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ZHONGYUN CHEN,[1,2] JIANGFENG HUANG,[1,2] XINYUAN HUANG,[1,2] XIUJUAN GAO,[1,2] YIFU ZHOU,[1,2] AND LING FU[1,2,3,*]

[1]*Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, Wuhan 430074, China*
[2]*MoE Key Laboratory for Biomedical Photonics, Huazhong University of Science and Technology, Wuhan 430074, China*
[3]*Advanced Biomedical Imaging Facility, Huazhong University of Science and Technology, Wuhan 430074, China*
[*]*Corresponding author:* *lfu@mail.hust.edu.cn*

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ZHONGYUN CHEN,[1,2] JIANGFENG HUANG,[1,2] XINYUAN HUANG,[1,2] XIUJUAN GAO,[1,2] YIFU ZHOU,[1,2] LING FU[1,2,3,*]

[1]*Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, Wuhan 430074, China*
[2]*MoE Key Laboratory for Biomedical Photonics, Huazhong University of Science and Technology, Wuhan 430074, China*
[3]*Advanced Biomedical Imaging Facility, Huazhong University of Science and Technology, Wuhan 430074, China*
**Corresponding author: lfu@mail.hust.edu.cn*

METHODS

1. Fiber supercontinuum generation

The pump laser source for fiber supercontinuum generation was a Ti:Sapphire laser (Insight X3, Spectra-Physics). Pulses (800 nm, 120 fs, 80 MHz) were coupled by an objective lens into a 5 cm-long photonic crystal fiber (SC-5.0-1040, NKT photonics). The input average power was 800 mW, and the output average power of PCF was 565 mW. The spectrum of supercontinuum (700-900 nm, Fig.1(b)) was measured using a spectrometer (USB2000+, Ocean Optics). The average power was 120 mW at the focal point of the imaging objective lens (for whole spectrum case). The transmittance of optical system for the whole fiber supercontinuum was 21.24%. For spectral windows tuning cases, we designed the grating patterns according to the spectral range and power. The power of three spectral windows was 40 mW in our experiments. Only a small fraction of the power of three spectral windows was used for imaging. According to the reference [1], the estimated maximal imaging depth was 100 μm. A wide, flat supercontinuum with high energy could be generated by adjusting the power, pumping wavelength, repetition rate, and pulse width of the input pulses, as well as selecting an appropriate PCF. Combining with the proposed spectral window tuning method, it is expected to have broad applications in deep intravital multicolor imaging.

2. The two-photon excitation spectra measurement

In order to ensure that fluorescent proteins with similar emission spectra have different optimal two-photon excitation wavelengths, we measured the two-photon excitation spectra of fluorescent proteins by labeling them in cells. We measured the fluorescence intensity of FPs as a function of wavelength in the range of 700-900 nm, the power of different wavelength was

identical during the experiment. We kept the laser power at moderate values to avoid saturation. The normalized two-photon excitation spectra are shown in Fig. S1(a).

3. Preparation of four-color B16 cells

To demonstrate the multiplexing imaging ability in live cells, we expressed four fluorescent proteins (green FPs: Clover, mAmetrine; red FPs: mKate and LSSmCherry) to various intracellular structures by transient transfection. Clover was expressed in the Golgi apparatus; mAmetrine was expressed in the endoplasmic reticulum (ER); mKate was expressed in mitochondria; and LSSmCherry was expressed in the entire cell. Plasmids pCAG-LSSmCherry-SiT15-Clover and pCAG-Mito-mKate-mAmetrine-ER are transfected into B16 cells, respectively. All transfections were performed by using liposome (Lipofectamine™ 3000, Thermo Fisher) and incubating for eight hours. After another 12 hours, two dishes of transfected B16 cells were mixed. The mixed cells were inoculated in confocal dishes and cultured at 37℃ in a 5% CO_2 incubator. The cells were used for imaging when growing the appropriate density.

4. Image processing

In imaging experiments, the grating patterns were predesigned and integrated into the imaging software. The three spectral windows were sequentially applied to imaging in cycles. The maximal power of three spectral windows was 40 mW, respectively. We used neutral density filters to attenuate the power to 5 mW for cell imaging here. Pixel dwell time ranged from 3-10 μs depending on the temporal and spatial resolution required for the application, typically resulting in a 0.8 s acquisition time for an image with 512×512 pixels. The transform rate of the different patterns is 60 Hz, which means the spectral windows tuning time is 16.67 ms. In the actual experiment, we set the tuning time of 50 ms to maintain the stability of the spectrum selection. The total imaging time consists of two parts: image acquisition (T_a) and spectral tuning (T_s), the total imaging time $T = 3T_a + 3T_s = 2.55$ s in Fig. 5 and Fig. S1.

5. Linear unmixing

The fluorescent intensity of fluorescent proteins (FPs) was changed when tuning the spectral windows (Fig. S1(b)). The problem was how to separate them in complex samples containing multiple cells expressing different FPs. Here we employed the linear spectral unmixing algorithm to separate FPs [2]. The linear unmixing algorithm utilized the pseudoinverse function of the JAMA matrix algebra library to determine the least-squares solution of the unmixing problem, which was available for free download from the National Institutes of Standard and Technologies. Using this linear unmixing algorithm, we obtained the unmixing image of four FPs, as well as a map of the residuals (Fig. S1(c)). The residuals were calculated by summing the intensities of all FPs for a given pixel and subtracting this from the unfiltered imaging. The residuals were very small and near to zero (Fig. S1(c)), which indicated that the different FPs were well separated. The results demonstrated the multiplexing imaging ability of our method.

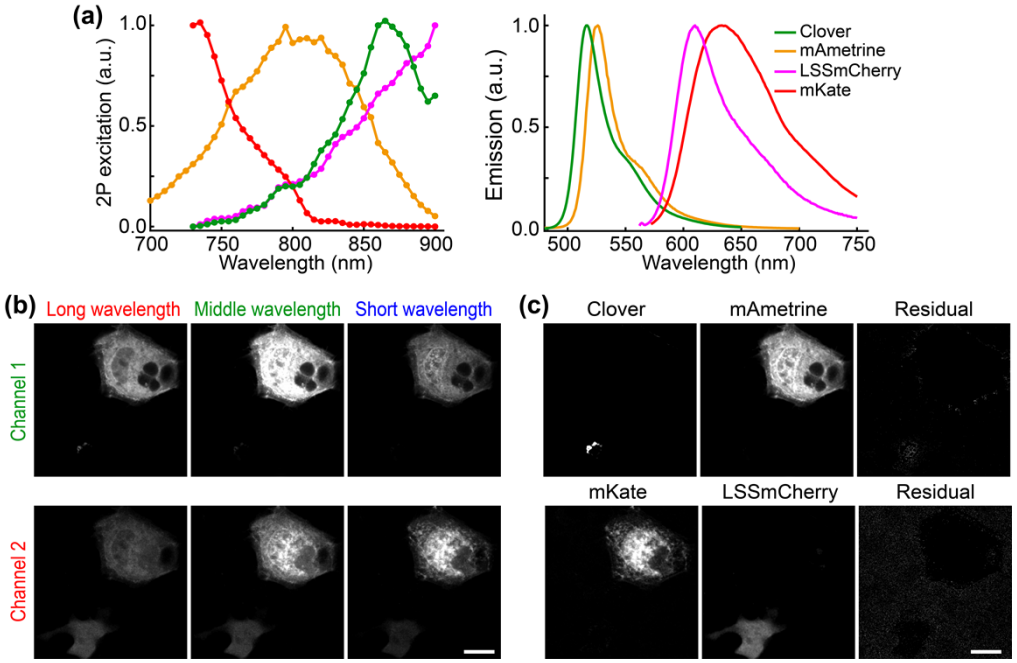

Fig. S1. (a) 2P excitation (left) and emission (right) spectra of four FPs. The 2P excitation spectra of Clover, mAmetrine, mKate, and LSSmCherry were measured by tuning wavelength of 100 fs Ti:Sa laser, the data of fluorescence emission spectra came from fpbase.org. (b) TPEF mixed images using three different spectral windows as excitation source. Images of the same channel were presented at the same contrast, which showed the fluorescent intensity as a function of spectral windows. (c) Spectrally unmixed gray image of cells and the unmixing residual. Scale bars: 20 μm.

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