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Letter

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High Affinity Neutral Bodipy Fluorophores for Mitochondrial Tracking

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KEYWORDS: Bodipy, fluorescent probes, mitochondrial staining, cytotoxicity

ABSTRACT: We report the first high affinity neutral Bodipy fluorophores for selective imaging of mitochondria with notable sensitivity (~100 nM) and insignificant cytotoxicity even at very high concentration (~100 μM), when tested against HeLa cells. Further, these fluorophores are chemically robust and require no special conditions for storage.

Mitochondria are unusual intracellular organelles involved in many cellular processes and play a key role in cell survival and death. Their main function, however, is to provide cellular energy in the form of ATP for various cellular needs. An imbalance in mitochondrial bioenergetics leads to formation of excessive reactive oxygen species (ROS), that in turn cause oxidative stress-mediated cellular responses. A wide range of human diseases, such as metabolic disorders, cancers, cardiovascular and neurological diseases are associated with abnormal functions in mitochondria.[1] Thus numerous therapeutic applications targeting the mitochondria have been developed and have been playing vital role in diagnosis and biomedical research.[2]

The morphological changes in mitochondria in living cells are typically discerned by fluorescence microscopy using mitochondria-selective dyes. A simple, yet efficient, approach to distinguish mitochondria from other organelles is to take the advantage of substantial negative electrochemical potential (130-170 mV, negative side) across the inner mitochondrial membrane. Based on this phenomenon, cationic lipophilic dyes tagged with triphenylphosphonium (TPP)[3], 5,5',6,6'-tetrachloro 1,1',3,3'tetraethyl-benzimidazol carbocyanine iodide (JC-1),[4] tetramethylrhodamine methyl ester (TMRM)[5] and several others were developed for studying mitochondrial physiology. Majority of the commercial dyes used for mitochondrial imaging include rhodamine,[6] and cyanine dyes[7] suffer from various trade-offs such as low specificity, inhibiting mitochondrial respiration and high toxicity.[8,9] However, most of the commercially available cationic probes are chemically unstable and thus require storage below -20 ^{o}C. It is a well-known fact that cationic molecules induce cytotoxicity by imposing mitochondrial membrane depolarization leading to unwarranted biological responses, thereby drastically limiting their applications. On the other hand, extensive studies on non-cationic mitochondrial probes have shown that they have a poor fluorescence image profile pertinent to explore for alternate fluorescent mitochondrial probes.[10-13] Neto *et al*. have succinctly discussed the relevance of neutral fluorophores by incorporating strong electron acceptor groups in the molecular skeleton. The low electron density (+δ) results in specific regime of a neutral structure, enabling the fluorophore to localize in the mitochondria.[14] Borondipyrromethene (Bodipy) derivatives show bright fluorescence, fair chemical stability and structural features with magnificient photophysical properties . They are often used as biological probes, fluorescent stains, and protein labelling reagents.[15] Also, various cationic-Bodipy dyes have been reported for staining mitochondria tagged with TPP,[16-19] *cis*-platin,[20] phosphonium,[21] and quarternized pyridine moieties.[22,23]

The present communication reports the design and synthesis of three neutral Bodipy derivatives tethered with phenoxymethylpyridine unit namely BIP, CF-MONO, and CF-BI (Figure 1), which provide highly efficient molecules for targeting mitochondria. To the best of our knowledge, this is the first report on highly efficient neutral Bodipy based specific mitochondrial staining.

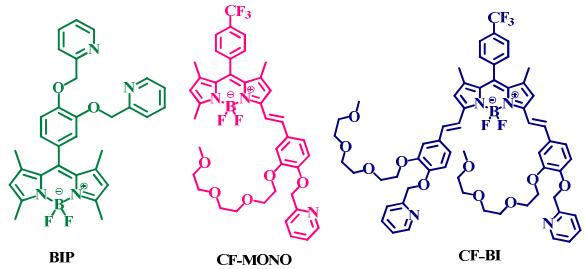
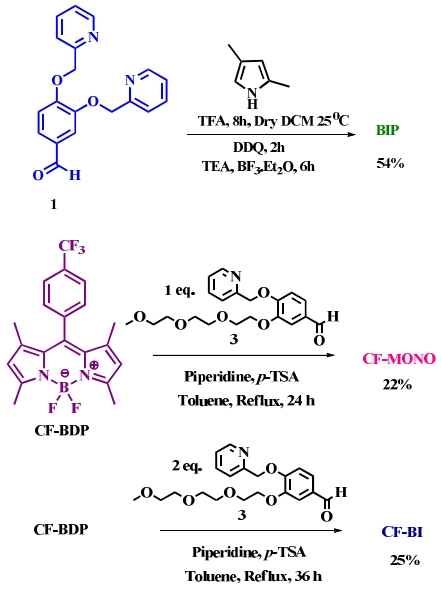


Figure 1. Molecular structures of Bodipy fluorophores

The probes CF-MONO and CF-BI contain oligoethyleneglycol moieties to maintain a hydrophilic−lipophilic balance and improved cell permeability. The electron accepting group -CF_3 (trifluoromethyl group) has been incorporated in the molecular structures in order to result

+δ character over a specific region, facilitating the fluorophore localization in mitochondria.

The choice of aldehydes **1** and **3** (Scheme 1) is the key for designing the present series of Bodipy based probes. The synthetic routes of compounds BIP, CF-MONO, and CF-BI were presented in Scheme 1. The compound BIP was synthesized by following classic one pot synthesis; by the acid catalyzed condensation of aldehyde **1** with 2,4 dimethyl pyrrole followed by the oxidation with DDQ to form dipyrrin, which in turn complexed with $BF_3.Et_2O$ in presence of triethylamine (see details in supporting information (SI).[24] The compounds CF-MONO and CF-BI were synthesized by Knoevenegal condensation of C_3/C_5 methyl groups of CF-BDP (for synthetic protocol see SI) with aldehyde **3** in presence of catalytic amount of piperidine and *p*-TsOH in toluene under reflux condition. The mono- and bi- condensations were controlled by adjusting mole ratio of the reactants and monitoring the reaction time, resulting CF-MONO and CF-BI derivatives in 22 and 25% yields respectively (Scheme 1) (details are in SI). All the compounds were characterized by NMR (^1H, ^{13}C) and HRMS (See SI).



Scheme 1. Synthetic route of Bodipy fluorophores

The photophysical properties of the Bodipy fluorophores were recorded in three solvents of varying polarity, including dicholoromethane (Figure S1, SI), acetonitrile (ACN, Figure 2) and dimethylformamide (Figure S1, SI) at room temperature.

The normalized absorbance and emission spectra of these fluorophores in acetonitrile are shown in Figure 2. All these dyes possess small Stokes shifts (<25 nm) and maintain similar photophysical properties in different solvents. Fluorescence quantum yields were calculated using fluorescence emission spectra, obtained from low concentration solutions (0.045-0.05 OD), by a standard relative method. Bodipy-CO_2H, Rhodamine B and Zinc tert-butyl phthalocyanine were used as reference for BIP, CF-MONO and CF-BI respectively.[25-27] The photophysical data are presented in Table1. Superior pH insensitivity and photostability was exhibited by the Bodipy fluorophores compared to the commercial Mitotracker Red CMXRos (Figures S2 and S3 in SI).

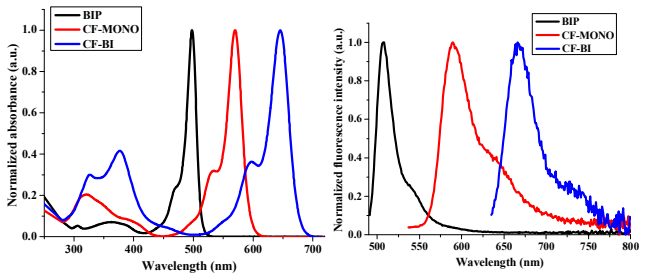


Figure 2. Normalized absorbance and emission spectra of Bodipy fluorophores in acetonitrile

Table 1. The photophysical properties of Bodipy fluorophores

Dye	$\Lambda_{max\ Abs}$[a]			$\Lambda_{max\ Ems}$[b]			ε[c]			Φ[d]
	DCM	ACN	DMF	DCM	ACN	DMF	DCM	ACN	DMF	ACN
BIP	502	498	501	514	510	515	1,37,671	89,562	81,707	0.51
CF-MONO	576	570	576	595	589	598	1,14,438	1,22,947	1,45,454	0.42
CF-BI	652	645	654	672	666	675	1,94,100	1,26,865	1,16,333	0.21

a. Absorption maximum; b. Fluorescence emission maximum (obtained by exciting at the absorption maximum of the dye); c. Extinction coefficient at absorption maximum; d. Fluorescence quantum yield calculated by a standard relative method using Bodipy-CO_2H, (ϕ = 0.57 in CH_2Cl_2), Rhodamine B (ϕ = 0.70 in CH_3OH), Zinc tert-butyl phthalocyanine (ϕ = 0.37 in benzene).

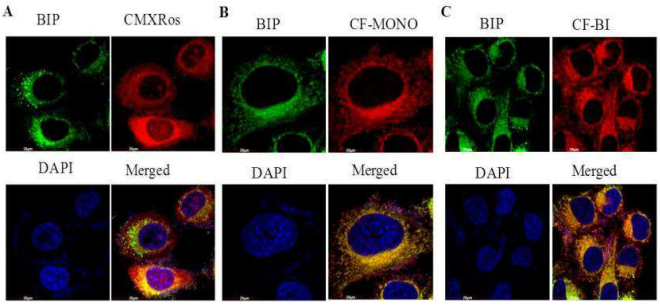


Figure 3. Mitochondrial staining by Bodipy fluorophores in HeLa cells: The cells were incubated with Bodipy fluorophores (100 nM) at 37 ^{0}C for 30 min. A) Green fluorescence indicates mitochondrial staining of BIP compound by confocal microscopy using FITC filter. Red fluorescence indicates mitochondrial staining of Mitotracker Red CMXRos (Thermofisher) by confocal microscopy by using rhodamine filter. Blue fluorescence indicates nuclear staining by DAPI. B) Same as A, except that red fluorescence indicates mitochondrial staining of CF-MONO compound by confocal microscopy by using rhodamine filter. C) Same as A, except that red fluorescence indicates mitochondrial staining of CF-BI compound by confocal microscopy by using rhodamine filter.

Fluorescence microscopy experiments were conducted in human cervical carcinoma (HeLa) cells to investigate The ability of Bodipy fluorophores to stain mitochondria was investigated in in human cervical carcinoma (HeLa) cells. The intracellular distribution was observed through colocalization imaging experiments. HeLa cells were incubated with these Bodipy fluorophores (100 nM) at 37 ^{0}C for 30 min. Figure 3 displays the fluorescence microscopic images of cells, and after coincubation with both BIP and Mitotracker^{TM} Red CMXRos, a commercially available mitochondria selective dye, from Thermofisher.

The results of confocal microscopy show that all the newly synthesized Bodipy fluorophores substantially stained the mi-

tochondria with marginal retention in the cytoplasmic compartment (Figure 3). However, no fluorescence was noticed in the nuclear compartment. In contrast, slight fluorescence was observed in the nuclear compartment of the cells with the commercially available Mitotracker Red CMXRos (Figure 3), implying that these Bodipy fluorophores are very specific in staining mitochondria in living cells.

The accumulation of these Bodipy fluorophores in the mitochondrial fraction was further confirmed by high resolution mass spectrometry (HRMS). For HRMS studies, HeLa cells were treated with 10 μM Bodipy fluorophores for 24 h and the cells were fractionated into cytosolic and mitochondrial fractions (see SI). From Table 2, it is evident that almost all (> 99%) the Bodipy fluorophores are localized in the mitochondrial fraction. HRMS spectra of mitochondrial and cytosolic fractions are included in SI.

Table 2. Cytosolic and mitochondrial uptake of Bodipy fluorophores (from HRMS)

Dye	Cytosolic Fraction (N=3) (nmole/μg protein)	Mitochondrial Fraction (N=3) (nmole/μg protein)
BIP	1.84 ± 0.91	6140.75 ± 300.94
CF-MONO	48.35 ± 9.2	6706.81 ± 163.09
CF-BI	0.0045 ± 0.81	4558.64 ± 229.0

We next studied the cytotoxicity of all these compounds in HeLa cells, treated at 100 μM for 24 h. None of the synthesized compounds caused any noticeable cell death. On the other hand, incubation of cells with 5 μM CMXRos resulted in significant cytotoxicity (Figure 4).

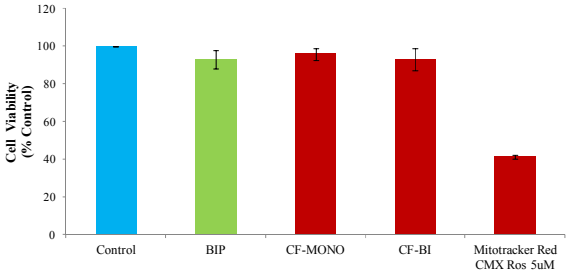


Figure 4. Effect of Bodipy fluorophores on cytotoxicity: HeLa cells were treated with Bodipy fluorophores (100 μM) and Thermofisher's Mitotracker Red CMXRos (5 μM) for 24h and cell viability was measured by SRB assay. Cell viability data above is an average of three independent experiments.

To examine whether the uptake of the dyes was dependent on the mitochondrial membrane potential,[28] cells were treated with *p*-trifluoromethoxyphenylhydrazone (FCCP) prior to the staining procedure. FCCP is a mitochondrial uncoupler that causes rapid acidification of the mitochondria and dysfunction of ATP synthase and reduces the mitochondrial membrane potential (MMP or Ψ_m). All the probes showed considerable reduction in the mean fluorescence intensity upon FCCP-induced MMP depolarization (Figure 5). The reduction in fluorescence for BIP, CF-MONO and CF-BI was 48%, 38 % and 38% respectively (Figure 6). This result indicates that these fluorophores definitely localize in mitochondria and its cellular uptake is sensitive to the mitochondrial membrane potential.

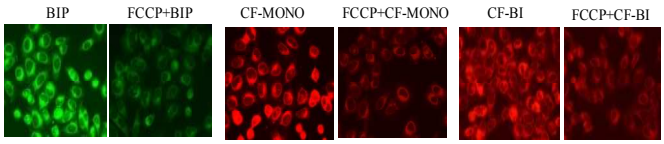


Figure 5. HeLa cells were pre-treated with FCCP (20 μM) for 8h before incubating with Bodipy fluorophores for 30 min (100 nM). Images were taken with fluorescence microscope equipped with Rhodamine and FITC filters.

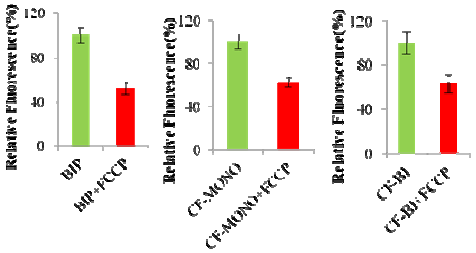


Figure 6. HeLa cells were pre-treated with FCCP for 8h before adding Bodipy fluorophores. Images were quantified with image proplus software.

In summary, highly specific neutral Bodipy-based fluorophores with exclusive affinity towards mitochondria were developed. The chemical robustness of these fluorophores requires no special conditions for storage. These Bodipy fluorophores present excellent mitochondrial targeting traits with exceptionally low cytotoxicity compared to commercially available mitochondrial probes. We believe, exploring and commercialization of such fluorophores is highly needed for better understanding of mitochondrial diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, characterization spectra (PDF).

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ABBREVIATIONS

ACN, acetonitrile; ATP, adenosine triphosphate; Bodipy, borondipyrromethene; $BF_3.Et_2O$, boron trifluoride diethyl etherate; DAPI, 4',6-diamidino-2-phenylindole; DCM, dichloromethane; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; DMF, dimethylformamide; FCCP, *p*-trifluoromethoxy phenylhydrazone; FITC, fluorescein isothiocyanate; HeLa, human cervical carcinoma; HRMS, high resolution mass spectrometry; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'tetraethyl-benzimidazolcarbocyanine iodide; MMP,

mitochondrial membrane potential; NMR, nuclear magnetic resonance; OD, optical density; ROS, reactive oxygen species; SRB, sulforhodamine B; TFA, trifluoroacetic acid; *p*-TsOH, *p*-toluenesulfonic acid; TMRM, tetramethylrhodamin emethylester; TPP, triphenyl phosphonium.

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