Review Article

Fluorescent Probes for Monitoring Cholesterol Trafficking in Cells

(cholesterol, sterol / cholesterol analogues / fluorescent probes / trafficking / cellular transport)

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Abstract. Cellular cholesterol plays fundamental and diverse roles in many biological processes and affects the pathology of various diseases. Comprehensive and detailed understanding of the cellular functions and characteristics of cholesterol requires visualization of its subcellular distribution, which can be achieved by fluorescence microscopy. Many attempts have been made to develop fluorescent cholesterol reporters, but so far, none of them seems to be ideal for studying all aspects of cholesterol management. To meet the requirements for the right probe remains a great challenge, and progress in this field continues. The main objective of this review is to not only present the current state of the art, but also critically evaluate the applicability of individual probes and for what purpose they can be used to obtain relevant data. Hence, the data obtained with different probes might provide complementary information to build an integrated picture about the cellular cholesterol.

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Abbreviations: ABC transporters – ATP-binding cassette transporters, ACAT – acyl-coenzyme A: cholesterol acyl-transferase, ATP – adenosine triphosphate, BChol – BODIPY-cholesterol, CTL – cholestatrienol, DHE – dehydroergosterol, DChol – dansyl-cholestanol, EGFP – enhanced green fluorescent protein, EN/LY – endosomes/lysosomes, ER – endoplasmic reticulum, ERC – endocytic recycling compartment, E4Pac – acetate form of the ergosterol analogue, Fc/SPH – free cholesterol/sphingolipid, GPI – glycophosphatidyl-inositol, HDL – high-density lipoprotein, HMG-CoA reductase – 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, ld phase – liquid-disordered phase, LDs – lipid droplets, LDL – low-density lipoprotein, lo phase – liquid-ordered phase, MβCD – methyl-β-cyclodextrin, MCS – membrane contact site, Mi – mitochondria, NPC – Niemann-Pick's disease type C, PM – plasma membrane, Scap – sterol regulatory element-binding protein cleavage-activating protein, SSD – sterol-sensing domain, STPs – sterol transfer proteins, TGN – trans-Golgi network, SR-BI – scavenger receptor, class B type 1.

Introduction

Cholesterol is an essential lipid constituent of eukaryotic cell membranes. It plays fundamental roles in diverse biological processes, such as membrane permeability, lateral lipid organization, signal transduction, and membrane trafficking (Gimpl and Gehrig-Burger, 2011; Maxfield and Wustner, 2012; Solanko et al., 2015). Cholesterol also serves as a precursor for synthesis of steroid hormones (Hu et al., 2010), bile acids (Javitt, 1994) and vitamin D (Prabhu et al., 2016). Cholesterol strongly influences the physical state of the membranes by regulating fluidity, increasing thickness, and reducing permeability for small water-soluble compounds (Gimpl and Gehrig-Burger, 2007; Yeagle, 1985). Cholesterol is non-randomly distributed in cells and membranes and participates in the formation of discrete membrane structures such as caveolae (Parton and Simons, 2007) and microdomains, called "lipid rafts" (Simons and Ikonen, 1997; Simons and Sampaio, 2011). Caveolae and lipid rafts are two distinct populations of free cholesterol, sphingolipid (FC/SPH)-rich cell surface microdomains. They differ in stability, shape, and the presence or absence of caveolin (present in caveolae) or GPI-anchored proteins (enriched in lipid rafts)(Fielding and Fielding, 2003). The membrane rafts compartmentalize cellular processes by forming platforms through protein-protein and protein-lipid interactions (Pike, 2006). It is widely believed that cholesterol helps to manage their delicate balance by preventing strong and stable interactions between acyl chains that would lead to a gel phase while also permitting the preferential interactions that drive demixing (Veatch and Keller, 2005; Levental et al., 2011; Levental and Veatch, 2016). However, despite the many cellular roles attributed to lipid rafts

and intensive research, the true nature of membrane organization in living cells still remains mysterious (Levental and Veatch, 2016).

The uneven distribution of cholesterol among intracellular membranes is demonstrated by the fact that although the endoplasmic reticulum (ER) is the site of cholesterol synthesis, it contains only about 0.5–1 % of cellular cholesterol, while plasma membranes account for 60–80 % of total cellular cholesterol (Lange et al., 1999; Maxfield and Wustner, 2002, 2012). Despite these remarkable differences in subcellular localization, cholesterol dynamically moves between various organelles. Cholesterol originating in dietary sources is transported in the plasma as a component of lipoproteins, either in the limiting phospholipid monolayer or in an esterified form as a component of the lipoprotein core (Solanko et al., 2015). It is internalized into cells by low-density lipoprotein (LDL)-receptor mediated endocytosis via clathrin-coated vesicles. The vesicles are transported to sorting endosomes, where LDL dissociates from the LDL receptor, which is recycled back to the plasma membrane (PM) via the endocytic recycling compartment (ERC) (Hao et al., 2002; Goldstein and Brown, 2009). Interestingly, some data show that cholesterol can move from the plasma membrane to the ERC also by a non-vesicular, ATP-independent process (Maxfield and Wustner, 2012). The recycling pathway, along with other mechanisms, ensures that the plasma membrane maintains its lipid and protein composition.

The majority of cholesterol is synthesized *de novo* from acetyl-CoA in ER and travels rapidly against a concentration gradient to the PM (Ikonen, 2008; Solanko et al., 2015). Cells maintain a gradient in sterol concentration between PM and the membrane-bound organelles by both vesicular and non-vesicular mechanisms (Prinz, 2007; Lev, 2012). Recently, the importance of non-vesicular routes of transfer, which are mediated by soluble carriers called sterol transfer proteins (STPs), has been accented (Wustner and Solanko, 2015; Tong et al., 2018). These carriers bind cholesterol in their hydrophobic pockets and facilitate its transfer across the aqueous cytosol (Luo et al., 2018). Accumulating evidence indicates that membrane contact sites (MCSs), regions where two distinct organelles are in close apposition to one another (Helle et al., 2013), facilitate STP-mediated cholesterol trafficking (Luo et al., 2018). Further information on the relationship between the sterol structure, its membrane properties, transfer between cell membranes, and underlying reason of differential concentration between intracellular and plasma membranes can be found in recent reviews (Mesmin et al., 2013; Iaea and Maxfield, 2015; Wustner and Solanko, 2015; Infante and Radhakrishnan, 2017; Ikonen, 2018; Litvinov et al., 2018; Luo et al., 2018).

Cholesterol homeostasis in cells is a well-coordinated machinery maintained by several mechanisms, including cellular uptake, transport, transcriptional control, synthesis, storage and efflux (Ikonen, 2008). Biosynthesis, as well as uptake of cholesterol from the plasma via circulating lipoproteins, involves several feedback loops that ensure the exact amount of cholesterol the cells need for their physiological function.

At cholesterol excess, cholesterol turnover is balanced by cholesteryl ester formation by enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT) in the ER. Cholesteryl esters are stored in cytosolic lipid droplets (LDs) or exported out of the cell (Luo et al., 2018). Cholesterol efflux can be a passive process involving simple diffusion via the aqueous phase and facilitated diffusion mediated by scavenger receptor class B, type 1 (SR-BI)(Phillips, 2014). The active pathways are mediated by the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 (Adorni et al., 2007; Phillips, 2014). High-density lipoprotein (HDL) particles with the principal component apolipoprotein A-I are appropriate acceptors of cholesterol in the extracellular medium (Phillips, 2014).

Mitochondria convert cholesterol to steroids, bile acids and oxysterols (Russell, 2003), and in addition to the ER are principal targets for intracellular homeostatic signalling (Steck and Lange, 2010). Cells employ membrane-embedded proteins of ER – Scap and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) – to monitor the levels of membrane sterols (Goldstein et al., 2006). These proteins share a polytopic intramembrane sequence called sterol-sensing domain (SSD) (Goldstein et al., 2006). In addition, six other proteins containing evolutionarily conserved SSDs emerged and their role was defined (Brown et al., 2018).

It is assumed that the endocytic recycling compartment (ERC) reflects the cholesterol content in the plasma membrane due to its continuous communication and exchange with PM via endocytic recycling pathway and due to its juxtaposition to ER with cholesterol-sensing proteins (Hao et al., 2002). Recent reports introduce the concept of so called “active membrane cholesterol” as the major pathway mediating cell cholesterol homeostasis. Active cholesterol serves as an upstream regulator and represents the fraction of cholesterol that exceeds the threshold of complexing capacity of the polar bilayer lipids. It exhibits elevated chemical activity and moves rapidly to intracellular membranes via diverse transport proteins, stimulating resident effectors to restore the plasma membrane cholesterol to its resting level (Steck and Lange, 2010; Lange and Steck, 2016).

Recently, a new emerging theme in the regulation of cholesterol homeostasis is the ubiquitination and degradation of key homeostasis players in response to specific signals (Sharpe et al., 2014).

Importantly, the imbalance in cholesterol homeostasis leads to pathological processes of atherosclerosis, and deregulated cholesterol trafficking is involved in the pathogenesis of neurodegenerative diseases including Niemann-Pick’s disease type C (NPC), Alzheimer’s disease (AD), Parkinson’s diseases (PD), and possibly Huntington’s disease (HD) (Arenas et al., 2017; Liu et al., 2010; Neefjes and van der Kant, 2014).

Since cholesterol plays so many indispensable roles and affects the pathology of various diseases, it is crucial to possess the right tools to monitor its intracellular dynamics and identify homeostatic disorders. Fluorescence microscopy is a valuable tool to visualize the cellular fate of many target molecules. However, as cholesterol and its physiological esters are not fluorescent, in principle, two different classes of fluorescent cholesterol reporters can be used for imaging by this technique: cholesterol-binding molecules and cholesterol analogues (Gimpl and Gehrig-Burger, 2007). An ideal cholesterol probe should exhibit properties similar to those of cholesterol, but it should not markedly disturb the membrane structure and should possess proper photo-physical properties for applications in live cell fluorescence microscopy (Marks et al., 2008). Development of such an analogue has been problematic, because the size, hydrophobicity and site of attachment of the fluorophore to the sterol molecule can affect how the probe molecule is partitioned in the membrane domains (Marks et al., 2008).

1. Cholesterol-binding molecules

Cholesterol-binding molecules are mainly represented by antibiotics such as polyenes (in particular filipin and its major component filipin III), and cytolysins.

1.1. Polyene antibiotics

Filipin III is a naturally fluorescent polyene antibiotic with antifungal properties (Fig. 1), which forms a complex with free (unesterified) cholesterol (Kruth, 1984). For its action, it requires a sterol partner with a free 3′-OH group, but a convincing mechanism of cholesterol binding remains an open question. Filipin binding perturbs the bilayer structure of membranes; therefore, it cannot be used on living cells. Although often used to determine total cholesterol distribution by microscopy, the main disadvantages of filipin are unfavourable spectroscopic properties (the excitation of the fluorophore is within the UV range), high bleaching rate of the fluorophore, and questionable specificity (Arthur et al., 2011). Moreover, the fluorescence of filipin represents the steady-state level of cholesterol but does not provide information about the inter-organelle sterol transport dynamics. It has also been reported that some sterol-containing membranes are not labelled by filipin (Severs and Simons, 1983). In addition, filipin is not able to detect sterol esters and distinguish endogenously synthetized from exogenous cholesterol.

Lately, filipin has been employed to selectively inhibit the rafts/caveolae endocytosis pathway or to investigate the involvement of lipid rafts in certain signalling processes (Schnitzer et al., 1994; Self et al., 2005). Filipin is also reported as a cholesterol competitor in binding assays or functional interaction studies to prove a putative cholesterol interaction of candidate proteins. Cholesterol-interacting receptors revealed a dose-dependent decrease in ligand binding in the presence of increasing concentrations of filipin (Pang et al., 1999).

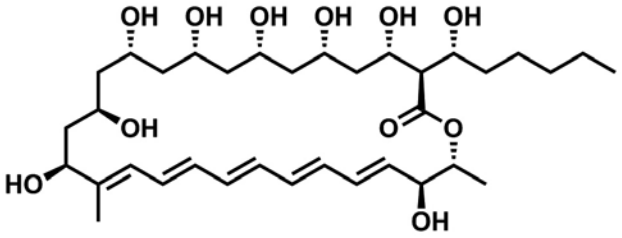

Fig. 1. Chemical structure of filipin III

The other polyene antibiotics such as nystatin and amphotericin B also form complexes with cholesterol that lead to ‘‘perforations’’ in the bilayer. These holes are small but large enough to allow permeabilization for ions such as sodium and potassium (Gimpl and Gehrig-Burger, 2007).

1.2. Cholesterol-dependent cytolysins

Cytolysins represent a large family of protein toxins produced by pathogenic gram-positive bacteria. Cytolysins are secreted as water-soluble monomers, which when bound to the target cell subsequently self-associate to form large aqueous pores in the bilayer. For pore formation, the presence of cholesterol in the target membrane is required, but its presence is not essential for binding of all cytolysins. Structural features of the cholesterol molecule required for interaction with the toxins include the 3β-OH group, the stereochemistry of the sterol ring system, and the isooctyl side chain (Gimpl and Gehrig-Burger, 2007). Perfringolysin O is one of the most studied cholesterol-binding cytolysins. Its C-terminal portion (designated D4 domain) is involved in cholesterol recognition and binding. A protease-nicked and biotinylated derivative of perfringolysin O (designated as BCθ-toxin) was developed to retain specific binding to cholesterol without cytolytic activity (Iwamoto et al., 1997). Conjugation of the D4 domain with a fluorophore or N-terminal-tagging with enhanced fluorescent protein (EGFP) seems to be a promising tool for analysing the dynamics of cholesterol-rich domains at the plasma membrane (caveolae/rafts) (Shimada et al., 2002) and intracellular cholesterol-rich domains (Sugii et al., 2003), but is not suitable for quantitative *in situ* determination of membrane cholesterol (Gimpl and Gehrig-Burger, 2011).

Visualization of cholesterol can also be achieved by using anti-cholesterol antibodies (Ohno-Iwashita, 2010) or by recombinant protein domains of toxins fused to photoactivatable fluorescent protein Dronpa (Mizuno et al., 2011).

2. Fluorescent cholesterol analogues

Despite certain limitations, so far developed fluorescent sterols represent a powerful approach to studying cholesterol behaviour in membranes and cells due to their sensitivity, time resolution, and multiplicity of measurable parameters (Gimpl and Gehrig-Burger, 2007).

Two major groups of fluorescent sterols have been recently recognized and used: (1) intrinsically fluorescent sterols (e.g., dehydroergosterol (DHE), cholestatrienol (CTL)) (Fig. 2B, C); and (2) extrinsic cholesterol probes with chemically linked fluorophores (NBD-cholesterol, dansyl-cholesterol, BODIPY-cholesterol, etc. (Figs. 3-5). These probes were extensively reviewed before (Gimpl and Gehrig-Burger, 2007, 2011; Maxfield and Wustner, 2012; Solanko et al., 2015); therefore, here they will be reported briefly with focus on those studied deeply. The specific advantages and limitations of these analogues will be discussed. Moreover, the lately synthesized analogues and new approaches to cholesterol detection will be shortly mentioned with awareness that additional studies are still needed to confirm their potential and faithfulness for broader application.

2.1. Intrinsically fluorescent cholesterol analogues

These analogues are natural compounds such as DHE and CTL, which are, in principle, the most suitable molecules to mimic cholesterol (Sezgin et al., 2016). These polyene sterols (P-sterols) contain additional conjugated double bonds in the steroid ring system (Fig. 2), giving them slight fluorescence in ultraviolet light. The main drawbacks of P-sterols are unfavourable spectroscopic properties including low quantum yield, excitation and emission in the UV region, and rapid bleaching rate. They can be inserted specifically into the plasma membrane or delivered to cells as part of lipoproteins for subsequent analysis of their transport itineraries and metabolism (Sezgin et al., 2016). Yet, cells must be loaded with a relatively high sterol concentration and it is quite possible that high concentrations force the sterol into pathways that are untypical of cholesterol (Gimpl and Gehrig-Burger, 2007).

Dehydroergosterol (DHE) is a sterol naturally occurring in yeast cells with intrinsic fluorescence due to the conjugated triene system. Its structure differs from cholesterol only in possessing three additional double bonds and a methyl group at C-24 (Fig. 2B). DHE is the closest analogue of ergosterol, from which it differs only by having one additional double bond in the ring system. DHE is surely one of the best-studied cholesterol probes, and in several respects it mimics cholesterol closely (Schroeder, 1984). Better corresponding distribution is obtained when monomeric DHE is incorporated into the plasma membrane from unilamellar vesicles by exchange/fusion or from DHE-methyl-β-cyclodextrin complexes (McIntosh et al., 2008). An important parameter for DHE wide use is also its easy preparation in stable form. However, DHE is a poor substrate for cholesterol oxidase; its esterification is slightly lower than that of cholesterol and fails to bind to several cholesterol-metabolizing proteins in the ER membrane (Mesmin et al., 2011; Pourmousa et al., 2014).

Cholestatrienol (CTL) is similar to DHE, from which it differs by the absence of double bond D22 and the methyl group at C-24 (Fig. 2C). It possesses an isooctyl side chain like cholesterol, and therefore resembles cholesterol better than DHE. It is regarded as a cholesterol analogue that mimics the membrane behaviour of cholesterol quite well (Robalo et al., 2013). Fluorescence properties and bleaching propensities of CTL are comparable with those of DHE.

New polyene sterols. Currently, a suitable strategy for improving existing intrinsically fluorescent sterol

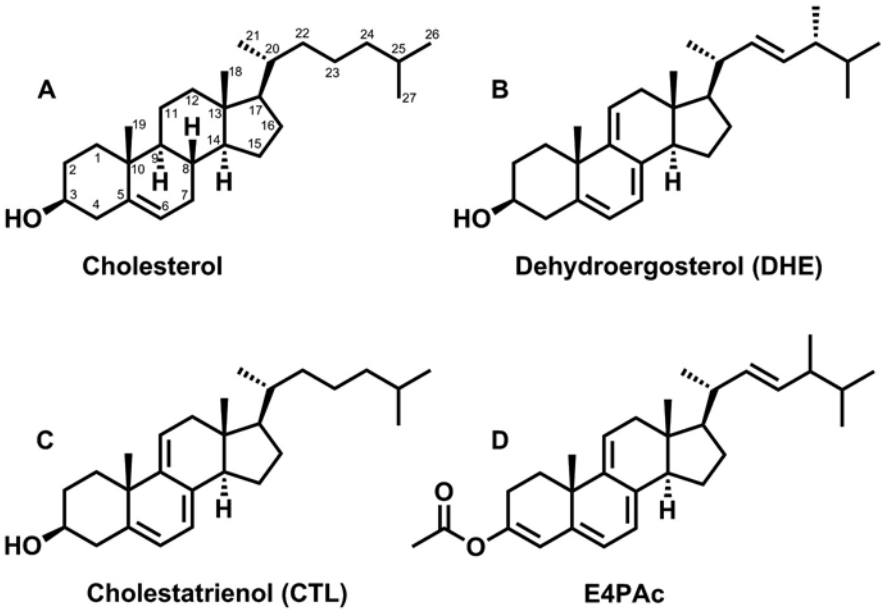

Fig. 2. Chemical structures of cholesterol (A) and polyene sterols: dehydroergosterol (B), cholestatrienol (C), and acetate form of ergosterol analogue, E4Pac (D)

probes relies on extending the conjugated system in the steroid ring system (Modzel et al., 2018). New polyene sterols containing four conjugated double bonds in the sterol ring system exhibit red-shifted excitation and emission by 20 nm and reduced photobleaching compared to DHE or CTL (Modzel et al., 2018). The red shift was even more pronounced when keto-enol tautomer equilibration was prevented by protecting the 3'-hydroxy group with acetate. The resulting acetate form of the ergosterol analogue, E4PAc (Fig. 2D), provides better live cell imaging quality than other polyene sterols (Modzel et al., 2018).

2.2. Extrinsic fluorescent analogues

Sterol analogues with chemically linked fluorophores exhibit much better fluorescence properties, so these probes can be applied at lower concentrations and allow pulse-chase experiments and/or imaging of sterol in living cells. Unfortunately, the attachment and orientation of the bulky reporter group may influence the characteristics of the analogue, such as its localization, interaction, and trafficking in cells. Such disturbances raise concerns about the reliability of extrinsic probes for cholesterol, and therefore it is important to get knowledge of such bias (Solanko et al., 2015; Sezgin et al., 2016).

NBD-tagged cholesterol analogues. Cholesterol tagged with a 7-nitrobenz-2-oxa-1,3-diazole (NBD) group at either carbon 22 or carbon 25 is designated as 22- and 25-NBD-cholesterol, respectively (Fig. 3A, B). Both analogues display similar environmental sensitivity of emission, suitable brightness, and a high bleaching propensity. They can be easily incorporated into cells with the help of β-cyclodextrin or serum albumin present in the medium and used for cholesterol trafficking studies (Solanko et al., 2015). Critical evaluation of both analogues revealed that they adopt a reverse (up-side-down) orientation and exhibit low ordering capacity and partitioning into the liquid disordered (Id) phase in ternary model membranes in contrast to cholesterol and intrinsically fluorescent sterols (Loura et al., 2001; Scheidt et al., 2003). Moreover, NBD-cholesterols have been shown to be mistargeted to cholesterol-poor mitochondria in CHO cells (Mukherjee et al., 1998), possibly as a consequence of metabolic trapping and conversion of the NBD group (Faletrov et al., 2013). Interestingly, in another cell type (L-fibroblasts), 22-NBD-cholesterol distributed similarly as dehydroergosterol (Frolov et al., 2000). The NBD group affects the cellular efflux of the tagged cholesterol, so that NBD-cholesterol is released from cells with rates higher than radioactively labelled cholesterol (Kheirolomoom and Ferrara, 2007). A relevant application of NBD-cholesterols seems to be in metabolic investigations such as studying the ACAT activity in cells (Solanko et al., 2015).

Dansyl-cholestanol (DChol) is a cholesterol analogue bearing the dansyl group attached at carbon 6 of cholesterol. Due to the specific linkage at carbon 6, the double bond of cholesterol is removed, and the resulting probe is dansyl-cholestanol (Wiegand et al., 2003) (Fig. 4). DChol has a broad excitation and emission spectrum with maxima around 336 and 522 nm, respectively. The fluorophore is small and exhibits relatively high quantum yield, but it is prone to photobleaching. The emission of DChol is environmentally sensitive and shifts toward lower wavelengths (around 509 nm) at neutral pH in polar solvents (Solanko et al., 2015). Like cholesterol, DChol has poor solubility in aqueous media; therefore, the complexation of DChol with methyl-β-cyclodextrin (MβCD) facilitates uptake and monitoring of the cholesterol behaviour in the membranes of living cells (Huang et al., 2010). It was shown that DChol preferentially localized in plasma membrane cholesterol-rich microdomains of living cells. The data obtained with DChol during microscopic imaging indicating its quality as a reporter for cholesterol were intensively dis-

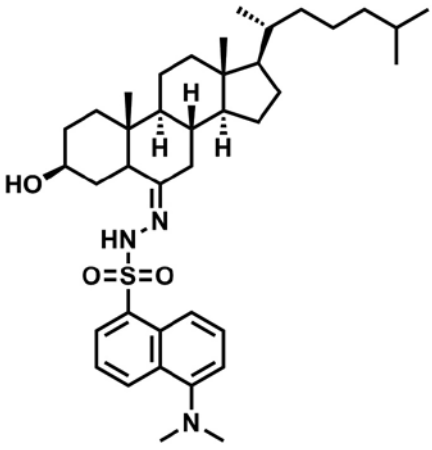

Fig. 4. Chemical structure of dansyl-cholestanol

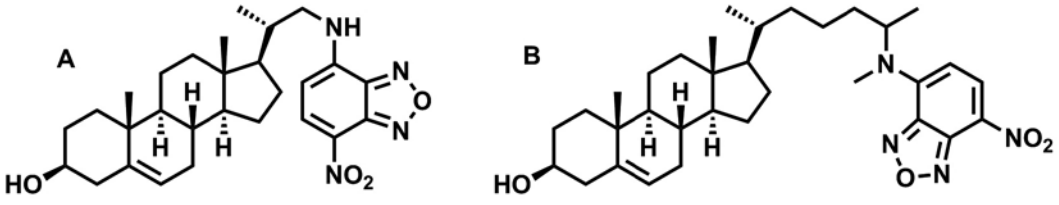

Fig. 3. Chemical structures of NBD-cholesterols

cussed previously (Gimpl and Gehrig-Burger, 2007). On the other hand, studies in model membranes demonstrated that DChol partitions with high preference into the cholesterol-poor liquid-disordered (ld) phase and co-localizes poorly with cholesterol-rich late endosomes/lysosomes in NPC1 disease cells (Sezgin et al., 2016). These results, together with the high bleaching propensity of this probe, make the use of DChol for live-cell imaging studies of cholesterol transport rather questionable.

BODIPY-cholesterol (BChol). Several analogues of cholesterol were synthesized in which the BODIPY fluorophore (4,4-difluor-4-bora-3a,4a-diaza-s-indacene) has been inserted into the aliphatic tail of the free sterol. The BODIPY moiety is relatively non-polar, allowing for insertion of the analogues into the hydrophobic interior of lipid membranes (Marks et al., 2008). It has low environmental sensitivity, high extinction coefficient and high quantum yield (Bergstrom et al., 2002). BODIPY-cholesterol (BChol) (Fig. 5), in which carbon 24 of cholesterol is linked directly to a BODIPY moiety (Li et al., 2006), exhibits similar physical behaviour to cholesterol, and hence became used by the research community (Holtta-Vuori et al., 2008, 2016; Solanko et al., 2013, 2015; Wustner et al., 2011, 2016). BChol is now available under commercial name TopFluor-Cholesterol (Avanti Polar Lipids). In model membranes, BChol partitioned into liquid-ordered (lo) domains, and the rate of desorption of BChol from monolayers was similar to that of cholesterol (Marks et al., 2008). However, several important differences compared to cholesterol were found, as BChol had no ordering effect on the fatty acyl chains in the same system, indicating that the attached BODIPY group interferes with cholesterol's ability to condense lipid membranes (Milles et al., 2013). The preferred targeting of BChol without esterification to lipid droplets (LDs) indicates that the BODIPY fluorophore affects the intracellular transport of BChol in cells with elevated fat content (Wustner et al., 2011). Some sterol transfer proteins do not recognize or transfer BChol (Wustner and Solanko, 2015). In addition, increased efflux of BChol from CHO cells to extracellular acceptors serum albumin or apolipoprotein A1, and decreased esterification were observed (Holtta-Vuori et al., 2008).

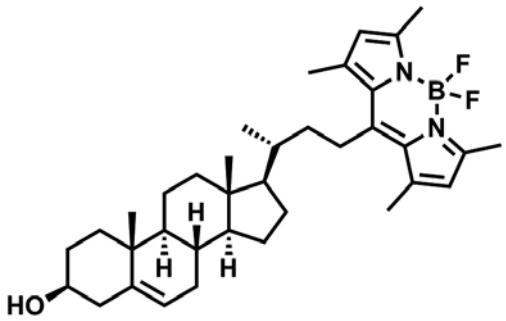

Fig. 5. Chemical structure of BODIPY-cholesterol (TopFluor-Cholesterol)

3. Update of cholesterol probe development

3.1. Novel BODIPY-cholesterol analogues

Recently, several novel BODIPY-cholesterol analogues were prepared using Suzuki or Liebeskind-Srogl coupling, implementing an aryl ring as a linker instead of an ester group (Liu et al., 2014). Various BODIPY scaffolds conjugated to sterol were evaluated for imaging and flow cytometry, and the results obtained with red-shifted BODIPY-cholesterol analogues indicated their potential for tracking cellular cholesterol pools (Liu et al., 2014).

3.2. Alkyne cholesterol analogues

These analogues aim to combine the best properties of intrinsically and extrinsically fluorescent derivatives. They carry the alkyne at the end of the cholesterol side chain (Fig. 6A) (Hofmann et al., 2014) or replace the axial methyl group at carbon 19 of the sterols (Fig. 6B) (Jao et al., 2015). They act as a substrate for the click chemistry reaction joining reporter molecules to their alkyne cholesterol targets. In microscopic studies, alkyne analogues can be imaged either via copper(I)-catalysed azide-alkyne cycloaddition (Jao et al., 2015), or via an azide coupled to biotin and subsequent incubation with a fluorescent streptavidin-conjugate or via an azide coupled to a fluorescent BODIPY-dye (Hofmann et al., 2014). All these procedures require cell fixation. Although the alkyne cholesterol analogues differ very little from cholesterol, the final visualization of sterol distribution is based on an extrinsic analogue in which a fluorophore becomes linked to the alkyne group. Accordingly, con-

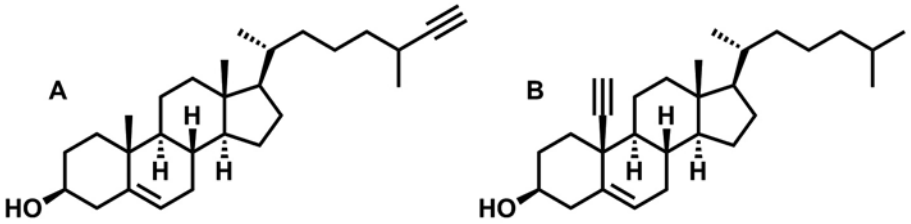

Fig. 6. Chemical structures of alkyne cholesterol analogues

cerns about the reliability of extrinsic fluorescence probes for cholesterol, as discussed in detail above, also applies to alkyne-tagged cholesterol analogues.

3.3. BODIPY-abiraterone acetate

Most recently described fluorescent abiraterone acetate derivative (designated FP-5, Fig. 7) looks, in comparison with commercially available probes, as a very promising probe for monitoring cholesterol trafficking and its disorders (Kralova et al., 2018). This heterocyclic sterol probe tagged with BODIPY exhibits superior and very fast labelling of cellular membranes followed by intracellular redistribution into organelles and vesicles. A transient appearance of the FP-5 signal in endoplasmic reticulum and mitochondria shortly after staining the plasmatic membrane seems to be in line with the concept of active (uncomplexed) cholesterol. According to this concept, an increased level of sterol at these organelles is sensed by a variety of sterol-sensitive proteins as a signal to reduce the cellular excess through homeostatic responses until the active fraction is removed (Steck and Lange, 2010; Lange and Steck, 2016). Accordingly, redistribution of the signal to lysosomes and its overall weakening due to sterol efflux is observed. In addition, this probe very effectively labels lysosomes in cells with strong accumulation of cholesterol due to pharmacological inhibition of cholesterol transport or due to mutation of NPC1 cholesterol transporter (Kralova et al., 2018). However, more comprehensive and detailed studies of membrane partitioning are still needed to support the general use of this probe.

Conclusions

Monitoring the cholesterol trafficking and disruptions of cholesterol homeostasis in various pathologies represents an important task of molecular biology. The currently used approaches to cholesterol imaging in cells by fluorescence microscopy are summarized in Table 1. In most cases, fluorescent analogues are used, but comparison of their performance in cellular assays revealed strong differences; some indicating proper performance in membrane-based experiments and others in intracellular trafficking assays. However, none displayed consistent results in all assays. While intrinsically fluorescent sterols usually mimic natural cholesterol much better than the extrinsically fluorescent ones, their main drawback is low molecular brightness, emission at UV light and requirement for specific detection equipment. A new hope for polyene sterols seems to lie in the strategy of extending the conjugated double bond system and conversing analogues into sterol derivatives with a blocked hydroxyl group. On the other hand, extrinsic fluorescent analogues display much better photophysical characteristics, but the presence of a bulky fluorophore seems to affect their distribution, interactions and trafficking. Recently developed alkyne cholesterol derivatives look very promising, but finally, the detected sterol probe is again an extrinsic cholesterol analogue. The rapid progress in synthesis of suitable cholesterol analogues such as BODIPY-abiraterone acetate in combination with fast improvement of live-cell imaging technology promise in-depth understanding of cholesterol trafficking and its alterations in human disease.

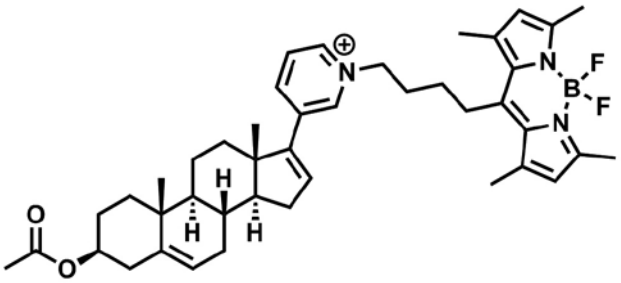

Fig. 7. Chemical structure of BODIPY-abiraterone acetate (FP-5)

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Table 1. Summary of commonly used fluorescent sterol probes

Cholesterol probes	Application/Localization	Advantages	Drawbacks
Filipin III	Screening assays; phenotyping of lysosomal storage disorders PM, EN/LY,	Quantitative measurements of cholesterol; partition into lo phase in model membranes	Requires fixation, cannot be used in living cells; does not bind esters; high photobleaching; UV optics; cross-reactivity to gangliosides
Fluorescently labelled perfringolysin O	Screening assays; assessment of transbilayer sterol distribution PM, lipid rafts	Assessment of threshold concentration of cholesterol in membranes	Not usable for inter-organelle transport, labelling of cholesterol poor organelles and for quantitative *in situ* determination of membrane cholesterol
DHE	Trafficking studies; membrane biophysics PM, ERC, TGN, LD	Closest analogue to ergosterol; gets bound and transferred by STPs; partition into lo phase in model membranes; ordered phospholipids fairly	Poor photophysical performance; poor substrate for ACAT1 cholesterol oxidase; fails to bind to cholesterol-metabolizing proteins in ER
CTL	Trafficking studies; membrane biophysics PM, ERC	Closest analogue to cholesterol; gets bound and transferred by STPs; partition into lo phase in model membranes; ordered phospholipids fairly	Poor photopysical performance; high bleaching
E4PAc	Live-cell imaging of sterol transport PM, sterol-rich endocytic vesicles	Reduced photobleaching, higher brightness; can be loaded from albumin complexes and imaged using a conventional DAPI filter set	Not fully characterized
NBD-cholesterols	Membrane biophysics; trafficking studies; metabolic investigations as ACAT activity in cells PM, LD, Golgi	Easily absorbed from media	Prone to bleaching; reverse orientation in the membrane; mistargeting to mitochondria; partition into ld phase in model membranes; not a substrate for STPs
Dansyl-cholestanol	Trafficking studies but questionable; ACAT activity PM, ER, LD	Small molecule; high brightness and good photostability	Partition into ld phase in model membranes; co-localizes poorly with cholesterol-rich LE/LYSs in NPC1 cells; prone to bleaching; not a substrate for STPs
BODIPY-cholesterol	Trafficking studies; cholesterol efflux EN, Golgi	High brightness and good photostability; partition into lo phase in model membranes	High affinity to lipid droplets; no ordering in model membranes; not a substrate for STPs
Alkyne cholesterols	Enzymatic assays; trafficking studies. PM, ER, Golgi, Mi	Mimic cholesterol well before click reaction; potential of various analytical enhancers to optimize fluorescence	Cannot be used in living cells; behaviour after click reaction is poorly defined
BODIPY-abiraterone acetate	Trafficking studies; lysosomal storage disorders ER, Mi, LY	Very fast cellular uptake without MβCD loading; bright fluorescence; mimics “active cholesterol“	Membrane biophysics and interactions with STPs not yet fully characterized

Adapted from Solanko et al. (2015) and Maekawa and Fairn (2014)

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