Supplementary Methods

Generation of circularly permuted yellow fluorescent protein

Two fragments of the EYFP coding sequence were amplified from the plasmid pEYFP-N1 (Clontech) by PCR with specific primers shown in Supplementary Table 1 online: (i) for fragment 1, Pr3 and Pr4; and (ii) for fragment 2, Pr5 and Pr6. Primers Pr3 and Pr6 cover overlapping parts of the gene, allowing complementary fragment annealing in the following elongation reaction. PCR products were mixed, annealed and elongated by PCR. The complete sequence of the resulting circularly permuted cpYFP was amplified by PCR with Pr4 and Pr5, digested using BamHI and HindIII restriction endonucleases, and cloned into the pQE30 plasmid. To optimize folding and chromophore maturation of cpYFP, several mutations were introduced into the coding sequence by site-directed mutagenesis: F46L, Q69K, V163A, S175G, H148D, and F64L. Mutation Y203F was also introduced by site-directed mutagenesis to visualize the protonated form of the cpYFP chromophore.

Protein expression and purification

For heterologous expression of proteins, full-length coding regions were cloned into the pQE30 vector (Qiagen). Proteins fused to an N-terminal 6xHis tag were expressed in E. coli and purified using TALON metal-affinity resin (Clontech) equilibrated with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM 2-mercaptoethanol. For expression in mammalian cells, the HyPer sequence was subcloned in the pEGFP-C1 vector (Clontech) in place of EGFP; and for mitochondrial targeting, the HyPer sequence was subcloned into the pECFP-Mito vector (Clontech) in place of ECFP. To avoid mistargeting, two tandem copies of the mitochondrial targeting sequence derived from the precursor of subunit VIII of human cytochrome C oxidase were inserted into the pECFP-Mito vector. The HeLa cell line was transfected with the resulting construct using the Lipofectamine™ 2000 (Invitrogen).

Fluorescence-activated cell sorting

COS-7 wild-type cells were transiently transfected with HyPer-C1 using the calcium phosphate method, and stable clones were selected from single cells using 1 mg/ml of G418. Cells were harvested by trypsinization and washed twice with FACS buffer (PBS containing 2% fetal bovine serum). H2O2 was added to the cell suspension 5 min before initiation of the flow. The 405- and 488-nm lasers of a CyAn ADP flow cytometer (DakoCytomation) were used for excitation, 530/540-nm emission filters were used for both excitation wavelengths. Data was analyzed using Summit software (DakoCytomation). 7-amino-actinomycin D was used to discriminate between live and dead cells.
PAGE and Western blot.

For polyacrylamide gel electrophoresis (PAGE) and Western blot, HeLa cells were solubilized with lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% NP40, the samples were boiled for 3 min, and equal (50 mg/well) amount of protein was analyzed on 12.5 % SDS–PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were incubated with a 1 : 1000 dilution of the rabbit polyclonal anti-Bcl-2 antibodies (Santa Cruz Biotechnology). Blots were further incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1 : 7500, and specific bands were visualized by chemiluminescence (ECL, Amersham International).

References