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Continuous Wave Diode Laser Induced Two-Photon Fluorescence Excitation of Three Calcium Indicators

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Continuous wave (CW) diode laser induced two-photon fluorescence spectra have been measured for calcium indicators of Indo-1, Oregon-green, and Fluo-3. The near 2.0 log-log power-dependence of fluorescence intensity versus incident laser power indicates that the fluorescence from these three calcium indicators was excited by two-photon absorption process of 809-nm laser light. Calcium-dependent emission spectra of Indo-1 and Oregon-green for two-photon excitation under wavelength of 809-nm have also been measured.

KEYWORDS: Two-photon fluorescence, calcium indicator, diode laser

1. Introduction

Since Webb and co-workers[1,2] and others[3–5] have shown that two-photon excitation can be used in fluorescence microscopy to provide localized excitation in the focal plane and increased the spatial resolution, two-photon microscopy and two-photon imaging techniques have attracted considerable interests in recent years. Calcium probes, which show a spectral response upon binding Ca^{2+}, have enabled researchers to investigate changes in intracellular free Ca^{2+} concentrations.[6] Lakowicz *et al.* have studied three-photon induced fluorescence of calcium probe Indo-1 and the possibility of multiphoton calcium imaging using Indo-1 under the excitation of picosecond pulsed lasers[7,8] However, to the best of our knowledge, there has no study focused on two-photon fluorescence excitation and two-photon imaging about calcium indicators by using continuous wave diode lasers. In this paper, we report two-photon fluorescence excitation of three calcium indicators, Indo-1, Oregon-green, and Fluo-3 by using a continuous wave diode laser with wavelength 809-nm as the exciting laser source. Two-photon fluorescence spectra have been measured for Indo-1, Oregon-green, and Fluo-3, and they were peaked around 475-nm, 525-nm, and 530-nm, respectively. The slopes of the log-log power dependencies for these three probes were all near 2.0 indicating that the fluorescence was excited by two-photon absorption process. Ca^{2+}-dependent two-photon fluorescence emission spectra of Indo-1 and Oregon-green were also measured.

2. Materials and Methods

The samples we used were Indo-1 (AM Easter), Oregon-green (potassium salt), and Fluo-3 (potassium salt) ordered from Molecular Probes, Inc. For each indicator, we made two samples, one was made by adding 20-μL calcium free ringer solution into 20-μL indicator solution with concentration of 1-mM, the other one was made by adding 20-μL ringer solution with calcium concentration of 1-mM into 20-μL indicator solution with concentration of 1-mM.

Our experimental setup was similar to the one previously described.[9] An optical trapping beam was derived from an AlGaAs laser diode (SDL 2352-H1) with maximum optical output power of 500-mW at wavelength 809-nm. The laser beam was first collimated by a special collimating lens, then circularized by a pair of anamorphic prisms. Passing through another lens and a long pass ($\lambda > 780$-nm) filter the beam was deflected by a dichroic beamsplitter, and then focused to its diffraction-limited spot-size ($2\omega_0 \sim 0.8$-μm) onto a sample using an oil-immersion, 100×, 1.25 N.A. microscope objective (Zeiss Neofluar). The fluorescence excited by this highly focused laser beam was collected by the same objective lens, passed through a dichroic mirror and a pinhole aperture, collimated with a beam expansion optics, passed through a short pass filter ($\lambda < 750$-nm), and directed onto a 300-groove/mm diffraction grating, which dispersed the optical signal. The signal was then focused onto an electrically CCD array. Spectra data, collected over 300-nm bandwidth, was acquired and analyzed using a personal computer.

3. Results and Discussion

Figure 1 shows the fluorescence spectra took from Indo-1 in calcium free ringer solution, Oregon-green in calcium saturated solution, and Fluo-3 in calcium saturated solution. The fluorescence spectra of Indo-1, Oregon-green, and Fluo-3 were peaked around 475-nm, 525-nm, and 530-nm, respectively. It is impossible for these spectra to be excited by one-photon absorption process of laser light with wavelength 809-nm, and they should be excited by two-photon absorption process or other higher nonlinear processes.

To further characterize the emissions of these three calcium indicators with 809-nm excitation we measured the log-log power dependencies of fluorescence intensity versus incident power. The slopes of log-log power dependence for Indo-1 in calcium free solution, Oregon-green in calcium saturated solution, and Fluo-3 in calcium saturated solution were 2.2, 1.9, and 2.0, respectively, and they were all near 2.0 indicating that the fluorescence from these three calcium indicators were excited by two-photon absorption process of 809-nm laser light. The results were shown in Fig. 2. The log-log power dependencies for Indo-1 in saturated calcium solution, Oregon-

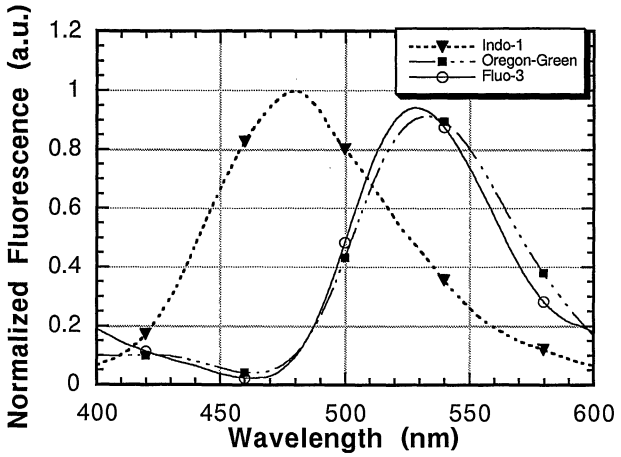

Fig. 1. Two-photon fluorescence spectra observed from Indo-1 in free calcium solution, Oregon-green in calcium saturated solution and Fluo-3 in calcium saturated solution excited by a cw diode laser with wavelength of 809-nm. The fluorescence spectra were peaked at 475-nm, 525-nm, and 530-nm for Indo-1, Oregon-green, and Fluo-3, respectively.

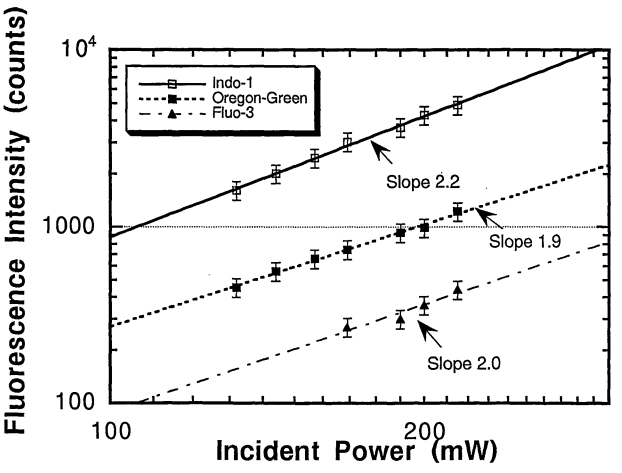

Fig. 2. Log-log power dependencies of fluorescence intensity versus incident laser power for Indo-1 in free calcium solution, Oregon-green calcium saturated solution, and Fluo-3 in calcium saturated solution under the excitation of 809-nm laser light. The integration time for the CCD array was 25-s.

green in calcium free solution, and Fluo-3 in calcium free solution were also measured and they were all near 2.0, the only difference was that the two-photon fluorescence signals from them were smaller than the ones shown in Fig. 2 under the same experimental conditions.

From the above experimental results, we know that we can observe two-photon fluorescence excitation under 809-nm laser illumination. The typical character of calcium indicator is the fluorescence intensity or shape changes with calcium concentration in intracellular or in solutions, Fig. 3 shows these changes for Indo-1 and Oregon-green under the excitation of 809-nm diode laser light. For the same incident laser power, the peak fluorescence intensity of Indo-1 in free calcium solution was 6 times of the peak intensity of Indo-1 in saturated calcium solution, and the peak fluorescence intensity of Oregon-green in calcium saturated solution was 4 times of the

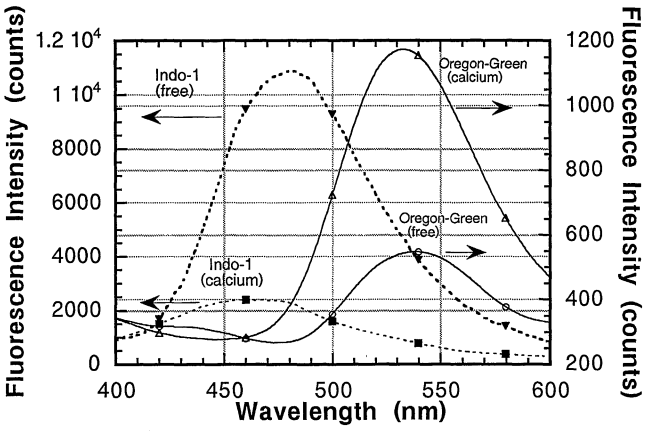

Fig. 3. Fluorescence intensity changes with calcium concentrations for Indo-1 and Oregon-green under the same incident power 190-mW. The integration time of the CCD array was 25-s.

peak intensity of Oregon-green in free calcium solution. The integration time for CCD array were all 25-s for the measured samples. Spectrum shapes have no clear difference for Oregon-green in calcium free solution and in calcium saturated solution, they both were peaked around 530-nm. However, the two-photon fluorescence spectrum of Indo-1 in saturated calcium solution had blue shift comparing to Indo-1 in free calcium solution. As discussed in reference,[6] two-photon fluorescence spectrum of Indo-1 should have peaks around 400-nm and 475-nm, the peak at 400-nm would increase with the increasing of calcium concentration under the excitation of 590-nm laser light. Under our experimental conditions of 809-nm excitation, there was no clear peak around 400-nm, as 400-nm wavelength is shorter than half wavelength of 809-nm, two-photon fluorescence can not be excited through 809-nm laser light.

4. Conclusion

In conclusion, we have demonstrated that we could observe the two-photon fluorescence spectra from calcium indicators Indo-1, Oregon-green, and Fluo-3 by using a cw diode laser as the exciting source. Fluorescence intensity changes for Indo-1 and Oregon-green with different calcium concentrations have been measured. If we could find suitable cw laser sources with half emission wavelengths just locating at the peak regions of one-photon absorption spectra of these indicators, the two-photon fluorescence signals from them will be enhanced a lot. As a result, a single cw diode laser beam can be used as a trapping source and a two-photon exciting source simultaneously to trap the biological samples and detect the calcium concentration changes of these trapped samples. Also, the above studies have suggested that it will be easy to take two-photon calcium images from biological samples by using pulsed laser sources with wavelength around 809-nm as the pulsed laser source has higher peak power.

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