PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2012, 39(10): 1012~1016
www.pibb.ac.cn

A Novel Fluorescent Protein Pair for Dual-color Two-photon Laser Scanning Microscopy*

YANG Song[1)], TENG Yan[2)], XU Ping-Yong[2)**]

([1)] *College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan* 430074, China;
[2)] *National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing* 100101, China)

Abstract Dual-color two-photon laser scanning microscopy is a useful method for simultaneously studying the expression, localization and trafficking of two different proteins in tissues. Because most two-photon microscopes only use a single wavelength excitation laser, simultaneously exciting multiple fluorescent proteins remains a challenge. Here, we present mAmetrine and mKate2, which can be used as a novel fluorescent protein pair in dual-color two-photon imaging by taking advantage of the large Stokes shift of mAmetrine and high brightness of mKate2. Both proteins have high two-photon absorption efficiencies and can be simultaneously excited at an optical wavelength of 765 nm. Dual-color two-photon imaging using this protein pair is highly effective in living cells.

Key words two-photon, dual-color, fluorescent protein
DOI: 10.3724/SP.J.1206.2012.00027

Two-photon (2P) laser scanning microscopy has been widely used since its invention approximately 20 years ago [1]. Compared with laser scanning confocal microscopy, it has many advantages, including reduced autofluorescence, deep tissue penetration and three-dimensionally localized excitation [2]. Most cellular proteins of interest can be easily labeled using green fluorescent protein (GFP) and its variants, allowing for convenient monitoring of the spatial distributions and dynamic movements of different proteins in tissues [3]. Dual-color or multicolor imaging is powerful to clarify the dynamic interactions, spatial distributions, co-localization of different proteins and subcellular structures in live tissues and whole animals. However, different fluorescent proteins have distinct optimal 2P excitation wavelengths, and most two-photon microscopes can only accommodate a single excitation wavelength at any given time. Therefore, it is challenging to simultaneously excite multiple fluorescent proteins using a single wavelength.

One method for simultaneously exciting multiple fluorescent proteins is with spectral un-mixing of overlapping emission profiles, which requires multiple detectors and sacrifices the imaging speed and sensitivity of two-photon microscopy. A simpler approach is to use a pair of FPs with a similar excitation wavelength but different emission spectra.

Recently, a red fluorescent protein, mKeima, was proposed that can be combined with EGFP for dual-color 2P imaging[4]. These proteins can be excited over a wavelength range of 810～1000 nm, and their fluorescence signals can be separated because of the extremely large Stokes shift (～180 nm) of mKeima[5]. However, this approach is limited because mKeima and EGFP have relatively low two-photon absorption efficiencies, or cross-sections, at the optimal excitation wavelength of 900 nm [4-6]. Alternatively, Shane *et al*. showed that tagRFP and mKalama1 can be

*This work was supported by grants from National Basic Research Program of China (2010CB912303), The National Natural Science Foundation of China (30870564, 30801416) and The Chinese Academy of Sciences Project (KSCX2-EW-Q-11).

**Corresponding author.
Tel: 86-10-64888524, E-mail: pyxu@moon.ibp.ac.cn

Received: January 12, 2012 Accepted: March 16, 2012

simultaneously excited at 780 nm and detected with appropriate band pass filters[7]. The limitations of this pair are that the blue emission of mKalama1 is not suitable for deep tissue imaging and that there is cross talk between mKalama1 and tagRFP because of the red shoulder in the mKalama1 emission spectrum. Another combination proposed by Shane *et al*. was using EBFP2-mcherry at 760 nm, but EBFP2 is also limited by its blue emission and a low cross-section (approximately 12 GM) at 760 nm[7].

In a paper recently published in *Nature Methods*, the two-photon absorption properties of a variety of fluorescent proteins over a wide range of wavelengths (650 ～1300 nm) have been characterized [8]. It is notable that many red fluorescent proteins, such as tagRFP and mKate2, have a high two-photon cross-section in the range of 700～800 nm due to a high-energy or short wave-length transition (s) of the chromophore. This absorption range is within the laser output range of mode-locked Ti:sapphire lasers, which are most commonly used in two-photon microscopes, and opens up the possibility for performing dual-color 2P imaging using lower power laser sources. mKate2 is a monomeric fluorescent protein with high-brightness, a far-red emission spectrum (MAX emission wavelength, 633 nm), excellent pH resistance and photostability which make it a superior fluorescent tag for imaging in living tissues[9]. mKate2 has been used as a protein tag in mammalian cell cultures, transgenic *X. laevis* embryos and liver cancer cell[9–11]. mKate2 is a red fluorescent protein, so we tried to find out a violet-excitable, blue or green fluorescent protein for pairing with mKate2 in dual-color 2P imaging. mAmetrine satisfies the requirement for that it is a large Stokes shift violet-excitable (MAX excitation wavelength, 406 nm) yellow fluorescent protein (MAX emission wavelength, 526 nm)[12]. It has been used with tdTomato as a FRET pair[12]. Based on the two-photon absorption properties of fluorescent proteins, we present mAmetrine and mKate2 as a novel fluorescent protein pair that can be excited by a single wavelength for dual-color two-photon microscopy. Both proteins have high cross-section and minimal cross talk in the emission spectrum, which make them suitable for dual-color two-photon imaging in living cells.

1 Materials and methods

1.1 Construction of expression vector

To construct pcDNA3.1-Golgi-mKate2, a cDNA fragment encoding mKate2 was PCR amplified from pmKate2-C (Evrogen Joint Stock Company, Moscow, Russia) and then fused to a targeting sequence that encodes the N-terminal 81 amino acids of human beta 1, 4-galactosyltransferase (pDsRed-Monomer-Golgi, Clontech Laboratories, Mountain View, USA) by fusion PCR. The fusion fragment was then inserted into the *Kpn* Ⅰ and *Eco*R Ⅰ sites of pcDNA3.1 (Invitrogen Life Technologies, New York, USA). The region of human beta 1, 4-galactosyltransferase contains the membrane-anchoring signal peptide that targets the fusion protein to the trans-medial region of the Golgi apparatus.

To generate the H2B-mAmetrine fusion protein, the EGFP coding sequence of pEGFP-N1 (Clontech Laboratories, Mountain View, USA) was replaced with mAmetrine, which was amplified from pBad-mAmetrine (a gift of Robert Campbell, Addgene plasmid #18084) to yield pmAmetrine-N1. Then, the coding sequence of histone H2B, which is a member of the core histones that package DNA in the nucleus, was subcloned into the *Nhe* Ⅰ and *Xho* Ⅰ sites of pmAmetrine-N1.

PCR fragments of mKate2 and mAmetrine were inserted into the *Eco*R Ⅰ-*Xho* Ⅰ and *Bam*H Ⅰ-*Eco*R Ⅰ sites of pcDNA3.1, respectively, to create the cytoplasmic expression vectors pcDNA3.1-mKate2 and pcDNA3.1-mAmetrine.

1.2 Cell culture and transfection

COS-7 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with heat-inactivated 10% (*v*/*v*) fetal bovine serum (FBS; Gibco, USA). Cells were maintained in a humidified incubator at 37℃ and 5% CO_2.

To obtain cells co-expressing Golgi-mKate2 and H2B-mAmetrine, the Golgi-mKate2 and H2B-mAmetrine plasmids were mixed and transiently transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen, New York, USA) in accordance with manufacturer's instructions. Six hours after transfection, the cells were trypsinized and re-plated on cover glasses.

Cells transiently transfected with either mKate2 or mAmetrine were subsequently mixed and re-plated following trypsinization. The cells were imaged approximately 24 h after being plated on cover glass.

1.3 Two-photon imaging

Two-photon imaging was performed with a

FV1000MAITAI two-photon laser-scanning microscope (Olympus Corporation, Tokyo, Japan) equipped with a 60 × water-immersion objective. To determine the optimal wavelength for two-photon excitation of both mKate2 and mAmetrine, the mixed cells expressing either mKate2 or mAmetrine were imaged simultaneously over a wavelength range from 740 nm to 800 nm at 5 nm steps.

To monitor the localizations of different organelles in the same cell, COS-7 cells co-transfected with Golgi-mKate2 and H2B-mAmetrine were excited at a single wavelength of 765 nm for dual-color imaging. Chroma filter sets included 495～540 nm band pass filter for mAmetrine and 600～660 nm band pass filter for mKate2.

1.4 Data analysis

The data were analyzed using Igor Pro6.11 (WaveMetrics, Portland, USA), SigmaStat (Aspire Software Internationa, Ashburn, USA) and ImageJ (National Institutes of Health, Wayne Rasband, USA) softwares.

2 Results

The crucial requirement for dual-color 2P microscopy is two fluorescent proteins that can be excited by a single wavelength at the same time, with fluorescence emission spectral profiles that are well separated. Here, we chose two fluorescent proteins with separated emission spectra and high cross-sections when excited at the same wavelength.

mKate2 is a red monomeric fluorescent protein with high-brightness, a far-red emission spectrum; mAmetrine is a large Stokes shift violet-excitable yellow fluorescent protein. The two-photon absorption property of mAmetrine shows that it can be excited at wavelengths between 600 nm and 800 nm; fortunately, mKate2 can also be excited at wavelength shorter than 800 nm [8]. The two-photon emission spectra of these proteins are similar to that of one-photon [8-9, 12]. The one-photon emission spectra of mAmetrine and mKate2 are shown in Figure 1a[9, 12]. The emission cross talk of mAmetrine and mKate2 is low at wavelengths longer than 610 nm. Therefore, band pass filters of 495～540 nm for mAmetrine and 600～660 nm for mKate2 were used respectively, as shown in Figure 1a.

We hypothesized that the high cross-sections and separated emission spectra of mAmetrine and mKate2 might make them a good pair for dual-color 2P microscopy. To determine the optimal wavelength for two-photon excitation of both mAmetrine and mKate2, we mixed cells expressing mAmetrine or mKate2 and tried to excite them at wavelengths from 745 nm to 800 nm at 5 nm steps at a constant power density. Figure 1b shows a series of images obtained at different excitation wavelengths. Excitation wavelengths from 750 nm to 780 nm allowed us to effectively visualize both mAmetrine and mKate2. As shown in

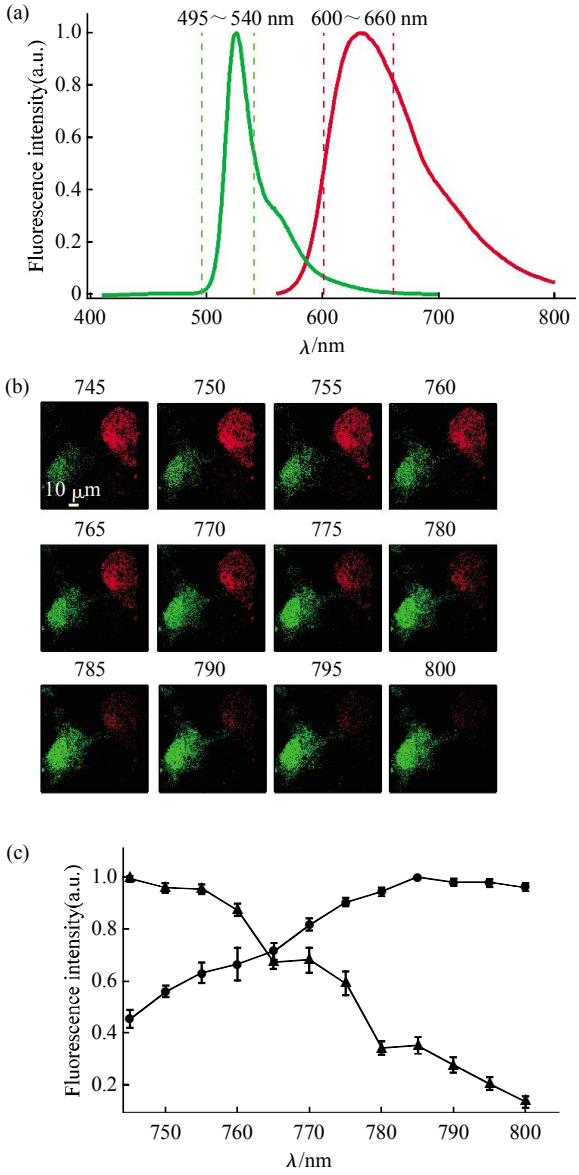

Fig. 1　mAmetrine and mKate2 as a protein pair for dual-color 2P imaging

(a) Emission spectra of mAmetrine and mKate2. The dashed lines indicate the band pass filters used for mAmetrine (green) and mKate2 (red) respectively. —: mKate2 Emission; —: mAmetrine Emission. (b) The cells were excited at the indicated wavelengths (nm). (c) The fluorescence intensity of mKate2 and mAmetrine at different excitation wavelengths. ▲—▲: mKate2; ●—●: mAmetrine.

Figure 1b, in contrast to the mAmetrine, the fluorescence intensity of mKate2 decreased with increasing wavelength. The excitation wavelength versus relative intensity curves of mAmetrine and mKate2 intersected at approximately 765 nm, at which point a good color balance of green and red was achieved, as shown in Figure 1c. In the following experiments, we chose 765 nm as the optimal excitation wavelength for dual-color imaging of different organelles in the same cell.

The two-photon absorption cross-section of mKate2 at this wavelength (765 nm) is comparable to that at 1100 nm, which is generally used as the optimal 2P excitation wavelength for mKate2[8]. The brightness of mAmetrine is also high at ~765 nm and is nearly the same as that of EGFP, which is approximately 30 GM[8]. These superior characteristics make it possible to label different organelles with mAmetrine and mKate2 in a same cell. As a proof-of-principle, we labeled the nucleus and Golgi of COS-7 cells with mAmetrine and mKate2, respectively, and excited the transfected cells at a wavelength of 765 nm. As shown in Figure 2, we can visualize both the nucleus and Golgi apparatus clearly. The results suggest that the mAmetrine and mKate2 pair can be simultaneously excited by a single wavelength and is appropriate for 2P dual-color imaging.

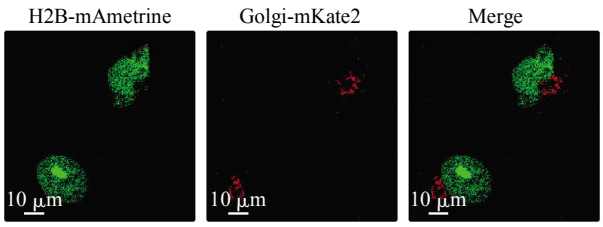

Fig. 2 Two photon dual-color imaging of COS-7 cells

COS-7 cells were transfected with mKate2 and mAmetrine targeted to the Golgi and nucleus, respectively, and excited at a wavelength of 765 nm.

3 Discussion

Identifying fluorescent protein pairs for dual-color two-photon imaging that have single wavelength excitation, well-separated emission spectra, good photostability and high two-photon brightness remains a challenge. One method is to use spectral un-mixing of overlapping emission profiles. However, this method is limited because it requires multiple detectors, which results in a loss of speed and sensitivity. A simple and effective implementation of dual-color 2P microscopy requires the use of fluorescent pairs that have similar absorption wavelengths but different emission spectra. Therefore, fluorescent proteins with specific 2P characteristic have been developed. Several pairs, such as EGFP-mKeima [4], mKalama1-tagRFP and EBFP2-mOrange [7], have been used in dual-color 2P imaging. However, their applications are limited due to either low 2P absorption or emission spectra cross talk.

In this study, we present mAmetrine and mKate2 as a protein pair that can be simultaneously excited by a single wavelength (765 nm) for dual-color 2P microscopy. This pair shows several advantages compared with the previously mentioned protein pairs. First, at an excitation wavelength of 765 nm, mAmetrine has cross-section of (~35 GM) and two-photon brightness of ~25 GM, which are both higher than those of mKalama1 (cross-section: ~30 GM at 770 nm and brightness: 11 GM) and EBFP2 (cross-section: 11 GM at 760 nm and brightness: ~8 GM)[8]. Although mKate2 has a lower cross-section (40 GM at 765 nm) and 2P brightness (~30 GM) than those of tagRFP (cross-section: ~300 GM at 770 nm and brightness: 130 GM) and mOrange (cross-section: ~65 GM at 760 nm and brightness: ~40 GM)[8], it is bright enough for live cell imaging and well balanced with the brightness of mAmetrine. Second, mAmetrine and mKate2 have less emission spectral overlap than that of mKalama1 and tagRFP. Moreover, the yellow emission spectrum of mAmetrine allows for deeper 2P imaging compared to the blue emission spectrum of mKalama1 and EBFP2. Finally, because the emission spectra of mAmetrine and mKate2 are yellow and far red, they can potentially be used for three-color 2P microscopy if a blue fluorescent protein, which has an emission spectrum separated from that of YFP and can be excited by wavelengths approximately 765 nm, can be developed.

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可用于双色双光子显微成像的新的荧光蛋白对 *

杨 松[1)] 滕 岩[2)] 徐平勇[2)**]

([1)] 华中科技大学生命科学与技术学院，武汉 430074；[2)] 中国科学院生物物理研究所，生物大分子国家重点实验室，北京 100101)

摘要 双色双光子激光扫描显微技术可以用来研究生物组织内两种不同蛋白质的表达、定位和示踪．由于大多数双光子显微镜一次只能提供一种波长的激发光，双色同时成像较难实现．mAmetrine 和 mKate2 作为新发现的荧光蛋白对可以用于双光子双色同时成像，这得益于它们各自的优势：mAmetrine 的斯托克斯位移和 mKate2 的高亮度．在 765nm 的波长激发时，它们的双光子吸收效率都很高．mAmetrine 和 mKate2 能够很好地用于双色双光子活细胞成像实验．

关键词 双光子，双色，荧光蛋白

学科分类号 Q6-33，Q-336 **DOI**: 10.3724/SP.J.1206.2012.00027

* 国家重点基础研究发展计划(2010CB912303), 国家自然科学基金(30870564, 30801416)资助项目和中国科学院知识创新工程重要方向性项目(KSCX2-EW-Q-11).

** 通讯联系人.

Tel: 010-64888524, E-mail: pyxu@moon.ibp.ac.cn

收稿日期：2012-01-12，接受日期：2012-03-16