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Integrated optical molecular imaging system for four-dimensional real-time detection in living single cells

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ABSTRACT

A novel, multifunctional optical imaging system was developed by integrating four-dimensional (4D) real-time confocal microscopy (RT-CM), multicolor total internal reflection microscopy (TIRFM), and Nomarski differential interference contrast (DIC) microscopy based on an epifluorescence microscope platform. A microcell incubator was combined with the imaging system for extended, real-time monitoring of living cells. The 4D images were generated by a combination of 3D images and multiple spatial or time images of a specimen, obtained at 10 nm intervals. Optical sectioning was accomplished with a *z*-motor, which obtained 4D information with sequential layered sections. The integrated imaging system showed excellent detection sensitivity at the single-molecule level and 3D-spatial resolution (20 nm *x*–*y* and 10 nm *z*-axis) without moving the cell sample. This could be a tool for obtaining crucial information needed to develop approaches for characterizing and understanding the dynamics of biomolecules and nanoparticles in individual living cells and molecular interactions at the single-molecule level.

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1. Introduction

Fluorescent labeling is a simple way to study the localization dynamics of specific molecules in living cells. Specially, epifluorescence microscopy is one of the most widely applied optical microscopic techniques in bioimaging research and includes Ca^{2+} oscillations (Ellis, 2001; Shotton, 1989) and Ca^{2+} waves (Shotton and White, 1989) applied in measuring many different types of cultured cells. Different fluorescence detection techniques such as confocal (Ellis, 2001; Shotton, 1989; Shotton and White, 1989; Villa and Doglia, 2004) and two-photon (Denk et al., 1990; Rocheleau et al., 2003) microscopy have been used to visualize and interrogate cellular compartments and the interior of biological tissues using diffraction-limited spatial resolution ($\sim\lambda/2$ nm). In addition, near-field scanning optical microscopy (Hausmann et al., 2006), total internal reflection fluorescence (TIRF) (Joos et al., 2006), and more recently, stimulated emission depletion (Willig et al., 2006), have allowed imaging of organelles with a spatial resolution that extends beyond the diffraction limit.

Recently, a combination of mechanobiology tools and advanced molecular imaging technologies including atomic force microscopy (AFM), magnetic twisting cytometry, and traction force microscopy has been used to study signaling by fluorescence imaging (Addae-Mensah and Wikswo, 2008). An integrated bioimaging system has been reported that uses total internal reflection in combination with fluorescence correlation spectroscopy to characterize the dynamic behavior and determine absolute concentrations of fluorescent molecules near or at the interface of a planar substrate and a solution (Lieto et al., 2003). A confocal-TIRF microscope with a high-aperture parabolic mirror lens was used in a theoretical study of TIRF microscopy (TIRFM) at a water-glass interface (Ruckstuhl and Seeger, 2003) and TIRF-DIC microscopy was used to image the cellular structures by DIC and fluorescent-nanoparticle techniques using TIRF (Lee et al., 2007, 2009). Fluorescence resonance energy transfer in combination with AFM was used for high-resolution fluorescence measurements (Vickery and Dunn, 2001). Trache and Meininger (2005) described the design and construction of a combination system with AFM, TIRF, and confocal microscopy. These tools have been widely used to measure the mechanical properties of cells and to probe the molecular basis of cellular mechano-transduction. However, imaging systems are limited by low temporal resolution and loss of structural information if the sample is higher than the *z*-piezo scanner can travel. They also have difficulty monitoring significant changes in cell shape or focal adhesion reorganization in living cells. Thus, most approaches to high-resolution optical microscopy using a hybrid instrument are hindered by the AFM requirement of one-half the space of the experimental volume (Binnig et al., 1986; Drake et al., 1989; Putman et al., 1992; Domke et al., 2000). Although several companies offer some integrated systems of confocal and TIRFM, two different types detectors (i.e., PMT and CCD camera) and two individual computers both for system controller and data acquisition

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are needed. Others companies provide only separated, individual systems (i.e., spinning disk confocal microscopy and TIRFM). However, the systems are not simultaneously operated as a combined system because the light paths and detectors of two systems are different and the confocal is motorized. They are also high-priced. In part because of these reasons, the integrated systems are not widely used (Supplementary Table S1).

Live-sample imaging has increasingly become a method for cell morphology and structural characterization. Since the early 1950s, cell culture systems have been designed in parallel with improvements in microscopy and cell culture techniques for drug- or gene-delivery studies that require hours of long-term observation (Christiansen et al., 1953). In addition, a cell culture incubator called a "ringcubator" on an inverted microscope (Heidemann et al., 2003), and an "open-microincubator" format on an upright microscope (Picard et al., 2010) have been created. However, observing dynamic biological phenomena in individual living cells on a broad range of time scales using integrated bioimaging system is still limited.

In this study, we developed an integrated molecular optical imaging system combined with real-time confocal microscopy (RT-CM), multicolor total internal reflection microscopy (MC-TIRFM), four-dimensional differential interference contrast (4D-DIC) and a microcell incubator. The integrated imaging system was based on a conventional fluorescence microscope and designed for visualization and dynamic studies of single molecules and nanoparticles in intact individual living cells. Real-time 4D imaging was achieved with a combination of 3D imaging and multiple images of the sample obtained with a 10 nm spatial interval or time, which was easily integrated using sequential layered sections to obtain 4D information. Without moving the living cell sample, observations of various characterizations, dynamics, and molecular interactions in a single living cell could be observed at the single-molecule level using the imaging system.

2. Materials and methods

2.1. Cell cultures

Cultures of HeLa cells (ATCC, Rockville, MD, USA) were plated as previously described (Lee et al., 2007), and grown in Dulbecco's modified eagles medium pH 7.4 (GIBCO, Gaithersburg, MD, USA) containing 10% fetal bovine serum (GIBCO) and 1× antibiotic–antimycotic (GIBCO). Cells were maintained in plastic tissue culture dishes (BD Biosciences, MA, USA) at 37 °C with a humidified atmosphere containing 5% CO_2. For single-cell imaging, cells were placed in 35 mm × 10 mm plastic tissue culture dishes (Suwon Plastic Labware, Korea) at 2×10^4 cells per bare cover glass (25 mm round No. 1, Deckglaser, Germany). Adherent cells were rinsed twice with Dulbecco's phosphate buffered saline (GIBCO) and nanoparticles (described below) were added to the cells immediately before experiments. Cover glasses with adherent cells were placed under the objective lens (APO 100×/1.65 Oil HR, Olympus) on a stage equipped with a microcell incubator (Live Cell Instrument, Seoul, Korea). All chemical instruments were sterilized by autoclaving.

2.2. Nanoparticles

Preparation of polyamidoamine (PAMAM) dendrimer nanoparticles was similar to previous descriptions (Choi et al., 2004; Lee et al., 2008). Briefly, PAMAM dendrimers modified by lysine (PAMAM-K) or arginine (PAMAM-R) entered different sites in living cells. Two types of modified PAMAM dendrimers labeled with fluorescent dyes emitting at different wavelengths

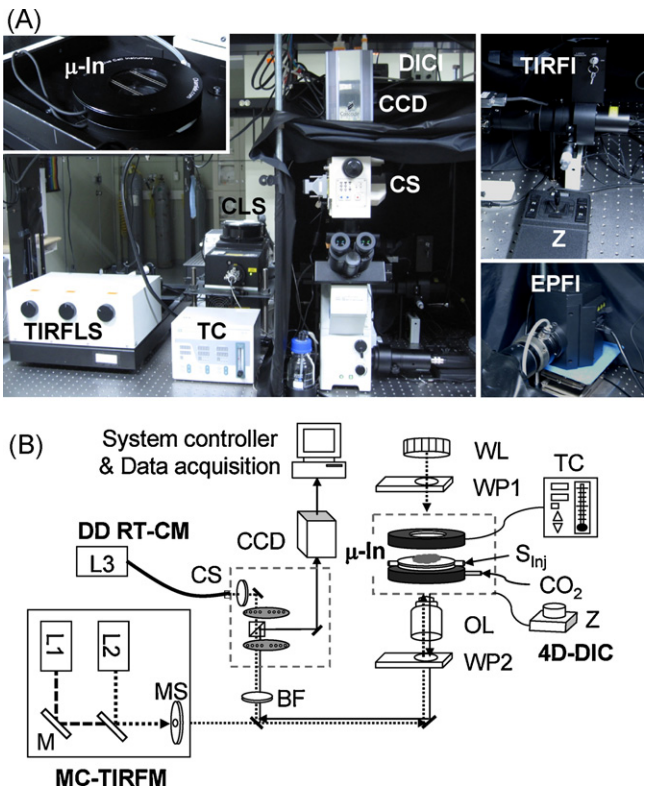

Fig. 1. (A) Integrated optical molecular imaging system and (B) schematic diagram. *Indicators*: DICI, DIC illuminator; TIRFI, TIRF illuminator; EPFI, epifluorescence illuminator; CLS, confocal laser source; TIRFLS, TIRF laser source; L, laser; M, mirror; MS, mechanical shutter; WL, white light; WP, Wollaston prism; TC, temperature controller; OL, objective lens; Z, z-motor; BF, bandpass filter; CS, confocal scanner; CCD, charge-coupled device; μ-In, microcell incubator; S_{inj}, sample injection; DD RT-CM, dual-disk real-time confocal microscopy.

(Alexa Fluor®488-labeled PAMAM-K and Alexa Fluor®633-labeled PAMAM-R) were mixed with plasmid DNA (pCN-Luci) to observe the pathways of the two types of nanoparticles simultaneously, using a single camera. Commercial nanoparticles (20-nm and 200-nm diameter, FluoSpheres carboxylate-modified microspheres, Molecular Probes, Eugene, OR, USA) were used to determine the detection limit in solution of the integrated system.

2.3. Integrated molecular imaging system with DIC, TIRFM, confocal and microcell incubator

The physical layout of the integrated system for real-time detection of single-molecules, nanoparticles, and living cells, and a schematic diagram of the construction of the hybrid 4D-DIC, MC-TIRFM, RT-CM, and microcell incubator with a conventional epifluorescence microscope are shown in Fig. 1. An Olympus IX71 inverted microscope (Olympus Optical, Tokyo, Japan) equipped with two DIC sliders (U-DICTS and IX2-AN, Olympus) and a *z*-motor (LEP MAC 5000, LUDL Electronic Products Ltd. NY, USA) used transmitted light to visualize nanoparticles and distinct cellular morphological features. The designed TIRFM objective (APO 100×/1.65 N.A., oil type, Olympus) had the high numerical apertures critical for proper laser alignment and specimen illumination. The 473-nm laser (SL-473 nm-50T, Shanghai Laser Century Technology Co., Ltd., China) and 633-nm laser (05-LHP-991, Melles Griot, Carlsbad, CA, USA) were used as light sources for multicolor TIRFM. A filter cube was composed of 520/15-nm and 670/40-nm bandpass filters in a Dual-View™ (Optical Insight, LLC, Tucson, USA). The optical system was integrated with the confocal system by

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