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Epicocconone, A New Cell-Permeable Long Stokes' Shift Fluorescent Stain for Live Cell Imaging and Multiplexing

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Epicocconone is a heterocyclic natural product from the fungus *Epicoccum nigrum* that fluoresces weakly in the green (520 nm). However, cells exposed to epicocconone rapidly absorb the dye and become bright orange fluorescent because the natural product reacts reversibly with proteins. The orange fluorescence is enhanced in lipophilic environments, allowing the visualization of membranous organelles and lipid rafts but does not stain oligonucleotides. As the unconjugated dye has no orange fluorescence, there is no need to wash out the excess fluorophore. Epicocconone is a neutral, non-toxic, small molecule that appears to diffuse readily into live of fixed cells without the need for permeabilization. These features enable the real-time imaging of live cells and the study of organelle movements. Cells stained with epicocconone are excitable by common lasers (UV, 405, 488, and 532 nm) and its long Stokes' shift allows multiplexing applications with more common short Stokes' fluorophores using a single light source.

KEY WORDS Epicocconone; fluorescence; long Stokes' shift; cytoplasmic stain; multiplexing; confocal laser scanning microscopy; live cell imaging .

INTRODUCTION

New fluorescent stains have been developed for their versatile and powerful detection capabilities in the areas of genomics, proteomics, and cellulomics [1, 2]. In the area of cell biology, fluorescent labeling of intracellular structures can be achieved using a variety of fluorescent probes ranging from organic fluorescent molecules [1–5] to fluorescently labeled antibodies [1,6] and co-expression of a GFP-fusion protein [7,8].

Each approach has advantages and disadvantages when applied to live cell imaging. Although small molecules are less likely to perturb cellular systems, many fluorescent small molecules are not cell-permeable [1] and some fluorescent species, e.g. quantum dots, have cytotoxic effects on cells under certain conditions [9]. In addition, common fluorescent molecules that are inherently fluorescent generally cause high fluorescence background originating from unbound dyes [1]. Although antibody based stains can be very specific, they are generally introduced into cells through permeabilization, and consequently have few practical applications in live cell imaging [2]. GFP fusion proteins for live cell imaging may generate concerns that the introduction of GFP (25–27 kD) to a target protein may have undesirable effects on cellular activities due to the relative large size of the fusion, which may be larger than the protein target [10]. A recent novel approach has been to genetically insert a small motif containing the –Cys-Cys-Xxx-Xxx-Cys-Cys– sequence and then exposing cells to non-fluorescent biarsenical derivatives of probes such as fluorescein or resorufin that become fluorescent once bound to the tetracysteine motif and can also be observed with an electron microscope [11]. However, although this approach avoids the large size of GFP, it still requires genetic manipulation of the cell by introduction of a strong metal binding site that could affect the function, distribution and fate of the tagged protein. In addition, the arsenes are toxic and can

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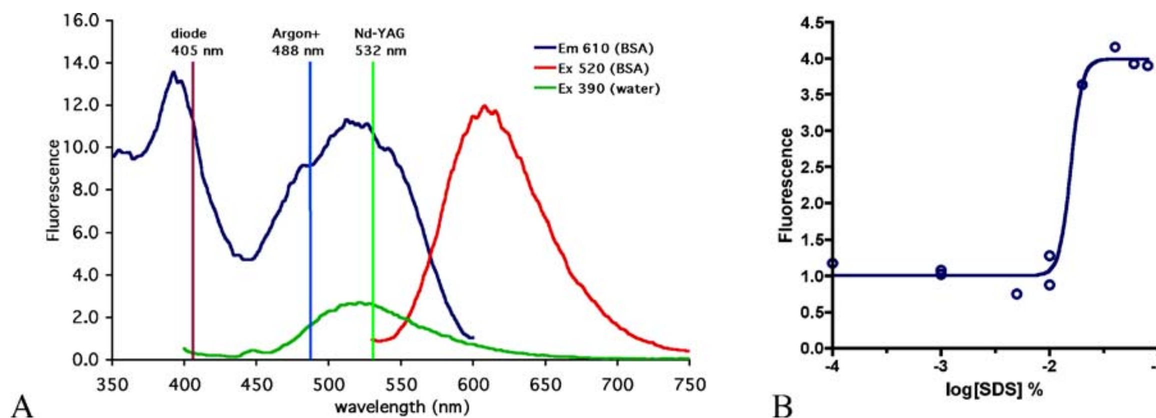

Fig. 1. Photophysical properties of epicocconone. (A) The fluorescence emission profile of epicocconone in water (excitation at 390 nm; green) and in the presence of BSA (excitation at 520 nm; red). The excitation profile (emission at 610 nm; blue) shows two maxima at 395 and 520 nm suitable for many common laser sources and UVA [20]. (B) Relative quantum yield of epicocconone (12 μM) with BSA (3 μM) with varying concentrations of SDS (PBS, pH 7.4) showing an increase in quantum yield in lipophilic environments. Above the CMC (0.7%), the quantum yield exponentially decays (data not shown).

cross-react with other cellular thiols requiring the addition of an antidote [11].

An ideal fluorescent stain for imaging intracellular organelles of live cells would have a specific reactivity so that it becomes brightly fluorescent against a low background, is cell permeable and small to avoid perturbing the cells, non-toxic and display good fluorescent properties such as a long Stokes' shift, high quantum yield and long emission wavelength to separate it from autofluorescence and minimize Rayleigh scattering [12]. For example, fluorescent styryl compounds such as the FM-series, become fluorescent only in a hydrophobic environment such as a membrane [13], generating little fluorescent background and have been used for staining membranes and studying vesicle trafficking, endocytosis and exocytosis [12,14]. Although the styryl dyes do have a long Stokes' shifts and good quantum yields in hydrophobic environments, they are not cell permeable because they have a permanent 2^+ charge. Many of the commercial products that can stain internal structures such as the plasma membrane [13], mitochondria [15], the endoplasmic reticulum [4], and the Golgi apparatus [4] use fluorescently labeled lipid analogs that, it has been suggested [16], perturb lipid phase separation and are ill suited for studying lipid microdomains. Development of fluorescent stains that are fully cell permeable and stain lipid organelles are thus still in demand and would provide researchers with useful alternatives to meet various needs and demands.

The fungus *Epicoccum nigrum* is known to be a source of fluorescent compounds [17] and a crude ethanolic extract from this fungus has been used in cell tracking studies in fluorescently labeled yeast cells [18] and *Giardia* cysts [19]. Epicocconone, an azaphilone natural product purified from this crude ethanol extract, is a water-soluble, low molecular weight (410 amu) molecule that has a weak green fluorescence in water ($\lambda_{em} = 520$ nm), which shifts to red ($\lambda_{em} = 610$ nm) with a concomitant increase in quantum yield in the presence of a protein (Fig. 1A) [20]. Epicocconone spontaneously conjugates to free amines of a protein source [21] to produce a highly fluorescent adduct that is stable in acid but is readily reversible by base ($k'_B = 1.03 \times 10^5$ s^{-1}). The adduct is excitable by common lasers such as solid-state violet (405 nm), argon ion (488 nm) or frequency doubled Nd-YAG (532 nm) [20,21] and additionally increases its quantum yield in lipophilic environments (Fig. 1B). The spontaneous conjugation of epicocconone to proteins, shift in the fluorescence emission and increase in quantum yield are desirable features of a fluorescent probe. Coupled with epicocconone's water solubility and cell permeability suggest this compound could be a very useful cellular stain with little or no background fluorescence. Here we describe the features of epicocconone as an intracellular, fluorescent stain for live cell imaging and provide initial results as to the relative specificity of the dye.

EXPERIMENTAL

Chemical and Reagents

Epicocconone was obtained from FLUOROtechnics Pty Ltd., Sydney, Australia (Epicocconone^{TM}; http://www.fluorotechnics.com/). Bovine serum albumin (BSA) was obtained from Sigma-Aldrich,

Sydney, Australia and sodium dodecyl sulfate (SDS) from USB, Cleveland, OH, USA. SYTOX-green (5 mM in DMSO), Hoechst 33342 (8 mM in H_2O), 3,3'-dihexyloxacarboncyanin iodide, $DiOC_6$ (1.7 mM in DMSO), 4-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a,-diaza-s-indacene-3-pentanoyl) sphingosine, BODIPY® FL C_5-ceramide (4 mM in DMSO) and AlexaFluor 488® lipid raft labeling kit were purchased from Molecular Probe (Eugene, Oregon, USA). The human colon cancer cell line HCT-116 (Australian Proteomic Analysis Facility, Sydney, Australia) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, NY, USA) containing 10% FBS. Fluorophores were diluted in phenol red-free Dulbecco's Modified Eagle Medium (DMEM, Gibco, NY, USA) containing 10% FBS for cell staining. For washing cells, Dulbecco's phosphate buffered saline (pH 7.4 ± 0.2; Gibco, NY, USA) was used. Trypsin-EDTA solution (Gibco, NY, USA) was used for detaching the cell monolayer from culture vessels. Cells were counted after staining with Trypan blue solution (0.4%, Sigma-Aldrich, Sydney, Australia) using the methods outlined in Freshey's manual [22]. For fixing cells, 4% formaldehyde (Sigma-Aldrich, Sydney, Australia) in Dulbecco's PBS was used. For microscopy, a glycerol-based mounting medium (50% glycerol in 20 mM phosphate buffer, pH 8.5) was used.

Fluoresccence Measurement of a Lipophilic Protein Stained with Epicocconone

A lipophilic environment was simulated in a solution containing BSA (3 μM) and varying concentrations of SDS (0–0.4%) in 96-well black plate (clear bottom, Greiner Bio-One, Germany). Epicocconone (12 μM) was added in phosphate buffered saline. Fluoresccence was measured after 30 min on a Typhoon™ 9200 scanner (Amersham Biosciences, Uppsala, Sweden). The scanner setting was 20 mW solid-state frequency doubled NdYAG laser (532 nm in wavelength) for excitation, 610 nm BP for emission, 100 μm for resolution and 500 V for PMT.

Culture Condition for Cell Lines and Preparation

HCT-116 cells were cultured in tissue culture flasks (75 cm^2, Costar, MA, USA) containing the normal DMEM medium (37°C, 5% CO_2, and 100% relative humidity) until ∼80% confluent. The spent medium was decanted from the culture dishes by pipetting. Cells were washed once with Dulbecco's PBS, detached by treatment of trypsin-EDTA solution for 5 min in the incubator, re-suspended in DMEM medium and seeded onto fresh culture vessels.

Trypan Blue Toxicity Testing

HCT-116 cells were cultured as described above and were seeded in duplicate at cell densities of approximately 1×10^5 cells per well in tissue culture multi-well plates (35 cm × 1.0 cm, Flow Laboratories, Virginia, USA) containing 2.5 mL of the normal DMEM medium, and grown for 1 day. The spent medium was decanted from the culture dishes by pipetting. Epicocconone, diluted in phenol red free DMEM to yield concentrations of 0, 0.5, 1, 2.5, 5, 7.5, and 10 μM, were carefully placed in the wells. Absolute cell numbers were determined at 0.5, 1, 2, and 24 hr intervals by counting cells on a hemocytometer using Trypan blue exclusion [22].

Staining Conditions

HCT116 cell monolayer was grown for 1 day in a CultureWell™ Chambered Coverglass (∼10^5 cells/mL/well, Molecular Probe, Oregon, USA). The spent medium in the chambered coverglass was carefully decanted by pipetting. The chambered coverglass was filled with 200 μL of the phenol-red free DMEM growth medium containing 5 μM epicocconone for 30-min-staining at room temperature (dark). Dual staining was achieved by counter-staining the cells with 1.25–2.5 μM SYTOX-green, 1–4 μM Hoechst 33342, 3.4 μM $DiOC_6$, 1 mg/mL cholera toxin subunit B (CT-B) from AlexaFluor 488® lipid raft labeling kit or 4 μM BODIPY® FL C_5-ceramide. These dual stains were used according to manufacture's instructions (Molecular Probes, Eugene, OR, USA).

The staining solutions were decanted from the chambered wells. The cell samples that were stained using Molecular Probes' stains were washed twice with Dulbecco's PBS by pipetting. Cells stained with epicocconone were not washed. The cells were then fixed in chilled 4% formaldehyde for 20 min at 4°C and washed twice with Dulbecco's PBS. The coverglass taken out from the chamber was mounted in the glycerol-based mounting medium onto a clean glass slide.

For live cell staining, HCT-116 cells (approximately 2×10^5 cells/mL) were seeded onto a 0.16–42 mm cover glass (POC chamber System, H. Saur, Reutlingen, Germany) that had been placed in tissue culture multi-well plate (6.0 cm × 1. 5 cm, Flow Laboratories, Virginia, USA) containing 2.5 mL of the DMEM growth medium, and grown for 1 day. One cover glass with the cell monolayer was aseptically transferred and placed into the POC cell chamber, and a stain-free growth medium (∼2 mL) was added to the temperature-controlled microscope chamber. The medium was replaced with 2.5 mL of the phenol red free growth medium containing 5 μM

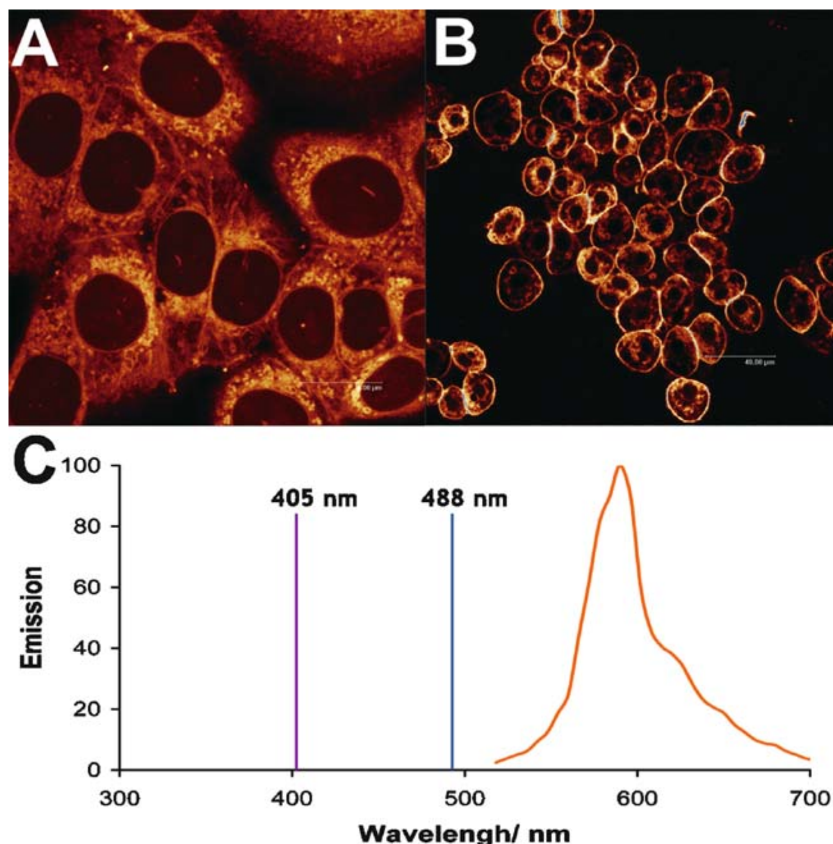

Fig. 2. Fluorescent staining of colon cancer cells with epicocconone. Epicocconone stains cellular proteins of fixed (A) or live (B) HCT116 cells orange against a low background. The XYλ scan (C) of fixed cells shows an emission centered on 590 nm with no fluorescence <540 or >650 nm when the sample was excited by an Ar ion laser (488 nm). Bar, 16 μm.

epicocconone. Images were obtained over a 30-min staining period.

Confocal Laser Scanning Microscopy

Confocal micrographs were obtained on TCS SP2 Confocal Laser Scanning system equipped with DM IRE microscope (Leica Microsystems, Heidelberg GmbH, Germany). Stained cells were viewed under the microscope with a ×100 (numerical aperture of 1.4) or with a ×63 (numerical aperture of 1.3) oil immersion objective.

A fluorescence emission profile of epicocconone-stained cells (fixed) was obtained by recording lambda series (XYλ) (TCS SP2 Leica Microsystems, user manual or http://www.sct.ub.es:802/PDF/Leica/Help/Tutorials/Lambda_Series.htm). A detection bandwidth of 500–700 nm was used for epicocconone. The stained cells were scanned with either a 405 nm diode laser or a 488 nm-Ar ion laser. A detection bandwidth of 420–700 nm (excitation 405 nm) was used for Hoechst 33342. The detection bandwidth for SYTOX, $DiOC_6$, CT-B conjugate and BODIPY® FL C_5-ceramide of 500–700 nm with excitation at 488 nm was used. Data from all images were processed using the manufacturer's "Quantify" software in the dye separation section.

Dual staining was performed with narrower detection bandwidths, for epicocconone (620–650 nm), Hoechst 33342 (450–490 nm), SYTOX-green, $DiOC_6$, BODIPY® FL C_5-ceramide (505–540 nm) and CT-B conjugate (500–520 nm). For single staining, 590–640 nm was used for epicocconone. A pinhole of 300 μm was used and each image (512 × 512 pixels) was recorded by XY single scan mode. The appropriated Z position was chosen to observe intracellular structures of the cells. The fluorescence images were displayed in blue (450–490 nm), green (505–540 nm) and orange (620–650 nm) color output. In order to ensure uniformity for comparisons of fluorescent

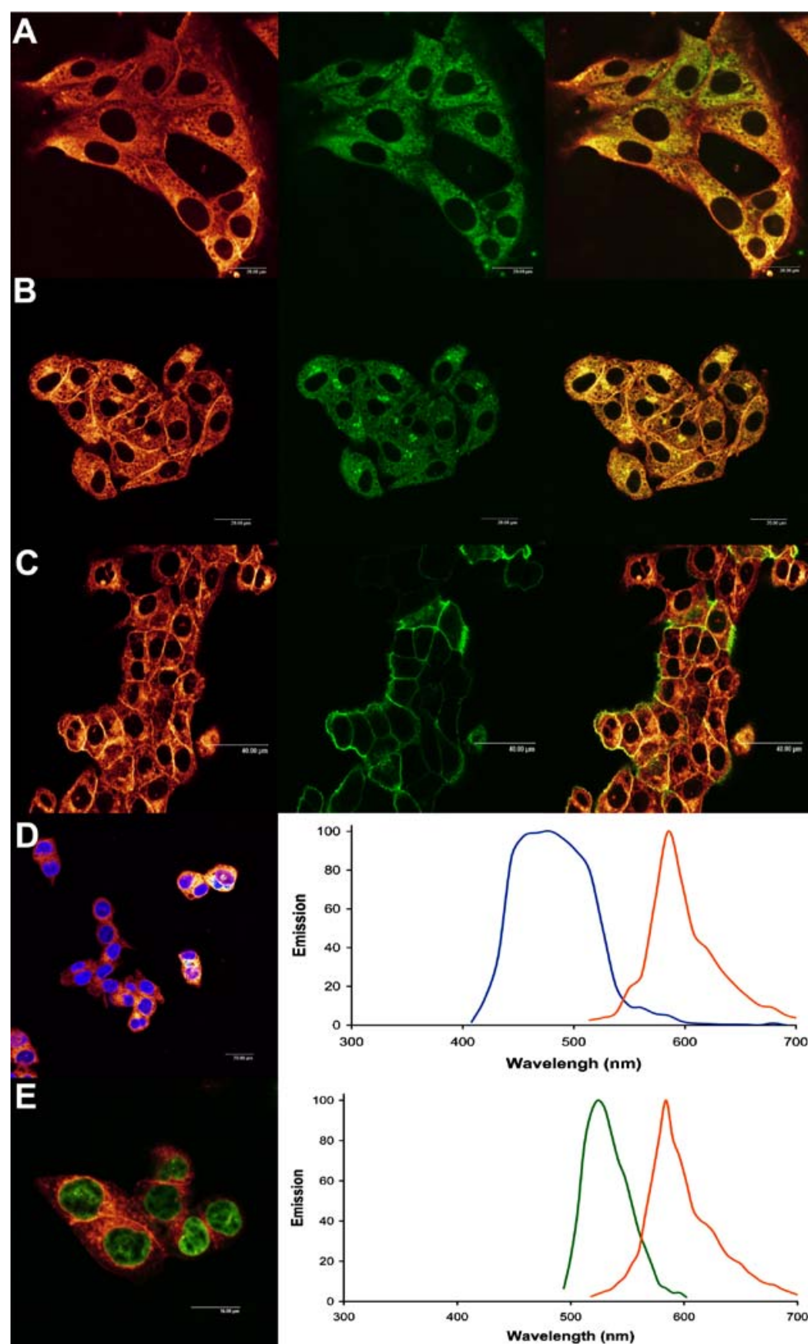

Fig. 3. Intracellular staining of lipophilic organelles. Confocal fluorescence images of HCT116 cells (fixed) that were dual stained with epicocconone (*left panels*) and $DiOC_6$ (A, *middle panel*), BODIPY® FL C_5-ceramide (B, *middle panel*) and lipid raft stain (C, *middle panel*). Overlayed images (*right panels*) shows similar staining patterns for epicocconone with all three specific stains but epicocconone additionally strongly stains the plasma membrane. In contrast, nucleic acid stains such as Hoechst 3342 (D) or SYTOX-green (E) show no overlap with epicocconone staining. Epicocconone can be multiplexed with blue (D) or green (E) fluorophores by excitation at 405 nm (D) or 488 nm (E). Bar, 20 μm except in D and E where the bar is 40 and 16 μm, respectively.

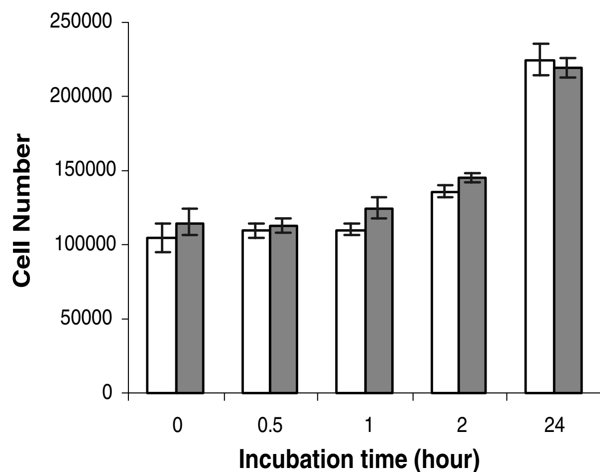

Fig. 4. Effect of Epicocconone™ on growth of HCT-116 cells and live cell staining with Epicocconone™. Counts of epicocconone-stained cells by using Trypan blue show that epicocconone has no significant effect on growth at the concentrations up to 5 μM over 24-hr incubation period. Unfilled columns represent cells that are not exposed to epicocconone and filled columns, cells exposed to epicocconone.

staining, all cell samples were analyzed the same day with the same laser scanning settings.

Images of live cells stained with epicocconone were recorded for 30 min using time image series (XY*t*) (Leica Microsystems, user manual or http://www.sct.ub.es:802/PDF/Leica/Help/Tutorials/Time_Image_Series.htm).

RESULTS AND DISCUSSION

Epicocconone has a low level of green fluorescence in pure water but becomes highly fluorescent in the presence of protein (Fig. 1A). The excitation profile of the protein reacted epicocconone (blue line in Fig. 1A) indicates that it can be excited by many commonly used laser sources [20] that facilitate its use in fluorescence microscopy. To simulate the environment of a cell, epicocconone was first added to a protein solution (BSA) with varying concentrations of lipid (SDS). The results (Fig. 1B) show that not only does the fluorescence emission shift from green to red/orange in the presence of protein but the quantum yield is increased in the presence of lipid, up until the CMC for SDS, after which the fluorescence intensity drops off dramatically. The fact that epicocconone fluorescence is sensitive to its environment suggested that it may be useful for staining cells and cellular features.

Live or fixed cells stained with epicocconone are highly orange fluorescent against a low fluorescent background even though excess fluorophore was not removed (Fig. 2). A maximum emission peak is obtained at 590 nm, blue shifted as compared to *in vitro* uses (Fig. 1A) with non-detectable fluorescence at 510–540 nm or 650–700 nm (Fig. 2C) when the stained sample was excited at either 405 or 488 nm. Elucidation of the mechanism of this long Stokes' shift fluorescence [21] provides the chemical background for epicocconone-staining: Epicocconone spontaneously conjugate to free amines of proteins, and the epicocconone–amine adduct (enamine) becomes highly orange/red fluorescent against very low (green) fluorescent background due to the formation of an internal charge transfer complex (ICT) that has a large dipole moment in the excited state. Like other ICT fluorophores, epicocconone fluoresces very brightly in hydrophobic environments but coupled with its long Stokes' shift suggests that epicocconone should be ideal for multiplexing with more common dyes.

Unlike the FM-dyes, which are amphophilic in nature, epicocconone is a small neutral molecule that rapidly crosses membranes, reversibly staining proteins with no background. Real-time confocal microscopy revealed that epicocconone stained the plasma membrane of live cells within 2 min, and that the cytoplasm is labeled to saturation within 15 min (data not shown). In the cells that are dual stained with epicocconone and $DiOC_6$ (an ER stain), an antibody-based lipid raft stain and BODIPY® FL C_5-ceramide (a Golgi stain) epicocconone is shown to concentrate in the same features (Fig. 3). However, epicocconone does not appear to stain nucleic acids as there is no overlap in staining between the DNA specific (Hoechst 33342) or general oligonucleotide (SYTOX green) stains (Figs. 3D–E). Epicocconone stains lipophilic organelles and the plasma membrane suggesting that this compound could be useful for imaging membranous, lipid-rich organelles in lipid trafficking study [12].

Because epicocconone is excitable by either at 405 or 488 nm ($\lambda_{ex} = 390/490$) and emits in the orange (590 nm) it can be multiplexed with shorter Stokes' shift dyes such as the blue-emitting Hoechst 33342 ($\lambda_{em} = 479$ nm, Fig. 3D) or a green-emitting SYTOX ($\lambda_{em} = 524$ nm, Fig. 3E). These results demonstrate that epicocconone could be multiplexed with a wide range of blue, green and yellow-emitting probes with a single laser source.

To be useful for live cell imaging, it is important that stains do not affect the viability or growth of cells. This can be a problem with many small molecules and quantum dots. However, we have found that epicocconone has no significant effects on the growth or viability of HCT116 cells at the staining concentration (5 μM) over a 24 hr period (Fig. 4). Similarly, a panel of 56 human cell lines [23] showed that epicocconone is not cytotoxic or inhibitory to growth (Table I) at concentrations ≤ 5 μM.

Table I. In Vitro Cytotoxicity Results Against 56 Benign and Malignant Cell Lines Showing Percentage Growth at Three Concentrations of Epicoccone [23]

	Percent growth at epicocconone				Percent growth at epicocconone		
Panel/cell line	0.5 μM	5 μM	50 μM	Panel/cell line	0.5 μM	5 μM	50 μM
Leukemia							
CCRF-CEM	110	95	5	SKMEL-2B	68	76	41
HL-60 (TB)	85	111	−17	SK-MEL-5	103	104	−6
K-562	100	141	3	UACC-257	94	94	57
MOL T-4	96	99	−26	UACC-62	92	89	−50
RPMI-8226	109	115	3				
SR	81	102	−8	*Ovarian cancer*			
				IGROV-1	98	98	12
Non-small cell Lung cancer				OVAR-3	68	78	−43
A549/ATCC	94	97	10	OVAR-4	104	100	51
EKVX	89	86	3	OVAR-5	83	85	−20
NCI-H226	86	66	−8	OVAR-6	91	93	17
HOP-62	94	96	1	SK-OV-3	72	62	−19
NCI-H322M	100	84	−15				
NCI-H460	93	90	11	*Renal cancer*			
NCI-H522	94	91	−22	786-0	95	102	9
				ACHN	95	94	87
Colon cancer				CAK1-1	97	80	−31
COLO 205	82	76	−49	RXF 393	85	86	15
HCC-2998	53	68	−28	SN 12C	87	80	−8
HCT-116	92	87	2	TK-10	90	73	20
HCT-15	92	93	73	UO-31	86	78	20
HT29	97	90	48				
KM12	96	87	−15	*Prostate cancer*			
SW-620	103	103	31	PC-3	76	109	35
				DU-145	91	94	20
CNS cancer							
SF-268	89	83	16	*Breast cancer*			
SF-295	74	56	−42	MCF7	95	87	16
SF-539	92	91	4	NCVADR−RES	100	95	−33
SNB-19	65	56	−32	MDA-MB-231/ATCC	76	70	−55
SNB-75	97	89	75	HS 578T	94	86	−15
U251	92	94	85	MDA-MB−435	94	82	−44
				BT-549	86	78	6
Melanoma				T-47D	52	45	−50
LOX IMV1	95	100	90				
MALME-3M	100	69	−8				
M14	99	83	−28				
SK-MEL-2	94	86	37				

A notable exception was the Breast cancer (T-47D) cell line that had a GI_{50} of 0.4 μM but showed no cytotoxicity (>20 μM).

The commercially available cell stain Epicocconone™ has a number of useful features for imaging live cell. When added to cells, epicocconone becomes bright orange fluorescent. This results in a very low background and negates the requirement to wash away unbound fluorophore. Live cells are readily permeable to epicocconone and do not require any pre-treatment to allow the stain to be taken up. Epicocconone does not affect growth of mammalian cells at concentrations similar to those used for staining. The long Stokes' shift of epicocconone enables multiplexing with a wide variety of common short Stokes' shift fluorophores. These technical features of epicocconone suggest it will bring benefits to live cell imaging and may find applications in staining lipophilic structures, membrane dynamics or tracking cellular trafficking.

Cells take up epicocconone by unfacilitated diffusion. Living or fixed cells are stained in a time-dependent manner, first the outer plasma membrane becomes flu-

orescent, especially at any cell–cell interfaces, then the lipophilic structures in the cytoplasm such as ER, Golgi and lipid rafts are stained. The nuclear membrane is stained but the nucleus remains completely unstained by epicocconone. The dynamic reversibility of the covalent derivatisation of proteins explains epicocconone low toxicity and suggests that cellular systems are minimally perturbed. The long Stokes' shift and relative specificity for proteins in a lipophilic environment mean that epicocconone can be multiplexed with many other more common fluorophores.

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