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ScienceDirect
Talanta 72 (2007) 1114–1122
Talanta
www.elsevier.com/locate/talanta

A method for visualization of biomolecules labeled by a single quantum dot in living cells by a combination of total internal reflection fluorescence microscopy and intracellular fluorescence microscopy

Zilin Fan, Wenrui Jin *

School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China

Received 19 September 2006; received in revised form 31 December 2006; accepted 6 January 2007
Available online 13 January 2007

Abstract

We developed a simple fluorescence microscopy for acquisition of high-resolution images of single quantum dots (QDs) labeled to biomolecules on apical plasma membrane, in cell interior and on basal plasma membrane of living cells. The method was a combination of total internal reflection fluorescence microscopy (TIRFM) at apical cell surface and intracellular microscopy coupled with focusing objective. Insulin conjugated to single QD (insulin-QD) was chosen as the model system. In order to bind insulin-QDs to insulin receptors on the plasma membrane through the interaction between insulin and its receptor, as well as internalize them, the cells attached on a coverslip were incubated with biotinylated insulin and QD-streptavidin conjugate at 37 °C. Next, fluorescent molecules in the cells were photobleached by illuminating the cells using a 100-W mercury lamp with the wavelengths from 460 to 490 nm. Then, the incident angle of a laser beam was adjusted to produce total internal reflection at the apical surface of a single cell. In this case, the insulin-QDs in the whole cell were excited, and the fluorescent molecules outside the cell were not illuminated. Finally, the images of single insulin-QDs on the apical plasma membrane, in the cell interior and on the basal plasma membrane of the cell were taken by focusing the objective to different positions, respectively. The resolution and contrast of the fluorescent spots in the images were much higher than those obtained by using epi-fluorescence microscopy and comparable to those obtained by using the conventional TIRFM. The method improved the image acquisition speed for the images on the apical and basal plasma membrane using the conventional TIRFM, and could acquire the high-resolution images in the cell interior quickly.

Keywords: Single-cell analysis; Single-molecule detection; Total internal reflection fluorescence microscopy; Epi-fluorescence microscopy

1. Introduction

Single-molecule detection (SMD) techniques are powerful tools for investigations of molecular mechanisms of biological reactions in life science [1–4]. Measurements in living cells [5] are essential, because it is almost impossible to refabricate the cellular conditions in vitro. A variety of SMD techniques have been extended to living cells [2,6,7]. Although fluorescence detection is very sensitive [8–11], reducing the high background from light scattering and fluorescence of biomolecules inside cells relative to the fluorescence signal of the molecules of interest is still a major problem for SMD. It is possible to obtain a significant reduction in both Rayleigh and Raman scattering by using ultrasmall probe volumes. Confocal fluorescence microscopy (CFM) with a high-sensitive avalanche photodiode as the detector can detect single fluorescently labeled biomolecules entering the probe volume within a living cell [12,13]. However, CFM cannot acquire images of single molecules in living cells except use of scan techniques. Although the conventional confocal laser scanning fluorescence microscopy (CLSFM) equipped with a photomultiplier tube as the detector can obtain fluorescent images of cells, it is impossible to acquire single-molecule images due to low sensitivity. In addition, the long data acquisition time of CLSFM renders impossible the rapid imaging on the time-scale of milliseconds. Besides intracellular component analysis [14], epi-fluorescence microscopy (Epi-FM) has been used in single-molecule imaging of living cells widely [15,16]. Usually, in this case, the images of single fluorescently labeled molecules are not very clear due to high background from the autofluorescence of cells. To avoid

* Corresponding author. Fax: +86 531 8856 5167.
E-mail address: jwr@sdu.edu.cn (W. Jin).

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doi:10.1016/j.talanta.2007.01.004

the autofluorescence of cells, a laser with a long wavelength (e.g., 633 nm) is used for excitation. The evanescent-wave excitation is an excellent technique to optically confine the excitation volume for SMD of living cells [2,7]. Total internal reflection fluorescence microscopy (TIRFM) based on the evanescent-wave excitation is one of the most popular techniques currently used to observe single molecules in living cells [17,18]. In this technique, an evanescent field is produced at the interface between two media of different refractive indices with an extremely thin penetration depth depending on the incident angle of light, the indices of refraction at the interface, and the light wavelength being used for excitation. Therefore, the illumination depths of 100–300 nm, even <100 nm, can be achieved easily. Because the evanescent-wave field is restricted to the region of the interface, only fluorescent molecules in the field are excited and imaged. Thus, the fluorescence or light scattering from intracellular molecules beyond the evanescent-wave field is negligible relative to the fluorescence of the biomolecules of interest. Therefore, TIRFM provides superior contrast as compared with other far-field microscopy techniques. Traditionally, its application is limited to the basal surface of cells, i.e., the interface between the coverslip and the cell attached on it [17–19]. For SMD at the basal cell surface, sometimes, the coverslip and the cell attachment may interfere with the movement and the interaction of molecules on the basal plasma membrane, as well as their response to external stimulations. Therefore, the results acquired from the images of the basal cell surface may be affected by some factors of non-cell characteristics. These problems can be avoided, when the images are obtained from the apical cell surface. Especially for polarized epithelial cells, imaging of the molecules on the apical plasma membrane is more important since the structure and molecular composition of the apical and basal plasma membranes are different [7]. Sako et al. used TIRFM to visualize dye-labeled epidermal growth factor receptors (EGFR) on the apical plasma membrane by adjusting the incident angle of light [20]. They also observed the labeled EGFR on the basal plasma membrane by careful adjusting the incident angle of light again. This adjusting procedure for both image at the apical cell surface and image at the basal cell surfaces took time. Additionally, a lot of biological process is not restricted at the cell surface, but proceeds in the whole cell from the cell surface to the cell interior. SMD techniques with high resolutions and acquirement speeds for visualization of single biomolecules from the plasma membrane to the cell interior in a single living cell should be developed.

Usually, the functional biomolecules in cells are natively non-fluorescent. Covalent labeling with fluorophores is a selection for detection of these molecules. Fluorescent dyes are frequently used to label the biomolecules. Recently, a novel fluorescent probe quantum dot (QD), a luminescent colloidal semiconductor nanocrystals, is employed with unique advantages such as size-dependent emission, very high and uniform brilliance, photostability over prolonged periods, broad and continuous excitation spectrum, very narrow Gaussian emission band, and high biochemical stability as well as low cell toxity [13,21,22]. When QDs are linked with bio-recognition molecules such as proteins [23,24], peptides [25,26] and nucleic acids [27,28], they will become promising attractive fluorescent probes in biological and medical fields such as bioassay, cell imaging and clinic diagnosis [28–32]. Especially, QDs are suitable for fluorescence imaging in living cells [13,21,33]. In this paper, we developed a simple and rapid high-resolution method to visualize QDs labeled to biomolecules at different positions in a single living cell. The method was a combination of TIRFM at the apical cell surface and the intracellular fluorescence microscopy (FM) coupled with objective focusing. The fluorescent images of single QDs labeled to biomolecules not only at both apical and basal plasma membrane, but also in the cell interior could be obtained. Insulin is an essential peptide hormone that modifies the expression or activity of a variety of enzymes and transport systems in nearly all cells. Therefore, insulin conjugated by a single QD (insulin-QD) was chosen as the model system. In this method, cells were incubated with biotinylated insulin and QD-streptavidin conjugate at 37 °C, to bind insulin-QDs to insulin receptors on the plasma membrane and internalize them. Before taking the fluorescent images other fluorescent molecules in cells were photobleached by illuminating the cells using a mercury lamp, to eliminate the background. After that, the incident angle of laser beam was adjusted to a value to produce total internal reflection (TIR) at the apical cell surface. The images of single insulin-QDs on the apical plasma membrane, in the cell interior and on the basal plasma membrane of the cell were taken by focusing the objective, respectively. The resolution and contrast of the fluorescent spots corresponding to single insulin-QDs in the images using the method described here were much higher than those obtained by using Epi-FM and comparable with those obtained by using conventional TIRFM. The method improved the image acquisition speed for both image at the apical cell surface and image at the basal cell surfaces by using the conventional TIRFM. Additionally, the method could acquire the high-resolution images in the cell interior quickly, which could not be taken by the conventional TIRFM, and no long wavelength excitation was needed.

2. Materials and methods

2.1. Materials

PRMI-1640 medium was purchased from HyClone (Logan, UT, USA); Trypsin (>250 N.F. units/mg) was from Amresco (Solon, OH, USA); Bovine calf serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China); insulin–biotin labeled bovine pancreas (insulin–biotin) was from Sigma (St. Louis, MO); QD605-Streptavidin conjugate (QD-streptavidin) was from Invitrogen (Eugene, OK, USA). Other reagents were of analytical grade and purchased from standard reagent suppliers. All aqueous solutions were prepared with doubly distilled water. Several steps described in our previous work [34] were taken to minimize contamination.

2.2. Cell culture and preparation

Human liver cell lines HL-7702 (Shanghai Cellular Research Institute, Shanghai, China) in a Petri dish, were cultured in the