

Imaging Lipid Rafts

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Lipid rafts are plasma membrane microdomains enriched in sphingolipids and cholesterol. These domains have been suggested to serve as platforms for various cellular events, such as signaling and membrane trafficking. However, little is known about the distribution and dynamics of lipids in these microdomains. Here we report investigations carried out using recently developed probes for the lipid components of lipid rafts: lysenin, a sphingomyelin-binding protein obtained from the coelomic fluid of the earthworm *Eisenia foetida*; and the fluorescein ester of poly(ethyleneglycol) cholesteryl ether (fPEG-Chol), which partitions into cholesterol-rich membranes. Lysenin reveals that the organization of sphingomyelin differs between different cell types and even between different membrane domains within the same cell. When added to live cells, fPEG-Chol is distributed exclusively on the outer leaflet of the plasma membrane and is clustered dynamically upon activation of receptor signaling. The surface-bound fPEG-Chol is slowly internalized *via* a clathrin-independent pathway into endosomes with lipid raft markers.

Key words: cholesterol, lipid probe, lipid rafts, membrane domain, sphingomyelin.

The biological membranes of eukaryotic cells contain thousands of different lipid species. These lipids serve to organize membranes into discrete, specific domains with distinct properties. Depending on their function, lipids are distributed to different particular cellular locations. For example, the membranes of different intracellular organelles have different lipid compositions from each other; and the distribution of lipids across the membrane bilayer differs markedly among them. Lateral segregation of lipids produces microdomains called lipid rafts, which can selectively incorporate and exclude proteins, and thereby govern protein-protein and protein-lipid interactions. (1–3). Lipid rafts have been suggested to play important roles in a variety of cellular functions such as signaling, adhesion, motility, and membrane trafficking (4, 5). In the case of membrane trafficking, lipid rafts were first proposed to mediate the sorting process that takes place in the *trans*-Golgi network, especially in polarized epithelial cells and neurons. Recent studies have suggested that lipid rafts may also be involved in clathrin-independent endocytic pathways of specific lipids and proteins. Since a lipid-based sorting mechanism may operate along with the membrane trafficking pathway, understanding the precise structure of the lipid microdomains should allow a more precise understanding of the molecular mechanisms of lipid domain-dependent membrane flow. It is also important to directly monitor the trafficking of lipid components in the micro-

domains. However, little is known at present about the functional or structural organization of the microdomains, in large part for lack of appropriate probes to pursue such investigations.

Probes for lipids are generally classified into two categories: fluorescent lipid analogues and lipid-binding molecules (including proteins such as antibodies and toxins). Fluorescent lipid analogues have proven particularly useful in membrane trafficking studies. However, it should be noted that many of the studies on sphingolipid trafficking have been performed with C6-NBD- or C5-BODIPY-labeled sphingolipids, in which the ceramide backbone is clearly distorted. These molecules are less hydrophobic than their naturally occurring counterparts and exhibit a generally weak affinity to liquid-ordered phases that are rich in sphingolipid and cholesterol in biomembranes. Lipid analogues that are partitioned into specific lipid membranes can be effectively employed as tools for imaging the specific lipid environment. Lipid-binding proteins are useful as specific probes because, in general, they have a high affinity for lipids. Since the molecular weights of most proteins are much higher than those of lipid molecules, probes may induce alteration of the membrane organization. In this respect, low molecular weight peptides or chemicals are more suitable, in that they minimize unexpected effects on cellular activities. To better understand the organization of lipids in biomembranes, potentially useful lipid probes need to be characterized in detail.

This review focuses on probes for sphingomyelin and cholesterol, important components of lipid rafts. First, we describe the characterization of lysenin, a sphingomyelin-binding toxin, and our recent attempt to elucidate the organization of sphingomyelin in cellular membranes using this probe. Second, we summarize various probes

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for cholesterol and describe our approach to investigate cholesterol trafficking using a fluorescein ester of poly (ethylene glycol)-derivatized cholesterol (fPEG-Chol).

Imaging sphingomyelin

Sphingomyelin is a major sphingolipid species in animal cells and a major lipid constituent of the plasma membrane, where it is concentrated in the outer leaflet (6–8). Although sphingomyelin is suggested to be important for the organization and function of lipid rafts, little is known about the distribution and trafficking of membranes containing sphingomyelin for lack of appropriate probes.

Several proteins that interact with sphingomyelin have been reported (9–13). Among them, lysenin, a pore-forming toxin, is known to be highly specific to sphingomyelin. Lysenin was isolated from the coelomic fluid of *E. foetida* as a 41-kDa protein that induces contraction of strips of isolated rat aorta (14, 15). Subsequently it was shown that lysenin induces hemolysis and is cytotoxic towards vertebrate spermatozoa as well as cultured mammalian cells (16, 17). Enzyme-linked immunosorbent assay (ELISA) revealed that lysenin binds specifically to sphingomyelin (17), but not to other phospholipids, glycosphingolipids, intermediates of sphingomyelin synthesis or sphingomyelin metabolites (18). Sphingomyelin shares a phosphocholine moiety with phosphatidylcholine, whereas its ceramide backbone is specific for sphingomyelin and glycosphingolipids. The ELISA results indicated that both the phosphocholine and the ceramide moieties are required for the sphingomyelin to be recognized by lysenin.

The specific binding of lysenin to sphingomyelin makes this protein a unique tool for examining the distribution of cell surface and intracellular sphingomyelin (17, 19). Niemann-Pick type A (NPA) cells are characterized by a deficiency in lysosomal acid sphingomyelinase and hence also by an intracellular accumulation of sphingomyelin (20). When NPA fibroblasts were fixed, permeabilized and labeled with lysenin, the cells displayed bright perinuclear lysenin labeling corresponding to sphingomyelin accumulation. Lysenin was applied to visualize the sphingomyelin on the surface of oligodendrocyte lineage cells. The results show that the content of sphingomyelin increases during cell differentiation (19). Lysenin has also been used to study the biosynthesis and transport of sphingomyelin. Selection of lysenin-resistant variants from Chinese hamster ovary cells yielded cell lines deficient in sphingolipid synthesis (21) and/or ceramide transport from the endoplasmic reticulum to the Golgi apparatus (22, 23).

The organization of sphingomyelin in the membranes is affected by other lipid constituents. Using model membranes, we examined whether lysenin distinguishes between alternative organizations of sphingomyelin. Naturally occurring sphingomyelin exhibits a relatively high transition temperature between gel and liquid-crystalline phases (36–40°C). Therefore, sphingomyelin is in either a gel or solid phase at physiological temperatures. Most sphingolipids also have high phase-transition temperatures and thus are in the solid phase under physiological conditions. In contrast, most glycerophospholipids are in a liquid-crystalline phase. Gel phase lipids, such as

sphingomyelin, are mixed well with one another, but not with liquid-crystalline lipids. Therefore, in the mixture of sphingomyelin and liquid-crystalline (disordered) lipids, such as dioleoylphosphatidylcholine (diC18:1 PC), sphingomyelin spontaneously forms clusters. In contrast, sphingomyelin and solid (ordered) lipids such as dipalmitoylphosphatidylcholine (diC16:0 PC) or glycosphingolipid are miscible with each other. Thus the local density of sphingomyelin is decreased in the presence of diC16:0 PC or glycosphingolipids. We found that lysenin bound to sphingomyelin/diC 18:1 PC giant unilamellar vesicles (GUVs), in which the sphingomyelin-rich phase and the diC18:1 PC-rich phase were clearly segregated. In contrast, lysenin did not bind to sphingomyelin/diC 16:0 PC GUVs, in which the lipids were uniformly distributed. By measuring the fluorescence resonance energy transfer between the tryptophan residues of lysenin and pyrene-labeled sphingomyelin, we observed that lysenin bound sphingomyelin/diC 18:1 PC vesicles. Replacing diC 18:1 PC with diC16:0 PC or galactosylceramide (GalCer) decreased lysenin binding to sphingomyelin. These results indicate that the binding of lysenin to sphingomyelin is dependent on the local density of the lipid, *i.e.*, lysenin-binding to sphingomyelin is increased as sphingomyelin forms clusters (Fig. 1). Isothermal titration calorimetry revealed that one lysenin molecule binds five sphingomyelin molecules. These observations suggest that the binding of lysenin to sphingomyelin is the result of specific stoichiometric sphingomyelin-lysenin complex formation (24).

The results indicate that lysenin is not only a lipid-specific protein but also a lipid organization-specific toxin. We used lysenin to study the heterogeneous organization of sphingomyelin at the mammalian cell surface (24). Epithelial cells contain two distinct plasma membrane domains; the apical domains that confront the external lumen, and the basolateral membranes that face the underlying cell layer (25, 26). Each plasma membrane domain has a specialized function and contains a distinct set of lipids and proteins. Interestingly, glycosphingolipids are highly enriched in the apical domain (27). A cultured epithelial cell line, MDCK (Madin-Darby canine kidney), was highly sensitive to lysenin when the toxin was added from the basolateral side, whereas the cells were resistant to apically added lysenin. Since the sphingomyelin content of the apical membrane is sufficient for lysenin recognition, it was hypothesized that the glycosphingolipid enrichment of the apical membrane resulted in a decrease in the local density of the sphingomyelin and thus endowed it with toxin resistance. To examine the effect of glycosphingolipid on the recognition of sphingomyelin by lysenin, we compared lysenin binding between a glycosphingolipid-deficient mutant melanoma cell line and its parent cells. GM95 is a mouse melanoma mutant, defective in ceramide glucosyltransferase I (CerGlcTI), which catalyzes the first step in glycosphingolipid synthesis (28). Lysenin brightly stained the cell surface of GM95 but not that of the parent. Consistent with cell labeling, GM95 was sensitive to lysenin-induced killing, whereas the parent cell was resistant to lysenin. The involvement of CerGlcTI in lysenin sensitivity was confirmed by the observation that the stable CerGlcTI transfectant of GM95 was resistant to lysenin.

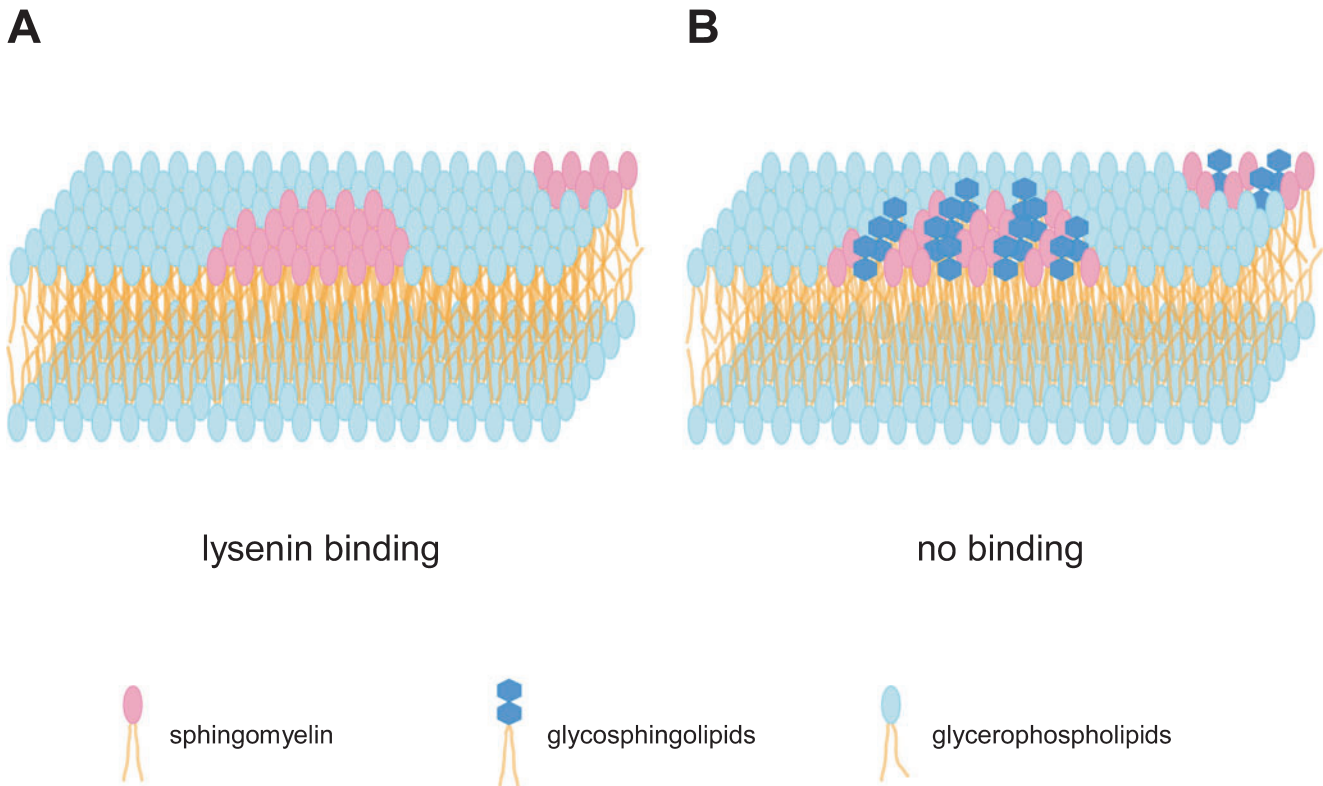


Fig. 1. **Lysenin recognizes the heterogeneous organization of sphingomyelin.** Lysenin binds membranes when sphingomyelin forms clusters (A). The presence of glycosphingolipids decreases the local density of sphingomyelin in the membranes and thus inhibits the binding of lysenin (B).

Detailed characterization showed that the observed difference between the mutant and parent melanoma cells is due to altered distribution of sphingomyelin rather than a change in the lipid content in the mutant. These results indicate that lysenin recognizes the heterogeneous organization of sphingomyelin in biomembranes and that the organization of sphingomyelin differs both between different cell types and also between different membrane domains within the same cell. Our results also suggest that sphingomyelin exists as tightly packed complexes consisting of a minimum of a few lipid molecules in biomembranes.

Imaging cholesterol

The content and distribution of cellular cholesterol are regulated dynamically by complex mechanisms. Inside mammalian cells, a graded level of free cholesterol is distributed in various membrane organelles with the highest accumulation in domains of post-Golgi membranes (29). In the plasma membrane, cholesterol is also a major component of lipid rafts (4, 5, 30, 31). Removal of cell surface cholesterol by methyl- β -cyclodextrin (M β CD) results in disintegration of these domains, affecting a range of diverse activities, such as signaling, adhesion, motility and membrane trafficking (32, 33).

Several probes have been used to image cholesterol-containing membranes. Filipin, a polyene antibiotic that forms a complex with free cholesterol, can be excited by UV. It has been successfully used to detect cholesterol in fixed cells. Filipin is not suitable for study of live cells,

since, in addition to its poor fluorescence properties, it exerts a cytotoxic side-effect by sequestering cholesterol.

Recently a novel protein probe derived from bacterial toxin, perfringolysin O (θ toxin) has been shown to selectively bind to cholesterol-rich membrane domains (34–37). θ toxin binds to membrane cholesterol and causes cell disruption. BC θ , a protease-nicked and biotinylated derivative of θ toxin, has the same binding specificity and the same affinity for membrane cholesterol as intact θ toxin, but causes no significant damage to membranes at 37°C or below. The C-terminal domain of the toxin is the smallest functional unit that has the same cholesterol-binding activity as the full-size toxin with structural stability (38). These θ toxin derivatives are used to detect cholesterol-rich domains in living cells.

Fluorescent cholesterol analogs offer a unique opportunity for the direct imaging of cholesterol trafficking. 25-Dehydroergosterol (DHE) is a naturally occurring fluorescent cholesterol analog. It has been used to follow the fate of unesterified sterol in living cells (39, 40). DHE is taken up by the nematode *C. elegans*, where this molecule was first found to accumulate in the pharynx, nerve ring and excretory gland cell, then in oocytes and spermatozoa (41). Although cholesterol can not be replaced by other sterols in mammalian cells, DHE applied in the form of a complex with M β CD appears to follow characteristic trafficking routes (39, 40). It has been shown that DHE is transported between the plasma membrane and intracellular compartments, such as recycling endosomes, in CHO cells. In addition to this vesicular route,

A

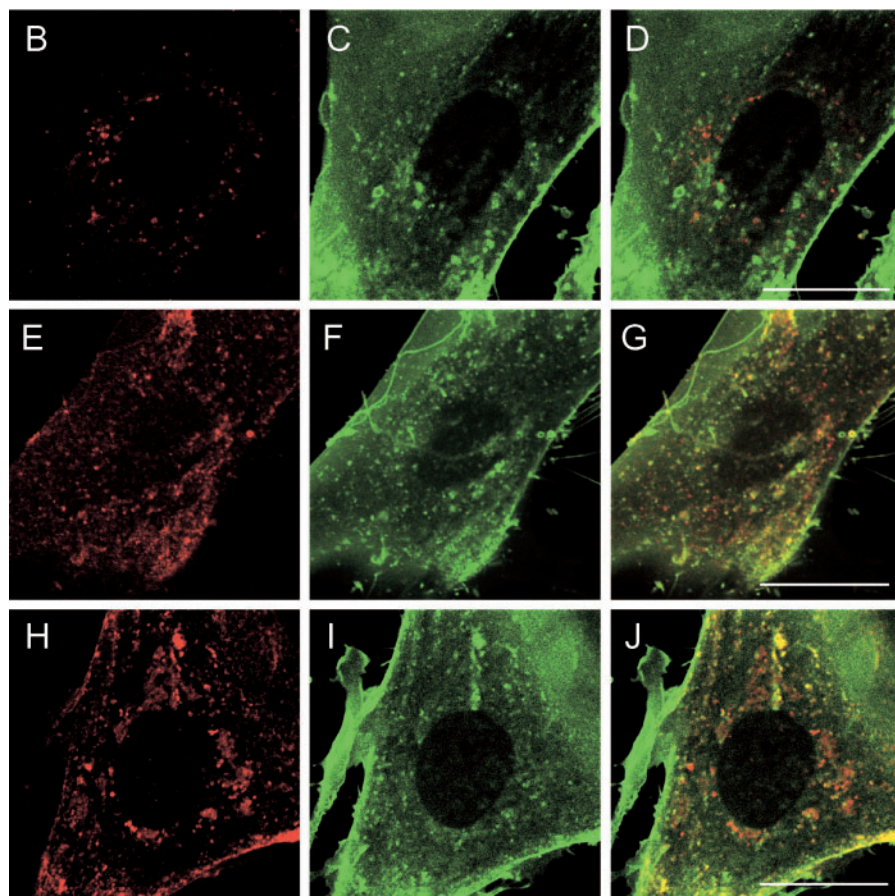
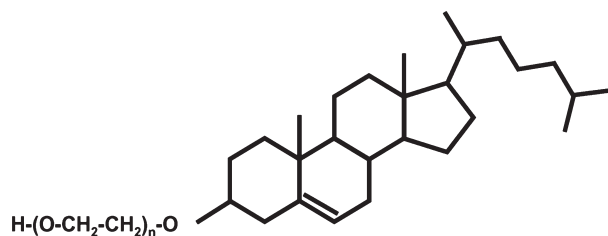


Fig. 2. PEG-Chol is internalized together with lipid raft components. A, structure of PEG-Chol. B–J, normal fibroblasts were incubated with $1 \mu\text{M}$ fPEG-Chol together with Texas Red transferrin (B–D), phycoerythrin (PE)-conjugated anti-CD59 monoclonal antibody (E–G) or AlexaFluor 594-labeled cholera toxin B subunit (H–J) for 5 min at room temperature. Cells were then washed and further incubated for 45 min (B–D, H–J) or 30 min (E–G) at 37°C . Cells were treated with ammonium chloride to enhance fluorescence before the images were taken. (B, E, and H) transferrin, anti-CD59 antibody, and cholera toxin B subunit fluorescence, respectively, (C, F, and I) fPEG-Chol fluorescence, (D, G, and J) overlay. Bar, $20 \mu\text{m}$.

DHE also exhibits non-vesicular trafficking, such as movement across the plasma membrane.

Recently, we synthesized a poly(ethylene glycol)-derivatized cholesterol and used this molecule to study cholesterol trafficking. Poly(ethylene glycol) cholesteryl ethers (PEG-Chols) are a unique group of non-ionic amphiphatic cholesterol derivatives (Fig. 2A) (42). These compounds are soluble in water but retain many of the structural aspects of cholesterol. Because of their low toxicity, various PEG-Chols were initially used *in vivo* to disperse otherwise water-insoluble antibiotics (43). We prepared a fluorescein ester of PEG-Chol that contains a fluorescein on the distal end of the PEG chain [fPEG-Chol, (42)]. A liposome-binding assay showed that the binding of fPEG-Chol to the membrane increased with increasing cholesterol content of the membranes. When fPEG-Chol pre-embedded in liposomes was incubated

with various liposomes, fPEG-Chol rapidly moved from cholesterol-poor liposomes to cholesterol-rich ones (44). These results indicate that fPEG-Chol was preferentially distributed to cholesterol-rich membranes. fPEG-Chol added to fixed and digitonin-permeabilized normal human skin fibroblasts stained the Golgi apparatus and intracellular small vesicles. The pattern of fPEG-Chol fluorescence was very similar to that of filipin. We then labeled Niemann-Pick type C (NPC) fibroblasts with fPEG-Chol. The hallmark of the NPC syndrome is the intracellular accumulation of unesterified cholesterol (45–47). fPEG-Chol brightly stained numerous perinuclear, cholesterol-rich compartments in NPC fibroblasts. Again the fluorescence was co-localized with filipin fluorescence. Because of its water-solubility and low toxicity characteristics, fPEG-Chol can be used to monitor the dynamics of cholesterol-rich membranes. When added to

live cells, fPEG-Chol is distributed exclusively on the outer leaflet of the plasma membrane and dynamically clusters upon activation of EGF receptor signaling (44).

Previously it was shown that PEG-Chol specifically inhibits clathrin-independent endocytosis (42, 48). However, endocytosis was not affected when cells were briefly incubated with a low concentration of fPEG-Chol (44). After labeling cells with fPEG-Chol for 5 min at room temperature, we chased the internalization of fluorescence at 37°C in the presence of rhodamine dextran. Most of the fPEG-Chol fluorescence stayed on the plasma membrane after 10 min of chase. After 60 min, fluorescent compartments surrounded the nucleus. fPEG-Chol further stained intracellular vesicles. Most of these vesicles were not co-localized with internalized rhodamine dextran. After 180 min, the Golgi apparatus was prominently labeled with fPEG-Chol, while the rhodamine fluorescence was distributed in endosomes/lysosomes. We then compared the internalization of fPEG-Chol with those of known raft markers (44). On incubation of cells at 37°C, fPEG-Chol co-localized with the lipid raft markers CD59 and GM₁ (Fig. 2, G and J), but not with internalized transferrin (Fig. 2D). Transferrin is internalized *via* clathrin-dependent endocytosis, whereas GPI-anchored proteins and cholera toxin are endocytosed by clathrin-independent mechanisms (49). These results suggest that the raft domains were internalized *via* a clathrin-independent pathway into acidic organelles. During prolonged incubation, fPEG-Chol was further transported to the Golgi apparatus.

Unlike filipin and DHE, fPEG-Chol is not permeable across membranes. This characteristic makes it possible to separately examine the cytoplasmic and exoplasmic cholesterol in biomembranes. We found that microinjected fPEG-Chol was highly accumulated in the Golgi in living normal and NPC fibroblasts (44). These results suggest that the Golgi apparatus exclusively exposes cholesterol-rich domains to the cytoplasm in both normal and NPC fibroblasts. This is in marked contrast to the bright late endosome/lysosome labeling of fixed and permeabilized NPC cells with fPEG-Chol. These results suggest that NPC cells accumulate cholesterol only on the luminal side of late endosomes/lysosomes. PEG-Chol thus provides a novel, sensitive probe for elucidating the dynamics of cholesterol-rich microdomains in living cells.

Conclusion and perspectives

Our knowledge of the organization and dynamics of the lipids in lipid rafts is still limited. To date, the cholera toxin B subunit, which specifically binds glycosphingolipid GM1, is almost the only tool available for the study of the lipids in lipid rafts. GM1 is not ubiquitous, and cholera toxin is reported to induce clustering of the lipids. Lysenin and cholesterol-specific probes certainly promise to be useful probes for future studies. These results with lysenin suggest the existence of different lipid domains with altered sphingomyelin density in biomembranes. Lipid heterogeneity of lipid rafts should be further examined using a variety of lipid probes. The need for small molecules able to recognize specific lipids or lipid environments is clear, but few such molecules are available. The development of new, improved probes is prerequisite for further understanding of lipid rafts.

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