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Supporting Information

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Water-soluble monofunctional perylene and terrylene dyes: powerful labels for single enzyme tracking

Supporting information

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Experimental section:

General: The solvents used are of commercial grade. N,N’-bis(2,6-diisopropylphenyl)-1,6,7,12-tetraphenoxyperylene-3,4:9,10 acid diimide **1** was supplied by BASF AG. Phospholipase A1 (PLA1) was a gift from Novozymes. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipid and used without further purification. 3,3'-dioctadecyloxacarbocyanine perchlorate (DIO) was supplied by Fluka (purity > 99.0%). 5-carboxyfluorescein diacetate single isomer (5-CFDA) was purchased from Molecular Probes. The solutions of 5-CFDA were kept in dimethylsulfoxide (DMSO) (Aldrich 99,9%). Tritom X-100 was purchased from Fluka. ATTO dye 647N NHS ester was supplied by ATTO-Tec.

Column chromatography was performed on silica gel (Geduran Si60, Merck). ^{1}H and ^{13}C NMR were recorded on Bruker Avanche 250, Bruker AMX 300, Bruker DRX 500 and Bruker Avanche 700. FD mass spectra were performed with a VG-Instruments ZAB 2-SE-FDP instrument. MALDI-TOF mass spectra were recorded on a Bruker MALDI-TOF spectrometer. UV/Vis spectra were recorded on a Perkin-Elmer Lambda 9, fluorescence spectra on SPEX Fluorolog 3 spectrometer. For the enzymatic measurements a Hepes buffer solution containing: 10 mM Hepes (Fluka, 99.5%); 30 μM calcium chloride (Sigma 99%); 10 μM EDTA (Sigma 95%); 150 mM sodium chloride (Sigma-Aldrich 99.99%) was used. For adjusting the pH until 8.0 a sodium hydroxide (Sigma-Aldrich 99.998%) solution was used.

The elemental analyses were performed by the Department of Chemistry and Pharmacy of the University of Mainz. Compound **6** was synthesized as described in

the literature.[1, 2] The synthesis of compounds **1**, **1a**, **1b**, **1c**, **2** and **4** is shown on figure S1.

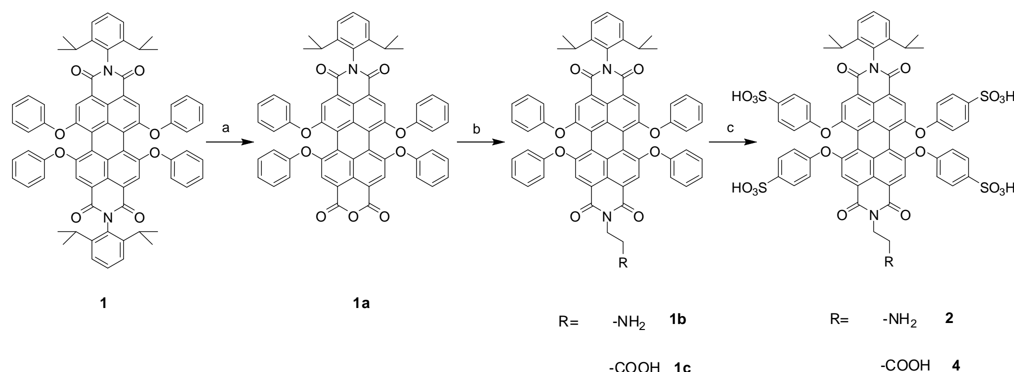

Figure S1. Synthesis of monofunctional water-soluble perylene derivatives.

N-(2,6-diisopropylphenyl)-1,6,7,12-tetraphenoxyperylene-3,4:9,10-tetracarboxy-9,10-monoanhydride-3,4-monoimide 1a:

To a solution of N,N'-bis(2,6-diisopropylphenyl)-1,6,7,12-tetraphenoxyperylene-3,4:9,10-tetracarboxydiimide (7.56 g, 7.00 mmol) in 2-propanol (1 L) was added KOH (148 g, 2.64 mol) dissolved in water (130 mL). The reaction was refluxed under argon at 110°C for 12 h. After cooling to room temperature the solution was poured into HCl/H_2O (60 mL/3.5 L). The precipitate was filtered and dried under vacuum at 60 °C. The solid was dissolved in acetic acid (100 mL) and refluxed at 80 °C for 30 min. Water (150 ml) was added; the precipitate was filtrated and purified over silica gel using dichloromethane as eluent. The product was obtained as a red solid in a yield of 24%. ^{1}H-NMR (250 MHz, DCM-d2 , 300 K): δ = 8.16 (s, 2 H), 8.15(s, 2 H), 7.47 (t, *J* = 7,2 Hz, 1H), 7.35 (d , 2H), 7.33-7.28 (m, 8H), 7.21-7.13 (m, 4 H), 7.03 – 6.97(m, 8H), 2.73-2.62 (sept, *J* = 6,8 Hz, 2H), 1.08 (d, *J* = 6,9 Hz, 12H); ^{13}C-NMR (300 MHz, DCM-d2, 300 K): δ = 163.54, 160.19, 156.17, 155.60, 146.33, 130.50, 130.42, 128.98, 125.29, 125.17, 124.38, 123.70, 122.49, 120.56, 120.39, 120.33, 118.79, 29.43, 24.04 ppm; UV-Vis ($CHCl_3$): λ_{max} (ε) = 443 (14100), 539 (23400), 577 (37300) nm; MS (FD): m/z (rel.int.) 920 $[M+H]^+$; elemental analysis calc'd (%) for $C_{60}H_{41}NO_9$: C 78.33, H 4.49, N 1.52; found C 78.36, H 4.47, N 1.62

N-(2,6-diisopropylphenyl)-N'-(4-aminoethyl)-1,6,7,12-tetraphenoxyperylene-3,4:9,10 tetracarboxydiimide 1b:

1a (200 mg, 0.2456 mmol) and 1,2-ethylenediamine (130 mg, 2.45 mol) were added to toluene (20 mL). The mixture was stirred under argon at 60°C for 3 h. Following the reduction of the solvent, the material was purified over silica gel using dichloromethane/acetone 9/1 and dichloromethane/ethanol 10/3 as eluent resulting in **1b** as a red solid (60% yield). ^{1}H-NMR (250 MHz, DCM-d2, 300 K): δ = 8.16 (s, 2 H), 8.15(s, 2 H), 7.47 (t, *J* = 12.5 Hz, 1H), 7.30 (m, 10H), 7.14 (m, 4 H), 6.98 (m, 8H), 4.16 (t, *J* = 12 Hz, 2H), 2.95 (t, *J* = 12.5 Hz, 2H), 2.65(m, 2H), 1.08 (d, *J* = Hz, 7.5H, 12H); ^{13}C-NMR (300 MHz, DCM-d2, 300 K): δ = 163.94, 163.83, 156.32, 156.14, 155.96, 146.55, 133.57, 131.62, 130.52, 129.95, 125.11, 125.08, 124.54, 123.37, 121.44, 121.16, 120.76, 120.63, 120.48, 120.44, 40.66, 31.16, 29.59, 25.34, 24.23 ppm; UV-Vis ($CHCl_3$): λ_{max} (ε) = 450 (12 348), 538 (21 708), 580 (35 991); Fluorescence ($CHCl_3$, excitation 566 nm) λ_{max} = 620 nm; MS (FD): m/z (rel.int.) 962 (100 %) [M+]; elemental analysis calc'd (%) for $C_{62}H_{47}N_3O_8$: C 77.33, H 4.88, N 4.36; found C 76.87, H 4.54, N 4.00

N-(2,6-diisopropylphenyl)-N'-(4-carboxyethyl)-1,6,7,12-tetraphenoxyperylene-3,4:9,10-tetracarboxydiimide 1c:

1a (500 mg, 0.59 mmol) and 4-aminopropionic acid (532 mg, 5.9 mmol mol) were added to propionic acid (50 mL). The mixture was stirred under argon at 140 °C for 3 h. After cooling the reaction mixture to room temperature, the product was precipitated with water (250 mL). The precipitate was filtered, washed with water and dried under vacuum. Further purification was done by column chromatography on silica gel with dichloromethane/acetone 9/1 and dichloromethane/ethanol 10/3 as eluent to afford **1c** in a yield of 65 %. ^{1}H-NMR (250 MHz, DCM-d2, 300 K): δ = 8.06 (s, 4 H), 7.37 (t, *J* = 7.5 Hz, 1H), 7.19 (m, 10H), 7.03 (m, 4 H), 6.87 (d, *J* = 7.5 Hz, 8H), 4.26 (t, *J* = 10 Hz, 2H), 2.59 (m, 4H), 1.00 (d, *J* = 7.5 Hz, 12H); ^{13}C-NMR (250 MHz, DCM-d2, 300 K): δ = 163.82, 156.37, 146.52, 130.50, 125.12, 125.06, 124.55, 123.35, 123.08, 121.33, 120.48, 31.99, 29.59, 24.22 ppm; UV-Vis ($CHCl_3$): λ_{max} (ε) = 450 (13 298), 536 (23 728), 580 (38 132); Fluorescence ($CHCl_3$, excitation 566 nm) λ_{max} = 620 nm; MS (FD): m/z (rel.int.) 990 (100 %) [M^+]; elemental analysis calc'd (%) for $C_{63}H_{46}N_2O_{10}xH_2O$: C 74.92, H 4.78, N 2.78; found C 74.38, H 4.78, N 2.58

N-(2,6-diisopropylphenyl)-N'-(4-aminoethyl)-1,6,7,12-tetra(4-sulfophenoxy)-perylene-3,4:9,10-tetracarboxydiimide 2:

1b (100 mg, 0.1 mmol) was added to concentrated sulfuric acid (3 mL). The flask was sealed, and the mixture was stirred at room temperature for 16 h. Water was slowly added to the flask and the resulted solution was dialyzed in water. The solution was freeze dried to give the product as a red solid in 97 % yield. ^{1}H-NMR (250 MHz, DMSO-d6, 300 K): δ = 7.97 (s, 2 H), 7.86 (s, 2 H), 7.67 (d, *J* = 7.5 Hz, 5H), 7.57 (d, *J* = 7.5 Hz, 5H), 7.41 (t, *J* = 12 Hz, 1H), 7.38 (d, *J* = 7.5, 2H), 7.01 (d, *J* = 7.5, 4H), 6.93 (d, *J* = 7.5, 4H), 3.08 (t, *J* = 7.5 Hz, 2H), 2.68 (sept, *J* = 10 Hz, 2H), 1.01 (d, *J* = 5 Hz, 12H) ;^{13}C-NMR (250 MHz, DMSO-d6, 300 K): δ = 163.61, 163.34, 157.38, 157.22, 155.42, 155.07, 146.02, 141.20, 141.10, 132.84, 132.33, 130.59, 129.38, 128.15, 128.03, 123.79, 123.42, 123.19, 121.35, 121.08, 120.89, 120.36, 119.07, 38.80, 37.92, 29.07, 23.08 ppm; UV-vis (H_2O): λ_{max} (ε) = 450 (10 850), 534 (21 825), 560 (22 243) ; Fluorescence (H_2O, excitation 566 nm) λ_{max} = 620 nm; MS (MALDI-TOF): m/z (rel. int.) = 1283 (100 %) [M^+]

N-(2,6-diisopropylphenyl)-N'-[N-(4-maleimidobutyryl)aminoethyl]-1,6,7,12-1,6,7,12-tetra(4-sulfophenoxy)-perylene-3,4:9,10-tetracarboxydiimide 3

To a solution of **2** (50 mg, 0.04 mmol) in dry DMF (3 mL) was a dded triethylamine (0.2 mL) and 4-Maleimidobutyric acid N-succinimidyl ester (GMBS) (10 mg, 0.035 mmol). After 5 h the solvents were removed under vacuum. Dichloromethane (10 mL) was added and the solution filtered. The solvent was removed under vacuum and the product was obtained as a red solid in 95% yield. ^{1}H-NMR (300 MHz, DCM-d2, 300 K): δ = 8.15 (s, 2 H), 8.13 (s, 2 H), 7.76 (m, 8H), 7.45 (t, *J* = 12 Hz, 1H), 7.48 (d, *J* = 12 H, 2H), 6.98 (m, 8H), 6.59 (s, 2H), 4.27 (t, *J* = 12 Hz, 2H), 3.53 (m, 2H), 3.41 (t, *J* = 15 Hz, 2H), 2.68 (sept, *J* = 15 Hz, 2H), 2.06 (t, *J* = 15 Hz, 2H), 1.74 (m, 2H), 1.07 (d, *J* = 9 Hz, 12H); ^{13}C-NMR (Spinecho, 300 MHz, DCM-d2, 300 K): δ = 173.34, 173.05, 171.50, 163.81, 163.62, 157.14, 157.08, 155.95, 146.58, 142.97, 142.84, 134.66, 133.44, 133.31, 131.52, 129.96, 128.67, 128.57, 124.54, 123.62, 121.55, 121.06, 119.83, 40.43, 38.65, 37.65, 33.78, 29.58, 26.05, 25.00, 24.25 ppm; UV-Vis (H_2O): λ_{max} (ε) = 450 (8 367), 534 (16 199), 566 (16 569); Fluorescence (H_2O, excitation 566 nm) λ_{max} = 620 nm; MS (MALDI-TOF): (100 %) [M^+] 1447

N-(2,6-diisopropylphenyl)-N'-(3-carboxyethyl)-1,6,7,12-tetra(4-sulfophenoxy)-perylene-3,4:9,10-tetracarboxydiimide 4:

1c (100 mg, 0.1 mmol) was added to concentrated sulfuric acid (3 mL). The flask was sealed, and the mixture was stirred at room temperature for 48 h. Water was slowly added to the flask and the resulting solution was dialyzed in water. The solution was freeze dried to give the product as a red solid in 97 % yield. ^{1}H-NMR (300 MHz, DMSO-d6, 300 K): δ = 7.92 (s, 2 H), 7.88 (s, 2 H), 7.66 (m, 4H), 7.60 (m, 4H), 7.40 (t, *J* = 12 Hz, 1H), 7.27 (d, *J* = 9 Hz, 2H), 6.98 (m, 8H), 4.19 (t, *J* = 12 Hz, 2H), 2.68 (sept, *J* = 10 Hz, 2H), 1.00 (d, *J* = 9Hz, 12H); ^{13}C-NMR (300 MHz, DMSO-d6, 300 K): δ = 172.25, 162.51, 162.14, 155.26, 155.07, 154.78, 145.43, 144.86, 132.33, 132.03, 130.45, 127.72, 127.69, 123.59, 122.48, 119.87, 119.08, 118.99, 118.88, 31.99, 28.19, 23.64 ppm; UV-Vis (H_2O): λ_{max} (ε) = 450 (11 454), 534 (22 510), 564 (25 059); Fluorescence (H_2O, excitation 566 nm) λ_{max} = 622 nm; MS (MALDI-TOF): m/z (rel. int.) = 1311 (100 %) [M^+]

N-(2,6-diisopropylphenyl)-N'-[3-(N-succinimidyl)carboxyethyl]-1,6,7,12-tetra(4-sulfophenoxy)-perylene-3,4:9,10-tetracarboxydiimide 5:

To a solution of **4** (50 mg, 0.038 mmol) in DMF (1 mL), dioxane (1 mL) and water (0.5 mL), was added diisopropylethylamine (DIPEA) (7.4 mg, 0.057 mmol) and N,N,N',N'-tetramethyl (succinimido) uronium tetrafluoroborate (TSTU) (14.3 mg, 0.0475 mmol). After 1 h the mixture was filtered, and the solvents removed under vacuum. Water (2 mL) was added and the solution freeze dried to give the product as red solid in 85% yield. ^{1}H-NMR (300 MHz, DMSO-d6, 300K): δ = 7.92 (s, 2 H), 7.88 (s, 2 H), 7.66 (m, 4H), 7.60 (m, 4H), 7.40 (t, *J* = 12 Hz, 1H), 7.27 (d, *J* = 9 Hz, 2H), 6.98 (m, 8H), 4.30 (t, *J* = 12 Hz, 2H), 2.76 (m, 4H), 2.68 (sept, *J* = 10 Hz, 2H), 1.00 (d, *J* = 9Hz, 12H); ^{13}C-NMR (300 MHz, DMSO-d6, 300 K): δ =169.95, 166.79, 162.58, 162.23, 154.87, 145.50, 144.90, 132.42, 132.10, 130.52, 127.78, 123.67, 123.04, 122.60, 119.96, 199.40, 119.28, 118.97, 53.49, 41.73, 28.28, 25.40, 25.18, 23.72, 18.04, 16.70, 12.35 ppm; UV-Vis (H_2O): λ_{max} (ε) = 450 (9 951), 534 (18 867), 566 (21 071); Fluorescence (H_2O, excitation 566 nm) λ_{max} = 620 nm; MS (MALDI-TOF): 1407 (100 %) [M^+]

N-(2,6-diisopropylphenyl)-N'-[5-(N-succinimidyl)carboxypentyl]-1,6,9,14-tetra(4-sulfonylphenoxy)-terrylene-3,4,11,12-tetracarboxidiimide 7:

To a solution of **6** (100 mg, 0.067 mmol) in DMF (1 mL), dioxane (1 mL) and water (0.5 mL), was added diisopropylethylamine (12 mg, 0.1 mmol) and TSTU (25 mg, 0.083). After 1 h the mixture was filtered, and the solvents removed under vacuum. Water (2 mL) was added and the solution was freeze dried to give the product as a blue-green solid in 80% yield. ^{1}H-NMR (700 MHz, DMSO-d6, 300 K): δ = 9.37 (s, 4H), 8.03 (s, 2H), 7.95 (s, 2H), 7.70 (d, *J* = 6.3 Hz, 4H), 7.67 (d, *J* = 6.3 Hz, 4H), 7.42 (t, *J* = 9 Hz, 1H), 7.29 (d, *J* = 9 Hz, 2H), 7.22 (m, 8H), 3.93 (m, 2H), 2.65 (sept., *J* = 13 Hz, 2H), 2.17 (t, *J* = 7.27 Hz, 2H), 1.58 (m, 2H), 1.50 (m, 2H), 1.29 (m, 2H), 1.01 (d, *J* = 6 Hz, 12H); ^{13}C-NMR (500 MHz, DMSO-d6, 300 K): 174.03, 168.53, 166.25, 162.22, 155.03, 153.60, 145.32, 144.69, 144.61, 130.52, 128.35, 128.22, 127.76, 125.36, 124.35, 123.48, 122.70, 121.95, 121.53, 118.28, 118.13, 40.33, 33.24, 30.99, 30.96, 28.19, 26.83, 25.74, 23.93, 23.49 ppm; UV-Vis (H_2O): λ_{max} (ε) = 426 (4 569), 638 (24 468); MS (MALDI-TOF): 1575 (100 %) [M^+]

N-(2,6-diisopropylphenyl)-N'-[5-(N-(2-(4-maleimidobutyryl)ethyl)-carboxy pentyl]-1,6,9,14-tetra(4-sulfonylphenoxy)-terrylene-3,4,11,12-tetracarboxidiimide 8:

To a solution of **7** (100 mg, 0.063 mmol) in dry DMF (3 mL) was added triethylamine (6.36 mg, 0.063 mmol) and 2-maleimidoethylamine trifluoroacetate salt (15 mg, 0.059 mmol). After 5 h the solvent was removed under vacuum. Dichloromethane (10 mL) was added and the solution filtered. The solvent was removed under vacuum and the product was obtained as a blue-green solid in 90% yield. ^{1}H-NMR (700 MHz, DCM-d2, 300 K): 9.15 (s, 4H), 8.19 (s, 2H), 7.82 (m, 8H), 7.46 (t, *J* = 9 Hz, 1H), 7.31 (d, *J* = 9 Hz, 2H), 7.15 (m, 8H), 7.03 (s, 2H), 3.91 (m, 2H), 3.36 (m, 4H), 2.65 (sept., *J* = 13 Hz, 2H), 2.17 (t, *J* = 7.27 Hz, 2H), 1.58 (m, 2H), 1.50 (m, 2H), 1.29 (m, 2H), 1.08 (d, *J* = 6 Hz, 12H); ^{13}C-NMR (700 MHz, DCM-d2, 300 K): 173.28, 171.53, 163.49, 162.89, 161.47, 161.28, 157.46, 157.42, 154.44, 154.42, 146.78, 143.21, 134.7, 129.03, 128.96, 128.94, 119.15, 116.86, 38.05, 37.98, 36.56, 30.29, 29.67, 26.08, 24.39 ppm; UV-Vis (H_2O): λ_{max} (ε) = 426 (2 782), 638 (16 633); MS (MALDI-TOF): 1665 (100 %) [M^++ 3Na^+]

Fluorescent labelling of phospholipase (PLA 1) with 5:

Phospholipase A1 (2.56 mg) in 10 mM succinic acid, 0,4M NH_4SO_4, pH 6 (400 µL) was reacted at 4 °C for one hour with **5** (1.5 mg) freshly dissolved in 0.01 M $NaHCO_3$. TentaGel HL NH_2 resin (Rapp Polymere GmbH) (20 mg) was added and the solution shaken for 5 min. The mixture containing the polymer resin was applied to a spin column and filtered. The colored solution containing the labeled enzyme was collected and quantified by 10% SDS-PAGE and by fluorescence correlation spectroscopy (FCS). Solution of the labeled phospholipase in 0.01 M $NaHCO_3$ was stored at -80°C.

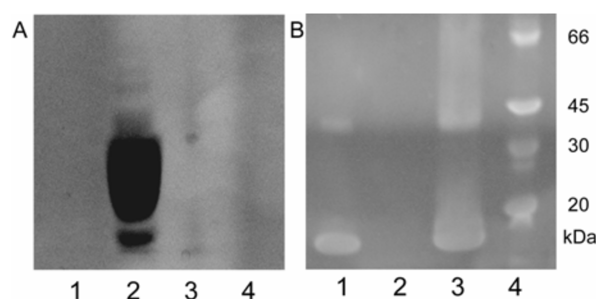

Figure S2. SDS-PAGE electrophoresis of the labeled enzyme. Panel A corresponds to UV-transillumination visualization and Panel B was stained with Coomassie blue. Lane 4 is the protein molecular weight standard. Lane 2 contains compound **5**. Lane 1 is the phospholipase and lane 3 the labeled phospholipase.

Fluorescence Correlation spectroscopy (FCS)

Fluorescence correlation spectroscopy was performed on Compound **5** itself and enzyme labeled with **5** in aqueous solutions. A green He-Ne laser (λ = 543 nm) was used as an excitation source, and the laser light was passed through a 543 nm interference filter. The excitation laser was reflected by a dichroic mirror (z543rdc) and focused into the sample solution by an objective (100x, NA 1.3, Olympus). The excitation power at the focal point was 16.3 µW. The fluorescence was collected by the same objective and filtered with two band pass filters (HQ607/28X, S600/40M). The fluorescence was detected by an avalanche photo diode (Micro Photon Devices, PDM 50CT) and the active area of the detector (50 µm) was used as a pinhole.

Calibration of the FCS setup was carried out with results of sulforhodamine101 aqueous solution. The diffusion time was estimated to be 30 µs and the form factor of

the focal point was to be 5.2 - 6.5 (see the next section). This form factor was used to analyze FCS curve of water soluble PDI and PDI **5** labeled enzyme.

Analysis of FCS curve

The following equation was used to analyze the FCS curve.

$$G(\tau) = 1 + \frac{1}{N}\left\{\frac{1}{(1+\tau/\tau_1)(1+\tau/\tau_1/w^2)^{1/2}}\right\} \quad \textbf{(1)}$$

Where N is number of molecule in the focal volume, w represents the form factor of the focal volume, and τ_D is FCS diffusion time. On this equation the effect of triplet formation is ignore.

The diffusion constant can be calculated from FCS diffusion time using by the following equation,

$$D = \frac{r_0^{\ 2}}{4\tau_D} \quad \textbf{(2)}$$

where r_0 is size factor of the focal volume and τ_D corresponds to the FCS diffusion time. Using the reference compound (the rhodamine derivative in this paper), it is possible to estimate the diffusion constant of the target sample trough the equation (3).

$$D = D_{ref} \times \frac{\tau_{ref}}{\tau_D} \quad \textbf{(3)}$$

(Using $D_{ref} = 3\times10^{-6}$ cm^2/s from http://www.biophysics.org/education/schwille.pdf)

This value is in good agreement with the diffusion coefficient calculated from the Stokes-Einstein equation and molecular weight of the enzyme

$$D = \frac{kT}{6\pi\eta a} \quad \textbf{(4)}$$

where k is the Boltzmann constant, T stands for temperature, η corresponds to viscosity of solution, and a is the radius of object. Once more, using a reference, the diffusion constant of the labeled PLA1 can be estimated by

$$D = D_{ref} \times \frac{a_{ref}}{a} \quad \textbf{(5)}$$

where the radius of enzyme and rhodamine were estimated to be $a \propto (30000)^{\frac{1}{3}} = 31$ and $a_{ref} \propto (606.72)^{\frac{1}{3}} = 8.5$ using their molecular weight, respectively.

The results are summarized in table S1.

Table S1. Diffusion time and constants for the reference and the target molecules

	Diffusion time (τ) (μs)	**Diffusion constant (*D*) (cm^2/s)**
Rodamine 101	30.0	3×10^{-6}
PDI **5**	39.7 ± 1.14	2.3 x 10-6
PDI **5 labelled** PLA1	90.1 ± 1.84	1×10^{-6}

Bulk activity measurements

Bulk activity was measured using SPEX FLUOROLOG 1500 (Spex Industries, Metuchen, NJ). The time base scan experiments were performed with 1Hz acquisition rate. The fluorescent product was excited at 500nm. The fluorescent emission was detected at 520nm.

Preparation of the layers

Phospholipid layers were prepared using the re-hydration method.[3] Briefly, a POPC solution containing 0.1 mol% DiO was spin-coated onto freshly cleaved mica (3000rpm for 40s). After drying under vacuum for 2h, the samples were hydrated by immersion in HEPES buffer (pH 8.0) followed by heating in an oven at 80 ◦C for 3 h. Mica was purchased from SPI supplies (V-4 grade).

Wide-field microscopy

Imaging of individual single enzymes labeled with **5** on the POPC bilayers has been performed using an inverted wide-field epi-fluorescence microscope (IX71, Olympus). The microscope was equipped with a 60x TIRF (total internal reflection fluorescence) objective and a cooled Electron Multiplying-CCD (cascade 512B, Princeton Instruments Inc.). The PLA1 labeled with **5** was excited with 1-5 kW/cm^2 of 532nm laser light (CDPS 532M-50, JDS Uniphase Co.). The enzyme labeled with ATTO 647N was excited using $1kW/cm^2$ of the 632.8 nm from He-Ne laser (1145P, JDSU & Research Electro-Optics). The POPC layers with DiO were imaged using 1-

100 W/cm^2 of the 488-nm line from an Ar^+ laser (Stabilite 2017, Spectra-Physics). The laser lines of two lasers (488 and 532, for PLA1 labeled with **5** or 488 and 633, for PLA1 labeled with ATTO 647N) were guided onto the sample through a dichroic mirror (z532rdc, or z633rdc, respectively, Chroma Technology, Inc.) and aligned in the total internal reflection mode (TIRF mode) in order to reduce background scattering form the aqueous solution. The emission from both the enzyme and the membrane were observed through a long-pass filter (HQ545LP for 532 nm and HQ645LP for 633 nm, Chroma Technology, Inc.). In such a way, most of the fluorescence form the POPC layers is rejected by the corresponding long-pass filter such that the fluorescence of individual enzymes can be detected with sufficient high signal-to-noise ratio. Movies were recorded at 20 frames per second over 1800 frames. Tracking of single enzyme molecules was performed using a home-made routine on Matlab®.[4]

PLA1 labelled with ATTO dye

The enzyme was labelled with ATTO 647N NHS ester by following the procedure suggested by the manufacturer (www.atto-tec.com). Specifically, the concentration of PLA1 solution was adjusted to 1.1 x 10^{-7}M in sodium bicarbonate buffer (pH 8.3). Two-fold molar excess of reactive dye were added to the solution and incubate for 1h at room temperature. The free dye was removed from the solution using a micro dialysis filter with a cut-off of 7,000 MWCO (Pierce).

Figure S3 is a typical fluorescence image of PLA1 with ATTO dye on a bilayer. 632.8 nm light was used to excite the dye molecules. The bright spots correspond to the labelled enzyme. As compared to PDI labelled PLA1 (Fig. 2 and 3), the spots are less bright and less clear in the image, which makes localization more difficult.

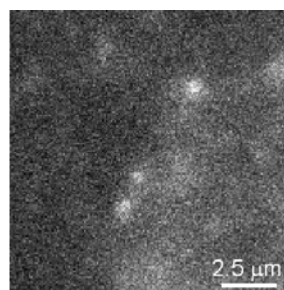

Figure S3. Fluorescence image of individual labelled enzymes on non-labelled POPC layers.

The survival time of the ATTO dye is less than 200 ms with about 1 kW/cm^2 of excitation power. This is considerably lower than the survival time of the PDI label (note that the ATTO is excited using a laser power about 5 times lower than the one use for Compound **5**). With such a short survival time, it is hard to track the diffusive

motion of the enzymes using single particle tracking. Therefore, such short-lived dye is not suitable for such experiments.

Supporting movie: This movie shows the calculated trajectory of a single labeled enzyme diffusing on labeled POPC multilayers.

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