

# Reduced nicotinamide adenine dinucleotide fluorescence lifetime separates human mesenchymal stem cells from differentiated progenies

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**Abstract.** The metabolic changes of human mesenchymal stem cells (hMSCs) during osteogenic differentiation were accessed by reduced nicotinamide adenine dinucleotide (NADH) fluorescence lifetime. An increase in mean fluorescence lifetime and decrease in the ratio between free NADH and protein-bound NADH correlated with our previously reported increase in the adenosine triphosphate (ATP) level of hMSCs during differentiation. These findings suggest that NADH fluorescence lifetime may serve as a new optical biomarker for noninvasive selection of stem cells from differentiated progenies. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2990752]

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Stem cells give rise to tissue progenitor cells, which can differentiate into specific progenies and have potential use in regenerative medicine, disease treatment, and developmental biology. Efforts have been made to search for reliable biomarkers to identify stem cells *ex vivo*<sup>1</sup> and *in vivo*<sup>2</sup> so as to gain a better insight into the biology and physiology of stem cells, as well as to increase the selection efficiency from a given cell pool. However, many of the markers are invasive even in *in vivo* imaging approaches because stem cells were preloaded *ex vivo* by radionuclide, ferromagnetic, or reporter labeling,<sup>2</sup> which decreases the clinical usefulness of these methods. Recently, a noninvasive biomarker using proton nuclear magnetic resonance spectroscopy (<sup>1</sup>H-MRS) has been identified for detection of neural stem and progenitor cells in the human brain *in vivo*.<sup>3</sup> Although the identity of this <sup>1</sup>H-MRS-detected biomarker is not known, it is suggestive of a metabolic profile of fatty acids. In fact, one generally accepted property of stem cells that differs from their differentiated progenies is a lower metabolic rate accompanied by a lower adenosine triphosphate (ATP) content.<sup>4</sup> The shift from anaerobic glycolysis to

the more efficient mitochondrial oxidative metabolism has been demonstrated in the differentiation of cardiomyocytes<sup>5</sup> and human mesenchymal stem cells (hMSCs).<sup>6</sup> The preference of stem cells to produce energy by glycolysis instead of oxidative phosphorylation is similar to that of cancer cells, which has been termed the Warburg effect.

Optical detection/imaging techniques have been employed to study cell metabolism in a noninvasive manner by monitoring the intrinsic fluorescence signal of reduced nicotinamide adenine dinucleotide (NADH), a key coenzyme in glycolysis and oxidative metabolism. Two measurement schemes are possible: fluorescence lifetime<sup>7</sup> and fluorescence intensity.<sup>8</sup> In the fluorescence lifetime measurement scheme, a fluorescence decay curve is typically fitted to a two-component exponential decay function  $F(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$ , where  $\tau_1$  and  $\tau_2$  correspond to the short and long fluorescence lifetimes of NADH and were reported to be  $\sim 400$  to  $500$  ps and  $\sim 2000$  to  $2500$  ps for free and bound NADH, respectively.<sup>7</sup>  $a_1$  and  $a_2$  are the corresponding relative amplitudes and  $a_1 + a_2 = 1$ . A mean fluorescence lifetime  $\tau_m$  is defined as  $\tau_m = (a_1\tau_1 + a_2\tau_2)$ . Increased  $a_1/a_2$  ratio and decreased  $\tau_2$  and  $\tau_1$ , and thus decreasing  $\tau_m$ , were reported in perturbed human breast cells that had increased NADH/NAD<sup>+</sup> ratio (decreased metabolism).<sup>7</sup> In the fluorescence intensity measurement scheme, NADH was often paired with another coenzyme in oxidative metabolism, flavin adenine dinucleotide (FAD), so that the oxidation-reduction state of cellular metabolism, NADH/FAD ratio, can be obtained. A decrease in the NADH/FAD ratio (increased metabolism) has been observed in MSCs after osteogenic differentiation for one week.<sup>8</sup> Based on these findings, we hypothesized that the increase of metabolism during stem cell differentiation can be detected by the changes of NADH fluorescence lifetime (i.e., increased  $\tau_m$  and decreased  $a_1/a_2$  ratio). If successful, NADH fluorescence lifetime change can be a new optical probe for selecting stem cells from differentiated progenies. Furthermore, stem cell differentiation provides an excellent model system to study NADH fluorescence lifetime change in the context of metabolic change from glycolysis to oxidative phosphorylation. In this letter, we report the time course of change in the NADH fluorescence lifetime in response to osteogenic differentiation of hMSCs. This has been previously characterized by researchers in our research teams at the biochemical and molecular biological levels regarding the changes of mitochondrial biogenesis and antioxidant enzymes.<sup>6</sup> Consistent with our hypothesis, we observed a decreased  $a_1/a_2$  ratio and increased  $\tau_m$  of NADH fluorescence lifetime during hMSC differentiation for up to 21 days using two-photon fluorescence lifetime imaging microscopy (FLIM).

Undifferentiated and osteogenically differentiated hMSCs were imaged with a two-photon laser scanning microscope and with a  $60\times 1.45$  NA PlanApochromat oil objective lens (Olympus Corp., Japan) as previously reported.<sup>9</sup> NADH fluorescence was excited at 740 nm by a Verdi pumped mode-locked femtosecond Ti:sapphire laser (Coherent, Inc., Santa Clara, California) at 76 MHz. The emitted fluorescent light was bandpass filtered at  $450 \pm 40$  nm (Edmund Optics, Inc., Barrington, New Jersey) at which NADH fluorescence emits

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maximally,<sup>7,8</sup> and was detected by a single-photon-counting photomultiplier tube (Hamamatus, Japan). Time-resolved detection was conducted by a single-photon-counting SPC-830 board (Becker & Hickl GmbH, Germany). Data were analyzed with the commercially available SPCImage software (Becker & Hickl GmbH) via a convolution of the two-component exponential decay function  $F(t)$  and the instrument response function (IRF), and then the convolved result was fitted to the actual data to derive lifetime parameters  $\tau_1$ ,  $\tau_2$ ,  $a_1$ ,  $a_2$ , and  $\tau_m$ . IRF was measured using a second-harmonic generated signal from a periodically poled lithium niobate crystal. Cell samples were prepared as described in our previous studies.<sup>6,9</sup> Bone marrow hMSCs were isolated and cultured in Iscove's modified Dulbecco's medium. hMSCs at a density of either 5000 or 1000 cells/cm<sup>2</sup> were seeded onto a 24-mm-diam round glass coverslip for 24 h to allow a good attachment of hMSCs onto the coverslip. Differentiation of hMSCs was induced by further incubating these attached hMSCs in the osteogenic induction medium. Before and on days 7, 14, and 21 post induction of osteogenic differentiation, samples of cells were imaged. All samples were washed twice using phosphate-buffered saline, and were then placed in a cell chamber containing 1 mL HEPES buffer as described previously.<sup>9</sup> All images were taken at 256 × 256 pixels resolution with an acquisition time of 900 s for sufficient photon counts (at least several hundreds) per pixel. FLIM images were acquired at 1 to 3 sites per coverslip within approximately 1 h. The average laser power measured at the focal plane of the microscope objective was ~5 mW, which was lower than the reported laser power that caused damage to biological samples. Additional measurements were performed by repeatedly imaging the same sample 2 to 4 times within 1.5 h to confirm that no optical damage was introduced to our samples.

Figure 1 shows representative images of the NADH fluorescence lifetime of undifferentiated hMSCs [Fig. 1(a)] and differentiated hMSCs at days 7 [Fig. 1(b)], 14 [Fig. 1(c)] and 21 [Fig. 1(d)], respectively, at the cell density of 5000 cells/cm<sup>2</sup>. Each pixel represents the mean fluorescence lifetime  $\tau_m$  and was color-coded between 500 (red) and 2000 ps (blue). Apparently, these images exhibited an NADH fluorescence lifetime shift (color changed) toward higher values during hMSC differentiation. The lifetime within a single cell was not homogeneous, for example, yellow, green, and blue colors were all seen in Fig. 1(c). Figure 1(e) depicts the normalized histograms of  $\tau_m$  shown in Figs. 1(a)–1(d). These histograms show that the peak of  $\tau_m$  distribution shifted from a lower value (~1000 ps) in hMSCs to a higher value (~1200 ps) in the 21-day-differentiated hMSCs. The full width at half maximum of each  $\tau_m$  histogram reflects the heterogeneous lifetime within an image that is in the range of ~450 to 600 ps. Similar images and histograms of  $\tau_1$ ,  $\tau_2$ ,  $a_1$ , and  $a_2$  were obtained using the same software (data not shown), and the corresponding mean value of each image was recorded for later averaging over multiple samples. The  $a_1/a_2$  ratio was calculated by dividing the mean value of the image of  $a_1$  by that of the image of  $a_2$ .

The changes in NADH fluorescence lifetime from undifferentiated hMSCs to differentiated osteoblasts were confirmed in a series of samples (Table 1 and Fig. 2). At a cell density of 5000 cells/cm<sup>2</sup>,  $\tau_m$  increased from 1022 ± 50 to

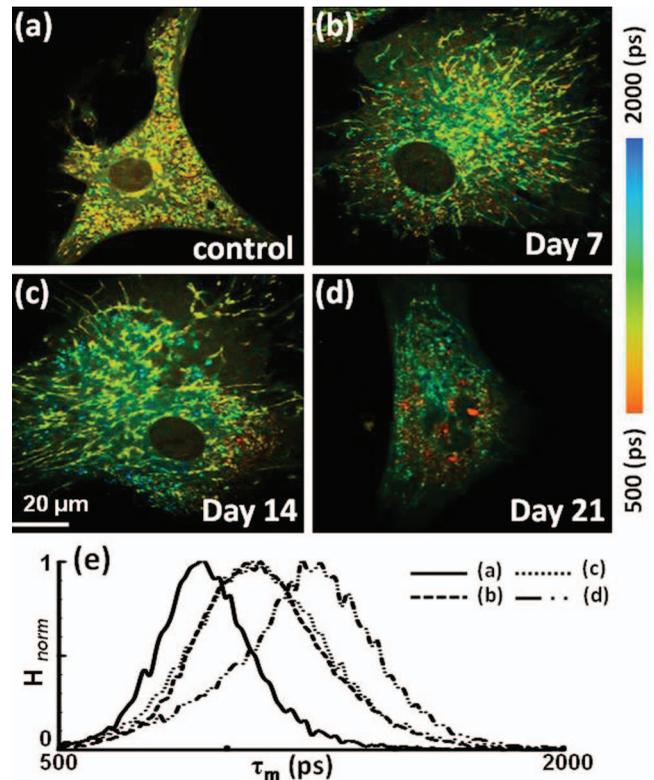


Fig. 1 Mean fluorescence lifetime images of NADH fluorescence in control (a) and differentiated hMSCs at 7 (b), 14 (c), and 21 (d) days. The fluorescence lifetime was color-coded between 500 (red) and 2000 (blue) ps. Normalized histograms ( $H_{norm}$ ) over the 256 × 256 pixels were plotted for comparison (e).

1200 ± 30 ps [Fig. 2(a), solid line], and the  $a_1/a_2$  ratio decreased from 3.00 ± 0.16 to 2.12 ± 0.24 [Fig. 2(b), solid line] when hMSCs differentiated up to 21 days. These changes were statistically different as judged by a two-tailed Student's  $t$  test ( $p$  values < 0.05) and marked in the figure.  $\tau_1$  and  $\tau_2$  did not show continuous increase or decrease, although the values of most of the differentiated hMSCs are statistically different from those of undifferentiated hMSCs. In this study, we used the same culture of bone marrow hMSCs as that used in our previous study, in which a continuously increased ATP level was reported during hMSC differentiation.<sup>6</sup> This ATP level change correlated well with the changes of  $a_1/a_2$  and  $\tau_m$  observed in this study, but not  $\tau_1$  and  $\tau_2$ .

Because stem cell density was reported to affect the cell metabolism and thus affect the NADH/FAD ratio,<sup>8</sup> we acquired additional data at 5 times lower cell density (1000 cells/cm<sup>2</sup>; Table 1 and Fig. 2) to see how the cell density influences NADH fluorescence lifetime and to test whether it affects the usefulness of NADH fluorescence lifetime technique in stem cell selection. Overall, we observed a similar trend of an increase in  $\tau_m$ , a decrease in  $a_1/a_2$ , and no continuous increase or decrease in  $\tau_1$  and  $\tau_2$  during hMSC differentiation. As expected, when the cell density is lower (lower metabolism), the  $\tau_m$  and  $a_1/a_2$  ratio tended to decrease and increase, respectively, although no change was seen in undifferentiated hMSCs. These changes between two cell densities at each time point of differentiation were not statistically different. We combined all data to increase the sample

**Table 1** The average values ( $\pm$ standard deviation) of mean  $\tau_m$  and  $a_1/a_2$  for hMSCs (controls) and differentiated hMSCs at 7, 14, and 21 days with higher (5000 cells/cm<sup>2</sup>) and lower (1000 cells/cm<sup>2</sup>) cell density, respectively, as well as the average values of  $\tau_m$  and  $a_1/a_2$  for all cells and the corresponding  $p$  value between controls and differentiated hMSCs.

		Controls	Day 7	Day 14	Day 21
5000 cells/cm <sup>2</sup>	$\tau_m$ (ps) (n)	1022 $\pm$ 50 (10)	1118 $\pm$ 33 (3)	1143 $\pm$ 36 (8)	1200 $\pm$ 30 (3)
	$a_1/a_2$	3.00 $\pm$ 0.16	2.71 $\pm$ 0.11	2.61 $\pm$ 0.21	2.12 $\pm$ 0.24
1000 cells/cm <sup>2</sup>	$\tau_m$ (ps) (n)	999 $\pm$ 70 (9)	1031 $\pm$ 62 (5)	1131 $\pm$ 62 (7)	1148 $\pm$ 63 (4)
	$a_1/a_2$	2.98 $\pm$ 0.40	2.97 $\pm$ 0.23	2.79 $\pm$ 0.35	2.30 $\pm$ 0.27
All cells	$\tau_m$ (ps) (n)	1011 $\pm$ 54 (19)	1064 $\pm$ 64 (8)	1147 $\pm$ 56 (15)	1165 $\pm$ 57 (7)
	$p$ value		0.039	1.30e-8	3.77e-6
	$a_1/a_2$	2.99 $\pm$ 0.25	2.87 $\pm$ 0.21	2.67 $\pm$ 0.31	2.24 $\pm$ 0.25
	$p$ value		0.24	1.85e-3	1.55e-6

number ( $n$ ) for a better representative result of average populations (Table 1). The results demonstrate that the average  $\tau_m$  and  $a_1/a_2$  values of hMSCs ( $n=19$ ) are significantly different from those of differentiated hMSCs except that the  $a_1/a_2$  ratio of 7-day-differentiated hMSCs is similar to that of hMSCs ( $p=0.24$ ).

We have demonstrated that the changes in the  $\tau_m$  and  $a_1/a_2$  ratio are correlated well with the metabolic changes during hMSC differentiation. The results of this study suggest that hMSCs and their progenies can be differentiated, based on their metabolic differences, by a robust noninvasive optical technique through monitoring the NADH fluorescence lifetime. Alternative to the NADH fluorescence intensity measurement scheme in stem cell detection,<sup>8</sup> a major advantage of the fluorescence lifetime measurement scheme is its insensitivity to the fluorescence intensity. Thus, clinical application of NADH fluorescence lifetime may be relatively easier than

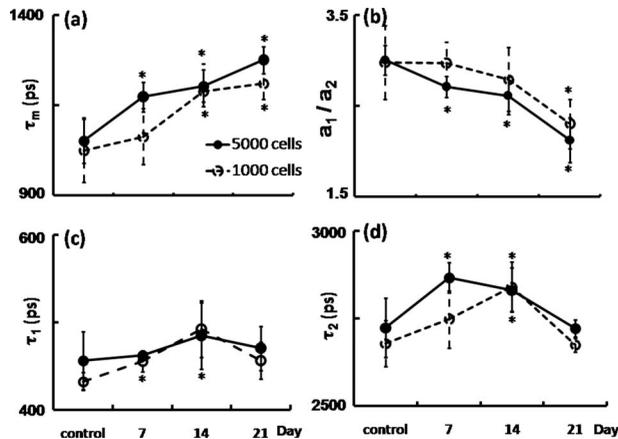
the fluorescence intensity measurement scheme regardless of the possible heterogeneity of the NADH spatial distribution.

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**Fig. 2** Plots of mean  $\tau_m$  (a),  $a_1/a_2$  (b),  $\tau_1$  (c), and  $\tau_2$  (d) from 3 to 10 experiments of samples in control and differentiated hMSCs. Two cell densities were used: 5000 cells/cm<sup>2</sup> (●, solid line) and 1000 cells/cm<sup>2</sup> (○, dashed line). The mean values of  $\tau_m$ ,  $a_1/a_2$ ,  $\tau_1$ , and  $\tau_2$  in differentiated hMSCs statistically different from those in controls were judged by a two-tailed Student's  $t$  test ( $p < 0.05$ ) and labeled (\*).