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Organization of inner cellular components as reported by a viscosity-sensitive fluorescent Bodipy probe suitable for phasor approach to FLIM

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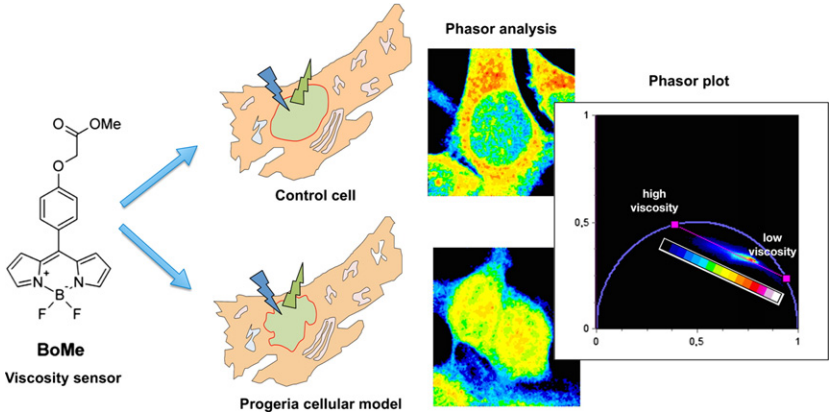
HIGHLIGHTS

- We developed a new Bodipy-based viscosity-sensitive molecular rotor, **BoMe**.
- We used **BoMe** to explore viscosity properties in cellulo, via FLIM phasor approach.
- **BoMe** binds to dsDNA both in vitro and in vivo.
- We compared intracellular viscosity maps in HGPS cellular model with control cells.
- A less viscous nucleoplasm environment in HGPS cell indicates less compact chromatin.

GRAPHICAL ABSTRACT



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ABSTRACT

According to the recent developments in imaging strategies and in tailoring fluorescent molecule as probe for monitoring biological systems, we coupled a Bodipy-based molecular rotor (**BoMe**) with FLIM phasor approach to evaluate the viscosity in different intracellular domains. **BoMe** rapidly permeates cells, stains cytoplasmic as well as nuclear domains, and its optical properties make it perfectly suited for widely diffused confocal microscopy imaging setups. The capability of **BoMe** to report on intracellular viscosity was put to the test by using a cellular model of a morbid genetic pathology (Hutchinson–Gilford progeria syndrome, HGPS). Our results show that the nucleoplasm of HGPS cells display reduced viscosity as compared to normal cells. Since **BoMe** displays significant affinity towards DNA, as demonstrated by an in vitro essay, we hypothesize that genetic features of HGPS, namely the misassembly of lamin A protein within the nuclear lamina, modulates chromatin compaction. This hypothesis nicely agrees with literature data.

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1. Introduction

In the last two decades, fluorescent molecules drastically increased our capacity to monitor biological systems at the cellular and subcellular level by fluorescence microscopy [1–3]. This prompted a strong effort towards the synthesis of new and ever more effective fluorescent probes tailored to a broad range of applications, including the characterization at nanoscale of the cellular environments where relevant biological processes take place [4–7]. In this context, ideal probes should combine good emission properties in the visible, high sensitivity towards specific molecular or physicochemical determinants, and low photo-toxicity. Another required feature is the capability to provide a *quantitative* fluorescence signal irrespective of excitation-light intensity or probe concentration [8]. Indeed, the control of probe concentration is very difficult to achieve in experiments targeting intracellular environments. This requirement is satisfied by λ-ratiometry, an imaging method based on the steady-state intensity ratio computed at two or more wavelengths [9]. Yet, λ-ratiometry requires fluorescent probes whose excitation and/or emission spectra are displaced by intermolecular interactions. Typically this is achieved by fusing together two emitting units with opposite optical behavior with respect to the parameter to be monitored [9]. As a consequence, the design and synthesis of such probes may be complex. Fluorescence lifetime imaging (FLIM) constitutes an excellent alternative to λ-ratiometry, given the strong sensitivity of fluorescence lifetime to biomolecular interactions conjugated with its independence from the probe concentration [10,11]. In most cases FLIM is carried out by recording the time decay of fluorescence in each image pixel by accumulating the stochastic time arrivals of single photons [12]. The main drawback of conventional FLIM is, however, the low number of photons per pixel (~500–1000) that can be collected in a typical measurement. This signal is barely enough to distinguish a single from a double exponential decay, which represents the minimum requirement to decide if at least two emitting species/states are concomitantly present in the same pixel. To address this issue, Clayton et al. [13] and Gratton et al. [14] developed the *phasor approach* to FLIM, a graphic method that adopts the perspective of *frequency domain* lifetime measurement [15]. In the phasor approach to FLIM, the emission decay in each pixel maps into a vector lying in a polar 2D plot (*phasor plot*), and sample regions associated with different lifetimes are immediately identifiable [16]. Additionally, combinations of different lifetimes follow simple vectorial addition rules, allowing for the calculation of the intensity fraction due to each component. Several groups, including us, applied successfully the phasor approach to FLIM to follow a broad range of intracellular processes including biomolecular interactions [17] and ion homeostasis [18].

Recently, we reported on the use of phasor approach to FLIM for monitoring local viscosity at intracellular level [19]. The reliable determination of micro/nanoviscosity in cell domains is particularly relevant from a biophysical perspective, since diffusion-controlled reactions and events are fundamental to many biological processes [20]. Fluorescent probes sensitive to local viscosity are referred to as *molecular rotors* [20]. Molecular rotors perceive local viscosity through intramolecular twisting motion(s) that occur at excited state [21]. Slowing of the twisting motions as viscosity increases leads to stronger emission and longer lifetime decay [22]. Most often a log–log relationship between viscosity and quantum yield or lifetime holds, as predicted by Forster and Hoffmann [23]. In our previous study [19], we demonstrated that the phasor plot is perfectly suited to determine local viscosity in cell domains after a reliable *in vitro* calibration, although our probe was characterized by a molecular structure that targeted it prevalently in mitochondria. In view of expanding our strategy to monitor intracellular lifetime, we here report on a different molecular rotor (**BoMe**) suitable for the phasor approach to FLIM. This rotor belongs to the 4,4′-difluoro-4-bora-3a,4a-diaza-*s*-indacene (Bodipy) family, has no net charge, and it carries a phenyl substituent in *meso* position (Scheme 1). As described for similar structures [7], the viscosity dependence of fluorescence emission and lifetime is attributable to the kinetically-restricted rotation of the phenyl group with respect to the dipyrrin core, which hampers non-radiative decay channels [24,25].

Notably, the present study demonstrates that **BoMe** is able to permeate living cells and stain most cell domains including the nucleoplasm, presumably at DNA level. On account of our long-standing interest in biological process occurring at the border and inside the cell nucleus [26–28], we here apply **BoMe** to monitor intranuclear viscosity in cells characterized by defective nuclear lamina (NL) assembly. NL is a fibrillar network located between the inner nuclear membrane of eukaryotic cells and chromatin-containing nucleoplasm [29]. For years, NL was thought to be essential solely to the nucleus integrity. Recent data, instead, indicate that NL plays a relevant role in many fundamental cellular functions [30]. The peculiar role of NL in cell and the quest for increasing knowledge about this structure is dramatically demonstrated by a wide range of inherited life-threatening diseases (laminopathies) that stem from sequence mutation in the proteins constituting NL, B-type lamins (LaB) and A-type lamins (LaA) [31]. One specific mutation in Lamin-A gene (LMNA) leads to nuclear architectural defects and is at basis of Hutchinson-Gilford progeria syndrome (HGPS), an extremely rare genetic disorder that causes premature, rapid aging shortly after birth [31,32]. Yet, while the genetic and protein processing defects in HGPS are clearly defined, the mechanism by which progerin mediates defects at cellular level are largely unknown. We shall show here that **BoMe** measures different viscosity values in the nucleoplasm of HGPS cells as compared to normal cells, thus suggesting modification in chromatin compaction as result of mutated LaA assembly. These results clearly highlight **BoMe** as a promising tool for biological studies addressing different cell compartments, particularly the nucleus.

2. Materials and methods

2.1. Synthesis of *BoMe*

Nomenclature of the synthetic intermediates refers to Scheme 1.

2.1.1. Methyl (4-formylphenoxy)acetate (*1*)

4-Hydroxybenzaldehyde (2.00 g, 16.4 mmol) was dissolved in acetone (45 ml). 2.78 g (20 mmol) of potassium carbonate and 2.1 ml (20 mmol) of methyl bromoacetate were added, and the resulting mixture was refluxed for 3 h. After, the reaction was cooled to room temperature, diluted with water and extracted with Et_2O. The organic phase was washed with water and brine, dried over Na_2SO_4 and concentrated under reduced pressure, obtaining a yellow oil that was treated with toluene and dried in *vacuum*. We obtained 2.85 g (14.7 mmol, 89% yield) of the desired product as a pale yellow solid.

^{1}H NMR (300 MHz, DMSO-*d6*) δ (ppm): 9.87 (s, 1H), 7.86 (d, J = 8.7 Hz, 2H), 7.12 (d, J = 8.7 Hz, 2H), 4.95 (s, 2H), 3.7 (s, 3H)

^{13}C NMR (300 MHz, DMSO-d6) δ (ppm): 191.9, 169.2, 162.9, 132.2, 130.6, 115.5, 65.1, 52.4

2.1.2. Methyl [4-(di-1H-pyrrol-2-ylmethyl)phenoxy]acetate (*2*)

476 mg (2.5 mmol) and 91 mg (0.3 mmol) of $InBr_3$ were introduced under nitrogen in a 50 ml three-necked round bottom flask containing 17 ml (0.25 mol) of freshly distilled pyrrole. The mixture was stirred at room temperature in the dark. After 3 h, 280 mg (6.9 mmol) of finely triturated solid NaOH were added to the solution. The reaction was stirred for further 45 min at the same temperature. The reaction mixture was filtered and the solution was evaporated under reduced pressure. The obtained residue was purified by flash chromatography on silica (eluent: cyclohexane/AcOEt), affording to 546 mg (1.8 mmol, 70% yield) of compound **2**, as a yellow oil. The intermediate was used directly in the subsequent reaction