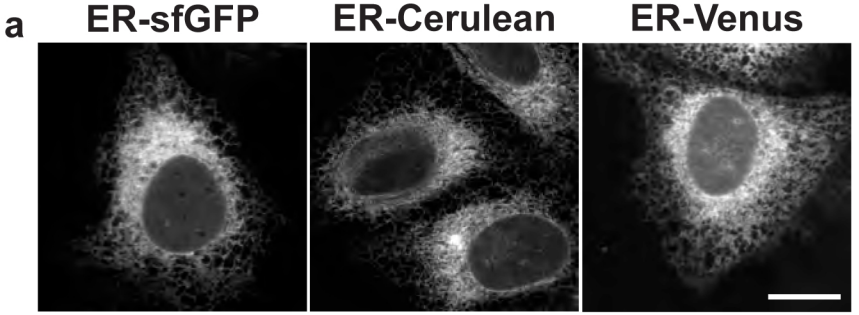
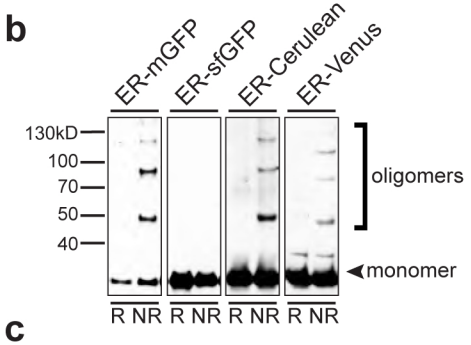
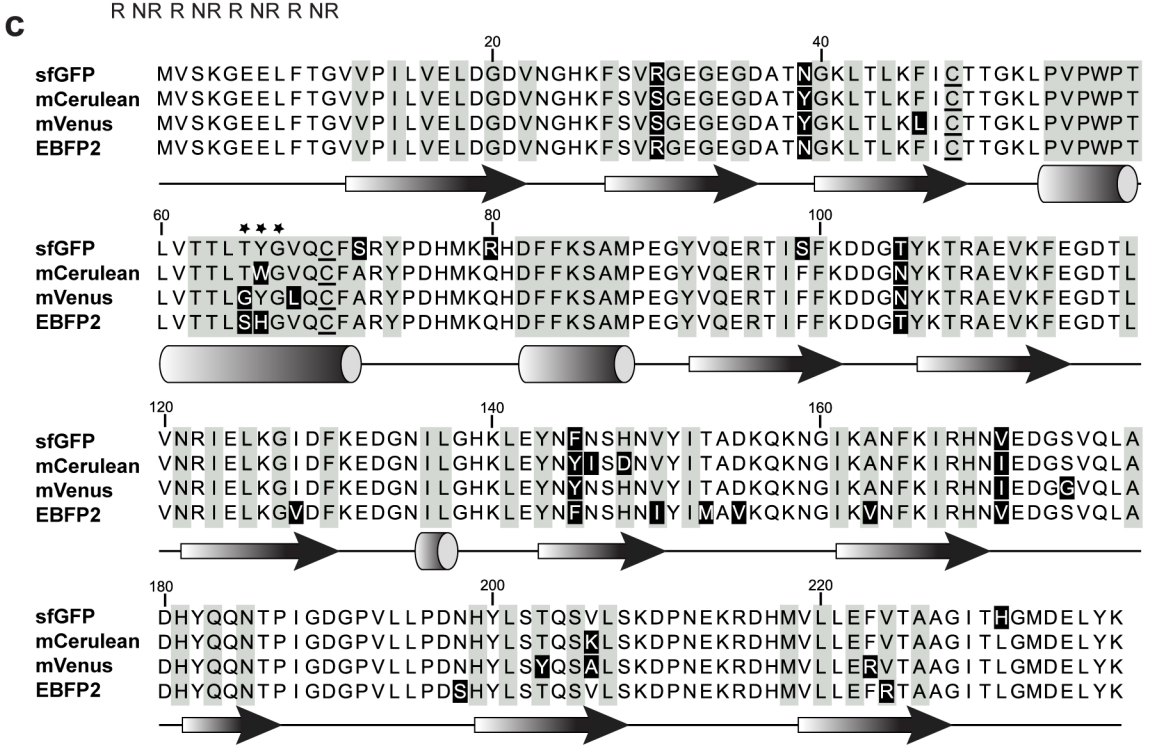
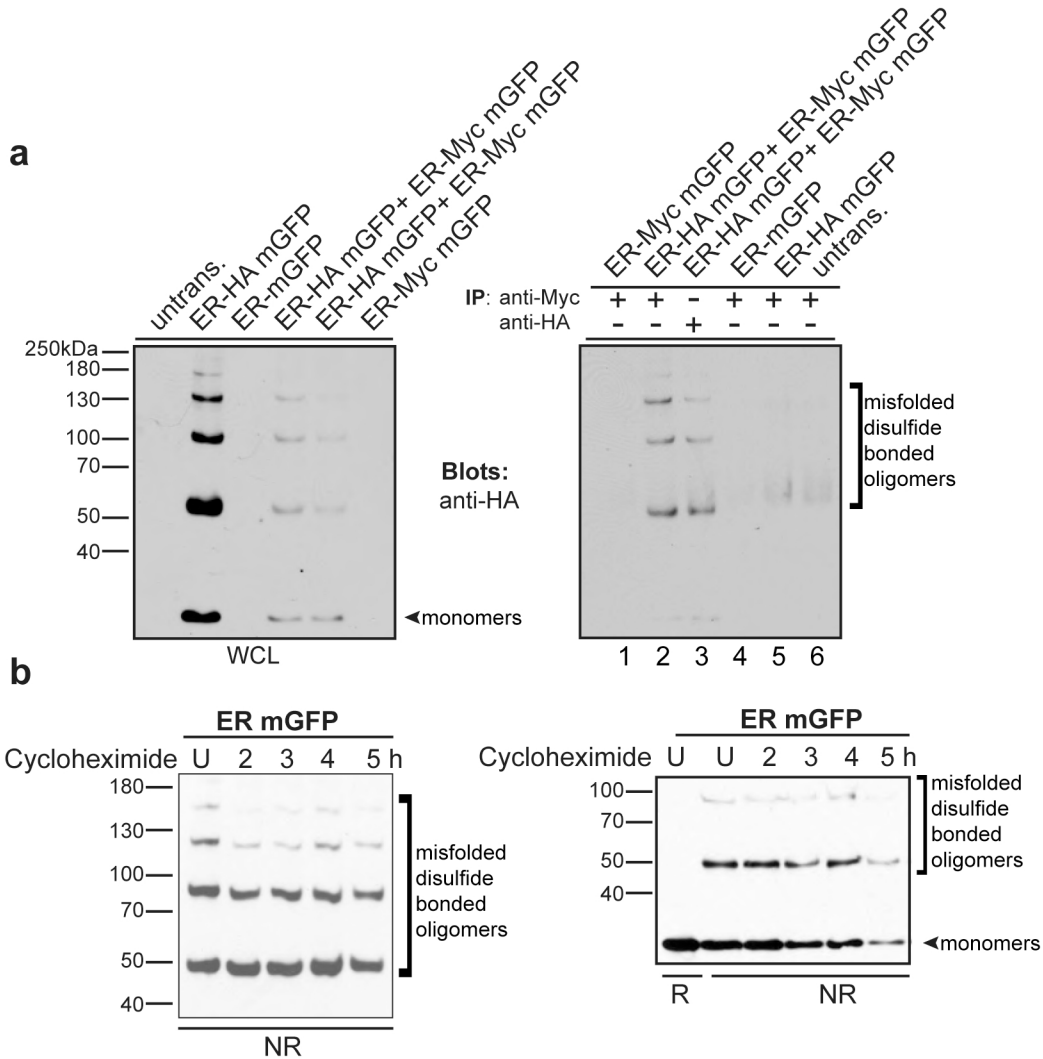


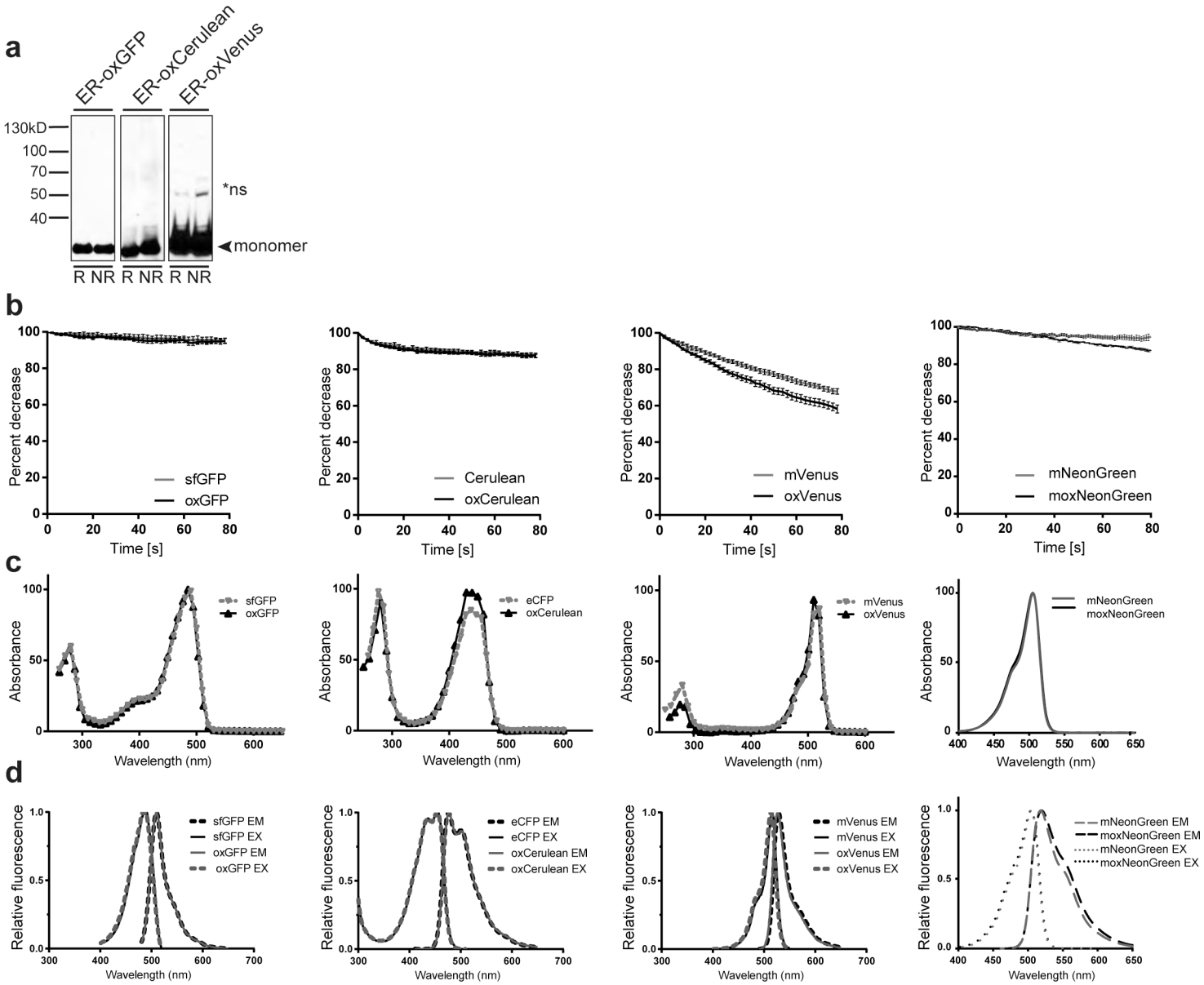




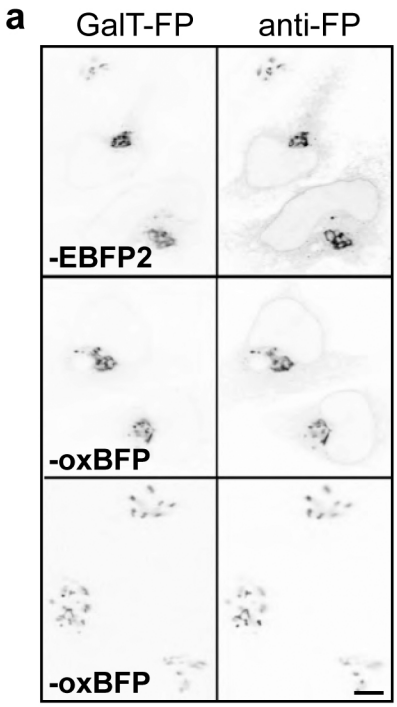
Supplementary Figure 1. ER localized-FPs (**a**) Representative image of transiently transfected U-2 OS cells expressing ER-sfGFP, -Cerulean, or -Venus, scale bar is 10 μm. (**b**) Immunoblot illustrates the tendency of ER-Cerulean and -Venus to oligomerize under NR conditions. (**c**) Amino acid sequence alignment and secondary structure of sfGFP, Cerulean, Venus and EBFP2. FP sequences are shown with the relative location of amino acids with secondary structures. Grey shading specifies inward facing residues within a correctly folded 11-β-strand barrel. Underlined residues denote location of cysteine residues, black highlights/white font denotes location of superfolder and cycle-3 mutations, and asterisk (*) identifies chromophore-forming residues.

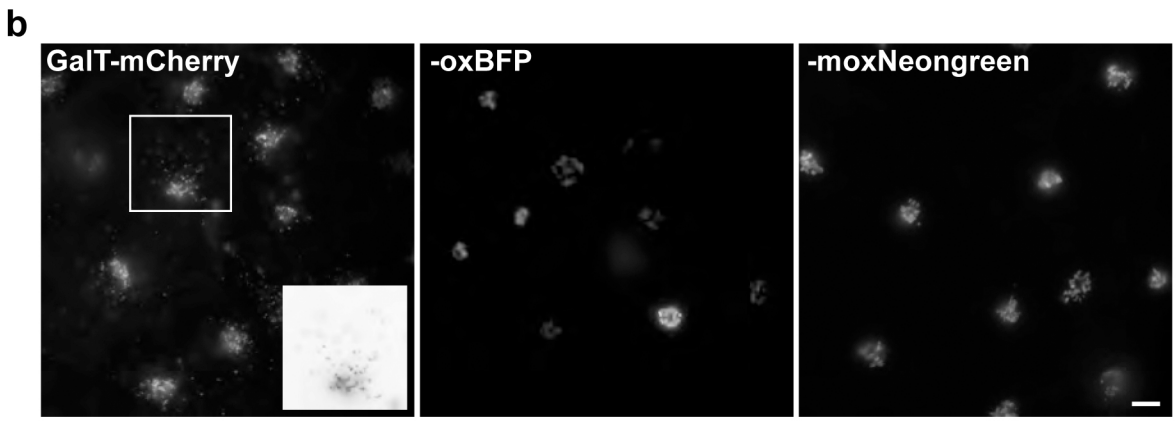


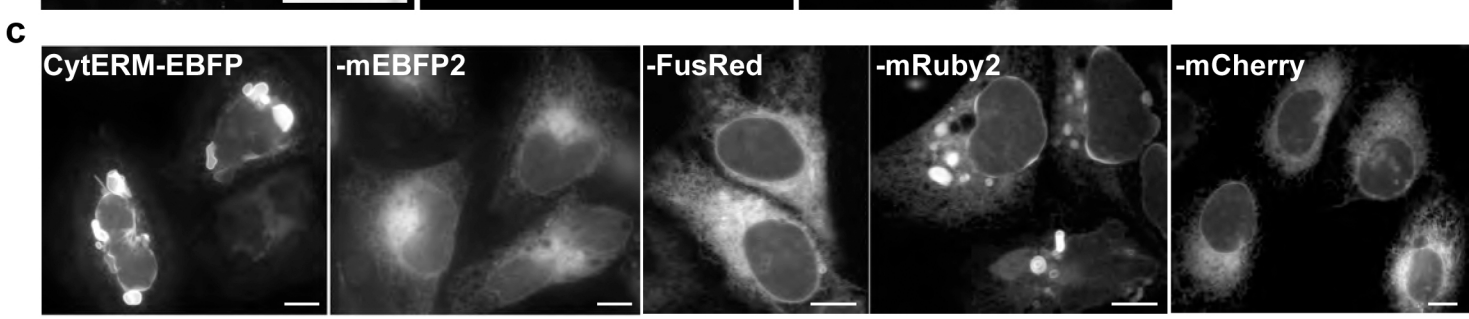
Supplementary Figure 2. FP Interchain disulphide bonds (**a**) Immunoprecipitation (IP) of U-2 OS cells expressing and co-expressing epitope-tagged (HA or Myc) versions of ER-mGFP. IP with anti-Myc antibody of whole cell lysates (WCL) of cells transiently co-expressing ER-HA-mGFP and -myc-mGFP (Lane 2) with anti-HA immunoblot indicate higher molecular weight bands correspond to the formation interchain disulphide bonds between GFP molecules. (**b**) U-2 OS cells transiently transfected with ER mGFP overnight and then treated (or untreated (U)) for indicated times with 50 μg/ml cycloheximide, treated with NEM 20 min before lysis, separated by SDS-PAGE, and immunoblotted with anti-GFP. There is no significant decrease or shift in higher molecular weight interchain disulphide bonded species (left) or the ratio of monomeric and dimeric species (right).



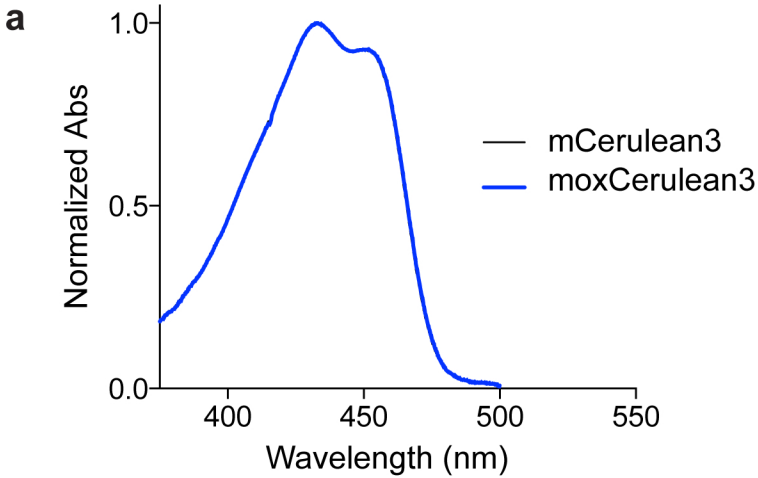
Supplementary Figure 3. oxFPs characterization (**a**) Immunoblot of U-2 OS cells transfected with ER -oxGFP, -oxCerulean, or -oxVenus, under NR conditions, the optimized, cysteine-less FPs do not form inappropriate disulphide bonds. (**b**) Optimized, cysteine-less variants, oxFPs (black line) maintain moderately comparable photostability properties under standard imaging conditions compared to parental versions (grey line). OxFPs (black data points) have comparable spectral characteristics to parental variants (grey data points). (**c**) Absorbance measurements and (**d**) fluorescence excitation and emission.

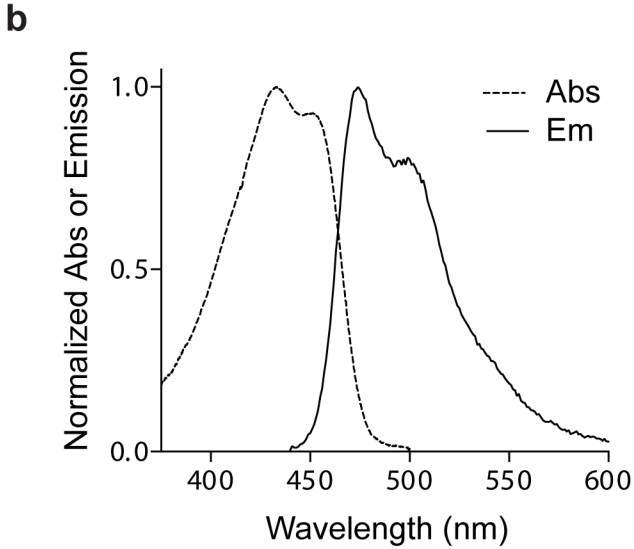




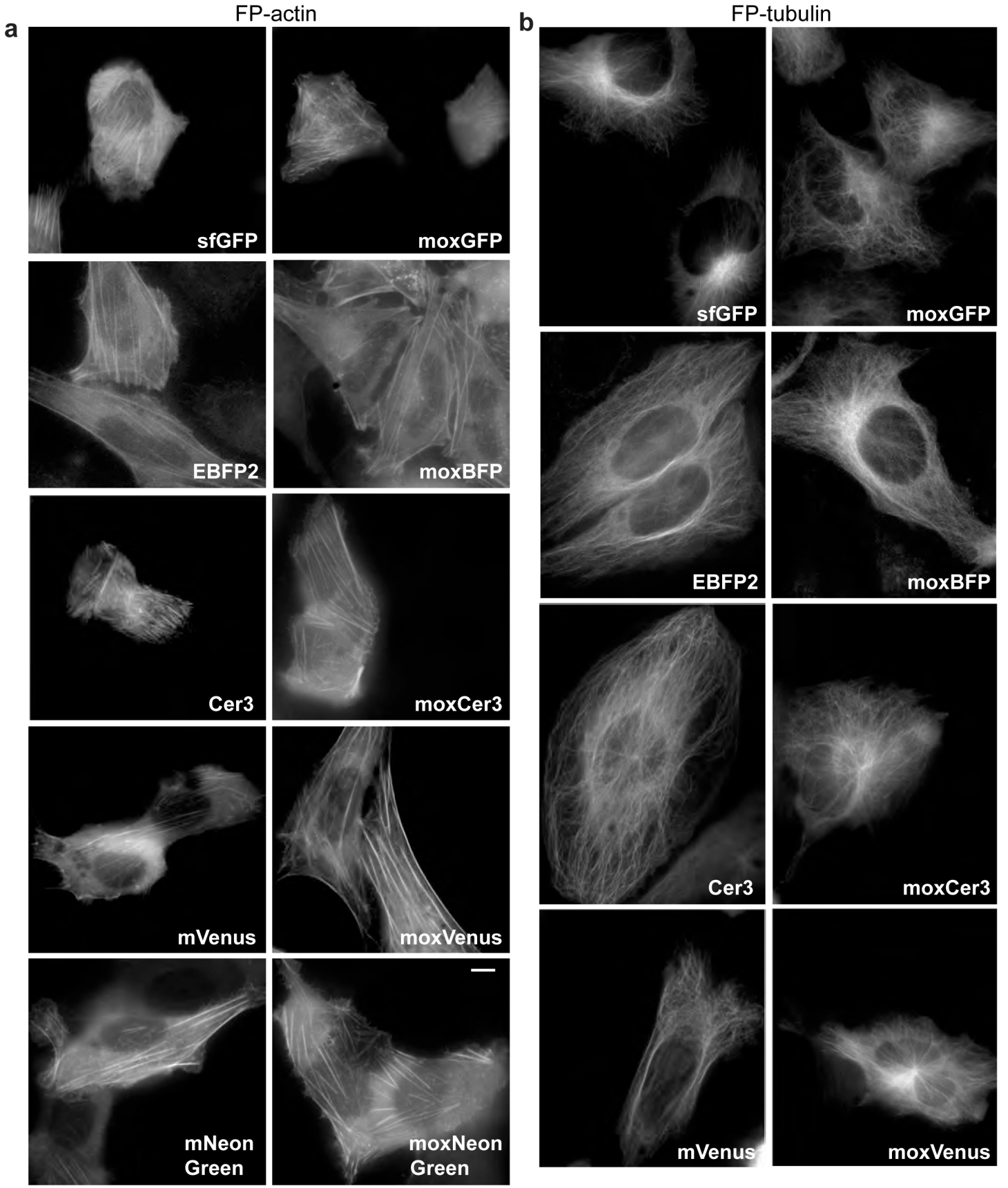


Supplementary Figure 4. FP non-covalent oligomers (**a**) Transiently transfected HeLa cells expressing indicated GalT-FP constructs. Similar to GalT-mGFP, GalT-EBFP2 exhibits a typical perinuclear GC distribution in the FP channel, but a significant dark ER pool is revealed in immunofluorescence images (top right panel). In contrast, ER fluorescence is rarely observed in either the FP channel or the immunofluorescence channel (see middle and bottom right panels of representative cells) in this experiment. (**b**) HeLa cells selected for stable expression of GalT-mCherry, -oxBFP, -sfGFP or moxNeonGreen. Inset, inverted image denoted by white box. (**c**) CytERM reporter reveals extensive OSER structures in live cells expressing CytERM-EBFP2 and -mRuby2. The monomerizing A206K mutation prevents OSER formation. FusionRed (FusRed) and mCherry also exhibit normal ER patterns consistent with being monomeric. Scale bars = 10 μm.

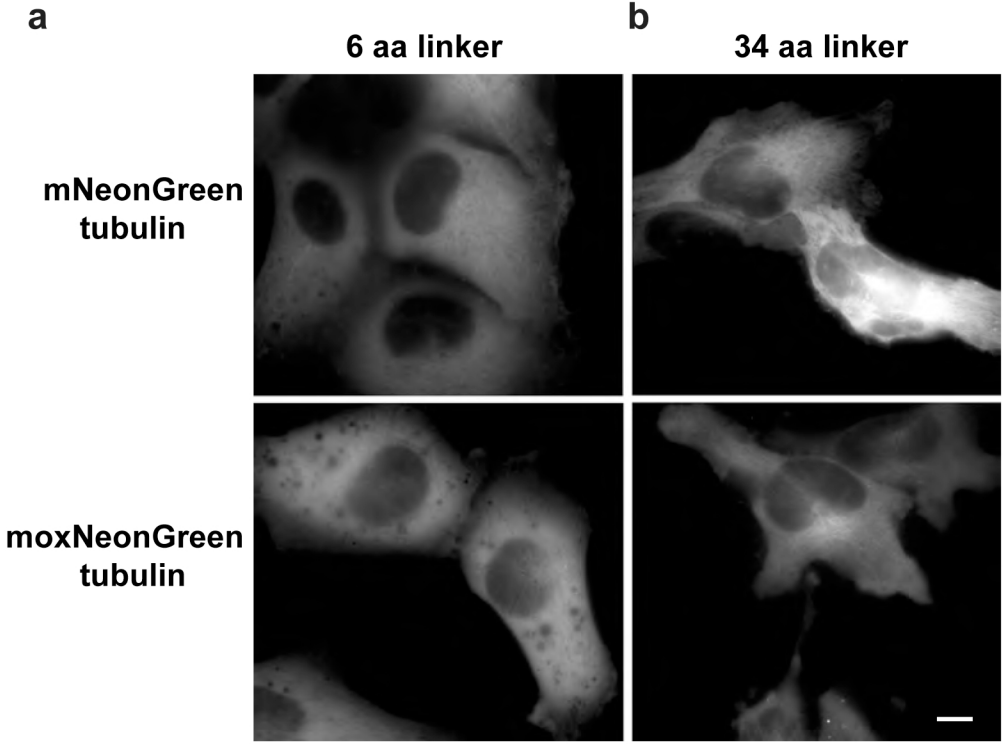




Supplementary Figure 5. Absorbance and emission spectra of purified moxCerulean3 (**a**) Normalized absorbance spectra of purified Cerulean3 and moxCerulean3. (**b**) Excitation and emissions spectra of moxCerulean3.



Supplementary Figure 6. Cytoplasmic moxFP fusions (**a**) Representative images of transiently transfected HeLa cells expressing parental FP- and paired moxFP-actin or (b) FP-tubulin fusions. Scale bars = 10 µm.



Supplementary Figure 7. moxNeonGreen-tubulin fusions (**a**) Representative images of transiently transfected HeLa cells expressing mNeonGreen or moxNeonGreen with 6 amino acid linker or (**b**) 34 amino acid linker. Scale bars = 10 μm.

Supplementary Table 1. Complete list of PCR primers

Mammalian cloning	
ER-FP	
sfGFP/oxGFP/EBFP2/oxBFP	F5' GCAATGGGCGGTAGGCG
	R5' GATCGCGGCCGCGTTACAATTCATCCTTATTAAGTTTGTGCCC
Cerulean/oxCerulean	F5' GATCACCGGTCGTGAGCAAAGGAGAGGAACTGTTC
	R5' GATCGCGGCCGCTTACAGCTCGTCCTTCTTATACAGCTCGTCCA TCCC
Venus/oxVenus	F5' GATCACCGGTCGCCACCATGGTGTCTAAAGGCGAG
	R5' GATCGCGGCCGCTTACAGCTCGTCCTTCTTATACAGCTCATCCA TTCC
Cyto-FP	
sfGFP/oxGFP/EBFP2/oxBFP	F5' GCAATGGGCGGTAGGCG
	R5' GATCGCGGCCGCTTAATTAAGCTTGTGCCC
Cerulean/oxCerulean	F5' GATCACCGGTCGCCACCATGGTGAGCAAAGGAGAGGAAC
	R5' GATCGCGGCCGCTTACTTGTACAGCTC
Venus/oxVenus	F5' GATCACCGGTCATGGTGTCTAAAGGCGAG
	R5' GATCGCGGCCGCTCACTTATACAGCTCATC
FP-actin/tubulin	
sfGFP/oxGFP/EBFP2/oxBFP	F5' ATCCGCTAGCGCTACCGGTCGCCACCATGGTGAGCAAGGGCGAGG
	R5' CTCGAGATCTGAGTCCGGACTTGTACAGCTCGTCCATGCCG
Cerulean3/oxCerlean3	F5' ATCCGCTAGCGCTACCGGTCGCCACCATGGTGTCAAAGGGCGAA GAGC
	R5' CTCGAGATCTGAGTCCGGACTTATACAGCTCGTCCATCCCCAG
Venus/oxVenus	F5' ATCCGCTAGCGCTACCGGTCGCCACCATGGTGTCTAAAGGCGAGGA ACTG
	R5' CTCGAGATCTGAGTCCGGA CTTATACAGCTCATCCATTCCCAGGG
mNeonGreen/moxNeonGreen	F5' ATCCGCTAGCGCTACCGGTCGCCACC ATGTCCTCAAAGGGAGAA GAAGACAAC
	R5' CTCGAGATCTGAGTCCGGA CTTATACAGTTCGTCCATCCCCATCAC
Epitope tag ER-FP	F5' GATCACCGGTCTACCCATACGACGTC
	R5' GATCACCGGTAGCGTAGTCTGGGAC
ER-split GFP	R5' GATCGCGGCCGCTTACAGCTCGTCCTTCTGCTTGTCGGC
Site-directed mutagenesis	
EBFP2	C70S- 5' CGGCGTGCAGTCGTTCGCCCGCTAC
	C70A- 5' GAGCCACGGCGTGCAGGCCTTCGCCCGCTACCCC

	C70V- 5’ GAGCCACGGCGTGCAGGCCTTCGCCCGCTACCCC
	F99S- 5’ CAGGAGCGCACCATCTCCTTCAAGGACGACGGC
	V163A- 5’ GAACGGCATCAAGGCCAACTTCAAGATCCGC
sfGFP	C48S- 5’ CCCTGAAGTTCATCAGTACTACCGGCAAGCTGCCC
	C70S- 5’ CGGCGTGCAGTCGTTCAGCCGCTAC
moxNeonGreen	C567T- F5' CTTTTGCTAAGCCTATGGCTGCAAAC
Monomerizing V206K	
EBFP2	5’ GAGCACCCAGTCCAAGCTGAGCAAAGAC
Cerulan3/oxCerulean3	5’ GTCCTTAGACAGCCTGGACTGATAGCTC
Venus/oxVenus	5’ GAGCTATCAGTCCAAGCTGTCTAAGGAC
Bacterial cloning	
sfGFP/oxGFP/EBFP2/oxBFP	F5’ GATCCCATGGGTATGGTGAGCAAGGGCGAGGAG
Cerulean/oxCerulean	F5’ GATCCCATGGGTATGGTGAGCAAAGG
Venus/oxVenus	F5’ GATCCCATGGGTATGGTGTCTAAAGGC
moxNeonGreen	F5' GATCCCATGGGT ATGTCCTCAAAGGG