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Forum Minireview

Optical Bioimaging: From Living Tissue to a Single Molecule: Calcium Imaging in Blood Vessel *In Situ* Employing Two-Photon Excitation Fluorescence Microscopy

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Abstract. Recent developments in optoelectronics permit real-time Ca^{2+} imaging of thin planes within cells utilizing laser scanning confocal microscopy (LSCM). However, a major complication associated with this imaging system involves increased phototoxicity with improved spatiotemporal resolution. Two-photon excitation microscopy (TPEM) helps to minimize phototoxicity due to the restriction of this technique to the volume proximal to the geometric focus of the light. In this study, the capability of Ca^{2+} imaging was investigated employing recently developed real-time TPEM, RTS2000MP (Bio-Rad, Tokyo) with a mode-locked Ti-sapphire laser. Z-axis resolution of RTS2000MP with high NA objectives defined as full-width at half maximum (FWHM) with a 0.5-μm fluorescent bead provided values nearly identical to those obtained with LSCM at a small pinhole (0.2 mm) (approximately 0.6 μm). When serial sectioning of 21 sequential images at 0.3-μm intervals in cultured endothelial cells loaded with calcein and tetramethyl-rhodamine methylester were performed with TPEM, the z-axis resolution was higher than that observed with LSCM; moreover, the photobleaching rate was significantly lower than that obtained with LSCM. Maximum fluorescence intensities were detected at 780 nm in excitation spectra of fluo-3 and fluo-4 Ca^{2+}-sensitive probes with TPEM. Fluorescence images in mouse arterial endothelial cells loaded with fluo-4 could be clearly visualized by TPEM *in situ*. Application of acetylcholine caused oscillatory increase in $[Ca^{2+}]_i$ of endothelial cells; subsequently, relaxation along the major axis of smooth muscle cells was evident. Furthermore, consecutive long-lasting experiments could be repeated with identical response in the same microscopic field. In conclusion, fluorescence imaging employing TPEM is useful for Ca^{2+} imaging in blood vessels *in situ*.

Keywords: Ca^{2+} imaging, intracellular free Ca^{2+} concentration, two-photon excitation, blood vessel, *in situ*

Introduction

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a pivotal role in the regulation of various cellular functions as an intracellular messenger system. Epifluorescence microscopy has become one of the most widely applied microscopic techniques in biomedical research, that is, such as with $[Ca^{2+}]_i$ measurements. Since the development of digital video imaging of $[Ca^{2+}]_i$, novel findings including Ca^{2+} oscillations (1, 2) and Ca^{2+} waves (3) have been described in many different cultured cell types. Recently, we observed Ca^{2+} spots as an elementary Ca^{2+}-influx event through mechanosensitive channels directly coupled with the initial step in mechanotransduction in cultured endothelial (4 – 6) and cultured lens epithelial cells (6, 7). The Ca^{2+} spots, which develop sporadically, exhibit a spatiotemporal pattern distinct from Ca^{2+} sparks, the elementary $[Ca^{2+}]_i$ release events from intracellular stores (8, 9). However, it has been reported that characteristics of receptors, ion channels and intracellular couplings in cultured or

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isolated cells are altered by enzymatic or mechanical treatments (10).

Phenotypic and functional heterogeneity of vascular endothelial cells in various types of blood vessels is thought to operate in an important physiological capacity. This type of heterogeneity has not been observed in cultured endothelial cells. Furthermore, Ca^{2+} mobilization in endothelial cells may be altered in pathological states such as hypertension and diabetes. However, such pathological change cannot be examined utilizing cultured endothelial cells. Therefore, investigation of the Ca^{2+} response to various stimuli *in situ* in intact cells, particularly in tissue in which intercellular communication between adjacent cells plays an important role in their function, that is, blood vessels, is of major significance. However, $[Ca^{2+}]_i$ in individual endothelial cells has yet to be measured due to the difficulty associated with separation of fluorescence of endothelial cells and smooth muscle cells *in situ* employing a digital video imaging system and conventional microscopy. In this respect, the thin optical sectioning capability of laser scanning confocal microscopy (LSCM) rejects light from out-of-focus planes, which permits imaging of $[Ca^{2+}]_i$ in individual cells *in situ* in optical sections approximately 1-μm-thick (11).

In practice, we have established an imaging analysis protocol with respect to $[Ca^{2+}]_i$ in individual endothelial cells *in situ* with LSCM (12, 13). However, LSCM led to increased photodamage with total time necessary to acquire images, especially during continuous imaging. On the other hand, two-photon excitation microscopy (TPEM), which displays several advantages in comparison to LSCM equipped with a one-photon excitation laser (14), is suitable for calcium imaging in blood vessels *in situ*. In this article, the basic performance and utility of TPEM as an imaging tool of $[Ca^{2+}]_i$ in individual cells *in situ* is described in comparison with similar aspects of LSCM employing a real-time confocal, multi-photon imaging system, RTS2000MP (Bio-Rad, Tokyo).

Fundamental principles of LSCM and TPEM

Fundamental principles of LSCM and TPEM are presented in Fig. 1. In LSCM, one-photon excitation laser light is condensed in a focal plane by an objective lens; additionally, light intensity decreases in inverse proportion to the square of the distance from the focal plane in the Z-axis direction. Fluorescence emission originating from the focal plane is passed through a small pinhole to a photomultiplier detector. However, fluorescence emission from above and below the focal plane is not focused on the pinhole; consequently, it is not transmitted to the detector. Thus, the diameter of the pinhole is a critical factor with respect to optical sectioning capability of LSCM. In TPEM, only at the point of focus is the photon density sufficient to elicit two-photon excitation. The intensity of two-photon absorption is in inverse proportion to the biquadrate of the distance from the focal plane in the Z-axis direction; intensity of two-photon absorption is in proportion to the square of the excitation light intensity. Therefore,

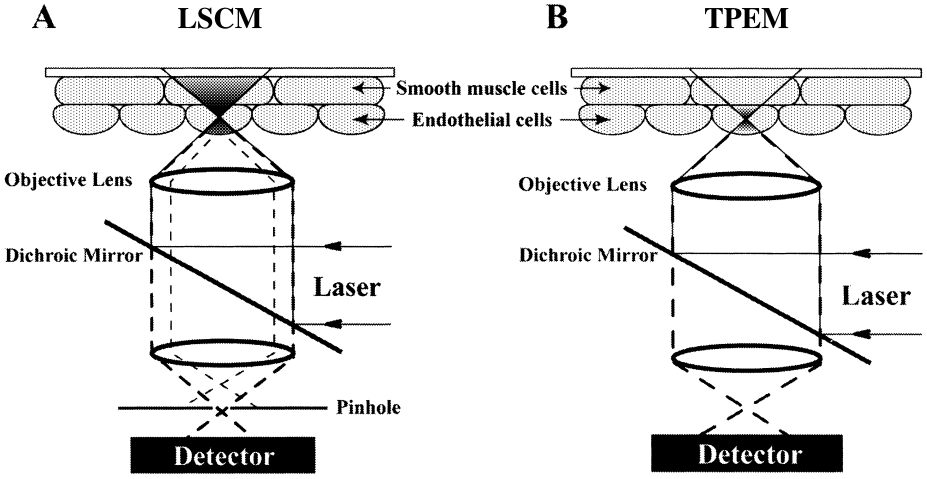

Fig. 1. Fundamental principles of LSCM (A) and TPEM (B) microscopy. In LSCM, one-photon excitation laser light is condensed in a focal plane (endothelial layer) by an objective lens; the light also excites upper (smooth muscle layer) and lower planes of the focal plane. However, fluorescence emission exclusively from the focal plane is detected through the pinhole. On the other hand, in TPEM, two-photon excitation laser light is condensed only in the focal plane, where the photon density is sufficiently high; moreover, emission from the plane can be acquired without through the pinhole.

the fluorophore is excited only in the focal volume where the photon density of the excitation light is highest; as a result, the excitation rate of the fluorophore decreases as the fourth power of the distance from the focal plane. The emitted photons originate exclusively from the focal plane. Therefore, the need to de-scan and de-image the spot in order to employ a pinhole is unnecessary.

Lateral and axial resolution of TPEM

Lateral and axial resolution of TPEM is slightly lower than those of LSCM when the pinhole is smallest; this phenomenon is attributable to the longer wavelength of the excitation light. In fact, a larger pinhole is useful in particular for imaging involving TPEM in order to acquire a brighter image and to increase the S/N ratio of the image. Therefore, spatial resolution of TPEM is actually identical to that of LSCM. Z-axis resolution of several water-immersion objective lenses was tested by z-axis scanning of 0.5-μm fluorescent beads (Molecular Probes, Inc., Eugene, OR, USA) at 760-nm excitation wavelength with a mode-locked Ti-sapphire laser, Tsunami (Spectra Physics, Tokyo), or at 488-nm excitation wavelength with a krypton-argon ion laser. Z-axis resolution, depicted as full-width at half maximum (FWHM), was dependent on NA of the objective lens utilized (Fig. 2). Maximum z-axis resolution was obtained from CFIPlanApo 60×WI (NA 1.2; Nikon, Tokyo), a water-immersion objective lens, among several object lenses applied; furthermore, the FWHM was estimated to be 0.63 μm with TPEM. This value was comparable to that observed with LSCM; moreover, it was estimated to be 0.46 and 0.72 μm at 0.2- and 0.5-mm pinhole diameter, respectively. In addition, the FWHM of CFIPlanFluor 20×MI (NA 0.75, Nikon) with TPEM (3.30 μm) was lower than that (3.59 μm) at 0.2-mm pinhole diameter with LSCM.

Photobleaching and photodamage of TPEM

Imaging employing LSCM involves a conical volume expanding above and below the focal plane, which constitutes the excitation volume, for each point of irradiation. Photobleaching and phototoxic products, such as highly reactive singlet oxygen from triplet states and free radicals, are generated within this volume where excitation occurs. On the other hand, in imaging with TPEM, excitation is restricted to the volume proximal to the geometric focus of the light; as a result, photobleaching and photodamage are also restricted. We subsequently tested how differences in photobleaching and photodamage are observed in practical fashion in imaging of cultured endothelial cells loaded

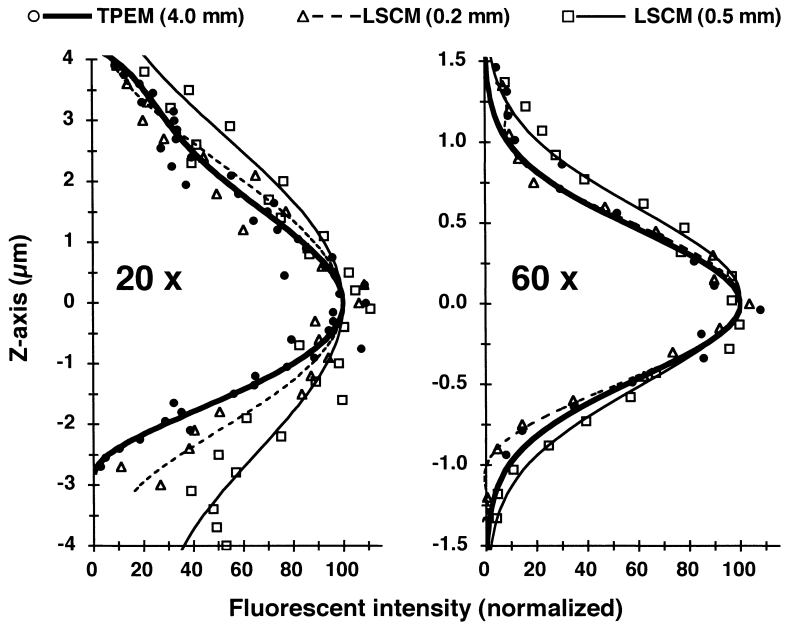

Fig. 2. Z-axis resolution using TPEM and LSCM. Z-axis scanning of 0.5-μm fluorescent beads was performed with sequential 0.1-μm steps at 760-nm excitation wavelength with a Ti-sapphire laser (Tsunami) (TPEM) or at 488-nm excitation wavelength with a krypton-argon ion laser (LSCM) employing CFIPlanApo 60×WI (NA 1.2, Nikon) and CFIPlanFluor 20×MI (NA 0.75, Nikon). In LSCM, 0.2- and 0.5-mm pinholes were utilized; additionally, a 4.0-mm pinhole was used in TPEM. Each approximate curve is fitted with a 5th- or 6th-degree equation for a square exponential function.

with calcein, an indicator of cell viability, and tetramethylrhodamine methyl ester (TMRM), which served as a mitochondrion-selective probe, in terms of LSCM and TPEM (Fig. 3).

Intensities of krypton-argon ion (488 and 568 nm) and Ti-sapphire lasers (770 nm) were adjusted in order to obtain the identical intensity of calcein fluorescence. Optical sectioning of cultured endothelial cells was effected with 21 images at 0.3-μm intervals. After the initial optical sectioning, the centers of cells were illuminated with the identical laser for 1 min; subsequently, identical optical sectioning was performed. TMRM images acquired with TPEM at the initial scanning exhibited greater sharpness than those acquired with LSCM at 0.2-mm pinhole diameter. In addition, intensities of calcein and TMRM images with LSCM at the second scanning decreased to 70% and 20% of those at the fist scanning, respectively, whereas intensities of these images with TPEM at the second scanning decreased only to less than 5% of those at the first scanning. These results suggest that photobleaching and photodamage caused by TPEM are clearly lower than those observed with LSCM. This restricted excitation associated with TPEM may be of great advantage with respect to imaging utilizing fluorescent probes *in situ* or in terms of consecutive long-lasting experiments.

Application of TPEM for measurement of Ca^{2+} response in endothelial cells of aortic strip *in situ*

We have established an imaging analysis technique for $[Ca^{2+}]_i$ in individual endothelial cells loaded with fluo-3/AM *in situ* involving LSCM (10). This method allowed distinction of Ca^{2+} signals from endothelial and smooth muscle cells of guinea pig artery *in situ*. However, consecutive real-time measurements of $[Ca^{2+}]_i$ were restricted within several minutes; furthermore, the repeated measurement in the same microscopic field was difficult due to cell damage induced by phototoxic products. Subsequently, we attempted to apply TPEM to imaging analysis of $[Ca^{2+}]_i$ in individual endothelial cells *in situ* in expectation of reduced cell damage as a consequence of phototoxic products. Two-photon excitation spectra of Ca^{2+} fluorescence probes, fluo-3/AM and fluo-4/AM, were tested employing TPEM as the Ti-sapphire laser (Tsunami), which is capable of emissions at wavelengths in the range of 700 – 1050 nm. Maximal excitation of flou-3 and fluo-4 was obtained at approximately 780 nm. This result reveals that the maximal excitation wavelength for flou-3 and fluo-4

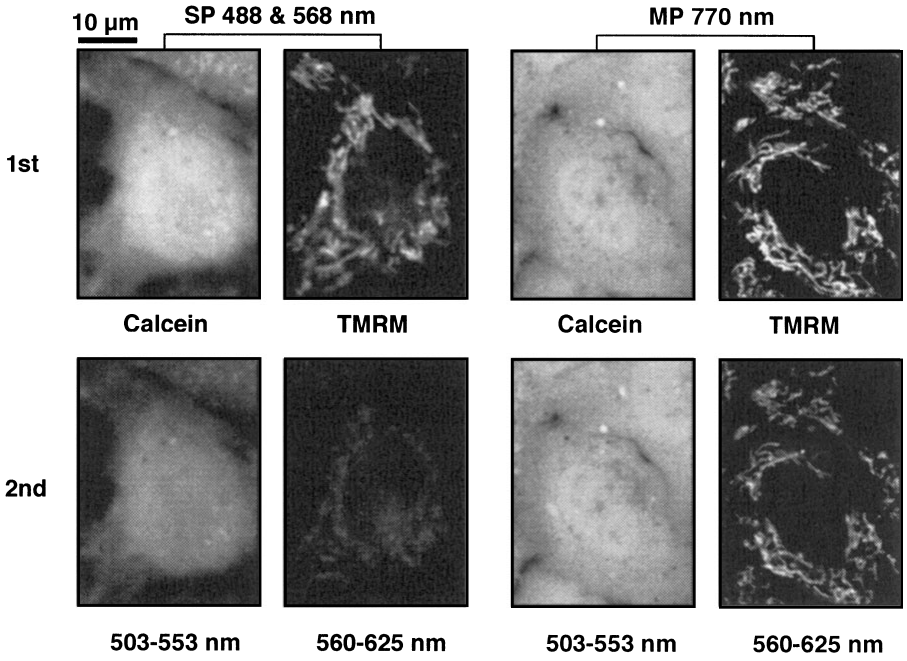

Fig. 3. Comparison of imaging of endothelial cells stained with calcein and TMRM with LSCM and TPEM. Cultured endothelial cells were loaded with 0.2 μM calcein and 2 μM TMRM for 20 min. The endothelial cells were washed and Z-axis scanning was conducted with each 0.3-μm step with LSCM (0.2-mm pinhole) at 488-nm excitation wavelength or TPEM at 780-nm excitation wavelength. Fluorescence of calcein and TMRM was directed to separate photomultipliers by a 560-nm long pass dichroic reflector through 525-nm (50-nm band pass) and 590-nm (70-nm band pass) barrier filters, respectively. Power of both lasers was adjusted to obtain the identical fluorescence intensity of calcein images. After the initial scanning, the center plane of the cells was excited with each laser for 1 min; the second scanning was then performed.

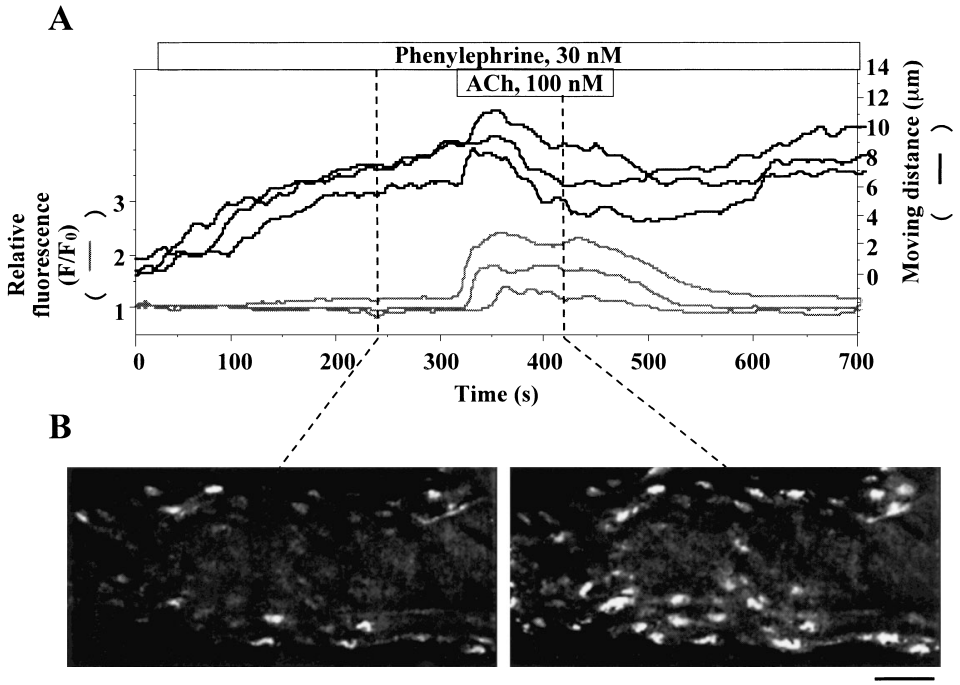

Fig. 4. Acetylcholine-induced Ca^{2+} response in endothelial cells and relaxation in mouse aortic strip contracted by phenylephrine. Mouse aortic strip, which was loaded with 10 μM fluo-4 for 1 h, was mounted in a specially designed parallel plate flow chamber. A: Time course of phenylephrine- and acetylcholine (ACh)-induced endothelial cell Ca^{2+} response (shaded line) and moving distance along major axis of smooth muscle cells in 3 different endothelial cells. B: Fluo-4 fluorescence images in the presence of phenylephrine (left) and phenylephrine and ACh (right). Scale bar, 50 μm.

with Ti-sapphire is considerably shorter than twice the maximal wavelength observed with SP.

Figure 4 shows the Ca^{2+} response to acetylcholine in endothelial cells in mouse aortic strip contracted by phenylephrine employing TPEM. Evaluation of contraction and the relaxation induced by phenylephrine and acetylcholine was possible with this imaging technique. When the extent of out of focus due to contraction or relaxation is too much to determine endothelial $[Ca^{2+}]_i$, the xy-image was acquired as averaged image of several focal planes (approx. 20 μm) by xyz-scanning using a piezoelectric actuator controlled by a function generator (13). Z-axis resolution using TPEN can be controlled by this imaging technique. Application of acetylcholine caused increase in $[Ca^{2+}]_i$ of endothelial cells, followed by relaxation along the major axis of smooth muscle cells. Furthermore, consecutive long-lasting experiments could be repeated at least four times with identical response in the same microscopic field (Fig. 4). In addition, rapid xy-scanning confirmed the induction of Ca^{2+} waves by acetylcholine. However, no intercellular relationship between adjacent cells was apparent. These data suggest that cell damage due to phototoxicity with respect to imaging with TPEM is lower than that observed with LSCM due to the restriction of two-photon excitation to the volume proximal to the geometric focus of the light. Furthermore, real-time imaging of the Ca^{2+} response in both endothelial and smooth muscle cells along the Z-axis would be possible if scanning were conducted with a real-time imaging system equipped with a piezoelectric actuator. The advantages of the TPEM imaging technique are of utmost importance for real-time imaging of ion kinetics under physiological conditions and for pharmacological evaluation *in situ*. In the future, we will attempt to clarify physiological and pharmacological roles of Ca^{2+} spots in terms of an elementary Ca^{2+}-influx event through mechanosensitive channels directly coupled with the initial step in mechanotransduction in endothelial cells *in situ* with the TPEM imaging modality.

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