Identification of source of calcium in HeLa cells by femtosecond laser excitation

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Abstract. Calcium is an important messenger in cells and whose store and diffusion dynamics at the subcellular level remain unclear. By inducing a controlled slow subcellular Ca^{2+} release through femtosecond laser irradiation in HeLa cells immersed in different media, cytoplasm is identified to be the major intracellular Ca^{2+} store, with the nucleus being the minor store and the extracellular Ca^{2+} also contributing to the total cellular Ca^{2+} level. Furthermore, Ca^{2+} released in either the cytoplasm or nucleus diffuses into the nucleus or cytoplasm, respectively, at different rates and influences the Ca^{2+} release in those regions. © *2010 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.3485741]

Calcium is a very important messenger in all cells and tissues, relaying information within cells to regulate their activities,[1] especially those related to apoptosis signaling.[2] It plays different roles within the cytoplasm, nucleus, and organelles. Free calcium in the nucleus affects gene transcription and cell growth significantly. The endoplasmic reticulum (ER) is recognized as the principal intracellular calcium store,[3] while mitochondria function as a modulator of calcium by their ability to release and uptake free calcium.[4] Calcium can be discharged from the nuclear envelope directly into the nucleoplasm,[5] and it can also diffuse into the nucleus from the cytoplasm through nuclear pores[6] or nuclear tubular structures by some special mechanisms. Yet despite these discoveries, the exact location of calcium stores remains uncertain. Although several possible Ca^{2+} stores have been proposed,[7] the traditional method of stimulating Ca^{2+} by chemicals such as InsP3 produces a global effect, and thus cannot identify the Ca^{2+} store at the subcellular level and pinpoint the pathway of Ca^{2+} diffusion into the nucleus. With the recent advances of lasers, the femtosecond (fs) laser has become a powerful and precise tool in biological research. A report in 2001[8] described the induction of a Ca^{2+} wave in a cell when it was irradiated by a finely focused fs laser beam, which activated both the intracellular store and the stretch-activated channel or other channels by light-induced pressure on the membrane, or by shock waves generated from the focal spot to cause an extracelluar Ca^{2+} influx.[9] The mechanisms were further investigated by another work in 2009,[10] which utilized the fs laser as a controllable Ca^{2+} stimulation in highly localized space and time. Inspired by these results, we also attempted to irradiate HeLa cells by a fs laser with the aim of pinpointing the exact location of the Ca^{2+} store within a cell. This becomes possible because the fs laser can provide a controllable and precise trigger of slow Ca^{2+} release at the subcellular level, thereby enabling a much easier observation of intracellular Ca^{2+} dynamics that cannot be achieved by traditional chemical stimulations.

In this study, HeLa cells obtained from American Type Culture Collection (Manassas, Virginia) were cultured in RPMI 1640 medium (Sigma Aldrich, Saint Louis, Missouri) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco, Invitrogen, Carlsbad, California) at 37 °C and 5% CO_2. They $(3 \times 10^5/ml)$ were seeded on a 35-mm petri dish with a glass slide (0.17 mm thick), stained with Fluo-4/AM [Invitrogen (Carlsbad, California), final concentration of 10 μM) for 40 min and washed two times by phosphate buffered saline (PBS) for Ca^{2+} study. The fluorescence was excited by a laser at 488 nm. The central wavelength of the fs laser [Calmar (Sunnyvale, California) FPL-04] employed in this study was 1554 nm, and the average power at the fiber output was about 100 mW at a pulse width of 170 fs and a repetition rate of 20 MHz. The fs laser was coupled into the microscope by a fiber collimator and a dichroic mirror, and then focused by a 40× objective (NA=1.0). The coupling efficiency of the system is around 60% and the pulse dispersion is around 150 fs. The full width at half maximum diameter of the focus was around 2 μm, and the focal plane was controlled by confocal scanning (Nikon C1 confocal microscope). The optical design is shown in Fig. 1(a). For clarity, we use $[Ca^{2+}]$, $[Ca^{2+}]_{NU}$, and $[Ca^{2+}]_{CY}$ to represent the intracellular Ca^{2+} concentration in the whole cell, the nucleus, and the cytoplasm, respectively.

The first group of HeLa cells (20 cells) was irradiated at the cytoplasm for 1 s, which elicited a slow release of Ca^{2+} whose diffusion pattern into the cell nucleus is shown in Fig. 1(b). The observed increase in fluorescence intensity subsequent to the laser excitation is consistent with the theory that any initial Ca^{2+} release can trigger more Ca^{2+} release from

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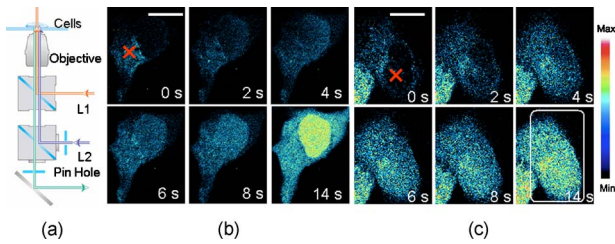
Fig. 1 (a) Optical setup of the experiment. L1: the fs laser at 1554 nm. L2: the laser at 488 nm for fluorescence excitation. Ca^{2+} scans versus time after the 1-s laser exposure (b) at cytoplasm and (c) at nucleus. In (c), the object appearing in the lower-left-hand corner is a neighbor cell. Red cross: exposure spot. Bar is 10 μm. (Color online only.)

the store, forming loop amplification[11] or Ca^{2+}-induced Ca^{2+} release (CICR).[12] Since the diffusion of Ca^{2+} requires a concentration gradient, $[Ca^{2+}]_{NU}$ should be less than $[Ca^{2+}]_{CY}$. This is examined in the next two figures. The reason for the apparently strong Fluo-4 fluorescence in the nuclear region

despite its lower Ca^{2+} concentration is that Fluo-4 excited at 488 nm gives a stronger fluorescence in the cell nucleus than in the cytoplasm.[13] Next, a second group of cells (20 cells) was irradiated by the fs laser for 1 s at the nucleus (guided by the white light image), and the corresponding Fluo-4 fluorescence is shown in Fig. 1(c). It is seen that the fs pulses also locally triggered a slow release of Ca^{2+} in the nucleus, which gradually diffused out into the cytoplasm. The diffused Ca^{2+}, on reaching the Ca^{2+} store in the cytoplasm, could cause it to release more Ca^{2+}. These results thus confirm that Ca^{2+} is stored in both the cytoplasm and the nucleus, which can diffuse into the nucleus and the cytoplasm, respectively, once its host store is released, and can influence the local store at the migrated site.

A third group of cells was incubated in Ca^{2+}-free HEPES buffer [concentration in mM: 1 EGTA, 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 10 Na^+−N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), final pH 7.2]. Femtosecond laser treatment was performed on the cells, after which the
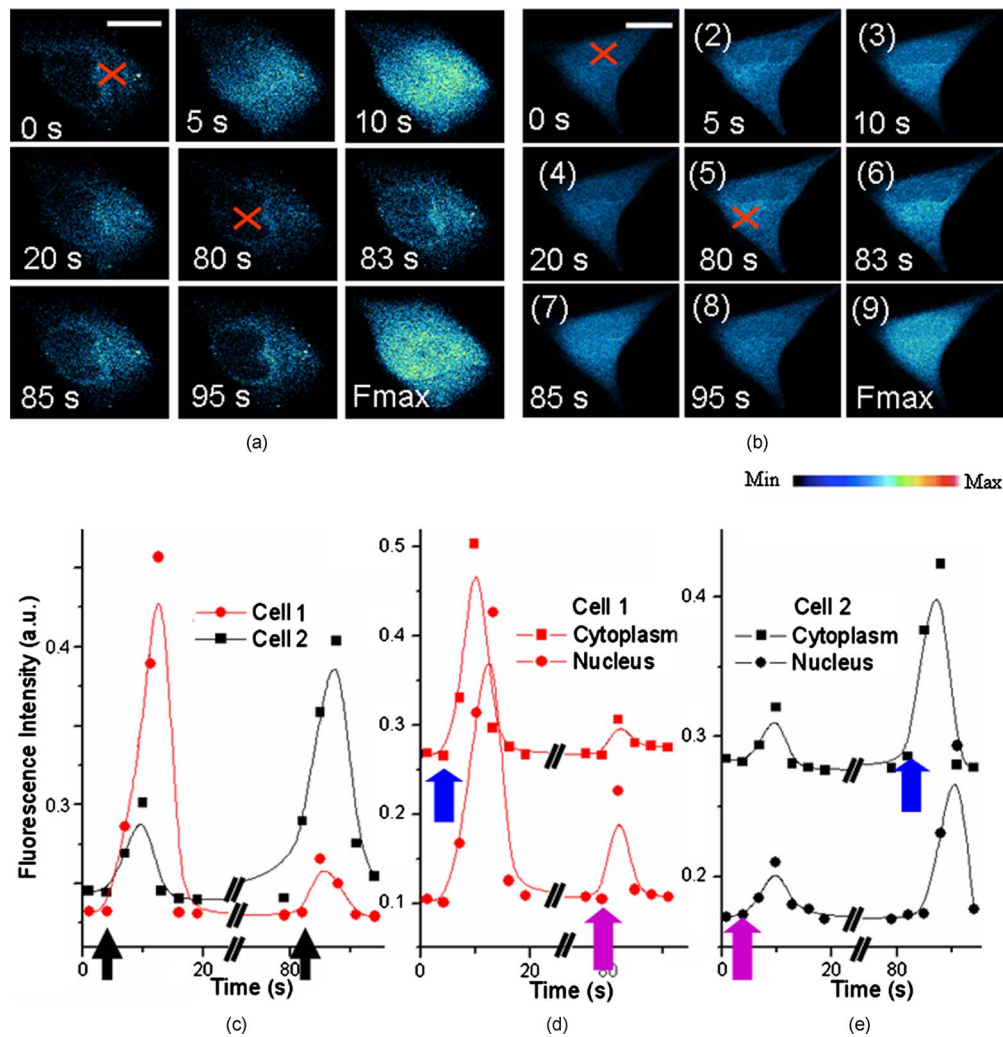
Fig. 2 Typical Ca^{2+} fluorescence intensity of cells after exposure by the fs beam for 1 s at 60 mW at the sample in Ca^{2+}-free buffer. (a) Cells were exposed at cytoplasm first and then at nucleus [cell 1 in (c)]. (b) Cells were exposed at nucleus first and then at cytoplasm [cell 2 in (c)]. Fmax is the maximum fluorescence resulting from ionomycin treatment (Fmax). Red cross: fs laser focusing spot. Bar is 10 μm. Fluorescence intensity versus time from the whole cell (c), from cytoplasm and nucleus separately in (d) cell 1 (n=5) and (e) cell 2 (n=5). Black arrow: irradiation instant. Blue arrow: irradiation at cytoplasm. Purple arrow: irradiation at nucleus. (Color online only.)

cell medium was replaced by PBS, and then ionomycin (2 μM) was added to work as ionophores by which Ca^{2+} can diffuse through cell membranes. Since the free Ca^{2+} concentration in PBS is around 2 mM, while it is only 1 to 100 nM inside cells, an extracellular Ca^{2+} influx into cells would occur. The maximum fluorescence intensity of Ca^{2+} (Fmax) can thereby be obtained for data normalization, where all fluorescence intensity is normalized as $F(t)/Fmax$. As shown in Fig. 2(a), the cells that were first exposed for 1 s at the cytoplasm, labeled cell 1 (5 cells), would release Ca^{2+} slowly which then started to decay after approximately 15 s. After 60 s, the cells were exposed again for 1 s, but at the nucleus. A slow rise in Ca^{2+} level was also observed, but the increase was much smaller. The measured fluorescence intensity versus time is shown in Fig. 2(c) cell 1, standard deviation less than 17% of the value at each point). To ensure that the low-intensity second peak in the curve is not caused by the depletion of Ca^{2+} store or fluorescence bleaching after the first laser exposure, another batch of cells from the same group, labeled cell 2, was irradiated by the fs laser first at the nucleus and then at the cytoplasm following the same protocol. The fluorescence scans of a typical cell 2 are shown in Fig. 2(b). Its intensity versus time plot is shown in Fig. 2(c) cell 2, standard deviation less than 20% of the value at each point). The Ca^{2+} rise due to the exposure at the cytoplasm is observed to be much higher than that caused by the exposure at the nucleus in both cells 1 and 2. Since there was no external source of Ca^{2+}, it can be concluded that the release of Ca^{2+} is much higher in the cytoplasm than in the nucleus. Consequently, one can infer that the Ca^{2+} store in the cytoplasm is larger than that in the nucleus.

The fluorescence intensities from the nucleus and cytoplasm regions were separately extracted from the scans and plotted as shown in Figs. 2(d) and 2(e) for cell 1 and 2, respectively. It is interesting to note that when the exposure was at the cytoplasm, there existed a delay of 2 to 3 s between the Ca^{2+} peak from the cytoplasm and that from the nucleus. On the contrary, there was no delay between the two Ca^{2+} peaks when the exposure was at the nucleus. Naturally, the observed delay is caused by the diffusion of Ca^{2+} from the cytoplasm to the nucleus, while the nonexistence of delay in the reverse direction may be due to a much faster diffusion mechanism from the nucleus to the cytoplasm. The asymmetric diffusion rates can arise from the regulation of proteins in the nuclear membrane acting as nuclear pore complexes.[14]

Next, we investigated the contribution of extracellular Ca^{2+} in the Ca^{2+} response elicited by the fs laser irradiation with the help of a Ca^{2+} channel blocker, nifedipine, which can block all Ca^{2+} channels in the membrane. At first, two groups of cells, labeled cells 3 and 4 (five cells in each group), were incubated in PBS and exposed by the fs beam for 1 s at the cytoplasm and the nucleus, respectively. According to the reported mechanism, the fs laser would release Ca^{2+} from the intracellular store and open up the Ca^{2+} channels in the cell membrane to let the extracellular Ca^{2+} diffuse into cells. This Ca^{2+} rise subsequently generated more Ca^{2+} by CICR, which would be taken up by mitochondria slowly. Then, nifedipine (100 nM) was added to the medium after 120 s, and the cells were incubated for another 40 min to block all Ca^{2+} channels in the cell membrane. The cells were subsequently irradiated

by the fs laser for a second time at the same location, but this time the extracellular Ca^{2+} could not diffuse into the cells. In the corresponding control groups, the cells were just exposed twice in PBS without any nifedipine addition as a reference. As in previous experiments, ionomycin (2 μM) was added at the end of the measurements to all cells to get the maximum fluorescence intensity of Ca^{2+}, Fmax, for data normalization. The Ca^{2+} scans of a typical cell 3 (irradiated at the cytoplasm) and cell 4 (irradiated at the cell nucleus) are respectively shown in Figs. 3(a) and 3(b). Their corresponding normalized fluorescence intensities are plotted in Figs. 3(c) and 3(d), respectively, where two peaks corresponding to the double exposures in each group are clearly seen (standard deviation less than 19% of the value at each point). By comparing the second peaks with the first ones in the control, there was obviously no bleaching effect on the fluorescence after the second laser exposure. Cells 3 and 4 behaved nearly the same and just like their control in the first irradiation event. It should be noted that the fluorescence peaks of cells 3 and 4 after the first irradiation event are around 70% of Fmax from Figs. 3(c) and 3(d). This is probably due to the balance achieved between the Ca^{2+} release triggered by the laser and amplified by CICR, and the Ca^{2+} uptake by mitochondria. The 1-s laser irradiation has been proven to be safe to the mitochondria,[15] so that they can still take up the extra Ca^{2+} to protect the cells. On the other hand, ionomycin treatment is quite different, as the high concentration of Ca^{2+} in the media freely diffusing into cells will depolarize all mitochondria and cause their dysfunction irreversibly. Consequently, even though the Ca^{2+} stores in the cytoplasm and nucleus are different, the intensities of the first fluorescence peaks from both groups of cells are almost the same at around 70% of Fmax.

After the addition of nifedipine, the fluorescence peak of Ca^{2+} in cell 3 is lower than that of the first peak by about 0.1-fold of Fmax. This reduction should correlate with the absence of influx of extracellular Ca^{2+}. On the other hand, cell 4 exhibits a second Ca^{2+} peak lower than that of the first peak by about 0.2-fold of Fmax, as shown in Fig. 3(d). This amount of reduction should also reflect the contribution due to extracellular Ca^{2+}. By comparing these two peaks, it can be inferred that the Ca^{2+} store in the nucleus is notably smaller than that in the cytoplasm. The fluorescence signals coming from the cytoplasm or nucleus were then separately extracted from cells 3 and 4, and they are very similar to the corresponding signals from the whole cells (data now shown). The results also exhibit the 3-s delay between the peak signals from the cytoplasm and from the nucleus when the diffusion is from the cytoplasm to the nucleus.

It should be noted that apart from the nucleus and cytoplasm, the nucleoplasmic reticulum developed from the nuclear membrane in the nucleus can also be a store of Ca^{2+}.[16] However, this structure is developed mainly during apoptosis, and the Ca^{2+} recorded there was found to be fluctuating in our previous work,[17] because the high Ca^{2+} concentration could induce mitochondria migration to uptake the Ca^{2+}. Therefore, our present study for live cells only focused on the generation of Ca^{2+} in the nucleus and the cytoplasm.

In conclusion, it was found that a controlled dose of fs laser irradiation at 1554 nm can induce a slow Ca^{2+} wave in HeLa cells. The Ca^{2+} increase arose from stores in both the
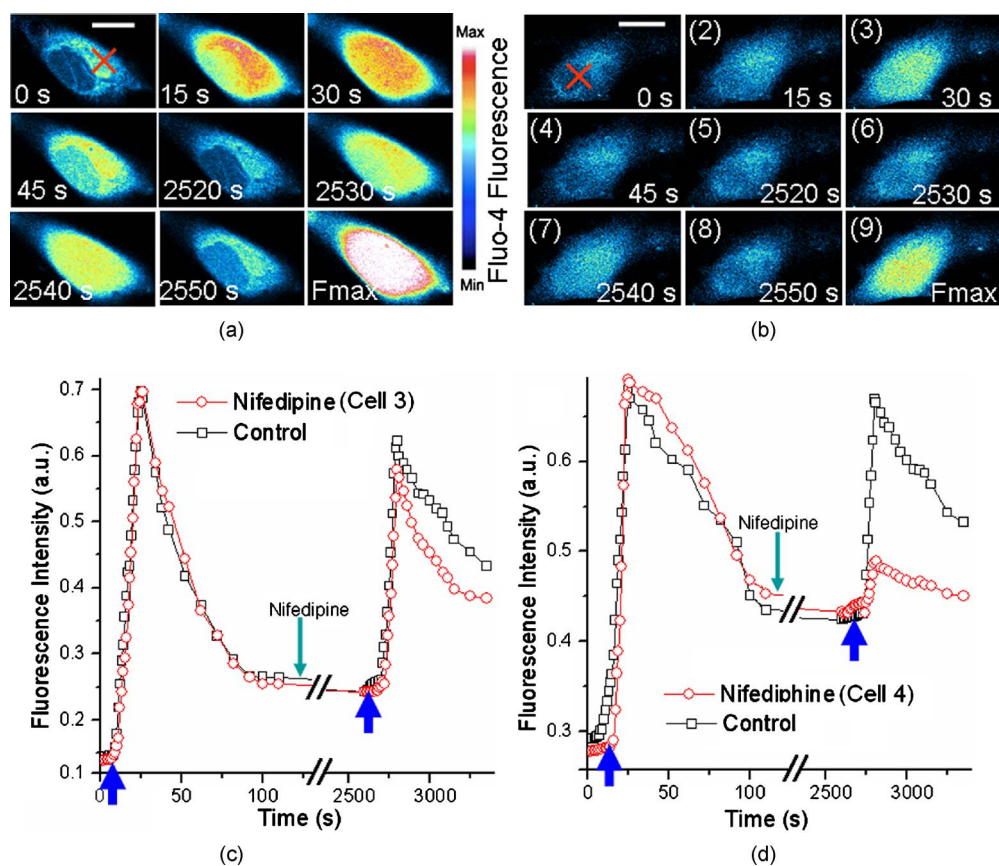
Fig. 3 Typical fluorescence scans of Fluo-4 by confocal microscope: (a) cell 3 and (b) cell 4. Fmax is the maximum fluorescence obtained after ionomycin treatment. Red cross: fs laser focusing spot. Bar is 10 μm. Normalized fluorescence intensity versus time is shown in (c) for cell 3 (n=5) and (d) for cell 4 (n=5). Blue arrow in (c) and (d): irradiation instant of cells 3 and 4. (Color online only.)

nucleus and cytoplasm, with the cytoplasm being the major Ca^{2+} store. Extracellular Ca^{2+} was also verified to contribute to the Ca^{2+} rise, whose contribution was more significant when the irradiation was focused on the nucleus. The Ca^{2+} diffusion patterns were experimentally recorded for excitation locations in both the cytoplasm and nucleus. A time delay of 3 s was observed when the excitation was on the cytoplasm between the peak Ca^{2+} signals measured at the cytoplasm, and at the nucleus due to the finite diffusion time from cytoplasm to nucleus. By contrast, no such delay is observed when Ca^{2+} diffused from the nucleus to the cytoplasm, presumably at a much faster rate. Hence, our technique provides a means for the slow and precise release of Ca^{2+} at the subcellular level. The subfemtoliter focus spot, laser power, duration of irradiation, and instant of irradiation can all be finely controlled, resulting in Ca^{2+} triggers of high spatial and temporal resolution. Last but not least, the technique is noncontact, noninvasive, free of any chemical compounds, and safe to cells, so that the dynamics of cells in live condition can be obtained.

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