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Time-lapse *in situ* fluorescence lifetime imaging of lipid droplets in differentiating 3T3-L1 preadipocytes with Nile Red

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ABSTRACT

To study the mechanisms of and conditions for adipogenesis, an accurate *in situ* observation tool is necessary to monitor the quantity of intracellular neutral lipids in differentiating preadipocytes. Although conventional fluorescence intensity imaging is a powerful tool for observing the formation and growth of an individual lipid droplet, it suffers from photobleaching and ambiguous autofluorescence or background signals from cells. In this paper, we present a fluorescence lifetime imaging microscopy (FLIM) technique that has the potential to quantify the ratio of neutral to polar lipids in a cell. Measurement of time-lapse FLIM images of differentiating 3T3-L1 cells that contained the Nile Red (NR) probe showed that the average lifetime of NR decreased from 4 ns in preadipocytes to 3 ns in fully differentiated adipocytes after 10 days of differentiation. This large change in the lifetime of NR can be used to monitor the early stages of adipogenesis, even when the lipid droplet is too small to be identified with a conventional microscope.

1. Introduction

Obesity is one of the greatest threats to the health of adults in industrialized countries because of its association with many metabolic diseases such as diabetes, atherosclerosis, and many types of cancer [1,2]. Because increased body weight is associated with the increase in the number and size of triglyceride-storing adipocytes in the human body, researchers are investigating the conditions and processes of adipocyte differentiation [3–7]. In addition to storing fat in lipid droplets (LDs), adipocytes play a role in energy homeostasis by secreting several proteins that regulate processes such as angiogenesis, hemostasis, immune function, blood pressure, and energy balance [8–10]. An *in vitro* study of 3T3-L1 cells is widely used to study adipogenesis because the cells differentiate into an adipocyte-like phenotype under appropriate conditions [6,11]. When 3T3-L1 cells differentiate into adipocytes, large LDs are synthesized, during which their size and number are monitored via various imaging techniques. Third-harmonic generation (THG) microscopy [12,13] and coherent anti-Stokes Raman scattering (CARS) microscopy [14,15] are two promising but complicated nonlinear optical imaging techniques that can image lipids without the need of stain. However, because nonlinear optical interaction is generally very weak, these two approaches suffer from low imaging signal intensity, resulting in slow frame rates.

Fluorescence microscopy is a widely used imaging technique for detecting lipids in a live cell. Nile Red (NR), DPH, DAPI, and BODIPY are fluorescent probes used in the imaging of LD *de novo* synthesis and fission during adipocyte differentiation [16,17]. Because adipocyte differentiation takes a few days, adipogenesis studies often require long-term observation, in which case photobleaching by a fluorescence dye [18] is a serious problem. Other obstacles to observing LDs in adipogenesis using fluorescence microscopy are unwanted fluorescence signals from membrane lipid bilayers and background autofluorescence signals. Because there are various lipids in the cell membrane, it is difficult to distinguish the LDs from the cell membrane using fluorescence intensity imaging, especially in the early stages of adipocyte differentiation.

The spectral properties of some fluorescent probes undergo large changes depending on the local environment [19,20]. When Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is attached to polar lipids such as phospholipids, its emission spectrum redshifts, and when attached to neutral lipids such as cholesterol or triacylglycerol (TAG), the emission spectrum

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blueshifts [21–24]. Because the absorption and emission spectra of NR are very sensitive to the local environment, various spectroscopic techniques have been used in lipid-sensing applications such as measurement of cholesterol content in cell membranes [25], differentiation of intracellular lipids in a cell [21,26,27], and monitoring of the biophysical properties of lipid membranes [20]. The electrons responsible for the absorption and emission of light in NR that labels phospholipids in the cell membrane have different binding potentials than those of NR that is attached to TAG in the LDs. These molecular structure differences can be effectively observed in the spectral domain of emitted fluorescence light [28,29].

The spectral absorption and emission properties of a fluorescence molecule in turn are coupled to the transition lifetime of an electron when it goes from an excited state to a ground state [19,30]. Therefore, the lifetime of an emitted photon of a fluorescence dye reflects the environmental conditions or energy states of the dye in a given medium. Fluorescence lifetime imaging microscopy (FLIM) is a two- or three-dimensional imaging method that produces a visual representation of the lifetimes of specific fluorescent probes and is used in the biosciences to measure the physical or chemical properties of a targeted molecule or its local environment [31–34]. Unlike conventional fluorescence microscopy, which displays the abundance of a fluorescent probe that labels a targeted cellular organelle, FLIM functionally maps environmental parameters such as viscosity, pH, temperature, hydration, binding of one protein to another, electrostatic potential, and molecular orientation order at the site of a fluorescent probe. Because the lifetime of a fluorescent probe is not affected by the abundance or the concentration of a fluorescence molecule, FLIM images do not deteriorate due to photobleaching; such deterioration is a serious problem in fluorescence intensity imaging.

In this paper, we propose the use of FLIM to monitor the procedures and conditions of adipogenesis. Over 10 days we captured a series of time-lapse FLIM images of the entire differentiation period of 3T3-L1 preadipocytes. The average fluorescence lifetime was calculated for every pixel in a FLIM image with a nonzero lifetime. We observed that as the 3T3-L1 cells differentiate, the LDs in the cells grow larger and the average lifetimes of the FLIM images monotonically decrease. There are reports on how the fluorescence lifetime of NR is affected by the targeted lipids in the cell membranes [35–39]. In a nonpolar medium, the emission spectrum of NR has a smaller Stokes shift, resulting in blueshifted peak intensity [21–24]. For a given fluorescent probe, the fluorescence lifetime associated with a blueshifted emission spectrum is shorter than that associated with a redshifted emission spectrum [28,40]. This explains the measured shift in the lifetime of NR from 4 to 3 ns as the quantity of neutral lipids increases during adipogenesis. Another report found that the fluorescence lifetimes of NR strongly depend on Young's modulus rather than on the polarity of the medium [41]. Our results suggest that fluorescence lifetime imaging can be an effective and powerful tool for monitoring LD formation, especially at the beginning of adipogenesis when droplet sizes are too small for identification with a conventional fluorescence microscope.

2. Materials and methods

2.1. Fluorescence lifetime imaging microscopy (FLIM) setup

A FLIM system can be implemented as either a wide-field imaging system or a point-scanning confocal system. A wide-field FLIM system with an arrayed detector has the advantages of fast detection speed and simple configuration; phase modulation [42,43] and gated imaging [44,45] are two techniques used in such a system. A confocal FLIM system has three-dimensional sectional imaging capability, higher spatial resolution, and less photobleaching. Time-correlated single-photon counting (TCSPC) is a well-established time-domain detection technique used in conventional confocal FLIM systems [33,46–49]. In this study, we used a homemade analog mean delay (AMD)-FLIM system, which is a time domain detection system.

Fig. 1 presents the block diagram of our AMD-FLIM system. It consists of four major parts: 1) a pulsed laser light source, 2) beam delivery and imaging optics, 3) light detector and analog signal processor, and 4) digitizing electronics and imaging display algorithms. The pulsed laser source is a gain-switched diode laser (PicoQuant, LDH-P-C-485) triggered by an external signal source with a 5-MHz repetition rate; it provides a 160-ps pulse at a wavelength of 479 nm and average power of 80 μW. The laser light is delivered through a single-mode fiber (SMF) with a collimator lens. The collimated laser light passes through an optical short-pass filter (SPF) with a cutoff wavelength of 485 nm to block unwanted spontaneous emission wavelength components in the gain-switched laser. A dichroic mirror with a cutoff wavelength of 505 nm is positioned after the optical SPF to eliminate the 479-nm excitation laser light from the fluorescence light signal from the probe. A resonant galvanometer mirror scanner (GSI, CRS4 KHz), oscillating at 4 kHz, is used for high-speed horizontal scanning and a nonresonant galvanometer (GSI, VM500S) is used for slow vertical scanning. The fast-scanned laser beam is delivered to an inverted microscope body (Olympus IX51) for imaging. To monitor the relative position of the scanning laser beam, which is focused on the sample via a 60× objective lens (Olympus, NA 0.8), there is a CCD camera in the microscope body for fluorescence intensity imaging. The fluorescence signal from the sample is transmitted through the dichroic mirror and is further filtered by an optical long-pass filter (LPF) with a cut-on wavelength of 490 nm.

The detection part of our AMD-FLIM system consists of a photomultiplier tube (PMT), a Gaussian low-pass filter (GLPF), an electronic amplifier, and a digitizer. The same external 5-MHz clock signal that triggers the pulsed laser source also triggers the digitizer. The electric pulse signal generated by the PMT (Hamamatsu, R7400U-20) is temporally broadened by a 10th-order GLPF custom-made with inductors and capacitors [47]. The 3-dB bandwidth of the GLPF is designed to be 100 MHz. The temporally broadened signal is amplified by a 1-GHz electric amplifier (Minicircuits, TB-409-74+), and then the amplified electric signal is digitized by an 8-bit data acquisition board with a 250-MHz sampling rate (National Instruments, PCI-5114).

2.2. Cell preparation and staining

3T3-L1 preadipocytes [American Type Culture Collection (ATCC) CCL 92.1] were grown to confluence as fibroblasts in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) at 37 °C in humidified air containing 5% CO_2. DMEM contained 10% fetal bovine serum (Gibco, Grand Island, NY), 1 μM dexamethasone (Sigma-Aldrich, St Louis, MO), 1 μg insulin/ml, and 10 μM rosiglitazone (Alexis Biochemicals, San Diego, CA). The 3T3-L1 cells were incubated for 2 days in a post-confluent stage (day 0). On day 2, cell differentiation was induced by adding a differentiation medium containing 5 μg insulin/ml, 0.5 μM dexamethasone, and 0.8 mM isobutyl methyl xanthine (Sigma-Aldrich, St Louis, MO) [50,51]. After the induction of differentiation, the cell culture medium was changed to DMEM with 10% FBS and was changed every 2 days for proper nutrition. The AMD-FLIM system, with NR as the fluorescent probe for lipid imaging, was used to observe the cells for 10 days, starting at day 0. A stock solution of NR (Sigma-Aldrich, St Louis, MO) was mixed with acetone to a concentration of 100 ng/ml.