Supporting Information

Single-Molecule Analysis of Lipid-Protein Interactions In Crude Cell Lysates

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SUPPLEMENTARY METHODS

Antibodies and Reagents. Antibodies were obtained from commercial sources as follows: biotinylated rabbit anti-GFP and mouse anti-GFP for immunoblotting from Rockland Immunochemicals; biotinylated mouse anti-S473 and rabbit anti-S473 for immunoblotting from Cell Signaling Technology. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1,2-dioleoyl-sn-clycero-3-phopho-(1′-myo-inositol-4′,5′-bisphosphate) (PIP_2), 1,2-dioleoyl-sn-clycero-3-phopho-(1′-myo-inositol-3′,4′,5′-trisphosphate) (PIP_3), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(Cap Biotinyl) (biotinPE) were purchased from Avanti Lipids Inc. Phosphatidylinositol-3-phosphate (PI(3)P) was from Echelon Biosciences Inc. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) was from Invitrogen. Purified GFP protein was from Abcam (ab84191).

Plasmids. The GFP-2xHrs-FYVE construct was previously reported by the Stenmark laboratory.[1] GFP-2xSpo20-PABD[2] was from the Du laboratory at The University of Texas Health Science Center at Houston. The following plasmids were obtained from Addgene: GFP-PLCd-PH (#21179),[3] 2xPH-PLCd-GFP (#35142),[4] GFP-Akt (#39531), and GFP-Akt-PH (#39533).[5] GFP-Akt mutants (T308D/S473D; T308A/S473A; T308A/S473D; T308D/S473A) were created using QuickChange II mutagenesis kit (Stratagene). GFP-1xSpo20-PABD and GFP-1xHrs-FYVE were created by inserting a single copy of Spo20-PABD and Hrs-FYVE, respectively, into a gateway destination plasmid pCDNA5 with AttR1/R2 recombination sites and GFP fused at the N-terminus.

Cell Lysis. HEK293 Cells seeded in 6-well plates were transfected at 70-80% confluence with various plasmids (3 µg each well) using PolyFect following the manufacturer's recommendation (Qiagen). After 24 hr, cells were wash twice in ice-cold PBS and re-suspended in 150 µL per well of detergent-free buffer (40 mM Hepes pH 8.0, 150 mM NaCl, 10 mM ß-glycerophosphate, 10 mM sodioum pyrophostate, 2 mM EDTA, 1x Sigma protease inhibitor cocktail). Cells were lysed by passing through a 25G1 needle eleven times (BD PrecisionGlide) followed by centrifugation for 20 min at 14,000xg to remove cell debris.

Western Blotting. Cell lysates were mixed with 2xSDS sample buffer and boiled for 5 min. Proteins were resolved on SDS-PAGE and transferred onto PVDF membrane (Millipore), followed by incubation with various antibodies. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer). Results were developed on x-ray films and scanned with an Epson scanner (Perfection 2400).

Vesicle Preparation. Lipids in chloroform (0.5 µmol total) were dried under nitrogen gas, and resuspended in 300 µL vesicle buffer (10mM Tris·HCl pH 8.0, 150mM NaCl) to a final concentration of 1.66 mM. Vesicles were formed by water bath sonication followed by probe sonication. Unilamellar vesicles were collected as the supernatant after ultracentrifugation at 194,398xg in a TLA100.3 rotor for 2hr at 25°C. Lipid compositions for the phosphoinositide vesicles were 5% PI(3)P, PIP_2, or PIP_3, 95% phosphatidylcholine (PC) as a carrier, 0.05% biotin-phosphatidylethanolamine (biotin-PE) for immobilization of the vesicles on imaging surface, and 0.1% C-18 DiD for

fluorescence imaging of the vesicles. The composition of PA vesicles was similar as above except with 20% PA and 80% PC. Vesicles were diluted 500-fold to yield ~700 vesicles per 4,500 μm^2, loaded into imaging chamber, and incubated for 20 min before loading of cell lysates.

Single-Molecule Co-localization. The percentage of colocalization between vesicles and proteins was quantified by determining the number of spots, S, and position of each DiD and GFP spot present in the same imaging region. Positions of DiD and GFP spots were determined to 2-pixel accuracy by fitting a Gaussian point spread function. Spots lying within 2-pixel distance (pixel size, 133 nm) were considered to be co-localized. The percentage of GFP-positive spots that overlapped with DiD spots was calculated. To determine false co-localization by chance, two different regions were imaged for DiD and GFP, and similar analysis was performed. Co-localization by chance was observed to be ~6%.

Single-Molecule Analysis for Assembly Plots. After addition of cell lysates into imaging chambers, several movies (frame rate = 100 msec) were recorded at different incubation times. The first 50 frames were taken with 640-laser excitation to localize DiD-labeled vesicles, followed by 488-laser excitation to detect GFP molecules. Single-molecule fluorescent traces obtained from the movies were divided in three different categories: empty vesicles (only DiD signal), bound vesicles (DiD and GFP signals), and non-specifically bound GFP (only GFP signal). The fraction of bound vesicles was calculated as a percentage of total labeled vesicles. GFP signal from the 'bound vesicles' category was further analyzed by Chung-Kennedy filtration algorithm to remove noise. Filtrated traces were manually scored for the number of bleaching steps to determine the number of GFP molecules per vesicle, N_{GFP}. The fluorescence traces were classified as having 1–10 bleaching steps. Number of GFP molecules per vesicle, $N_{GFP,}$ at different incubation times was used to build the assembly plots as a distribution of total labeled vesicles. To determine the stoichiometry of vesicle-free proteins, GFP-LBDs in cell lysates were captured using biotinylated GFP antibodies immobilized to the surface via biotin-NeutrAvidin interaction. Copy number of GFP was determined by single-step photobleaching analysis and classified as having one to four bleaching steps or was discarded if no clean bleaching steps could be identified.

SUPPLEMENTARY FIGURES

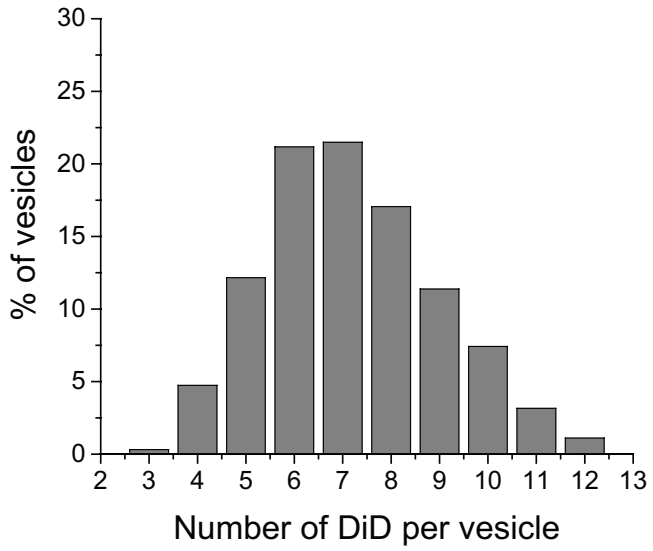

Figure S1. Number of DiD molecules per vesicle. Vesicles (containing biotin-PE) were captured on the surface via biotin-NeutrAvidin interaction. The number of DiD molecules was determined by fluorescent photobleaching analysis after laser excitation at 640 nm.

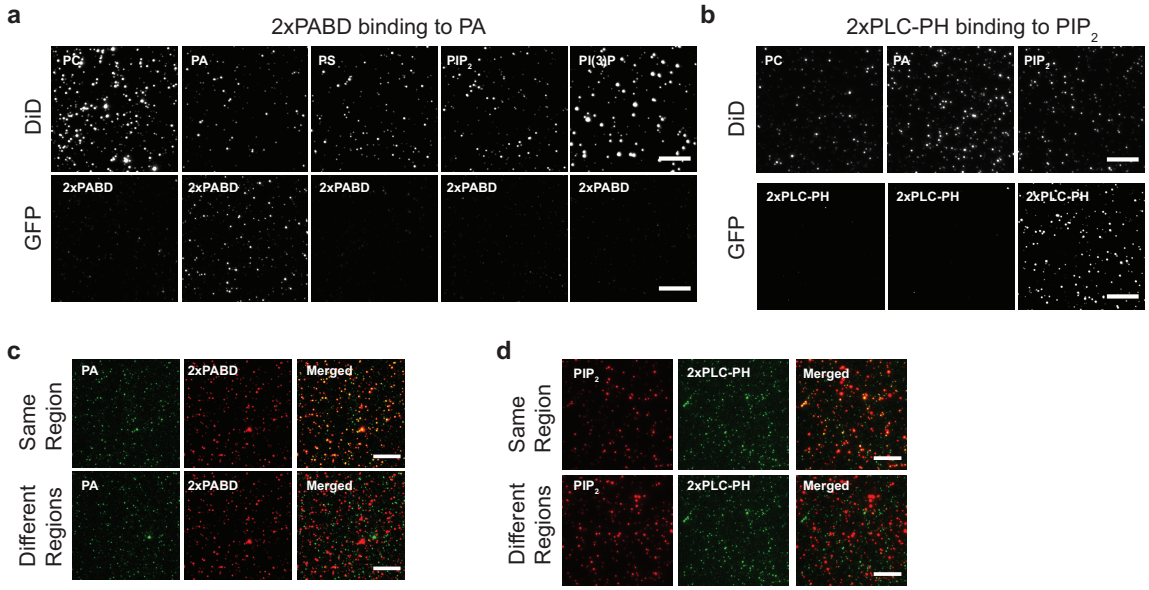

Figure S2. Binding of tandem-repeat LBDs to their target lipids. TIRF images for LBD-vesicle pairs are shown. Vesicles were detected via DiD, and LBDs via GFP. (a) 2xPABD and PA. (b) 2xPLC-PH and PIP_2. (c,d) Overlay of LBDs and vesicles from the same region or different regions. Scale bars: 10μm.

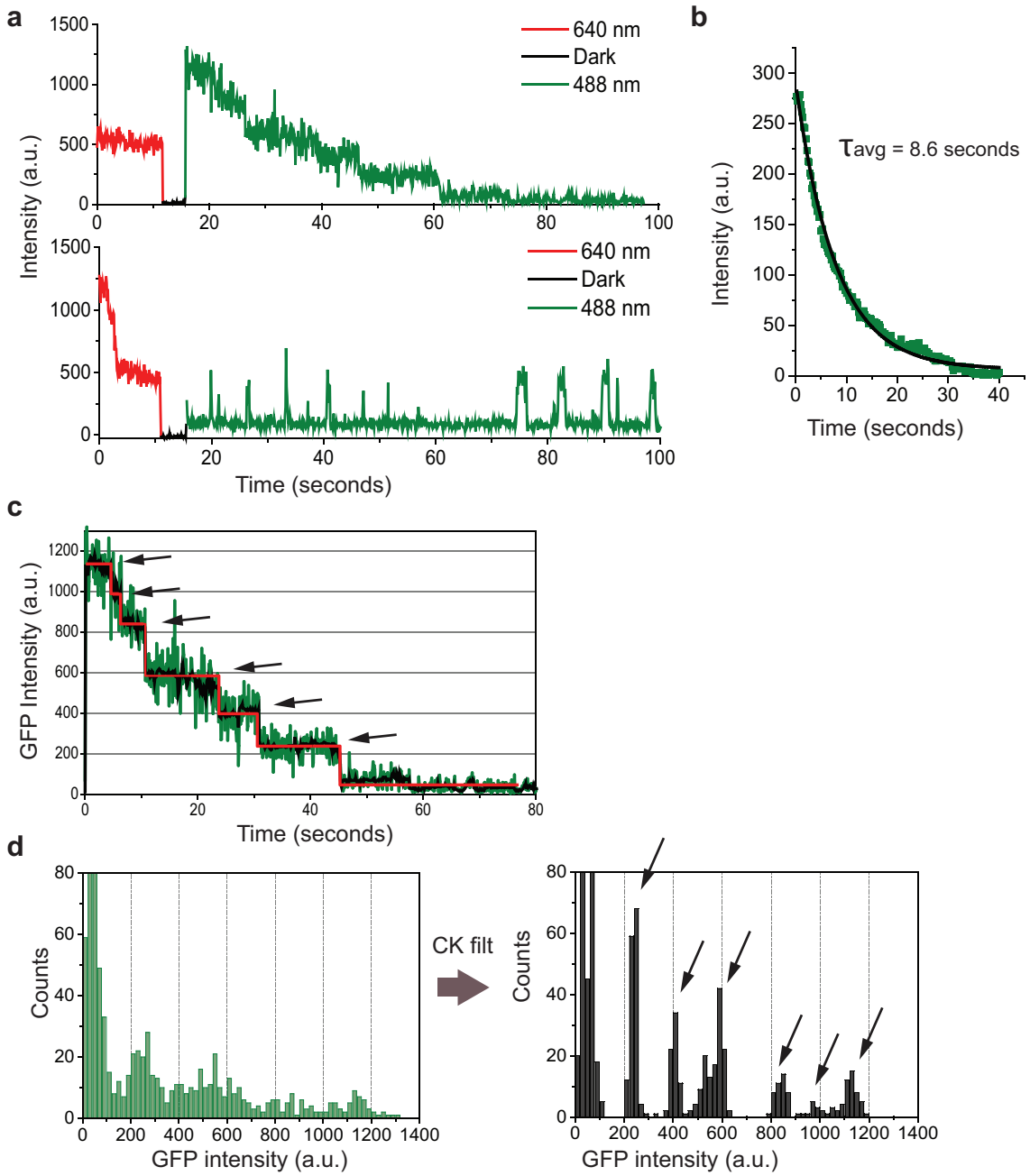

Figure S3. Framework for data analysis. (a) Single-molecule fluorescent traces of DiD-labeled vesicles (red lines, 640 nm excitation) and GFP-LBD (green lines, 488 nm excitation) are shown for stable (top panel) and transient (bottom panel) interactions. (b) GFP photobleaching curve. (c) Identification of 6 photobleaching steps (arrows indicate photobleaching steps) after CK filtration (red line). Raw single-molecule traces are shown in green. (d) Histogram of GFP fluorescence intensity before (left) and after (right) CK filtration. Arrows indicate photobleaching steps.

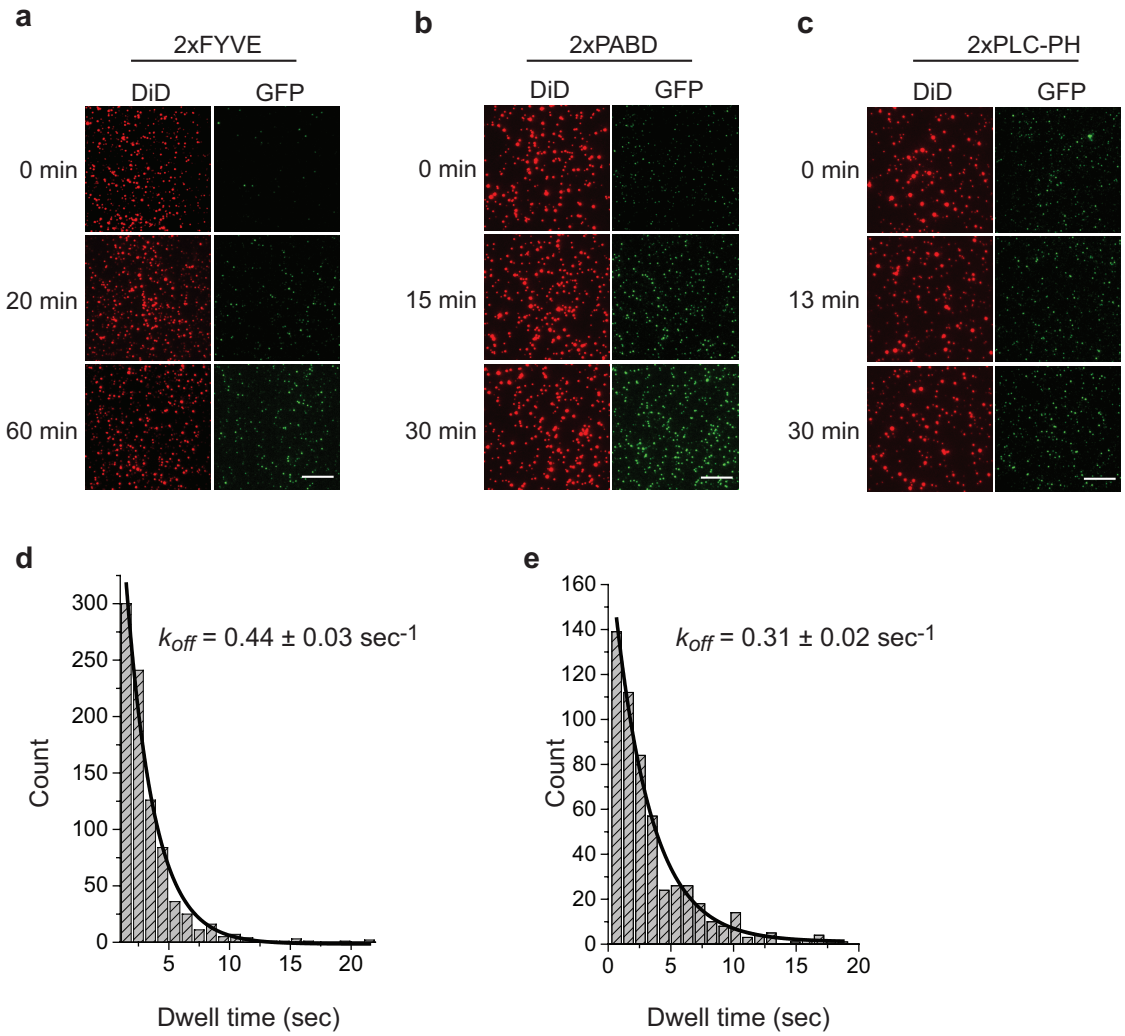

Figure S4. Binding of tandem LBDs to their target lipids. (a) TIRF images of DiD vesicles and GFP-2xFYVE bound to PI(3)P vesicles at 3 incubation times. (b) Similar to (a), for binding of 2xPABD to PA vesicles. (c) Similar to (a), for binding of 2xPLC-PH to PIP_2 vesicles. Scale bars: 10µm. (d, e) Dwell time histograms of (d) 2xFYVE transiently bound to PI(3)P and (e) 2xPLC-PH transiently bound to PIP_2 were fitted to exponential decay to yield dissociation rate constants (k_{off}).

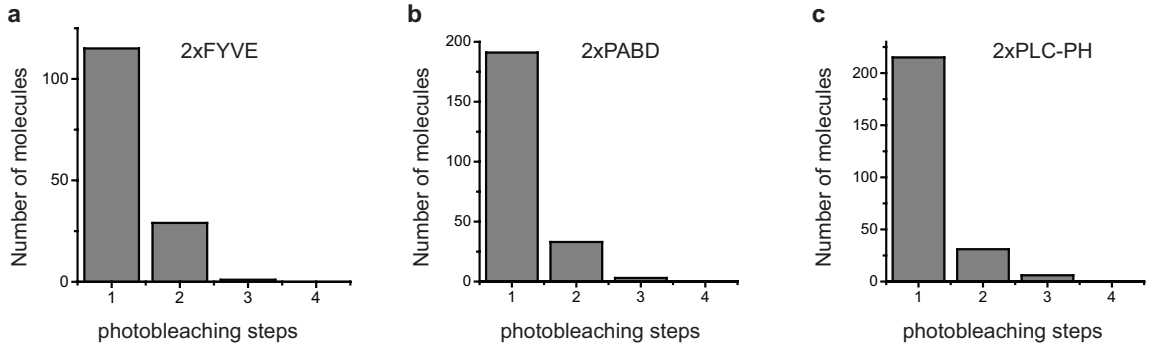

Figure S5. **Tandem-repeat LBDs are mostly monomeric.** GFP-fusion of 2xFYVE (a), 2xPABD (b), and 2xPLC-PH (c) were captured from cell lysates using a biotinylated anti-GFP antibody, followed by GFP photobleaching step analysis.

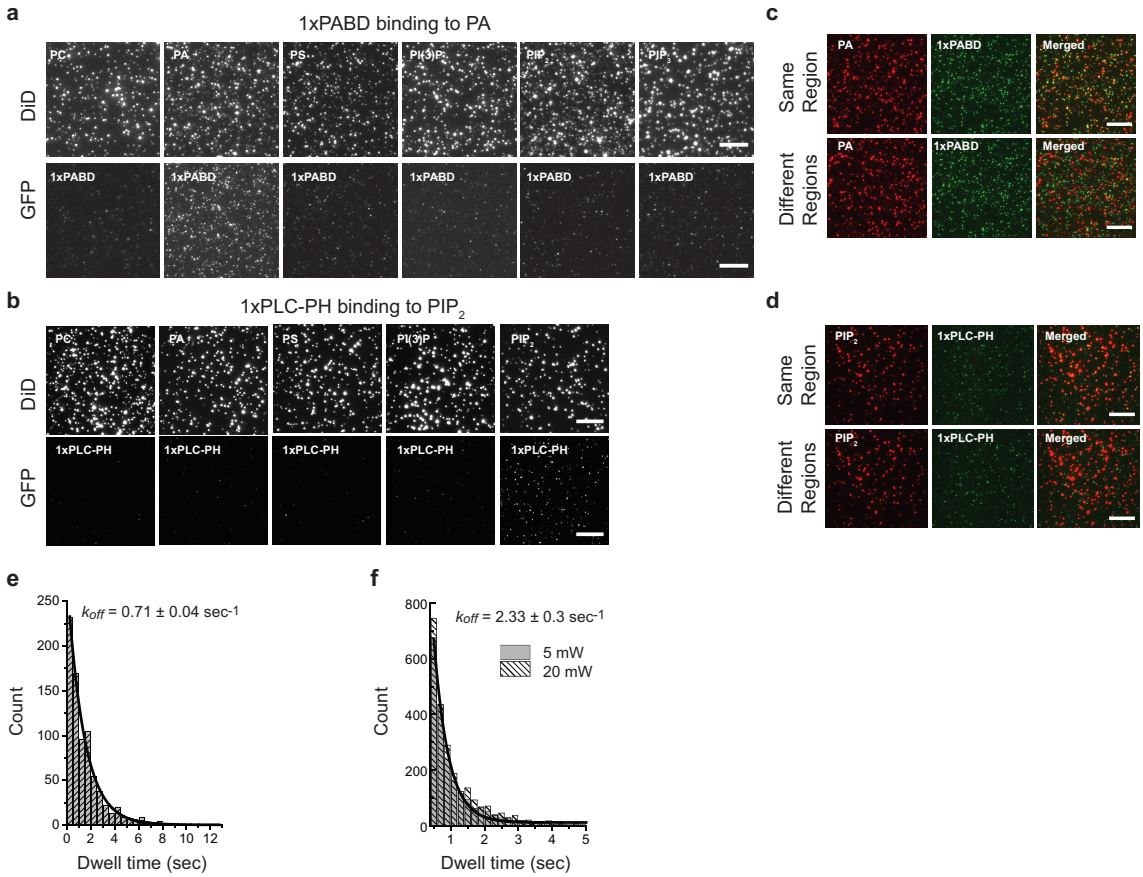

Figure S6. Binding of single-copy LBDs to their target lipids. TIRF images for 1xLBD-vesicle pairs are shown. Vesicles were detected via DiD, and 1xLBDs via GFP. (a) 1xPABD and PA. (b) 1xPLC-PH and PIP_2. (c,d) Overlay of 1xLBDs and vesicles as indicated from the same region or different regions. Scale bars: 10µm. (e) Dwell time histogram of 1xPABD transiently bound to PA was fitted to exponential decay to yield dissociation rate constant (k_{off}). (f) Similar to (e) for 1xPLC-PH transiently bound to PIP_2.

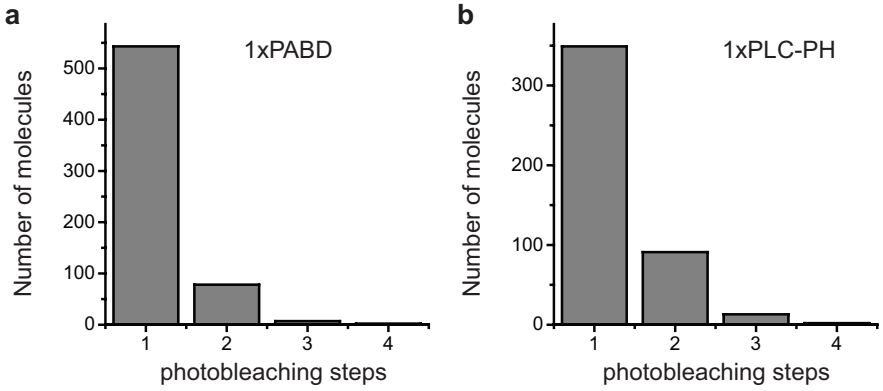

Figure S7. Single-copy LBDs are mostly monomeric. GFP-fusion of 1xPABD (a) and 1xPLC-PH (b) were captured from cell lysates using a biotinylated anti-GFP antibody, followed by GFP photobleaching step analysis.

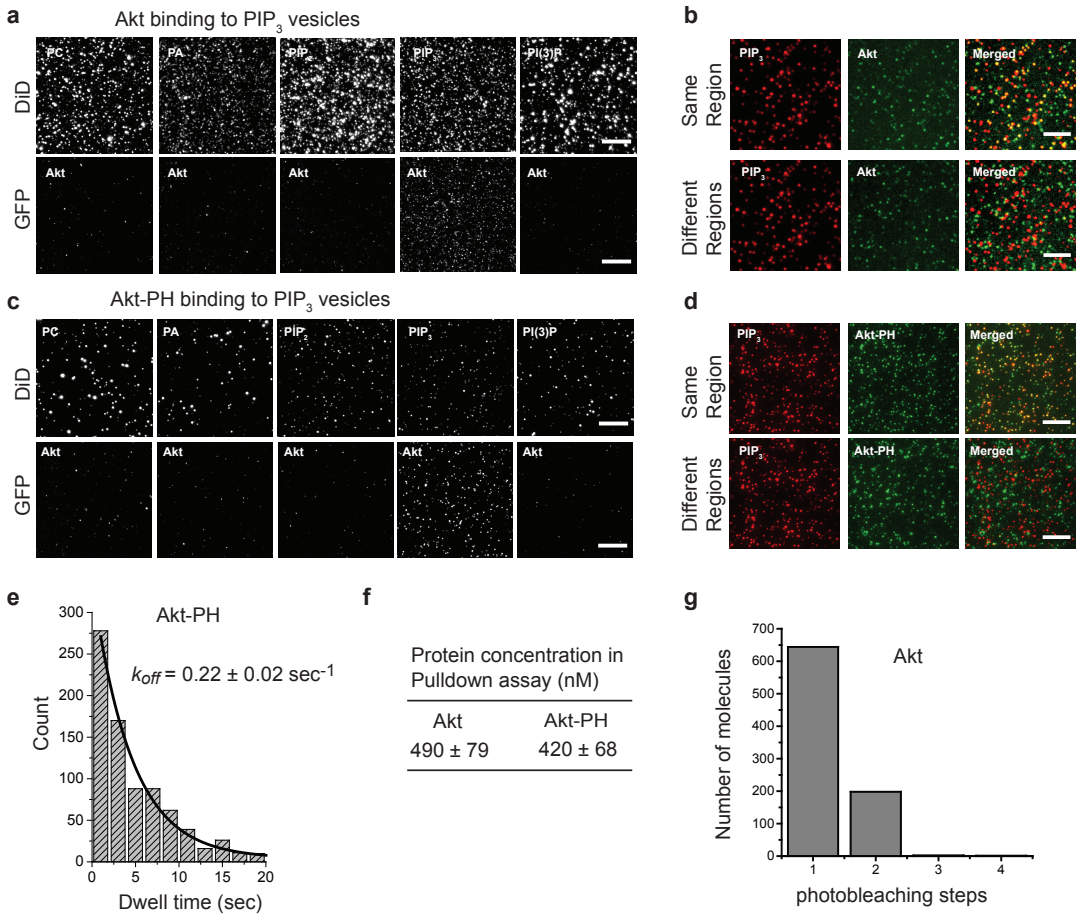

Figure S8. Binding of Akt and Akt-PH to PIP_3 vesicles. (a) TIRF images for Akt (GFP) and PIP_3 (DiD) vesicle are shown. (b) Overlay of Akt and PIP_3 images from the same region or different regions. (c) TIRF images for Akt-PH (GFP) and PIP_3 (DiD) vesicle are shown. (d) Overlay of Akt-PH and PIP_3 images from the same region or different regions. (e) Dwell time histogram of Akt-PH transiently bound to PIP_3 vesicles were fitted to exponential decay to yield the dissociation rate constant (k_{off}). (f) The concentrations of Akt and Akt-PH in the pulldown assays were determined by single-molecule pulldown of GFP, with purified recombinant GFP as a reference. (g) GFP-Akt captured by anti-GFP antibody was analyzed for photobleaching steps. Scale bars: 10μm.

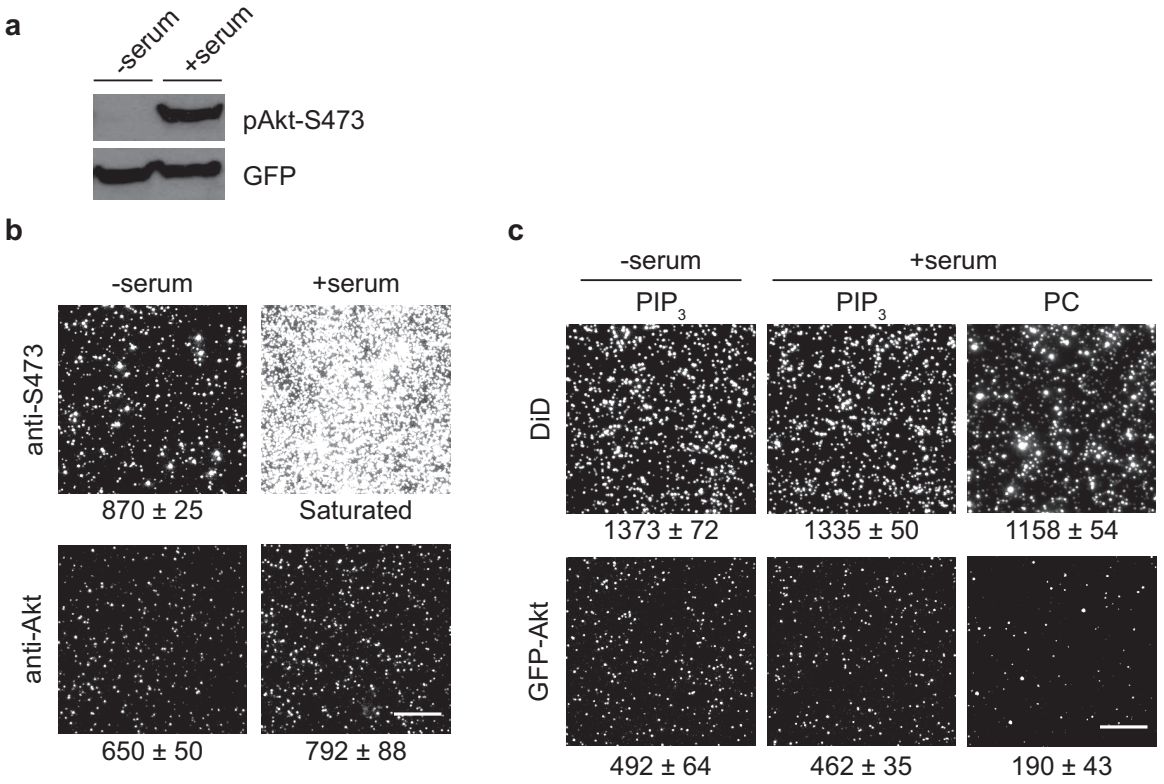

Figure S9. Serum stimulation does not affect Akt-PIP_3 interaction. Cells were serum-starved overnight and then stimulated with 10% FBS for 30 min prior to lysis. (a) Expression and S473 phosphorylation of GFP-Akt were determined by western blotting. (b) Akt was captured from cell lysates by either an anti-pS473 antibody or an anti-GFP antibody. (c) Akt was captured from cell lysates by PIP_3 vesicles, with PC vesicles as negative control. The average number of fluorescence spots per imaging area ± SD (n=10 images) is shown under each image.

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