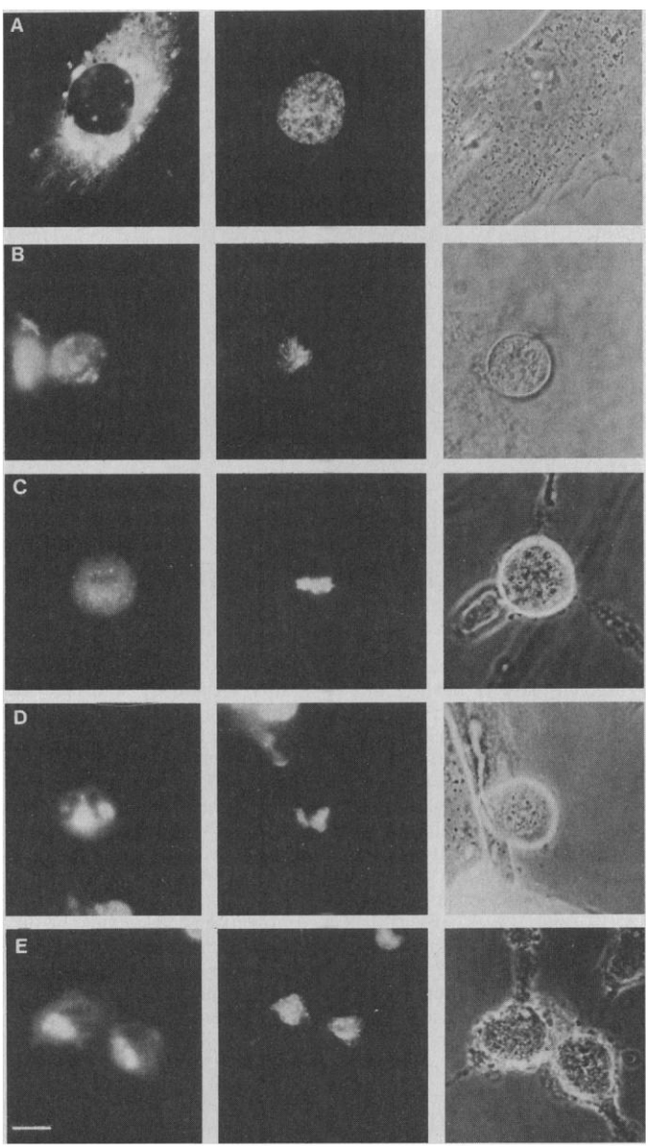


Fig. 3. Appearance of the Golgi apparatus in living cells during the cell cycle. Human skin fibroblasts were stained with C_6-NBD-ceramide–containing liposomes and the vital chromosome dye Hoechst 33342 (*21*). They were then photographed under optics appropriate for NBD- (left) or Hoechst 33342- (middle) fluorescence, and under phase-contrast (right). (A) Interphase; (B) prophase; (C) metaphase; (D) anaphase; and (E) late telophase. Scale bar, 10 μm.

reticular nature could be observed. In the presence of 10 μM Colcemid (Fig. 2D), the fluorescence was scattered throughout the cytoplasm, consistent with the morphology of the Golgi apparatus in fixed, Colcemid-treated cells (*19*).

Finally, we asked whether the distribution of the Golgi apparatus in living cells at different mitotic stages would parallel that of fixed cells in which the Golgi becomes fragmented and almost disappears during metaphase (*6, 20*). Human skin fibroblasts were labeled with C_6-NBD-ceramide and chromosomes were vitally stained with Hoechst 33342 (*21*). During interphase (Fig. 3A) the Golgi had its distinctive, threadlike appearance. As the cell rounded up in prophase (Fig. 3B), the Golgi was visible as scattered punctate regions in the cytoplasm. At metaphase (Fig. 3C), these regions were reduced to infrequent, pinpoint areas of fluorescence. The background fluorescence appeared to have increased because of the rounding of the cell. During anaphase (Fig. 3D), the Golgi apparatus reappeared close to the separating chromosomes. Finally, in late telophase (Fig. 3E), the characteristic perinuclear structure reappeared.

In conclusion, C_6-NBD-ceramide was distributed in the same manner as traditional Golgi stains in various cell types and during mitosis or drug treatment. The advantages of rapidity and ease of labeling permitted immediate visualization of alterations in Golgi morphology in living cells.

NAOMI GELLER LIPSKY
RICHARD E. PAGANO
Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210

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10. C_6-NBD-ceramide was synthesized and purified by modifying the procedure of Y. Kishimoto [*Chem. Phys. Lipids* **15**, 33 (1975)]. We used NBD-aminocaproic acid (Avanti Biochemicals), and doubled the sphingosine concentration.
11. Lipid vesicles were formed (*9*) by ethanol injection of C_6-NBD-ceramide and dioleoylphosphatidylcholine (in a molar ratio of 20/80) in 10 mM Hepes-buffered calcium- and magnesium-free Puck's saline and dialysis at 2°C overnight against this buffer. The vesicle preparation was diluted with 18 mM HMEM to a final concentration of 5 to 10 nmol of C_6-NBD-ceramide per milliliter.
12. A solution of defatted BSA (0.68 mg/ml; Sigma) in HMEM was added to a tube containing desiccated C_6-NBD-ceramide for a final concentration of 10 nmol of C_6-NBD-ceramide per milliliter. The tube was immersed in a bath sonicator for 5 to 10 seconds to obtain a clear solution.
13. Although the distribution of fluorescence obtained by both methods was identical, the uptake of C_6-NBD-ceramide may occur by different mechanisms in the two procedures. In liposome-cell incubations at 2°C, uptake of C_6-NBD-ceramide is solely by net transfer (*9*). Therefore, this method may be preferable when the route of uptake must be known. In C_6-NBD-ceramide–BSA incubations, the route of incorporation is unknown and may involve an endocytic pathway, resulting in the uptake of both BSA and ceramide by the cells. However, this method was rapid and convenient for use with cells whose morphology changed during incubations at low temperatures.
14. Fluorescence microscopy was carried out with a Zeiss IM-35 inverted microscope, equipped with filter packs (Zeiss 487717 and 487715) that allowed no crossover between NBD and rhodamine fluorescence. For visualization of Hoechst 33342 fluorescence, Zeiss filter pack 487702 was used.
15. Cells were fixed as described by G. Griffiths, P. Quinn, and G. Warren [*J. Cell Biol.* **96**, 835 (1983)] and stained for thiamine pyrophosphatase (*4*), except that cover slips were inverted (*20*) in the reaction medium for 3 hours at 37°C. No product was formed in the absence of substrate, and the presence of C_6-NBD-ceramide had no effect on the reaction.
16. Fluorescent antibody staining of the Golgi apparatus was carried out as described (*8*) with a monoclonal antibody (53FC3) directed against a Golgi membrane protein found in rodent cells (*6*).
17. BHK-21 and 3T3 cell lines were obtained from American Type Culture Collection (Rockville Md.). Human cell line GM302 was obtained from the Genetic Mutant Cell Repository (Camden, N.J.). Other established cell lines (B104 CHO, LA-23, MDCK, MDBK, NB77, PTK2 and Vero) were supplied by B. Geiger (Weizmann Institute, Israel). Primary cultures of chick sympathetic ganglia and chick myotubes were supplied by M. Tamkun and D. Fambrough (Carnegie Institution, Baltimore, Md.) rat hepatocytes by S. Yedgar (Hebrew University, Israel), and a newt intestinal cell preparation by L. Epstein (Carnegie Institution).
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21. GM302 cells were first incubated with C_6-NBD ceramide–containing vesicles for 1/2 hour at 2°C, then washed and incubated in medium containing Hoechst 33342 (2.5 μg/ml) for 3 minutes at 37°C [M. E. LaLande, V. Ling, R. G Miller, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 36 (1981)].
22. We thank J. Gall and L. Epstein for helpful discussions, A. Novikoff and P. Novikoff (Albert Einstein College of Medicine, New York for the gift of thiamine pyrophosphate and advice on the staining procedure, and B. Burke (Johns Hopkins School of Medicine, Baltimore Md.) for providing the monoclonal antibody Supported by grant GM-22942 from the U.S Public Heath Service and by a postdoctoral fellowship (GM-08848) from the National Institutes of Health (N.G.L.).

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Trigeminal-Taste Interaction in Palatability Processing

Abstract. *Peripheral transection of the sensory branches of the trigeminal nerve in rats unbalanced palatability, selectively reducing the ingestive actions elicited by preferred tastes but leaving unchanged the aversive actions elicited by unpreferred tastes. The reduction in the number of positive ingestive actions occurred even though the capacity to emit these actions remained unimpaired. These findings show that there is an interaction between somatosensation and gustation in the processing of palatability.*

Trigeminal deafferentation, which removes somatosensation selectively from the mouth and face, severely disrupts food intake in rats, cats, and pigeons (*1–6*). The disruption is not attributable solely to a simple incapacity to execute required movements, but also to reduced responsiveness to food, reduced probability of initiating a meal (*1, 2*), and disrupted dietary selection of protein and carbohydrate nutrients (*3*). Deafferentated rats are hypophagic in response to food pellets, and can be maintained best on highly preferred foods (such as moist cereal mash).

The severity and nature of the feeding deficit produced by deafferentation have led to the suggestion that trigeminal oro sensation contributes more to the motivational control of ingestive behavio than does gustatory information (*1*); conversely, it has been suggested that trigeminal deafferentation leaves a rat ab normally sensitive to the perceived palatability of its diet (*1*), possibly becaus remaining gustatory cues are more sa

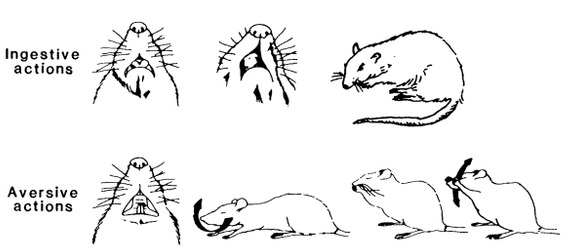

Fig. 1. Taste-elicited actions Ingestive actions are rhythmi tongue protrusion, nonrhyth mic lateral tongue protrusion and paw licking. Aversive ac tions are gaping, headshaking face washing, and forelim flailing.