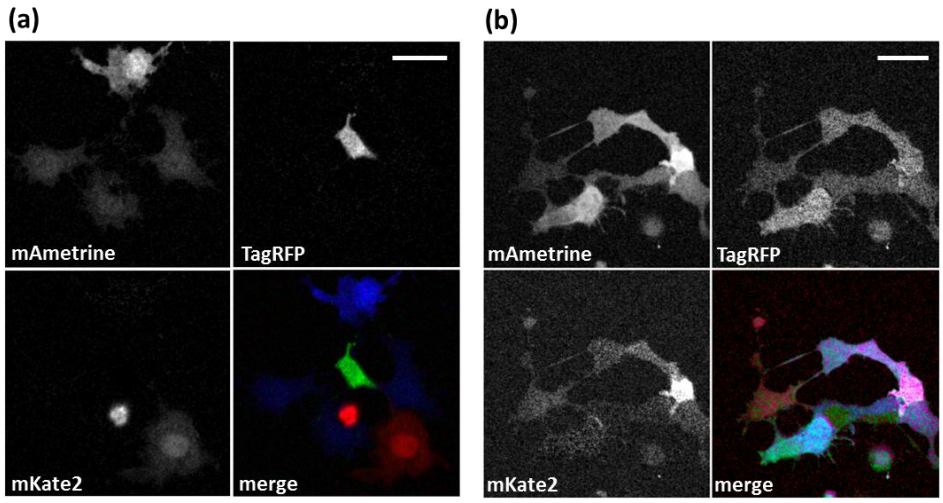

Fig. 4. Linear unmixing. (a) Signal of mAmetrine, TagRFPt and mKate2 from Fig. 3 (middle and right columns) is restored in three separate images. (b) Pseudo-colored merge image of a sample co-expressing all three FPs after linear unmixing. In (a) and (b), mAmetrine, TagRFPt and mKate2 are pseudo-colored in blue, green and red in the merge image, respectively. Gamma correction (0.5) was applied to both panels in order to better present the dimly labeled cells. Scale bars are 20 μm.

5. Discussion and Conclusions

Using a broadband femtosecond laser and ultrafast phase-shaping techniques, we have developed a two-photon imaging modality allowing selective excitation of three FPs with distinct fluorescence spectra. By combining this with linear unmixing from two PMT channels, the fluorescence signal of each of the three FPs was restored with high separation. With two phase-shaped pulses, a total of four raw images were taken by two PMTs, which allows unmixing of as many as four FPs with distinct fluorescence spectra. Addition of more PMTs would permit either detection of more FP species or improved separation and reduced noise in the unmixing results [54, 55]. The use of a single broadband laser not only reduces the overall cost of the system, but also avoids the alignment complications of other multi-color 2PFM setups, allowing easy adaptation to most biomedical laboratories. Since phase-shaping provides versatile narrow excitation selectivity within the laser spectrum [33], selective FP excitation is only limited by the laser bandwidth. Using emerging laser sources with ultra-broad bandwidths, such as the VENTEON systems (VENTEON Laser Technologies GmbH), would easily expand our setup to nearly all available FPs [30] with precise selective excitations [56]. Such excitations could also be tailored to ensure equal fluorescence signal for the FPs of interest. In addition, phase-shaping may offer reduced photodamage in some imaging contexts. Depending on sample composition, photodamage may scale linearly (for example in pigment-rich tissues) or nonlinearly with peak intensity [57, 58]. Phase-shaped pulses designed for high 2PF signal from the FPs of interest have lower peak intensities than TL pulses and should provide reduced photodamage in samples where two and three photon absorption processes are the dominant photodamage mechanisms.

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