C11-BODIPY\textsuperscript{581/591}, AN OXIDATION-SENSITIVE FLUORESCENT LIPID PEROXIDATION PROBE: (MICRO)SPECTROSCOPIC CHARACTERIZATION AND VALIDATION OF METHODOLOGY

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Abstract—C11-BODIPY\textsuperscript{581/591} is a fluorescent ratio-probe for indexing lipid peroxidation and antioxidant efficacy in model membrane systems and living cells, with excellent characteristics: (i) emission in the visible range of the electromagnetic spectrum, with good spectral separation of the nonoxidized (595 nm) and oxidized (520 nm) forms; (ii) has a high quantum yield and because of this, low labeling concentrations can be used, ensuring minimal perturbation of the membrane whilst retaining favorable signal to noise ratios; (iii) has a good photo-stability and displays very few fluorescence artifacts; (iv) is virtually insensitive to environmental changes, i.e., pH or solvent polarity; (v) is lipophilic and as such easily enters membranes; (vi) once oxidized, C11-BODIPY\textsuperscript{581/591} remains lipophilic and does not spontaneously leave the lipid bilayer; (vii) C11-BODIPY\textsuperscript{581/591} localizes in two distinct pools within the lipid bilayer, a shallow pool at 18 Å and a deep pool at \(< 7.5 \text{ Å}\) from the center of the bilayer; (viii) is not cytotoxic to rat-1 fibroblasts up to 50 μM; (ix) is sensitive to a variety of oxy-radicals and peroxynitrite, but not to superoxide, nitric oxide, transition metal ions, and hydroperoxides per se; (x) its sensitivity to oxidation is comparable to that of endogenous fatty acyl moieties. © 2002 Elsevier Science Inc.

Keywords—Fluorescence, C11-BODIPY\textsuperscript{581/591}, Probe, Oxidative stress, Radical, Fluorescence microscopy, Lipid peroxidation, Rat-1 fibroblast, Vesicle, Comparative study, Free radicals

INTRODUCTION

Over the past few decades, an increasing body of evidence has been accumulated implicating the involvement of reactive oxygen species (ROS) in the pathophysiology of many diseases, including various forms of nonhormone-dependent cancers [1], atherosclerosis [2,3], ischemic reperfusion injury [4], neurodegenerative diseases [5,6], and even some factors underlying the ageing process itself [7,8]. These species are highly reactive free radicals or give rise to the formation of free radicals via Fenton reactions, which subsequently are able to damage or modify a myriad of biomolecules, such as proteins, DNA, and lipids [9,10]. The polyunsaturated fatty acids (PUFA) in lipids are opportune targets for ROS. Peroxidation of lipids has far-reaching consequences, well beyond the biomembrane itself. Oxidation reactions in biomembranes lead to amplification of ROS, changes in membrane fluidity, loss of compartmentalization and plasma-membrane integrity, disturbance of ion-gradients, impairment of lipid-protein interactions, modification of nucleic acids and proteins through the release of reactive aldehydes, release of bioactive products that influence gene transcription and signal transduction, and fatty acid hydroperoxides released are potent inhibitors of mitochondrial oxidative phosphorylation (see [10–13]). In order to maintain homeostasis, an elaborate network of detoxifying enzymes and low molecular weight antioxidant molecules countermands oxidant activities. Disturbance of this intricate balance between oxidant activities and antioxidant capacity may induce damage to biomolecules. In nutritional research, antioxidants are commonly used to modulate the consequences of oxidative stress in organisms and to maintain the quality of lipid-containing food products [14]. Not surprisingly, oxidative stress, the involvement of lipid per-
oxidation in deleterious reactions within cells, and the prevention thereof by nutritional antioxidants have been the focus of attention of research for many years.

Because lipid peroxidation and antioxidant detoxifying processes are complex and multi-factorial, various techniques have been developed to measure lipid peroxidation and related indices (compared in [15–19]). However, many of these techniques are complicated, destructive, require elaborate sample preparation, are consequently time consuming, and do not provide information about oxidant activities at the (sub)cellular level. To circumvent these problems, investigators turned their attention to fluorescent reporter molecules that could accurately report the damage inflicted to biomolecules. Lately, several new assays have been introduced to index lipid peroxidation or antioxidant efficacy in model membranes and living cells without the need for extensive sample preparation. Besides the most commonly used fluorochrome, cis-parinaric acid [4,20–27], a flow-cytometric method using undecylamine fluorescein as a reporter molecule [28,29], an assay utilizing radical induced changes in fluorescence anisotropy of diphenylhexatriene propionic acid in vesicles [30], and the exploitation of excimer formation of hexadecanoyl-BODIPY-FL in erythrocytes [31] have been reported and used successfully. Whilst some properties of these fluorochromes, such as photo-stability, unfavorable emission in the UV range (cis-parinaric acid), the necessity for high probe concentrations, and heterogeneous probe distribution, limit their applicability in living cells and medium-throughput screening of antioxidants, these assays have proven to be promising and indicate the direction in which the oxidative stress field is moving, i.e., towards single-cell biochemistry.

In concordance with these new developments, we recently introduced a method based on the fluorescent reporter molecule, C11-BODIPY 581/591 [32,33]. BODIPY probes are a newly introduced class of fluorescent molecules that fluoresce in the visible range of the electromagnetic spectrum and have outstanding fluorescent properties [34,35]. C11-BODIPY 581/591 is a fluorescent fatty acid analogue with fluorescent properties in the red range of the visible spectrum (emission maximum 595 nm), allowing its application in fluorescence microscopy. Upon free radical-induced oxidation, its fluorescent properties shift from red to green. This characteristic is highly advantageous, since ratio-imaging of oxidant activities at the (sub)cellular level now becomes feasible. Furthermore, the fluorescent properties of C11-BODIPY 581/591 allow the use of this probe in fast- and medium-throughput screening of antioxidants in living cells and model membranes in a multiwell/fluorescence reader approach [32,33,36]. This greatly enhances the flexibility in screening phytochemicals, extracts, and combinations of antioxidants for their antioxidant efficacy and bioremedial effects.

Since C11-BODIPY 581/591 not only offers the perspective to detect lipid peroxidation and evaluate the efficacy of antioxidants in “medium-throughput” screening, but also the visualization of oxidation processes at the (sub)cellular level, a thorough validation of methodology seems to be warranted in order to exclude fluorescence-associated artifacts and misinterpretation of results. With the present study, the first of two validation studies, we initiated an extensive validation and characterization of the use of C11-BODIPY 581/591 as a versatile and valid marker for lipid peroxidation or the evaluation of antioxidant efficacy in model membrane systems and living cells.

MATERIALS AND METHODS

Chemicals

Peroxyinitrite and the PTS1-eYFP construct were a kind gift from Dr. W. H. Koppenol (ETH, Zürich, Switzerland) and Dr. T. B.Dansen (Dept. Biochemistry of Lipids, Utrecht University, The Netherlands), respectively. 2,2’-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) was obtained from Wako Pure Chemical Industries (Neuss, Germany) and 2,2’-Azobis(2-aminodipropylpropane dihydrochloride) (AAPH) was acquired from Polysciences (Warrington, PA, USA). N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanol) spingosine (BODIPY-FL C5-ceramide), 4,4-difluoro-5-(4-phenyl-1,3-butanediyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY 581/591), 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 505/515), 5-dodecanoylamino fluoroscein (C12-fluorescein), cis-parinaric acid (cis-PnA), rhodamine 123, propidium iodide, LysoTracker green, S-nitroso-N-acetylpenicillamine (SNAP), and the CyQuant cell proliferation kit were obtained from Molecular Probes (Leiden, The Netherlands). Fluorescein was purchased from Fisher Scientific (’s Hertogenbosch, The Netherlands). All probes were stored at −20°C under nitrogen in the dark. The concentrations of C11-BODIPY 581/591 and cis-PnA stock solutions in ethanol were determined by measuring the absorption on a Hitachi U-2000 dual beam spectrophotometer at 581 nm (ε0 = 139,444 1 mol−1 cm−1 [35]) and 324 nm (ε0 = 78,000 1 mol−1 cm−1 [37]), respectively. Egg-phosphatidylcholine (egg-PC), 1,2-dioleoyl-phosphatidylcholine (DOPC), 1-stearoyl, 2-oleoyl-phosphatidylcholine (SOPC), 1,2-dilinoleoyl-phosphatidylcholine (DLPC), 1,2-dioleoyl-(TEPPO) phosphatidylcholine (TEPPO-PC), and 1-palmitoyl-2-[5- or 12-doxyl]-stearoyl-phosphatidylcholine (5-SLPC or 12-SLPC), were purchased from Avanti Polar Lipids.
Preparation of preoxidized C11-BODIPY581/591

C11-BODIPY581/591 (batches of 40 μM) was oxidized to complete-ness at 37°C in UV-grade ethanol with 20 mM cumene hydroperoxide (CumOOH) and 250 μM CuSO4. The progression in oxidation was assessed fluoro-metrically as described under fluorometry. Subse-quentl-y, the product was extracted according to Bligh and Dyer [40] to remove polar components. The extracts were dried under nitrogen, dissolved in chloroform/methanol (3:1 v/v) and subsequently purified by thin layer chromatography (TLC) on Merck Kieselgel DC-fertig plates (chloroform:methanol:glacial acetic acid: H2O (90:40:12:2 v/v/v/v)). The oxidized probe was re-covered from the silica by extraction according to Bligh and Dyer and finally dissolved in UV-grade ethanol.

Staining of cellular organelles

Cells were incubated in DMEM with 1 μM of the appropriate organelle probe at 37°C for 20 min. Mitochondria were stained with rhodamine 123 and lysosomes with LysoTracker green. Staining of Golgi apparatu-rus was performed by incubating with 100 nM BODIPY-FL C2-ceramide in DMEM at 37°C until suf-ficient green staining of the Golgi apparatus was ob-served. Subsequently, growth medium was refreshed to abolish the influx of probe and to allow any probe that re-mained in the cytosol to incorporate into the organelle. Peroxisomes were labeled by transfecting rat-1 fibro-blasts with 1 μl PTS1-eYFP and 5 μl Fugene, 24 h before fluorescence microscopic evaluation. Labeling with nonoxidized and preoxidized C11-BODIPY581/591 was performed as described previously. Imaging was per-formed in PBS+ that contained 1 μM propidium iodide to evaluate membrane integrity.

Cell fractioning

The fibroblast plasma-membrane was isolated using the gas-dissection technique as previously described by Langer et al. [41] and Post et al. [42]. Briefly, a disc with attached cells was placed at the center of a mobile platform inside the dissection chamber. The platform, with disc, was then elevated to make firm contact with the protruding inner horizontal gas nozzle. Subsequently, the inlet valve was opened rapidly (<1 s) to allow the entry of nitrogen gas at a pressure of 1900–2000 psi. As the N2 stream bursts radially over the surface of the monolayer, the upper surface of the cells is sheared open, the cellular material is blown out, and the plasma-membrane is left in a fenestrated layer and, in some areas, in a wrinkled or rolled form attached to the disks.

Mitochondria were isolated from rat-1 fibroblasts cul-tured in 75 cm² flasks and preincubated with C11-BODIPY581/591 as described before. Cells cultured in flasks were treated with trypsin and resuspended in se-
rum-free DMEM. Cells were washed twice with PBS. After centrifugation, the cell pellet was resuspended in an ice-cold hypotonic buffer (RSB; 10 mM NaCl, 1.5 mM MgCl$_2$, and 12.5 mM Tris-HCl, pH 7.5) to allow the cells to swell for 5–10 min. Cells were disrupted in a Dounce homogenizer for 15 min at 17,000 × g to the supernatant for 15 min at 17,000 × g to allow the cells to swell for 5–10 min. Cells were disrupted in a Dounce homogenizer and immediately added to an ice-cold 2 × MS buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, and 2.5 mM EDTA, pH 7.5) to a final concentration of 1 × MS. The homogenate was centrifuged twice at 13,000 × g for 5 min. After centrifuging the supernatant for 15 min at 17,000 × g, the pellet (mitochondrial fraction) was resuspended in PBS.

Lipids and probe were extracted according to Bligh and Dyer [40].

Cells were rinsed twice with PBS at least 24 h before use on coverslips (diameter 24 mm).

**Extraction of lipids and phospholipid phosphorous analysis**

Cells were extracted for 60 min at ambient temperature with isopropanol (0.05% butylated hydroxytoluene) as described before [21,42]. Total phospholipid content was determined according to Rouser et al. [43]. Prior to determining the phospholipid content, isopropanol extracts were dried under nitrogen and extracted according to Bligh and Dyer [40] to eliminate nonphospholipid phosphate. The blue color developed was measured at 700 nm with a Hitachi U-1100 spectrophotometer.

**Confocal laser scanning microscopy (CLSM)**

For fluorescence microscopy, cells were plated at least 24 h before use on coverslips (diameter 24 mm). Cells were rinsed twice with PBS and subsequently samples were placed in the temperature-controlled coverslip holder (37°C). Images were taken with a Leica TCSN confocal laser-scanning system on an inverted microscope DMIRBE (Leica Microsystems GmbH, Heidelberg, Germany) with an argon-krypton laser as excitation source. The green and red fluorescence signals were acquired simultaneously using double wavelength excitation (laser lines 488 and 568 nm) and detection (emission bandpass filters 530/30 and 590/30).

**Fluorometry and determination of probe depth by fluorescence parallax**

Fluorescence measurements were carried out at ambient temperature in a cuvette equipped with a magnetic stirring device using a Quantum Master spectrophotometer (PTI, Surbiton, Surrey, UK). Excitation and emission bandpasses were set to 1 nm and 10 nm, respectively. Fluorescence was measured directly in the SUV solution. Samples from cell experiments were standardized to 1 ml isopropanol after evaporating the cell extracts to dryness under nitrogen and lipid extraction according to Bligh and Dyer [40]. Measurement of cis-PhA fluorescence was carried out at wavelengths 324/413 nm, as described previously [21]. Fluorescence of C11-BODIPY$_{581/591}$ was measured by simultaneous acquisition of the green (485/520 nm) and red signal (581/595 nm). Alternatively, a Tecan multiwell spectrophotometer (Tecan, Goring-on-Thames, Reading, UK) was used to measure fluorescence in 96 well plates. C11-BODIPY$_{581/591}$ red fluorescence was detected using excitation and emission bandpass filters of 590/15 and 635/20 nm, respectively. The CyQuant green fluorescence was acquired using 485/20 excitation and 535/25 emission bandpass filters.

Depth measurements were performed as described by Kaiser and London [44]. Briefly, samples containing 0.1 μM BODIPY derivatives incorporated into 200 μM SUVs composed of DOPC or DOPC with 15 mol% spin-labeled PC (5-SLPC, 12-SLPC or TEMPO-PC) in PBS (pH 7.2) were prepared by octylglucoside (OG) dilution [45]. Fluorescence in the presence (F) and absence (F$_0$) of quenchers was measured at ambient temperature using a Spex Fluorolog spectrofluorometer operating in ratio mode. The excitation and emission bandpasses were set to 2.3 nm to prevent overloading of the photo-multiplier tube. Using F/F$_0$ values, the distance of fluorochromes from the center of the bilayer was calculated according to the parallax equation [44]:

$$z_{cl} = L_{c1} + (-\ln(F/F_0)/\pi C - L_{c2}^2)/2L_{c21}$$  \hspace{1cm} (1)

where $z_{cl}$ is the distance from the center of the bilayer, $F$ is the fluorescence intensity (F/F$_0$) in the presence of the shallow quencher, $F_0$ is the fluorescence intensity (F/F$_0$) in the presence of the deep quencher, $L_{c1}$ is the distance of the shallow quencher from the center, $L_{c2}$ is the distance between the shallow and deep quenchers, and $C$ is the quencher concentration in molecules/Å$^2$.

**Assessing C11-BODIPY$_{581/591}$ cytotoxicity with a CYQUANT cell proliferation assay**

Cells were plated in Nunc 96 wells plates at an initial density of 2500/cm$^2$ and grown for a maximum of 6 d in the presence of 0, 1, 15, 25, and 50 μM C11-BODIPY$_{581/591}$. Cell proliferation was evaluated experimentally by determining the apparent partition coeff
C11-BODIPY$_{581/591}$, a lipid peroxidation probe

Monolayer experiments

Monolayer experiments were essentially performed as described by Leenhouts et al. [49]. Surface tension was measured by the Wilhelmy plate method in Teflon troughs at ambient temperature, using a paper plate. Increasing amounts of egg-PC stock solutions containing 0, 0.5, 1, and 2 mol% nonoxidized or oxidized C11-BODIPY$_{581/591}$ in chloroform/methanol (3:1 v/v) were spread on the PBS sub-phase. Changes in surface tension were subsequently evaluated for 20–30 min (per spread) with an Ankersmit CAHN microbalance.

Fatty acid analysis

The fatty acid composition was determined by capillary gas chromatography using a Perkin Elmer 8500 gas chromatograph equipped with a CP-Wax 52 CB capillary column (Chrompack, Middelburg, The Netherlands; length 50 m, diameter 0.32 mm). The oven temperature was increased between 180°C and 200°C by 10°C/min, kept isothermal for 6 min, increased to 220°C by 10°C/min, kept isothermal for 5 min and finally raised to 235°C. The injector and detector temperature were both 285°C. Stearic acid in SOPC was used as a nonoxidizable internal standard. Fatty acids were transmethylated to fatty acid methyl esters (FAMEs) with 14% boron fluoride in methanol according to Morrison and Smith [50].

Image processing, curve fitting and statistical analysis

The images were processed with Scion Image 4.02 (ScionCorp, Frederick, MD, USA) as previously described [32,33]. Curve fitting and numerical analysis of initial peroxidation rates (IPR) were performed with Igor Pro (WaveMetrics, OR, USA) as outlined in [21,33]. Standard procedures were used to calculate means and standard deviations.

RESULTS

C11-BODIPY$_{581/591}$, a ratio probe for lipid peroxidation: oxidant specificity and sensitivity to oxidation

Boron dipyrromethene difluoride (BODIPY)-based dyes are a relatively new class of fluorochromes that display a bright green fluorescence in the range of 450 to 550 nm [35]. C11-BODIPY$_{581/591}$ is a fatty acid analogue in which the boron dipyrromethene difluoride core is substituted with a phenyl moiety via a conjugated diene interconnection (Fig. 1A, insert). Because of this extended conjugation, the intact C11-BODIPY$_{581/591}$ displays a bright red fluorescence with an emission maximum at 595 nm. Figure 1A shows that C11-BODIPY$_{581/591}$ incorporated into egg-PC vesicles is oxidation-sensitive when challenged with oxidizing species, with the diene interconnection as the most likely target for oxidation. The initial red fluorescence at 595 nm decreases and the emission shifts to considerably shorter wavelengths, with a maximal emission at 520 nm and a shoulder at 545 nm, as the oxidation of C11-BODIPY$_{581/591}$ progresses.

To accurately determine oxidant activities in living cells, reporter molecules for lipid peroxidation should preferentially be sensitive to a variety of reactive oxygen species, but not to hydroperoxides per se. Because various radical-generating systems are commonly used in oxidative stress research, we evaluated C11-BODIPY$_{581/591}$’s sensitivity to a variety of oxidizing species in egg-PC SUVs. Figure 1B shows that C11-BODIPY$_{581/591}$ is exclusively sensitive to free radical species formed from hydroperoxides, but not to hydroperoxides per se, as deduced from the absence of any decline in red fluorescence when the transition metal ion is not present. Furthermore, C11-BODIPY$_{581/591}$ is insensitive to superoxide generated from Xanthine/Xanthine oxidase and to nitric oxide (SNAP), but distinctly sensitive to peroxynitrite, which reacts rapidly with the probe. No significant degradation of the probe was induced by transition metal ions and as such, no perturbation of observations occurs when these ions are used to induce formation of free radicals from hydroperoxides. In addition, curve 1 in Fig. 1B illustrates that during fluorometric acquisition of C11-BODIPY$_{581/591}$, photobleaching effects are low. Conversely, photobleaching does occur during microscopic evaluation of samples and should be taken into account.
To assess if C11-BODIPY\textsuperscript{581/591} is sensitive to chain propagation reactions, mixed SUVs of SOPC with increasing fractional amounts of DLPC (0–100\%) were made. From the results presented in Fig. 1C, it is clear that the rate of oxidation increases when increasing amounts of PUFA are present in the mixed SUVs. It cannot be ruled out that increasing amounts of DLPC in the SUV influence the water penetrability of the bilayer because of a higher curvature of the outer leaflet and that the increase in oxidation rate is a reflection of an enhanced contact between the oxidant (AAPH) and the PUFA or C11-BODIPY\textsuperscript{581/591}. Conversely, from the results in Fig. 1B and C as a whole, it is likely that C11-BODIPY\textsuperscript{581/591} is oxidized by chain-propagating species such as peroxyl and alkoxyl species, and not only directly from the oxidation-initiating species.

Previously, we reported that C11-BODIPY\textsuperscript{581/591}’s sensitivity to oxidation is in the same order of magnitude as the sensitivity of lipid-arachidonate in rat-1 fibroblasts and cultured myocardial cells [32]. To investigate the sensitivity of C11-BODIPY\textsuperscript{581/591} to free radical-mediated degradation compared with endogenous fatty acyl moieties under more controlled conditions, we compared C11-BODIPY\textsuperscript{581/591}’s oxidation with linoleoyl-PC (also containing two unsaturated bonds) and \textit{cis}-parinaric acid (the most commonly used fluorescent reporter molecule) in SUVs. Figure 2 shows the degradation of C11-BODIPY\textsuperscript{581/591}, \textit{cis}-PnA and linoleate in mixed DLPC/SOPC vesicles induced by 500 \textmu M AAPH. Upon oxidation, a decrease in C11-BODIPY\textsuperscript{581/591} red fluorescence and a concomitant increase in green fluorescence are noticed, with equal rates of fluorescence decay and increase. From the peroxidation rates (Fig. 2B), it is apparent that C11-BODIPY\textsuperscript{581/591} is approximately a factor 5 less sensitive to oxidation compared with the widely used reporter molecule \textit{cis}-parinaric acid and at least 2.3 times more sensitive than lipid-linoleate. It may be concluded that C11-BODIPY\textsuperscript{581/591}’s sensitivity to oxidation is indeed in the same order of magnitude as the sensitivity of endogenous lipid-PUFA, which corroborates the findings in cultured cells reported previously [32].

### Cytotoxicity and stability of C11-BODIPY\textsuperscript{581/591}

Since high concentrations of fatty acids in biomembranes generally lead to perturbation of the bilayer and subsequently cellular metabolism is adversely affected, we evaluated if prolonged exposure to high concentrations of C11-BODIPY\textsuperscript{581/591} resulted in cytotoxic effects by assessing if cells were able to proliferate in the presence of increasing concentrations of C11-BODIPY\textsuperscript{581/591}. Cells were grown in 96 well plates in the presence of 0, 1, 5, 15, 25, and 50 \textmu M C11-BODIPY\textsuperscript{581/591} at an initial
density of 2500/cm² for a maximum of 6 d. Cell proliferation was assessed fluorometrically with a CyQuant cell proliferation kit. The results in Fig. 3 demonstrate that proliferation progressed normally, because no significant deviation from results with unlabelled control cells occurred and thus cytotoxicity is apparently low.

Fluorometric evaluation of cellular extracts from cells cultured for 24 h in the presence of C11-BODIPY°581/591°, under normal culturing conditions, showed no significant oxidation of the probe. Microscopic and fluorometric evaluation of living rat-1 fibroblasts and neonatal rat cardiomyocytes labeled with C11-BODIPY°581/591° did not reveal any destabilization of the fluorescence signal in the absence of oxidative stress other than that associated with photo-bleaching. This indicates that the probe is not rapidly β-oxidized, dissimilar to cis-parinaric acid, which is rapidly degraded through β-oxidation in myocardial cells [4,21]. Furthermore, separation of the various lipid classes on HPLC with simultaneous UV and fluorescence detection showed that, under our labeling conditions and in rat-1 fibroblasts, no metabolism of C11-BODIPY°581/591° to lipids occurs (all data not shown).

(Sub)cellular distribution of C11-BODIPY°581/591° and possible fluorescence artifacts

Fluorescence microscopic evaluation of rat-1 fibroblasts labeled with 1 μM C11-BODIPY°581/591° in DMEM (stock prepared in fetal calf serum) for 30 min at 37°C shows that C11-BODIPY°581/591° is distributed heterogeneously throughout the cell, with predominant staining in the perinuclear region and possibly mainly mitochondria (Fig. 4A and B). In various other regions of the cell, labeling seems to be low or virtually absent. However, image enhancement of those regions with low labeling efficiencies, at the expense of over-illumination of the perinuclear region, shows additional membranous structures (Fig. 4B) with morphologies reminiscent of endoplasmic reticulum and plasma-membrane. No detectable cytosolic or nuclear staining, either from the intact C11-BODIPY°581/591° or the oxidized C11-BODIPY°581/591°, was observed (see Figs. 4, 5, and 6). Microscopic evaluation during a 2 h period of labeled rat-1 fibroblasts did not reveal any probe redistribution to such an extent that the probe becomes distributed more homogenously.

In order to further assess the (sub)cellular distribution of C11-BODIPY°581/591° and to determine if C11-BODIPY°581/591° preferentially incorporates into mitochondrial membranes, a series of colocalization studies with organelle-specific live cell probes were performed.
Mitochondria were stained with rhodamine 123, lysosomes with LysoTracker green, and Golgi apparatus was stained with BODIPY-FL C5-ceramide in DMEM at 37°C until sufficient green staining of the organelle was observed. Since BODIPY-FL C5-ceramide is known to form red excimers (dimers of green excited state and nonexcited state BODIPY-FL C5-ceramide monomers) [51] with emission maxima that coincide with the emis-
The formation of BODIPY-FL C₅- ceramide excimers was excluded by exciting at 488 nm and acquisition of the red channel (590/30 nm) at maximum gain prior to incubation with C11-BODIPY 581/591. Because there is no probe available to stain the peroxisomes of living cells, rat-1 fibroblasts were transfected with a construct of enhanced yellow fluorescent protein coupled to the peroxisomal target sequence (PTS1-eYFP) 24 h prior to microscopic evaluation. The micrographs in Fig. 5 show predominant and significant C11-BODIPY 581/591 staining of the perinuclear region with no specific preference for any particular organelle.

With varying labeling efficiencies throughout the cell, it may be concluded that C11-BODIPY 581/591 is distributed heterogeneously. However, because the emissions of the oxidized and nonoxidized form are spectrally well separated (Fig. 1A), ratio imaging allows the simultaneous detection of both forms (Fig. 4C, left panel). It is possible to determine the fraction oxidized probe in...
every pixel of a micrograph by dividing the green image
by the sum of the red and green images (Fig. 4C, right panel). In this way heterogeneous probe uptake and
distribution are effectively eliminated. Therefore, the
extent of oxidation in membranes with low labeling
efficiencies can still be evaluated (Fig. 4C, right panel).

It is apparent from Fig. 5 that relatively compact
perinuclear structures are pronouncedly stained. High
probe concentrations in this region could give rise to
unwanted fluorescence artifacts. At high mol fractions,
three different mechanisms may lead to nonlinearity in
the concentration-fluorescence relationship: (i) Excimer
formation [31,35]. The fluorescence emission of these
excimers is red-shifted with respect to that of the mono-
mers. Red excimer fluorescence of oxidized C11-
BODIPY\(^{581/591}\) could seriously interfere with the data
interpretation. (ii) Self-quenching; a bimolecular process
that reduces the fluorescence quantum yield without

![Fig. 6. Evaluation of excimer formation by oxidized C11-BODIPY\(^{581/591}\). (A) Emission spectra of preoxidized C11-BODIPY\(^{581/591}\)
in 250 \(\mu\)M egg-PC vesicles with excitation at 485 nm. (B) Rat-1 fibroblasts overloaded for 1 h with 30 \(\mu\)M preoxidized
C11-BODIPY\(^{581/591}\). Exclusion of propidium iodine throughout the experiment indicated plasma-membrane integrity. After addition
of a few drops Triton X-100, nuclear staining becomes visible (insert). (C) Photobleaching (586 nm) of several cells labeled with 10
\(\mu\)M C11-BODIPY\(^{581/591}\) and subsequent acquisition of green and red fluorescence. Images were corrected for the difference in
excitation efficiency.](image)
changing the fluorescence emission spectrum; it therefore tends to occur when high loading concentrations or labeling densities are used. (iii) Resonance energy transfer; the nonoxidized form of C11-BODIPY<sub>581/591</sub> quenches the fluorescence of the oxidized form by absorbing its excitation energy.

In order to exclude the formation of excimers after oxidation of C11-BODIPY<sub>581/591</sub>, the concentration of oxidized C11-BODIPY<sub>581/591</sub> necessary to form excimers was determined by incorporating increasing amounts of preoxidized C11-BODIPY<sub>581/591</sub> (0–20 mol%) into 250 μM egg-PC vesicles. Subsequently, emission spectra were recorded at 485 nm excitation. The results depicted in Fig. 6A show that excimer formation of oxidized C11-BODIPY<sub>581/591</sub> occurs from 5 mol% upward. Initially, at low mol%, a normal spectrum of oxidized C11-BODIPY<sub>581/591</sub> is visible with the maximum at 520 nm and a shoulder at 545 nm. As the concentration increases (> 0.5 mol%), the shoulder clearly becomes a peak and subsequently an additional emission peak appears at 590 nm (at arrow). The increased emission at 545 nm may be attributed to excimer formation induced by the green peak at 520 nm, whilst the additional excimer emission maximum at 590 nm virtually coincides with the emission maximum of the nonoxidized form of C11-BODIPY<sub>581/591</sub>. Thus, if artificial membrane systems are used, excimer formation can be excluded when using labeling conditions well below 0.1 mol%.

Since brightly stained organelles, such as mitochondria, could potentially contain high mol fractions intact C11-BODIPY<sub>581/591</sub>, it cannot be excluded that excimer formation increasingly perturbs measurements as the oxidation of C11-BODIPY<sub>581/591</sub> progresses. To assess this possibility, CLSM control experiments were carried out. In the first approach, rat-1 fibroblasts were incubated with 30 μM preoxidized C11-BODIPY<sub>581/591</sub> in DMEM at 37°C for 1 h to attempt to induce red excimer formation. Alternatively, a region of cells incubated with nonoxidized C11-BODIPY<sub>581/591</sub> was photo-bleached through prolonged exposure to excitation light at 568 nm. Subsequently, images were taken by exciting only with the 488 nm laser and acquisition at 530 and 590 nm. Figure 6B and C both show significant green emission from the oxidized C11-BODIPY<sub>581/591</sub>, but no red signal in any of the cells. Membrane integrity was assessed by propidium iodine exclusion. Only after addition of a few drops Triton X-100 did nuclear staining occur (see insert, Fig. 6B), indicating that the plasma-membrane was intact throughout the experiments.

To evaluate self-quenching induced perturbation of measurements with C11-BODIPY<sub>581/591</sub> under our labeling conditions, small unilamellar vesicles (250 μM egg-PC) with various amounts of both the nonoxidized and oxidized probe (0–1 mol%) were prepared by ethanolic co-injection into PBS. Figure 7A shows that the first deviation from linearity occurs at 0.12 mol% of both C11-BODIPY<sub>581/591</sub> forms. The maximal amount of C11-BODIPY<sub>581/591</sub> present in cellular structures relative to phospholipid under our labeling conditions was assessed by cell fractioning in which the plasma-membrane and mitochondria were isolated from rat-1 fibroblasts labeled with 1, 5, or 10 μM C11-BODIPY<sub>581/591</sub>.
for a maximum of 1 h at 37°C. Our results show that under these labeling conditions, not more than 0.021 ± 0.004 mol% C11-BODIPY581/591 was found in mitochondria and a factor 11-15 less in the plasma-membrane. Consequently, under our labeling conditions (maximum of 10 μM for 30 min), staining of even the brightest organelles occurs well below the range in which self-quenching or excimer formation occurs.

**Sensitivity to environmental changes**

Changes in the environmental conditions, such as pH and solvent polarizability (solvent in this context includes interior regions of cells, proteins, membranes, and other biomolecular structures), have been shown to potentially induce changes in a fluorochrome’s excitation and emission spectra. Solvent polarizability has been exploited to measure alterations in membrane fluidity during lipid peroxidation with a variety of fluorochromes, including 1-pyrenedodecanoic acid, 1,6-diphenyl-1,3,5-hexatriene, and anthroyloxy fatty acids [35]. While sensing changes in membrane fluidity when the fluorochrome itself is not oxidatively degraded is advantageous, in the case of C11-BODIPY581/591, which directly reports on lipid peroxidation through direct oxidation of the probe, this feature is highly undesirable. Possible problems with data acquisition could arise from shifts in either the excitation or emission spectrum as the oxidation of the probe progresses. Therefore, we recorded excitation and emission spectra in a variety of solvents, ranging from relatively polar to highly apolar (methanol → benzene) [52]. The results in Fig. 7B show only marginal shifts in emission spectra of 9 nm and 5 nm (methanol to benzene), for nonoxidized and oxidized C11-BODIPY581/591, respectively. As expected, shifts in excitation spectra were even smaller than the shifts in emission spectra (data not shown). Apparently, C11-BODIPY581/591 is only mildly affected by environmental polarizability and as such no perturbations in data acquisition should be expected.

It is well documented that during oxidative stress, changes in intracellular pH can affect interaction of a fluorochrome with ions can dramatically affect its fluorescence quantum yield. Because C11-BODIPY581/591 does not contain protolytically ionizable substituents (with the exception of the terminal carboxyl group, which does not participate in the conjugated system of the BODIPY core), it is not expected that pH changes modulate its fluorescence quantum yield. However, it cannot be excluded that oxidation of C11-BODIPY581/591 introduces ionizable substituents. To exclude perturbations by pH changes, the pH sensitivity of C11-BODIPY581/591 was measured in egg-PC SUVs (0.05 mol% C11-BODIPY581/591) prepared in PBS ranging from pH 4 to 10. No intensity changes were observed, indicating that both the oxidized and nonoxidized C11-BODIPY581/591 are insensitive to changes in pH (data not shown).

**Probe lipophilicity, membrane localization, and probe leakage**

Lipophilicity was assessed by determining the partition coefficients of both nonoxidized and oxidized C11-BODIPY581/591 in octanol/water at pH 7.4 (log \( P_{OW} \)), which is a commonly used measure for lipophilicity. Various concentrations of both oxidized and nonoxidized C11-BODIPY581/591 dissolved in octanol were added to three different ratios of octanol-PBS and placed on a shaker for 24 h at ambient temperature in the dark. In addition, because partition coefficients in octanol/water are no true reflection of the partitioning of a particular substance into a biomembrane with many different classes of lipid, various degrees of fluidity, and the presence of membrane proteins, partitioning of the probes was also determined in white erythrocyte ghosts. The results are depicted in Table 1. Results based on log \( P_{OW} \) values show that C11-BODIPY581/591 is lipophilic at pH 7.4 (log \( P_{OW} > 2.3 \)) and that upon oxidation its lipophilicity is not dramatically changed (Table 1). The log \( P_{OW} \) of BODIPY505/515 was measured to determine the lipophilicity of the BODIPY-core itself, which is clearly lipophilic. Furthermore, the impact of the carboxyl-group on the partition coefficient is negligibly low, as demonstrated by the log \( P_{OW} \) at pH 6 (protonation of the carboxyl-group). Comparing these results with the results in white erythrocyte ghost membranes, a significantly higher partition coefficient is found in biological membranes, as expected. In general, oxidized C11-BODIPY581/591 is slightly less lipophilic compared with the intact C11-BODIPY581/591.

Because slight differences in partitioning between nonoxidized and oxidized C11-BODIPY581/591 occurred, a difference in localization of the probe within the lipid bilayer might occur. To assess how deep C11-BODIPY581/591 penetrates into the lipid bilayer, fluorescence parallax measurements were carried out [44,53]. Substitution of the measured ratio of the fluorescence intensities in the presence (\( F \)) and absence (\( F_0 \)) of the quencher into the parallax equation (Eqn. 1) yields the calculated BODIPY probe distance from the center of the bilayer. The strongest quenching occurred with the TEMPO labeled lipids (Table 2), indicating a relatively shallow location, in concordance with the results reported for other BODIPY-acyl probes [44]. An intermediate strength quenching by the 12-SLPC suggests a deep subpopulation (< 7.5 Å), as reported previously by Kaiser and London for various BODIPY probes linked to fatty acids near the end of the fatty acyl chain [44]. These
results are relatively straightforward in the case of the oxidized C11-BODIPY$_{581/591}$. However, the nonoxidized C11-BODIPY$_{581/591}$ is a relatively large and extended fluorochrome and as such it is difficult to accurately determine its quenching by spin-labeled lipids. Overall, the results seem to indicate that the oxidized C11-BODIPY$_{581/591}$ occupies a more shallow location in the vicinity of the polar head-group region of the bilayer compared with its intact form. A shift towards the polar head-group region might possibly facilitate probe leakage. However, as stated previously, fluorescence microscopic observations did not show any detectable probe leakage over a 2 h period in cells stained with C11-BODIPY$_{581/591}$, unlike our observations in 5-dodecanoylaminofluorescein-labeled cells.

By performing surface tension measurements in C11-BODIPY$_{581/591}$ containing egg-PC monolayers, we aimed to substantiate our observations experimentally by determining if either C11-BODIPY$_{581/591}$ or its oxidation products (0, 0.5, 1, and 2 mol%) could spontaneously leave lipid monolayers. Surface tension in the monolayer was measured by the Wilhelmy plate method in Teflon troughs at ambient temperature, using a paper plate and PBS as the sub-phase. The results for 1 mol% probe, as

Table 1. Partition Coefficients of BODIPY Probes in Octanol-water and White Erythrocyte Ghosts

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Log P$_{ow}$ pH6</th>
<th>Log P$_{ow}$ pH 7.4</th>
<th>Log P$_{WEG}$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>C11-BODIPY$^{581/591}$</td>
<td>2.93 (± 0.09)</td>
<td>2.53 (± 0.30)</td>
<td>4.34 (± 0.09)</td>
</tr>
<tr>
<td>C11-BODIPY$^{581/591}$ (oxidized)</td>
<td>2.77 (± 0.05)</td>
<td>2.49 (± 0.04)</td>
<td>4.01 (± 0.03)</td>
</tr>
<tr>
<td>BODIPY$^{505/515}$</td>
<td>4.26 (± 0.23)</td>
<td>4.29 (± 0.41)</td>
<td>n.m</td>
</tr>
</tbody>
</table>

a Relative to PBS pH 7.4. Standard deviations in parenthesis. n.m. = not measured.

Table 2. Fluorescence Parallax a Depth Measurements in DOPC Vesicles at pH 7.2 and Surface Tension Measurements in Egg-PC Monolayers of Oxidized and Nonoxidized C11-BODIPY$_{581/591}$

<table>
<thead>
<tr>
<th>Quencher</th>
<th>F/F$_0$ nonoxidized</th>
<th>F/F$_0$ oxidized</th>
<th>$\sigma = \frac{dy/dt}{d\gamma_i} (\times 10^{-3} s^{-1})$</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence parallax a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMPO-PC</td>
<td>0.50</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-SLPC</td>
<td>0.67</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-SLPC</td>
<td>0.60</td>
<td>0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zcf (Å)</td>
<td>18.80</td>
<td>20.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface tension measurements b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11-BODIPY$^{581/591}$ (nonoxidized)</td>
<td>3.12</td>
<td>0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11-BODIPY$^{581/591}$ (oxidized)</td>
<td>3.44</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg-PC c</td>
<td>3.38</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescein d</td>
<td>52.21</td>
<td>1.75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a F and F$_0$ are fluorescence intensities in the presence and absence of 15% quenchers, respectively. Zcf is the distance from the center of the bilayer. Results are from duplicates.

b $\sigma$ represents the time dependent change in surface tension (mN/m.s) as a function of the initial surface tension ($\gamma_i$) for 1 mol% fluorochrome.

c Negative and d positive control.
presented in Table 2, indicate that both forms do not spontaneously leave the monolayer. Conversely, the positive control, with an estimated log P_{OW} of −0.26 (of every three molecules, one resides in the octanol phase and two in the water phase), induces significant changes in surface tension as it leaks from the monolayer. In general, our measurements showed that even at high mol% no significant change in surface tension compared with negative control (egg-PC) could be detected, irrespective of the oxidation state of C11-BODIPY^{581/591} (data not shown). This indicates that neither C11-BODIPY^{581/591} nor its oxidation products are able to spontaneously leak from the lipid bilayer.

**DISCUSSION**

The development of new assays for measuring lipid peroxidation or evaluating antioxidant efficacy in living cells is important to allow a better understanding of the pathogenesis of lipid peroxidation or the bioremedial effects exerted by antioxidants. Preferentially, these assays should be uncomplicated, sensitive, accurate, and easy to perform with high throughput. Furthermore, since a myriad of biochemical changes are believed to be related to lipid peroxidation, measurement of the average lipid peroxidation over an entire cell population may obscure significant variations in lipid peroxidation among cells. This is clearly demonstrated by Fig. 4C, where several cells show resistance to oxidative stress, whereas others are clearly oxidized in distinct regions. Therefore, methods that allow the evaluation on a cell-by-cell basis would be highly advantageous.

Fluorescent probes are highly suitable to detect particular components of complex biomolecular interactions and processes in cellulo, with excellent sensitivity and selectivity. In combination with suitable detection of fluorescence signals, such as CLSM or flow cytometry, fluorescence measurements per cell are possible, allowing subpopulations within a large sample to be identified and quantitated. In addition, the use of a multiwell/fluorescence reader approach allows screening of large numbers of components, such as antioxidants, for their bioremedial effects in diseased or abnormal cells. Fluorometric detection of lipid peroxidation is most commonly performed with cis-parinaric acid as a reporter molecule. Although this natural fatty acid has been used successfully to elucidate a variety of processes associated with lipid peroxidation in living cells [4,21,24,27], isolated organelles [20,26] and model membrane systems, and has been fully validated [21], several intrinsic molecular properties prevent its application in fluorescence microscopy and multiwell fluorescence readers. cis-Parinaric acid is sensitive to dioxygen, fairly photolabile, and undergoes photodimerization when exposed to intense illumination, all resulting in loss of fluorescence that is not associated with degradation by ROS. Furthermore, its fluorescence excitation and emission in the near-UV range of the electromagnetic spectrum hinders its applicability in fluorescence microscopy. Recently, we introduced C11-BODIPY^{581/591} as a fluorescent ratio probe for measuring and visualizing lipid peroxidation in living cells [32,33]. This new probe overcomes many of the aforementioned disadvantages associated with cis-parinaric acid. Nonetheless, it is imperative that the probe is fully validated, in particular if health claims are to be made based on this new methodology.

C11-BODIPY^{581/591} is an oxidation-sensitive fluorescent fatty acid analogue that shifts its fluorescence from red to green when challenged with oxidizing species (Figs. 1A and 4C). C11-BODIPY^{581/591} is sensitive to a variety of oxidizing species. Studies with various oxidants show that C11-BODIPY^{581/591} is oxidatively degraded by free radical species and peroxynitrite. However, the probe is insensitive to superoxide, nitric oxide, transition metal ions, and hydroperoxides, if no transition metal ions are present. The latter property is advantageous, since the reporter molecule should only report on active ROS activities. Furthermore, because transition metal ions are commonly used to initiate the formation of free radicals, direct degradation of C11-BODIPY^{581/591} by these ions would be highly undesirable. C11-BODIPY^{581/591}'s inertness to superoxide and nitric oxide should be expected, since both free radical species are incapable of reacting with polyunsaturated fatty acids that contain bis-allylic hydrogen with much lower bond energies compared with the C-H bond energies in the diene interconnection of C11-BODIPY^{581/591} [10,12,54]. Given that the radical generating systems used produce a variety of oxy-radicals, it may be concluded that C11-BODIPY^{581/591} is sensitive to hydroxyl, alkoxy, and peroxyl radicals. Interestingly, C11-BODIPY^{581/591} seems to have a preference for the latter two species, as deduced from a relatively mild oxidation by the hydroxyl radical, which could be advantageous in reporting on peroxidation chain propagation reactions (Fig. 1C). Previously we reported that C11-BODIPY^{581/591} is insensitive to peroxynitrite, as deduced from studies with SIN-1 [32]. SIN-1 produces superoxide and nitric oxide, and it is presumed that copious amounts of peroxynitrite are formed by recombination reactions. Apparently, the formation of peroxynitrite from SIN-1 is inefficient and as such, no effects were noticed previously. The results in the current study show that C11-BODIPY^{581/591} is distinctly sensitive to peroxynitrite-mediated degradation. The shape of the decay curve in Fig. 1B indicates that oxidation of C11-BODIPY^{581/591} is rapid within the first 10 min. However, after this initial exponential decrease, the curve levels off at 60% and continues with a slope
C11-BODIPY$^{581/591}$, a lipid peroxidation probe

Comparable to the photobleaching curve. This suggests that no chain propagation occurs. Whether this indicates that peroxynitrite reacts directly with C11-BODIPY$^{581/591}$ and to what extent C11-BODIPY$^{581/591}$ can report on in cellulo formation of peroxynitrite remains to be established and is currently under investigation.

Comparison of the oxidative sensitivity of C11-BODIPY$^{581/591}$ with the commonly used cis-PnA and lipid derived linoleate showed that C11-BODIPY$^{581/591}$ is 2.3 times more sensitive to oxidation compared with linoleate and about 5 times less sensitive compared with cis-PnA. This is favorable, since we argued previously that the high sensitivity of cis-parinaric acid could lead to an overestimation of the damage inflicted to lipids [21]. Conversely, this feature is not necessarily a disadvantage when evaluating attenuation of lipid peroxidation by antioxidants. Nonetheless, the fact that C11-BODIPY$^{581/591}$ is only slightly more sensitive compared with endogenous PUFA allows its application in both the evaluation of antioxidant efficacy and the indexing of lipid peroxidation without the risk of misinterpretation of data.

Microscopic evaluation of C11-BODIPY$^{581/591}$ stained cells showed that the probe is distributed heterogeneously throughout cellular membranes, with a predominant staining of the perinuclear region. Colocalization studies revealed no specific preference for any particular organelle, as mitochondria, peroxisomes, lysosomes, and Golgi apparatus all showed considerable staining and organelle-specific probes colocalized with C11-BODIPY$^{581/591}$ (Fig. 5). Plasma-membrane staining was low, albeit not absent (Fig. 4B). This was further confirmed by isolating mitochondria and the plasma-membrane from labeled rat-1 fibroblasts. The results show that mitochondrial membranes contain a maximum of 0.02 mol% C11-BODIPY$^{581/591}$ relative to phospholipid, whilst the plasma-membrane contains a factor 15 less. Because the oxidized and nonoxidized forms of C11-BODIPY$^{581/591}$ are spectrally well-separated (Fig. 1A), simultaneous acquisition of the green and red emission allows ratio imaging (Fig. 4C, left panel). By calculating the fraction oxidized probe from the green and red images, variations in probe uptake between cells and heterogeneous distribution within cells, as well as instrumental factors such as illumination stability, are effectively eliminated. In addition, this approach allows the evaluation of oxidation of membranous structures with relatively low labeling, such as the plasma-membrane (Fig. 4C, right panel).

Evaluation of cell proliferation showed that no negative effects were induced by concentrations up to 50 μM C11-BODIPY$^{581/591}$ for a maximum of 6 d in culture. This indicates a low cytotoxicity, unlike cis-PnA, which rapidly becomes cytotoxic at these high concentrations in rat-1 fibroblasts (unpublished results), fetal rat astrocytes [55], and neonatal rat cardiomyocytes [4]. cis-Parinaric acid has even been suggested as a suitable sensitizing agent in enhancing lipid peroxidation in malignant cells [55]. Furthermore, no enzymatic oxidation, degradation, or metabolism to lipids of C11-BODIPY$^{581/591}$ seems to occur in rat-1 fibroblasts or neonatal rat cardiomyocytes. This contrary to cis-PnA, which has been shown to metabolically incorporate into the major lipid classes of various cell types [4,21,27] and is distinctly sensitive to β-oxidation in myocardial cells, requiring inhibition of the mitochondrial fatty acid import mechanism with DL-aminocarnitine [4,21].

Fluorochrome fluorescence and detectability is often dependent on environmental factors such as background fluorescence, light scattering, photobleaching, solvent polarity, pH changes and fluorochrome-fluorochrome interactions. Signal distortion caused by autofluorescence in cells can be kept to a minimum by using probes that can be excited at longer wavelengths (> 500 nm). With this respect, C11-BODIPY$^{581/591}$ is highly suitable and we observed no significant interference by autofluorescence when using C11-BODIPY$^{581/591}$ in rat-1 fibroblasts or neonatal rat cardiomyocytes. Furthermore, light scattering by dense media, such as concentrated vesicle solutions, is much reduced at longer wavelengths. Photobleaching of C11-BODIPY$^{581/591}$ is virtually absent when performing measurements in fluorometers and fluorescence readers, as illustrated by curve 1 in Fig. 1B. However, under high-intensity illumination conditions in the CLSM, photobleaching of C11-BODIPY$^{581/591}$ becomes the main factor limiting fluorescence detectability and should be taken into account. Fortunately, a strategic choice of microscope settings during time-course experiments, i.e., laser power, exposure time and frequency, magnification, and detector amplification, combined with evaluating part of the sample which has not been exposed to excitation light allows correction for photoinduced probe oxidation. Although we did not expect C11-BODIPY$^{581/591}$ to be highly sensitive to solvent polarizability or pH changes [35], oxidation of C11-BODIPY$^{581/591}$ might induce molecular changes or introduce substituents, which potentially could drastically influence its sensitivity to environmental changes. Our results show that neither the nonoxidized nor the oxidized forms of C11-BODIPY$^{581/591}$ are sensitive to changes in environmental parameters. The excitation and emission spectra, as well as the fluorescence quantum yield are not significantly influenced by changes in pH or solvent polarity. These characteristics are favorable, since environmental changes will not influence the detection or the quantification of C11-BODIPY$^{581/591}$ oxidation.

Since significant fluorescence emanated from compact structures in the perinuclear region, interference by
fluorochrome-fluorochrome interactions, such as self-quenching and excimer formation, could potentially perturb observations and results. BODIPY-based fluorochromes are known to form red shifted excimers [31,35]. If oxidized C11-BODIPY$^{581/591}$ is present in high fractional concentrations and forms excimers in particular organelles as oxidation progresses, the observer could perceive this phenomenon as if these organelles are resistant to oxidation, when in fact they are completely oxidized, but display red excimer fluorescence induced by increasing amounts of green oxidized BODIPY monomers. Self-quenching would initially induce an exponential decrease followed by an increase in fluorescence as self-quenching is lifted (normally only exponential decays are observed). As the amount of intact probe in the biomembrane is gradually diluted through oxidation and the effective concentration drops below the self-quenching concentration, a subsequent normal exponential decrease in fluorescence is observed. These artifacts could seriously perturb the experimental results and could lead to misinterpretation of data. Therefore we investigated if self-quenching or excimer formation occurred under our labeling conditions (maximum of 10 $\mu$M C11-BODIPY$^{581/591}$ for 30 min). The results in model membranes, as depicted in Figs. 6A and 7A demonstrate that self-quenching occurs above 0.12 mol% C11-BODIPY$^{581/591}$ and excimer formation from 0.5 (green-yellow excimer) to 5 mol% oxidized C11-BODIPY$^{581/591}$ upward (red excimer). Isolation of the plasma-membrane and mitochondria (highly fluorescent) showed that under our labeling conditions a maximum of 0.02 mol% C11-BODIPY$^{581/591}$ is present in brightly fluorescent cellular structures. These results and the microscopic evaluation of excimer formation as depicted in Fig. 6B and C effectively exclude the occurrence of these fluorescence artifacts under our labeling conditions. In addition, the fact that very low labeling concentrations can be used with favorable signal to noise ratios is a major advantage, since this ensures minimal perturbation of the biomembrane.

Microscopic evaluation of cells stained with C11-BODIPY$^{581/591}$ showed that C11-BODIPY$^{581/591}$ readily incorporates into membranes and a good labeling is obtained within 30 min. Its lipophilicity is demonstrated by the partition coefficient in octanol/water (log $P_{\text{ow}} = 2.5$). Partitioning of the probe into white erythrocyte membranes is much higher, presumably because various domains in the lipid bilayer provide a better environment for C11-BODIPY$^{581/591}$ compared with octanol. Furthermore, interaction with membrane proteins cannot be excluded to contribute significantly to C11-BODIPY$^{581/591}$ partitioning. The oxidized form of C11-BODIPY$^{581/591}$ is only moderately more polar than the intact C11-BODIPY$^{581/591}$, as deduced from both the small difference in log P values and the monolayer studies. The latter investigation showed that both forms of C11-BODIPY$^{581/591}$ do not spontaneously leak from membranes, although active removal in living cells by, for instance, Multi-Drug Resistance complexes cannot be excluded. Conversely, routinely performed microscopic observations showed no leakage over a 2 h period.

Fluorescence parallax studies demonstrated that C11-BODIPY$^{581/591}$ localizes in two distinct populations within the lipid bilayer. The dominant population localizes shallow at approximately 18 Å from the center of the bilayer and presumably loops back towards the water interface, whilst the deep population remains deeply embedded within the lipid bilayer. The depth of the deeper C11-BODIPY$^{581/591}$ population suggests that the conformation of the acyl chain is near normal when C11-BODIPY$^{581/591}$ locates deeply. These results are in concordance with the results found for several BODIPY fluorochromes attached to fatty acyl chains, as reported by Kaiser and London [44]. Oxidized C11-BODIPY$^{581/591}$ shows a slight shift towards the polar head group region (± 20 Å), which could be an indication of a marginal increase in polarity due to scission of particular apolar substituents or the introduction of polar substituents. However, this has no dramatic influence on the partition coefficient (Table 1) or probe leakage, as deduced from the monolayer experiments (Table 2). Thus, despite the fact that the predominant population of C11-BODIPY$^{581/591}$ localizes near the polar head group region of the bilayer, the fact that C11-BODIPY$^{581/591}$ can exist in a deeply buried form makes acyl-linked BODIPY probes superior to classical NBD or dansyl acyl-linked membrane probes [44].

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ABBREVIATIONS

AAPH—2,2’-Azobis(2-amidinopropane dihydrochloride)

BODIPY—Boron dipyrromethene difluoride

cis-PnA—cis-Parinaric acid

CLSM—Confocal laser scanning microscopy

CumOOH—Cumene hydroperoxide

DOPC—1,2-Dioleoyl-phosphatidylcholine

DLPC—1,2-Dilinoleoyl-phosphatidylcholine

IPR—Initial peroxidation rate

MeO-AMVN—2,2’-Azobis(4-methoxy-2,4-dimethylvaleronitrile)

PUFA—Polyunsaturated fatty acid

ROS—Reactive oxygen species

SIN-1—3-Morpholinosydnonimine-hydrochloride

5-SLPC—1-Palmitoyl-2-(5-doxyl)-stearoyl-phosphatidylcholine

12-SLPC—1-Palmitoyl-2-(12-doxyl)-stearoyl-phosphatidylcholine

SNAP—S-nitroso-N-acetylpenicillamine

SOPC—1-Stearoyl, 2-oleoyl-phosphatidylcholine

SUV—Small unilamellar vesicle

TEMPO-PC—1,2-Dioleoyl-(TEMPO)phosphatidylcholine