SCOTfluors: Small, Conjugatable, Orthogonal, and Tunable Fluorophores for In Vivo Imaging of Cell Metabolism

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Abstract: The transport and trafficking of metabolites are critical for the correct functioning of live cells. However, in situ metabolic imaging studies are hampered by the lack of fluorescent chemical structures that allow direct monitoring of small metabolites under physiological conditions with high spatial and temporal resolution. Herein, we describe SCOTfluors as novel small-sized multi-colored fluorophores for real-time tracking of essential metabolites in live cells and in vivo and for the acquisition of metabolic profiles from human cancer cells of variable origin.

Metabolites are essential biochemical components, with their transport and localization regulating most biological functions. Despite advances in fluorescence imaging to label biomolecules,[1] there are few approaches to image small metabolites in live cells and intact organisms. Most metabolites do not contain groups that allow direct visualization and need to be modified with exogenous chromophores. However, fluorescent labels, in particular red and near-infrared (NIR) fluorophores, are bulky structures that can impair metabolic traffic within cells. Our group has recently developed fluorogenic amino acids to label peptides without affecting their properties.[2] Herein, we describe a new strategy for direct imaging of essential metabolites in live cells and in vivo using small-sized multi-color fluorophores.

Since the report by Ghosh and Whitehouse,[3] nitrobenzodioxazole (NBD) has been widely used because of its small size and neutral character. These properties have facilitated labeling biomolecules with retention of their native properties.[4] However, NBD (λem ≈ 540 nm) is incompatible with other green fluorescent reporters (e.g., GFP) and has limited application for in vivo use. To address these shortcomings, herein we report a collection of fluorophores, named SCOTfluors, with tunable emission covering the entire visible spectrum. SCOTfluors include the smallest fluorophores emitting in the NIR window (650–900 nm) reported to date (Figure 1).

Figure 1. General synthetic procedure, structures, and spectral properties of SCOTfluors. a) Values determined in EtOH. b) QY in dioxane using acridine orange, fluorescein, rhodamine 101, and Cy5 as standards.
Several strategies have been described to optimize the optical properties of fluorophores for live-cell imaging.\[3\] For instance, the replacement of oxygen atoms with geminal dimethyl groups in rhodamine and fluorescein produced red-shifted fluorophores with enhanced properties for bioimaging.\[5\] We envisioned that the synthesis of nitrobenzodiazoles with different groups bridging the nitroaminoaniline core would render multi-color fluorophores with tunable emission and enhanced capabilities for metabolite imaging in live cells.

The preparation of SCOTfluors was achieved in two synthetic steps from the common intermediate 1 (Figure 1). First, the aminoaniline core was cyclized with different bridging groups. All these reactions proceeded similarly for fluoride and chloride derivatives (full list of analogues in the Supporting Information). Second, halogenated compounds (2, Figure 1) underwent substitution with primary and secondary amines to render the final fluorophores (3-7, Figure 1). Triazole derivatives (4) were synthesized by reaction with sodium nitrite in acidic media at r.t., thio derivatives (5) were obtained by condensation with N-thionylaniline under heating, and selenium analogues (6) were prepared by reaction with SeO\textsubscript{2} under reflux in EtOH. Finally, carbon derivatives (3 and 7) were synthesized by Cu-catalyzed coupling using linear and cyclic ketones, respectively.

We examined the optical properties of SCOTfluors and compared them to the original NBD (Figure 1 and Figure S1). With the exception of triazoles (4), all compounds showed longer emission wavelengths than NBD, long Stokes shifts (around 80–100 nm), solvatochromic properties (Figure S2), and good photostability (Figure S3). Among SCOTfluors, Se- and C-bridged derivatives display red and NIR emission, respectively, likely owing to reduced HOMO–LUMO gaps.\[2,6b,c,7\] To the best of our knowledge, this is the first example of C-bridged nitrobenzodiazoles as fluorophores with NIR emission. Furthermore, C-bridged derivatives are readily accessible through one-step coupling of aminoaniline 1 with different ketones, representing a new platform for the direct synthesis of small NIR fluorophores. SCOTfluors proved compatible for experiments in live cells, showing no significant cytotoxicity in HeLa cells (Figure S4).

Then, we examined the properties of SCOTfluors for imaging the trafficking of essential metabolites under physiological conditions. Sphingolipids are critical components of biologically active cells and tissues,\[10\] although few have been reported for in vivo use. We synthesized compound 9 (Figure S3) as an in vivo-compatible glucose analog by conjugation of the nitrobenzoselenadiazole 6 with 2-deoxyglucose amine. Notably, we performed the reaction with chloride and fluoride derivatives of 6 and observed increased reactivity and recovery for the latter (Figure S9). Compound 9 showed emission around 605 nm with a remarkable Stokes shift of 115 nm (Figure S10), enabling multiplexed imaging with blue and green fluorescent proteins (i.e., BFP and GFP, Figure 3A–F). We examined the transport of 9 in HeLa cells

in emission (Figure 2B and Figure S7). We exploited this property to visualize the recycling of ceramide 8 in real time in human A549 cells using fluorescence confocal microscopy.

At short times (i.e., 15 min), the ceramide 8 was mainly found at the Golgi apparatus around the ER, as shown by high co-localization with ER Tracker Green (R = 91%) but not LysoTracker Blue (R = 26%). Time-lapse imaging demonstrated that the ceramide 8 translocated to the recycling lysosomes after 3 h (Figure 2C) as highlighted by the increased co-staining with LysoTracker Blue (R = 75%). Notably, these observations agree with prior reports of lipid mobilization,\[9\] and could not be obtained with a fluorescein ceramide analog (Figure S8). These results confirm the suitability of our approach to prepare neutral NIR-fluorescent probes to image biolipid function in cells.

We also examined whether SCOTfluors could be used to image in vivo tissues with high metabolic activity. Fluorescent deoxyglucose tracers can monitor glucose uptake in metabolically active cells and tissues,\[10\] although few have been reported for in vivo use. We synthesized compound 9 (Figure S3) as an in vivo-compatible glucose analog by conjugation of the nitrobenzoselenadiazole 6 with 2-deoxyglucose amine. Notably, we performed the reaction with chloride and fluoride derivatives of 6 and observed increased reactivity and recovery for the latter (Figure S9). Compound 9 showed emission around 605 nm with a remarkable Stokes shift of 115 nm (Figure S10), enabling multiplexed imaging with blue and green fluorescent proteins (i.e., BFP and GFP, Figure 3A–F). We examined the transport of 9 in HeLa cells...
transfected with EGFP-tagged GLUT4, the main glucose transporter in mammalian cells. Fluorescence microscopy showed the uptake of 9 in GLUT4-EGFP and co-localization with the transporters (Figure 3A–C). Notably, the uptake of 9 was blocked by competition with excess glucose (Figure 3D–F) and was increased by pre-treating HeLa cells with insulin (11) (Figure 3H and Figure S11). These results confirm that compound 9 is a functional substrate of GLUT4 and that enables dual tracking of glucose uptake and its transporters under physiological conditions. Finally, we tested compound 9 in vivo in zebrafish embryos to visualize regions of high glucose uptake. In vivo administration and imaging of compound 9 in wildtype zebrafish embryos indicated bright red fluorescence staining in regions of the developing brain (e.g., midbrain and hindbrain; Figure 3J), highlighting the role of deoxyglucose to recognize GLUT2 transporters. Altogether, compound 9 can be used to image glucose uptake in vivo and to perform non-invasive studies of glucose transport in whole organisms.

Next, we used SCOTfluors to prepare the first red-fluorescent analogue of lactic acid, an essential metabolite in muscle, blood, and cancer cells. Lactic acid is known as a carbon source in cancer cells and its uptake in tumours has been recently linked to aggressive oncological behaviour, yet little is known about its traffic and diffusion inside cancer cells. We conjugated the nitrobenzoselenadiazole 6 with l-isoserine to produce compound 10 (Figure 4A, $\lambda_{em} \approx 605$ nm) as a probe to study the transport of lactic acid in live cells. First, we confirmed increased uptake in hypoxic (1% $O_2$) versus normoxic (20% $O_2$) cells, since lactic acid can accumulate in environments with low concentrations of oxygen (Figure 4B).
We performed flow cytometry analysis to observe that hypoxic cells were significantly brighter than normoxic cells after incubation with the same concentration of compound 10. We also performed competition assays between 10 and excess of lactic acid in normoxic cells, which markedly reduced the fluorescence staining, suggesting a common transporter for compound 10 and lactic acid in live cells (Figure 4B). Encouraged by these results, we used total internal reflection fluorescence (TIRF) microscopy to image the real-time diffusion of lactic acid in normoxic and hypoxic cancer cells with super-resolution. For these studies, we used HeLa cells that had been treated or not with dimethyloxalylglycine (DMOG), a permeable prolyl 4-hydroxylase inhibitor that upregulates hypoxia-inducible factors.

We tracked the paths of over 1000 individual particles in both untreated (i.e., normoxic) and DMOG-treated (i.e., hypoxic) cells after incubation with compound 10 and measured their respective intracellular diffusion coefficients (Figures 4D-E and Figure S12, Movies 1–4). Remarkably, particles in hypoxic cells showed higher mean diffusion coefficients than in normoxic cells, as well as a reduction of the slow diffusion species. Altogether, these results suggest that hypoxic tumors might display faster recycling rates for intracellular lactic acid than normoxic tumors and demonstrate the utility of compound 10 as a new probe for imaging lactic acid metabolism in live cells with high spatiotemporal resolution.

Finally, given the multi-color capabilities and high stability of SCOTfluors under physiological and oxidative environments (Figure S13), we employed them to analyze the metabolic profiles of human cells from different origin. The groups of Chang and Rotello previously reported the metabolic profiles of human cells from different origin. The authors acknowledge funding from Medical Research Scotland (S.B.: 879-2015), MSCA Individual Fellowship (A.F.: 704912), OPTIMA (N.D.B.: EP/L016559/1), Wellcome Trust Sir Henry Dale Fellowship (Y.F.: 100104/Z/12/Z) and the Spanish Ministry of Science, Innovation and Universities (J.L.A, A.D.: CTQ2017-85378-R). M.V. acknowledges funds from ERC Consolidator Grant (77143), Biotechnology and Biological Sciences Research Council (BB/M025160/1) and the Royal Society (IECR3/170132). The authors thank the technical support from the Flow Cytometry and the Confocal Advanced Light Microscopy units at the University of Edinburgh.

**Conflict of interest**

The University of Edinburgh has filed an invention disclosure form to protect part of the technology described in the study.

**Keywords:** cancer - fluorescence - imaging agents - metabolites - super-resolution imaging


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Small fluorophores: A family of small multi-color benzodiazole fluorophores for non-invasive imaging of essential metabolites in live cells and in vivo is described.