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Double labelling of intracellular mitochondria and nucleolus using thiophene pyridium salt with high quantum yield as biosensor and its application in stimulated emission depletion nanoscopy

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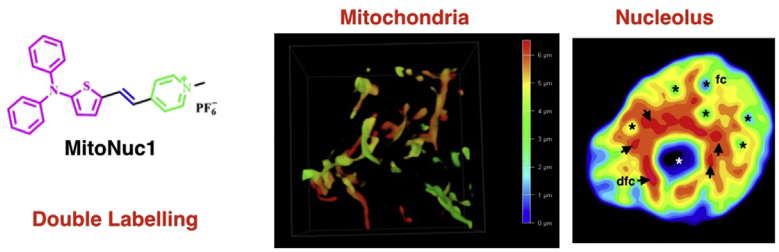
HIGHLIGHTS

- A small water-soluble pyridium based compounds with two-photon activity.
- High sensitivity and selectivity against RNA.
- This probe render double labelling of mitochondria and nucleolus with superb photo-stability and non-invasiveness.
- Its application of revealing mitochondrial and nucleolar ultrastructure under STED was displayed.

GRAPHICAL ABSTRACT



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ABSTRACT

Probe for dual-site target distinct subcellular compartments from cytosol and nucleus is an attractive approach, however, which was scarcely reported. Herein, a series of small-molecular thiophene pyridium salt derivatives (**MitoNuc1-4**) possessing water-soluble, high quantum yield and two-photon activity were rationally designed, and their structures were crystallographic confirmed. Systematic photophysical and biological imaging property investigations were carried out for them. It was found that **MitoNuc1-4** exhibit two-photon absorption properties in the near infrared region, and **MitoNuc1** has membrane permeability and cationic nature, rendering it to be double labelling of mitochondria and nucleolus in living cells with superb photo-stability and non-invasiveness. It also demonstrated that **MitoNuc1** in living cells can monitor mitochondrial division in real time and revealed nucleolar ultrastructure under stimulated emission depletion nanoscopy.

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1. Introduction

Labelling subcellular organelles in living samples under laser microscopy are of great importance and have become a universal strategy in biological related research [1–3]. In particular, its power

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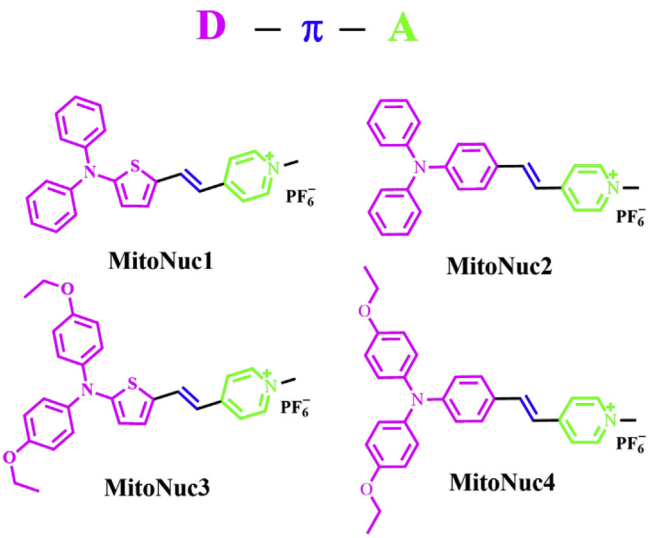
has been further extended to super resolution (SR) fluorescence microscopy [4,5], such as stimulated emission depletion (STED) microscopy (or nanoscopy) [6,7], which offers the resolution down to hundreds nanometers (e.g. ~ 200 nm for green fluorescent protein, GFP). Whereas SR applications were somehow limited in fixed or transfected samples, as well as suffering the risk of alternation the very ultrastructure using concentrated markers during such process [8].

In living system, individual organelle (e.g. mitochondria, ribosome, nucleus and nucleolus) is given priority to specific roles, whereas there is little doubt that each of them are inextricably linked either in physical or functional manner. For instance, nucleolus, ribosomes, endoplasmic reticulum and mitochondrial etc. Are all involved and closely correlated during the course of synthesising quaternary structural protein from single amino acid [9]. It is known that mitochondria play essential roles in many biological functions and service as a 'power house' for other organelles; while nucleolus functions as storeroom of nuclear RNA. It is believed that the nucleolus number and size directly attribute for intracellular protein synthesis capability (or activity) [10]. In order to study the complexities and the coordination of biological process involving mitochondria and nucleolus as the above mentioned in living system, it is generally considered that double or even triple labelling of distinct organelles are necessary. Thus, small-molecule imaging probes are attractive and versatile tools for studying living system due to their mild toxicity, easy penetration and sensitive detection. Consequently, several efforts were assigned to such purpose and a number of them became commercially available [11,12]. Due to diverse nature of each intracellular component, varied targeting moieties were well developed. For example, triphenylphosphonium could be accumulated in mitochondria, and morpholine could specifically label acidic lysosomes [3,13]. However, double or triple labelling of each subcellular organelles require multiple steps of staining or washing, subsequently, introduce unexpected toxicity, cell stress and signal crosstalk. One probe for multiple organelles labelling is definitely ideal, yet rarely existed.

Within varied developed fluorescent probes, pyridinium salts, with both cationic and anionic units, have been extensively investigated in the nonlinear optical (NLO) materials, as well as considered to be potential candidates for two-photon microscopy [14–18]. Pyridinium salt derivatives possess an overall positive charge, which facilitates the permeation through the plasma membrane into mitochondria of cells and have a high tendency to genetic substances including DNA and RNA [19,20]. However, a survey of recent literature revealed that pyridinium salt derivatives possess low quantum yield [21–23], which may not favour for biological laser microscopy and its extended SR utilities. Furthermore, thiophene derivatives have also attracted much attention due to the rich electronic feature of the thiophene moiety [24,25]. Thiophene unit acts as a π-conjugated bridge, which owns higher stability than the double bond, and higher electron delocalization than benzene. Therefore, it can be used to construct molecular optoelectronic functional materials. It was proposed that the derivatives containing a thiophene unit should result in large molecular hyperpolarizability and charge transfer, which are necessary for the photophysical properties and NLO responses [26,27].

Bearing these in mind, pyridium based biosensor **MitoNuc1-MitoNuc4** have been rationally designed and synthesised (Scheme 1 and Fig. S1), its double labelling purpose would be achieved *via* following considerations: (1) The triphenylamine/diphenylamine thiophene group and ethyoxyl moiety acts as donor (D) and N-methyl pyridinium moiety acts as acceptor (A) to form a D-π-A system, resulting in strong and concomitant optimization of optical performances [20]. (2) Ethyoxyl was also attached to the triphenylamine group to enhance its electron-donating ability and improve solubility of the compounds. (3) The pyridium salts, PF_6^- anion, as well as thiophene group should make the designed compounds to be good water-soluble, photostability and biocompatibility. The results confirmed that compound **MitoNuc1** displayed good water solubility and relative high quantum yield, and possessed high affinity with RNA *in vitro*. Further *in cellulo* studies observed mitochondria and nucleolus double labelling under confocal microscopy with superb biocompatibility and photonstability. We further showed **MitoNuc1** is capable of displaying mitochondria and nucleolus ultrastructure in living system under stimulated emission depletion (STED) nanoscopy. Such outcome offered valuable tools as intracellular double labeling probe to monitor mitochondrial and nucleolar dynamic functions simultaneously in living system.



Scheme 1. The title compounds of **MitoNuc1**, **MitoNuc2**, **MitoNuc3 and MitoNuc4.**

2. Experimental sections

2.1. Materials and apparatus

All chemicals and solvents were dried and purified by the standard methods. ^{1}H NMR and ^{13}C NMR spectra were carried out on a Bruker 400 Ultrashield spectrometer using DMSO-d_6 as the solvent. MALDI-TOF mass spectra were recorded on a Bruker Autoflex III Smartbeam. IR spectra were obtained on a Nicolet FT-IR 870 SX spectrophotometer with KBr pellets.

UV-*vis* absorption spectra and one-photon fluorescence spectra were carried out on a SHIMADZU UV-3600 spectrophotometer and HITACHI F-7000 spectrophotometer, respectively. The two-photon cross-section was measured by two-photon excited fluorescence method and Z-scan method at femtosecond laser pulse and Ti: sapphire system (680–1080 nm, 140 fs) as the light source.

2.2. Synthesis

The corresponding aldehyde derivatives [28–31] (10 mmol) and M (2.35 g, 10 mmol) were dissolved in 50 mL ethanol, and then added 5 drops piperidine, refluxed for 8 h. The mixture was cooled to room temperature and filtered, washed twice with ethanol. Pure solid was collected. Single crystals of **MitoNuc2-MitoNuc4** suitable for X-ray diffraction analysis were obtained.

MitoNuc1: Yield: 78%, Mp: 220 °C. ^{1}H-NMR (DMSO-d_6,

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