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online data

SUPPLEMENTARY ONLINE DATA

Far-red fluorescent tags for protein imaging in living tissues

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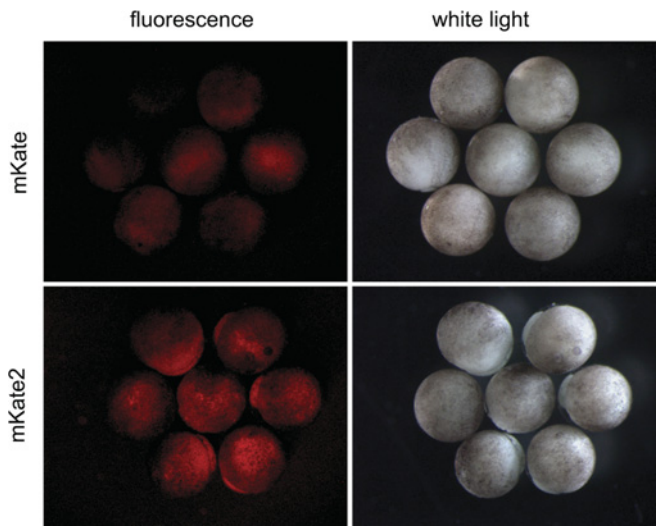

Figure S1 Comparisonof mKate and mKate2 in developing *X. laevis* embryos

At the stage of two blastomeres, embryos were microinjected with mKate-N1 and mKate2-N vectors. Living embryos were photographed from the animal pole side at the middle gastrula stages (10.5 h after fertilization).

EXPERIMENTAL

Cloning and gene construction

Synthetic DNA oligonucleotides for cloning and mutagenesis were purchased from Integrated DNA Technologies or Evrogen. PCR products and products of restriction digests were purified by gel electrophoresis and extraction using the QIAquick™ gel extraction kit (Qiagen). Plasmid DNA was purified from overnight cultures by using the QIAprep Spin Miniprep kit (Qiagen). Restriction endonucleases were purchased from Invitrogen or New England Biolabs. Sequencing was used to confirm the complete cDNA sequences for all fluorescent protein variants and fusion constructs (Florida State University Bioanalytical and Molecular Cloning DNA Sequencing Laboratory or Evrogen). Site-directed mutagenesis was performed by overlap-extension PCR [1] with primers containing the appropriate target substitutions. The Diversity PCR Random Mutagenesis kit (Clontech) was used for random mutagenesis in conditions optimal for seven mutations per 1000 bp. For bacterial expression, a PCR-amplified BamHI/HindIII fragment encoding a fluorescent protein variant was cloned into the pQE30 vector (Qiagen). For expression in eukaryotic cells, a PCR-amplified AgeI/NotI fragment encoding a fluorescent protein variant was swapped for TurboGFP within the pTurboGFP-N or pTurboGFP-C vectors (Evrogen). To generate fusion vectors, the appropriate cloning vector and an EGFP fusion vector were digested, either sequentially or doubly, with the appropriate enzymes and ligated together after gel purification. Thus, to prepare mKate2 N-terminal fusions, the following digests were performed: human non-muscle α-actinin, EcoRI and NotI (vector source, Tom Keller, Florida State University); human cytochrome *c* oxidase subunit VIII, BamHI and NotI (mitochondria, Clontech); human zyxin, BamHI and NotI (Clare Waterman-Storer, National Institutes of Health, Bethesda, MD, U.S.A.); rat α-1 connexin-43, EcoRI and BamHI (Matthias Falk, Lehigh University, Bethlehem, PA, U.S.A.); human H2B, BamHI and NotI (George Patterson, National Institutes of Health); N-terminal 81 amino acids of human β-1,4-galactosyltransferase, BamHI and NotI (Golgi, Clontech); human microtubule-associated protein EB3, BamHI and NotI (Lynne Cassimeris, Lehigh University); human vimentin, BamHI and NotI (Robert Goldman, Northwestern University Medical School, Chicago, IL, U.S.A.); human keratin 18, EcoRI and NotI (Open Biosystems); chicken paxillin, EcoRI and NotI (Alan Horwitz, University of Virginia, Charlottesville, VA, U.S.A.); and human lysosomal membrane glycoprotein 1, BamHI and NotI (LAMP1; George Patterson). To prepare mKate2 C-terminal fusions, the following digests were performed: human β-actin, NheI and BglII (Clontech); human α-tubulin, NheI and BglII (Clontech); human light chain clathrin, NheI and BglII (George Patterson); human lamin B1, NheI and BglII (George Patterson); PTS1 (peroxisomes), AgeI and BspEI (Clontech); human annexin (A4), AgeI and BspEI (Alen Piljic, EMBL, Heidelberg, Germany); human RhoB GTPase with an N-terminal c-Myc epitope tag (endosomes), AgeI and BspEI (Clontech); and the 20-amino-acid farnesylation signal from c-Ha-Ras, AgeI and BspEI (membrane, Clontech); mouse VASP, NheI and BglII (Robert Fischer, National Institutes of Health). DNA for mammalian transfection was prepared using either the Plasmid Midi or Maxi kit (Qiagen).

Characterization of fluorescent proteins *in vitro*

Proteins fused to the N-terminal polyhistidine tag were expressed in *E. coli* XL1 Blue strain (Invitrogen). The bacterial cultures were centrifuged at 4000 ***g*** for 20 min, and the cell pellets were resuspended in 20 mM Tris/HCl (pH 7.5) and 100 mM NaCl and lysed by sonication. The recombinant proteins were purified using TALON™ metal-affinity resin (Clontech) followed by a desalting step over gel-filtration columns (Bio-Rad Laboratory).

[1] Sergey Lukyanov and Dmitriy Chudakov have interest in Evrogen JSC. mKate2, Katushka2 and tdKatushka2 are the property of Evrogen JSC.
[2] To whom correspondence should be addressed (email ChudakovDM@mail.ru).

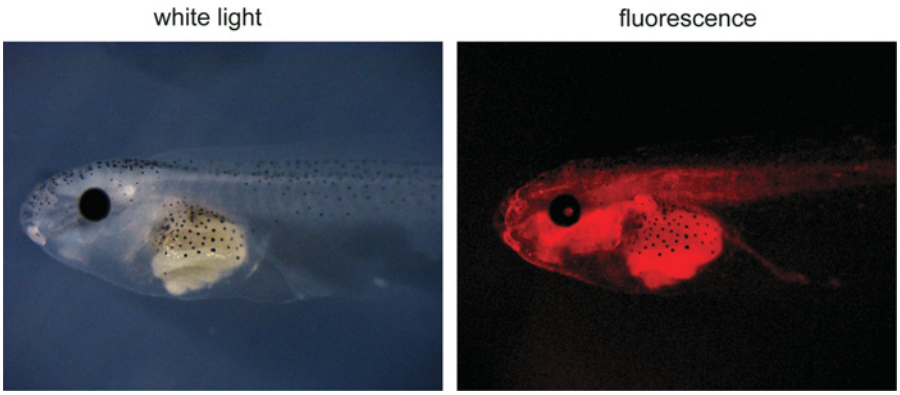

Figure S2 Expression of mKate2–zyxin in *X. laevis* embryos

Expression of mKate2–zyxin under CMV promoter control in the transgenic *X. laevis* embryo of stage 47–48 (7 days). Despite quite intensive and ubiquitous expression of mKate2–zyxin, the embryos look normal and healthy. The embryo is shown from the left-hand side, with the dorsal side at the top.

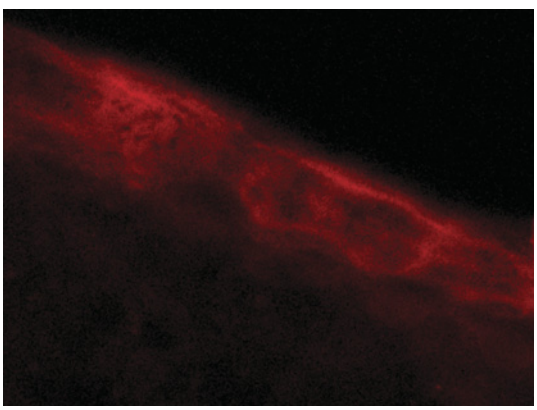

Figure S3 Expression of mKate2–zyxin in epidermal cells of *X. laevis* embryo

The epidermal cells of stage 35 transgenic embryo, expressing mKate2–zyxin under the control of the CMV promoter, as it is seen on the transverse section of the embryo. mKate2–zyxin is localized primarily in the submembrane space of the epidermal cells, where it presumably interacts with the membrane-associated cytoskeleton proteins.

Absorption spectra were recorded with a Beckman DU520 UV–visible spectrophotometer. A Varian Cary Eclipse fluorescence spectrophotometer was used for measuring excitation–emission spectra. All measurements were performed in 20 mM Tris/HCl (pH 7.5) and 100 mM NaCl. For molar absorption coefficient determination, we relied on measuring mature chromophore concentration. Proteins were alkali-denatured with an equal volume of 2 M NaOH, and absorbance spectra were measured immediately. It is known that alkali-denatured DsRed-like chromophore converts into the GFP (green fluorescent protein)-like one [2], with an absorption coefficient of 44 000 $M^{-1} \cdot cm^{-1}$ at 452 nm under these conditions. Based on the absorption of the native and alkali-denatured proteins, molar absorption coefficients for the native states were calculated. For determination of the quantum yield, the fluorescence of the mutant variants was directly compared with that of mCherry (quantum yield 0.22), and corrected to the area of the peak. pH titrations were performed by using a series of buffers in the range 3–10. For each pH value, an aliquot of purified protein was diluted in an equal volume of the corresponding buffer solution, and the fluorescence brightness was measured after 1 h of incubation at room temperature (25 °C). For accuracy, the actual pH was measured in each sample using a microelectrode (Sartorius). Gel filtration was performed on a 1 cm × 90 cm Econo-column (Bio-Rad Laboratories) packed with Sephacryl-S200 (Amersham Biosciences), equilibrated with 25 mM Tris/HCl (pH 7.5) and 150 mM NaCl. To measure the maturation rate, transformed *E. coli* cells (XL1 Blue strain) were grown overnight in LB (Luria–Bertani) supplemented with 200 mg/l ampicillin and 2 % D-glucose. Tubes were filled to the rim and sealed upon 1 h of induction to restrict oxygen availability. The bacterial cultures were centrifuged at 4000 ***g*** for 15 min, and the cell pellets were resuspended in 20 mM Tris/HCl (pH 7.5) and 100 mM NaCl and lysed by sonication. The recombinant proteins were purified using TALON™ metal-affinity resin (Clontech). Maturation was performed at 37 °C, in 35 mM KCl, 2 mM $MgCl_2$, 50 mM Tris/HCl (pH 7.5) and 1 mM dithiothreitol. A Varian Cary Eclipse fluorescence spectrophotometer was used for measuring maturation kinetics.

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