Fluorescent protein FRET pairs for ratiometric imaging of dual biosensors

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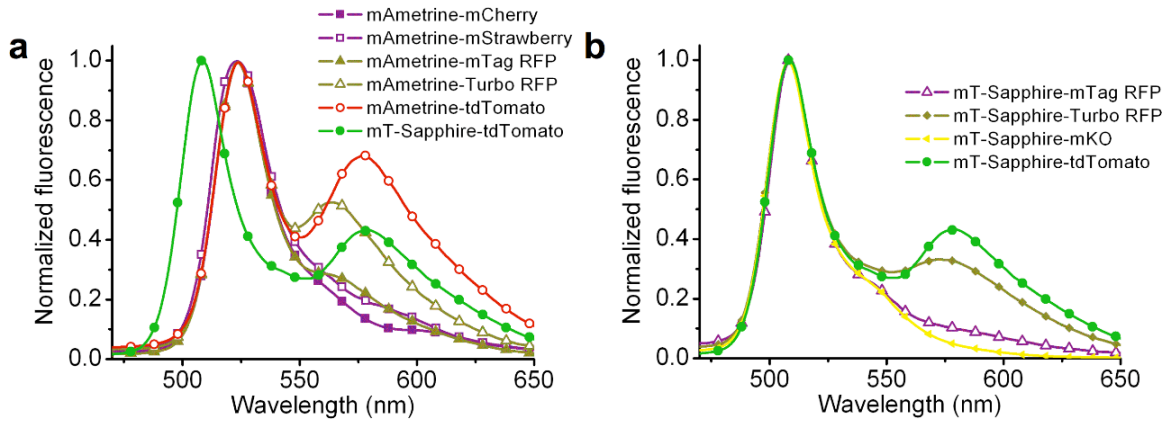
Supplementary figures and text:

Note: Supplementary Movies 1–3 are available on the Nature Methods website.

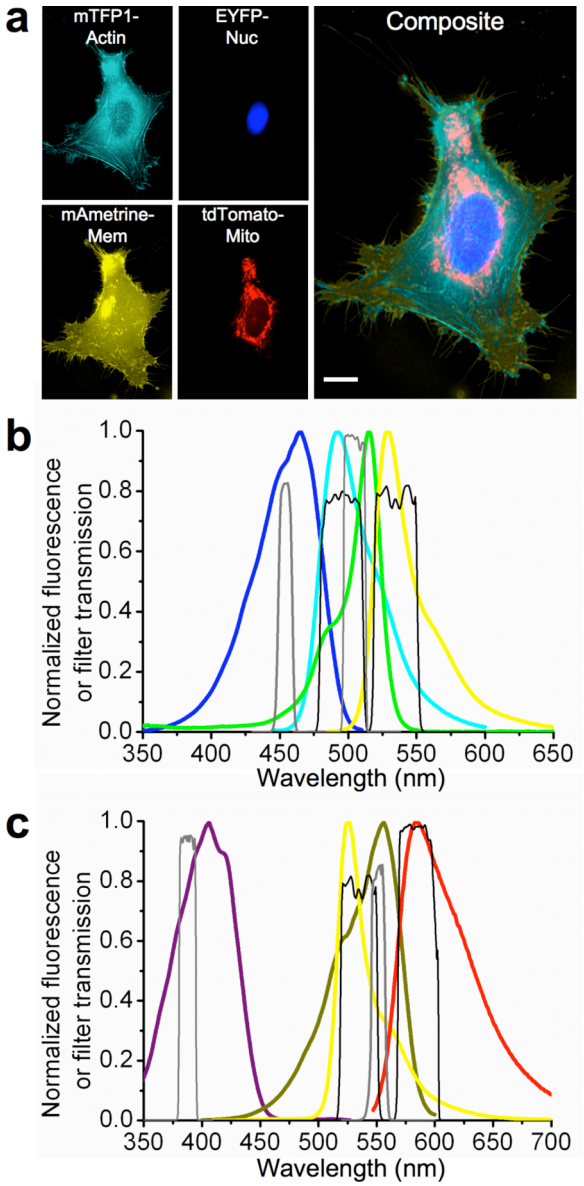
Supplementary Figure 1



Supplementary Figure 1 | Testing OFPs and RFPs as FRET acceptors in live cells.

Shown are the fluorescence emission spectra for a series of analogous FRET constructs in live cells. (**a**) mAmetrine as the donor fluorescent protein. mT-Sapphire-tdTomato is also shown for the sake of reference. (**b**) mT-Sapphire as the donor fluorescent protein.

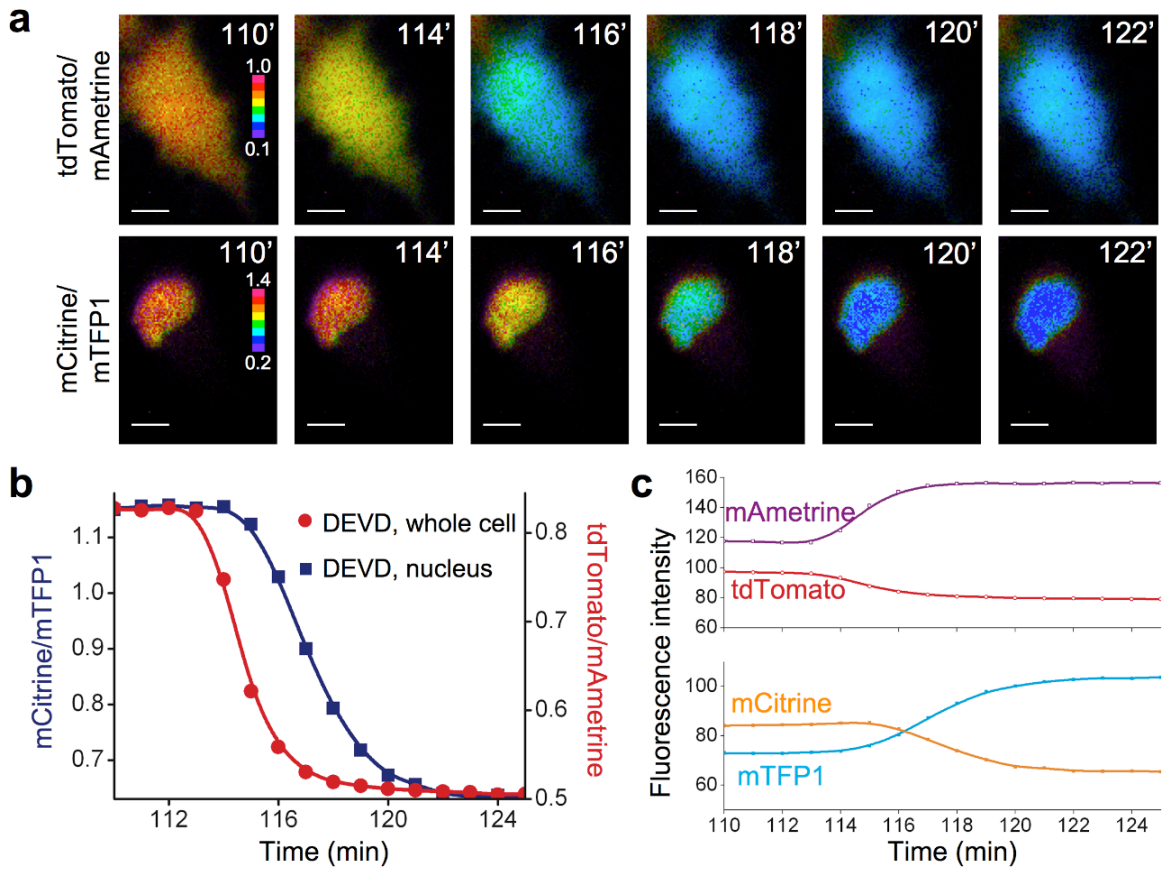
Supplementary Figure 2



Supplementary Figure 2 | Four-color widefield imaging.

(**a**) HeLa cell expressing mAmetrine, mTFP1, EYFP, and tdTomato in targeted fusion constructs. Scale bar = 10 μm. The filter sets employed are represented in panels **b** and **c** and detailed in **Supplementary Table 1**. (**b**) Fluorescence excitation and emission spectra of mTFP1 (blue and cyan) and mCitrine (green and yellow). The transmission profiles of the excitation (gray) and emission (black) filters are also represented in panels **b** and **c**. (**c**) Fluorescence excitation and emission spectra of mAmetrine (violet and yellow) and tdTomato (brown and red).

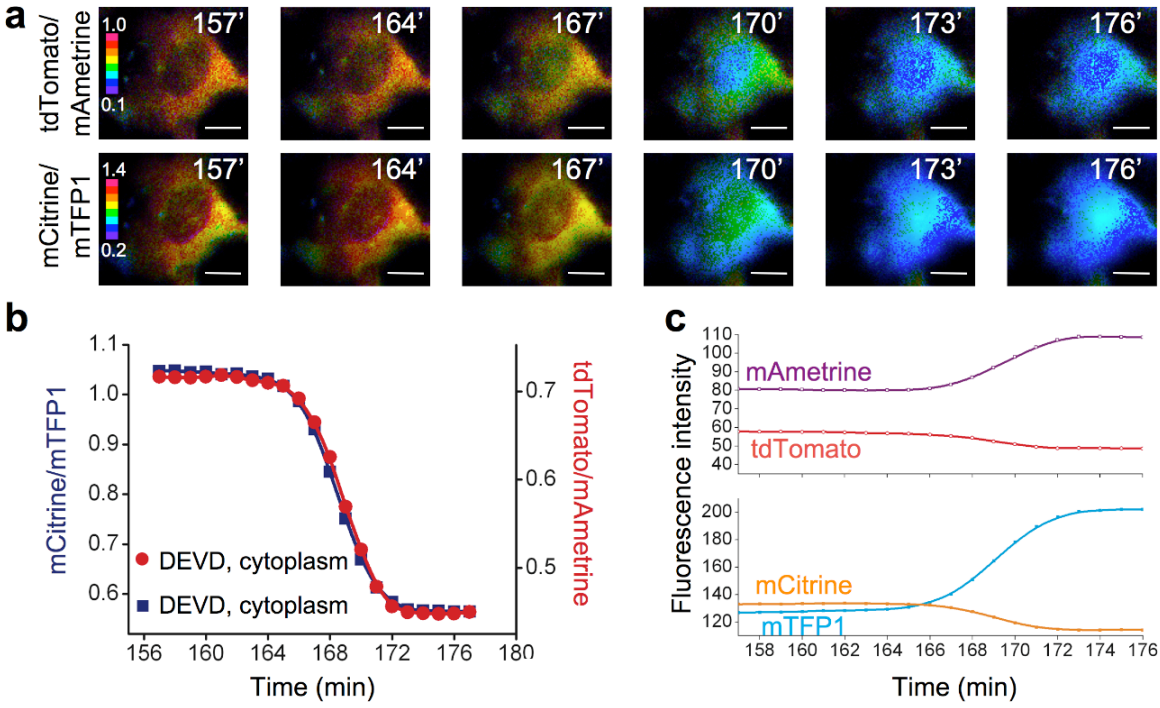
Supplementary Figure 3



Supplementary Figure 3 | Live cell imaging with dual FRET pairs.

(**a**) Pseudocolored ratio images of a representative staurosporine-treated HeLa cell expressing dual caspase biosensors. In this particular experiment, mCitrine-DEVD-mTFP1 is localized to the nucleus and mAmetrine-DEVD-tdTomato is expressed with no targeting and is rapidly exchanging between nucleus and cytoplasm (from **Supplementary Movie 2**). Scale bar = 10 μm. (**b**) Ratios plotted versus time for the cell shown in panel **a**. Results for 11 individual cells are summarized in **Supplementary Table 2** online. (**c**) Intensities in the 4 emission channels for the cell shown in panel **a**.

Supplementary Figure 4



Supplementary Figure 4 | Live cell imaging with dual FRET pairs.

(**a**) Pseudocolored ratio images of a representative staurosporine-treated HeLa cell expressing dual caspase biosensors. In this particular experiment both mCitrine-DEVD-mTFP1 and mAmetrine-DEVD-tdTomato are expressed with a single copy of a C-terminal nuclear export sequence (from **Supplementary Movie 3**). Proteolysis releases mAmetrine and mCitrine that rapidly diffuse into the nucleus. This explains the decreased tdTomato/Ametrine ratio and increased mCitrine/mTFP1 ratio observed in the nucleus at the 170-minute time point. Scale bar = 10 μm. (**b**) Cytoplasmic ratios plotted versus time for the cell shown in panel (**a**). (**c**) Cytoplasmic intensities in the 4 emission channels for the cell shown in panel (**a**).

Supplementary Table 1 | Filters used for widefield dual FRET or four-color imaging.

Protein	Filters		
	Donor (Ex; Em; dichroic)	**Acceptor (Ex; Em; dichroic)**	**FRET (Ex; Em; dichroic)**
mTFP1 (donor) mCitrine (acceptor)	3RD450-460 [a]; HQ495/30m [b]; 470dcxr [b]	FF01-504/12 [c]; ET535/30m [b]; 515dclp [b]	3RD450-460 [a]; ET535/30m [b]; 470dcxr [b]
mAmetrine (donor) tdTomato (acceptor)	FF01-387/11 [c]; ET535/30m [b] 470dcxr [b]	3RD550-560 [a]; FF01-585/29 [c]; 565dclp [b]	FF01-387/11 [c]; FF01-585/29 [c]; 470dcxr [b]

[a] Omega Optical Inc. part number. [b] Chroma Tech. Corp. part number. [c] Semrock part number.

Supplementary Table 2 | Single-cell caspase-3 responses for staurospaurine-induced apoptosis for various experimental protocols. Time 1 indicates the time elapsed between the addition of staurospaurine and the midpoint of the FRET change for mAmetrine-tdTomato FRET pair. Time 2 is the midpoint of the FRET change for mTFP1-mCitrine FRET pair.

Experiment	Time 1 (min)	Time 2 (min)	Δ*t* (min)
mAmetrine-DEVD-tdTomato-NES and mCitrine-DEVD-mTFP1-NLS	128.2	131.3	3.1
	138.5	139.2	0.7
	131.5	134.8	3.3
	130.9	131.9	1.0
	137.4	138.0	0.6
	142.1	146.2	4.1
	114.0	115.5	1.5
	144.2	145.4	1.3
	141.2	144.5	3.3
	110.3	112.9	2.6
	142.9	143.3	0.4
	153.7	156.3	2.6
	147.2	148.5	1.3
	148.7	152.6	3.9
	150.8	156.9	6.1
	152.8	158.0	5.2
	157.7	160.5	2.8
		Average	**2.6 +/- 1.6**
mAmetrine-DEVD-tdTomato and mCitrine-DEVD-mTFP1-NLS	109.1	111.2	2.1
	126.5	128.2	1.7
	138.6	141.4	2.8
	124.4	127.8	3.4
	124.5	125.5	1.0
	124.1	125.1	1.0
	131.1	133.5	2.4
	134.1	138.3	4.2
	120.7	122.7	2.0
	135.7	137.7	2.0
	152.4	153.6	1.2
		Average	**2.2 +/- 1.1**
mAmetrine-DEVD-tdTomato-NES and mCitrine-SASG-mTFP1-NES	183.2	NA[a]	NA
	153.5	NA	NA
	170.2	NA	NA
mAmetrine-SASG-tdTomato-NES and mCitrine-DEVD-mTFP1-NES	NA	150.0	NA
	NA	138.1	NA
	NA	114.4	NA

[a] NA, not applicable.

SUPPLEMENTARY METHODS

General methods and materials

Synthetic DNA oligonucleotides for cloning and library construction were purchased from Integrated DNA Technologies (Coralville, IA). PCR products and products of restriction digests were purified by gel electrophoresis and extraction using either the GenCatch™ gel extraction kit (Epoch Biolabs, TX) or the QIAquick™ gel extraction kit (QIAGEN, Valencia, CA). Plasmid DNA was purified from overnight cultures by using the GeneJET™ Plasmid Miniprep Kit (Fermentas, ON) or the QIAprep Spin Miniprep kit (QIAGEN). Restriction endonucleases were purchased from Invitrogen, Fermentas, or New England Biolabs. Dye terminator cycle sequencing using the DYEnamic ET kit (Amersham Biosciences) was used to confirm the complete cDNA sequences for all fluorescent protein variants and fusion constructs. Sequencing reactions were analyzed at the University of Alberta Molecular Biology Service Unit or the Florida State University Bioanalytical and Molecular Cloning DNA Sequencing Laboratory. All filters for fluorescence screening and imaging were purchased from Chroma Technology (Rockingham, VT), Omega Filters (Brattleboro, VT) and Semrock (Rochester, NY).

Construction of 10-AA linker FRET constructs for spectral imaging

To construct mAmetrine and mT-Sapphire fusions to tdTomato, mCherry, TurboRFP, TagRFP, and mStrawberry for FRET spectral imaging, the donor fluorescent protein genes (mAmetrine and mT-Sapphire) were amplified with a 16-nucleotide 5' primer that adds an EcoRI site immediately before three nucleotides comprising the Kozak sequence (ACC; threonine) preceding the start codon, and a 3' primer containing the C-terminal 25 nucleotides of the appropriate fluorescent protein followed by the sequence: TCCGGACTCAGATCCCCACCGGTCGCCACC, containing an AgeI site. The PCR products were sequentially digested with AgeI and EcoRI and ligated into a similarly digested Clontech-style N1 cloning vector containing the appropriate acceptor to yield the amino acid sequence 'SGLRSPPVAT' flanked by mAmetrine or mT-Sapphire on the 5' terminus and by an orange or red fluorescent protein (i.e. tdTomato, mCherry, TurboRFP, TagRFP, mKO, or mStrawberry) on the 3' terminus.

Construction of fusion proteins for 4-color widefield imaging

The pEYFP-Nuc vector was from Clontech and mTFP-actin has been described previously[1]. The tdTomato-Mito vector was constructed by fusing the mitochondrial targeting sequence derived from the precursor of subunit VIII of human cytochrome C oxidase to the N-terminus of tdTomato. To construct the mAmetrine-Mem vector, the sequence of the N-terminal 20 amino acids of neuromodulin (targeting to plasma membrane and intracellular membranes) was first assembled by PCR with Nhe1 and Xho1 restriction sites on 5' or 3' ends, and mAmetrine was amplified with suitable primers to produce Xho1 and BamH1 restriction sites at the 5' and 3' ends, respectively. The digested PCR products were ligated into pEYFP-Nuc vector digested with Xho1 and BamH1 in a three-part ligation. To prepare cDNA for transfection, all plasmids were purified by Plasmid Midi kit (Qiagen).

Construction of caspase-3 sensors

To construct the genes encoding the caspase-3 sensors, mAmetrine and mCitrine were PCR amplified with a 5' primer containing an Xho1 site and a 3' primer that appended the DNA sequence encoding 'LGGT' followed by a Kpn1 site. The genes encoding tdTomato and mTFP1 were PCR amplified with a 5' primer that appended a Kpn1 site followed by DNA encoding 'GSGSGDEVDG', and a 3' primer with an EcoR1 site. PCR products were digested with the appropriate restriction enzymes and plasmid pBAD/His B was digested with Xho1 and EcoR1. Three-part ligations were performed with the digested mAmetrine PCR product, the digested tdTomato PCR product and the digested plasmid or the digested mCitrine PCR product, the digested mTFP1 PCR product and the digested plasmid. DNA was purified from transformed bacteria and the sequence confirmed by DNA sequencing. The gene products for these sensors consist of the amino acid sequences 'LGGTGSGSGDEVDG' flanked by either mCitrine (1-230) and mTFP1 (1-231) or mAmetrine (1-230) and tdTomato (1-476).

A similar three-part ligation method was used to create the nucleus targeted caspase-3 sensor mammalian expression vectors. The procedure described above was modified such that the outside primers encoded 5' Nhe1 and 3' Xho1 sites rather than 5' Xho and 3' EcoR1 sites. Furthermore, the target plasmid was pEYFP-Nuc (Clontech) rather than pBAD/His B. To construct the expression vector with no targeting, a 3' BamH1 site was used in place of the 3' Xho1 site and the pEYFP-Nuc plasmid was digested with Nhe1/BamH1 rather than Nhe1/Xho1. Digestion of pEYFP-Nuc with Nhe1/BamH1 removes the nuclear localization sequence normally

present in this plasmid. To append a C-terminal nuclear export sequence, a 3' primer encoding the amino acid sequence 'LPPLERLTL' peptide followed by a BamH1 site was used.

Control FRET constructs that could not be cleaved by caspase-3 (DEVD sequence replaced with SASG) were constructed by replacing the 5' primer used to amplify tdTomato and mTFP1 with a new primer appended a Kpn1 site followed by DNA encoding 'GSGSGSASGG'. All other primers and ligations for plasmid construction were identical to those described above. This procedure produced the FRET constructs mAmetrine-SASG-tdTomato-NES and mCitrine-SASG-mTFP1-NES.

Spectral imaging of FRET constructs

mAmetrine and mT-Sapphire FRET constructs were imaged and the spectra recorded using an Olympus FV1000 laser scanning confocal microscope equipped with 405 nm and 543 nm lasers and a diffraction grating-based spectral imaging detector system. Log phase HeLa (CCL-2) cells were plated directly into Delta-T imaging chambers and were transfected at 60% confluency as described above. Between 36 and 48 hours after transfection, the chambers were bathed in fresh media, transferred to a stage adapter on the confocal microscope, and maintained at 37 °C under a 5% CO_2 for the duration of the experiments. After examination of general cell viability and fluorescent protein expression at low (10× - 20×) magnification, selected candidates were chosen for further analysis at higher magnification (40× Plan Apo). Spectra of all FRET constructs were recorded with a 405 nm diode laser over the range of 450-720 nm (2 nm bandwidth) at 3% of total laser output power (15 μW), a 4x zoom setting, 100 μs dwell time, and a fixed pinhole size of 550 μm. The emission spectra are provided in **Supplementary Fig. 1**.

4-color widefield imaging

HeLa cells in 35 mm imaging dishes were transfected by the method described previously[1] with a total of 4 μg of plasmid DNA encoding, EYFP-Nuc, mTFP-actin, tdTomato-Mito, and mAmetrine-Mem (1 μg × 4). The next day the culture medium was exchanged with Hank's buffered salt solution (HBSS) and then the cells were imaged on an Axiovert 200M (Zeiss) microscope equipped with a 75 W xenon-arc lamp, 40× objective lens (NA = 1.3, oil) and a 25% neutral density filter in the light path. Exposure times were adjusted between 200 ms to 3 s to obtain suitable intensities in each channel. A representative cell is shown in **Supplementary Fig.**

2a.The filters used for imaging are represented in **Supplementary Fig. 2b,c** and detailed in **Supplementary Table 1**.

Widefield imaging of staurospaurine-induced apoptosis

The plasmids encoding caspase-3 sensors and control FRET constructs were purified using the Plasmid Midi kit (Qiagen). HeLa cells in 35 mm imaging dishes were transfected with 0.5 μg mCitrine-mTFP1 cDNA and 3.0 μg mAmetrine-tdTomato cDNA by the method described previously[1]. Between 36 to 60 hours post-transfection, staurospaurine was added into culture medium to the final concentration of 2 μM. After incubated for another 100 mins, cells were taken out from the incubator and washed 3× with HBSS. Cells were then maintained in HBSS and subjected to imaging on an Axiovert 200M microscope (Zeiss) equipped with a 75 W xenon-arc lamp and 20× objective lens (NA = 0.75, air). For a typical experiment, 1 second exposure time for each channels, 1 minute intervals between every measurements of all 4 channels, and a 25% neutral density filter were used. The excitation, dichroic and emission filters used for imaging are detailed in **Supplementary Table 1**. Cells were maintained at 37 °C during all imaging experiments.

Control experiments were performed by coexpressing either mCitrine-DEVD-mTFP1-NES and mAmetrine-SASG-tdTomato-NES or mCitrine-SASG-mTFP1-NES and mAmetrine-SASG-tdTomato-NES. Live cell imaging and induction of apoptosis by staurosporine was performed exactly as described above.

Image processing

QEDImaging Invivo (Media Cybernetics), NIS-Elements (Nikon), FluoView (Olympus), and Simple PCI (Hamamatsu) software was used for all data collection and camera and microscope control. ImagePro Plus 6.0 (Media Cybernetics) was used to prepare the composite image shown in **Supplementary Fig. 2**. ImageJ[2] was used for processing of dual FRET caspase-3 imaging. A custom macro (available upon request) was used to create the ratiometric and intensity scaled time-lapse sequences represented in **Supplementary Movies 1, 2, and 3**. **Supplementary Movie 1** is the ratiometric time-lapse movie of the cell shown in **Figure 2**; **Supplementary Movie 2** is the ratiometric time-lapse movie of the cell shown in **Supplementary Fig. 3**; and **Supplementary Movie 3** is the ratiometric time-lapse movie of the cell shown in **Supplementary Fig. 4**.

REFERENCES

1. Ai, H.W., Henderson, J.N., Remington, S.J. & Campbell, R.E. *Biochem. J.* **400**, 531-540 (2006).
2. Abramoff, M.D., Magelhaes, P.J. & Ram, S.J. *Biophotonics International* **11**, 36-42 (2004).

SUPPLEMENTARY NOTE 1

Methods and Results for Evolution and Characterization of mAmetrine

Directed evolution of mAmetrine

Our initial library had been designed with the goal of identifying avGFP variants with a violet-excitable blue-fluorescing chromophore that was protonated in both the ground and excited states[1]. Starting from *Aequorea* EGFP, we created a gene library in which positions Thr65, Thr203, and Ser205 were randomized. During exhaustive screening of this initial library we made the unexpected identification of EGFP Thr65Ser/Thr203Phe that exhibited strong violet absorption and strong yellow fluorescence at 520 nm. A subsequent library with randomization of Ser147 and His148 resulted in the identification of the additional Ser147Val/His148Trp mutations that contributed a further red-shift to 526 nm. To further improve the brightness of this protein we undertook many rounds of library creation and screening. Libraries were created by randomly substituting all 20 amino acids at a single position, or by randomly substituting a subset of all amino acids at a single position, or by employing random mutagenesis of the whole gene. Positions targeted for randomization included 30, 39, 63, 64, 72, 105, 145, 153, 163, 171, 175, and 181. In each round of this process, libraries were created as described above and screened as described below. This process was halted only when no further improvement had been observed for several rounds of screening. The final variant resulting from this process was designated mAmetrine (**Fig. 1**).

Gene libraries with saturation or semi-saturation mutagenesis at a particular residue and libraries of randomly mutated genes were constructed as previously described[1,2]. PCR products were digested with Xho1 and EcoR1 and ligated into similarly digested pBAD/HisB vector (Invitrogen). Electrocompetent *Escherichia coli* strain DH10B (Invitrogen) was transformed and plated on Luria-Bertani (LB)/agar plates supplemented with ampicillin (0.1 mg/ml) and L-arabinose (0.02%). Plates were incubated for 14 h at 37 °C prior to screening.

The screening system has been described previously[1,2]. Colonies were illuminated with light from a 175 W xenon-arc lamp (Sutter) that had been passed through a 375 to 415 nm or a 490 nm to 510 nm bandpass filter. Colony fluorescence was imaged with a Retiga 1300i 12-bit CCD camera (QImaging) fitted with either a 440 to 480 nm or a 520 to 550 nm emission filter. For each plate of colonies, 3 images were acquired: excite 375-415 nm, emit 440-480 nm; excite

490-510 nm, emit 520-550 nm; and excite 375-415 nm, emit 520-550 nm. Colonies with the highest ratio of fluorescence intensity in the latter image to fluorescence intensity in the other 2 images were picked. These clones were cultured overnight in 4 ml of LB medium containing ampicillin and L-arabinose. The following day 0.1 ml of each culture was dispensed into a 96-well plate (Nunc) and the full emission spectra of each variant measured with a Safire2 plate reader (Tecan). Variants with the most intense emission peak were used as templates in the subsequent round of library construction.

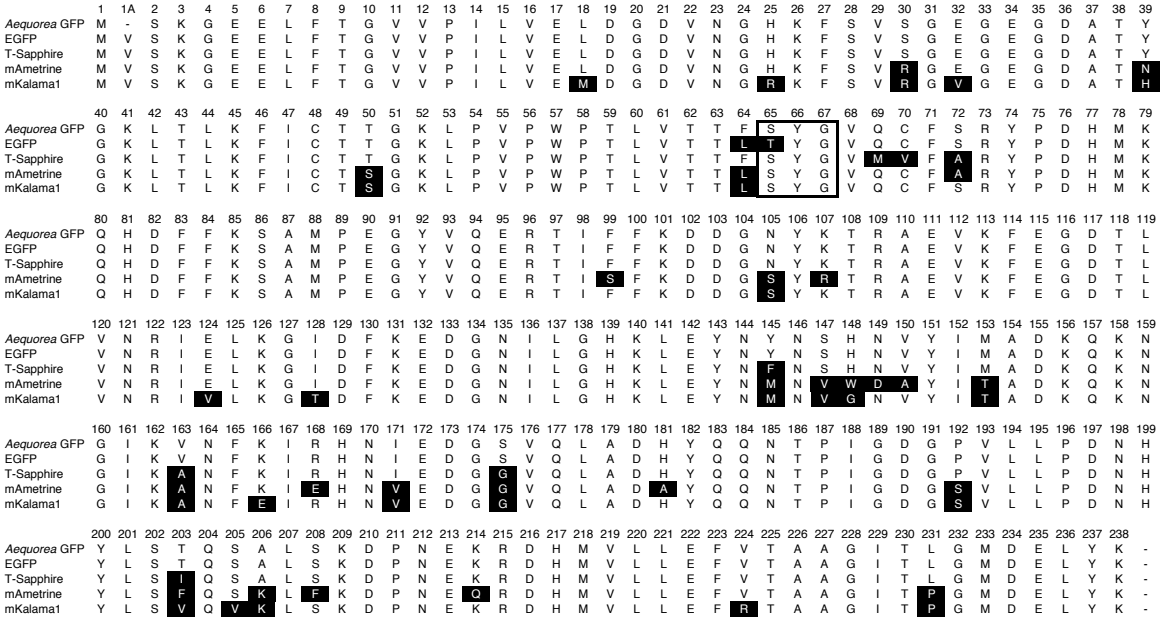


Figure 1 | Sequence alignment of mAmetrine and related variants.

Sequences included in the alignment are wild-type *Aequorea victoria* GFP[3], EGFP[4], T-Sapphire[5], mAmetrine, and mKalama1[1]. Mutations relative to *Aequorea* GFP are indicated as white text on a black background. The chromophore precursor residues are boxed.

Protein characterization

mAmetrine was purified as described in **Supplementary Methods**. Absorption spectra were recorded on a DU-800 UV-visible spectrophotometer (Beckman). A monochromator-based Tecan Safire2 plate reader was used to acquire the fluorescence excitation and emission spectra. The excitation and emission maxima are 406 nm and 526 nm, respectively. The quantum yield of

mAmetrine was measured using quinine sulfate in 0.1 M H_2SO_4 as the reference standard[6] and was found to be 0.58. The extinction coefficient of mAmetrine was measured by the base-denaturation method[7, 8] and was found to be 45,000 $M^{-1}cm^{-1}$.

To measure the pH-dependence of mAmetrine fluorescence, purified protein was dialyzed into 5 mM Tris-HCl buffer (pH 7.5). This solution was diluted 100-fold into a series of buffers (200 mM sodium phosphate and 200 mM imidazole) that had been adjusted to pH values ranging from 2.5 to 9.0. The fluorescence of each solution was measured with a fluorescence plate reader. As shown in **Fig. 2a**, mAmetrine has an apparent fluorescence pKa of 6.0

In order to determine the relative rates of in vitro protein maturation, a modified version of a previously reported method was used[9]. Samples of mAmetrine, mT-Sapphire, and mCitrine were heated to 95 °C in a denaturing and reducing buffer (8 M urea, 10 mM sodium hydrosulfite in PBS) for 5 min. The samples were cooled to room temperature, another 5 mM freshly dissolved hydrosulfite was added, and the samples were heated to 95 °C for another 5 min. Protein maturation was initiated by 300-fold dilution into 3 ml renaturation buffer (5 mM $MgCl_2$ in PBS, pH 7.4). The recovery of fluorescence at 37 °C was followed using a QuantaMaster spectrofluorometer (Photon Technology International) equipped a temperature controlled cuvette holder. During the recovery time, protein solutions were stirred with a magnetic bar at ~ 500 rpm and cuvettes were not capped. As shown in **Fig. 2b**, mAmetrine, mT-sapphire and mCitrine have maturation half-times of 108 s, 970 s, and 690 s respectively.

To elucidate the oligomeric structure of mAmetrine, gel filtration chromatography was performed using an Amersham HiLoad 16/60 Superdex 75 pg size exclusion column with 100 mM phosphate running buffer (pH 7.5). The UV-vis detector on the AKTAbasic HPLC system can monitor multiple wavelengths and was used to detect absorbance at 400 nm, 510 nm and 560 nm for mAmetrine, mCitrine, and tdTomato, respectively. The upper trace of **Fig. 2c** shows a co-injection of mAmetrine and mCitrine. The lower trace of **Fig. 2c** shows a co-injection of mAmetrine and tdTomato. The co-elution of mAmetrine and mCitrine in the upper trace clearly demonstrates that mAmetrine is a monomer as expected for an *Aequorea* GFP variant.

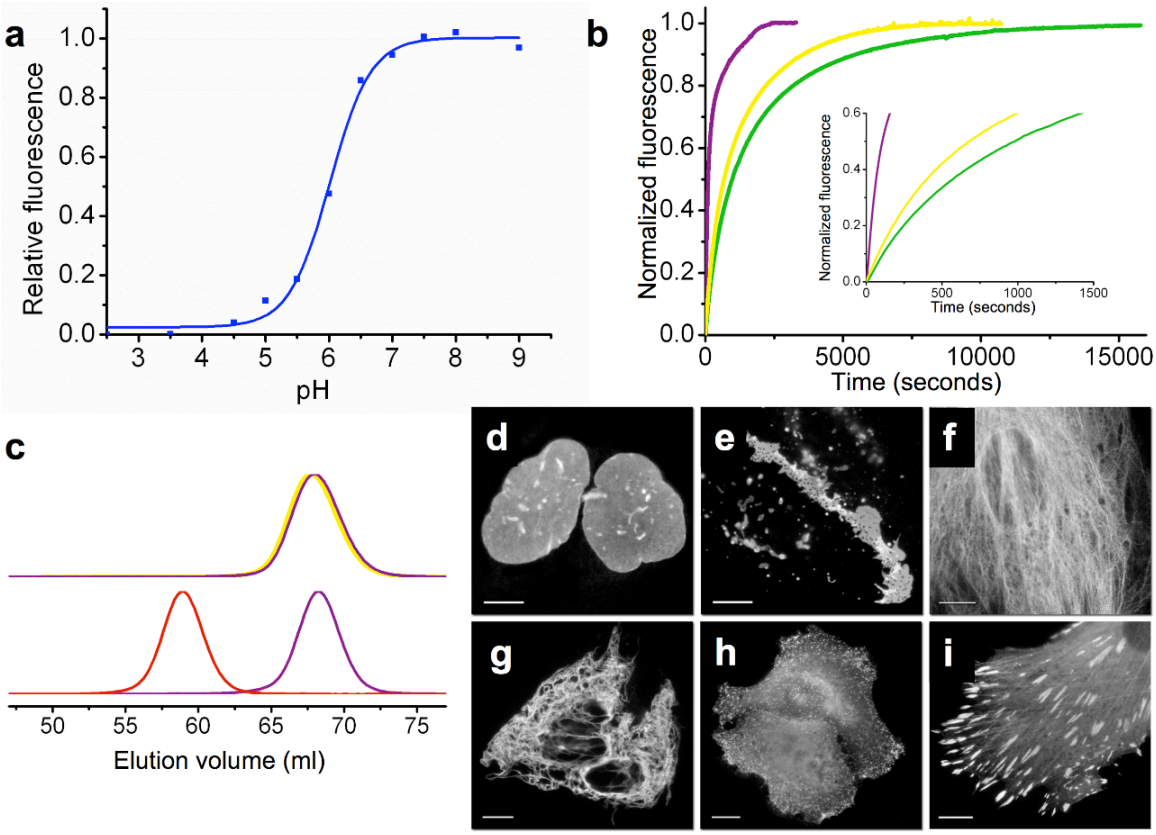


Figure 2 | Characterization of mAmetrine.

(**a**) The pH dependence of mAmetrine fluorescence. (**b**) Maturation rate profiles of mAmetrine (violet), mT-sapphire (green) and mCitrine (yellow) normalized to final fluorescence intensity. Inset shows the first 1500 s of the time course. (**c**) Gel filtration analysis of mAmetrine (detection at 400nm, violet line) using mCitrine (detection at 510 nm, yellow line) and tdTomato (detection at 560 nm, red line) as mass standards. (**d-i**) Imaging of mAmetrine as fusions with: (**d**) lamin B1; (**e**) connexin43; (**f**) tubulin; (**g**) vimentin; (**h**) clathrin; and (**i**) paxillin. Scale bar = 5 μm.

Construction of fusion proteins for imaging and photostability measurements

To create the mAmetrine fusion protein vectors, the gene encoding mAmetrine was PCR amplified with a 5' primer encoding an AgeI site and a 3' primer encoding either a BspEI (C1 cloning vector) or Not1 (N1 cloning vector) site. The purified and digested PCR products were ligated into similarly digested EGFP-C1 and EGFP-N1 (Clontech) cloning vector backbones, to create mAmetrine-C1 and mAmetrine-N1, respectively. To generate fusion vectors, the appropriate cloning vector and a previously constructed EGFP fusion vector were digested, either sequentially or doubly, with the appropriate enzymes and ligated together after gel

purification. Thus, to prepare mAmetrine N-terminal fusions, the following digests were performed: human H2B, BamHI and NotI (George Patterson, NIH); rat α-1 connexin-43, EcoRI and BamHI (Matthias Falk, Lehigh University); chicken paxillin, EcoRI and NotI (Alan Horwitz, University of Virginia); human vimentin, BamHI and NotI (Robert Goldman, Northwestern University). To prepare mAmetrine C-terminal fusions, the following digests were performed: human α-tubulin, NheI and BglII (Clontech); human light chain clathrin, NheI and BglII (George Patterson, NIH); human lamin B1, NheI and BglII (George Patterson, NIH).

Imaging of mAmetrine fusion proteins

HeLa cells (ATCC CCL-2 and CCL-2.2) were transfected with mAmetrine fusion vectors using Effectene (1 μg DNA/ml) (Qiagen) for routine visual examination of morphology and live cell imaging using widefield fluorescence illumination. 24 hours post-transfection, the cells were trypsinized and seeded into either Delta-T imaging chambers or 35-mm Petri dishes containing an 18 × 18 mm #1.5 coverslip and allowed to grow for another 24-36 hour period. Cultures in the Petri dishes were then washed with PBS, fixed with 2% paraformaldehyde (EMS; Hatfield, PA) in PBS and washed again with 4 exchanges of PBS to completely remove fixer. The adherent cells on coverslips were then treated with 0.1M glycine in PBS to reduce autofluorescence and mounted using gelvatol (25% w/v polyvinyl alcohol in 40% v/v glycerol/PBS). The mounted coverslips were examined at high magnification (100× Plan Apo) to verify correct localization of the fusion protein. As shown in **Fig. 2d-i**, the localization of all fusion constructs was consistent with the pattern expected for an *Aequorea* GFP variant. This conclusion was verified by comparison to cells expressing analogous EGFP fusions that were imaged under identical conditions.

For live cell imaging, the adherent cultures in Delta-T chambers were placed in a stage adapter (Bioptechs) on a Nikon TE2000 inverted microscope with a heated lid (Bioptechs) and equilibrated at 37 °C under a 5% CO_2 for 30 minutes to 1 hour. Cells expressing the fusion construct were imaged with a Cascade II or Retiga EXi (QImaging; Vancouver, CA) camera system using Nikon NIS-Elements software. HeLa S3 (ATCC CCL-2.2) fusions of mAmetrine to human histone H2B were observed to complete all stages of mitosis (data not shown).

Photostability measurements in live cells

For determination of photostability of fluorescent proteins in live HeLa cells, N-terminal fusions

of each fluorescent protein were constructed to human histone H2B with a 6 amino acid linker in order to restrict fluorescence to the nucleus. This system closely approximates the dimensions of the aqueous droplets of purified fluorescent proteins commonly used for *in vitro* widefield measurements[10]. Log phase HeLa (S3; ATCC 2.2) were transfected with the appropriate H2B construct using Effectene (1 μg DNA/ml; Qiagen) and maintained in a 5% CO_2 incubator for 24-36 hours in Delta-T (Bioptechs) imaging chambers prior to conducting photobleaching measurements. The chambers were then transferred to a specialized stage adapter (Bioptechs) on a Nikon TE2000 widefield inverted microscope equipped with an X-Cite 120 metal-halide lamp (EXFO; Mississauga, Ontario, CA), and imaged at low magnification (20×) to ensure cell viability. Selected regions containing 3-10 nuclei were photobleached without neutral density filters using a 40× Plan Fluor (numerical aperture, NA = 0.75) objective, recording images in 1-second intervals using a Cascade II or QuantEM camera system (Photometrics; Tucson, AZ) in direct readout mode (bypassing the electron multiplier circuitry). Filter sets used for widefield photobleaching were provided by Omega: mAmetrine and mT-Sapphire, XF76; mTFP1, XF114-2; EYFP, mCitrine, XF104-2; EGFP, XF100-2; tdTomato, XF108-2. Light power at the objective output was measured with a FieldMaxII-TO power meter (Coherent; Santa Clara, CA) equipped with a high-sensitivity silicon/germanium optical sensor (Coherent, OP-2 Vis). Widefield photobleaching raw data was collected with NIS-Elements software (Nikon) and analyzed with Simple PCI software (Hamamatsu). The corrected curves are shown in **Fig. 3a** and numerical values are provided in **Table 1**.

For laser scanning confocal microscopy photobleaching measurements, cells in Delta-T chambers were transferred to a Bioptechs stage adapter on an Olympus FluoView 1000 microscope. Photobleaching was conducted using a 40× oil immersion objective (Olympus UPlan Apo, NA = 1.0) using laser lines (405 nm, diode; 458, 488, 514, argon-ion; 543, HeNe) adjusted to an output power of 50 μW. The instrument was set to a zoom of 4×, a region of interest of 341.2 μm^2 (108 × 108 pixels), a photomultiplier voltage of 650 V, and an offset of 9% with a scan time of 0.181 seconds per frame. Nuclei having approximately the same dimensions and intensity under the fixed instrument settings were chosen for photobleaching assays. Photobleaching curves were adjusted for the time to bleach the proteins from an emission rate of 1000 photons/second/molecule to 500 photons/second/molecule as previously described[10]. The corrected curves are shown in **Fig. 3b** and numerical values are provided in **Table 1**.

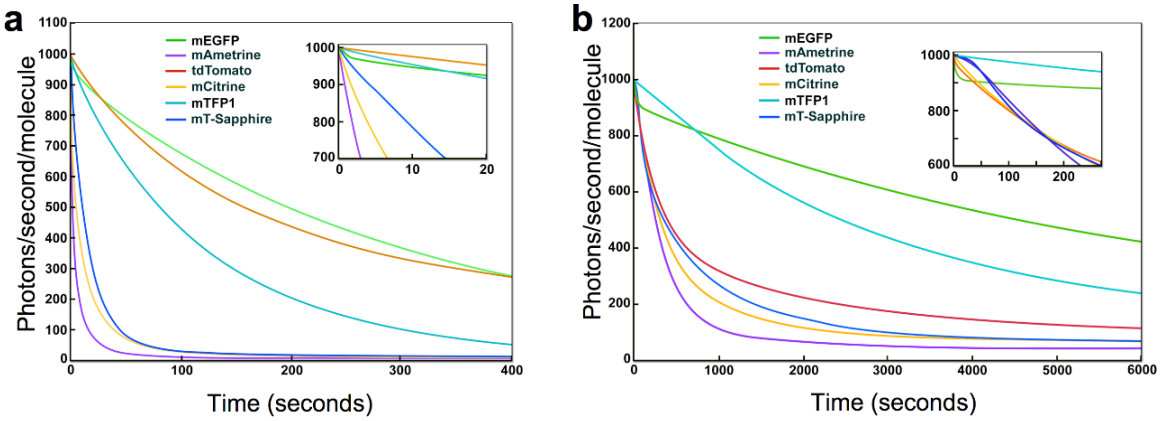


Figure 3 | Corrected photobleaching curves for fluorescent proteins used in this work. (**a**) Photobleaching curves with widefield (metal-halide lamp) illumination. (**b**) Photobleaching curves with confocal (laser scanning) illumination. Numerical values are provided in **Table 1**.

Table 1 | Photobleaching rates for FPs used in this work. Curves are shown in **Fig. 3**.

Protein	Widefield				Confocal (@ 50 μW)		
	Filter Set	Power (mW)	Uncorrected $t_{0.5}$ (s)	Corrected[a] $t_{0.5}$ (s)	Laser line (nm)	Uncorrected $t_{0.5}$ (s)	Corrected[a] $t_{0.5}$ (s)
mAmetrine	Omega XF 76	46.6	12	2.8	405	95	281
mT-Sapphire	Omega XF 76	46.6	62	12	405	143	397
mCitrine	Omega XF 104-2	30.6	22	6.6	514	42	349
tdTomato	Chroma HQ TRITC	89.5	68	159	543	35	419
mTFP1	Omega XF 114-2	65.7	108	82	458	395	2721
EGFP	Omega XF 100-2	57.3	332	198	488	1087	5008

[a] Time to bleach from an emission rate of 1000 photons/second/molecule to 500 photons/second/molecule

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SUPPLEMENTARY NOTE 2

Inter-FRET pair correction factors for dual FRET imaging

The dual FRET imaging experiments described in this manuscript require the use of two excitation filters (**Supplementary Table 1**); one that primarily excites mAmetrine and one that primarily excites mTFP1. However, there is some degree of cross-excitation between the dual FRET pairs (**Table 1**). That is, the filter set used to excite mAmetrine gives some excitation of mTFP1 (14% of the mTFP1 excitation as measured by convolving and integrating the excitation spectrum of mTFP1 for each filter), and the filter set used to excite mTFP1 gives some excitation of mAmetrine (2% of the mAmetrine excitation as measured by convolving and integrating the excitation spectrum of mAmetrine for each filter).

Table 1 | Relative emission intensities for each protein when imaged with each of the filter sets listed in **Supplementary Table 1**. For each protein the intensity provided by each filter set has been divided by the intensity provided by the recommended filter set.

Protein	Filter Set[a]					
	ex TFP em TFP	**ex TFP em Cit**	**ex Cit em Cit**	**ex Ame em Ame**	**ex Ame em Tom**	**ex Tom em Tom**
mTFP1	**1.0**	0.390	0.054	0.055	0.008	< 0.001
mCitrine	0.002	0.057	**1.0**	0.010	0.002	< 0.001
mAmetrine	0.001	0.020	< 0.001	**1.0**	0.264	< 0.001
tdTomato	< 0.001	< 0.001	0.001	< 0.001	0.001	**1.0**

[a] Abbreviations: ex = excitation, em = emission, TFP = mTFP1, Cit = mCitrine, Ame = mAmetrine, and Tom = tdTomato.

Fortunately, due to differences in fluorescent protein emission profiles, this cross-excitation is much less of a problem than it might first appear and it can be accounted for with a straightforward mathematical correction factor. Applying this correction to the images acquired in this work did not significantly change the timing or magnitude of the FRET changes. However, we anticipate that in cases where the expression levels of the two FRET pairs are less evenly matched, and/or the magnitude of the FRET changes are much smaller than in this work, application of the correction factor might be critical for the correct interpretation of the ratio changes.

Since the emission filter for both mAmetrine and mCitrine are the same, the experimental fluorescence intensities can be calculated using the following equations:

$$I_{Cit}^{raw} = I_{Cit}^{cor} + 0.02 * I_{Ame}^{cor}$$

and

$$I_{Ame}^{raw} = I_{Ame}^{cor} + 0.14 * I_{Cit}^{cor}$$

where I_{Ame}^{raw} and I_{Ame}^{cor} are the experimental and corrected intensities for mAmetrine and I_{Cit}^{raw} and I_{Cit}^{cor} are the respective intensities for mCitrine. Rearranging these equations gives the following relationships between the corrected intensities and the raw intensities:

$$I_{Cit}^{cor} = 1.003 * I_{Cit}^{raw} - 0.020 * I_{Ame}^{raw}$$

and

$$I_{Ame}^{cor} = 1.003 * I_{Ame}^{raw} - 0.14 * I_{Cit}^{raw}$$

These correction factors can be used to scale each image in a time-lapse series on a time-point by time-point basis. It is important to note that these simple correction factors are specific for the fluorescent proteins and filters used in this work but are general with respect to the FRET construct and FRET efficiency. Furthermore, the simplicity of these correction factors stems from the fact that mAmetrine and mCitrine use the same emission filter. If this were not the case (e.g. if mAmetrine was substituted with T-Sapphire and a green emission filter was used), these correction factors would be significantly more complex. Both the mTFP1 channel and the tdTomato channel are free from bleed-through from other fluorescent proteins (**Table 1**).