Supporting Information for

A Chemoenzymatic Strategy for Imaging Cellular Phosphatidic Acid Synthesis

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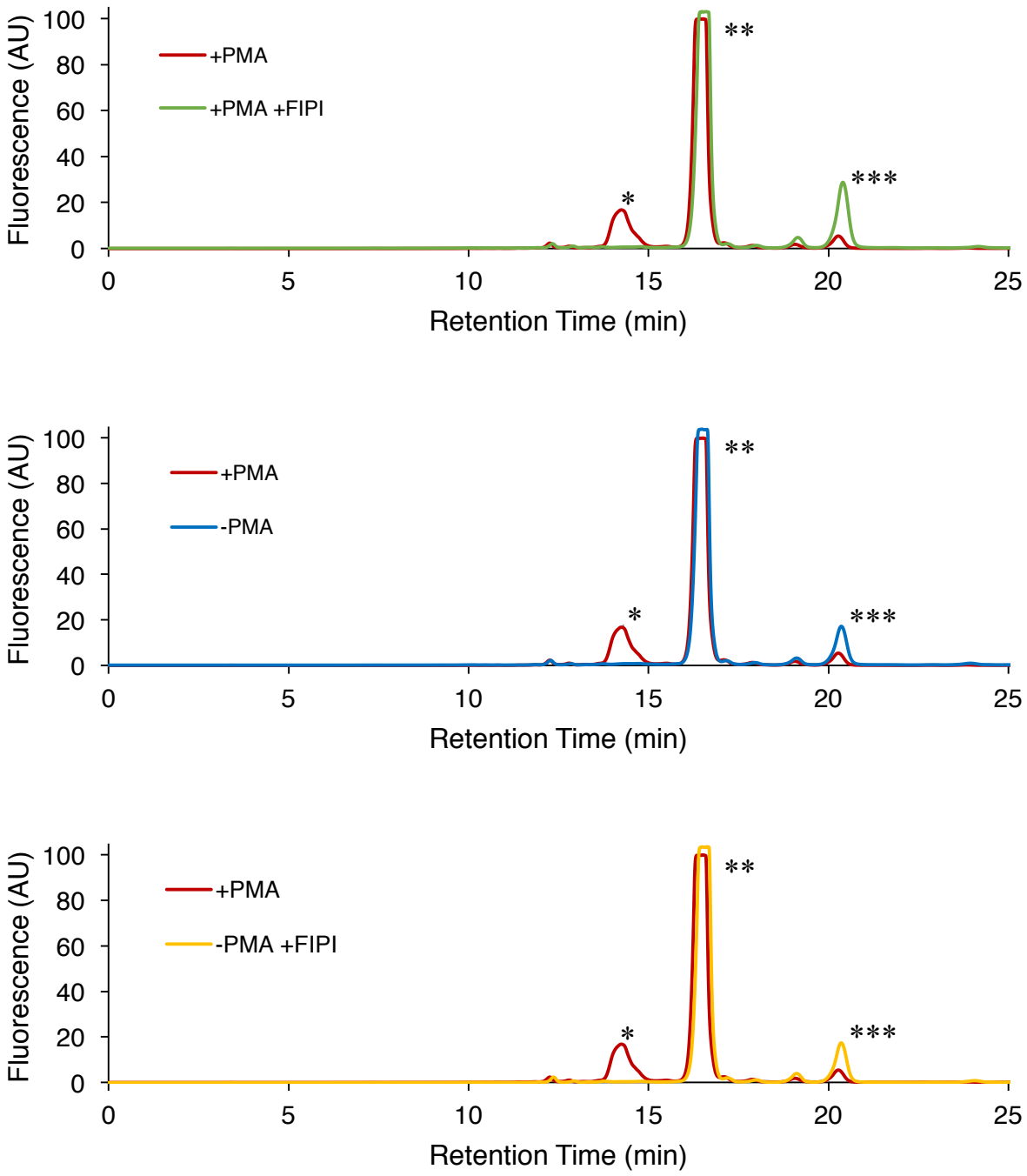

Figure S1: Hexynol-based labeling is PMA-dependent and FIPI-sensitive. HeLa cells were treated with 750 nM FIPI (green, yellow lines) or vehicle (blue, red lines) for 30 min, then 12.9 mM hexynol for 20 min, and then stimulated with 100 nM PMA (red, green lines) or control (blue, yellow lines) for 20 min. Following lysis and lipid extraction, samples were labeled with Az488 by CuAAC and then analyzed by HPLC with fluorescence detection. The +PMA/–FIPI experimental sample (red) is shown pairwise with each negative control. *, Az488-labeled PA analogs; **, unreacted Az488; ***, triazole derived from Az488 reaction with free hexynol.

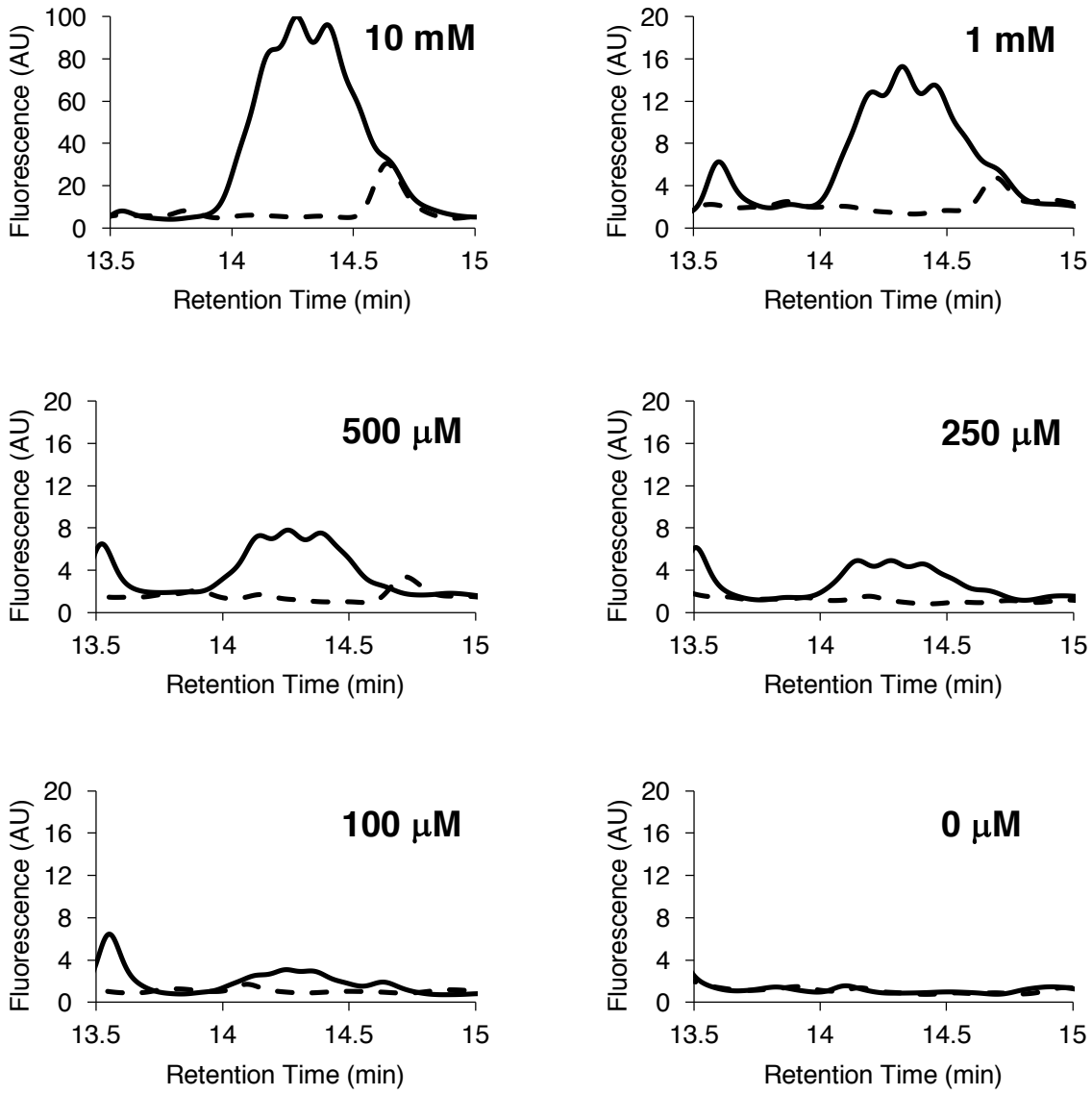

Figure S2: Hexynol-based labeling is detectable at concentrations as low as 100 μM. HeLa cells were treated with 750 nM FIPI (dotted lines) or vehicle (solid lines) for 30 min, then the indicated concentration of hexynol for 20 min, followed by stimulation with 100 nM PMA for 20 min, lysis, lipid extraction, CuAAC labeling with Az488, and analysis by HPLC with fluorescence detection.

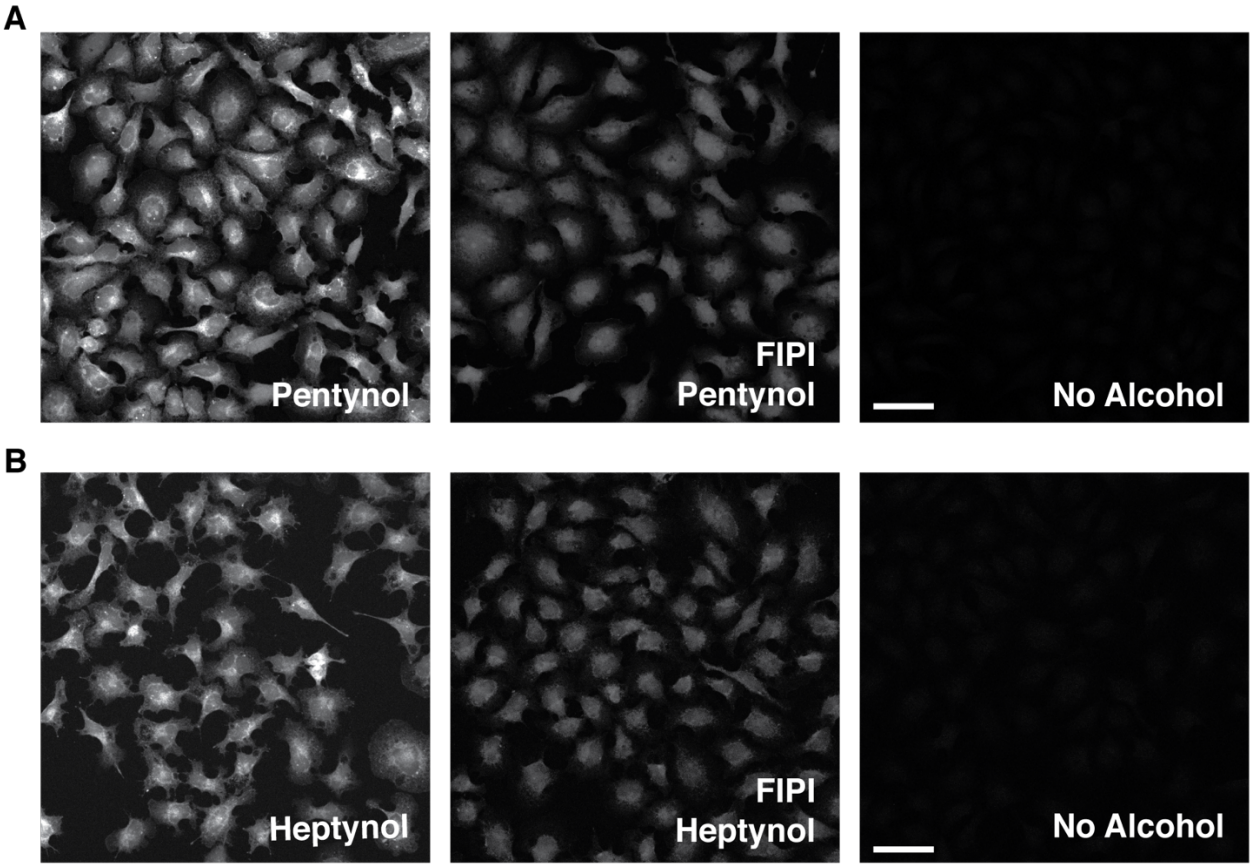

Figure S3: Pentynol and heptynol can also be used to image sites of PLD-dependent PA synthesis. HeLa cells were treated with 750 nM FIPI (middle panels) or vehicle (left panels) for 30 min, then 10 mM pentynol (A, left), heptynol (B, left), or no alcohol (right panels) for 20 min, and then stimulated with 100 nM PMA for 20 min. Cells were fixed with paraformaldehyde, rinsed with PBS, labeled with Az488 by CuAAC, rinsed, mounted, and imaged by confocal microscopy. Shown are maximum intensity z-projection images of a z-stack. Scale bars: 50 μm.

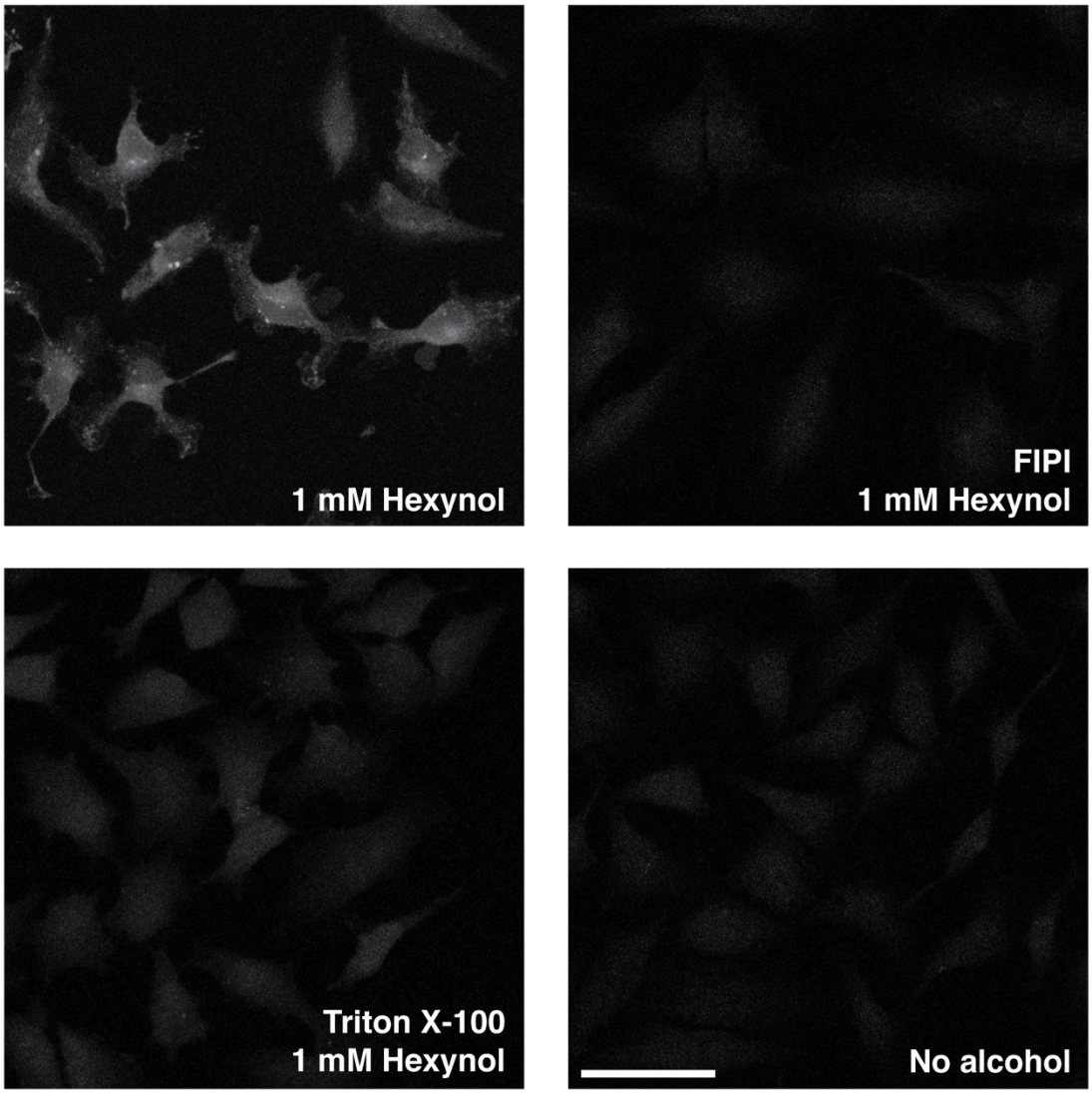

Figure S4: Hexynol-based labeling of PLD-mediated PA synthesis is detergent-sensitive. HeLa cells were treated with 750 nM FIPI (top right panel) or vehicle (all other panels) for 30 min, then 1 mM hexynol or no alcohol as indicated for 20 min, and then stimulated with 100 nM PMA for 20 min. Cells were fixed with paraformaldehyde, and rinsed with PBS. The cells shown on the lower left were additionally permeabilized for 10 min with 0.1% Triton X-100 in PBS followed by three rinses with 1X TBS. All cells were then labeled with Az488 by CuAAC, rinsed, mounted, and imaged by confocal microscopy. Shown are maximum intensity z-projection images of a z-stack. Scale bar: 50 μm.

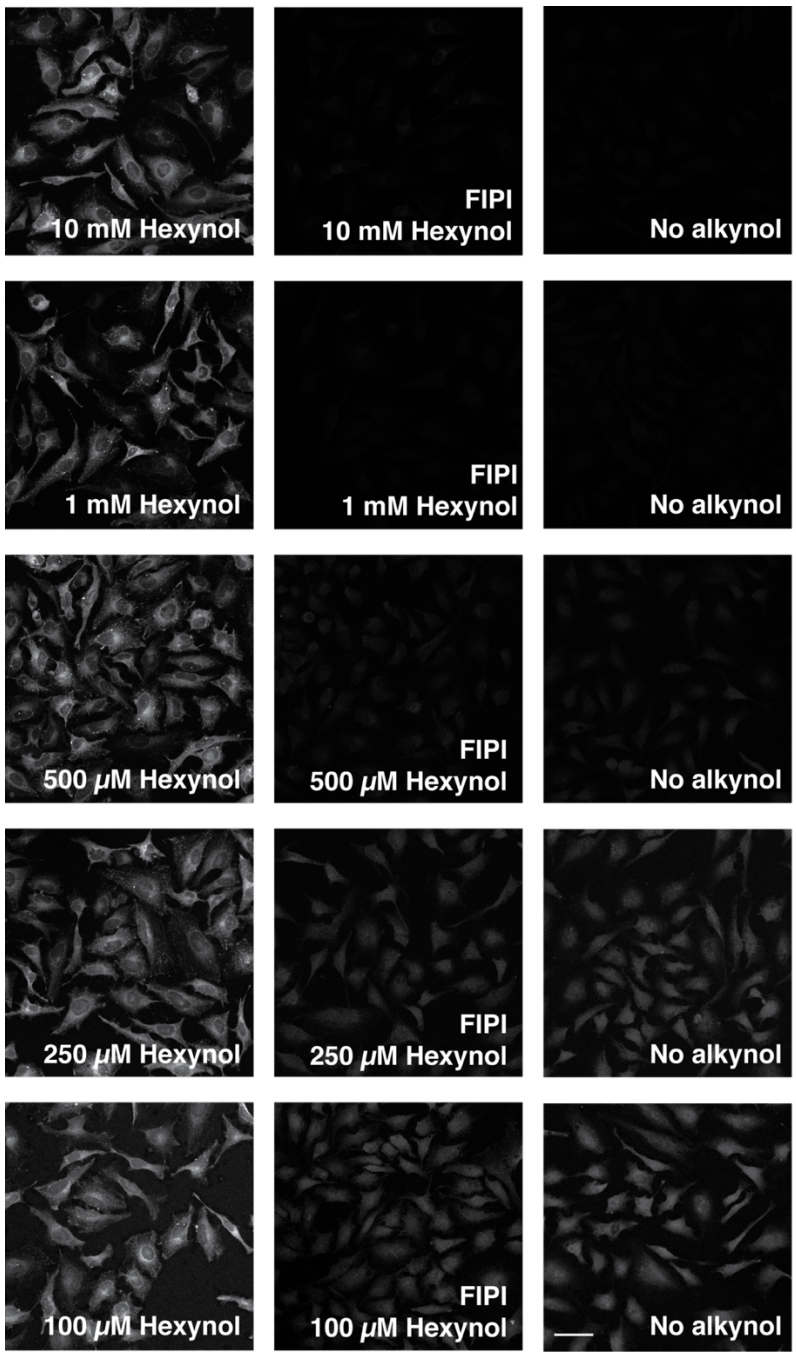

Figure S5: Hexynol-based labeling is detectable by imaging at concentrations as low as 100 μM. HeLa cells were treated with 750 nM FIPI (middle panels) or vehicle (left and right panels) for 30 min, then the indicated concentration of hexynol (left panels) or no alcohol (right panels) for 20 min, and then stimulated with 100 nM PMA for 20 min. Cells were fixed with paraformaldehyde, rinsed with PBS, labeled with Az488 by CuAAC, rinsed, mounted, and imaged by confocal microscopy. Shown are maximum intensity z-projection images of a z-stack. Note that for each row of images, microscope acquisition settings (i.e., laser power, PMT gain) were kept constant, but acquisition settings were optimized for each hexynol concentration (and its corresponding controls). Scale bar: 50 μm.

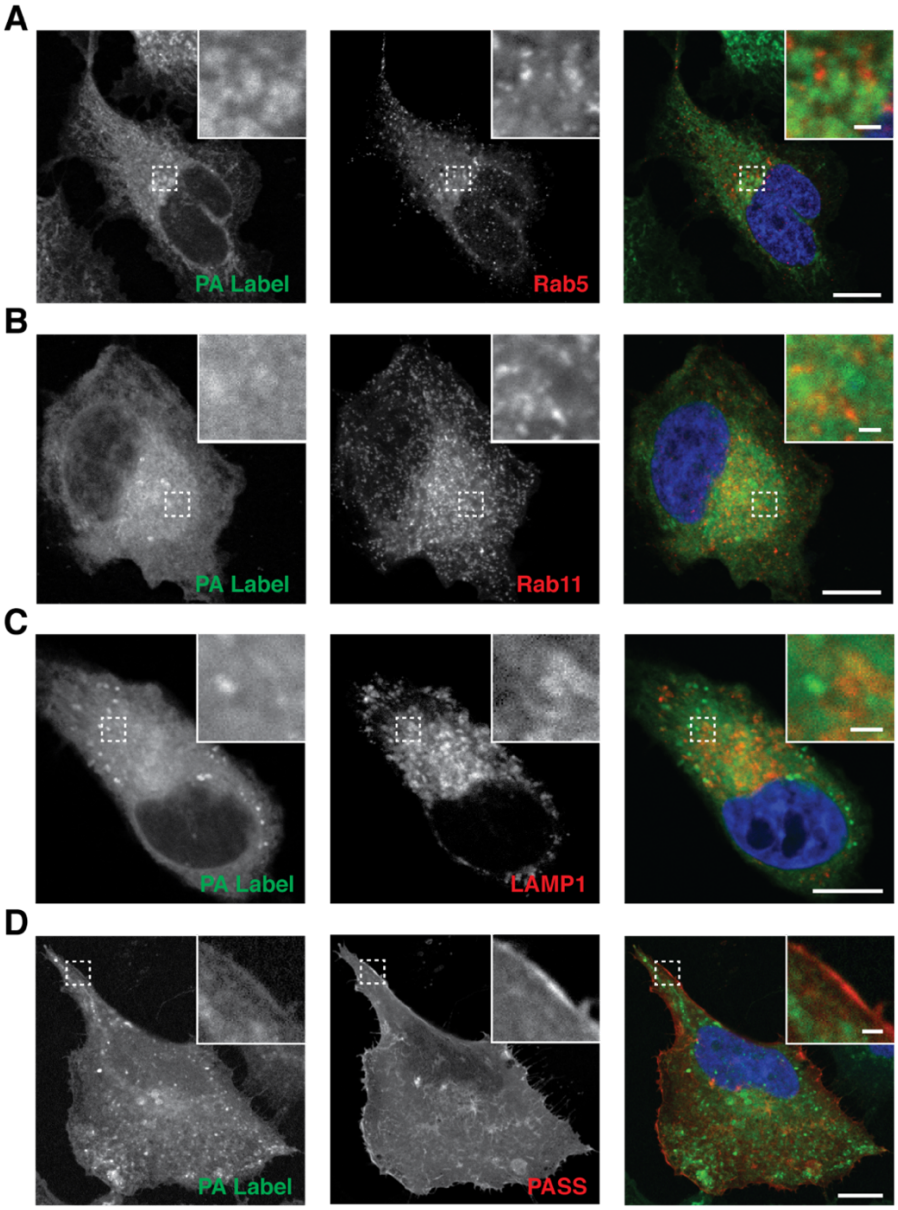

Figure S6: Colocalization analysis reveals minimal overlap of hexynol-based PA label with markers of endosomes and lysosomes and partial overlap with a genetically encoded PA biosensor. HeLa cells were transfected with plasmids encoding the early endosomal marker Rab5-GFP (A), the recycling endosomal marker Rab11-GFP (B), the late endosomal/lysosomal marker LAMP1-mRFP (C), or the genetically encoded PA biosensor mRFP-PASS (D). After 24 h, cells were labeled with 1 mM hexynol for 20 min and then stimulated with 100 nM PMA for 20 min. Cells were fixed with paraformaldehyde, rinsed with PBS, labeled with azido tetramethylrhodamine (Az545) (A and C) or Az488 (B and D) by CuAAC, rinsed, mounted in medium containing DAPI, and imaged by confocal microscopy. Shown are single z-plane images. For ease of interpretation, Az488- and Az545-derived fluorescence (PA Label) is shown green in the merged image and GFP- or RFP-derived fluorescence is shown in red in the merged image. DAPI is shown in blue. Insets denote boxed area a higher magnification. Scale bars: 10 μm, insets 1 μm.

Table 1: High-resolution ESI-MS analysis of lipid extracts from cells labeled as described in Figure 3[a].

Lipid identity[b]	Pentynol		Hexynol		Heptynol	
	Observed	*Expected*	*Observed*	*Expected*	*Observed*	*Expected*
32:1	1287.73059	1287.72973	1301.74663	1301.74538	1315.76208	1315.76103
32:0	1289.74364	1289.74538	1303.75938	1303.76103	1317.77364	1317.77668
34:2	1313.74451	1313.74538	1327.76138	1327.76103	—	—
34:1	1315.76084	1315.76103	1329.77649	1329.77668	1341.77130	1341.77668
36:4	—	—	1351.76729	1351.76103	1343.79201	1343.79233
36:3	—	—	1353.77509	1353.77668	1365.77789	1365.77668
36:2	1341.77707	1341.77668	1355.79326	1355.79233	1367.79940	1367.79233
36:1	1343.78748	1343.79233	1357.80672	1357.80798	1371.82018	1371.82363

[a] HeLa cells were treated with 12.9 mM of the indicated alkynol (pentynol, hexynol, or heptynol) for 20 min, followed by stimulation with PMA for 20 min. Following lysis and lipid extraction, samples were labeled with Az488 by CuAAC and analyzed by UHPLC-ESI-MS.
[b] Fatty acyl composition (number of carbons:degrees of unsaturation).

Materials and Methods

General materials and methods

All chemical reagents were analytical grade, obtained from commercial suppliers, and used without further purification, unless otherwise indicated. Where indicated, solvents were degassed by sparging with argon for 10 min. 2-Propyn-1-ol was purchased from TCI America; 3-butyn-1-ol, 4-pentyn-1-ol, and 5-hexyn-1-ol were purchased from BeanTown Chemical, 6-heptyn-1-ol was purchased from Ark Pharm; 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Echelon Biosciences, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) was purchased from Cayman Chemical; phorbol 12-myristate 13-acetate (PMA) was purchased from Santa Cruz Biotechnology; tetrakis(acetonitrile)copper(I) tetrafluoroborate, Az488 (Azide-fluor 488), Az545 (Azide-fluor 545), and *Arachis hypogaea* PLD were purchased from Sigma-Aldrich. Az488 was further purified by reverse-phase HPLC to yield single isomers. HPLC analysis was performed on a Shimadzu LC-20AR HPLC equipped with an SPD-20AV UV/Vis detector, an RF-20A fluorescence detector, and an ES industries Epic silica 3 μm 120 Å 25 cm x 4.6 mm column. Electrospray ionization-mass spectrometry (ESI-MS) analysis was performed on a Dionex UltiMate 3000 UHPLC system coupled to a Thermo Q-Exactive orbitrap mass spectrometer.

Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), and 0.05% trypsin-EDTA were purchased from Corning. Fetal bovine serum (FBS), Lipofectamine 2000, NBD-C6-ceramide, and ProLong with DAPI were purchased from Thermo Fisher. Imaging was performed on a Zeiss LSM 800 confocal laser scanning microscope equipped with 20X 0.8 NA and 40X 1.4 NA Plan Apochromat objectives, 405, 488, 561, and 640 nm solid-state lasers, two GaAsP PMT detectors.

In vitro PLD reaction with DOPC

An Eppendorf tube was charged with 10 μL of an 8 mg/mL chloroform solution of DOPC. The chloroform was removed under a stream of air. The DOPC was then dissolved in 1.5 μL of 50 mM SDS, 3 μL of 1 M sodium acetate (pH 5.6) and 19.5 μL of 118.5 mM alkynol in water. Subsequently, 3 μL of 500 mM calcium chloride and 2 μL of deionized water were added and the solution was vortexed. Finally, 1 μL of a freshly made solution of *Arachis hypogaea* PLD (2 U/μL) was added, and the reaction was placed at 30 °C for 90 min. The reaction was then diluted with 70 μL of PBS, 250 μL of methanol, 250 μL of chloroform, and 125 μL of 20 mM acetic acid. This solution was vortexed for 1 min and then centrifuged for 2 min at 16,000 x g. The organic layer was then removed, an additional 250 μL of chloroform was added and the sample was vortexed and centrifugated as before. The two organic layers were then combined and dried under a stream of N_2.

The functionalized lipid products were then subjected to CuAAC labelling as described by Thiele *et al*[1]. Briefly, to the lipid residue in a 1.5 mL Eppendorf tube was added 7 μL of degassed chloroform followed by 30 μL of a reaction master mix (2.95 μL of 11.72 mM Az488 in DMSO, 78 μL of 10 mM $[acetonitrile]_4CuBF_4$ in degassed methanol, and 312 μL of degassed ethanol). The tube was briefly flushed with argon and placed in a 42 °C water bath, such that the top half of the tube is not submerged. After 5 h, the reactions were diluted with 113 μL of a chloroform:methanol:water (73:23:3) mixture and filtered (0.45 μm) for HPLC analysis.

Cell culture

HeLa cells were maintained in a 5% CO_2, water-saturated atmosphere and grown in media containing DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Cell densities were maintained between 10^5 and $1.6x10^6$ cells/mL.

Plasmids

The sources of plasmids were the following: LAMP1-mRFP, mCherry-OMP25TM, GalT-GFP, and Lyn_{11}-mRFP were obtained from P. De Camilli (Yale University, New Haven, CT); STIM1-mRFP was obtained from Barbara Baird (Cornell University, Ithaca, NY); Rab5-GFP and Rab11-GFP were obtained from Yuxin Mao (Cornell University, Ithaca, NY); mRFP-PASS was obtained from Guangwei Du (University of Texas, Houston, TX).

Labeling cellular PA synthesis for HPLC analysis

HeLa cells (400,000 cells) were plated in a 60 mm dish with 2 mL media. The cells were allowed to grow overnight (12–16 h) prior to experimental treatment. Cells were treated with FIPI to a final concentration of 750 nM from a stock of 750 μM in DMSO or 0.1% DMSO vehicle and incubated for 30 min. Following the 30 min FIPI treatment the media was exchanged for 2 mL of imaging buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $CaCl_2$, 100 μM $MgCl_2$, 1 mg/mL BSA, and 1 mg/mL glucose) containing FIPI or DMSO as appropriate and also the appropriate alkynol at the indicated concentration (100 μM to 12.9 mM) and incubated for a further 20 min. Cellular PA production was then stimulated by the addition of 100 nM PMA, and the cells were incubated for a further 20 min. The buffer was then aspirated, and the cells were rinsed with 1 mL of PBS. Cells were scraped off of the dish, after the addition of 1 μL of 200 μM NBD-ceramide to act as an internal control for normalization, and the lipids were extracted using an adaptation of the method described by Bligh and Dyer[2]. Briefly, 100 μL of PBS, 250 μL of methanol, and 125 μL of 20 mM acetic acid were added to the dish to aid in removing the cells and the suspension placed in an Eppendorf tube. Subsequently, 250 μL of chloroform were added, and the mixture was vortexed for 1 min and then centrifuged at 16,000 x g for 2 min. The organic layer was transferred to a clean Eppendorf tube, and the aqueous layer was extracted again with an additional 250 μL of chloroform by repeating the vortexing and centrifugation. The organic layers were combined and dried under a stream of N_2. The lipid extracts were then subjected to CuAAC labelling as described in the previous section.

Analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (80:19.5:0.5) and solvent B was chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a nonlinear, exponential-based gradient from 0 to 65% solvent B over 18 min (Shimadzu pump B curve value of -6).

Imaging of cellular PA production

HeLa cells (60,000 cells per well) were plated on 12 mm glass coverslips in 12-well cell culture multiwell dishes and allowed to grow in media overnight. After 24 h, cells were labeled with appropriate combinations of FIPI, alkynol, and PMA (or controls) as described above. Following the 20 min PMA stimulation, the cells were then rinsed three times with PBS, fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature, and labeled with Az488 according to a protocol described by Jao *et al.*[3]. Briefly, following fixation, the cells were rinsed three times with PBS and two times with Tris-buffered saline (TBS). Cells were then labeled by

CuAAC for 1 h at room temperature in the dark by deposition of the cover slip on top of a 50 μL droplet of CuAAC reaction mixture, which was prepared immediately prior to use and consisted of these reagents added in the following order: 100 mM Tris pH 8.5, 5 μM Az488 (from a 5 mM DMSO stock solution), 1 mM $CuSO_4$ (from a freshly made 20 mM deionized water stock), 50 mM sodium ascorbate (from a freshly made 500 mM deionized water stock). After the CuAAC reaction, the cells were then rinsed three times with TBS, three times with 500 mM sodium chloride, and three times with TBS. The coverslips were then mounted on glass microscope slides in ProLong with DAPI and allowed to solidify in the dark overnight at room temperature.

For colocalization experiments, 100,000 cells were seeded 1 d prior to transfection and transfected with the appropriate plasmid using Lipofectamine 2000 according to the manufacturer's instructions. The alkynol labeling was performed 24 h after transfection as described above. For samples transfected with a GFP-labeled protein, Az545 was used in place of Az488.

Image analysis was performed using the Zeiss Zen Blue 2.1 and FIJI software packages. Images shown in Figures 4B, S3, S4, and S5 are maximum-intensity z-projection images, and all colocalization images (Figures 4C and S6) are single z-plane images. Note that for ease of interpretation, in the merge images, all alkynol-derived fluorescence (whether it is derived from Az488 or Az545) is colored green and organelle markers (whether they are from GFP, RFP, or mCherry) are colored red.

Supplemental References

[1] C. Thiele, C. Papan, D. Hoelper, K. Kusserow, A. Gaebler, M. Schoene, K. Piotrowitz, D. Lohmann, J. Spandl, A. Stevanovic, et al., *ACS Chem. Biol.* **2012**, *7*, 2004–2011.

[2] E. G. Bligh, W. J. Dyer, *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

[3] C. Y. Jao, M. Roth, R. Welti, A. Salic, *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 15332–15337.