Electronic Supplementary Information

Ambient Condition Oxidation in Individual Liposomes Observed at the Single Molecule Level

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Contents

Materials

All chemicals were used as received without additional purification. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(biotinyl) sodium salt (biotin-DPPE) lipids were purchased from Avanti Polar Lipids (Alabaster, AL). 2,2,5,7,8-Pentamethyl-6-chromanol (PMHC), (±)-α-tocopherol, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (ABAP) were purchased from Sigma-Aldrich. Chloroform and acetone (HPLC grade) and HEPES buffer were purchased from Fischer Scientific. 5 M NaCl stock solution was purchased from Life Technologies (Ambion® brand). Water (molecular biology grade) was acquired from Thermo Scientific HyClone. (South Logan, UT). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) was purchased from Invitrogen Canada (Burlington, ON). Vectabond™ was purchased from Vector Laboratories (Burlington, ON). Poly(ethylene glycol) succinimidyl valerate, MW 5000 (mPEG-SVA) and biotin-PEG-SVA were purchased from Laysan Bio Inc. (Arab, AL). Streptavidin protein was purchased from Life Technologies (Burlington, ON). H_2B-PMHC was prepared as previously reported.[1]

Liposome preparation

Lipid films were prepared by mixing lipid solutions of DOPC and biotin-DPPE lipids (1 mol% vs. DOPC), with (±)-α-tocopherol in the case of the ABAP oxidation experiment, all dissolved in chloroform. Dye solutions (H_2B-PMHC in acetonitrile or DiD in EtOH) were also added to the solution. When liposomes containing PMHC were prepared, H_2B-PMHC was dissolved in an acetonitrile solution containing 2 mM PMHC to prevent premature probe oxidation. Concentrations of antioxidants and dyes per liposomes were calculated by assuming 75000 DOPC lipid molecules per liposome.[2] The lipid solution with additives was then dried under a stream of argon gas to produce a thin film and solvents were completely removed *in vacuo*. The dry lipid film was hydrated by the addition of HEPES buffer (10 mM HEPES, 150 mM NaCl, pH = 7.3) which contained 200 μM of Trolox when desired, to give 20 mM DOPC solutions. After five freeze-thaw cycles (freeze in dry ice, thaw in a sonicator bath) the hydrated lipids were extruded through a 100 nm polycarbonate membrane to yield small unilamellar vesicles. Care was taken to keep the liposomes under dark conditions during the longest steps, namely removal of trace solvents and the freeze/thaw cycles. Exposure to ambient light did not exceed 10 minutes during liposome preparation. Liposome solutions were diluted 2000x with the appropriate HEPES buffer and immediately flowed onto prepared coverslips for confocal imaging.

Coverslip preparation

The coverslips were prepared as described previously.[3] Coverslips (Fischerfinest #1, Fisher Scientific) were first cleaned in piranha solution (1:3 30% H_2O_2:concentrated H_2SO_4 by volume) for 1 h, followed by rinsing with water and acetone. Clean coverslips were then immediately treated with Vectabond™/acetone 2% v/v solutions for 5 min. Amino-modified coverslips were then rinsed with H_2O

and stored for usage within a week. Further surface modifications were made by a mixture of mPEG-SVA and biotin-PEG-SVA in order to prevent non-specific binding and create a platform onto which liposomes could be immobilized. More precisely, clean coverslips were masked with patterned silicone films (Grace Bio-Labs Inc., Bend, OR) and the unprotected area was exposed to a 25% w/w mPEG-SVA aqueous solution containing 0.25% w/w biotin-PEG-SVA and 0.1 M sodium bicarbonate for 3-8 hours. The silicone templates were removed, the excess PEG solution rinsed with water, and the coverslips dried under a N_2 stream. Sample chambers were formed by depositing a pre-drilled adhesive polycarbonate gasket (Grace Bio-Labs Inc.) on the PEG-functionalized side of a coverslip. Silicone inlet and outlet ports (Grace Bio-Labs Inc.) were glued on top of the chambers. Streptavidin (0.2 mg/mL) was flowed in the assembled chamber, and then rinsed with the appropriate HEPES buffer. Finally, a small volume (typically ~ 20 μL) of the liposome sample (ca. 130 pM liposome concentration) was then flowed in the sample chamber and rinsed when adequate surface density was achieved.

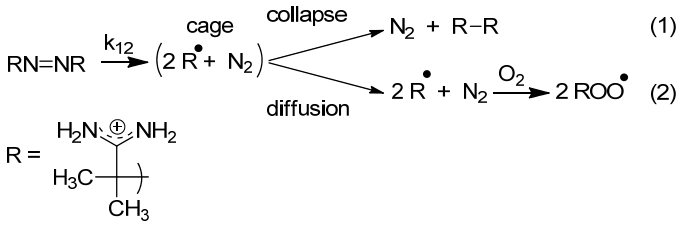
Scanning confocal microscopy imaging of single liposomes

Confocal imaging was performed on an Olympus IX-71 inverted microscope equipped with a closed-loop nanopositioning stage (Nano LP100, Mad City Labs, Madison, WI) used for imaging and sample positioning. Samples were excited by CW laser emission from either the 514 nm line from an Ar^+ laser (43 series, Melles Griot) or the 633 nm output from a HeNe laser (Melles Griot). The linearly polarized laser beams introduced *via* a single mode fibre optic were circularly polarized by a quarter-wave plate and directed by a dichroic beamsplitter (z514-633rdc, Chroma, Rockingham, VT) to the sample through a high numerical aperture oil immersion objective (Olympus U PLAN SAPO 100X, N.A. = 1.40). Fluorescence emission was collected through the same objective, split into two channels by a second dichroic beam splitter (640DCXR Chroma, Rockingham, VT) and directed to two avalanche photodiode detectors (Perkin Elmer Optoelectronics SPCM-AQR-14, Vaudreuil, QC). A 514nm or 633nm holographic Raman notch filter (Kaiser Optical Systems, Ann Arbor, MI) was placed in front of the APDs to block residual laser excitation. The H_2B-PMHC channel emission (λ < 640 nm) was cleaned by a 565/70m bandpass filter (Chroma, Rockingham, VT) while the DiD channel emission (λ > 640 nm) was cleaned by a 685/80m bandpass filter (Chroma, Rockingham, VT). Images were acquired under ambient conditions, with no added imaging buffers.

ABAP oxidation experiment

Following the procedure outlined in the Liposome preparation section, liposomes containing 100 (0.13 mol% vs. DOPC) H_2B-PMHC, 200 (0.27 mol%) DiD, 1600 (2.13 mol%) PMHC, 200 (0.27 mol%) (±)-α-tocopherol and 750 (1 mol%) biotin-DPPE were prepared. The dried lipid film was hydrated with HEPES buffer to which 200 μM of Trolox was added. Following immobilization on the microscope coverslip, HEPES-only buffer was flowed over the sample to remove Trolox from the solution. A buffer consisting of 10 mM PBS (5 mM KH_2PO_4, 5 mM K_2HPO_4, 140 mM NaCl, pH = 7.2) buffer was then flowed to remove HEPES from the solution before inducing oxidation by flowing a 0.25 M ABAP solution in PBS.

PBS buffer was chosen as solvent for the ABAP-induced oxidation as HEPES is a free radical scavenger. The salt concentrations of the PBS buffer were chosen to yield an isotonic solution to the HEPES buffer solution. Images were acquired using laser powers of 300 W/cm^2 at 514 nm and 4 W/cm^2 at 633 nm.



Scheme S1: Production of peroxyl radicals ($ROO^{\bullet}$) following thermolysis of ABAP. ABAP initially decomposes in a first order reaction to yield two geminate carbon centered radicals which may either recombine (Equation 1) or diffuse away (Equation 2). In air equilibrated solutions radicals that escape cage recombination will readily trap molecular oxygen[4] to yield two water soluble peroxyl radicals.

Ambient oxidation experiments

Following the procedure outlined in the Liposome Preparation section, liposomes containing 100 (0.13 mol% vs. DOPC) H_2B-PMHC, 50 (0.07 mol%) DiD and 750 (1 mol%) biotin-DPPE molecules per liposome were prepared. 1600 (2.13 mol%) PMHC molecules per liposome were also added in samples that contained PMHC. The dried lipid film was hydrated with HEPES buffer, which may also contain 200 μM of Trolox depending on the desired conditions. Images were acquired using laser line powers of 6400 W/cm^2 at 514 nm and 200 W/cm^2 at 633 nm, when needed. The images used for determining the center of Gaussian distributions of fluorescence intensity ratios (see Figure S7) were only scanned once with the 514 nm laser excitation during data acquisition.

H_2B-PMHC and DiD spectral discrimination

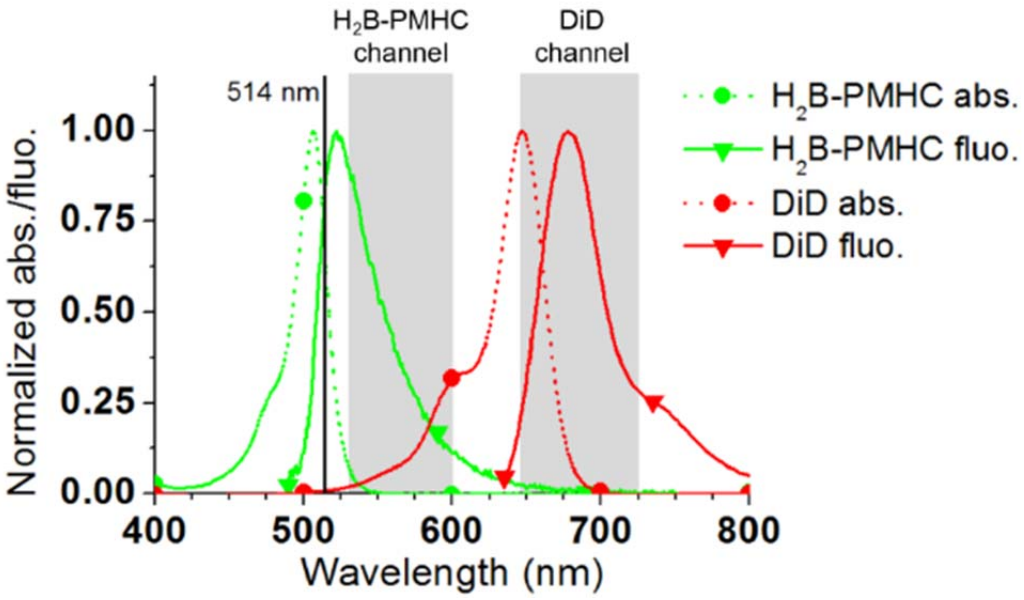


Figure S1. Absorbance (dotted line) and fluorescence (solid line) spectra of H_2B-PMHC$_{(ox)}$ (green) and DiD (red). H_2B-PMHC and DiD show good spectral separation and their emission can be split and detected by two separate channels (gray areas). The 514 nm output of an Ar^+ laser was used to excite H_2B-PMHC near its absorbance maximum. This laser line further enables direct excitation of DiD at the blue edge of its absorbance spectrum. 7% H_2B-PMHC to DiD channel crosstalk and negligible DiD to H_2B-PMHC crosstalk was observed with our setup.

H_2B-PMHC fluorescence enhancement over time under oxidative conditions

To determine the fluorescence intensity enhancement of H_2B-PMHC, we first compared the H_2B-PMHC emission intensity from all individual liposomes before and during ABAP exposure. Liposomes were identified from their DiD emission in the red channel with a custom MATLAB routine.

Alternatively, we also calculated the fluorescence intensity enhancement from the average of the intensity ratio of H_2B-PMHC versus DiD ($I_{H_2B\text{-}PMHC}/I_{DiD}$) recorded for each individual liposome, determined before and during ABAP exposure (see below). Importantly, the same pixel regions (same liposomes) were used in the green and red channels to calculate the intensity ratio of H_2B-PMHC versus DiD. This ratiometric method is preferred as it corrects for effects such as dye partitioning heterogeneity due to liposome size distribution. The immobilized liposomes were initially incubated in HEPES buffer which was rinsed away by PBS buffer and subsequently 0.25 M ABAP in PBS was flowed over the liposomes at a rate of 5 μL/min to initiate the oxidation of H_2B-PMHC. The fluorescence intensity ratios are determined by the mean value of a distribution of $I_{H_2B\text{-}PMHC}/I_{DiD}$ fitted by a Gaussian function (Figure S5B). Time points correspond to the onset of each image acquisition, which takes about 5 minutes, and time = 0 minutes corresponds to when the ABAP flow was started.

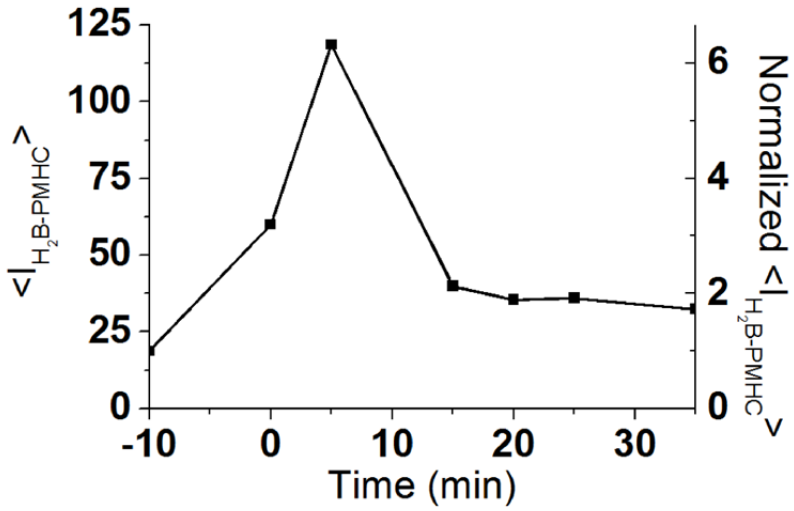


Figure S2. H_2B-PMHC fluorescence intensity as a function of time under oxidative conditions. The representative average intensity is determined by averaging the intensity of all liposomes identified from the DiD channel.

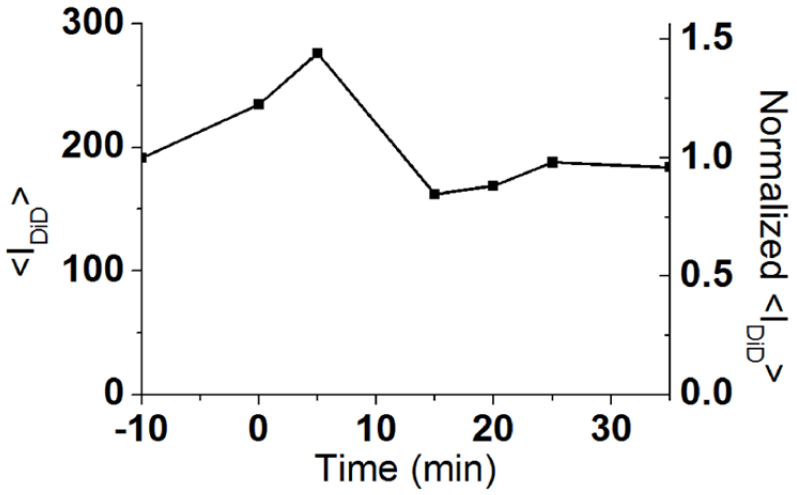


Figure S3. DiD fluorescence intensity as a function of time under oxidative conditions. The representative average intensity is determined by averaging the intensity of all liposomes identified from the DiD channel.

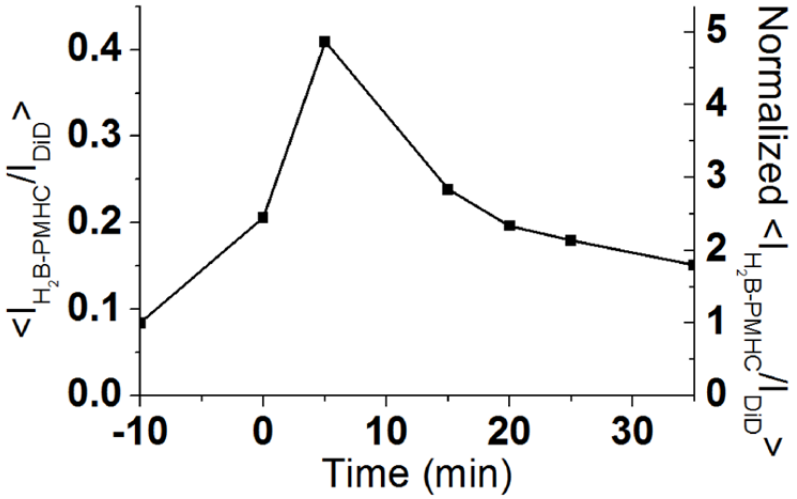


Figure S4. H_2B-PMHC/DiD fluorescence intensity ratio as a function of time under oxidative conditions. The representative average value of H_2B-PMHC/DiD fluorescence intensity is determined from the mean value of Gaussian distributions of the H_2B-PMHC/DiD intensity ratio calculated for each liposome.

Comparison of linear and Gaussian fitting of individual liposome data

To determine the ratio of H_2B-PMHC/DiD intensity, we initially plotted intensities of H_2B-PMHC versus those of DiD for individual liposomes (583 liposomes in total, **+PT** case). However, this analysis is sensitive to outliers. The initial data was fit by a linear regression model, yielding a slope of 0.163. The slope of the linear fit when excluding 3 outliers decreased 39% to 0.117.

We preferred a Gaussian fitting as the method to extract representative averages for all the different samples. Here the H_2B-PMHC/DiD intensity ratios of individual liposomes were used to construct a histogram fitted with a Gaussian function. The mean value of the H_2B-PMHC/DiD intensity ratio resulting from the Gaussian fits did not vary regardless of whether outliers are included or excluded.

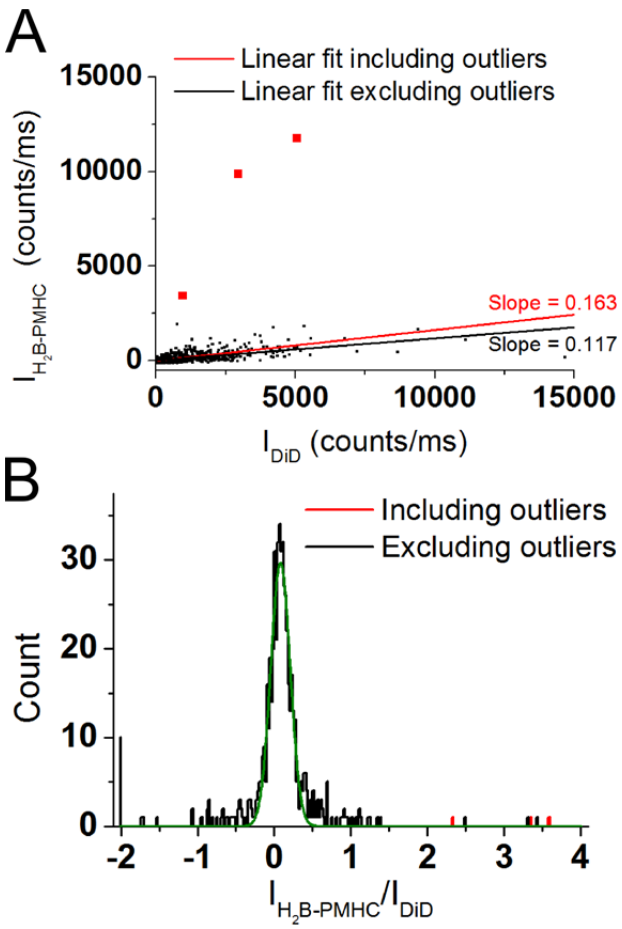


Figure S5. (A) Scatter plot of Intensities of H_2B-PMHC versus DiD for individual liposomes fitted with a linear regression. Outliers in red squares are shown. (B) Histogram of the H_2B-PMHC/DiD intensity ratios of individual liposomes constructed from the same data set as in (A) but now fitted to a Gaussian distribution. Both Gaussian fits (green lines) overlap perfectly. Outliers are shown as red data points on the right.

Gaussian fitting of fluorescence intensity ratios recorded under oxidation conditions induced by ABAP

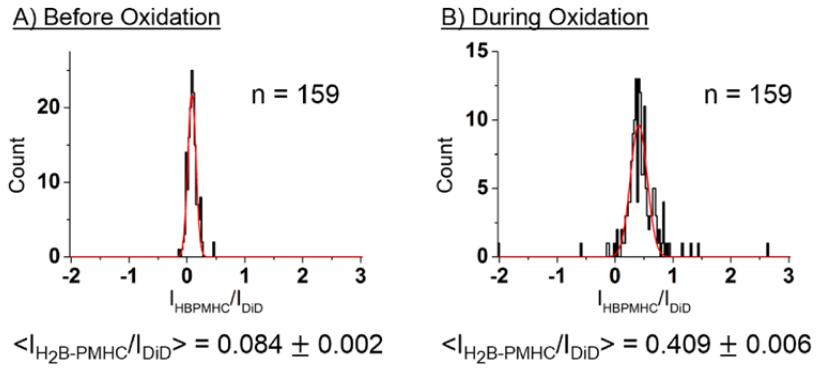


Figure S6. Histogram of $I_{H_2B\text{-}PMHC}/I_{DiD}$ ratios for individual liposomes before (A) and during (B) exposure to ABAP. The number of liposomes analysed and the mean value of Gaussian fits (red lines) ± fitting error are also indicated.

Gaussian fitting of fluorescence intensity ratios recorded under ambient conditions and different antioxidant loads

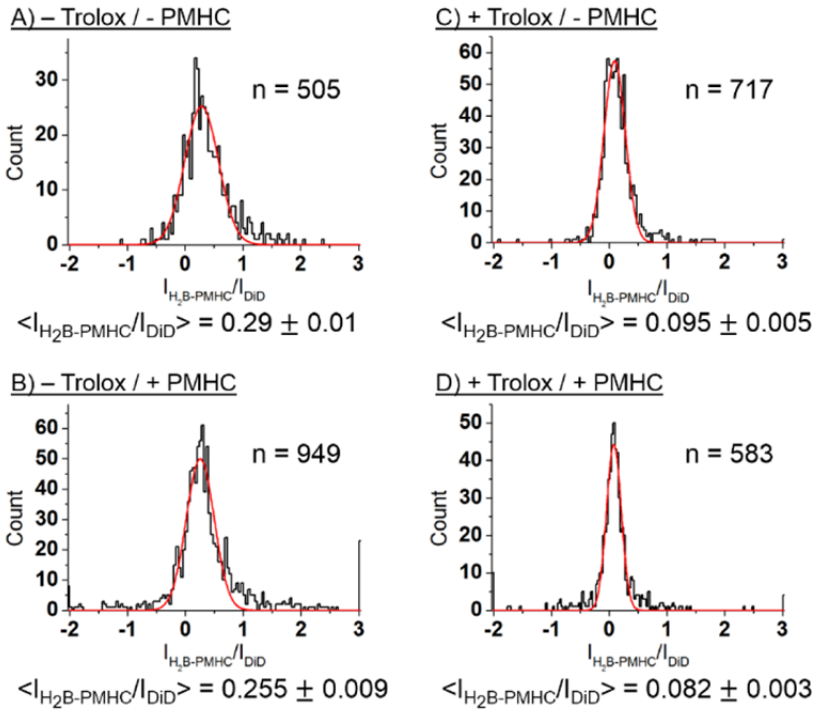


Figure S7. Histogram of $I_{H_2B\text{-}PMHC}/I_{DiD}$ ratios for individual liposomes imaged under ambient conditions and in the presence of different antioxidants added during sample preparation. The number of liposomes analysed and the mean value of Gaussian fits (red lines) ± fitting error are also indicated.

Confocal fluorescence intensity images recorded under ambient conditions and different antioxidant loads

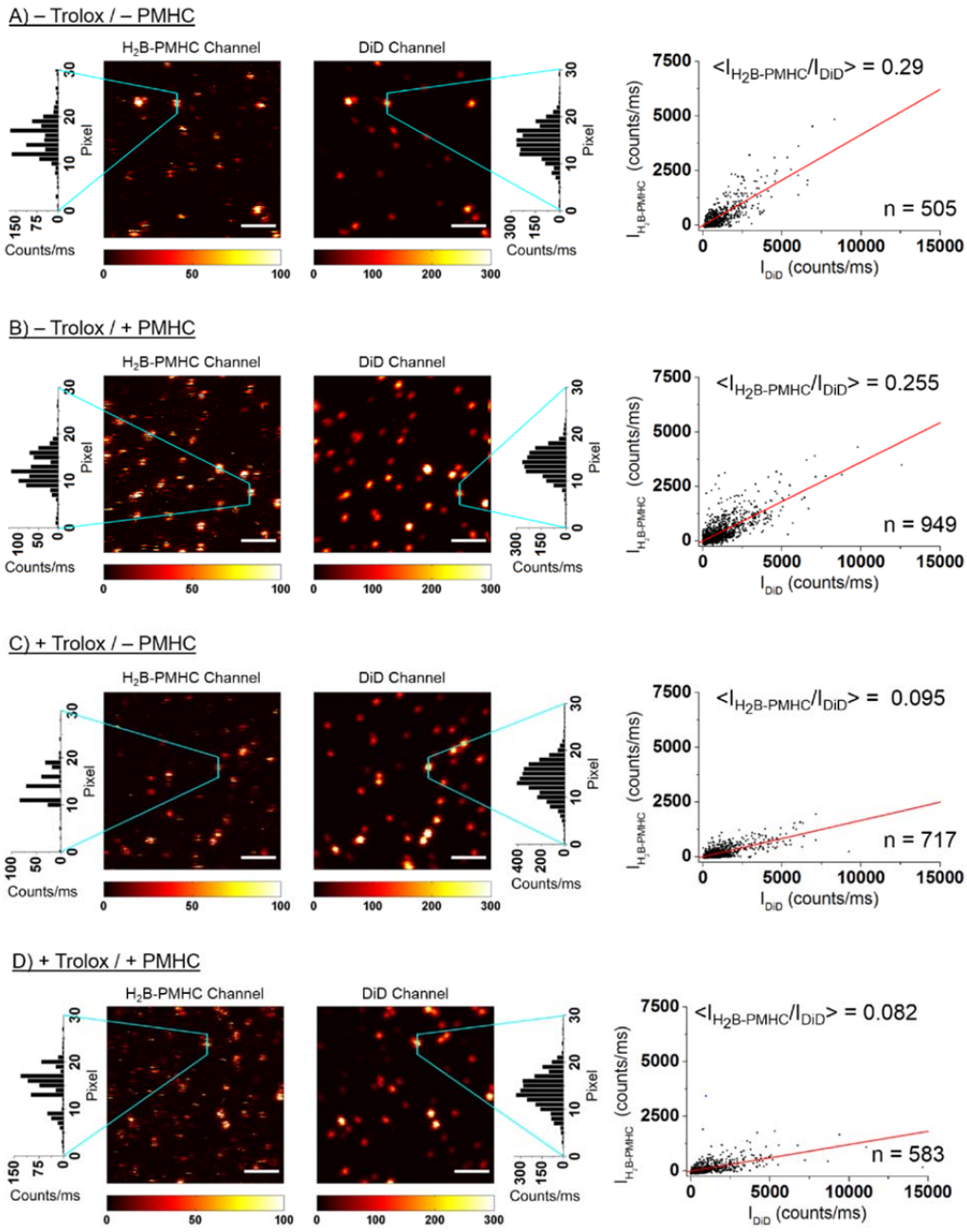


Figure S8. Fluorescence intensity images corresponding respectively to the H_2B-PMHC channel and the DiD channel, obtained for a sample prepared (A) with no additional antioxidants (**-PT**); (B) with PMHC only (**+P**); (C) with Trolox only (**+T**); and (D) with both antioxidants (**+PT**). Linescans are shown for a single molecule beside the images and their positions on the images are indicated by a cyan line. White scale bars are 2 μm. The right panel displays scatter plots showing the correlation of H_2B-PMHC vs. DiD fluorescence intensity, where linear fits serve as a guideline. Mean values of fluorescence intensity ratios obtained by Gaussian analyses, and the total number of liposomes analysed are further listed.

Effect of PMHC on H_2B-$PMHC_{ox}$: preoxidized H_2B-PMHC is not reduced by PMHC

We conducted fluorescence intensity measurements of H_2B-$PMHC_{ox}$ in the presence of a large excess of PMHC to establish whether preoxidized, emissive dye may be reduced by PMHC regenerating non-emissive H_2B-PMHC. Such a process would lead to a drop in the emission intensity reflected as a static (not dynamic) quenching. Steady-state and time-resolved emission experiments were conducted to establish whether fluorescence quenching was static or dynamic (the latter is expected based on intermolecular photoinduced electron transfer from the chromanol moiety in PMHC to the photoexcited BODIPY dye in H_2B-$PMHC_{ox}$).

Following addition of increasing amounts of PMHC we observed a drop in the fluorescence intensity of preoxidized H_2B-PMHC. The H_2B-$PMHC_{ox}$ solution was prepared by oxidation of 1.7 μM H_2B-PMHC with cumyloxyl radicals generated upon the irradiation at 263 nm of a 100 μM dicumylperoxide solution in acetonitrile. The initial intensity divided by the intensity in the presence of increasing amount of quencher PMHC (I_0/I), when plotted vs the PMHC concentration, followed a Stern-Volmer (linear) correlation as expected from a quenching process, with K_{SV} = 29.1 M^{-1} (Figure S9 below). The same correlation was observed when the analysis involved the ratio of initial fluorescence decay lifetime over the fluorescence decay lifetime in the presence of increasing amount of quencher PMHC, that is τ_0/τ plotted vs. [PMHC]. These results points to a purely dynamic fluorescence quenching mechanism. The lack of static quenching (drop in H_2B-$PMHC_{ox}$ intensity with no change in its observed fluorescence decay lifetime) rules out the occurrence of any thermal reduction of H_2B-$PMHC_{ox}$ by PMHC. In summary, there is no evidence of redox reactions under these conditions.

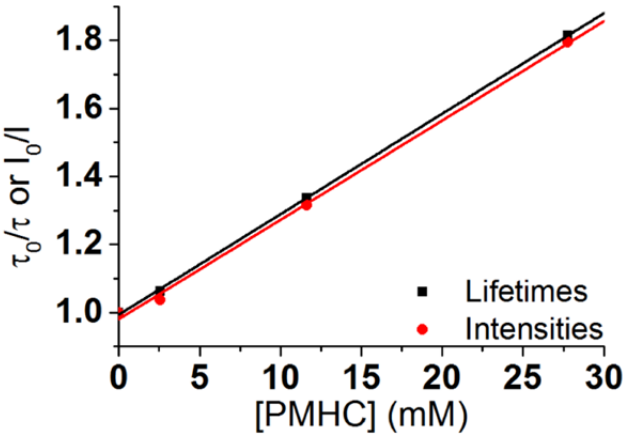


Figure S9. Stern-Volmer analysis of H_2B-$PMHC_{ox}$ fluorescence intensity upon addition of increasing amounts of PMHC in acetonitrile solutions. Fluorescence intensities were integrated over the range of 500 - 675 nm, with λ_{ex} = 480 nm. Fluorescence lifetimes are the intensity-weighted averages of biexponential fits ($\chi^2 < 1.2$). The fluorescence lifetime measurements were carried out using a Picoquant Fluotime 200 time-correlated single photon counting setup employing an LDH 470 ps diode laser (Picoquant) with excitation wavelength at 467 nm as the excitation source. The laser was controlled by a PDL 800 B picosecond laser driver from Picoquant. The excitation rate was 10 MHz, and the detection frequency was less than 150 kHz. Photons were collected at the magic angle.

Confocal imaging of regions that have been pre-irradiated with 633 nm laser light

We conducted imaging experiments in order to rule out photosensitization of ROS upon direct excitation of DiD occurring either when absorbing ambient light during liposome preparation. Specifically, we imaged a large 90 x 90 μm^2 area once it had been pre-irradiated in predefined regions with the 633 nm (200 W/cm^2) or the 514 nm 6400 W/cm^2) laser output in the absence of any external antioxidant (**-PT**). Three sectors of 30 x 30 μm^2 along one diagonal were irradiated for increasing periods of time (increasing number of scans from 1 to 3) with 633 nm laser light. Three sectors of 30 x 30 μm^2 along the other diagonal were irradiated for increasing periods of time with 514 nm laser light (increasing number of scans from 1 to 3). Next the whole 90 x 90 μm^2 area was imaged upon exciting at 514 nm. We did not observe increased emission arising from oxidized probes in regions excited with 633 nm laser light only. In turn, regions excited with increasing amounts of 514 nm showed a drop in intensity arising from sample photobleaching. We conclude that exposure to 633 nm laser excitation has no significant effects under our experimental conditions. The potential photosensitization of ROS by DiD is not a prevalent phenomenon, thus the contribution of photogenerated ROS to the observed intensity enhancement of H_2B-PMHC is negligible.

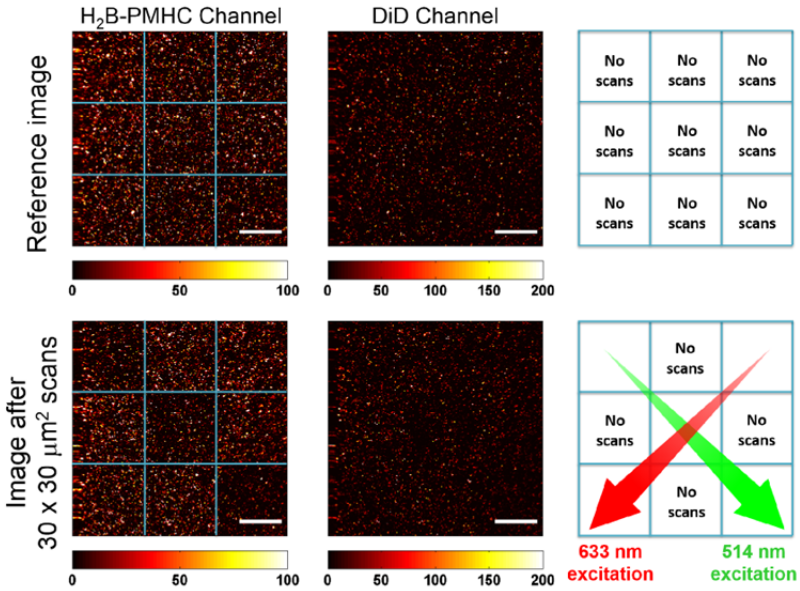


Figure S10. Confocal images (90 x 90 μm^2 regions) acquired under 514 nm illumination. Top panels represent a region that has not been exposed to laser irradiation prior to the full region scan. The image in the bottom panel was acquired after the irradiation of predetermined regions along the diagonals for increasing periods of time, as depicted in the right grid. Irradiated sections in the top row of the grid were scanned once by laser, while the middle and bottom sections were scanned 2 times and 3 times, respectively. Scanning conditions: 1 ms dwell time/pixel, 256 x 256 pixels/scan; same as imaging conditions. Scale bars are 18 µm.

Ensemble irradiation of DiD under maximum power

Liposomes containing H_2B-PMHC and DiD, without added antioxidants (**-PT**), were irradiated in a fluorimeter with fully open excitation slits (5 mm wide, gives 20 nm bandpass), exciting at the DiD absorbance maximum. We observed that extended (up to 30 minutes) irradiation results in an increase (ca. 2 fold) of H_2B-PMHC fluorescence intensity. No changes were observed in a sample that was kept in the dark for the same amount of time. This suggests the potential influence of, presumably, photogenerated ROS species in oxidation of liposome suspensions. The illumination intensity at 647 nm (4.5 mW/cm^2) used was roughly 40-fold higher than the ambient lighting in our laboratory (0.12 mW/cm^2).

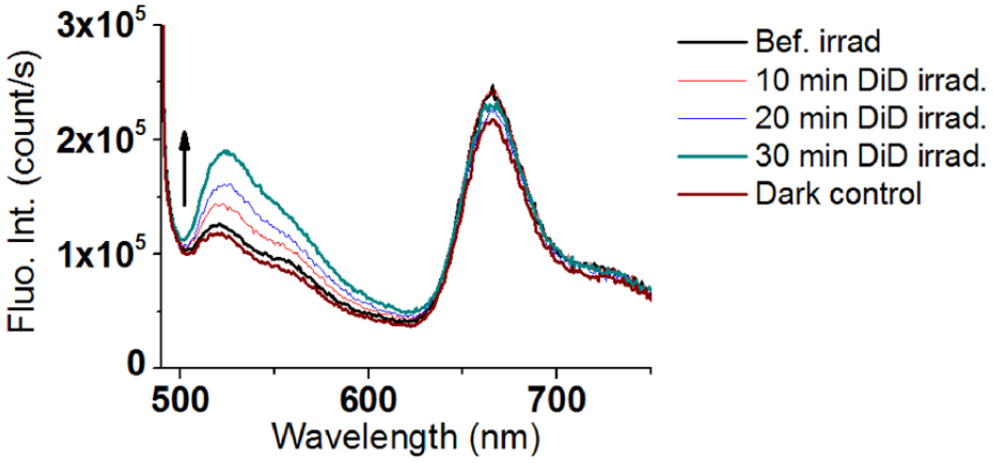


Figure S11. Ensemble irradiation of **–PT** sample. DiD was irradiated at the maximum of its absorbance (647 nm) for periods of 10 minutes. Emission spectra were acquired afterwards. Emission spectra were acquired with λ_{ex} = 475 nm.

Number of fluorescent bursts in individual liposomes

Monitoring the H_2B-PMHC fluorescence over time allowed us to further investigate the observed blinking dynamics. Interestingly, in all cases many more fluorescence bursts were recorded within a 3 minutes observation period than the expected number of embedded H_2B-PMHC molecules (i.e. 100) per liposome (Table S1). A MATLAB algorithm was written to quantify fluorescence bursts from time trajectories binned at 5 ms per data point. Fluorescence burst events were counted as such only if the fluorescence intensity in the H_2B-PMHC channel crossed a threshold and remained above it for at least two consecutive data points. The threshold used for analysis was the average of the sum of the mean background intensity (lowest intensity level) plus five times its standard deviation under each conditions. The background intensity-time trajectory was acquired in a region with no liposomes. Importantly, only one event is counted when the intensity above the threshold drops for one or two data points below the threshold and returns. This avoids counting as multiple events a noisy fluorescence burst that lies near the threshold, similar to the 'interpeak' correction method used by Terentyeva et al.[5] The number of fluorescence bursts counted provides a lower estimate since any jump in the fluorescence intensity that crosses the threshold is counted as a single event, regardless of the fluorescence intensity change (e.g. many dyes becoming simultaneously fluorescent).

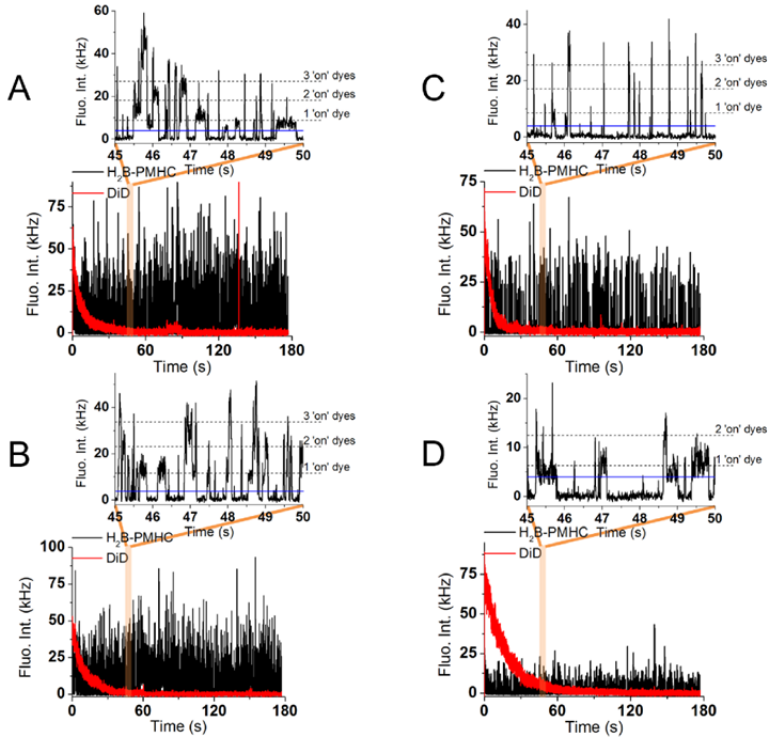


Figure S12. Fluorescence intensity-time trajectories recorded for single liposomes prepared with different antioxidant additives: (A) no antioxidants (**-PT**); (B) PMHC only (**+P**); (C) Trolox only (**+T**); and (D) both PMHC and Trolox (**+PT**). Trajectories were specifically chosen on the basis of having initial DiD intensities close to 50 kHz, i.e. they correspond to liposomes of similar size. The black lines display the intensity from the H_2B-PMHC channel and the red lines display the intensity from the DiD channel. Trajectories are from 3 minutes acquisitions while insets show a 5 s expanded region, indicated in full-length trajectories by a shaded orange area. The blue line in the insets corresponds to the threshold applied in order to count fluorescence burst events. Dashed black lines correspond to the observed intensity steps corresponding to 1, 2 or 3 H_2B-PMHC$_{ox}$ simultaneously fluorescing. Data points are binned to 5 ms.

Summary of antioxidant additive effects

Table S1. Effects of added antioxidants on ambient oxidation processes.

	Antioxidants			
	- PT	**+ P**	**+ T**	**+ PT**
$\langle I_{H_2B\text{-}PMHC}/I_{DiD} \rangle$[a]	0.29 [0.25 0.32]	0.255 [0.24 0.28]	0.095 [0.08 0.11]	0.082 [0.07 0.10]
Fluorescence bursts in 3 min representative intensity-time trajectory of a liposome[b]	638	659	282	364
<Fluorescence bursts> in 3 min intensity-time trajectory of background[c]	3	75	5	11

[a]Mean values of Gaussian distribution of individual liposome intensity ratios obtained from confocal images. 95% confidence intervals as determined by Bootstrap analysis (10k resamples) using the bias corrected percentile method in MATLAB are indicated in square brackets. [b]Number of fluorescence bursts in liposomes of similar sizes (similar initial DiD intensity) were determined from a single 3 minute long trajectory for each condition (shown in Figure S11), where the laser was parked on a region containing a liposomes as determined from the DiD channel image under 633 nm excitation. [c]The average number of bursts was calculated for 1 or 2 separate 3 minutes trajectories acquired by parking the laser on a region containing no liposomes as determined from the absence of DiD emission under 633 nm excitation.

References

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