Supporting Information for

Clickable substrate mimics enable imaging of phospholipase D activity

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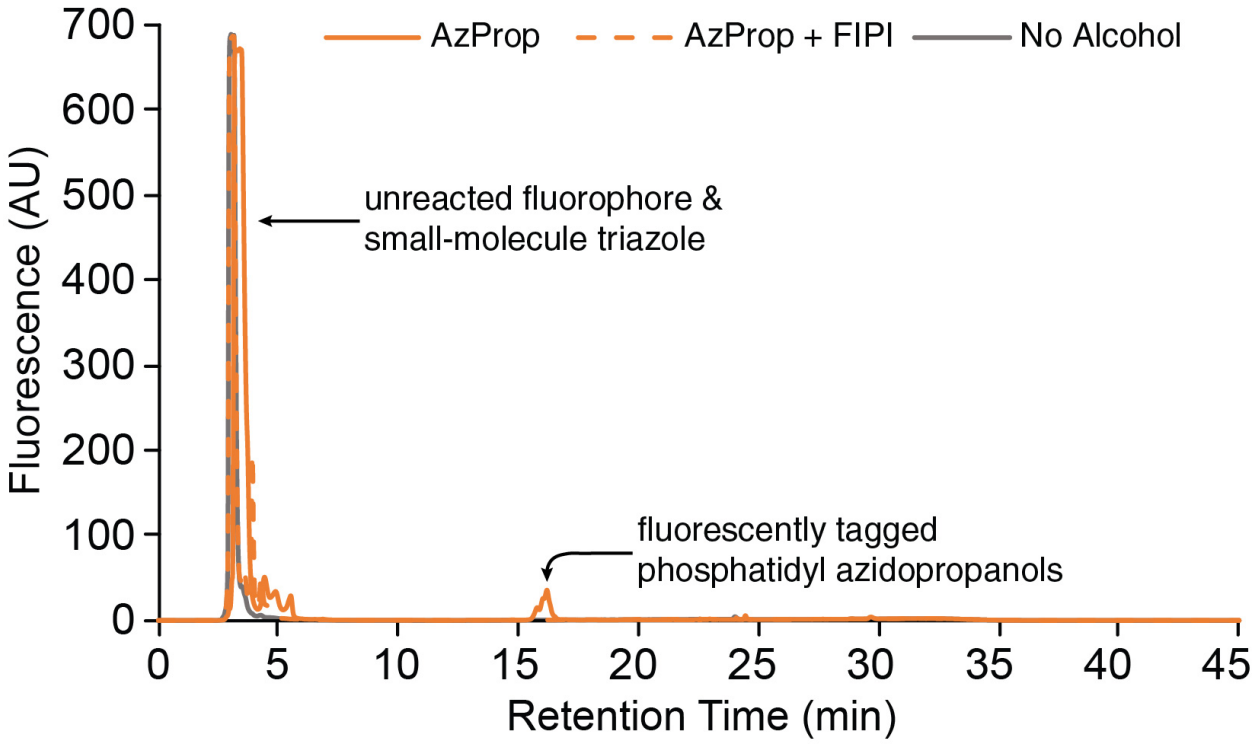

Figure S1. Representative full chromatogram of cellular lipid extracts after labeling with AzProp (from Figure 1B). HeLa cells were first treated with DMSO (solid lines) or a PLD inhibitor (FIPI (750 nM, dashed line) for 30 min, then with AzProp (1 mM, orange lines) or vehicle (B, gray line) for 20 min, and then stimulated with PMA (100 nM) for 20 min, followed by lipid extraction, SPAAC tagging with BODIPY-cyclooctyne **1**, and analysis by fluorescence-coupled HPLC. Fluorescence intensity is shown on the y-axis in arbitrary units (AU).

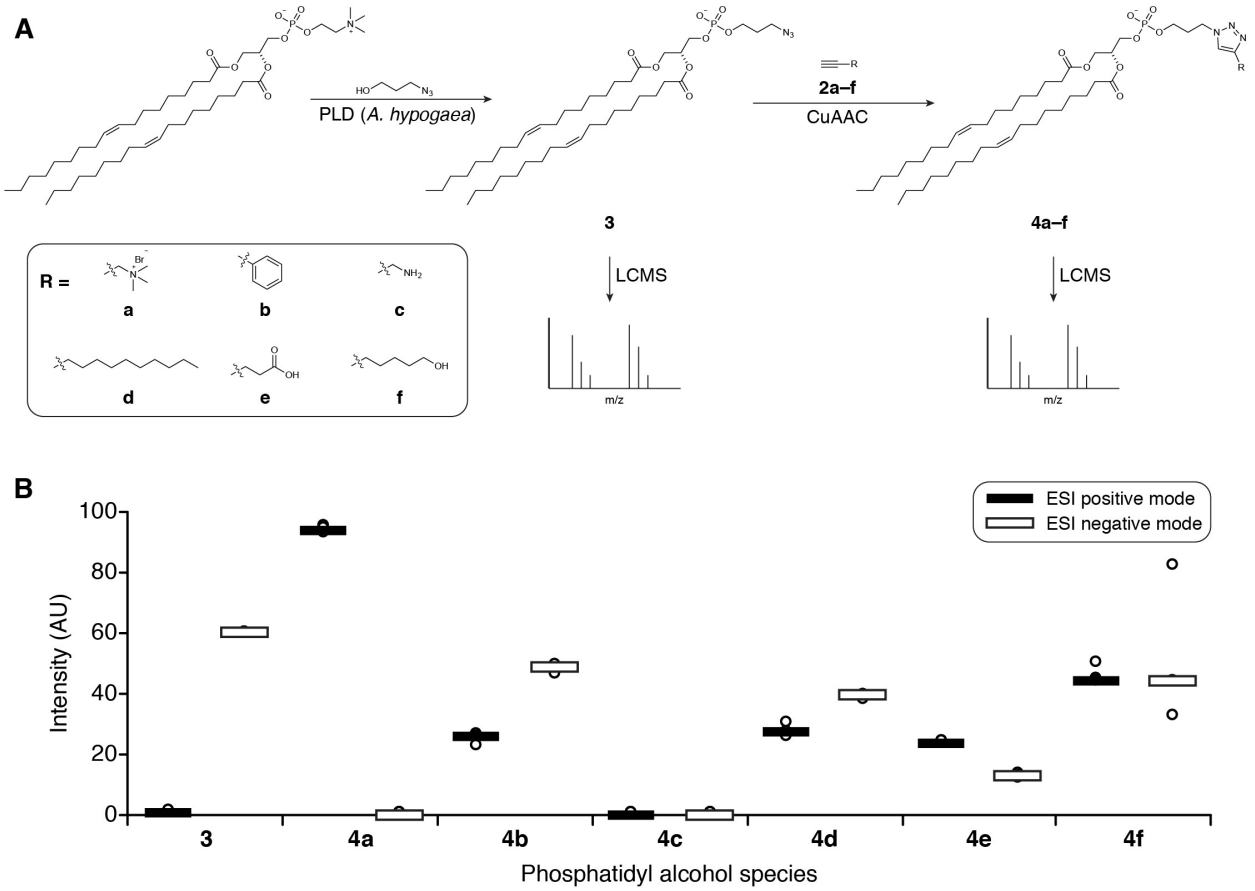

Figure S2. Screen of alkynes for enhancing phosphatidyl azidoalcohol detection by ESI–TOF MS. (A) Schematic of approach. Phosphatidyl azidopropanol standard **3** was synthesized by an in vitro chemoenzymatic reaction between 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and AzProp catalyzed by PLD from *A. hypogaea*. The lipid product (**3**) was either analyzed directly by ESI–TOF MS or further reacted by CuAAC with one of six alkyne probes (**2a–f**), and the corresponding lipid triazole products (**4a–f**) were subsequently analyzed by ESI–TOF MS. (B) Quantification of relative amounts of **3** and **4a–f** by ESI–TOF MS (area under the curve of the relevant extracted ion chromatogram, denoted as intensity and provided in arbitrary units (AU)). Lipids were analyzed in ESI positive mode (n = 3, black bar represents mean) and ESI negative mode (n = 3, white bar represents mean), n = 3 technical replicates. For calculated and observed masses for **3** and **4a–f**, see **Table S1**.

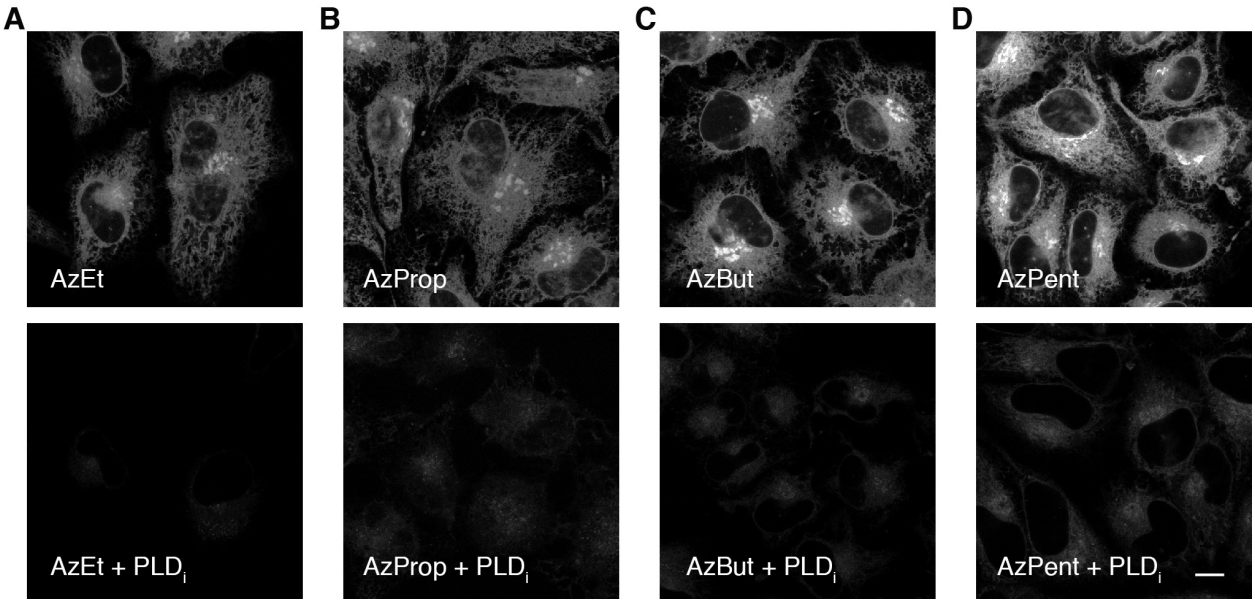

Figure S3. Evaluation of panel of azidoalcohols for imaging of PLD-mediated PA synthesis. HeLa cells were first treated with PLD_i (FIPI, 750 nM, bottom panels) or DMSO vehicle (top panels) for 30 min followed by the indicated azidoalcohol ((A) AzEt, (B) AzProp, (C) AzBut, (D) AzPent, all at 1 mM) for 20 min, and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with BODIPY-cyclooctyne **1** (2 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy. Shown are representative z-slices. Scale bar: 10 μm.

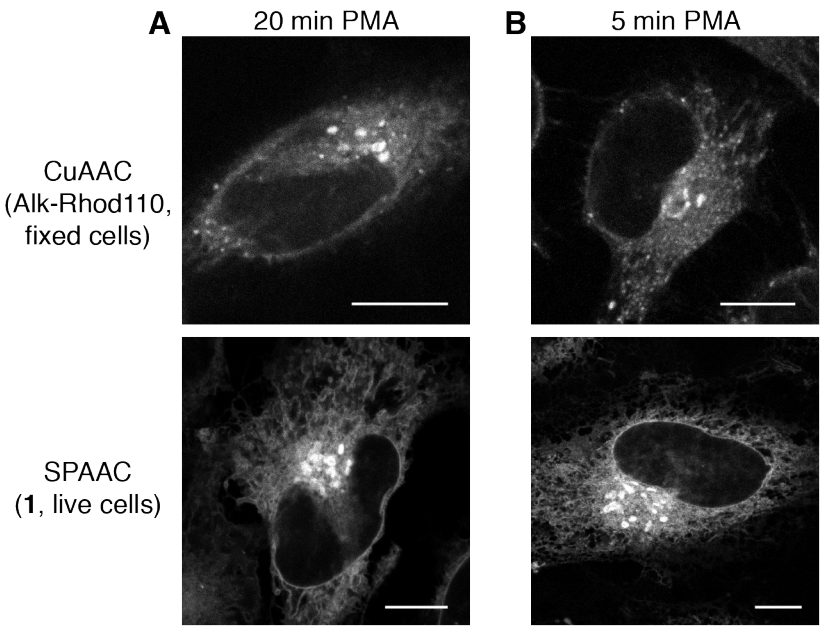

Figure S4. IMPACT using either SPAAC in live cells or fixation followed by CuAAC results in a similar subcellular distribution of the fluorescent lipid reporter. HeLa cells were treated with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for (A) 20 min or (B) 5 min. Following stimulation, cells were labeled either (top) by fixation with paraformaldehyde and tagging via CuAAC with an alkyne-rhodamine 110 conjugate (Alk-Rhod110) or (bottom) by tagging via SPAAC in live cells with **1** (1 μM) for 10 min, rinsed for 15 min. Cells were then imaged by confocal microscopy; shown are single z-slices. Scale bars: 10 μm.

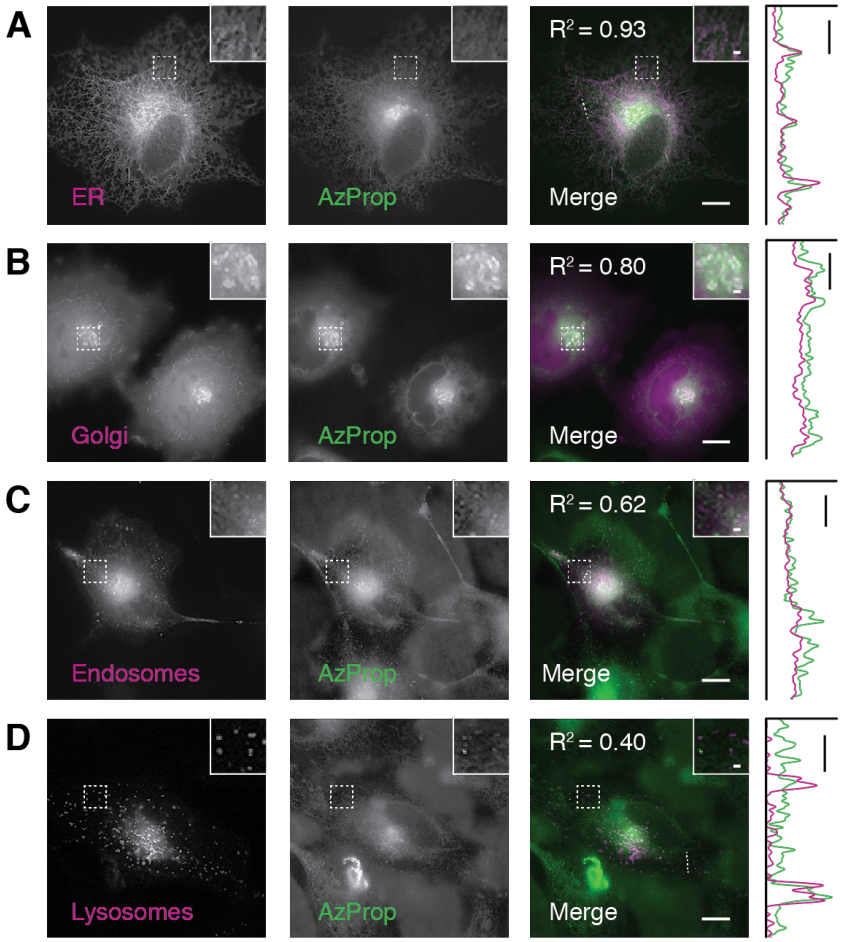

Figure S5. Colocalization analysis of IMPACT labeling with organellar markers using SR-SIM. HeLa cells were transfected with the indicated plasmids to mark different organelle compartments: (A) STIM1-mRFP (ER), (B) mCherry-P4M (Golgi), (C) Rab5-mRFP (Endosomes), and (D) LAMP1-mRFP (Lysosomes). In all cases, the cells were labeled, 24 h post-transfection, by IMPACT as follows. Cells were treated with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by super-resolution structured illumination microscopy. Shown are single z-slices of organelle marker fluorescence (left panels), IMPACT-derived fluorescence (AzProp, middle panels), and merge (right panels), with zoomed-in regions indicated by the dotted outline shown in the upper right corners. Colocalization is demonstrated by intensity plots along a one-dimensional profile corresponding to the dashed line in the merged image. Pearson correlation coefficients (R^2) are provided to aid in interpreting colocalization of the two markers. Scale bars: 10 μm; insets and one-dimensional profiles, 1 μm.

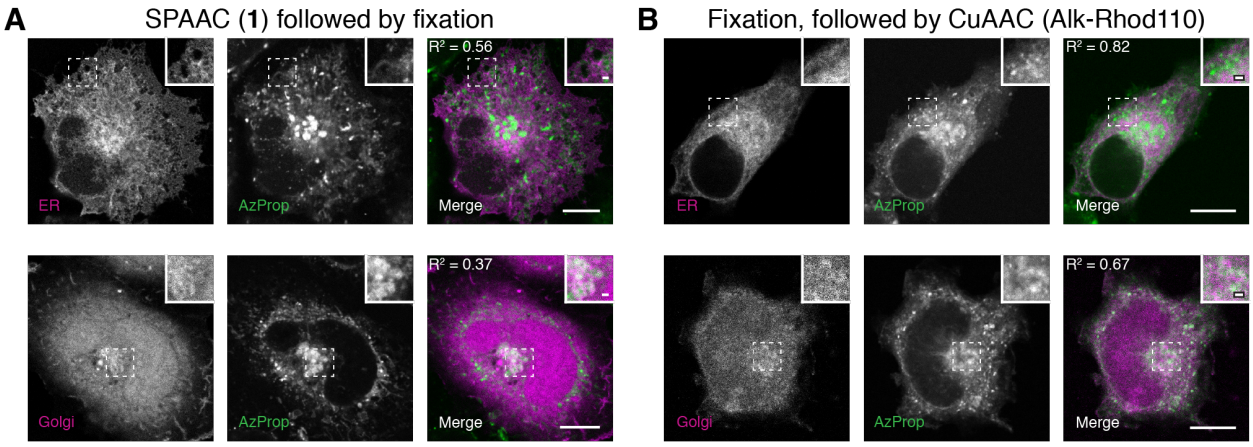

Figure S6. Colocalization analysis of IMPACT labeling in fixed cells comparing SPAAC labeling prior to fixation and CuAAC labeling following fixation. HeLa cells were transfected with the indicated plasmids (ER, STIM1-mRFP; Golgi, mCherry-P4M), and after 24 h they were labeled by IMPACT. Cells were treated with AzProp (1 mM) for 20 min, and then stimulated with PMA (100 nM) for 20 min. (A) Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and fixed with paraformaldehyde prior to imaging via confocal microscopy. (B) Cells were then fixed with paraformaldehyde and labeled with Alk-Rhod110 via CuAAC before imaging via confocal microscopy. Shown are single z-slices, with zoomed-in regions indicated by outline shown in the upper right corner. Pearson correlation coefficients (R^2) are provided to aid in interpreting colocalization of the two markers. Scale bars: 10 μm; insets 1 μm

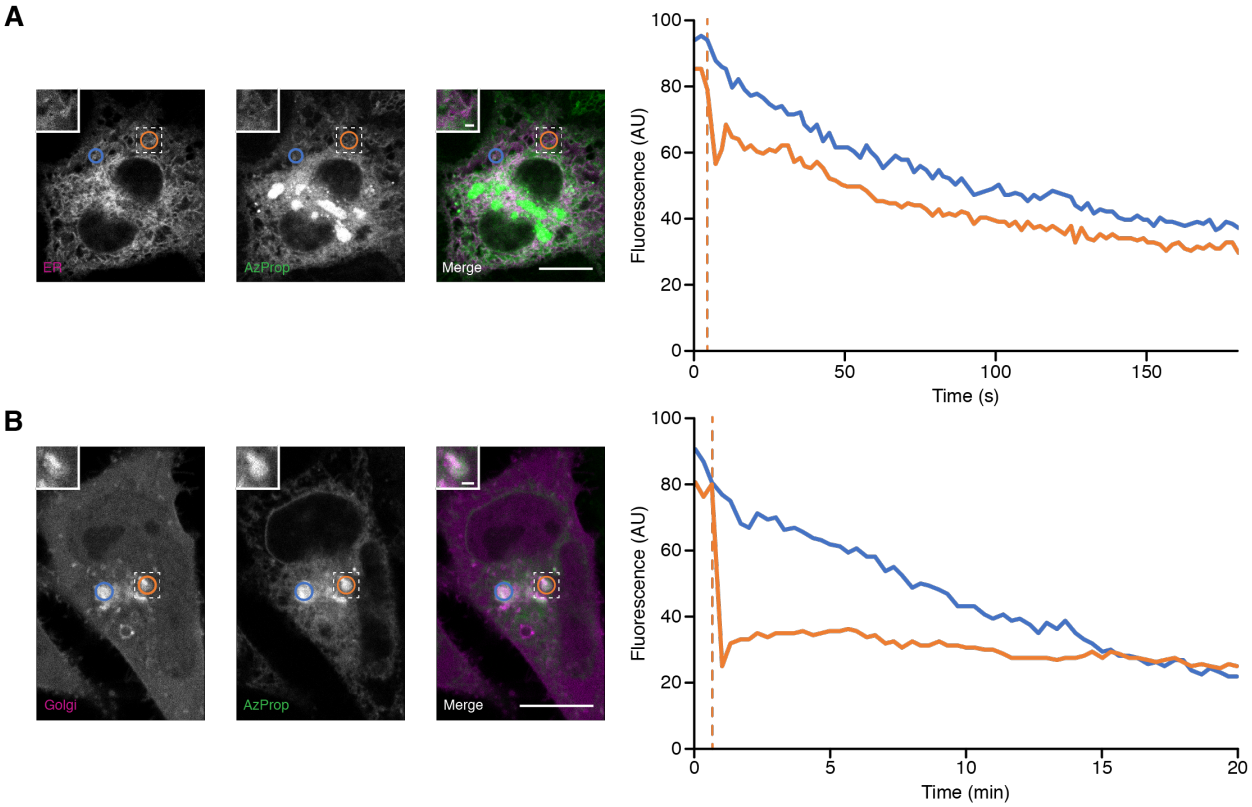

Figure S7. Fluorescence recovery after photobleaching (FRAP) studies of the IMPACT label. HeLa cells expressing markers for the (A) ER (STIM1-mRFP) or (B) Golgi apparatus (mCherry-P4M) were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min and rinsed for 15 min. The images shown are single z-slices of the cells taken at the outset of the FRAP experiment. The orange circle represents the region to be photobleached (also shown in the inset image), and the blue circle is a region of similar intensity that will not be photobleached. For the FRAP experiment, time-series were acquired by confocal microscopy, with photobleaching of the region circled in orange occurring in between the third and fourth frames of both time series. In (A), the interval in between frames was 2.01 s, with photobleaching occurring after 6.03 s, and in (B), the interval in between frames was 20 s, with photobleaching occurring after 1 min. Photobleaching was performed using 488 nm laser irradiation at 100% intensity. See Movies S3 and S4 for the complete time-series. The traces show fluorescence (indicated in arbitrary units (AU)) in the bleached region (orange trace for orange-circled region) and non-bleached region (blue trace for blue-circled region), with bleaching indicated by the dotted orange line. We note that we selected different time intervals for these two experiments such that they could be as short as possible in (A) due to the rapid recovery of fluorescence observed in the ER-derived signal, and longer in (B) due to the longer recovery time of Golgi-derived fluorescence and the photobleaching of BODIPY fluorophore due to acquisition (apparent in the decreases in the blue curves). We also note that in (A), the images are displayed using fluorescence settings in which some regions of the IMPACT label appear overexposed in order to facilitate observation of the lower intensity of the signal in the desired regions. Scale bars: 5 μm, inset 1 μm.

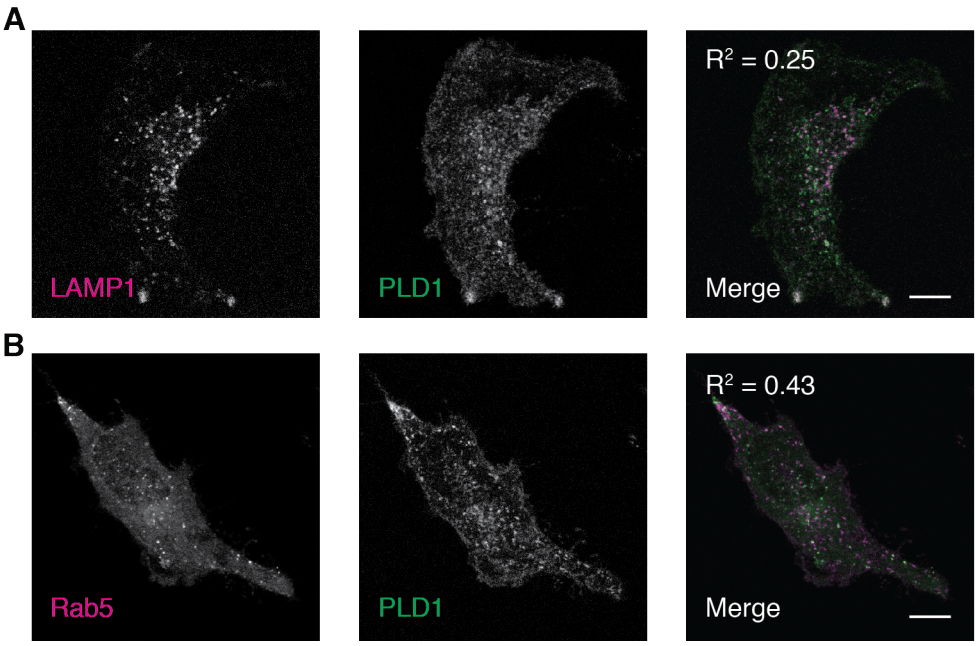

Figure S8. Colocalization analysis of mCherry-PLD1 with lysosomal and endosomal markers. HeLa cells were transfected with the indicated plasmids: (A) LAMP1-mRFP and GFP-PLD1; (B) Rab5-GFP and mCherry-PLD1. After 24 h, the cells were imaged by confocal microscopy. Shown are representative z-slices. For clarity, PLD1 fluorescence is shown in green and organelle markers are shown in magenta in the merged images (right), where colocalization appears as white. Pearson correlation coefficients (R^2) are provided to aid in interpreting colocalization of the two markers. Scale bars: 10 µm.

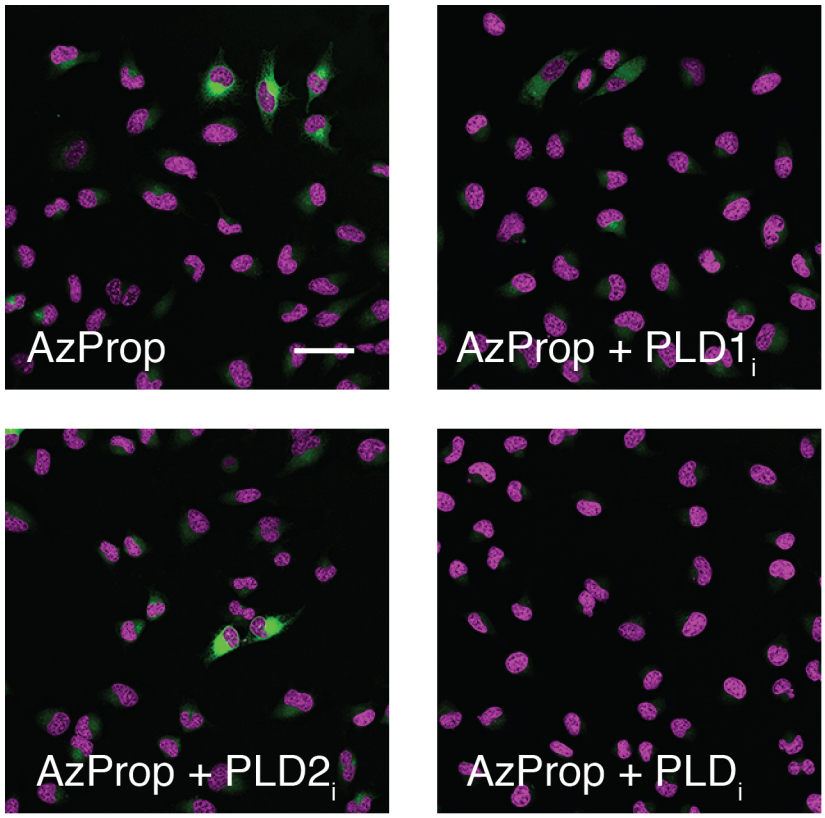

Figure S9. Higher exposure images of AzProp-labeled HeLa cells reveal heterogeneity of unstimulated PLD activity. HeLa cells were first treated with the indicated PLD inhibitor (PLD_i (FIPI), 750 nM; $PLD1_i$ (VU0359595), 250 nM; $PLD2_i$ (VU0364739), 350 nM) or DMSO vehicle for 30 min, followed by AzProp (1 mM) for 120 min, rinsed, labeled by SPAAC with **1** (1 μM) for 10 min, rinsed, stained with Hoechst 33342 to mark nuclei for 15 min, and imaged by confocal microscopy. Single z-slices are shown, with AzProp fluorescence is green and Hoechst 33342 in magenta. Scale bar: 50 μm.

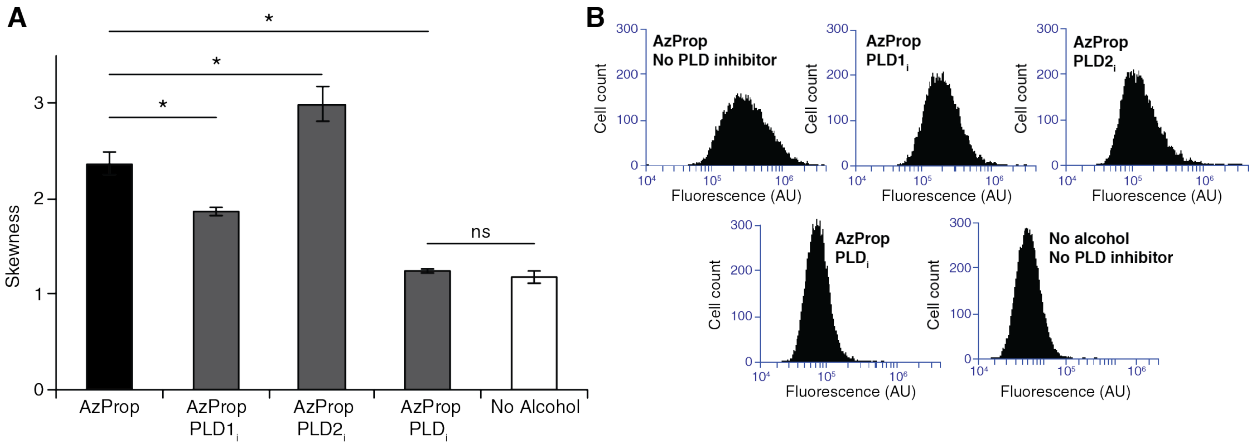

Figure S10. Quantatitive population analysis of cellular heterogeneity in unstimulated PLD activity. HeLa cells were first treated with the indicated PLD inhibitor (PLD_i (FIPI), 750 nM; $PLD1_i$ (VU0359595), 250 nM; $PLD2_i$ (VU0364739), 350 nM) or DMSO vehicle for 30 min, followed by addition of AzProp (1 mM) or no alcohol for 120 min. Cells were then rinsed and incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and analyzed by flow cytometry. (A) Mean skewness for each treatment condition. n = 21 (no inhibitor), 18 (PLD_i, $PLD1_i$, and $PLD2_i$), 12 (no alcohol) biological replicates. *, $p < 0.01$; ns, not significant. (B) Representative population histograms for each treatment condition demonstrating the positive skew of the populations.

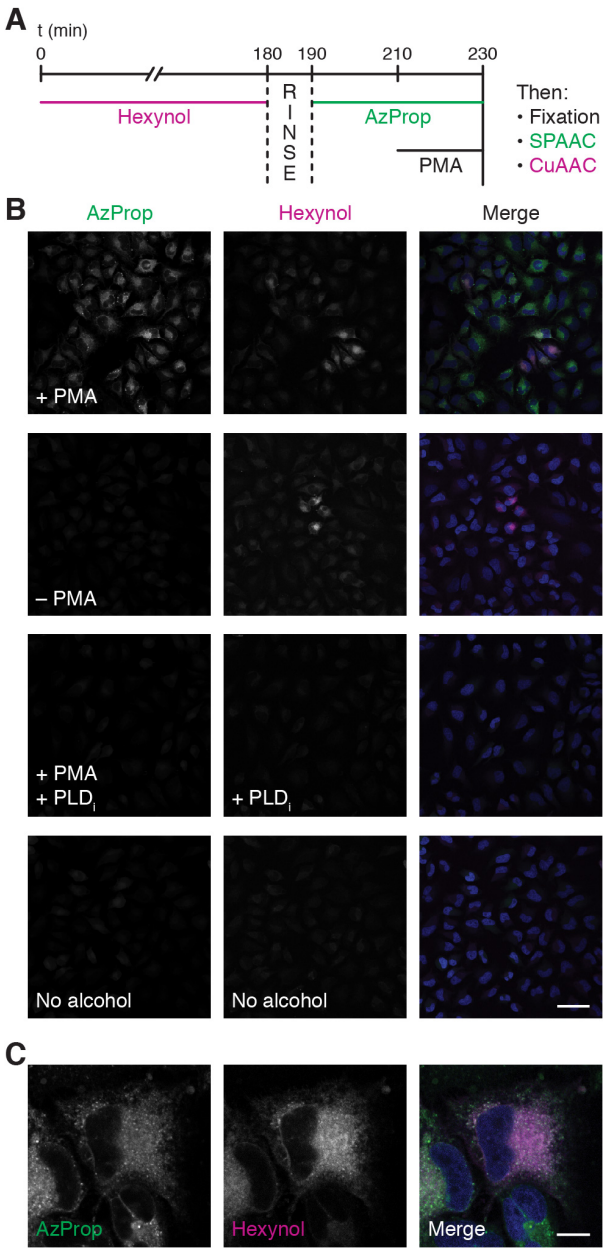

Figure S11. Sequential, two-color imaging of basal and PMA-stimulated PLD activity using labeling with hexynol followed by AzProp. (A) Schematic of experimental setup. HeLa cells were treated with hexynol (1 mM) for 180 min, rinsed for 10 min, and then treated with AzProp (1 mM) for 20 min. Where indicated, cells were then stimulated with PMA (100 nM) in the presence of AzProp for 20 min. Cells were then fixed with paraformaldehyde, sequentially labeled first by SPAAC with **1** and then by CuAAC with an azido tetramethylrhodamine conjugate, mounted in medium containing DAPI, and imaged by confocal microscopy. Where PLD_i is indicated in (B), cells were incubated with FIPI (750 nM) for 30 min prior to hexynol labeling and throughout both alcohol labeling steps. (B–C) Confocal images of cells labeled as described above. In merged images, AzProp-derived fluorescence is in green, hexynol-derived fluorescence is magenta, DAPI is blue; colocalization of AzProp and hexynol appears as white. Shown are single z-slices at low (B) and high (C) magnification. Scale bars: 50 μm (B), 10 μm (C).

Table S1. Masses of phosphatidyl alcohols **3** and **4a–f**.

Phosphatidyl alcohol	Calculated Mass (ESI +)	Observed Mass (ESI +)	Calculated Mass (ESI –)	Observed Mass (ESI –)
3	784.5599	784.5595	782.5454	782.5455
4a	881.6491	881.6489	879.5852	–
4b	886.6069	886.6062	884.5923	884.5919
4c	839.6021	839.5999	837.5876	837.5832
4d	950.7321	950.7318	948.7175	948.7173
4e	882.5967	882.5965	880.5822	880.5816
4f	896.6491	896.6484	894.6432	894.6345

Table S2. Masses of identified phosphatidyl alcohol species from cellular labeling with AzProp and CuAAC tagging with **2a**.

Lipid Species	Calculated Mass	Observed Mass
30:1	799.5709	799.5703
30:0	801.5865	801.5875
32:2	825.5865	825.5867
32:1	827.6022	827.6030
32:0	829.6178	829.6170
34:2	853.6178	853.6179
34:1	855.6335	855.6325
36:5	875.6022	875.6002
36:4	877.6178	877.6180
36:3	879.6335	879.6324
36:2	881.6491	881.6486
36:1	883.6648	883.6616
36:0	885.6804	885.6686

Table S3. Masses of identified phosphatidic acid species from cellular extracts.

Lipid Species	Calculated Mass	Observed Mass
30:1	617.4183	617.4165
30:0	619.4339	619.4333
32:2	643.4339	643.4344
32:1	645.4496	645.4495
32:0	647.4652	647.4648
34:2	671.4652	671.4653
34:1	673.4809	673.4809
36:5	693.4496	693.4500
36:4	695.4652	695.4653
36:3	697.4809	697.4804
36:2	699.4965	699.4963
36:1	701.5122	701.5122
36:0	703.5278	703.5191

Legends for Movies S1–S9

Movie S1. Z-stack of colocalization analysis of AzProp labeling with an ER marker. HeLa cells were transfected with STIM1-mRFP, a marker of the ER. After 24 h, the cells were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy. Shown in the movie is a z-stack. Phosphatidyl alcohol fluorescence is shown in green and STIM1 is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

Movie S2. Z-stack of colocalization analysis of AzProp labeling with marker of the Golgi apparatus. HeLa cells were transfected with mCherry-P4M, a marker of structures positive in phosphatidylinositol 4-phosphate, principally the Golgi apparatus and the plasma membrane[1]. After 24 h, the cells were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy. Shown in the movie is a z-stack. Phosphatidyl alcohol fluorescence is shown in green and P4M is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

Movie S3. Time-lapse assessing fluorescence recovery after photobleaching of IMPACT label in the ER. HeLa cells expressing a marker of the ER (STIM1-mRFP) were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy for a total of 220 s with an interval of 2.01 s in between each frame. Photobleaching occurs in between the third and fourth frame. The orange circle indicates the bleached region while the blue circle was monitored, but unbleached (see Figure S7 for traces of fluorescence intensity in these regions). We note that images are displayed using fluorescence settings in which some regions of the IMPACT label appear overexposed in order to facilitate observation of the lower intensity of the signal in the desired regions. Scale bar: 5 μm.

Movie S4. Time-lapse assessing fluorescence recovery after photobleaching of IMPACT label in the Golgi apparatus. HeLa cells expressing a marker of the Golgi apparatus (mCherry-P4M) were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy for a total of 20 min with an interval of 20 s in between each frame. Photobleaching occurs in between the third and fourth frame. The orange circle indicates the bleached region while the blue circle was monitored, but unbleached. Scale bar: 5 μm.

Movie S5. Time-lapse of colocalization analysis of AzProp labeling with a lysosomal marker. HeLa cells were transfected with LAMP1-mRFP, a marker of lysosomes and late endosomes. After 24 h, the cells were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy, with images acquired at the indicated intervals using two-channel, line-switching mode. Phosphatidyl alcohol fluorescence is shown in green and LAMP1 is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

Movie S6. Time-lapse of colocalization analysis of AzProp labeling with a marker of early endosomes. HeLa cells were transfected with Rab5-mRFP, a marker of early endosomes. After 24 h, the cells were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy, with images acquired at the indicated intervals using two-channel, line-switching mode. Phosphatidyl alcohol fluorescence is shown in green and Rab5 is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

Movie S7. Time-lapse of colocalization analysis of AzProp labeling with mCherry-tagged PLD1. HeLa cells were transfected with mCherry-PLD1. After 24 h, the cells were labeled with AzProp (1 mM) for 20 min and then stimulated with PMA (100 nM) for 20 min. Cells were then incubated with **1** (1 μM) for 10 min, rinsed for 15 min, and imaged by confocal microscopy, with images acquired at the indicated intervals using two-channel, line-switching mode. Phosphatidyl alcohol fluorescence is shown in green and PLD1 is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

Movie S8. Time-lapse of colocalization analysis of GFP-PLD1 with a lysosomal marker. HeLa cells were transfected with GFP-PLD1 and LAMP1-mRFP. After 24 h, the cells were imaged by confocal microscopy, with images acquired at the indicated intervals using two-channel, line-switching mode. For clarity, PLD1 is shown in green and LAMP1 is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

Movie S9. Time-lapse of colocalization analysis of mCherry-PLD1 with a marker of early endosomes. HeLa cells were transfected with mCherry-PLD1 and Rab5-GFP. After 24 h, the cells were imaged by confocal microscopy, with images acquired at the indicated intervals using two-channel, line-switching mode. For clarity, PLD1 is shown in green and Rab5 is shown in magenta in the merged images (right), where colocalization appears as white. Scale bar: 10 μm.

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. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Echelon Biosciences, 5-fluoro-2-indolyl deschlorohalopemide (FIPI) was purchased from Cayman Chemical; phorbol 12-myristate 13-acetate (PMA) was purchased from Santa Cruz Biotechnology; *Arachis hypogaea* PLD, azido tetramethylrhodamine (Azide-fluor 545), and alkyne-rhodamine 110 (Fluor 488-Alkyne/Alk-Rhod110) were purchased from Sigma-Aldrich. HPLC analysis was performed on a Shimadzu LC-20AR HPLC equipped with an SPD-20AV UV/Vis detector, an RF-20A fluorescence detector, and a Phenomenex Luna silica 3 μm 100 Å 25 cm x 4.6 mm column. LCMS analysis was performed on an Agilent 6230 electrospray ionization–time-of-flight (ESI–TOF) MS coupled to an Agilent 1260 HPLC equipped with a Zorbax Rx-Sil normal phase silica column (2.1 x 50 mm, 1.8 μm). Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), and 0.05% trypsin-EDTA were purchased from Corning. Fetal bovine serum (FBS) and Lipofectamine 2000 were purchased from Thermo Fisher. Confocal 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, and two GaAsP PMT detectors. Super-resolution structured illumination microscopy (SR-SIM) imaging was performed on a Zeiss ELYRA super-resolution microscope equipped with a 40X 1.4 NA objective and 405, 488, 561, and 640 nm solid-state lasers. Image analysis was performed using the Zeiss Zen Blue 2.3, Zeiss Zen Black, and FIJI/ImageJ software

packages. Flow cytometry was performed on a BD Accuri C6 flow cytometer, and analysis was performed using the BD Accuri C6 analysis software.

Synthetic procedures

3-azido-1-propanol[2], BODIPY-cyclooctyne **1**[3], and *N*,*N*,*N*-trimethylprop-2-yn-1-aminium bromide **2a**[4] were prepared according to literature procedures. 2-azidoethanol[5], 4-azido-1-butanol[6], and 5-azido-1-pentanol[6] were prepared using a method analogous to that described in ref. 2.

Cell culture

HeLa cells were maintained in a 5% CO_2, water-saturated atmosphere at 37 °C and grown in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Cell densities were maintained between 10^5 and $1.6x10^6$ cells/mL.

Plasmids

Plasmids were obtained from the following sources: STIM1-mRFP was obtained from Barbara Baird (Cornell University); mCherry-P4M-SidM was a gift from Tamas Balla (a marker of structures positive in phosphatidylinositol 4-phosphate, principally the Golgi apparatus and the plasma membrane[1]; Addgene plasmid # 51471); Rab5-GFP and Rab5-mRFP were obtained from Yuxin Mao (Cornell University); LAMP1-mRFP was obtained from Pietro De Camilli (Yale University). mCherry-PLD1 and GFP-PLD1 were made by amplifying a PLD1 ORF clone (NCBI accession # BC068976) from the ORFeome 8.1 library (a gift of Haiyuan Yu, Cornell University)

and inserting the gene into the mCherry-C1 and GFP-C1 vectors (Clontech) using SalI and ApaI restriction sites.

PLD1 cloning primers

Sense: CAAGTCGACATGTCACTGAAAAACGAGCCACG

Antisense: CAGGGCCCTTAAGTCCAAACCTCCATGGGCA

Generation of phosphatidyl alcohols by *in vitro* PLD reactions

A 1.5 mL conical 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 AzProp 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 and saved, an additional 250 μL of chloroform was added to the remaining aqueous layer, and the sample was vortexed and centrifuged as before. The two organic layers were then combined and dried under a stream of N_2.

Samples for analysis by LCMS were subjected to CuAAC labeling based on a modified version of the method described by Thiele et al.[7] 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 (e.g., for 10 reactions, master mix contains 1 mg of alkyne **2a–f** (representing a substantial molar excess in

all cases), 60 μL of 10 mM [acetonitrile]$_4$CuBF$_4$ in degassed acetonitrile, and 240 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 was 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 analysis. Phosphatidyl alcohols were detected by direct injection onto an Agilent 6230 TOF–MS using chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5) as the carrier solvent. For detection, the MS was equipped with a dual ESI source operating in positive mode, acquiring in extended dynamic range from m/z 100–1700 at one spectrum per second; gas temperature: 325 ºC; drying gas 10 L/min; nebulizer: 20 psig; fragmentor 300 V. Separate injections were also analyzed in negative mode, where the MS settings were identical to those described above with the following changes: dual ESI source in negative mode; fragmentor 250 V.

Detection of cellular PLD activity by HPLC analysis

HeLa cells (500,000) were seeded in a 60-mm dish and allowed to grow overnight. Cells were first treated with the indicated PLD inhibitor (FIPI, 750 nM; VU0359595, 250 nM; VU0364739, 350 nM) from 100–1000X DMSO stock solutions or the appropriate amount of DMSO for 30 min in Tyrode's-HEPES buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/mL glucose, 1 mg/mL bovine serum albumin, 20 mM HEPES, pH 7.4). The indicated azidoalcohol was then added directly to the buffer to a final concentration of 1 mM, and the cells were incubated for a further 20 min. For PMA stimulation experiments, PLD activity was then stimulated by the addition of PMA to a final concentration of 100 nM, and the cells were again incubated for a further 20 min. For basal PLD activity experiments, PMA treatment was omitted and total azido alcohol treatment was 2 h. The treatment buffer was then aspirated, and the cells

were rinsed with 1 mL cold PBS. The cells were then scraped from the dish and subjected to a modified Bligh-Dyer extraction. In brief: 100 μL of cold PBS, 125 μL of cold acetic acid (20 mM in water), and 250 μL of cold methanol were added to the aspirated dish. The cells were then scraped off the dish and transferred to 1.5 mL conical tubes. To the suspension was added 250 μL of chloroform. The mixture was then vortexed for 1 min and centrifuged at 16,000 x g for 2 min. The organic layer (bottom) was removed and placed in a separate conical tube, and a further 250 μL chloroform was added to the remaining aqueous layer. The vortexing and centrifugation steps were repeated, and the two organic layers were combined and dried under a stream of N_2.

Samples for analysis by HPLC with fluorescence detection were subjected to SPAAC reaction with BODIPY-cyclooctyne **1** (72 μM in a 24:6:7 mixture of ethanol:acetonitrile:chloroform) overnight at 42 °C. These reactions were then diluted to a total volume of 200 μL by addition of 128 μL of a 73:23:3 mixture of chloroform:methanol:water and filtered (0.45 μm). Analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (95:7:0.5) and solvent B was chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a linear gradient from 100% A to 100% B over 25 min.

Samples for analysis by LCMS were subjected to CuAAC labeling based on a modified version of the method described by Thiele et al.[7] Briefly, to the lipid residue in a 1.5 mL conical tube was added 7 μL of degassed chloroform followed by 30 μL of a reaction master mix (e.g., for 10 reactions, master mix contains 1 mg of alkynyl ammonium salt **2a** (representing a substantial molar excess), 60 μL of 10 mM $[acetonitrile]_4CuBF_4$ in degassed acetonitrile, and 240 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 was 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 analysis. LCMS analysis was performed using normal phase HPLC with a binary gradient elution system where solvent A was chloroform:methanol:ammonium hydroxide (85:15:0.5) and solvent B was chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5). Separation was achieved using a linear gradient from 100% A to 100% B over 9 min. Phosphatidyl alcohol species were detected using a dual ESI source operating in positive mode, acquiring in extended dynamic range from m/z 100–1700 at one spectrum per second; gas temperature: 325 ºC; drying gas 10 L/min; nebulizer: 20 psig; fragmentor 300 V. Endogenous phosphatidic acid species were detected using identical MS settings as described above with the following changes: dual ESI source in negative mode; fragmentor 250 V.

Live-cell imaging and flow cytometry

HeLa cells (150,000 cells) were seeded in a 35-mm, glass-bottomed imaging dish (MatTek) and allowed to grow overnight. The cells were then transfected with the indicated plasmid using Lipofectamine 2000 as per the manufacturer's instructions. After 24 h, the cells were treated with the indicated PLD inhibitor (FIPI, 750 nM; VU0359595, 250 nM; VU0364739, 350 nM) from DMSO stock solutions (100–1000X) or the appropriate amount of DMSO alone for 30 min in Tyrode's-HEPES buffer. To the buffer was then added the indicated azidoalcohol to a final concentration of 1 mM, and the cells were incubated for a further 20 min. For PMA stimulation experiments, PLD activity was then stimulated by the addition of PMA to a final concentration of 100 nM, and the cells were again incubated for a further 20 min. For basal PLD activity experiments, PMA treatment was omitted and total azido alcohol treatment was 2 h. The cells were then rinsed three times with PBS and subjected to reaction with BODIPY-cyclooctyne **1** (1 or 2

μM in Tyrode's-HEPES buffer) for 10 min at 37 °C. Following the reaction, the cells were rinsed three times with PBS and placed in 2 mL of Tyrode's-HEPES buffer. Excess unreacted fluorophore was allowed to wash out of the cells for 15 min by incubating cells again at 37 °C, and cells were then immediately imaged by confocal microscopy. Multicolor images and time-lapse images were obtained in two-channel, line-switching mode. Z stacks were taken with 0.45 μm sectioning. In FRAP experiments, bleaching was performed using the 488 nm laser at 100% power, 1 μs dwell time, five iterations. SR-SIM images were processed using default parameters in the Zeiss Zen Black software, and a channel alignment was performed using data acquired on the same instrument of 0.5 μm multi-channel beads. For flow cytometry analysis, cells were labeled as described above, and following the final rinse, cells were resuspended in PBS + 0.5% FBS, kept at 4 ºC, and analyzed by flow cytometry, gating on at least 10,000 live cells as ascertained by forward/side scatter analysis.

Fixed-cell imaging

For experiments shown in Figure S6A, cells were transfected with the appropriate plasmid and labeled using the live-cell SPAAC protocol as described above but, prior to imaging, fixed in 3.7% paraformaldehyde for 20 min, rinsed three times with PBS, mounted in ProLong Diamond with DAPI, and allowed to solidify in the dark overnight. For images in the top panels of Figure S4 and in Figure S6B, HeLa cells (40,000 cells) were seeded on 12 mm glass coverslips in a 12-well plate and allowed to grow overnight. After 12 h, the cells were treated with the indicated azidoalcohol to a final concentration of 1 mM, and the cells were incubated for a further 20 min. PLD activity was then stimulated by the addition of PMA to a final concentration of 100 nM, and the cells were again incubated for a further 20 min. The cells were rinsed three times with PBS

and fixed in 3.7% paraformaldehyde for 20 min. Following fixation, the cells were rinsed three times with PBS and twice with TBS. CuAAC labeling was then performed as previously described[8], 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 alkyne-rhodamine 110, 1 mM $CuSO_4$, and 50 mM sodium ascorbate. The cells were rinsed three times with TBS, three times with 500 mM sodium chloride, and three times with PBS. The cover slips were then mounted on glass microscope slides in ProLong Diamond with DAPI and allowed to solidify in the dark overnight at room temperature.

Two-color labeling of basal and stimulated PLD activity for imaging

For experiments shown in Figures 4 and S11, HeLa cells (40,000 cells) were seeded on 12-mm round cover slips (#1.5) in the well of a 12-well dish and allowed to grow overnight. After 24 h, the cells were treated with the indicated PLD inhibitor (FIPI, 750 nM; VU0359595, 250 nM; VU0364739, 350 nM) from DMSO stock solutions (100–1000X) or the appropriate amount of DMSO alone for 30 min in media. To the cells was then added the indicated alcohol (AzProp or hexynol) to a final concentration of 1 mM, and the cells were incubated for 3 h. The media was aspirated and the cells were rinsed three times with PBS, then for 10 min at 37 ºC with Tyrode's-HEPES buffer, and then three more times with PBS. The second alcohol (hexynol or AzProp, 1 mM) was then added to the cells in Tyrode's-HEPES for 20 min, and, where indicated, PMA was then added to the cells in the alcohol solution for a further 20 min. The cells were rinsed three times with PBS and fixed in 3.7% paraformaldehyde for 20 min. After three rinses with PBS, the cells were subjected to SPAAC reaction by deposition of the cover slip on top of a 50 μL droplet of **1** (1 μM in PBS) for 10 min at room temperature in the dark. Following the reaction, the cells

were rinsed three times with PBS and twice with TBS. CuAAC labeling was then performed as previously described[8], 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 azido tetramethylrhodamine, 1 mM $CuSO_4$, and 50 mM sodium ascorbate. The cells were rinsed three times with TBS, three times with 500 mM sodium chloride, and three times with PBS. The cover slips were then mounted on glass microscope slides in ProLong Diamond with DAPI and allowed to solidify in the dark overnight at room temperature.

Statistical analysis

Graphing and statistical analysis was performed in Microsoft Excel and RStudio. Skewness calculations were carried out using the moments package in RStudio following data processing. For skewness calculations, the raw FACS data was processed by gating on live cells as ascertained by forward/side scatter and further removal of data points outside the (population mean ± 4(standard deviation)). Error bars represent SEM, and all experiments were performed in at least three biological replicates.

Safety statement

No unexpected or unusually high safety hazards were encountered.

Supporting Information References

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