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Carboxy-eosin as a marker for correlative light - electron microscopic imaging of newly synthesized *in vivo* DNA

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ABSTRACT

Here we report carboxy-eosin as a fluorescent marker for *in vivo* visualization of DNA in the correlative (fluorescence and electron) biological microscope. For that purpose a fluorescent thymidine analog 2',4',5',7'-tetrabromofluorescein-aminoallyl-dUTP (eosin-aminoallyl-dUTP) was synthesized and incorporated into the genome of *Escherichia coli*. The fluorescent dye eosin contains four bromine atoms, which enable electron spectroscopic imaging in the transmission electron microscope. Labeled cells were prepared and directly observed by fluorescence and electron microscope without any preliminary fixation step. We demonstrated that eosin-aminoallyl-dUTP is a suitable marker for imaging of cell's ultrastructures by correlative biological microscopy. Copyright © 2010 VBRI press.

Keywords: Carboxy-eosin, correlative microscopy, Hilbert differential contrast TEM, ESI

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Introduction

Electron spectroscopic imaging (ESI) is a powerful microscopic technique that provides a way to map and localizes cell's ultrastructures according to their chemical content. During the past two decades various intracellular studies have been performed by using ESI to visualize particular molecules or ultrastructures within living cells. For example, DNA visualization in yeast cells have been achieved by phosphoprus mapping (P), lipopeptides in limphocytes through fluorine (F) mapping, calcium (Ca) in mouse neurons, nuclear structure in cultured cells through carbon (C) and nitrogen (N) mapping and etc. [**1-5**]. In these studies internal elements intrinsically involved in the biomolecules are utilized to identify ultrastructure or localize biomolecules. Until now, almost no study has been applied ESI to identify and localize biomolecules in the living cells through elements artificially introduced from outside as fluorescent tags with non-invasive manner, which is subject of this report.

We synthesized and incorporated into cell genome the fluorescent marker 5-carboxyeosin (or 5-carboxy-2',4',5',7'-tetrabromofluorescein), which contains four atoms bromine. The bromine is a suitable element for ESI, because it has strong energy loss edge in the electron energy loss spectroscopy comparatively [**6**]. In order to achieve our goal the fluorescent dye is coupled to aminoallyl-dUTP. The obtained fluorescent derivative is a natural precursor of thymidine triphosphate. It is incorporated *in vivo* into DNA by endogenous enzyme DNA polymerase. The mechanism for recognizing and repairing of unnatural DNA residues do not prevent progress around the cell cycle [**7**]. In such way, we are able to observe and map newly synthesized eosin-labeled nucleic acid of E. coli by combination of ESI and fluorescence microscope (FM) techniques. The fluorescent strands or spots in the cell can be followed by FM. On other hand, the four heavy bromine atoms of the marker enhance the contrast of DNA in the electron microscope, and enable to visualize its natural shape in the cell by ESI.

Experimental

^{1}H NMR analysis. The ^{1}H NMR spectra were recorded in NaOD/D_2O and DMSO-d6 solvents as indicated at 300 and 400 MHz. Chemical shifts are reported in ppm downfield from TMS (δ).

Materials

For the synthesis of 5-carboxyeosin were used the reagents as follow: resorcinol, trimellitic anhydride, bromine, sodium hydroxide (all from Wako) and methanesulfonic acid (Aldrich). The coupling reagents and Aminoallyl-dUTP were supplied from Molecular Probes, Invitrogen (A21664).

Synthesis

5-Carboxyeosin-aminoallyl-dUTP was prepared following the synthetic scheme as shown on **Fig. 1**. At stage ***1*** 4-carboxyphtalic anhydride (10.0 g, 0.05 mol) was added to a solution of resorcinol (11.4 g, 0.26 mol) in 1 M methanesulfonic acid (100 ml, 1.5 mol). An air condenser was attached to the flask and the reaction was heated at 85 °C in an open vessel for 24 hours. [**8**] After cooling to room temperature the reaction mixture was poured into an ice bath yielding a precipitate. The precipitate was collected and dried in an oven at 200 °C. Later 22.2 g residue was recrystallized from MeOH-hexanes (2:1) to give 5-carboxyfluorescein methanesulfonic acid aduct. It was added to a solution of 4.3 M NaOH and the color changed to dark red. To the obtained solution conc. HCl was added dropwise. This step gave green precipitate of 5-carboxy fluorescein that was collected by filtration: ^{1}H NMR (300 MHz, NaOD/D_2O): δ 6.56 (d, *J* = 2.47 Hz, 2H), 6.66 (dd, *J* = 2.21, 9.36 Hz, 2 H), 7.33 (d, *J* = 7.71 Hz, 1 H), 8.07 (dd, *J* = 1.65, 7.84 Hz, 1 H), 8.26 (d, *J* = 1.93 Hz, 1 H).

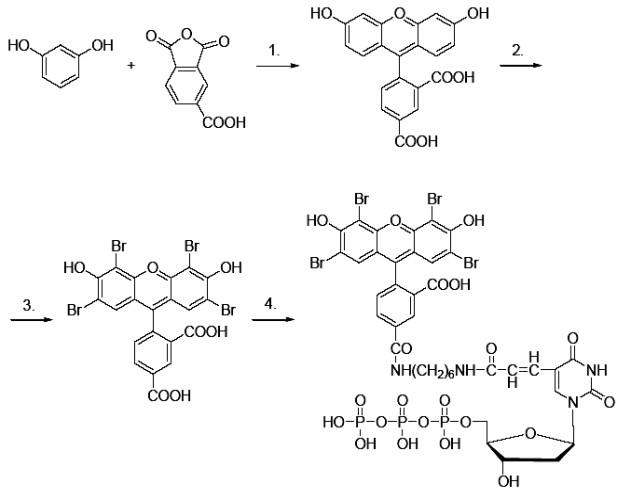

Fig. 1. Reaction scheme for synthesis of 5-carboxyeosin-aminoallyl-dUTP

5-Carboxyfluorescein (0.5 g, 1.33 mmol) was dissolved in a hot mixture of 6 ml (stage ***2***) aq sodium hydroxide (4 mmol). At stage ***3*** bromine (2.7 g, 33.8 mmol) and 5 ml H_2O were added into the 5-carboxyfluoresceine mixture. Addition of aq. NaOH into 5-carboxyfluoresceine solution leads to decolarization. After addition of glacial acetic acid (dropwise, 1.9 ml, 7.9 mmol) into 5-carboxyfluoresceine the reaction mixture was refluxed for 3 hours [**9**]. After cooling down 5-carboxy-eosin is crystallized: ^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.23 (s, 2 H), 8.40-8.29 (m, 3H), 7.53 (2dd, 2H).

To a solution of 5-carboxyeosine (0.1 g, 0.115 mmol) in anhydrous DMF (1.1 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC, 0.027 g, 0.135 mmol) followed by *N*-hydroxysuccinimide (HOSu, 0.016 g, 0.135 mmol) [**10**]. The reaction was covered with foil (stage ***4***), stirred under Ar and monitored by TLC (phase-reverse, 40 % CH_3CN / 60 % H_2O with 0.1 % formic acid). Aminoallyl-dUTP (Invitrogen) was dissolved in sodium borate buffer (1 ml, 0.1 M, pH 8.5) and 5-carboxyeosin-N-hydroxysuccinimide ester (3.2 mmol) was dissolved in amine-free DMF (100 ml). Both solutions were combined and the reaction mixture was kept at room temperature for 3h. The solution was filtrated and applied to a high load 16/10 Sepharose column. The labeled

triphosphate was purified using the following HPLC program: flow rate 4 ml/min; 0-5 min 100 % buffer A; 5-30 min increase of buffer B from 0 to 28 % (buffer A, 10 % CH_3CN in H_2O; and buffer b, 1.5 M LiCl in H_2O). The product-containing fractions were combined and sodium phosphate buffer (1 ml, 20 mM, pH 7.2) was added. The buffer solution was concentrated and poured into aceton/ethanol 3:1. After centrifugation, the precipitate was washed and dried in a vacuum and redissolved in sodium phosphate buffer (20 mM, pH 7.2). The labeled triphosphate (1.5 mmol, 50 %) was kept in solution at -20 °C.

Preparation of mediums: *Medium A* contains 5 ml M9 Medium, 0.5 % glucose, 0.4 % casamino acid, 50 μg/ml Trp, 10 μg/ml thymine; *Medium B* contains M9 medium, 0.5 % glucose, 0.4 % casamino acid, 50 μg/ml Trp and 10 μg/ml 5-carboxyeosin-aminoallyl-dUTP. Medium C contains 0.5 % glucose, 0.4 % casamino acid and 50 μg/ml Trp. Bacteria culture is inoculated into *Medium A* at 37 °C. At mid log phase 100 μL cells were washed by Medium 9, repeat twice. Then the cells were suspended into Medium B, and incubated for at least 120 min. After incubation (10-60 min), thymine was added into the culture medium (500 μg/ml, final concentration). The cells were observed directly in fluorescence and electron microscope without any preliminary fixation. Phase-contrast images were captured with a Hamamatsu-CCD attached to a Nikon Diaphot-200 microscope, fitted with a heated (Bioptech) 60xPlanApo objective and stage (Zeiss). High-resolution 300 kV transmission electron microscope (JEOL 3100FFC) equipped with EELS and phase plate was used for observation and ESI analysis.

Results and discussion

Incorporation of Eosin-aminoallyl-dUTP into the genome of Escherichia coli

The *C*-5 position of the pyrimidine derivative aminoallyl-dUTP is not involved in Watson-Crick base-pairing and so interferes little with probe hybridization [**11**]. The aminoallyl linker between the fluorescent dye and the nucleotide is designed to reduce the fluorophore's interaction with enzymes or target binding sites. In addition this nucleotide contains 10 atoms spacer that further separates the dye from the base. The number in the product's name (eosin-10-aminoallyl-dUTP) indicates the number of the spacer atoms in the chain. Longer spacer typically results in brighter conjugates and increased accessibility for secondary detection reagents.

Fluorescence microscopy analysis of cells labeled with 5-Carboxyeosin- aminoallyl-dUTP

The fluorescent properties of 5-carboxyeosin (Ex/Em = 527 / 556 in aqueous solution) enable to observe and study the shape of newly synthesized DNA in stained cells. As we could see on **Fig. 2** the stained divided cell is emitting red fluorescence color. However, the obtained daughter cells are continuing to divide, because their DNA are concentrated in the cell ends. This circumstance is an evidence for the nontoxicity of applied marker. It gives the opportunity to study various kinds of intercellular physiological processes at low or high resolution level in combination with the electron microscope.

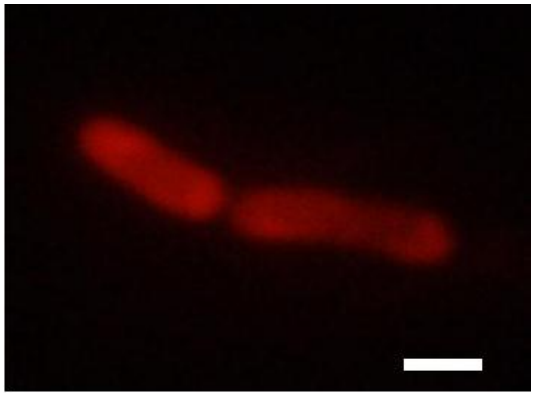

Fig. 2. Fluorescent micrograph of divided Escherichia coli stained with 5-carboxy-2',4',5',7'-tetrabromofluorescein. Scale bar = 10 μm.

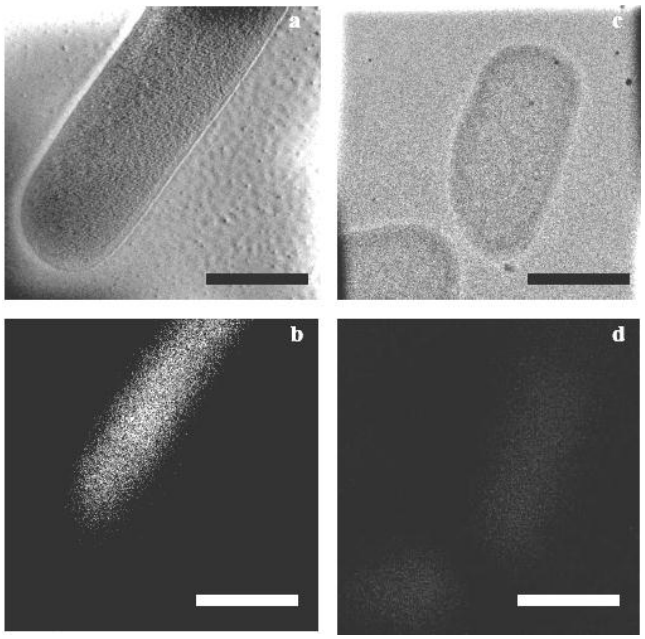

Fig. 3. HDC-TEM observation and electron spectroscopic imaging of E.coli. (a) High-contrast image of labeled with carboxy-eosin cell and (b) ESI and bromine elemental mapping of the same cell. Control experiment of unlabeled cell observed in (c) HDC-TEM mode and (d) ESI at the same condition. Scale bar = 1 μm.

HDC-TEM observation and high-resolution ESI analysis of stained Escherichia coli

The stained bacteria were observed in Hilbert differential contrast transmission electron microscope (HDC-TEM) developed by Nagayama et al. (2008) [**12**] (*see* **Fig. 3a**). It enables visualization of many heretofore "invisible" substances, including those that are soft, fragile or hydrous. As it is seems the cell is surrounded by smooth cell walls. The energy filter (Gatan), which is installed in the TEM machine, allows recording electron energy loss spectra (EELS) of the observed specimen. Standard preliminary processing procedures were performed on each collected spectrum and bromine was identified in the cells by its

characteristic $M_{4,5}$ edge in the energy-loss spectrum. The images were taken after ionization edge of bromine; then two additional images were recorded at energy losses smaller than ionization edge. In such way an ESI bromine elemental map was formed (*see* **Fig.3b**). The ESI revealed the distribution of the covalently-bound element in the cell genome with high resolution.

Thus we were able to observe the native shape of DNA in rapidly frozen E. coli cell (closed to living state) at electron microscopic level. HDC TEM observed cells that had incorporated 5-carboxyeosin-aminoallyl-dUTP exhibit electron dense areas at the location corresponding to fluorescently labeled marker. On **Fig. 3c** is presented an electron micrograph of unstained cell as a control experiment. The detailed ultrastructure inside the cell is not well visualized as in the labeled one. However, we could not obtain any EELS signal and respectively bromine ESI image (**Fig. 3d**) of the non stained bacteria.

Conclusion

5-Carboxyeosin-aminoallyl-dUTP is a suitable marker for *in vivo* staining of DNA in the correlative microscopy. It is non toxic for the cell and enables long time observation of the intracellular physiological processes. We conclude that the method promises to identify and study the structural changes of the *in vivo* nucleic acid at low (fluorescence) and high (electron) microscopic resolution.

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