Supporting Information

Cellular Internalization of $LiNbO_3$ Nanocrystals for Second Harmonic Imaging and the Effects on Stem Cell Differentiation

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Table S1. Normalized hyperpolarizabilities ⟨d⟩ of different nonlinear nanocrystals and bulk crystals. Data from (Ref [7]).

	$LiNbO_3$	$BaTiO_3$	$KNbO_3$	KTP	ZnO
$\langle d \rangle_{nanoparticle}$	4.8	4.6	3.4	1.4	1.9
$\langle d \rangle_{bulk}$	17.3	15.3	14.1	7.6	2.8

If the nanocrystals were excited with laser intensity of I_ω, the SHG intensity $I_{2\omega}$ can be expressed as

$$I_{2\omega} = G(NT\langle\beta^2\rangle)I_\omega^2$$

, where G is an experimental constant, N is the LN concentration and T an internal field factor. ⟨β⟩ is the nanoparticle effective hyperpolarizability, which can be expressed as

$$\langle\beta\rangle = \langle d\rangle V$$

, where V is the volume of nanocrystals. In the above formula, brackets indicate isotropic orientational averaging. Since LN has the highest nonlinear coefficients, the SHG intensity from the LN nanocrystals would be the highest compared with other nonlinear materials listed above, regardless of the factor of nanoparticle volume.

Cellular Internalization of LN in rMSCs by FFT analysis. A two-dimensional Fast Fourier transform (FFT) was used to characterize the square regions for the quantification of the orientation of actin fibers and SHG nanocrystals. First, the fluorescent image of actin and the corresponding SHG image were transferred into 8-bit gray format in ImageJ (National Institutes of Health) and their brightness and contrast were adjusted automatically. The images were then processed with 2D FFT function provided by the software. The FFT output images contained gray-scaled pixels distributed in oval patterns that reflected the degree of actin or LN nanocrystal orientation. The pixel intensities from the FFT images were summed radially from 1 to 360 degree. The sum brightness value was normalized to the minimum value and then plotted versus the angle from a vertical line.

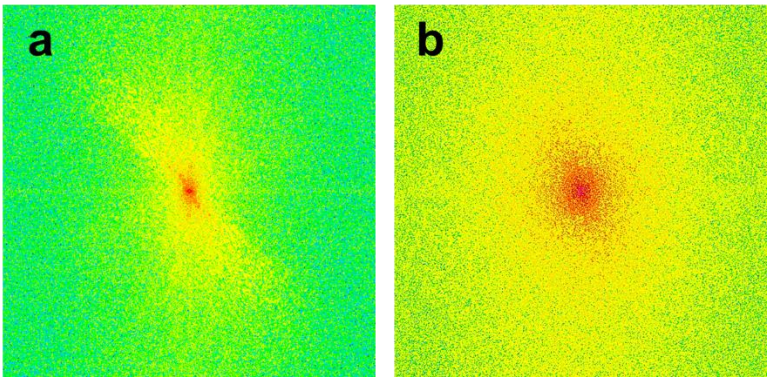

Figure S1. FFT output images generated from (a) fluorescent image of rMSCs with immunostained actin and (b) SHG image of rMSCs labeled with LN nanocrystals. Gray-scale pixel intensity was color-coded with the 16-color look-up-table (LUT) using ImageJ software.

Anti-bleaching ability of LN nanocrystals. The SHG imaging was conducted with a TPLSM equipped with a tunable Ti:Sapphire femtosecond laser (Mai Tai, Spectral-Physics). After 24 h of incubation with a particle concentration of 50 μg/mL, the cells were also labeled with Alexa Fluor 633 phalloidin, an organic dye for staining actin. Bleaching tests were carried out by exposing the LN and the organic dye under 910 nm (~300 mW) and 633 nm (~30 mW) laser for 500 s. As shown in **Figure S2**, the SHG signals from LN were stable in intensity whereas the emission intensity for the organic dye dropped quickly, even though the intensity of the exciting laser for LN-labeled cells was ten times higher than the one for the organic dye. The results indicate the potential use of LN nanocrystals for long-term tracking of stem cells.

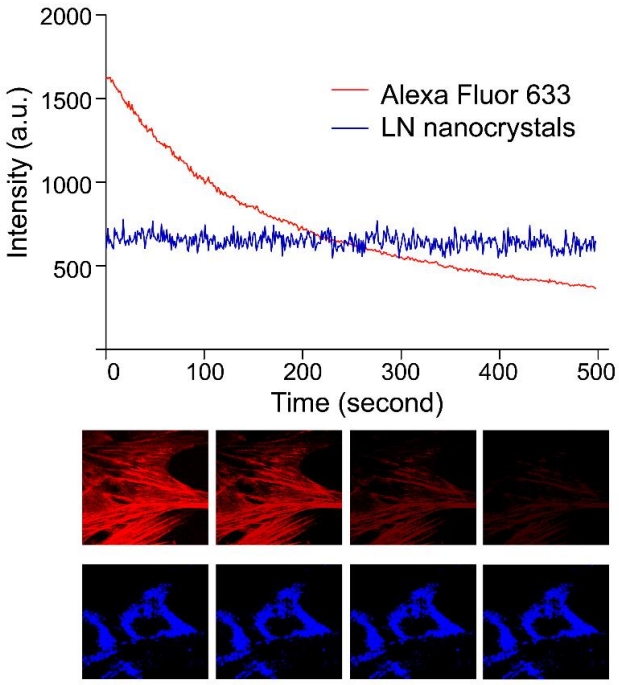

Figure S2. Bleaching behavior of LN nanocrystals compared with Alexa actin dye under NIR laser. Images below the graphic were taken after 50, 100, 300 and 500 seconds of 633 nm and 910 nm laser exposure (laser power: ~30 mW at 633nm; ~300 mW at 910 nm). Emission filter: 640-680 nm for Alexa Fluor 633 and 420-460 nm for SHG.

Cytocompatibility of LN nanocrystals. A Cell Count Kit-8 (Dojindo Molecular Technology) was used to quantitatively evaluate cell viability after 24 and 72h of culture while rMSCs were treated with 10, 50, and 100 μg/mL concentrations of LN nanocrystals. First, 500μL of serum-free L-DMEM medium plus 10% substrate solution was added to each well. The resultant production of water-soluble formazan dye was formed after 4 hours of incubation at 37°C and measured at a wavelength of 450 nm by a microplate reader (MULTISKAN MK3, Thermo, USA). The samples were analyzed in triplicate for each group. To further verify the results of the CCK-8 assay, rMSCs treated with 50 μg/mL LN nanocrystals for 24 and 48 h were stained using a LIVE/DEAD Kit (Invitrogen) according to the manufacturer's protocol. The cells were washed with PBS buffer and incubated with 1 mL of PBS containing 2 μL of ethidium homodimer-1 and 0.4 μL of Calcein AM. The cells were observed by fluorescence microscopy (Leica, Germany) after 30 min of incubation.

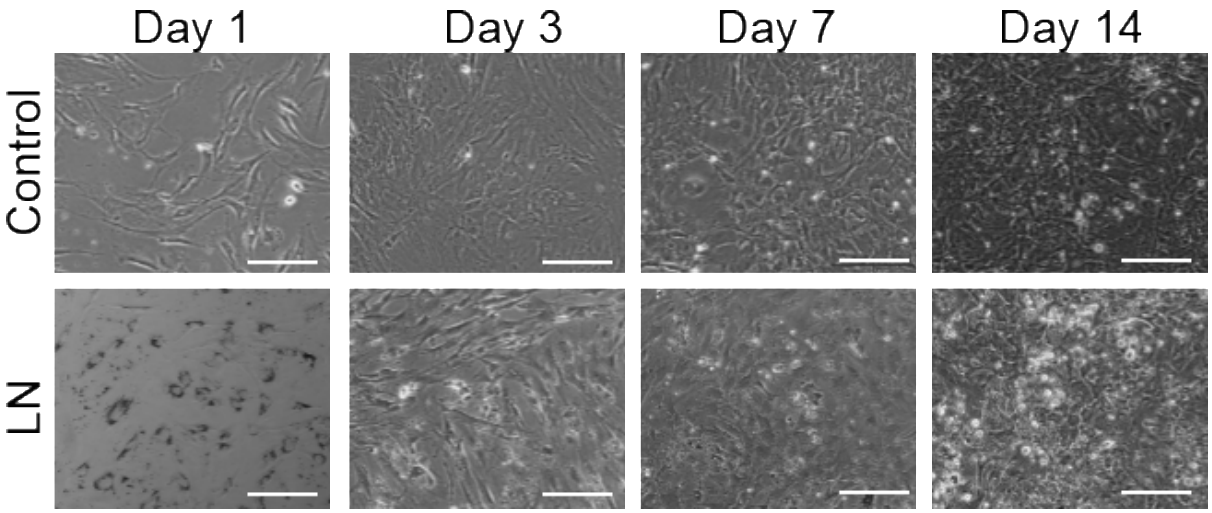

Figure S3. Bright field images of rMSCs treated with 0 μg/mL (control) and 50 μg/mL LN after osteogenic differentiation at day 1, 3, 7, and 14, respectively. The mineral deposit formation can be clearly recognized on day 14. Bar: 200 μm.

Table S2 Sequences of qPCR primers used in this paper.

Gene	Forward primers (5’-3’)	Reverse primers (5’-3’)
GAPDH	GCCTCGTCTCATAGACAAGATGGT	GAAGGCAGCCCTGGTAACC
OPN	TCCTGTCTCCCGGTGAAAGT	GGCTACAGCATCTGAGTGTTTGC
OCN	AAGCCCAGCGACTCTGAGTCT	CCGGAGTCTATTCACCACCTTACT
Runx2	AATGCCTCCGCTGTTATG	TTCTGTCTGTGCCTTCTTG

The effects of LN nanocrystals on the adipogenic differentiation of rMSCs. To determine the extent of adipogenic differentiation after 14 days of culture in adipogenic differentiation medium, rMSCs were stained with Oil Red O and the lipid contents were quantified. rMSCs were washed twice and stained with 0.6% (w/v) Oil Red O (Sigma, USA) solution (60% isopropyl alcohol, 40% water) for 30 min. For quantification of Oil Red O, rMSCs were washed with PBS three times to remove background staining and then isopropyl alcohol was added to resolve Oil Red O. Absorbance at 510 nm was measured on a microplate spectrophotometer. The intracellular lipid content of rMSCs treated with 50 μg/mL LN was normalized to the control group (0 μg/mL LN).

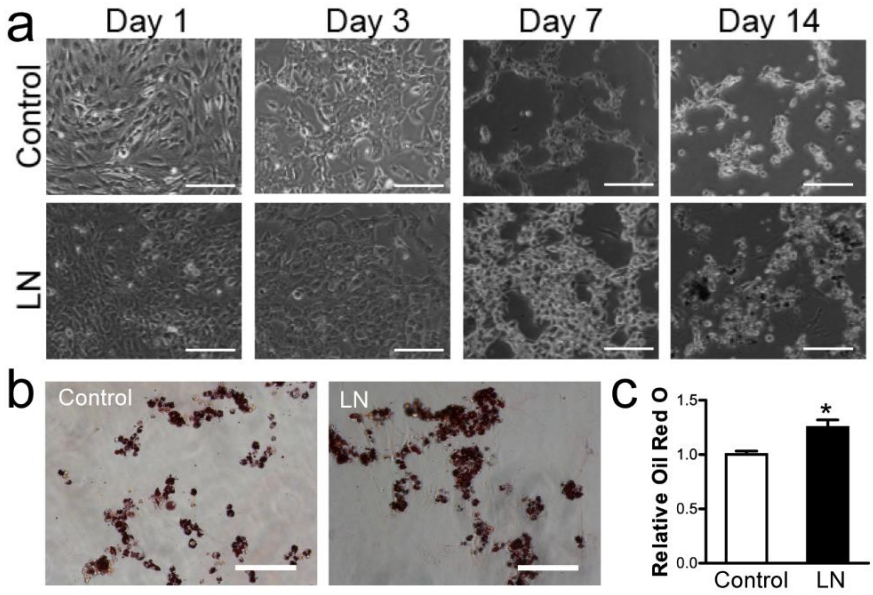

Figure S4. (a) Bright-field images of rMSCs treated with 0 μg/mL (control) and 50 μg/mL LN after 1, 3, 7, and 14 days in culture with adipogenic differentiation medium. The lipid formation can be clearly recognized on day 14. (b) Oil Red O staining of rMSCs with or without LN after adipogenic differentiation for 14 days. Bar: 200 μm. (c) Quantification of Oil Red O in (b). *$P<0.05$, $n=3$. Bar: 200 μm.