Published in final edited form as:
Chembiochem. 2011 September 19; 12(14): 2120–2121. doi:10.1002/cbic.201100365.

Selective Imaging of Mitochondrial Surfaces with Novel Fluorescent Probes

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Keywords

bioconversions; fluorescent probes; high-throughput screening; mitochondria; organelles

Organelles play critical roles in cellular function, and thus imaging of specific cellular organelles and tracking the status of their components by fluorescence cellular imaging is vital.[1–3] Selective staining of organelles helps to identify the location of particular proteins or targets of interest in cells and provides information about the functional and structural dynamics of cellular components. Although a diverse array of organelle-selective small-molecule dyes and fluorescent-protein-based markers are commercially available, there is a growing demand for more sensitive and selective fluorescent molecules for biological applications, particularly in order to better understand complex cellular processes in vivo. In this highlight, we shall briefly introduce how novel, organelle-specific fluorescent molecules can be discovered and optimized by using cell-based high-throughput screening (HTS), and we will use Kawazoe et al.'s recent report[4] of a mitochondrial, surface-specific fluorescent probe as an example.

Mitochondria are found in nearly every cell in the body and are known as cellular "powerhouses" because they generate and supply the cells' energy. Given the importance of this metabolic role, it is perhaps not surprising that recent discoveries have implicated mitochondrial defects in a wide range of diseases, including cancer, diabetes, autoimmunity, and aging.[5–7] To elucidate the cellular and molecular mechanisms of mitochondrial processes, several fluorescent dyes have been developed.[8] Mitochondrion-specific, small-molecular fluorescent dyes may be categorized into two groups based on their mechanism of accumulation in mitochondria. One group consists of lipophilic, cationic dyes, such as the analogues of rhodamine, rosamine, styryl, and carbocyanide dyes. Since mitochondria have a highly negative mitochondrial membrane potential (MMP), lipophilic, positively charged dyes that penetrate the cellular membrane (due to their lipophilicity) are readily sequestered by a functioning mitochondrion through a trans-membrane potential difference of the positively charged dyes and the negatively charged MMP. Such dyes have been widely used to study the MMP and the distribution of mitochondria in living cells. These dyes reversibly accumulate in mitochondria and can be subsequently washed out of the cells when the MMP is lost, except for rosamine derivatives, which contain a mildly thiol-reactive chloromethyl moiety (e.g., MitoTracker probes). The second group of dyes accumulate in mitochondria largely independently of MMP; examples are 10-*N*-nonyl acridine orange (NAO) and

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MitoTracker Green FM. These dyes selectively accumulate in mitochondria by a specific interaction with mitochondrial membrane proteins and/or lipids. Alternatives to small-molecule dyes are fluorescent proteins, such as green fluorescent protein (GFP) or red fluorescent protein (RFP), that can be fused to mitochondrial-targeting sequences.[9] While these fluorescent proteins provide the advantage of imaging live cells, they can perturb mitochondrial function and require engineered cell lines.

It is difficult to visualize and quantify mitochondrial structure and dynamics simultaneously in living cells by using a single type of probe. Typically, different types of lipophilic cationic dyes are used in combination with MMP-independent dyes or proteins to explore the relationships between MMP and mitochondrial morphology as well as to screen the effects of drugs that alter mitochondrial function. Therefore, to elucidate molecular mechanisms more precisely and to assess the effects of a compound on a mitochondrially mediated pathway, there is an increasing demand to develop novel, fluorescent probes specific for mitochondria that act through different mechanisms.

Combining cell-based HTS of large chemical libraries with microscopy is widely used in the discovery of new, fluorescent, small molecules.[10, 11] Nowadays, HTS can be done at a reasonable cost and provide moderate accuracy. Kawazoe et al. recently carried out cell-based screening of 12 000 small molecules with aromatic structures and identified 31 compounds as potential cellular probes for live-cell imaging applications.[4] One of the hit compounds, **1**, exhibited a distinct cellular staining pattern and an analogue of **1**, molecule **2**, provided a simpler and smooth staining pattern in confocal microscopic images (Figure 1A). When molecule **2** was used with commercially available organelle-specific dyes such as LysoTracker Red, Mito-Tracker Red, and ER-tracker Blue-White DPX (all from Invitrogen) to costain HeLa cells, Kawazoe et al. found that the Mito-Tracker Red staining was completely surrounded by staining from molecule **2** (Figure 1B). After investigating fractionated organelles from stained cells by SDS-PAGE, the authors confirmed that molecule **2** specifically stains the mitochondrial surface. Further structure analysis revealed that the weakly fluorescent molecule **2** underwent structural bioconversion within the cells and cyclized to the highly fluorescent molecule **3** in the mitochondrial matrix (Figure 1A). Molecule **3** is the key dye that generates strong fluorescent signals on the mitochondrial surfaces by targeting an unknown molecular target. In addition, it was verified that the staining properties of molecule **2** are MMP-independent and similar in a range of cell types.

Although this study successfully demonstrated how to select, improve, and characterize organelle-specific dyes from a small-molecule library by using a cell-based fluorescence HTS, careful validation studies are still needed to verify and optimize the lead compound, especially when a dye is selected for identifying the subtle structure of cells, such as the mitochondrial membrane. In many cases, as shown in Figure 1B, it is difficult to clearly distinguish vague borders in cells such as the mitochondrial matrix from the inner and outer membranes simply because of the limited resolution of standard confocal microscopy. New technologies like stimulation emission depletion (STED) or super resolution microscopy can overcome the resolution limit and can be used to verify compounds like **3**. In addition, the exact mechanism of bioconversion from molecule **2** to **3** in cells and molecule **3**'s direct molecular targets need to be identified. Discovery of this new cell-permeable dye that enables live-cell imaging of mitochondrial surfaces will, in combination with other conventional fluorescent molecules, certainly improve traditional visualization studies of mitochondrial dynamics.

The most disadvantageous, or perhaps advantageous, aspect of cell-based HTS is that the target is not known during the initial experimental phase. As described by Kawazoe et al., many biologically useful fluorescent dyes can be accidently discovered by using this

technique. Once hit compounds are found to be reasonably specific to a certain biological target in cells, the specificity and fluorescent properties of lead compounds can generally be improved by structure-based rational design and synthesis. However, several aspects of optical dyes besides specificity and fluorescent intensity should be characterized and optimized.[12] For example, aqueous solubility, cellular membrane permeability and uptake, autofluorescence, quantum yield, and photobleaching properties are important to the design. Since most fluorescent dyes can induce phototoxicity as a by-product of the photobleaching process, their cytotoxicity under fluorescent microscopy should be carefully examined. In addition, the effects of dyes on physiological or structural function of the target organelle should be considered. As mentioned above, a successful fluorescent dye for specific organelle imaging, such as one that images mitochondrial surfaces in live cells, should have many additional aspects fully characterized. Kawazoe et al.'s article demonstrates that cell-based HTS of aromatic-enriched chemical libraries by fluorescence microscopy is useful for the discovery of a new, cell-permeable, fluorescent dye with unexpected but interesting and practical properties.

Acknowledgments

This work was supported by the Intramural Program of the National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health. We thank Dr. Henry S. Eden for proofreading the manuscript.

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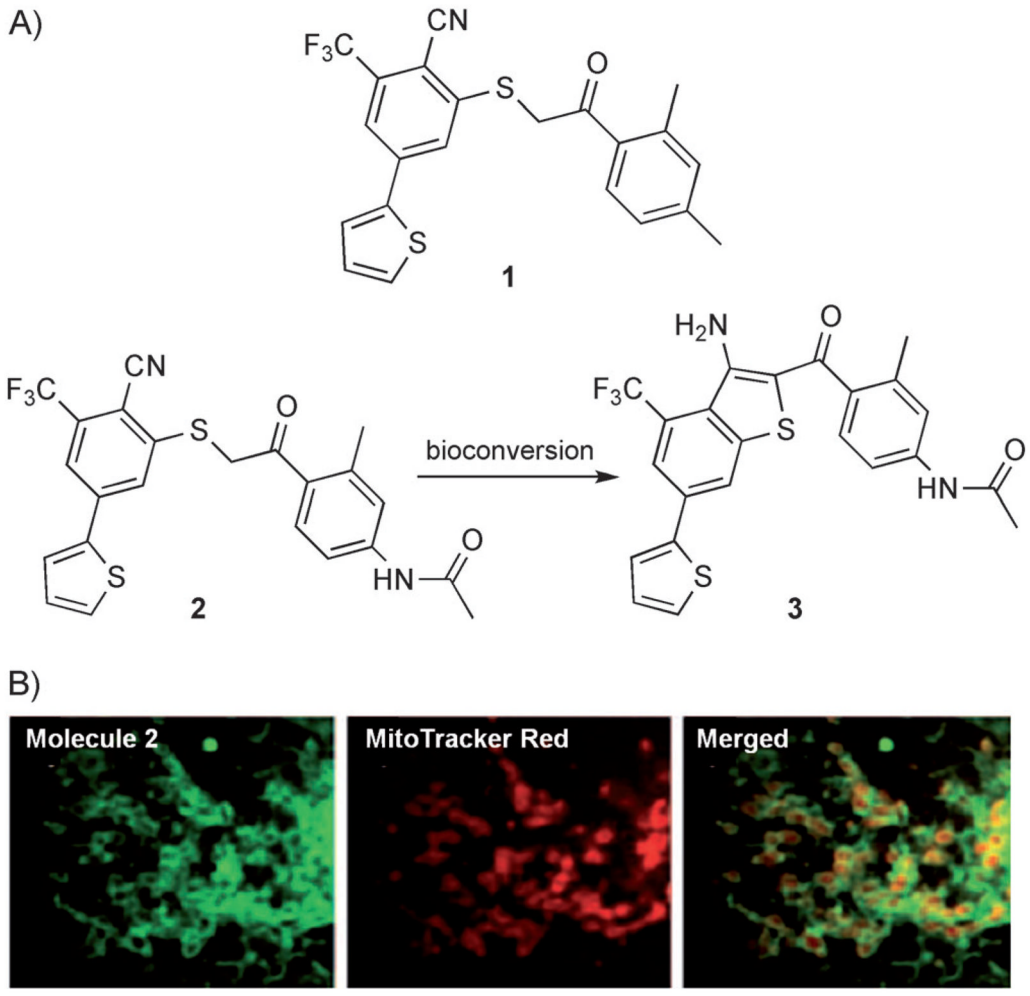

Figure 1.

A) Chemical structures of molecules **1**, **2**, and **3**. Molecule **2** is weakly fluorescent and can be cyclized to its metabolite molecule **3**, which is highly fluorescent, by in situ bioconversion in the cells. B) Confocal images of HeLa cells treated with **2** or MitoTracker Red and the merged image. This figure is reproduced with permission from ref. [4]. Copyright: Wiley-VCH Verlag GmbH & Co. KGaA, 2011.