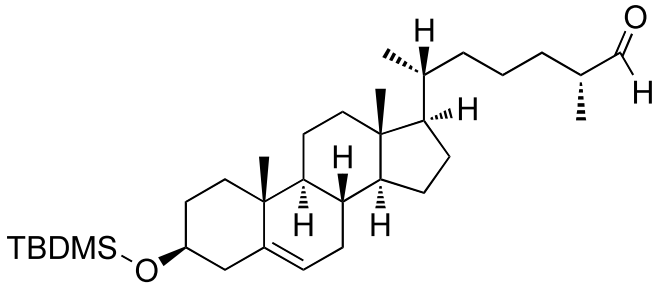
Supplmental text

Chemical syntheses

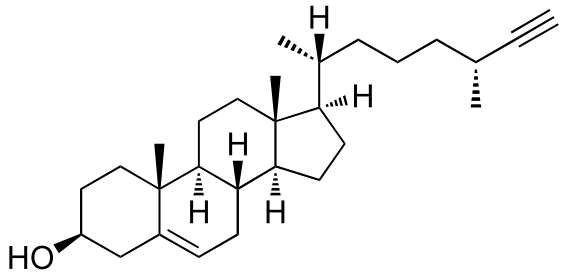
(i) Synthesis of **(25*R*)-3β-(*tert*-Butyldimethylsilyloxy)cholest-5-en-26-al**



A mixture of 250 mg (0.48 mmol) (25*R*)-3β-(*tert*-Butyldimethylsilyloxy)cholest-5-en-26-ol (synthesized as described (1); compound 14 of this ref.), 600 mg (2.78 mmol) pyridiniumchlorochromate, 3 g powdered molecular sieves and 8 ml dichloromethane was stirred at room temperature for 30 min. The solvent was evaporated, the residue extracted with 8 ml hexane/ethyl acetate 6/1 and purified by silica gel column chromatography using hexane/ethyl acetate 6/1 as a solvent to yield 180 mg product.

1H-NMR (400 MHz, $CDCl_3$): 9.56 ppm (d, 1H, 26-C*H*O), 5.26 (m, 1H, 6-C*H*), 3.42 (m, 1H, 3-C*H*), 1.04 (d, 3H. 27-CH_3), 0.94 (s, 3H, 19-CH_3), 0.85 (d, 3H, 21-CH_3), 0.82 (s, 9H, Si-C(CH_3)$_3$), 0.61 (s, 3H, 18-CH_3), 0.0 (s, 6H, Si-(CH_3)$_2$)

(ii) Synthesis of **27-alkyne cholesterol**; (25*R*)-25-ethinyl-26-nor-3β-hydroxycholest-5-en



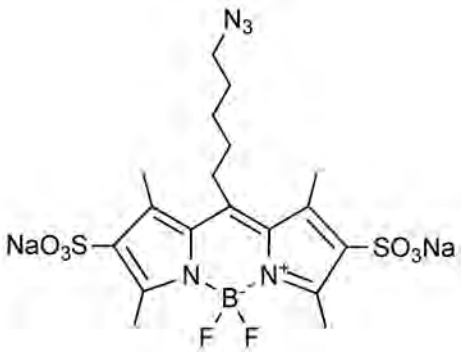
A mixture of 180 mg (0.35 mg) (25*R*)-3β-(*tert*-Butyldimethylsilyloxy)cholest-5-en-26-al, 110 mg (0.58 mmol) Dimethyl-(1-Diazo-2-oxopropyl)phosphonate (Bestmann-Ohira reagent, TCI), 80 mg (0.6 mmol) potassium carbonate and 6 ml MeOH/THF 1/1 was stirred for 5 h at 45°C. The solvent was evaporated, the remnant dissolved in hexane/ethyl acetate 5/1 1 and purified by silica gel column chromatography using hexane/ethyl acetate 6/1 as a solvent to yield 170 mg protected alkyne of 90% purity. This material was dissolved in 10 ml THF and 2 ml of 4N HCl were added. After 10 min, the deprotection reaction was stopped by addition of an excess of solid sodium carbonate. Ether (50 ml) was

added and the mixture washed twice with 10 ml of brine. The solvent was evaporated and the remnant purified by silica gel column chromatography using hexane/ethyl acetate 3/1 as a solvent to yield 120 mg of final product.

1H-NMR (400 MHz, $CDCl_3$): 5.34 ppm (m, 1H, 6-C*H*), 3.50 (m, 1H, 3-C*H*), 2.40 (m, 1H, 25-C*H*), 2.015 (d, J=2.6 Hz, 1H, Ethinyl-C*H*), 1.14 (d, 3H. 27-CH_3), 0.99 (s, 3H, 19-CH_3), 0.91 (d, 3H, 21-CH_3), 0.66 (s, 3H, 18-CH_3),

Elemental analysis was performed with a Vario EL apparatus:
$C_{28}H_{44}O$; Calculated (C, 84.79; H, 11.18); Detected (C, 84.76; H, 10.92)

(iii) Synthesis of **azido-sulfo-Bodipy (**8-(5-Azidopentyl)-4,4-difluor-1,3,5,7-tetramethyl-4-bora-3a,4a-*s*-indacene-2,6-disulfonic acid disodium salt):



8-(5-Bromopentyl)-4,4-difluor-1,3,5,7-tetramethyl-4-bora-3a,4a-*s*-indacene (200 mg, 0.5 mmol, synthesized as described (2)) was treated for 2 h with a solution of 70 mg sodium azide in 3 ml DMSO. Water (3 ml) was added and the mixture was extracted with 5 ml dichloromethane. The dichloromethane phase was washed with water and with brine and dried over magnesium sulfate. A solution of 90 µl chlorosulfonic acid in 3 ml of dichloromethane was added at -20°C. After 20 min, the precipitate was collected by centrifugation and dissolved in 10 ml 3% aqueous sodium hydrogencarbonate. Unsulfonated material was back-extracted using DCM and the product was purified in 5 batches on preparative scale RP18 column with a gradient of 0-70% methanol. Yield 110 mg, containing 90% product and 10% impurities, mainly mono-sulfonated material.

1H-NMR (D_2O) 3.18 (t, 2H, C5'H_2), 2.91 (m, 2H, C1'H_2), 2.57 (s, 6H, C10,11H_3), 2.52 (s, 6H, C9,12H_3), 1.55-1.35 (m, 6H, C2'-4'H_2).

Cell collection and lipid extraction for MS analysis. Cell incubated with medium containing de-lipidated FCS and 10 µg/mL sterols for 48 h were washed twice with PBS containing 10 mg/mL fatty acid free BSA (Applichem) and once with PBS. Cells were scraped in 155 mM ammonium acetate and dried overnight in a speedvac. The dry weight was determined before addition of 4 mL of chloroform/methanol 2/1. To extract lipids samples were kept at 4°C in darkness for 12 h. An internal standard (d4-24OH-cholesterol) was added and both phases washed repeatedly. The combined organic phases were evaporated and lipids redissolved in 1 ml n-hexane before solid-phase extraction.

Solid-Phase Extraction. Sterols were separated from oxysterols using a 100 mg Chromabond silica cartridge (Marchery-Nagel), pre-conditioned with 3 mL n-hexane. Sterols were eluted with 8 mL hexane/isopropanol 199/1; oxysterols with 5 mL hexane/isopropanol 70/30 (3). The combined fractions were evaporated, lipids redissolved in 1 mL methanol, and internal standards (epicoprostanol, 5α-cholestane) added.

Lipid silylation and analysis by gas-liquid chromatography (GLC). After removal of solvent the sterols were converted to trimethylsilyl ethers by treatment with 500µL pyridine/hexamethyldisiliazane/trimethylchlorosilane 9/3/1 at 90°C for 90 min. The reagent was evaporated, the residue redissolved in n-decane and transferred to autosampler vials. Cholesterol and alkyne cholesterol were quantified against 5α-cholestane using flame ionization detection (GLC-FID); cholesterol precursors and cholestanol against epicoprostanol; oxysterols against d4-24OH-cholesterol both using GLC-MS.

Gas-liquid chromatography-flame ionization detection (GLC-FID).
A GLC-system (HP 6890 series ll plus) was operated in the split less mode (injection temperature 280°C) using hydrogen (flow: 0.8 mL/min) as carrier gas. Silylated sterols were separated on a DB-XLB (30m x 0.25mm i.d. x0.25µm film; J&W, Palo Alto, CA, USA) using following temperature gradient: 150°C for 3 min, heating by 30°C/min up to 290°C (kept for 30min). Quantification was performed as described (4), but for 24-OH-alkyne cholesterol its ratio to d4-24-OH-cholesterol was determined.

Gas-liquid chromatography-mass spectrometry (GLC-MS). A GLC-system (HP 6890N) was used with helium (flow: 0.8 mL/min) as carrier gas and a modified temperature gradient: 150°C for 3 min, 30°C/min to 290°C (kept for 20 min), and 30°C/min to 300°C (kept for 4 min). Data were collected by selective ion monitoring (Agilent, 5973, operated using the ChemStation Software G1701DA version D.03.00) and full scan mode (range, *m/z* 50-650) was used for compound and structure identification. Silylated sterols were monitored using the following masses: epicoprostanol (*m/z* 370), 5α-cholestane (*m/z* 372), lathosterol (*m/z* 458), lanosterol (*m/z* 393), dihydrolanosterol (*m/z* 395), desmosterol (*m/z* 441), 24-OH-cholesterol (*m/z* 413), cholesterol (*m/z* 458), alkyne cholesterol

(*m/z* 468), 24-OH-alkyne cholesterol (*m/z* 466), d4-24-OH-cholesterol (*m/z* 416). Peak integration was performed manually and sterols were quantified using calibration curves of the internal standards. Characteristic fragment ions of 24-OH-alkyne cholesterol (*m/z* 141, 337, 426, 515, 556) were used for structural identification as qualifier ions (4).

Analysis of cholestenones by gas-liquid chromatography-mass spectrometry (GLC-MS). From lipid extracts the solvent was evaporated before addition of internal standards, 10 µg 5α-cholestane and 1 µg epicoprostanol. To eliminate residual sucrose 3 mL water were added before extraction twice with 3 mL cyclohexane as follows: vortex 30 s, centrifugation 2,000xg 10 min. The cyclohexane phases were combined in a new glass tube. To measure the free sterols, 1 mL of the organic phase was dried and lipid silylation performed as above, but using a 3/2/1 reaction mix. Our silylation conditions for cholest-4-ene-3-ones were optimized for complete conversion of the keto- into its enol-tautomer, cholest-2,4-diene-3-ol. To measure the total sterols after alkaline hydrolysis, the remaining 5 mL of the organic phase were dried and incubated with 1 mL of 1 M NaOH in 90% ethanol at 63°C for 1 h. After saponification and addition of 0.5 mL water the neutral sterols were extracted twice as above before lipid silylation as above. All residuals were redissolved in n-decane and transferred to autosampler vials. GLC-MS analysis was performed as above but using a modified temperature gradient: 150°C for 3 min, 20°C/min to 290°C (kept for 15 min). The injector and detector transfer line heater temperatures were set to 280°C; the MS source to 230°C; multiplier voltage to ~1800 V. Electron impact ionization was employed at 70 eV. Selected ion monitoring (SIM) was performed by cycling the quadrupole mass filter between the chosen *m/z* values at a rate of 2.0 cycles/s. Complete SIM parameters and retention times of the analytes are shown in Supplemental Table 1.

Supplemental References:

1. Martin, R., A. W. Schmidt, G. Theumer, T. Krause, E. V. Entchev, T. V. Kurzchalia, and H. J. Knolker. 2009. Synthesis and biological activity of the (25R)-cholesten-26-oic acids--ligands for the hormonal receptor DAF-12 in Caenorhabditis elegans. *Org Biomol Chem.* **7**: 909-920.
2. Heisig, F., S. Gollos, S. J. Freudenthal, A. El-Tayeb, J. Iqbal, and C. E. Muller. 2013. Synthesis of BODIPY Derivatives Substituted with Various Bioconjugatable Linker Groups: A Construction Kit for Fluorescent Labeling of Receptor Ligands. *J Fluoresc.*
3. Dzeletovic, S., O. Breuer, E. Lund, and U. Diczfalusy. 1995. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. *Anal Biochem.* **225**: 73-80.
4. Lutjohann, D., M. Stroick, T. Bertsch, S. Kuhl, B. Lindenthal, K. Thelen, U. Andersson, I. Bjorkhem, K. K. Bergmann, and K. Fassbender. 2004. High doses of simvastatin, pravastatin, and cholesterol reduce brain cholesterol synthesis in guinea pigs. *Steroids.* **69**: 431-438.

Supplemental Legends:

Supplemental Figure S1. Analyses of chemically pure sterols. (A) Synthetic alkyne cholesterol was analyzed by TLC after click-reaction to a fluorogenic coumarin reporter (lane 2) and compared to a control click-reaction lacking lipid (lane 1). A major band corresponding to alkyne cholesterol (aChol), a faint band (asterisk, presumably an auto-oxidation product), and a background band (bg) from the coumarin reporter, also found in the control sample, were detected. A TLC plate and a profile plot of lane 2 are shown. (B-E) Alkyne cholesterol was also subjected to gas-liquid chromatography-mass spectrometry (GLC-MS). Panel (B) shows its fragmentation pattern, which was compared to that of cholesterol (C). Characteristic fragment ions of both lipids are labeled and depicted with abbreviated *m/z* numbers (D&E). The respective molecule peaks are *m/z* 468.4 and 458.5 for alkyne cholesterol and cholesterol, respectively. (F&G) Comparison of 1H-NMR spectra of alkyne-cholesterol (F) and authentic commercial cholesterol (G). Only the region between 0.6 and 2.6 ppm is shown; the low-field region is identical for both compounds. In (F), note the loss of the 26-CH3 signal, the shift of the 27-CH3 signal, and the doublet of the ethinyl-H. The single asterisk denotes the water peak in the commercial cholesterol sample, the double asterisk an impurity (less then 1% of all H-signal) by remnants of the cleaved, water-soluble protecting group.

Supplemental Figure S2. Primary cells from rat brain utilize alkyne cholesterol to generate cellular metabolites. Primary rat astrocytes (A), hippocampal neurons (B) or oligodendrocytes (C) were incubated in the respective culture medium containing 12 µM of alkyne cholesterol. After the indicated times lipids of washed cells were extracted and analyzed by TLC for fluorescent metabolites, which were identified by comigrating lipid standards. Relative amounts of cellular alkyne cholesterol esters (CE) and alkyne cholesterol (chol) were determined by fluorography. Asterisks denote lipid amounts below the detection limit. X denotes unidentified lipids, potentially oxysterols, for which we have not yet synthesized an according lipid standard. Data are mean, (A) n=3; (B) n=2, or (C) single determinations.

Supplemental Figure S3. Analysis of cellular sterols by gas-liquid chromatography-mass spectrometry (GLC-MS). Cells transiently expressing CYP46A1 were incubated with medium containing delipidated FCS and 10 µg/mL alkyne sterols for 48 h. After extraction and silylation lipids were analyzed. Panel (A) shows a total ion current (TIC) chromatogram recorded while scanning the *m/z* range 50-600 with four peaks highlighted. Panel (B) shows a selected-ion monitoring (SIM) chromatogram for *m/z* 458, 468, 413, 466 representing cholesterol, alkyne cholesterol, and fragments of 24-OH-cholesterol and 24-OH-alkyne cholesterol, respectively. In (C) a fragmentation analysis of 24-OH-alkyne cholesterol in the corresponding peak from (A) is shown. The lipid was positively identified by comparing its fragmentation pattern to that of cell-derived 24-OH-cholesterol (D) from the corresponding peak in (A). Characteristic fragment ions of both lipids are labeled, some of which differ by 10, the mass of the alkyne moiety. The molecule peaks for 24-OH-alkyne cholesterol and 24-OH-cholesterol are *m/z* 556.5 and 546.5, respectively.

Supplemental Figure S4. Detection of sterol-bound filipin. (A&B) Analysis of cellular sterol localization using filipin detection. Cells incubated with alkyne cholesterol or carrier in delipidated medium for 16 h were fixed and probed for sterols using filipin. The distribution was visualized by microscopy and (A) epifluorescence images or (B) projections of z-stacks are shown. Bars, 20 μm. (C) Spectroscopic analysis of the filipin UV-absorption in dependence of a sterol binding partner. Upon presence of cholesterol or alkyne cholesterol, but not cholesteryl stearate or carrier, the filipin spectrum shows a characteristic shift indicating a molecular interaction of the sterol with the polyene probe.

Supplemental Figure S5. Analysis of plasma membrane sterols. (A) Confocal-like micrograph of cells focusing on the plasma membrane (PM) near the level of the support. Cells incubated with alkyne cholesterol in delipidated medium for 16 h were fixed and probed for sterols that were detected after click-reaction using an azide coupled to a Bodipy-dye (BDY). The distribution was visualized using structured illumination microscopy. Note the PM staining, which is best seen in flat cell areas (asterisk), often decorated by PM-blisters, blebbed during fixation. Also cell-cell-contacts (arrowheads) are positively stained. Bars, 10 μm. (B) Quantitative analysis of PM sterols. Cells incubated for 16 h in medium containing delipidated serum and equal amounts of alkyne cholesterol and deuterated d6-cholesterol (total 10 μg/mL) were chased in medium lacking lipids for 1 h to achieve a steady-state distribution of the provided sterols. Samples were either fixed for 1 h using glutaraldehyde to arrest cellular lipid transport or left untreated. All samples, living or fixed, were incubated with cholesterol oxidase at 37C for 30 min before lipid extraction. Extracts were divided and deuterated sterols quantified by MS, alkyne sterols by MS and also by TLC. aCE, alkyne cholesterol ester; aChol, alkyne cholesterol; aCO, alkyne cholest-4-en-3-one; d6-CE, d6-cholesterol ester; d6-CO, d6-cholest-4-en-3-one; n.d., not determined.

Supplemental Figure S6. Analysis of sterol distribution by cellular fractionation. Cells incubated for 16 h in medium containing delipidated serum and alkyne cholesterol were washed, harvested, homogenized and centrifuged to obtain a post-nuclear supernatant (PNS). The PNS was loaded onto a continuous sucrose gradient before velocity centrifugation. From the top, 12 fractions (1 mL each) were collected and lipids and proteins separated. (A) After click-reaction the lipids were analyzed by TLC and quantified by fluorometry and colorimetry. (B) A line plot of the relative gradient distribution of alkyne sterols and the respective normal sterols is shown. (C) The protein content of the fractions was analyzed by Western blotting for the distribution of marker proteins of various cell organelles. aCE, alkyne cholesterol ester; aChol, alkyne cholesterol; CE, cholesterol ester; ER, endoplasmic reticulum; mito, mitochondria; PM, plasma membrane.

Supplemental Movie 1. Cellular distribution of alkyne cholesterol imaged by fluorescence microscopy after click-reaction using an azide coupled to a Bodipy-dye. A z-stack of individual micrographs is shown with individual slices separated by 0.24 μm. Bar, 20 μm.

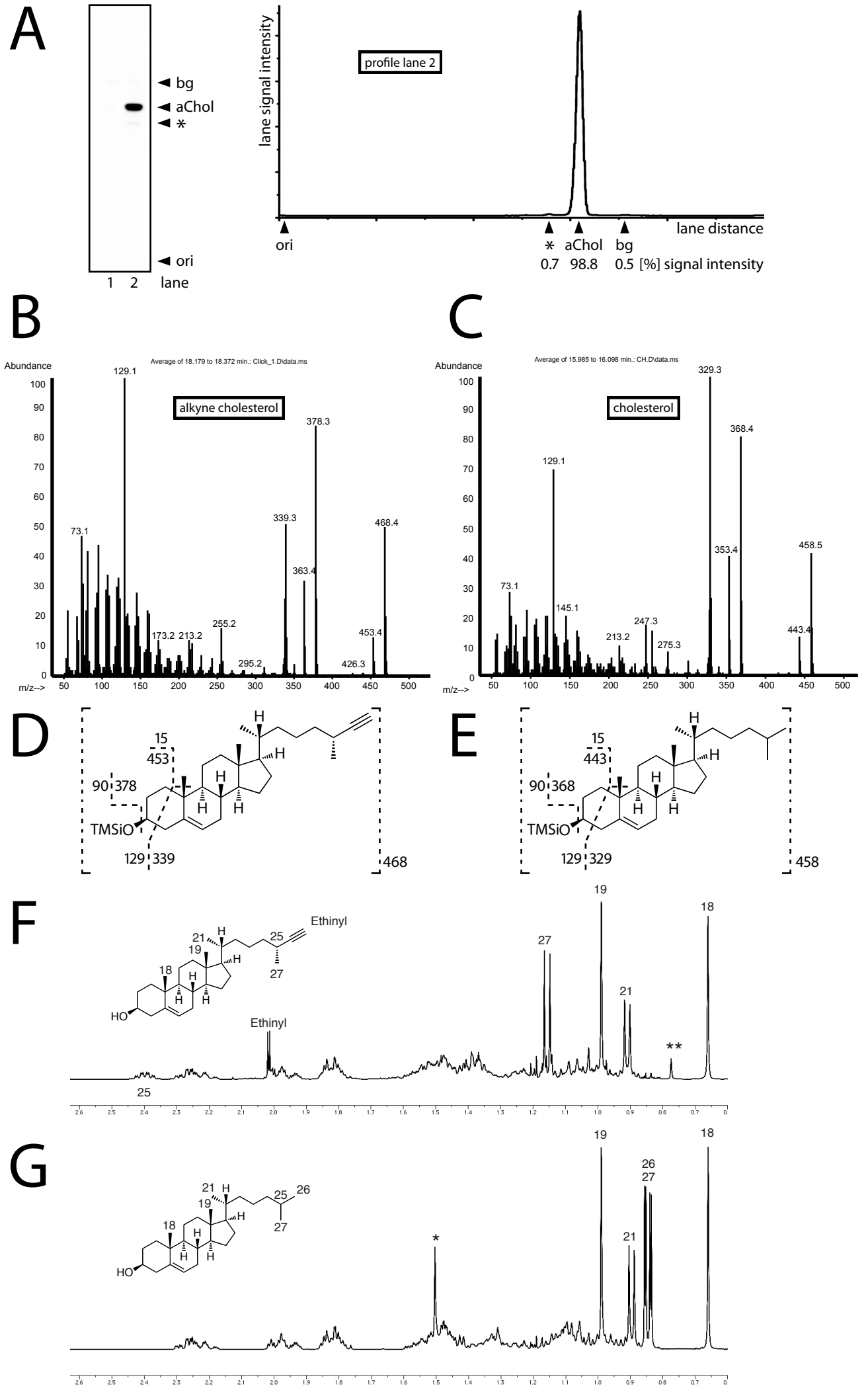
Supplemental Movie 2. Cellular distribution of alkyne cholesterol imaged by fluorescence microscopy after click-reaction using an azide coupled to biotin and detected by Alexa-488-streptavidin. A z-stack of individual micrographs is shown with individual slices separated by 0.24 μm. Bar, 20 μm.

Supplemental table 1. MS-SIM conditions for the GLC–MS analysis of sterols and cholestenones.

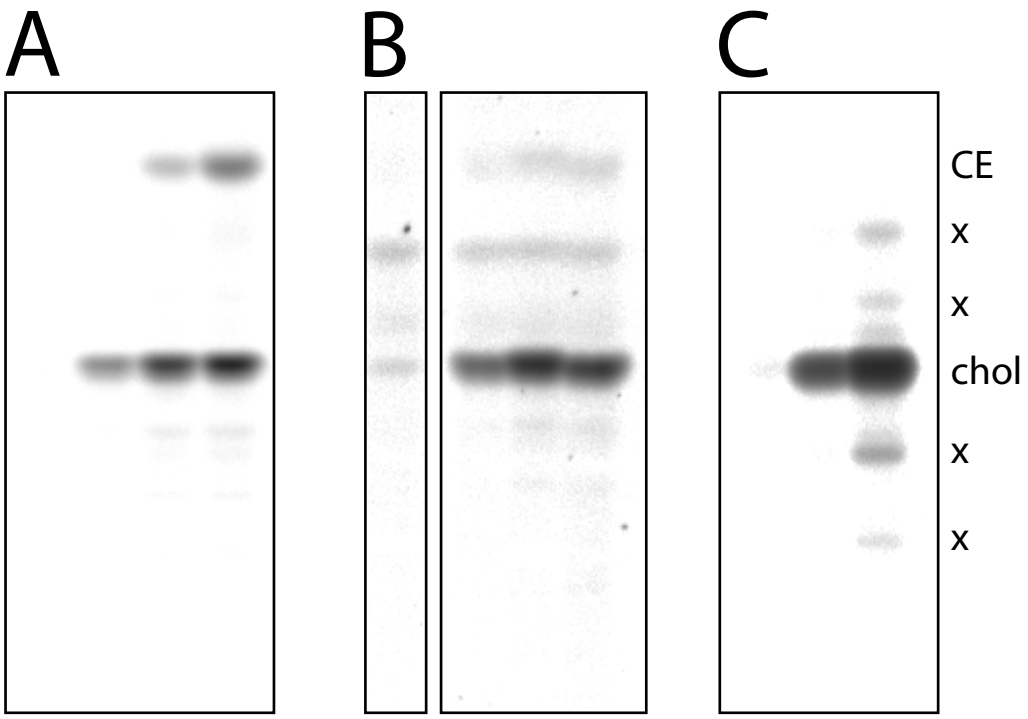
analyte		retention time	target ion (m/z)	analyzed as	compound
epicoprostanol		13.44	370.4	TMS-ether	internal standard
cholesterol		14.83	458.5	TMS-ether	sterol
[$^2H_6$]cholesterol		14.65	464.5	TMS-ether	deuterated sterol
cholest-2,4-dien-3ß-ol		15.81	456.5	TMS-ether	sterol
[$^2H_6$] cholest-2,4-dien-3ß-ol		15.70	462.5	TMS-ether	deuterated sterol
alkyne cholesterol		16.46	468.5	TMS-ether	sterol
alkyne cholest-2,4-dien-3ß-ol		18.11	466.5	TMS-ether	sterol
cholestenone		20.28	384.4	underivatized	sterol

TMS, trimethylsilyl

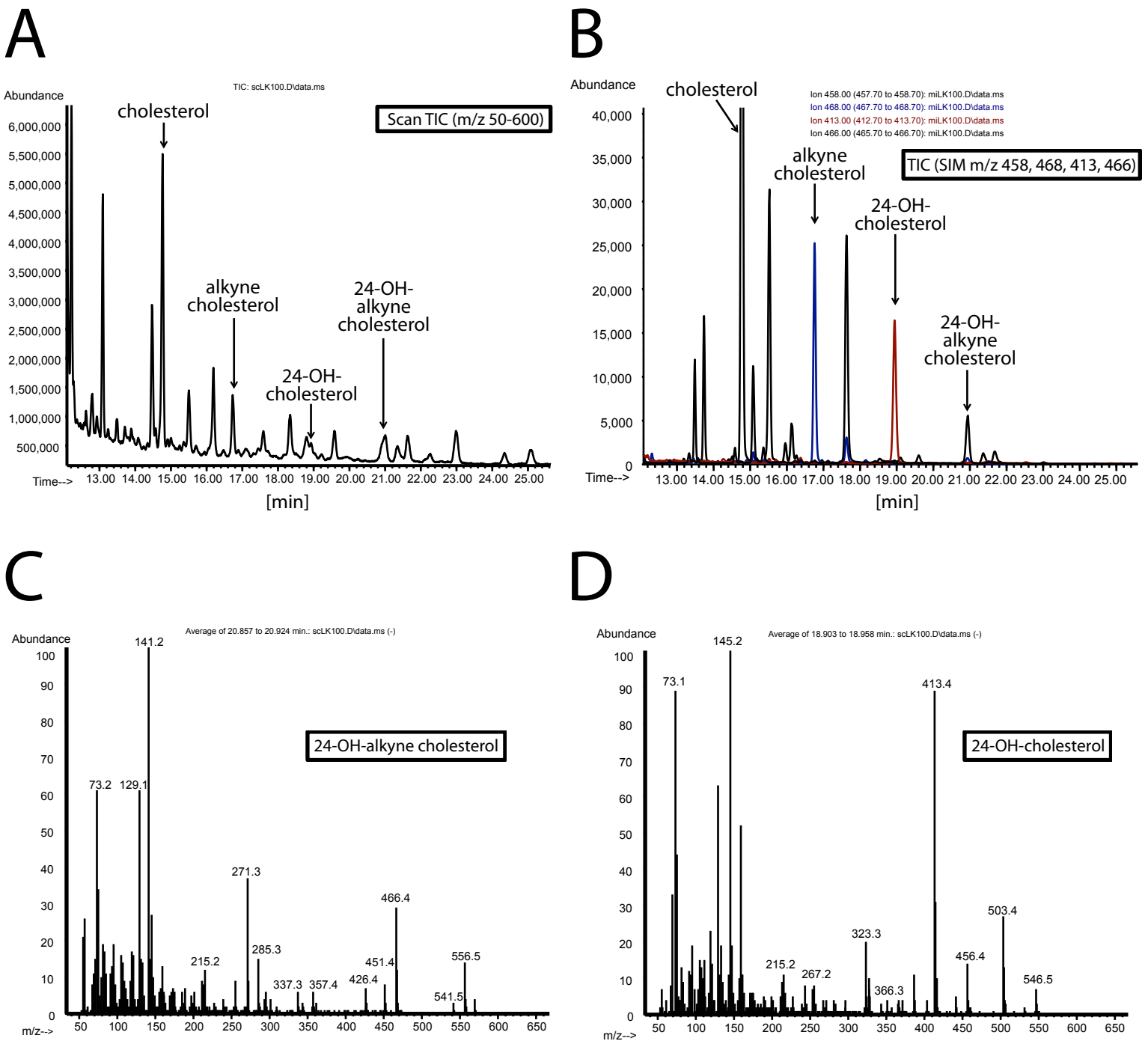
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

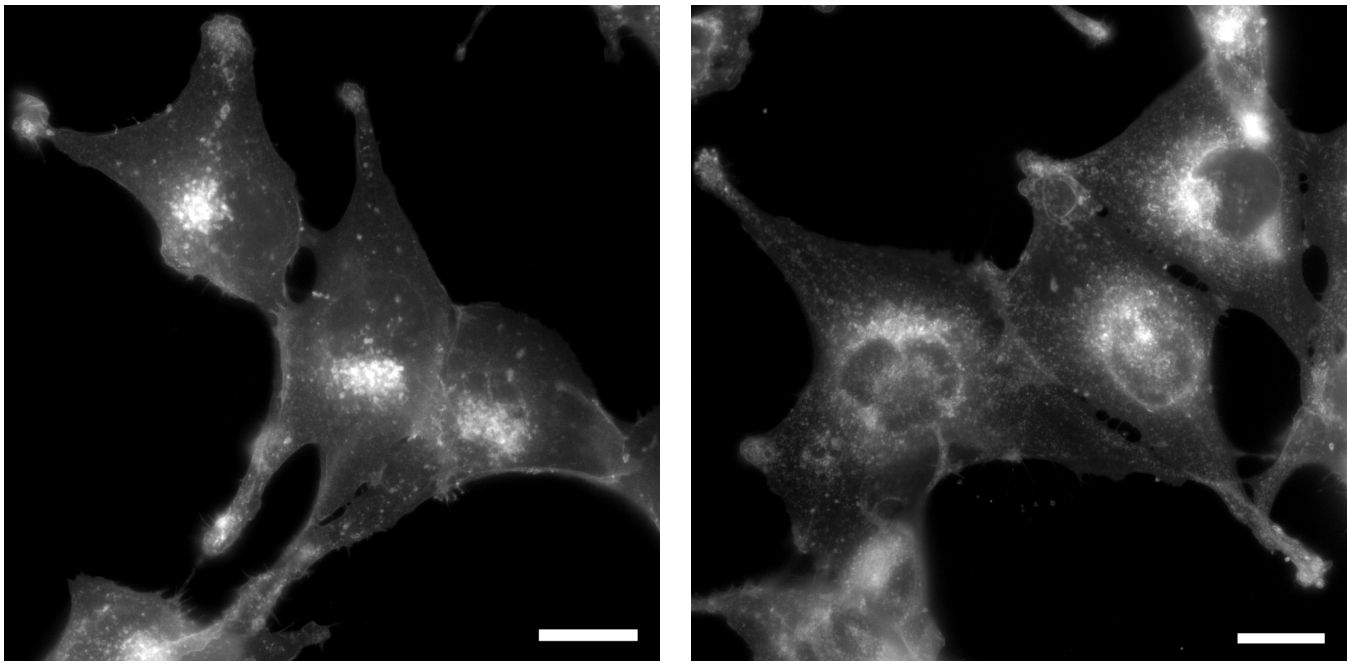


Supplemental Figure 4

A

no sterol supplement

+ alkyne cholesterol



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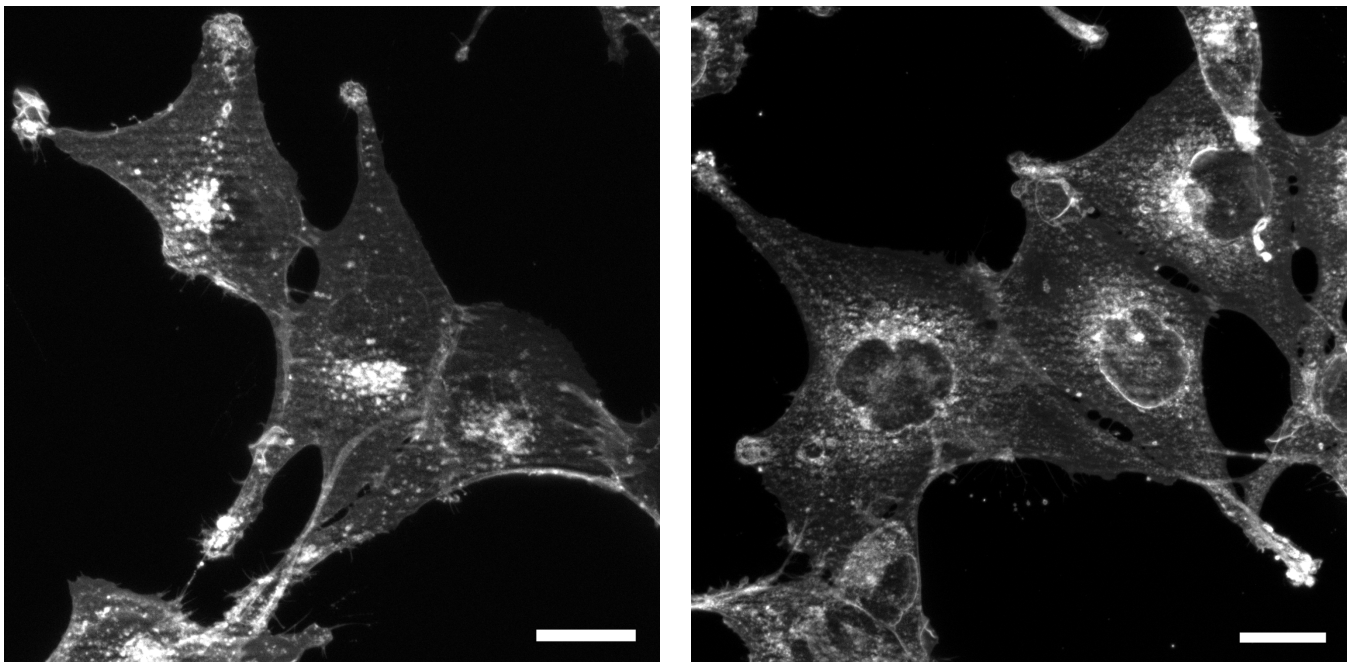

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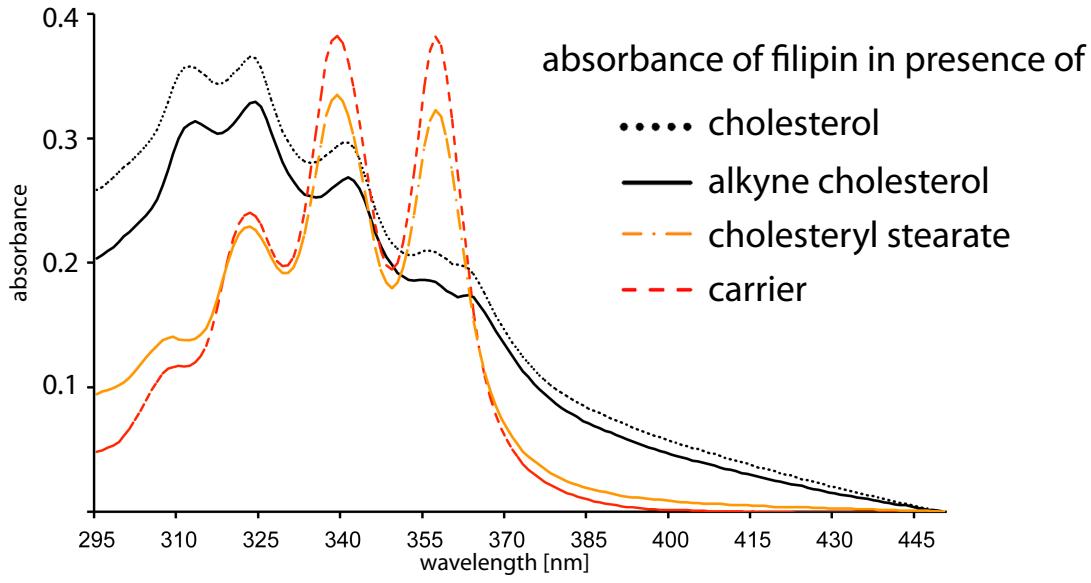
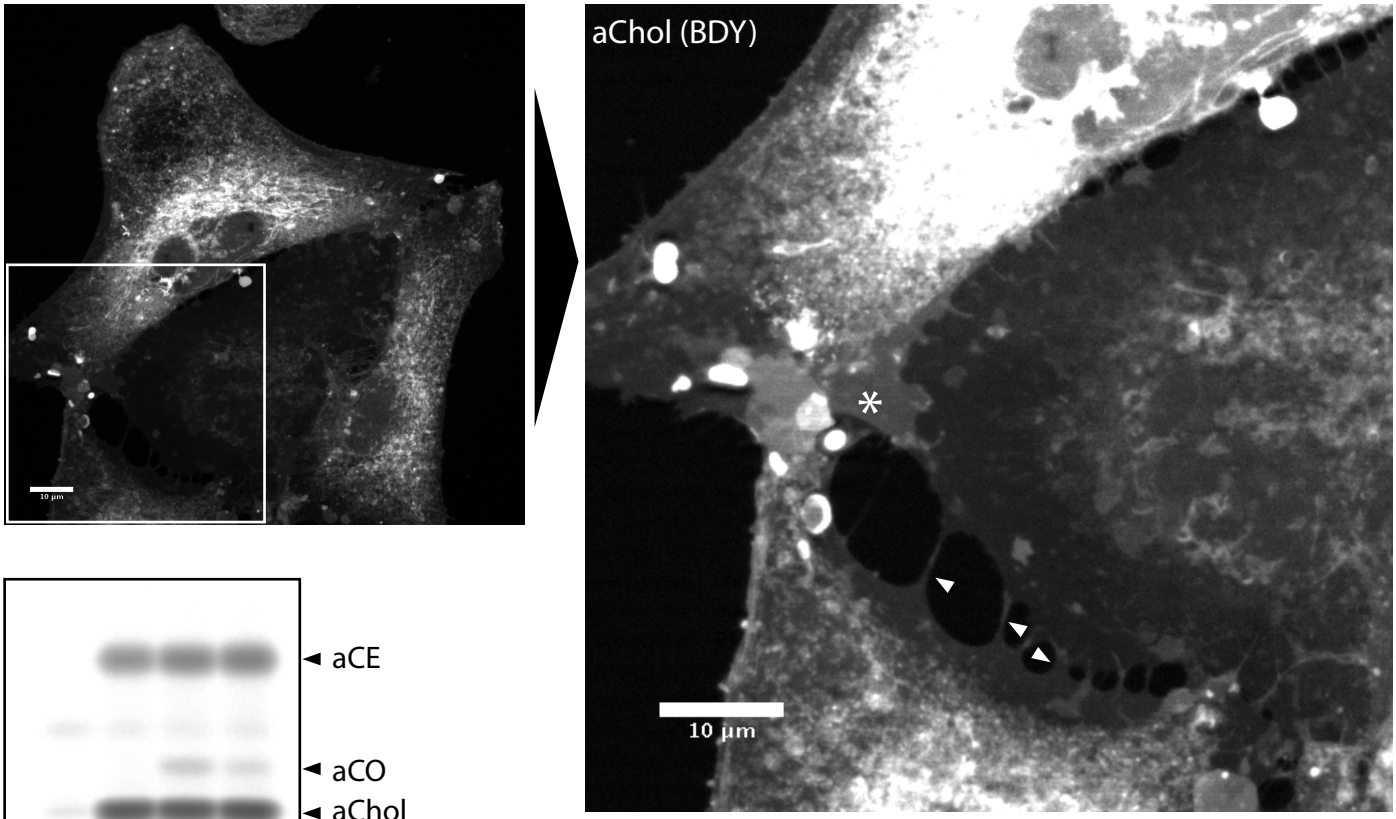
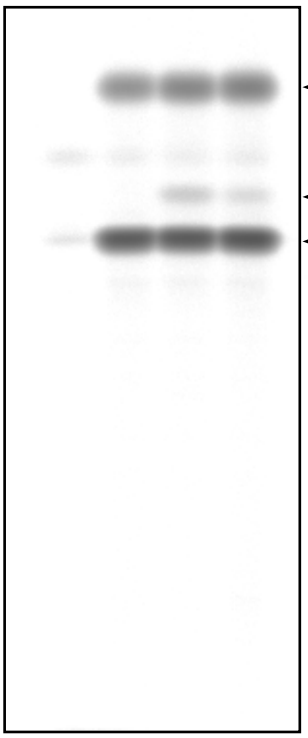


Supplemental Figure 5

A



B



1	2	3	4	lane number
+	+	+	+	cells
+	+	+	+	click reaction
–	+	+	+	alkyne cholesterol fed
–	–	+	+	d6-cholesterol fed
–	–	–	+	fixation
–	–	+	+	cholesterol oxidase

				fluorometry signal intensities [% of total lane signal]
–	19.5	24.7	25.3	aCE
–	0.0	1.9	0.9	aCO
–	80.5	73.4	73.8	aChol

				mass spec quantification [% of the respective sterol precursor]
–	n.d.	1.25	0.60	aCO
–	n.d.	1.50	0.92	d6-CO

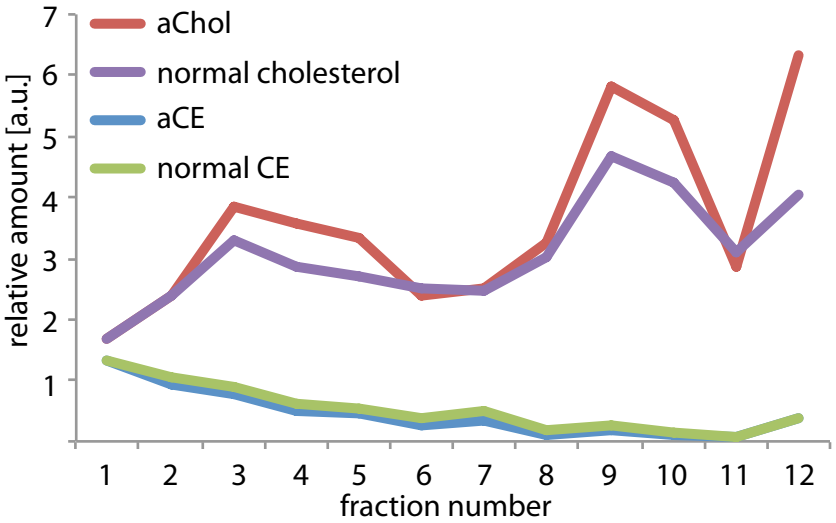
				mass spec quantification [% of total d6-sterol]
–	n.d.	15	22	d6-CE
–	n.d.	85	78	d6-cholesterol

Supplemental Figure 6

A

1	2	3	4	5	6	7	8	9	10	11	12	fraction number	
1.32	0.95	0.79	0.50	0.46	0.26	0.35	0.11	0.19	0.09	0.07	0.37	aCE [nmol]	fluorometry quantification (calibrated against internal alkyne lipid standard)
1.67	2.38	3.84	3.58	3.35	2.41	2.50	3.25	5.83	5.26	2.86	6.34	aChol [nmol]	
1.32	1.05	0.88	0.61	0.55	0.38	0.52	0.20	0.28	0.12	0.07	0.37	normal CE [a.u.]	colorimetry quantification (relative; set to match value from lane 1 of fluorometry)
1.67	2.37	3.28	2.87	2.71	2.51	2.45	3.01	4.66	4.22	3.08	4.03	normal chol. [a.u.]	

B



C