Cellular cholesterol and how to find it

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Abstract

Cholesterol is an essential component of eukaryotic cellular membranes. Information about its subcellular localization and transport pathways inside cells are key for the understanding and treatment of cholesterol-related diseases. In this review we give an overview over the most commonly used methods that contributed to our current understanding of subcellular cholesterol localization and transport routes. First, we discuss methods that provide insights into cholesterol metabolism based on readouts of downstream effects such as esterification. Subsequently, we focus on the use of cholesterol-binding molecules as probes that facilitate visualization and quantification of sterols inside of cells. Finally, we explore different analogues of cholesterol which, when taken up by living cells, are integrated and transported in a similar fashion as endogenous sterols. Taken together, we highlight the challenges and advantages of each method such that researchers studying aspects of cholesterol transport may choose the most pertinent approach for their problem.

Introduction

Cholesterol serves important roles as a precursor for the synthesis of steroid hormones, bile acids [1] and vitamin D, but is also an essential constituent of eukaryotic cellular membranes [2]. Membranes share a common structural organisation of phospholipid bilayers, with the hydrophobic tails buried in the membrane and the polar head groups exposed to aqueous environments. Cholesterol inserts into these bilayers, with its polar hydroxyl group close to that of the phospholipid, to maintain membrane integrity, modulating rigidity and permeability. The lipid composition, including cholesterol content, is a major determinant of membrane function, governing physical properties such as permeability and curvature as well as influencing the recruitment and activity of associated membrane proteins that provide specialised functions [3].

Given that cholesterol has such key fundamental biological functions, intracellular cholesterol accumulation is associated with organelle dysfunction and disease [4]. Cellular cholesterol levels are kept within a narrow range by sophisticated homeostatic pathways that predominantly occur in the endoplasmic reticulum (ER), the site of sterol synthesis. Accordingly, the cholesterol content of ER membrane is maintained at relatively low levels (3-6% of ER lipid molecules are cholesterol), with newly synthesised cholesterol rapidly redistributed to other organelles or esterified for storage in lipid droplets [2]. The plasma membrane contains the highest cholesterol concentration (30-40% mol%, [5]), where it limits permeability of the phospholipid bilayer separating the intracellular contents from extracellular milieu.

Both vesicular and non-vesicular transport between organelles facilitates the heterogeneous distribution of cholesterol among intracellular membranes. Membrane cholesterol content increases through the secretory pathway and budding of cholesterol-rich vesicles from the trans-Golgi network (TGN) contributes to ER to plasma membrane sterol transport. Another source of cellular cholesterol is endocytosed low density lipoprotein (LDL) which is hydrolysed by acid lipases in the endocytic pathway to release free cholesterol. Recycling endosomes, rich in LDL-derived cholesterol as well as endocytosed plasma membrane cholesterol, can also deliver cholesterol to the plasma membrane. Membrane contact sites, where the membranes of two organelles are closely apposed, provide sites for non-vesicular lipid exchange. Since cholesterol levels are low in the ER relative to other membranes, newly synthesised sterol is generally transported against the concentration gradient, therefore requiring energy to achieve this transport to already cholesterol-rich organelles. This can be achieved via a lipid counter exchange mechanism at membrane contact sites where sterol transport proteins exchange ER-derived cholesterol for another lipid to be processed in the ER [6]. For example at the Golgi:ER interface, a PI4P gradient is maintained by Sac1-mediated PI4P dephosphorylation in the ER, promoting Golgi to ER PI4P transport (ref: https://pubmed.ncbi.nlm.nih.gov/24209621/). This phospholipid transport is achieved by lipid transfer proteins and the energy released from PI4P hydrolysis drives counter transport of cholesterol against the concentration gradient from ER membrane (approximately 5mol% cholesterol) to the Golgi (approximately 10mol% cholesterol). Cholesterol's high affinity for saturated lipids likely facilitates the process (ref: https://pubmed.ncbi.nlm.nih.gov/476099/), since ER membrane rich in unsaturated lipids is a permissive environment for sterol loading of lipid transfer protein, while the Golgi lipid environment, rich in saturated lipids such as glycophosphlipids and sphingolipids, favours sterol unloading.

Non-vesicular pathways can also mediate the transport of LDL-derived cholesterol from the endocytic pathway to ER [7–10], occurring in this case along the concentration gradient. However, since the lipid transfer proteins implicated in this process can transport phospholipids as well as sterol, a phospholipid exchange mechanism may also operate to drive sterol unloading in the ER membrane.

While major advances have been made in our understanding of intracellular cholesterol transport and distribution, measurement of membrane cholesterol content remains challenging, in part due to the potential for cholesterol to rapidly and spontaneously desorb from membranes [11]. Probes that detect endogenous cellular cholesterol have proved extremely useful in studying cholesterol distribution in health and disease, but are limited by sensitivity to membranes with relatively high cholesterol content. Cholesterol analogues can provide an alternative, complementary approach to bypasses the sensitivity issues of cholesterol-binding probes, but are also not without limitations. The high concentrations typically required to visualise naturally occurring fluorescent sterols may result in atypical behaviour, while the chemical reporters of synthesized sterol analogues may also influence behaviour. Since cholesterol undergoes organelle-specific modifications, for example esterification in the ER, measurement of cholesterol modification can be informative. Here we discuss the tools currently available for the analysis of intracellular cholesterol transport and distribution.

Endogeneous cholesterol

Indirect sterol sensing

Transport to the ER.

The ER is responsible for biosynthesis, distribution and storage of cholesterol, therefore it is not surprising that cholesterol sensing also occurs at the ER.

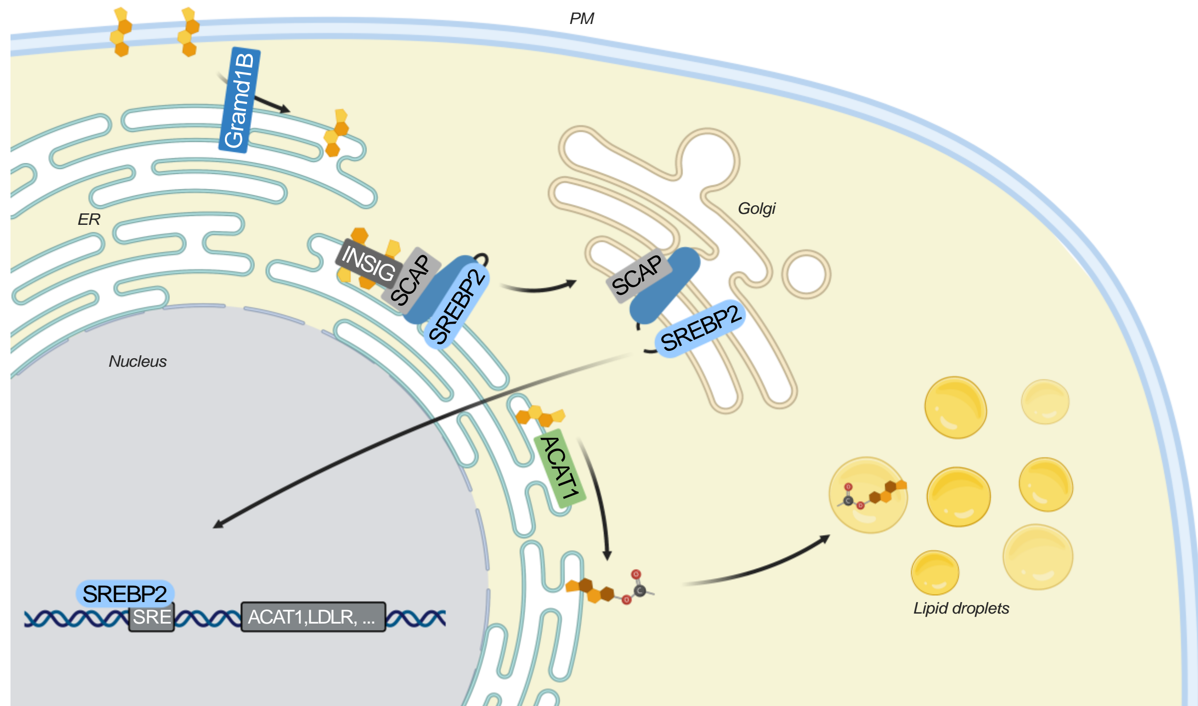

Figure 1: Cholesterol sensing and esterification. Schematic overview of the SREBP-dependent steps involved in the cellular response to increasing cholesterol levels in the ER membrane. Sterol sensing by INSIG results in the release of SREBP2 and the SREBP-cleavage activating protein (SCAP) from the ER membrane, followed by SREBP2-cleavage in the Golgi. In the nucleus, cleaved SREBP2 binds to the sterol recognition element (SRE), leading to a downregulation of key factors in sterol biosynthesis and sterol uptake, while increasing esterification of cholesterol by upregulating ACAT1 and subsequent storage in lipid droplets. Figure created with Biorender.com

The transcription factor sterol regulatory element-binding protein 2 (SREBP2) is the master regulator of a transcriptional program governing cholesterol biosynthesis [12]. A two pass transmembrane polypeptide, SREBP2 requires import to the Golgi where it can be activated through proteolysis, releasing the N-terminal transcription factor domain to translocate to the nucleus and transactivate its target genes through the binding of the sterol regulatory elements (SRE) on theirs promoters [13]. SREBP2 exit from the ER is regulated by its binding partner, SREBP-cleavage activating protein (SCAP) and interaction between SCAP and the ER resident protein insulin-induced gene (INSIG). As illustrated in Figure 1, when bound to INSIG, SCAP anchors SREBP2 to the ER [14], whereas dissociation from INSIG promotes export of the SCAP-SREBP complex to the Golgi, recruiting the coatomer II export machinery [15]. SCAP and INSIG are two sterol-sensing proteins and their interaction is dependent on the availability of cholesterol (SCAP) [16] and oxysterol (INSIG) on the ER membranes, making SREBP2 processing exquisitely sensitive to sterol fluctuation. Indeed, ER membrane

cholesterol levels of less than 5 mol% of total ER lipids is sufficient to trigger SREBP2 cleavage [17]. Since the full length and processed forms of SREBP2 have very different molecular weight (120-125 and 64-66 kDa respectively) Western blot analysis of SREBP2 provides a simple qualitative indirect indication of the cholesterol bioavailability of the cell: In steady-state condition the full length protein, should be preponderant, while after sterol depletion the cleaved nuclear form is more abundant.

A different approach that takes advantage of the SREBP2 regulatory pathway to monitor intracellular cholesterol level is the construction of reporter systems relaying SREBP2 transcription factor activity. Based on this principle, an endogenous reporter system was recently developed using CRISPR technology to knock-in the fluorescent protein Clover into the highly SREBP2 responsive 3-hydrocy,3-methyl-glutaryl CoA synthase (HMGCS1) locus to obtain a HMGCS1-Clover fusion protein [18]. The authors employed this cellular model in combination with genome-wide CRISPR screening to identify novel cholesterol regulators. Cells that were highly positive for the reporter after a cycle of sterol starvation and repletion were enriched in guide RNA for genes with a role in rebalancing ER cholesterol levels. A similar approach was developed exploiting the endogenous regulation of LDL receptor (LDLR) by SREBP2 in place of an exogenous reporter. As an SREBP2 transcriptional target, the LDLR is upregulated when ER sterols are low (eg under conditions of sterol starving) and downregulated by the arrival of cholesterol in the ER. Coupling a genome-wide CRISPR screening with the selection of the cells unable to turn on the negative feed-back of LDLR expression after sterol depletion and LDL refeeding enabled identification of genes required for cholesterol mobilization [19].

Excess cholesterol in the ER is esterified by the ER-resident enzyme ACAT, for storage in lipid droplets [20]. Thus cholesterol esterification can also be used as a measure of transport to the ER. Pulse-chase experiments measuring incorporation of radiolabelled cholesterol or oleic acid into cholesterol esters, can be used to monitor esterification, with free and esterified cholesterol separated by thin-layer chromatography (TLC) after cellular lipid extraction [21]. Gas and liquid chromatography coupled with mass spectrometry are widely used to misure cholesterol and cholesterol esters and although providing a good separation of cholesterol from other interfering species resulting more reliable and sensitive than enzymatic methods, they required sample derivatization to increase ionization efficiency of free cholesterol and isotopically labelled cholesterol as internal standards (https://doi.org/10.1093/oxfordjournals.jbchem.a127988) (10.14233/ajchem.2014.15780)(10.1016/S0378-4347(00)81515-7). The development of ambient ionization mass spectrometry (AIMS) techniques that operate at atmospheric pressure, like direct analysis real time spectrometry (DART), although not as sensitive as gas chromatography, opened the possibility for rapid and less laborious cholesterol quantification useful for fast quantitative screening (10.1021/acs.analchem.7b00943). Matrix-assisted laser desorption/ionization (MALDI-MSI) and Desorption electronspray ionization mass spectrometry imaging (DESI-MSI) offer the possibility to determine the spatial distribution of cholesterol sulfate or cholesterol directedly on tissue sections with a resolution of 30 and 200 µm. (10.1007/s00216-011-5562-6)(10.1021/ac901003u). A recent study used electrospray ionising tandem mass spectrometry (ESI-MS/MS) to quantify lipids in mammalian cell membranes, comparing the plasma membrane and total cellular lipidomes. As expected the plasma membrane was enriched in cholesterol (up to 40% mol

%). REF: Symons JC et al, 2021, https://pubs.rsc.org/en/content/articlehtml/2021/sm/d0sm00404a)

Fluorimetric methods can provide an additional alternative approach. The widely used Amplex red assay measures cellular cholesterol with high sensitivity [22]. Oxidation of free cholesterol by cholesterol oxidase yields hydrogen peroxide, which reacts with Amplex red to produce resorufin, a highly fluorescent product that can be readily quantified on a plate-reader. Measurement of free cholesterol following hydrolysis of cholesterol ester by cholesterol esterase gives readings for total cellular cholesterol. Subtracting free (without cholesterol esterase) from total cholesterol provides a readout of esterification.

Plasma membrane cholesterol.

Radiolabelling coupled with plasma membrane fractionation has helped inform our appreciation of the high cellular content of the plasma membrane. A quicker method relies on cholesterol oxidase treatment replacing a streroid hydroxyl group by a keto group, coverting cholesterol to cholestenone [23]. Sphingomyelinase treatment in fixed cells digests plasma membrane sphingomyelin, rendering free cholesterol at the plasma membrane accessible to cholesterol oxidase. Oxidation of the fluorescent cholesterol analogue DHE can be measured by spectrofluorimetry to reflect plasma membrane cholesterol content. Intracellular cholesterol was also found to be oxidised following hypotonic incubation, but not in gluteraldehyde-fixed cells treated with sphingomyelinase. Cholestenone is highly mobile in membranes and influences cholesterol distribution and in living cells, cholesterol oxidation affects membrane organisation, reducing membrane order.

Mitochondrial cholesterol

In steroid-producing cells, cholesterol is converted to the steroid precursor pregnenolone. Therefore, the conversion of cholesterol to pregnenolone can be used to measure mitochondrial cholesterol import. This can be mimicked in non-steroidogenic cells by expression of a mitochondria-targeted fusion construct of the P450 side chain cleavage enzyme CYP11A1, ferredoxin reductase and ferredoxin [24]. In cells expressing this construct, cholesterol trafficked to mitochondria is converted to pregnenolone at the inner mitochondrial membrane. Mitochondrial cholesterol import can be measured by analysis of pregnenolone formation by ELISA or radioimmunoassay of the medium.

Sterol binding probes

Filipin

Visualization of the intracellular localization of endogenous cholesterol was over the years achieved using a variety of sterol-binding biomolecules. The most well-known sensor Filipin is an intrinsically fluorescent polyene macrolide antibiotic [25] whose binding to cellular membranes depends on the presence of cholesterol [26,27]. Filipin binds to unesterified cholesterol [28,29] and is therefore well suited for the visualization of free cholesterol without staining a potential surplus of cholesterol in esterified form that is stored inside lipid droplets. Filipin has been used extensively in the study of Niemann-Pick disease type C (NPC), greatly advancing our understanding of the cellular basis for this disease [30–32]. In NPC cells cholesterol is trapped inside the lysosome which in turn also decreases the amount of free cholesterol in the plasma membrane (see Figure 2B). Using Filipin in an automated microscopy screening to detect the amount of free cholesterol in the PM,

multiple compounds could be identified that partially rescued the NPC disease phenotype [33–35]. To this day Filipin is used in the clinical diagnosis of NPC [32,36,37] and as a tool to unravel the mechanisms of sterol transporters acting at membrane contact sites. [38]. For instance, the lysosomal transmembrane protein STARD3 (MLN64) was found to promote cholesterol import into the lysosome from the ER [39] as well as cholesterol export from the lysosome to mitochondria through the use of Filipin [40,41]. This transport route might also be part of a rescue route for excessive lysosomal cholesterol accumulation, since the presence of STARD3 promotes an increase in contact site formation between these two organelles in NPC1 deficient cells [8]. In the same study, Filipin staining revealed that knock-down of the ER resident sterol transporter Gramd1B results in lysosomal cholesterol accumulation. Since Gramd1B and NPC1 co-localize at ER-lysosome contact sites, this might indicate a role of Gramd1B in non-vesicular cholesterol transport from the lysosome to the ER.
The mechanism of Filipin binding to cholesterol is not fully understood to this day. Additionally, it has become apparent that Filipin also binds other lipids, in particular the ganglioside GM1 [42]. This is important to consider when interpreting Filipin stained micrographs, especially from tissues with high abundance of GM1, such as the nervous system. Unfortunately, Filipin binding also perturbs the bilayer structure which leads to cell lysis, therefore limiting the application of Filipin to visualization of cholesterol in fixed cells. Imaging of intracellular Filipin can be additionally restricted by its low photostability, which makes sample handling quite delicate when intended for use in quantitative image analysis. While optimal imaging of Filipin requires the use of UV lasers or excitation lamps, due to its excitation maximum at 360 nm, using a 405 nm laser for excitation is also possible [8,43], albeit with compromises in signal intensities. Altogether, these drawbacks made the search for better suitable cholesterol probes the focus of many efforts.

Cholesterol dependent cytolysins (CDCs)

A different class of sterol probes are based on cholesterol-dependent cytolysins. Several pathogenic bacteria and fungi have evolved to secrete pore-forming toxins as a way to lyse host cells for access to their nutrients and defense against phagocytosis. A number of these toxins bind to cholesterol-rich regions in the plasma membrane [44]. This property has been exploited in the generation of cholesterol-sensors based on cytolysins summarized in Figure 2.

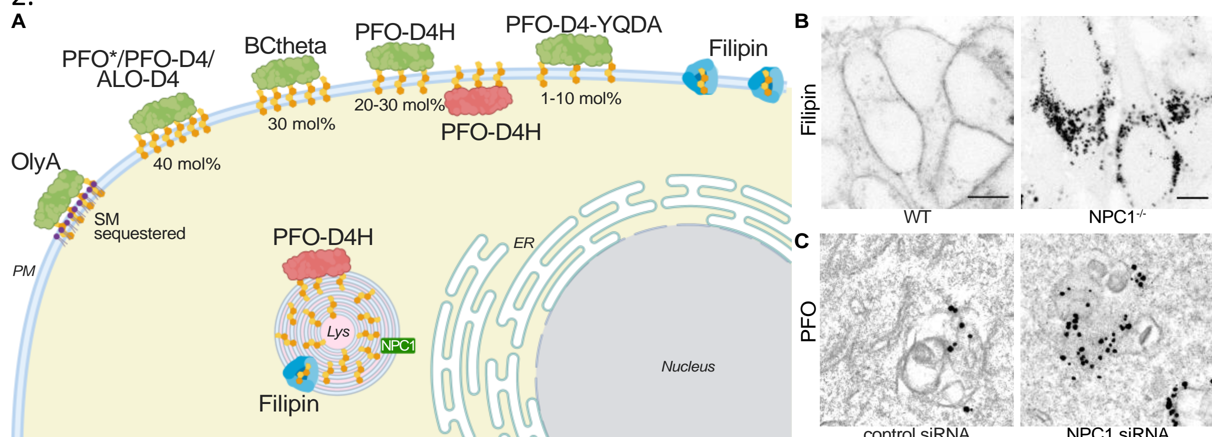

Figure 2: Cholesterol detection by sterol binding probes. A) Schematic overview of cholesterol binding properties of cholesterol-dependent cytolysins and Filipin, created with Biorender.com B) Exemplary Filipin

staining of WT and NPC1$^{-/-}$ HeLa cells. Filipin reveals a striking accumulation of unesterified cholesterol in the lysosomes of NPC1$^{-/-}$ cells. C) Application of PFO for the detection of cholesterol by immuno-electron microscopy in HeLa cells treated with control and NPC1 siRNA.

Perfringolysin O (Theta toxin) ~53 kDa

One of the most well-known members of cholesterol dependent cytolysins (CDCs) is perfringolysin O (PFO), historically known as theta-toxin. However, PFO only binds to cellular membranes which exhibit cholesterol concentrations of higher than 35-40 mol% [45,46]. This high threshold results in poor staining of even the PM, where cholesterol levels are highest. In isolated membranes of the endoplasmic reticulum (ER) however, this threshold concentrations seems to be much lower, around ~5 mol% [47]. This apparent discrepancy could be explained by the modulating effect of membrane phospholipids on PFO-cholesterol binding. In vitro reconstitution experiments showed that liposomes featuring more ordered acyl-chains exhibit much higher detection threshold of PFO towards cholesterol (cite). In cells, PFO has been used successfully to identify regions of intracellular cholesterol accumulation using GST-tagged PFO in immuno-fluorescence, immuno-electron microscopy and cellular ELISA analysis [8,48,49].

However, due to its cytolytic activity, application of native PFO is limited to *in vitro* studies or labelling of fixed cells. To circumvent this problem, three major strategies were developed which aimed to inhibit assembly and pore formation [50–52].

Ohno-Iwashita et al. were able to significantly reduce pore formation by partial proteolysis and subsequent methylation of theta toxin (PFO) [50,53]. The resulting derivative, called MCtheta, causes no membrane damage even at 37°C and reduced the binding threshold to ~30 mol%. MCtheta could also be further functionalized via biotinylation, yielding BCtheta [54]. Labeling of cell surfaces or cryosections of cells with BCtheta and subsequent anti-Biotin staining for electron microscopy revealed that more than 80 % of cholesterol detected in the endocytic pathway is present at multivesicular bodies [55,56]. Labeling of fixed cells with BCtheta showed that cholesterol binding of PFO derivatives follows a different behavior than Filipin, as it is completely abolished when PM cholesterol is reduced by cyclodextrin extraction [57]. Filipin staining however, is partially retained even at lower cholesterol concentrations, since binding does not behave in a threshold dependent manner. Together, this indicated that PFO might bind to a specific subpopulation of cholesterol [46].

Instead of using partial proteolysis, Hotze et al. created a mutated version of full length PFO which is less capable of pore assembly at lower temperatures [51]. The resulting mutant PFO* (Y181A, C459A) is easier to prepare than BCtheta and able to label live cells at low temperature without causing cell lysis. Using radioactive 125I-PFO* labelling of live cells Das et al. were able to identify three different pools of cholesterol being present in the cell. Quantification of bound 125I-PFO* revealed that besides the easily accessible population of cholesterol in the PM, a second population only becomes accessible for binding after treatment with sphingomyelinase[58]. The third pool is comprised of essential cholesterol, which stays inaccessible to PFO even after SMase treatment. Another study utilizing heavy isotope labeled PFO* was performed by He et al., who used nanoscale secondary ion mass spectrometry (NanoSIMS) imaging [59] to visualize 15N-PFO* bound to the cell. The resulting images achieved a lateral resolution of 70 nm and showed preferential binding to microvilli that was eliminated after treatment with cyclodextrin.

In a third approach to reduce toxicity, the minimal domain of PFO necessary for cholesterol binding was identified [52]. Much like the native full-length protein, the binding of this much

smaller (13 kDa) C-terminal domain 4 (PFO-D4) to cholesterol rich membranes requires a similar threshold concentration of ~40 mol% [60]. Recombinant, fluorescently-tagged PFO-D4 has proven useful in multiple assays ranging from super-resolution microscopy visualizing cholesterol-rich microdomains in the plasma membrane [61] to microscopy-based screenings identifying chemical compounds which decreased plasma membrane cholesterol levels [62]. Using flow cytometry, the binding of EGFP-PFO-D4 to cells can be used as a readout to quantify PM-cholesterol levels. Using this approach, a decrease in PM-cholesterol as a consequence of an increased cholesterol import to the lysosomes could be detected in STARD3-overexpressing cells [39,63].

Since PFO-D4 is not able to cross the plasma membrane, several studies also made use of in cell expression of fluorescence tagged PFO-D4 and achieved cholesterol staining of the cytosolic leaflet of the plasma membrane [64] and of the outside of endosomal vesicles [65]. The latter study visualized for the first time the endo-lysosomal accumulation of endogenous cholesterol inside of live NPC-diseased neurons. Going further, the authors were able to demonstrate that the defective long-term potentiation in NPC-deficient neurons is caused by a lack of cholesterol re-distribution between plasma membranes and endosomes.

Efforts to reduce the binding threshold of PFO-D4 resulted in a mutant termed D4H which binds at ~20-30 mol% cholesterol [66,67]. This new threshold enabled a more extensive use of D4H in cytosolic expression for staining of internal membranes, which rarely exhibit cholesterol levels of 40 mol%. In an initial study, Maekawa and Fairn were able to discriminate between cholesterol rich regions of the cytosolic and the exofacial leaflets of the plasma membrane by combining intracellular D4H expression with labelling of the cell surface by recombinant D4 [67].

In the yeast S. pombe, fluorescent D4H was used for tracking of sterol flow between PM and endosomes inside living cells. In a remarkable study, the authors discovered that inhibition of actin assembly triggered sterol redistribution from PM to endosomes and further showed that the lipid transfer protein Ltc1 is involved in this process [68]. In mammalian cells, PFO-D4H was used to study a family of sterol transporters called GRAMD1 [69]. Here, PFO-D4H showed an increase of the accessible cholesterol pool in the PM of cells lacking all three members of the GRAMD1-family [69]. By introducing additional ER-PM contact sites through the artificial recruitment of GRAMD1B, this effect could be reversed and cholesterol levels at the PM were reduced. In combination with additional results, this data helped to unravel how free cholesterol in the PM is sensed by GRAMD1 proteins and imported into the ER membrane.

Through the addition of further point mutations, the binding threshold of D4H to cholesterol was lowered to 10 mol% [70] with binding starting to occur *in vitro* at cholesterol concentrations as low as 1 mol%. The performance and specificity of the D4H was further improved by addition of solvatochromic fluorophores that change their spectral properties based on their surroundings. Combining incubation with extracellular probes and microinjection of intracellular probes of this design revealed a striking asymmetry in plasma membrane, where cholesterol in the PM was estimated to be ~12-fold higher in the exofacial leaflet. The validity of these results, however were later challenged, as the intracellular probe (DAN-D4) was shown to have a lower preference for membranes mimicking the cytoplasmic leaflet of the plasma membrane [71]. This might just be due to the nature of the fluorescent dye, since exogenously expressed mCherry-D4 does in fact bind to intracellular membranes [64,65,67,71,72]. However, while genetically encoded

sensors for phospholipids have been used for a long time [73], as of now there is not enough conclusive evidence that in-cell expression of D4 does not have perturbing effects on cholesterol homeostasis [67,69 (revision notes)]. This goes to show that the search for a probe that can reliably detect cholesterol inside of living cells is far from over.

Anthrolysin O

Another cholesterol-dependent cytolysin, originally extracted from gram-positive bacteria, is called Anthrolysin O (ALO). In order to reduce cytotoxicity, again the c-terminal domain 4 (ALO-D4) was characterized as the minimal necessary domain for cholesterol binding, with a threshold of ~40 mol% [60,74]. Like PFO-D4, recombinant, fluorescently tagged ALO-D4 has been used for the measurement of cell surface cholesterol levels. Applied to human red blood cells, ALO-D4 exposed a striking variation of plasma membrane cholesterol levels among individuals [75], while ALO-D4 measurements on primary mouse hepatocytes revealed a role for the ER-resident StarD5-protein in maintenance of PM cholesterol levels [76]. A different approach to visualize cholesterol distribution on the cell surface uses heavy isotope labeled 15N-ALO-D4 and nanoSIMS imaging [59,77]. Using this method, an expansion of PM cholesterol pools under conditions of Gramd1 sterol transporter downregulation was observed [77]. Recent studies investigating the downstream effects of ALO-D4 labelling in living cells revealed that ALO-D4-bound cholesterol is trapped at the plasma membrane [78]. This offered a different use of ALO-D4 as tool to deplete ER cholesterol levels without altering the total cholesterol levels [79]. In contrast to previously used strategies that target cholesterol export from lysosomes (i.e. pharmacological inhibition, NPC1 knock-down), this new approach allowed a more in-depth analysis of intracellular transport processes. This revealed that a small but steady stream of PM to ER cholesterol transport is necessary for cholesterol homeostasis. Trapping this small fraction of cholesterol resulted in SREBP activation, leading to increased LDL uptake and cholesterol synthesis [78].

Ostreolysin A

A third cytolytic toxin in use for the study of PM cholesterol is Ostreolysin A (Oly A) that can be extracted from oyster mushrooms. It was shown to bind and lyse liposomes which contain both cholesterol and sphingomyelin [80]. However as it is only able to form pore complexes with its co-factor Ply B, OlyA can be used for studies on living cells when recombinantly expressed and purified by itself [81]. In initial studies, fluorescently-tagged OlyA revealed a cell surface staining that was distinct from both cholesterol sphingomyelin markers, suggesting OlyA detects regions of high cholesterol/sphingomyelin that are not accessible by other probes [82]. Indeed, further studies showed that Oly A binds and stabilizes microdomains of SM-sequestered pool of cholesterol which only becomes accessible to PFO-D4 or ALO-D4 after sphingomyelinase treatment [83,84]. So analogous to ALO-D4, OlyA can be used as a tool to trap PM cholesterol of the second, sequestered pool, which results in the depletion of the accessible pool of cholesterol and downstream activation of the SREBP2 pathway [83].

Theonellamides

Theonellamides (TNMs) are a group of potent sterol-binding antifungal bicyclic peptides, derived from marine sponges of the genus *Theonella* [85,86]. Similar to Filipin, TNMs bind exclusively to unesterified 3β-hydroxysterols like cholesterol and ergosterol [85]. Binding to

membranes was shown to occur at a cholesterol concentration of as little as 10 mol% [87]. Fluorescent TNM derivatives recognize cellular cholesterol and offer an alternative probe for visualizing subcellular cholesterol distribution in fixed cells, as well as visualizing the exofacial cholesterol content of the plasma membrane in living cells [88]. Theonellamide labelled with BODIPY was also effective for visualization of cholesterol by cryo-immunoEM, using anti-BODIPY antibody [89]. For staining applications TNMs look promising in replacing some of the much larger sterol-binding proteins mentioned above, but due to the required isolation from natural extracts, they are currently less broadly accessible.

Cholesterol analogues

Fluorescent cholesterol analogues

Instead of relying on probes that bind to endogenous cholesterol and might thereby reduce its mobility, a different technique to study intracellular cholesterol transport makes use of directly detectable cholesterol analogues. These detectable cholesterol analogues must of course mimic the protein binding and lipid organization in biological membranes as closely as possible. In the following paragraphs we want to present some successfully applied cholesterol derivates, which helped to unravel many intricate processes and protein lipid interactions *in vitro* and *in vivo*.

DHE

Dehydroergosterol (DHE) is a naturally occurring and inherently fluorescent analogue of cholesterol known for closely mimicking endogenous cholesterol in multiple respects: DHE exhibits comparable flip-flop rates as cholesterol in transbilayer distribution experiments [90]. After loading of the PM with DHE, it is internalized into the endocytic recycling complex from where it can be exported in an energy dependent manner and redistributed inside the cell [91]. The same import into recycling endosomes can be observed when DHE is delivered as an oleate ester inside of LDL particles [92]. When additional cholesterol is given to the cell, DHE exported from the endocytic network can be esterified at the ER membrane and stored inside lipid droplets (LD). Quantification of DHE storage in lipid droplets via fluorescence microscopy [93–95] or quantification of total DHE-esterification levels via HPLC analysis [93–97] can be used as a readout for efficient cholesterol export from the endocytic network. Furthermore, owing to DHE's inherent fluorescence, it can be used as a FRET donor-acceptor pair together with tryptophan [98]. This allows the detection of sterol-protein interactions and the identification of potential cholesterol transporters [96]. Multiple studies also used DHE in a FRET-based *in vitro* assay to quantify the cholesterol transport efficiency of various proteins of interest [99–102]. This led to numerous new insights about how cholesterol transport at membrane contact sites functions and what the molecular basis for cholesterol binding is. However, due to the lackluster spectral properties of DHE, its application has been mostly focused on *in vitro* studies. The very low brightness, fast photobleaching and emission maximum in the UV range make detection quite challenging. Furthermore, studies using DHE in yeast cells require incubation under hypoxic conditions in order to avoid aerobic sterol exclusion [93–97,103]. Nevertheless, due to the highly physiological behavior of DHE, its application allows a high level of confidence in the biological validity of the obtained results.

BODIPY-Chol (TopFlour-Chol)

In an effort to improve the spectral properties of fluorescent sterol analogues, cholesterol was labelled with Bodipy at carbon 24, which resulted in a 1000-fold brighter probe compared to DHE [104]. BODIPY-cholesterol (BChol, also known as TopFluor-Cholesterol or Bodipy-Cholesterol-2) incorporates into model membranes in a similar way as cholesterol [105,106] and also behaves similarly in living cells [107]. It is readily taken up and transported into the endocytic vesicles [108] when given to the plasma membrane. Due to its high brightness and photostability, BChol is well suited for diffusion and interaction dynamics studies using fluorescence correlation microscopy (FCS) [106]. It has also succefully been applied for two-photon polarization micrscopy [106]. However for application in super-resolution imaging via stimulated emission depletion microscopy (STED), BChol proved to be less suitable than other fluorophores, requiring gated detection and relatively high laser powers [43,106]. High-resolution imaging of intracellular BChol distribution can however be achieved using electron microscopy, since BODIPY is compatible with the DAB photooxidation method [109]. Delivery of BChol into cells can be achieved either via cyclodextrin-complexation and integration into the PM or by using BODIPY-labelled LDL particles [110]. Unfortunately, intracellular trafficking of BChol was shown to diverge from natural cholesterol in several respects. A direct comparison of the esterification levels of BChol and 3H-Cholesterol showed that under conditions of high lipid storage esterification of BChol is about 4-fold lower than the esterification of 3H-Cholesterol [107]. Furthermore, experiments in an NPC disease model known for lysosomal cholesterol accumulation showed only limited colocalization of BChol with lysosomal markers under standard assay conditions [43]. However when using longer incubation times or delivery of esterified BChol in LDL-particles [111], BChol was also found enriched in NPC lysosomes. This observed delay could be attributed to an artificial enrichment of BChol to lipid droplets induced by the Bodipy-moiety itself [104,112,113]. It has also become apparent that not all known sterol transporters bind to BChol. The cytosolic sterol transport protein STARD4 for instance is not able to transport BChol [114] while having no troubles with transporting DHE [115]. Overall, while BChol is an excellent marker for following cholesterol diffusion at the plasma membrane or in *in vitro* systems, using BChol for intracellular sterol transport assays must be treated with caution.

Isotope labelled analogues and precursors

A complementary approach to fluorescence-based localization of cholesterol derivates are radiolabeled sterols which can passively be detected and quantified while exhibiting perfectly physiological behavior.

Radioactive cholesterols has been used to study intracellular localization for many decades [116] and is used to this day in clinical diagnosis to measure the cholesterol efflux capacity in cardiovascular disease prevention [117]. In pulse chase experiments, the amount of internalized, transported or metabolized 3H cholesterol can be measured by scintillation counting. Using subcellular fractionation, the internalization into the endocytic recycling complex (ERC) and efflux back to the plasma membrane can be quantified [91]. Since radioactively labelled cholesterol exhibits the exact same properties as cholesterol it is also perfectly suited for binding and transport assays [40]. Making use of this, cholesterol binding sites and saturation kinetics of NPC1 could be determined [118,119]. Furthermore, the authors of this study quantified the transfer rate of 3H-cholesterol by NPC1 *in vitro*.

They found both cholesterol extraction from a liposomal membrane and delivery to it to be greatly increased by the addition of NPC2, demonstrating its importance for successful NPC1 function [120].

Another method based on the quantification of radiolabeled sterols uses isotope labeled precursor molecules of cholesterol biosynthesis. Studies using 3H- or 14C-acetate [121,122] were able to reveal the kinetics of cholesterol synthesis and transport to the plasma membrane. In yeast cells, 3H-methionine can be used as a precursor for ergosterols. This allows for an easier chase than with acetate as it is incorporated at a later step in ergosterol biosynthesis [38]. Extracting newly synthesized 3H-ergosterol from the plasma membrane of yeast cells after pulsing them with 3H-methionine [93,94,96,103] revealed a fast transport from the ER to the PM, that is independent of the secretory pathway [38], indicative of the actions of dedicated cholesterol transfer proteins acting at this contact site.

While these radioactive analogues provide the highest level of endogenous behavior, their application brings some hazards with respect to radioactivity and their application is limited to endpoint measurements, due to a lack of real-time detection methods. Also, imaging of radioactive compounds inside of cells, while possible [123], is severely limited and cannot be combined with other fluorescence based microscopy techniques.

Non-radioactive isotope labelled analogues on the other hand have been proven useful during recent advances in mass spectrometry imaging [124,125]. Using nanoscale secondary ion mass spectrometry imaging (NanoSIMS) and simultaneous detection of ^{15}N and ^{18}O labelled lipids, cholesterol distribution in the plasma membrane was shown to be independent of sphingolipid domains [126]. Due to current efforts by M. Kraft and her colleagues, this method might even be combined with fluorescence microscopy in the not so distant future [127].

Raman active analogues

Another spectroscopic method that allows direct imaging of small biomolecules *in vivo* is Raman spectroscopy. Like infrared spectroscopy, Raman imaging is based on introducing molecular vibrations and measuring the difference in energy, taken up by the vibration system. Since Raman microscopy is insensitive towards water and many biological molecules already contain Raman-active molecular bonds, detection can even be performed on unperturbed living cells. When imaging these cells excitation can be fine-tuned to a specific type of bond like the aliphatic C-H bonds of lipids for instance [128]. However, low sensitivity and slow imaging speeds make it difficult to directly visualize endogenous lipid molecules. Nevertheless, Raman imaging was successfully used in visualizing endogenous cholesterol crystals in atherosclerotic lesions [129,130]. Problems with low signal intensities can be overcome by incorporating cholesterol analogues with stronger Raman signal intensities (such as alkynes, diynes or deuterium) or by using advanced Raman imaging techniques [131]. Labeling cells with hexa-deuterated cholesterol (D6-Chol) allowed the tracking of cholesterol uptake, metabolism and storage in lipid droplets [132]. Similar visualization of lipid droplets in fixed cells was achieved using alkyne-tagged sterols [133,134]. Due to a shortening of the frame time, which was permitted by the development of a tagged cholesterol with a much stronger Raman-signal (phenyl-diyne cholesterol, PhDY-Chol) imaging in live cells was made possible [135]. After addition to the cell PhDY-Chol is incorporated in the plasma membrane and can later be found inside lipid droplets. The signal inside lipid droplets was shown to be dependent on esterification by ACAT1, in

contrast to LD staining with BODIPY-cholesterol. Making use of an engineered yeast strain that is able to produce highly-deuterated cholesterol (D38-Cholesterol) [136], Alfonso-García and her colleagues were able to obtain highly-resolved images which not only offered information on localization, but also on esterification status of the detected cholesterol molecule itself [137]. This allowed them to uncover a heterogeneity among lipid droplets, that is not only based on preferential sequestration of either triglyceride or cholesterol esters, but also distinguishes between enrichment of esterified or free cholesterol.

Functionalized analogues

Photoactivatable and clickable Cholesterol

A novel strategy that combines the advantages of fluorescent cholesterol analogues (easy detection and visualization) and radioactive analogues (more physiological behavior), uses minimally modified lipid derivatives that can easily be further functionalized using click chemistry [138–140].

The introduction of an alkyne moiety into a lipid allows the easy attachment of a secondary molecule from a wide selection of detection, visualization or affinity markers. Cholesterol derivatives that are modified this way are readily taken up and metabolized by living cells and can be imaged with high resolution after being fixed and subjected to click-labelling with a fluorescent marker [141,142]. Indeed, in our lab we found clickable cholesterol to be a useful tool to follow cholesterol metabolism, in particular esterification, by thin-layer chromatography (see Figure 3).

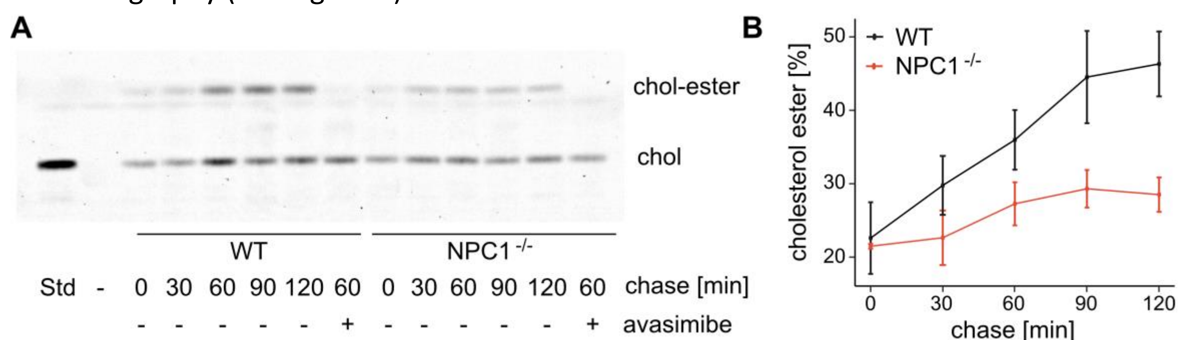

Figure 3: A) Thin-layer chromatographic analysis of HeLa cells pulsed with clickable cholesterol for 20 min and chased in serum-free medium for the indicated times. The extracted lipids were clicked to a fluorogenic fluorophore and separated on TLC silica gel plates. In two condititions, the Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor avasimibe was added to the cells in order to confirm the identity of the upper band as cholesterol ester. B) Quantification of TLC esterification experiments. The fluorescence of the cholesterol ester band over the sum of both bands is shown as means of three independent experiments ± *sem* for WT and NPC1$^{-/-}$ HeLa cells.

Using clickable cholesterol in a pulse-chase approach, we could detect a cholesterol esterification defect in NPC1$^{-/-}$ cells that we attributed to delayed transport of the cholesterol probe from lysosomes to the ER.

However, in order to make efficient use of the affinity tagging option for the study of lipid-protein interactions, a stronger binding between the lipid and its interacting protein is necessary. This could be achieved by the additional introduction of a photoactivatable diazirine group into the clickable cholesterol that enables covalent crosslinking even of transient interacting proteins [143]. The practicality of cholesterol derivatives with such a modification for crosslinking had already been established prior [144,145] and was used to

identify interaction partners *in vivo* [146–148]. There, validation of a crosslink was however based on the detection of the radioactive labeled cholesterol analogue in immunoprecipitations and was therefore limited to a predefined set of proteins of interest per study. In contrast, the later combination of the click and photo-crosslinking approaches in a single molecule by Hulce and his colleagues allowed them to create a proteome wide map of cholesterol-protein interactions [143]. By “clicking” a biotin to their cholesterol derivative, they were able to selectively enrich all proteins that were crosslinked to the cholesterol and identify them via mass spectrometry. Their dataset even provided information about the binding sites of the proteins through the identification of the crosslinked peptides. This compound and other photoactivatable and clickable cholesterols [149–151] have since been used for the same purpose in yeast cells [152], to study the interaction of a viral protein with cholesterol in the host cell membrane [153] and to quantify cholesterol accumulation in lysosomal contact site defective cells [8].
The application of these bifunctional cholesterol analogues is highly versatile due to the range of available clickable groups and can be easily combined with most protein-based assays due to the crosslinking capabilities. This provides a highly flexible toolset for the study of lipid-protein interactions as well as for fluorescence or Raman microscopy.

In summary, the distribution of cellular cholesterol is tightly controlled and differs vastly between organelles. Several approaches exist to determine the subcellular localization of cholesterol with each their own strengths and weaknesses. We have divided the available methods into three major groups: First, several indirect methods were introduced which investigate downstream processes such as SREBP2 activation or LDL-receptor expression. The advantages of not interfering with cellular processes are juxtaposed with the loss of temporal and spatial resolution of not monitoring cholesterol directly. Secondly, cholesterol-binding molecules such as Filipin or sterol-binding toxins are highly useful in investigating endogenous cholesterol levels in cells and tissues and were used with great success in the study of cholesterol-related diseases. They also allow for a much more rapid investigation of cholesterol-modulating treatments compared to indirect methods. However, their application is limited to membranes with relatively high cholesterol content. Here, ongoing research and the discovery of mutants with a lower binding threshold will certainly increase the scope and utility of these molecules. And thirdly, modified cholesterol analogues proved useful in visualizing cholesterol transport and metabolism with highest time resolutionand were successfully used in determining biophysical features of cholesterol in membranes. Here, the impact of the chemical modifications on the behaviour of the probes compared to natural sterols should be kept in mind when interpreting the results. Given that each of the discussed tools exhibits unique disadvantages, it is good practice to apply various approaches to the same question for cross-validation. Overall, knowing about the respective advantages and drawbacks of each method, scientists can choose the method or combination of methods most suited to answering their research question in order to further advance the field of cholesterol-mediated biology.

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