Technical Advancements

Real-Time Two-Photon Microscopy and Its Application for In Situ Imaging

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The recent developments in fluorescent-probes and laser technologies have provided us opportunities to observe the biological functions in living organs and tissues under a microscope. Although single-photon excitation confocal microscopy is an indispensable tool for observing these specimens, the observable depth of this microscopy is still not enough to analyze the functions of whole parts of organs in living organisms. To observe the deeper parts, we have attempted to develop new real-time two-photon excitation microscopy equipped with a microlens-arrayed multipinhole scanning device. Using this real-time two-photon microscope, we examined the dynamics of the intracellular calcium concentration ([Ca\(^{2+}\)]) in the cardiac myocytes of the rat whole heart to understand their physiological functions and mechanisms. We could observe Ca\(^{2+}\) waves on the surface in the whole heart. However, we still need higher-energy laser power for two-photon excitation and visualization of [Ca\(^{2+}\)] dynamics in the deeper parts of the whole heart.

Key words: Ca\(^{2+}\) wave, Whole heart, In situ imaging, Real-time confocal microscopy, Two-photon excitation microscopy

I. Introduction

Advances in the technology of microscopy enables us to visualize spatiotemporal events, such as intracellular calcium concentration ([Ca\(^{2+}\)]) dynamics in living cells. However, most investigations have been done with cultured or isolated cells as an experimental model, and consequently provided only a limited insight into the mechanisms that operate in tissues or organs in situ. Application of such technologies to intact tissues or organs will provide us more detailed information to understand cellular functions. A confocal fluorescence microscope has been widely used to observe those specimens, where the optically sectioned three-dimensional images can be obtained without mechanically slicing the specimen. However, the observable depth is still not enough to analyze the functions of whole parts of organs or tissues because the single-photon excitation confocal microscope visualizes the specimen as shallow up to 50 μm. Two-photon excitation microscopy is expected to be a promising technique to observe biological specimens in deeper areas because of its inherent advantages such as three-dimensional resolution, less photobleaching above and below the observing plane, and long depth penetration with near-infrared light excitation [14, 16]. Near-infrared radiation of the two-photon excitation microscopy is applied in the spectral range from 700 nm to 1100 nm. This range is referred to as the ‘optical window’ of cells and tissues owing to the lack of efficient endogenous absorbers in this spectral range and the subsequent high light penetration depth of the order of a few millimeters in most tissues.
We will first explain the principle of the two-photon excitation, next illustrate the instrumentation of our new real-time two-photon excitation microscope, and then describe our results on the \([\text{Ca}^{2+}]_i\) dynamics in the cardiac myocytes in the rat whole heart using real-time confocal single- or two-photon excitation microscopes. Finally we explain the limitation of real-time two-photon excitation microscopy at the moment.

II. Principle of the Two-Photon Excitation

In two-photon excitation, the wavelength of excitation light is about twice as long as that for single-photon excitation (Fig. 1A), resulting in the reduction of light scattering within a sample up to 1/16. Since the probability of two-photon excitation is proportional to the square of the excitation intensity, the fluorescence emission can be obtained only in the focal volume of an objective lens which illuminates the specimen. This localization of the fluorescence emission brings about three-dimensional resolution, the same as that in a confocal laser scanning microscope, and reduces photobleaching on the out-of-focus planes (Fig. 1B).

III. Real-Time Scanning System

A two-photon excitation microscope was realized with the recent development of ultra-short pulse high-power laser systems [2, 3]. Since the two-photon excitation microscopy requires a tightly focused laser illumination, a single-focus scanning system has usually been used with a pair of two galvanometer mirrors. This system is disadvantageous when the required image-acquisition rate exceeds the video-rate, because fluorescence flux obtainable within a frame decreases proportionally with the increase in scanning speed. Accordingly, we used a microlens-array disk to produce multiple foci to increase the fluorescence flux and avoid the reduction of it even with the higher image acquisition rates [1, 7–10, 15].

The microlenses are arranged in Nipkow-disk configuration that gives uniform illumination onto the specimen by its rotation [19]. The rotation of the microlens array brings video-rate or faster scanning on the observing plane. Since the actual scanning speed of foci is not as fast as that using the single-focus scanning, one can increase the image acquisition rate without causing degradation of signal-to-noise ratio of images, cell damages and nonlinear optical phenomena arising with higher excitation intensity [4]. We also introduced a pinhole-array disk on the focal plane of the microlenses, which realizes the confocal detection of fluorescence in the two-photon multifocus microscope. The use of the pinhole-array disk effectively functions to increase the lateral and the axial resolution and to enhance the contrast of images by reducing fluorescence scattered within a specimen and that from reabsorption [7, 8]. Those advantages with the confocal detection appear more effectively in fluorescence images from deeper parts of a specimen.

IV. Real-Time Two-Photon Excitation Microscope

An optical setup of the new real-time two-photon excitation microscope equipped with a microlens- and pinhole-array scanning disks is shown in Figure 2. A mode-locked Ti:Sapphire laser (Tsunami, Spectra Physics, wavelength=800 nm, pulse width=80 fs, repetition rate=82 MHz) was used for a light source for fluorescence excitation. The microlenses and the pinholes are arranged in a helical order to achieve uniform illumination on a specimen and display 12 images with one rotation of the disks. Each laser beam from the microlens-array disk passes through each pinhole and is focused on a specimen by a water immersion objective lens (Olympus, 0.8 NA, 40×) and excite fluorescence by two-photon absorption. The fluorescence from the specimen is collected by the same objective lens and reflected by a dichroic mirror, which is placed between the two disks and has around 95% reflectivity at 400–580 nm wavelength of light, into an intensified CCD camera (Hamamatsu, C2400-35). In principle, simultaneous rotation of the two disks scans a specimen in 3 ms at 1800 rpm. However, in our current setup, the image acquisition time is limited to 33.3 ms.

Fig. 1. A: Energy transition diagram of a fluorescent molecule under light absorption and emission in single-photon excitation (left) and two-photon excitation (right). B: The spatial distribution of fluorescence induced by single-photon excitation (left) and two-photon excitation (right).
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V. Ca\textsuperscript{2+} Imaging in a Whole Heart with Real-Time Two-Photon Excitation Microscopy

The functional role of Ca\textsuperscript{2+} waves in the heart in situ is poorly understood, because Ca\textsuperscript{2+} waves have been studied mostly in enzymatically isolated cells \[5, 17, 18\]. We developed a system for in situ imaging of [Ca\textsuperscript{2+}], equipped with a multipinhole-type confocal scanning device, which enabled us to visualize real-time X–Y images of Ca\textsuperscript{2+} waves \[11, 12\]. Using this system on Langendorff-perfused rat hearts, we observed [Ca\textsuperscript{2+}], dynamics in the in situ whole heart of a rat with real-time confocal single-photon excitation microscopy. The heart was loaded with a fluorescent Ca\textsuperscript{2+} indicator (fluo-3/AM). The motion artifact on the image was prevented by 2,3-butanedione monoxime (BDM). Figure 3 shows that Ca\textsuperscript{2+} waves propagated to the neighboring cells in the rat whole heart with real-time confocal single-photon excitation microscopy. Brighter parts in the images show higher [Ca\textsuperscript{2+}], in the cell.

Using our in situ imaging system in combination with real-time two-photon excitation microscopy, we attempted to visualize [Ca\textsuperscript{2+}], dynamics in deeper parts of a rat whole heart \[6, 9, 10\]. Figure 4 shows [Ca\textsuperscript{2+}], dynamics in the anterior wall of the right ventricle of the rat whole heart. The total excitation power at the focal plane of the objective lens was 150 mW. In Figure 4, an intracellular Ca\textsuperscript{2+} wave propagating from the lower right to the upper left is shown. Although Ca\textsuperscript{2+} waves in the surface of the rat whole heart could be observed with real-time two-photon excitation microscopy, we were not able to visualize the [Ca\textsuperscript{2+}], dynamics in the deeper parts of the rat whole heart.

VI. The Advantage and Limitation of Real-Time Two-Photon Excitation Microscopy at Present

Fluorescence imaging through the pinhole-array is expected to bring twice the resolution of non-confocal system \[13\]. Although, even without a detection pinhole, the two-

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Fig. 2. An optical setup of a real-time two-photon excitation microscope with a microlens- and pinhole-array scanner. This diagram is simplified for convenience of explanation. In this optical system, a laser beam from Ti:Sapphire laser comes into a microlens-array disk and is focused onto a pinhole-array disk. Each focused light passes through each pinhole and is focused in a specimen by an objective lens so that the pinholes are imaged on a focal plane of the objective lens as excitation points of fluorescence. Fluorescence from a specimen is introduced onto an intensified CCD camera by a dichroic mirror that is placed between the two disks.

Fig. 3. Sequential confocal single-photon fluorescence X-Y images (every 100 ms) of Ca\textsuperscript{2+} waves in the whole heart of a rat. Ca\textsuperscript{2+} wave initiated at the center of the frame shows transverse ((d) through (g)) and longitudinal ((e) through (i)) propagation to adjacent cells.
photon excitation microscopy has the three-dimensional resolution similar to that of single-photon excitation confocal microscopy, its lateral resolution is almost the same as that of conventional fluorescence microscopes because of the use of near-infrared light for excitation. The scattered fluorescence blurs the images in observing both shallow and deep parts of the specimen. Comparisons between the images obtained by the confocal and the non-confocal system revealed that the use of the pinhole array is useful irrespective of the observation depth, and works more effectively for observing stronger scattering specimens. The elimination of the scattered fluorescence enables the observation of deeper parts of the specimen. These advantages brought by a confocal pinhole do not appear in a typical two-photon excitation microscope with single-focus scanning, except for the high spatial resolution.

Under experimental conditions for observing [Ca\(^{2+}\)], the excitation rate of fluo-3 in each focus is estimated to be about 6% of its two-photon absorption cross-section, NA of our used objective lens and the excitation power. This excitation rate is not high enough for this application. In addition, the field of view in our microscope is not wide enough. Currently the penetration depth, the excitation rate and the field of view discussed above are limited by the maximum output power of the used Ti:Sapphire laser. The maximum output power of a commercially available Ti:Sapphire laser is about 2 W, which is still not high enough in our calculation. For the further advance of a multipoint two-photon excitation microscope, the developments of a Ti:Sapphire laser with higher output and a fluorescence dye with high two-photon absorption cross-section are required.

VII. Conclusion

We have attempted to visualize the in situ dynamics of [Ca\(^{2+}\)] of working heart using real-time confocal microscopy. Higher spatial resolution of the image was also assessed by application of two-photon excitation microscopy equipped with Nipkow disk. Although higher depth discrimination was attained by the two-photon excitation system, we still have problems such as an inability of visualization on deeper parts due to the lower energy of laser power. Therefore, a Ti:Sapphire laser with higher output and fluorescence dyes with higher absorption in two-photon excitation are needed for us to visualize the biological signaling in situ.

VIII. Acknowledgements

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IX. References

8. Fujita, K., Nakamura, O., Kaneko, T., Oyamada, M., Takamatsu, T. and Kawata, S.: Real-time imaging of two-photon induced fluorescence with a microlens-array scanner and a regenerative Fig. 4. Sequential confocal two-photon fluorescence X-Y images (every 100 ms) of Ca\(^{2+}\) wave in the whole heart of a rat. Ca\(^{2+}\) wave propagates from the lower right to the upper left.


