Supplemental Materials

Molecular Biology of the Cell

Guan et al.

Supporting Information

Live Cell Multi-Photon Fluorescence Correlation Spectroscopy with an Improved Large Stokes Shift Fluorescent Protein

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mKeima Plasmids

Table S1 – Plasmids used for mKeima variants in this study

Name	Backbone	Insert	Reference
pRS415-P_{Gal1}	CEN-ARS, LEU2	no	Ref[1]
pRSET	P_{T7}, 6xHis, EK-site		Life Technologies
(YMaM679)*	pRS415-P_{Gal1}	mKeima	this study
(YMaM680)*	pRS415-P_{Gal1}	mKeima4.15	this study
(YMaM681)*	pRS415-P_{Gal1}	mKeima8.5	this study
pJag795	pRSET-C	hmKeima	this study
pJag797	pRSET-A	hmKeima4.15	this study
pMaM329	pRSET-A	hmKeima8.5	this study

*Plasmids were constructed by homologous recombination of a PCR product with a gapped plasmid directly in yeast

Table S2 – Yeast strains used in this study

Name	Strain background	Genotype/Plasmid	Reference
ESM356-1	FY1679	MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63	Ref[2]
EMS356-1	FY1679	pRS415-P_{Gal1}	this study
YMaM679	ESM356-1	pRS415-P_{Gal1}-mKeima	this study
YMaM680	ESM356-1	pRS415-P_{Gal1}-mKeima4.15	this study
YMaM681	ESM356-1	pRS415-P_{Gal1}-mKeima8.5	this study

1. Mumberg, D., Müller, R. & Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156,** 119–122 (1995).
2. Rogowska-Wrzesinska, A. *et al.* Comparison of the proteomes of three yeast wild type strains: CEN.PK2, FY1679 and W303. *Comp. Funct. Genomics* **2,** 207–225 (2001).

FCS and FCCS Plasmids

Table S3. All plasmids used in FCS/FCCS measurement:

Number	Backbone	Expression	Bacterial Resistance
pJag85	EYFP-C1	ECFP	Kanamycin
		mCerulean (aquired plasmid)	
pJag724	EYFP-C1	mTFP	Kanamycin
	pmTurquoise2-C1	mTurquoise2 (aquired plasmid)	
pJag410	EYFP-C1	mKeima	Kanamycin
	pLSSmKate2 c1	LssmKate2 (aquired plasmid)	
pJag813	pcDNA3.1-	hmKeima4.15	Ampicillin
pJag812	pcDNA3.1-	hmKeima8.5	Ampicillin
pJag830	pcDNA3.1-	TFP	Ampicillin
pJag838	pcDNA3.1-	TFP-(GGGS)2-TFP	Ampicillin
pJag798	pcDNA3.1-	TFP-P2A-TFP	Ampicillin
pJag800	pcDNA3.1-	TFP-(GGGS)2-FKBP12-P2A-TFP-(GGGS)2-FRB	Ampicillin
pJag833	pcDNA3.1-	FKBP12-(GGGS)2-hmKeima8.5	Ampicillin
pJag824	pcDNA3.1-	TFP-(GGGS)2-hmKeima8.5	Ampicillin
pJag840	pcDNA3.1-	TFP-P2A-hmKeima8.5	Ampicillin
pJag850	pcDNA3.1-	hmKeima8.5-(GGGS)2-FKBP12-P2A-TFP-(GGGS)2-FRB	Ampicillin

Table S4. PCR Oligonucleotides for DNA construction:

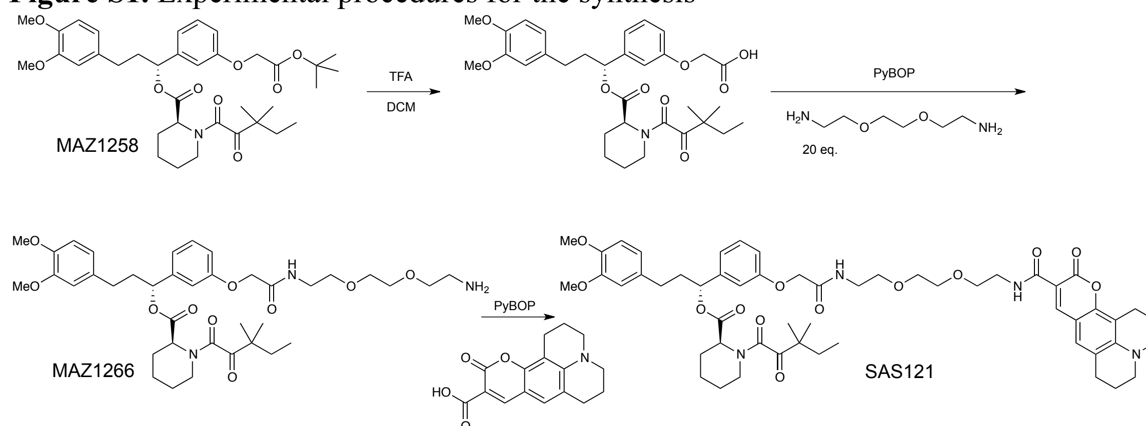
Plasmids	Fragments	Primers
TFP-pcDNA	NheI-TFP-HindIII	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-TATTATAAGCTTTTACTTGTACAGCTCGTCCATGCC-3'
hmKeima8.5-pcDNA	NheI-hmKeima8.5-HindIII	Forward: 5'-ATAATA GCTAGCGCTGCCACCATGGTGAGCGTGATCGCCGAG-3'
		Reverse: 5'-TATTATAAGCTTTTAGCCCAGCAGGGGGTG-3'
TFP-(GGGS)2-TFP-pcDNA	NheI-TFP-(GGGS)2	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCCTTGTACAGCTCGTCCATGCC-3'
	(GGGS)2-TFP-HindIII	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-TATTATAAGCTTTTACTTGTACAGCTCGTCCATGCC-3'
TFP-P2A-TFP-pcDNA	NheI-TFP-EcoR1	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-TATTATGAATTCCTTGTACAGCTCGTCCATGCC-3'
	BamHI-TFP-HindIII	Forward: 5'-ATAATA G'GATCCAGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-TATTATAAGCTTTTACTTGTACAGCTCGTCCATGCC-3'
TFP-(GGGS)2-FKBP12-P2A-TFP-(GGGS)2-FRB-pcDNA	NheI-TFP-(GGGS)2	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCCTTGTACAGCTCGTCCATGCC-3'
	(GGGS)2-FKBP12-EcoRI	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTGGAGTGCAGGTGGAAACC-3'
		Reverse: 5'-TATTATGAATTCTTCCAGTTTTAGAAGCTCCACATCGAA-3'
	BamHI-TFP-(GGGS)2	Forward: 5'-ATAATA G'GATCCAGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCCTTGTACAGCTCGTCCATGCC-3'
	(GGGS)2-FRB-HindIII	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTTCTAGAATCCTCTGGCATGAGATG-3'
		Reverse: 5'-TATTATAAGCTTTTATCTAGTCTTTGAGATTCGTCGGAA -3'
FKBP12-(GGGS)2-hmKeima8.5-pcDNA	NheI-FKBP12-(GGGS)2	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGGAGTGCAGGTGGAAACC-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCTTCCAGTTTTAGAAGCTCCACATCGAA-3'
	(GGGS)2-hmKeima8.5-HindIII	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTGTGAGCGTGATCGCCGAG -3'
		Reverse: 5'-TATTATAAGCTTTTAGCCCAGCAGGGGGTG-3'
TFP-(GGGS)2-hmKeima8.5-pcDNA	NheI-TFP-(GGGS)2	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCCTTGTACAGCTCGTCCATGCC-3'
	(GGGS)2-hmKeima8.5-HindIII	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTGTGAGCGTGATCGCCGAG-3'
		Reverse: 5'-TATTATAAGCTTTTAGCCCAGCAGGGGGTG-3'
TFP-P2A-hmKeima8.5-pcDNA	NheI-TFP-EcoR1	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-TATTATGAATTCCTTGTACAGCTCGTCCATGCC-3'
	BamHI-hmKeima8.5-HindIII	Forward: 5'-ATAATA G'GATCCAGTGAGCGTGATCGCCGAG-3'
		Reverse: 5'-TATTATAAGCTT TTAGCCCAGCAGGGGGTG-3'
hmKeima8.5-(GGGS)2-FKBP12-P2A-TFP-(GGGS)2-FRB-pcDNA	NheI-hmKeima8.5-(GGGS)2	Forward: 5'-ATAATAGCTAGCGCTGCCACCATGGTGAGCGTGATCGCCGAG-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCGCCCAGCAGGGGGTG-3'
	(GGGS)2-FKBP12-EcoRI	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTGGAGTGCAGGTGGAAACC-3'
		Reverse: 5'-TATTATGAATTCTTCCAGTTTTAGAAGCTCCACATCGAA-3'
	BamHI-TFP-(GGGS)2	Forward: 5'-ATAATA G'GATCCAGTGAGCAAGGGCGAGGAG-3'
		Reverse: 5'-ACTTCCACCGCCGCTGCCACCTCCCTTGTACAGCTCGTCCATGCC-3'
	(GGGS)2-FRB-HindIII	Forward: 5'-GGAGGTGGCAGCGGCGGTGGAAGTTCTAGAATCCTCTGGCATGAGATG-3'
		Reverse: 5'-TATTATAAGCTTTTATCTAGTCTTTGAGATTCGTCGGAA-3'

Drug Synthesis Methods

MAZ1258 and MAZ1889 were synthesized as reported previously (Keenan, T.; Yaeger, D. R.; Courage, N. L.; Rollins, C. T.; Pavone, M. E.; Rivera, V. M.; Yang, W.; Guo, T.; Amara, J. F.; Clackson, T.; et al. Synthesis and Activity of Bivalent FKBP12 Ligands for the Regulated Dimerization of Proteins. *Bioorg. Med. Chem.* **1998**, *6*, 1309–1335).

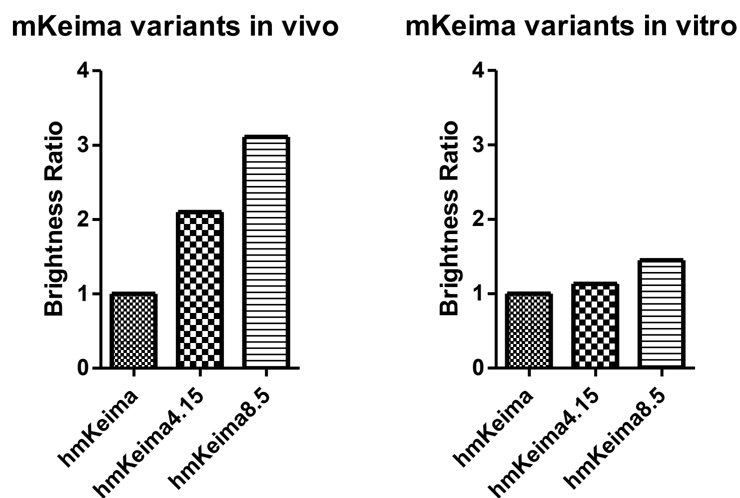
SAS121 was synthesized following the same synthetic strategy with minor modifications. Briefly, MAZ1258 was treated with trifluoroacetic acid to yield the free carboxylic acid and coupled with excess 2,2’ -(ethylenedioxy)- bis(ethylamine) to afford the amine functionalized intermediate MAZ1266. MAZ1266 was coupled with Coumarin 343 using PyBOP and purified via reverse phase Flash Chromatography (Biotage, 12 g C18 column; linear gradient of 5−100% MeCN/H_2O) to yield the desired product.

Figure S1. Experimental procedures for the synthesis



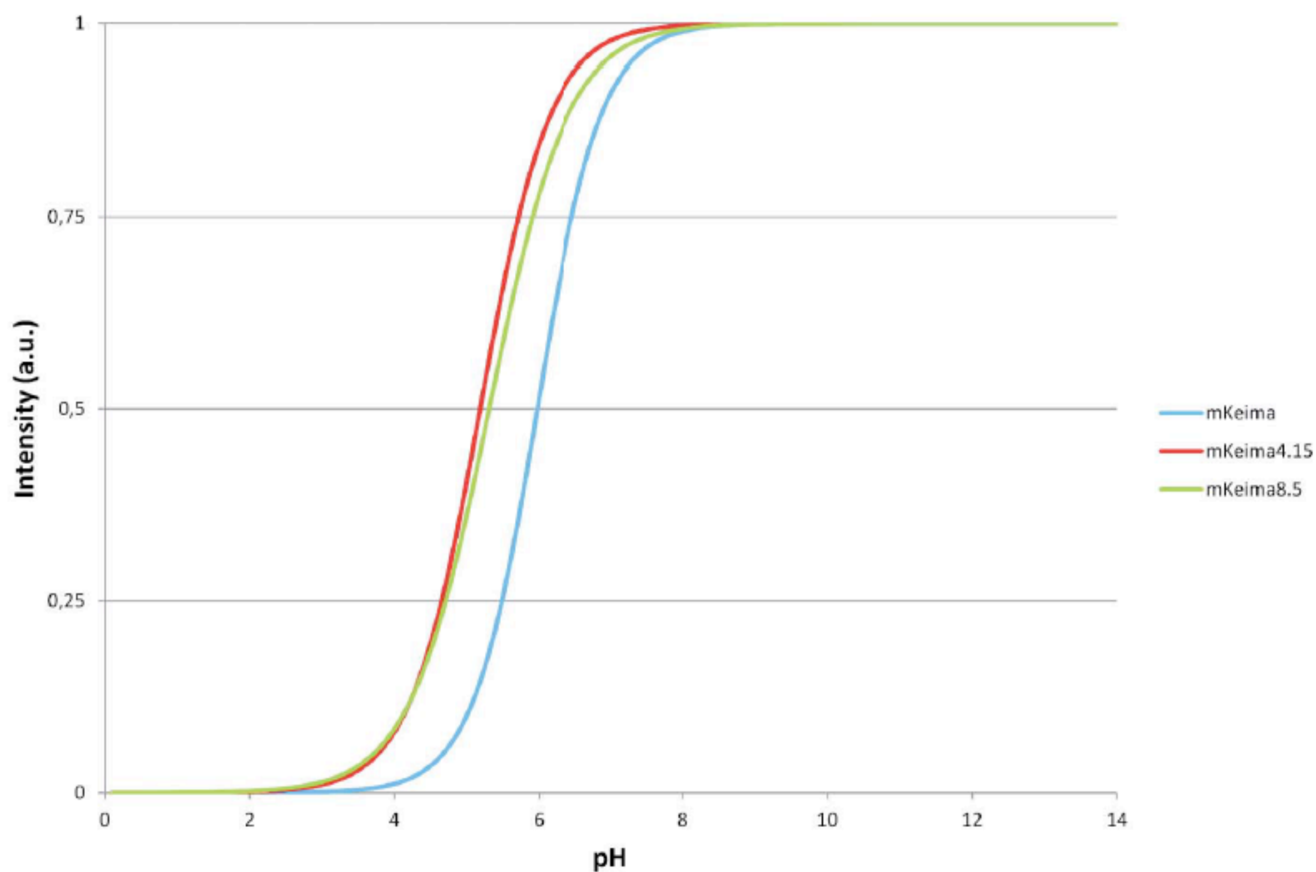
Brightness Comparison in vivo and in vitro

Figure S2. The brightness ratio between hmKeima variants in ptk cells and cell lysate were compared. hmKeima4.15 and hmKeima8.5 are brighter than hmKeima in vivo than in vitro. The unexpected decrease of brightness in vitro is related to the change in pH (Violot S, Carpentier P, Blanchoin L, Bourgeois D. Reverse pH-dependence of chromophore protonation explains the large Stokes shift of the red fluorescent protein mKeima. J Am Chem Soc. 2009 Aug 5;131(30):10356-7).



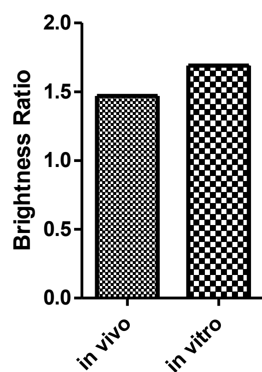
pKa-value of mKeima and variants

Figure S3. pKa-value of mKeima and variants



TFP Brightness Comparison in vivo and in vitro

Figure S4. The brightness ratio of mTFP1 and mTFP1-$(GGGS)_2$-mTFP1 in ptk cells and cell lysate were compared. The ratio in vitro is higher than in vivo.



hmKeima8.5 photophysics in living cell

Figure S5. The photophysics of hmKeima8.5 in living cells. (left) The diffusion coefficients measured in live cells expressed with hmKeima8.5 at four different power levels. (right) the average of normalized FCS curves of hmKeima8.5 freely intracellular diffusion at four different power levels. There are no obvious changes of the curve shape or shift of the correlation time indicating there is no anomalous photophysical property, specifically a significant dark state or triplet relaxation contribution, detectable under these laser power range which is relevant for intracellular measurements.