A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum

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We report a monomeric yellow-green fluorescent protein, mNeonGreen, derived from a tetrameric fluorescent protein from the cephalochordate Branchiostoma lanceolatum. mNeonGreen is the brightest monomeric green or yellow fluorescent protein yet described to our knowledge, performs exceptionally well as a fusion tag for traditional imaging as well as stochastic single-molecule superresolution imaging and is an excellent fluorescence resonance energy transfer (FRET) acceptor for the newest cyan fluorescent proteins.

Since the initial cloning of Aequorea victoria green fluorescent protein (avGFP) over 20 years ago, fluorescent proteins have become staples of biological imaging. After an initial flurry of activity leading to the development of avGFP variants in the blue to yellow-green wavelength range1, the bulk of subsequent fluorescent protein research has focused on expanding the fluorescent protein color palette into the red region and improving the brightness and performance of these longer-wavelength variants2, along with more recent major improvements to cyan variants of avGFP3–5.

As green and yellow variants of the original avGFP perform very reliably for most applications, recently less effort has been placed on developing new fluorescent proteins in the green region of the spectrum. However, there is still room for improvement of green and yellow fluorescent proteins, both for routine imaging as well as more specialized applications such as FRET6,7.

Most newly cloned green and yellow fluorescent proteins are not subjected to additional engineering simply because of their lack of improved properties relative to existing proteins. Thus, we were intrigued that a yellow fluorescent protein from B. lanceolatum (LanYFP, Allele Biotechnology; GenBank EU482389) exhibits an unusually high quantum yield (~0.95) and extinction coefficient (~150,000 M⁻¹ cm⁻¹), making it a very attractive candidate for additional development. Size-exclusion chromatography revealed that, like the vast majority of naturally occurring fluorescent proteins, LanYFP is a tetramer (Supplementary Fig. 1); we set out to monomerize it using a directed evolution approach that has proved highly successful for the development of many other fluorescent proteins8–10.

Guided by structural modeling of the wild-type LanYFP tetramer using the I-TASSER (iterative threading assembly refinement) structure-prediction and RosettaDock computational-docking servers11,12, we selected side chains for substitution following the well-established approach of introducing positive charges at key interface positions, followed by structure-guided directed evolution to rescue fluorescence (Supplementary Discussion, Supplementary Figs. 2–5 and Supplementary Data 1,2). The final mutant, designated mNeonGreen, contains 21 substitutions relative to tetrameric LanYFP (F15I, R25Q, A45D, Q56H, F67Y, K79V, S100V, F115A, I118K, V140R, L144T, D156K, T158S, S163N, Q168R, V171A, N174T, I185Y and F192Y), in addition to the appended enhanced GFP (EGFP)–type termini9. Based on our models, these substitutions are distributed over the A-B interface (I118K and N174T), the A/C interface (V140R, L144T, D156K, T158S, Q168R and F192Y), additional external regions (R25Q, A45D and S163N) and inside the beta-barrel (F15I, Q56H, F67Y, K79V, S100V, F115A, T141S, M143K, V171A and I185Y). We verified the monomeric status of mNeonGreen by size-exclusion chromatography (Supplementary Fig. 1). A sequence alignment of LanYFP, dLanYFP and mNeonGreen is available in Supplementary Figure 6.

Our initial characterization of mNeonGreen revealed sharp excitation and emission peaks (506 nm and 517 nm; Supplementary Fig. 7 and Table 1) somewhat blue-shifted relative to those of the original tetrameric LanYFP, placing mNeonGreen wavelengths roughly midway between typical green and yellow fluorescent protein wavelengths. Thus, mNeonGreen may be imaged with essentially no loss of emission photons using standard green fluorescent protein band-pass or long-pass filter sets, or can be separated from cyan fluorescent protein signals with yellow fluorescent protein filter sets with only minimal reduction in collection efficiency. mNeonGreen is also the brightest monomeric green or yellow fluorescent protein yet described to our knowledge. Its high quantum yield and extinction coefficient (Table 1) make it between 1.5 and 3 times as bright as most commonly

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used green and yellow fluorescent proteins. Its photostability is
slightly higher than that of mEGFP under widefield illumina-
tion (Table 1 and Supplementary Fig. 8) but somewhat lower
for laser illumination (~40% of mEGFP; Supplementary Fig. 9),
well within a practical range for most imaging applications. Its
fluorescence pKₐ of ~5.7 is similar or superior to most modern
green and yellow fluorescent proteins. mNeonGreen does not
exhibit any measurable sensitivity to Cl⁻ ions (data not shown).
The oxygen-dependent maturation time of mNeonGreen was too
short to measure using standard methods (Online Methods
and Supplementary Discussion), but based on our observations we
estimated it to be <10 min at 37 °C.

After we engineered mNeonGreen, Clover, a new bright yellow-
green fluorescent protein derived from avGFP, was reported².
Clover has very similar excitation and emission peak wavelengths
to those of mNeonGreen and is nearly as bright, with slightly lower
quantum yield and extinction coefficient. However, mNeonGreen
has the notable advantages of superior photostability (about threefold
greater than Clover under both widefield and laser illumination),

improved acid tolerance (mNeonGreen pKₐ of 5.7 versus 6.2
for Clover) and faster maturation (Table 1). We additionally
determined that mNeonGreen exhibits a substantially greater
degree of monomeric character than Clover (Online Methods,
Supplementary Discussion and Supplementary Table 1).

To determine the performance of mNeonGreen as a fluorescent
probe in live-cell imaging, we constructed vectors encoding fusions
to both N and C termini of the fluorescent protein. All fusions
localized as expected, and mNeonGreen exhibited character typical
of monomeric fluorescent proteins in ‘difficult’ fusions, including
histone H2B, connexins 26 and 43, and α-tubulin (Fig. 1 and

Figure 1 | Fluorescence imaging of mNeonGreen (mNG) fusion vectors.
(a–t) Fusions to the C terminus of mNG (linker length is indicated
between mNG and the name of the fusion partner): mNG-12–Annexin A4
(a; human; plasma membrane); mNG-18–β-actin (b; human; actin
cytoskeleton); mNG-20–β-catenin (c; mouse; tight junctions);
mNG-5–CAAX (d; 20-amino-acid farnesylation signal from c-Ha-Ras;
plasma membrane); mNG-10–CAF1 (e; mouse chromatin assembly factor);
mNG-10–Caveolin1 (f; human); mNG-14–RhoB (g; human; endosome);
mNG-10–Fascin (h; human; actin bundling); mNG-7–fibrillarin (t; human;
nucleolus); mNG-14–FilaminA (j; human; actin cytoskeleton); mNG-20–LAMP1
(k; rat; lysosomal membrane glycoprotein 1; lysosomes); mNG-15–clathrin
(l; human; clathrin light chain B); mNG-14–myotilin (m; human; actin
filaments); mNG-19–PCNA (n; human; replication foci); mNG-10–plastin
(o; human; actin binding); mNG-7–Rab4a (p; human; endosomes); mNG-7–
LC3B (q; rat; light chain; autophagosomes); mNG-18–talin (r; mouse; focal
adhesions); mNG-35–tubulin (s; human; microtubules); mNG-14–ZO1
(t; human; tight junctions). Constructs in c and t were expressed in
Madin-Darby canine kidney (MDCK; American Type Culture Collection,
CCL-34) cells, and all other constructs were expressed in HeLa CCL2
(American Type Culture Collection) cells. Scale bars, 10 μm.

Table 1 | Physical and optical data

| Protein          | Refs. | λₑ (nm) | λₘ (nm) | e (μm⁻¹ cm⁻¹) | φ | Brightness | Photostability | pKₐ | Maturation (min) |
|------------------|-------|---------|---------|---------------|---|------------|----------------|-----|-----------------|
| LanYFP           | j     | 513     | 524     | 150           | 0.95 | 424 | ND            | 3.5  | ND              |
| dLanYFP          | j     | 513     | 524     | 125           | 0.90 | 335 | ND            | ND   | ND              |
| mNeonGreen       | j     | 506     | 517     | 116 ± 4       | 0.80 ± 0.016 | 276 | ND            | 158 ± 13 | 5.7  | <10             |
| Clover           | 7     | 505     | 515     | 111           | 0.76 | 251 | 50            | 6.2  | 30              |
| YPet             | 2     | 517     | 530     | 104           | 0.77 | 238 | 49            | 5.6  | ND              |
| mCitrine         | 2     | 516     | 529     | 77            | 0.76 | 174 | 49            | 5.7  | ND              |
| mVENUS           | 2     | 515     | 528     | 92            | 0.57 | 156 | 15            | 6.0  | ND              |
| EYFP             | 2     | 514     | 527     | 83            | 0.61 | 151 | 60            | 6.9  | ND              |
| mEmerald         | 2     | 487     | 509     | 57            | 0.68 | 116 | 101           | 6.0  | ND              |
| sfGFP            | 2     | 485     | 510     | 83            | 0.65 | 161 | 157           | 5.5  | ND              |
| mWasabi          | 15    | 493     | 509     | 70            | 0.80 | 167 | 93            | 6.5  | ND              |
| mAG              | 16    | 492     | 505     | 42            | 0.81 | 100 | ND            | 6.2  | ND              |
| mEGFP            | 2     | 488     | 507     | 56            | 0.60 | 100 | 150           | 6.0  | 25              |

ND, not determined.

*Given reference is the source of data unless otherwise noted. βExcitation maximum. λEmission maximum. βExcitation coefficient determined by alkali-denaturation method. βFluorescence
quantum yield. βProduct of φ and e, expressed as a percentage of mEGFP brightness. βTime to photobleach from 1,000 to 500 photons s⁻¹ per molecule in live cells under widefield arc-lamp
illumination (Online Methods). βpH at which fluorescence intensity is 50% of its maximum value. βTime for fluorescence to reach its half-maximal value after exposure to oxygen at 37 °C. βThis
study; ± values indicate s.d. (n = 4 for e and φ; n = 20 for photostability) shown for mNeonGreen data. βPhotostability measurements of Emerald and mEmerald have historically been difficult to
replicate, especially using purified proteins, because of the presence of a fast initial photobleaching component¹⁶, and so the additional Aequorea-derived GFPs, superfolder (sf)GFP and mEGFP,
have been included for comparison. Note that in our hands, mEmerald performs substantially better as a fusion tag than sfGFP in most experiments. ℓData from ref. 7.
Supplementary Figs. 10–11]. Fusions of mNeonGreen with signal peptides and targeting proteins confirmed expected localization patterns in the cytoskeleton (β-actin, Lifeact, fascin, cortactin, plastin (fimbrin), MAP Tau, light chain myosin, myosin IIA, EB3, TPX2 and myotilin), intermediate filaments (keratin and vimentin), the Golgi complex (sialyltransferase, gal-T and mannosidase II), the nuclear envelope (lamin B1), nuclear pores (Nup50), nucleus (CAFI), endoplasmic reticulum (calnexin and calreticulin), the plasma membrane (annexin A4, CAAX, transferrin receptor and C-src), nucleoli (fibrillarin), mitochondria (pyruvate dehydrogenase and TOMM20), endosomes (Rab4a, Rab5a and RhoB GTase), autophagosomes (LC3), centromeres (CENPB), tight junctions (β-catelin, VE-cadherin and ZO1), DNA replication foci (PCNA), lysosomes (LAMP1), auto peroxisomes (peroxosomal membrane protein), various vesicles (clathrin and caveolin) and focal adhesions (α-actinin, talin, focal adhesion kinase, filamin A, VASP, paxillin, vinculin and zyxin). We observed all phases of mitosis in fusions of human histone H2B to either the N terminus or the C terminus of mNeonGreen (Supplementary Fig. 11).

Owing to its high brightness and ability to be driven into a temporary dark state with high-power laser illumination, we reasoned that mNeonGreen would also be an excellent tag for single-molecule superresolution imaging of fusion proteins. We performed stochastic single-molecule superresolution imaging of zyxin, β-actin, keratin and myosin IIA fusions with mNeonGreen, Clover, mEGFP and mEmerald. In almost all cases, the performance of mNeonGreen in this mode of superresolution imaging was superior to the performance of other fluorescent proteins tested in terms of the number of molecules localized, the most critical metric of this imaging mode (on the order of twofold greater than Clover and fourfold greater than mEGFP or mEmerald on average, Supplementary Figs. 12–15).

Because of its high extinction coefficient and quantum yield, we expected that mNeonGreen would be a good FRET acceptor from cyan fluorescent proteins and a good FRET donor to red fluorescent proteins, as has been demonstrated recently for the Clover-mRuby2 pair7. Notably, in our tests of FRET pairs, a direct fusion of mNeonGreen to mTurquoise3 achieved substantially greater FRET efficiency than identical constructs using mVenus13 or Clover as acceptors (Supplementary Table 2). When used as a FRET donor to mRuby2, mNeonGreen achieved FRET efficiency similar to that of Clover in an otherwise identical construct (Supplementary Table 2 and Supplementary Fig. 16). When measured using fluorescence lifetime imaging (FLIM; Online Methods), the mTurquoise-mNeonGreen pair exhibited markedly greater FRET efficiency than the Clover-mRuby2 pair (32% versus 12%; data not shown). Moreover, for purposes of FLIM-FRET, mTurquoise is a superior donor because of its single-exponential fluorescence lifetime, versus the two-component fluorescence decay of Clover (and mNeonGreen), making the mTurquoise-mNeonGreen pair preferable to Clover-mRuby2 (Supplementary Table 3). Thus, we expect that mNeonGreen will be an excellent choice for a broad array of FRET applications.

As a representative of the evolutionarily distant cephallochordate fluorescent protein lineage14, LanYFP has low sequence identity to any fluorescent protein whose structure has been solved. However, modeling of its tetrameric configuration using structure prediction and protein–protein docking algorithms provided sufficient information to identify the side chains to target for monomerization and to guide subsequent directed evolution. The resulting monomeric variant, mNeonGreen, has optical properties that are superior to those of the most commonly used green and yellow fluorescent proteins, and is expected to be highly useful for general imaging, FRET probe design and single-molecule superresolution imaging. Moreover, as it has so little sequence identity in common with other frequently used fluorescent proteins, mNeonGreen will be an attractive target for antibody development and should be amenable to orthogonal immunoprecipitation experiments along with jellyfish-derived and coral-derived fluorescent proteins.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. mNeonGreen, GenBank KC295282.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.I. cloned the original gene encoding LanYFP from B. lanceolatum, performed initial characterization of the protein, and identified the substitutions I118K (dimerizing) and N174I (folding enhancement). G.G.L. performed the majority of library construction, E. coli expression experiments, and protein purification. A.C., J.W. and Y.N. performed initial cloning and library construction for screening dimeric variants. R.N.D. performed FLIM–FRET experiments. J.R.A. performed single-molecule superresolution imaging experiments. M.W.D., P.J.C., M.A.B. and B.R.S. constructed mammalian expression and fusion plasmids, performed fixed- and live-cell imaging and FRET experiments, and helped write the manuscript. J.W. contributed to writing and editing the manuscript and supported the project. N.C.S. designed and planned the project, performed library design and screening, optical characterization and size-exclusion experiments, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cloning, protein expression and purification. All fluorescent protein–coding sequences were inserted between BamHI and EcoR1 sites in the constitutive expression vector pNCS, which encodes an N-terminal 6His tag and linker. Sequences for all primers used in this study are listed in Supplementary Table 4. Fluorescent proteins were expressed in E. coli strain NEBTurbo (New England BioLabs) or Mach1 (Invitrogen) by growing cultures in 2x YT (yeast extract and tryptone) medium supplemented with ampicillin overnight at 37 °C with shaking at 250 r.p.m. Fluorescent proteins were purified by Ni2+–affinity chromatography as previously described9. Proteins were eluted in 50 mM Tris pH 7.5 or 50 mM sodium phosphate buffer pH 7.5 containing 250 mM imidazole. For all additional characterization experiments, eluted fluorescent proteins were buffer-exchanged using Amicon Ultra0.5 10 kDa MWCO ultrafiltration units (Millipore) into the same buffer without imidazole. Proteins were stable when stored at 4 °C indefinitely or when frozen at −20 °C or −80 °C.

Directed evolution. Multiple rounds of directed evolution and screening were performed as previously described9,10, with a summary of techniques provided here. Screening of fluorescent protein–expressing E. coli colonies was done by eye using a blue LED lamp and long-pass yellow filter. For each round of directed evolution, 1–3 of the brightest clones from the previous round were used as the template for construction of randomly mutagenized libraries using the GeneMorph II kit (Agilent Technologies). Mutagenic PCR conditions were chosen such that the library would contain an average of 2–4 mutations per clone. Of the brightest clones identified by random mutagenesis, 20–30 were sequenced, and any clones containing mutations predicted to revert the oligomeric state of the protein were rejected. The remaining substitutions present in clones from each round of random mutagenesis were used to guide selection of codons to target in a subsequent round of directed mutagenesis of the same template(s). For each such directed mutagenesis library, the selected codons were partially or fully randomized by overlap-extension PCR using degenerate primers, producing library sizes typically between 500 and 25,000 unique clones. The brightest clones from directed mutagenesis were sequenced, optically characterized and evaluated for their oligomeric state by size-exclusion chromatography (see below). Clones that exhibited superior optical properties while maintaining the desired oligomeric state were used as the input for the next round of directed evolution.

Optical characterization. For spectroscopy measurements, all samples and buffers were filtered or centrifuged immediately before use. Purified fluorescent protein samples were diluted into 10 mM Tris pH 7.4 buffer, and fluorescein (Sigma) was diluted into 0.1 M NaOH. Absorbance measurements were collected with a Cary Bio 100 UV-Vis Spectrophotometer (Varian Inc.). Fluorescence measurements were collected with a Cary Eclipse Spectrophotometer (Varian Inc.). All measurements for absorbance were immediately preceded with a measured baseline with the appropriate blank buffer. Fluorescence pKs values were determined by measuring fluorescence emission of heavily diluted (~10 nM), purified, dialyzed fluorescent protein samples in 100 mM mixed citrate-Tris-glycine buffer with pH ranging from 3 to 11.

Size-exclusion chromatography. Purified and dialyzed fluorescent protein samples were diluted into 50 mM Tris-HCl pH 7.5, 100 mM NaCl and filtered through 0.2-µm filters immediately before injection into a Shimadzu Nexera UHPLC equipped with a Waters BEH200 (1.7 µm pore size) 4.6 mm × 150 mm size-exclusion column and 4.6 mm × 30 mm guard column. Samples were run in the same buffer at a flow rate of 0.3 ml per minute for a total run time of 20 min. Fluorescence of the eluted protein was detected with an RF-20Axs fluorescence detector (Shimadzu) with 480 nm and 540 nm excitation, and 530 nm and 620 nm emission wavelengths. Each LanYFP or variant sample was co-injected with mCherry9, which had been purified under identical conditions and which served as a monomeric size standard. A control run of mCherry alone produced no bleed-through into the yellow emission channel.

Quantum yield (φ) and extinction coefficient (ε). A sample of fluorescent protein or fluorescein was prepared (typically at a concentration of approximately 5 µM, giving a peak absorbance value of ~0.5) and its full absorbance spectrum was measured with 0.5-nm step size. This was done in the same cuvette to be used for fluorescence spectra measurement. Identically absorbing solutions (target OD ≤ 0.05) were separately prepared in quadruplicate for the fluorescent protein and fluorescein (φ = 0.925)19 and their emission spectra were measured with 488-nm excitation. The excitation and emission bandwidths were 2.5 nm and 5 nm, respectively, with 1.0-nm step size. Emission was collected for 490–750 nm, and the integrated intensities for each sample were calculated using the fluorimeter’s software. The average integrated intensities and their associated absorbance values were used to calculate quantum yields as previously described6.

Absorbance spectra of purified fluorescent protein samples were measured in quadruplicate (0.5-nm step size) and were used to determine the mean peak absorbance value. A baseline absorbance spectrum was then measured with buffer diluted 1:1 with 2 M NaOH (1 M final concentration). A double-concentration sample was prepared in half the cuvette volume, mixed 1:1 with 2 M NaOH, and its absorbance was measured immediately. Data were acquired for NaOH-denatured protein between 430 nm and 460 nm (with a peak at ~447 nm) and a full UV-visible light
Excitation and emission. For excitation spectra, fluorescence emission was monitored at 535 nm. For emission spectra, fluorescence excitation was 465 nm. A 5.0 nm band-pass window was used for scanning across wavelengths in both cases.

Maturation. For oxygen-dependent maturation experiments, sequence encoding mNeonGreen was subcloned into a modified pBAD vector and grown as described, with flasks sealed with a neutral density (ND32) filter in the light path. For each bleaching experiment, data were analyzed using Simple PCI software (Hamamatsu).

Photobleaching. Laser-scanning confocal and widefield microscopy photobleaching experiments were performed as described previously, using fusions of the appropriate fluorescent protein to human histone H2B to allow for localized fluorescence in the nucleus. HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 12.5% FBS (HyClone). The cells were then seeded onto 35 mm Delta T dishes (Bioptechs) for live-cell imaging. Approximately 24 h after being seeded, cells were then transfected with 1 µg of DNA using Effectene (Qiagen) and maintained in a 5% CO2 incubator for at least 24 h before imaging.

We measured widefield photobleaching curves using a Nikon TE2000 inverted microscope with a Nikon Plan Fluorite 40× dry objective (numerical aperture (NA) = 0.85). The excitation light source was an X-Cite Exacte metal halide lamp (Lumen Dynamics). Illumination power at the objective with a Brightline FITC-HQY filter cube (Chroma) in place was verified to be 4.3 mW using a Newport 1908-C optical power meter. During initial scanning of the culture dish for suitable regions to bleach, the illumination power was attenuated using a neutral density (ND32) filter in the light source. For each bleaching measurement, we selected a region of the culture dish containing 10–20 bright nuclei in a single field. After locating a suitable region, we removed the neutral density filter from the light path and collected 4,700 images over 15 min with an exposure time of 65 ms per frame, using a QImaging Retiga EXi camera (Photometrics) coupled to NIS-Elements software (Nikon). Sufficient fields were bleached such that a total of at least 30 nuclei could be averaged for each bleaching experiment. Data were analyzed using Simple PCI software (Hamamatsu).

Confocal photobleaching measurements were collected on an Olympus FV1000 confocal microscope with an Olympus PLAPO 40× oil-immersion objective (NA = 1.0). A 488-nm Argon-ion laser line (Melles Griot) was confirmed to be attenuated to an output power of 1005 µW at the objective with a FieldMax II-TO power meter (Coherent). The microscope was set to a zoom of 2×, a pinhole size of 500 µm, a photomultiplier voltage of 450 V, an offset of 8 V and a scan time of 4 µs/pixel. Emission was collected with a region of evenly bright nuclei was located. The laser power was raised back to 1,005 µW, and each region was photobleached continuously for ~8 min for a total of 300 frames, with multiple regions being bleached to ensure data for 30 nuclei. Raw data were collected with the FluoView software (Olympus) and then analyzed with Simple PCI software (Hamamatsu).

All photobleaching data were scaled to represent the equivalent of an emission rate of 1,000 photons/s per fluorescent protein chromophore at time zero as previously described, a condition which produces a half-time of 150 s for mEGFP.

Acceptor photobleaching FRET. FRET constructs of mTurquoise–mNeonGreen, mTurquoise–mVenus, mTurquoise–Clover, mCerulean–mVenus, mNeonGreen–mRuby2 and Clover–mRuby2 all contained a 10-amino acid linker SGLRSPPVAT between fluorescent proteins. Acceptor photobleaching measurements were performed on an Olympus FV1000 confocal microscope with a UPLAPO 40× oil immersion objective (NA = 1.0), a Zeiss Elyra PS.1 inverted research microscope operated in laser-scanning confocal mode with a PlanApo 63× oil-immersion objective (NA = 1.4) and a Zeiss LSM 5 Live with a PlanApo 63× oil-immersion objective (NA = 1.4). For measurements performed on the Olympus FV1000, a 515-nm argon-ion laser line was used with a 458/515 nm dichroic mirror to excite and photobleach the mNeonGreen or mVenus in each FRET pair. Emission during acceptor photobleaching was collected in one channel spanning 528–553 nm to ensure bleaching of all fluorescence. For each of the cyan-yellow fluorescent protein FRET constructs, a 405-nm diode laser line was used with a 405/488 nm dichroic for excitation of the cyan fluorescent protein with one emission channel spanning 450–485 nm. The detector gain was set to 685 V, the offset was set to 8 V and the scan speed was set to 8.0 µs/pixel. Each experiment was performed with a pinhole size of 500 µm. Measurements performed on the Zeiss Elyra PS.1 used a 561-nm helium-neon laser line used with a 488/561 nm dichroic mirror to excite and photobleach mRuby2 and a 514-nm argon-ion laser line to photobleach mNeonGreen or Clover in each FRET pair. Emission was collected in one channel spanning 568–638 nm for mRuby2 and 516–586 nm for mNeonGreen to ensure bleaching of all fluorescence. For cyan-yellow fluorescent protein pairs, a 405-nm diode laser line was used with a 405/488 nm dichroic for excitation of the cyan fluorescent protein with one emission channel spanning 436–488 nm. For yellow-red fluorescent protein pairs, a 488-nm Argon-ion laser line was used with a 488/561 nm dichroic for excitation of the yellow fluorescent protein with one emission channel spanning 496–553 nm. The detector gain was set to 700 V, the zoom was 2.0, the scan speed was 1.6 µs/pixel and the pinhole size was 600.9 µm for both FRET pairs. Measurements performed on the Zeiss LSM 5 Live used a 56-nm helium-neon laser line with a NFT565 beam-splitter paired with a band-pass filter BP575-615 to excite and photobleach mRuby2, and a 489-nm Argon-ion laser line paired with a 405 + 532 nm beam-splitter.
and band-pass filter BP495-555 to excite and image the yellow fluorescent protein donors.

For measurements of FRET efficiency in live cells, a full-view image of the donor fluorescence was acquired before and after acceptor photobleaching of the entire cell. A region of interest (ROI) was drawn over identical areas of the cell in each image, and the average intensities of these regions were calculated using the microscope’s software. The following formula was used to calculate the FRET efficiency of each construct: FRET efficiency = 1 − (average intensity donor before AP ÷ average intensity donor after AP)², where AP is acceptor photobleaching.

For measurements of FRET efficiency in fixed cells, a full view image of the donor fluorescence was acquired before and after acceptor photobleaching. An ROI was drawn over an evenly bright part of the cell, and acceptor was photobleached. The average intensities of these regions were calculated using the microscope’s software, and the above FRET efficiency formula was again used to calculate FRET efficiency.

**Frequency-domain fluorescence lifetime measurements.**

The fluorescence lifetime measurements were made using a ISS ALBA FastFLIM system (ISS Inc.) coupled to an Olympus IX71 microscope equipped with a 60× water-immersion objective lens (NA = 1.2). A stage top environmental control system (Pathology Devices Inc.) maintained temperature at 36 °C and CO2 at 5%. A 5 mW, 448-nm diode laser was modulated by the FastFLIM module of the ALBA system at the fundamental frequency of 20 MHz (ref. 24). The modulated laser is coupled to the ALBA scanning system, which is controlled by the VistaVision software (ISS Inc.). The fluorescence signals emitted from the specimen are routed by a beam-splitter through the 530/43 nm (acceptor emission) and the 480/40 nm (donor emission) band-pass emission filters. The signals are then detected using two identical avalanche photodiodes (APD). The phase delays and modulation ratios of the emission relative to the excitation are measured at seven modulation frequencies (20 MHz, 40 MHz, 60 MHz, 80 MHz, 100 MHz, 120 MHz and 140 MHz) for each pixel of an image.

The system was calibrated with the 50 µM Coumarin 6 dissolved in ethanol (lifetime 2.5 ns) to provide the software with a reference standard to estimate the lifetime values from the experimental data24. Additionally, a second reference standard, 10 mM HPTS (8-hydroxyxyprene-1,3,6-trisulfonic acid) dissolved in phosphate buffer (PB) pH 7.8 (lifetime of 5.4 ns) was used to check that the system was accurately reporting the fluorescence lifetime of a known sample. The distribution of the lifetimes for all the pixels in the image was determined using the phasor (polar) plot method25,26. For live-cell imaging, transfected cells grown in chambered cover glass (two-well, Thermo Scientific) were identified by epifluorescence microscopy, and then imaged by frequency-domain FLIM using the 448-nm laser line. The laser power was adjusted to achieve ~100,000 counts per second in the donor emission channel, and frame averaging was used to accumulate approximately 200 peak counts per pixel. The data were analyzed with the VistaVision software (ISS Inc.) using a region average for each selected square ROI (typically 1–2 µm).

**Fusion plasmid construction.** Expression plasmids encoding mNeonGreen with multiple cloning sites at the N or C terminus (Clontech-style “N1” and “C1” vectors) were constructed by insertion of a PCR-amplified mNeonGreen coding sequence between either AgeI and BspE1 (C1) or AgeI and NotI (N1) of the pEGFP-N1 and pEGFP-C1 vector backbones (Clontech). To construct each fusion vector used in this study, the mNeonGreen N1 or C1 plasmid and a corresponding pre-existing EGFP or mEmerald fusion plasmid were digested with appropriate enzymes, the restriction fragments were purified from agarose gels and the fragments were ligated together.

We performed the following restriction digests to prepare mNeonGreen fusion constructs in the mNeonGreen C1 plasmid (amino acid (aa) linker length shown in parentheses for each construct): annexin A4 (12 aa), coding sequence cloned between NheI and BspE1 sites (cDNA source: A. Piljic, European Molecular Biology Laboratory, Heidelberg, Germany; Genbank accession NM_001153.3); β-actin (7 aa), coding sequence cloned between NheI and BglII (human β-actin; cDNA source: Clontech; NM_001101.3); β-catenin (20 aa), coding sequence cloned between XhoI and BamHI (mouse β-catenin; cDNA source: Origene; NM_001165902.1); 20-aminoc acid farnesylation signal from c-Ha-Ras (CAAX; 5 aa), coding sequence cloned between AgeI and BspE1 (c-Ha-Ras; cDNA source: Clontech; NM_001130442.1); CAF1 (10 aa), coding sequence cloned between AgeI and BspE1 (mouse chromatin assembly factor; cDNA source: A. Gunjan, Florida State University, Tallahassee; NM_013733.3); caveolin 1 (10 aa), coding sequence cloned between NheI and BglII (human caveolin 1; cDNA source: Origene; NM_001753.4); human RhOB GTPase (14 aa), coding sequence cloned between NheI and BspE1 (endosome; cDNA source: Clontech; NM_0040402.2); fascin (10 aa), coding sequence cloned between BspE1 and BamHI (human fascin; cDNA source: Origene; NM_003088.2); fibrillarin (7 aa), coding sequence cloned between AgeI and BspE1 (fibrillarin; cDNA source: Evrogen; NM_0014363.3); filamin A (14 aa), coding sequence cloned between BspE1 and HindIII (human filamin; cDNA source: D. Calderwood, Yale University; NM_0014563.3); rat lysosomal membrane glycoprotein 1 (20 aa), coding sequence cloned between BmHI and NotI (LAMP1; cDNA source: G. Patterson, US National Institutes of Health; NM_012857.1); human light chain clathrin (15 aa), coding sequence cloned between NheI and BglII (human clathrin light chain; cDNA source: G. Patterson; NM_001834.2); human myotilin (14 aa), coding sequence cloned between AgeI and BspE1 (MYOT; cDNA source: Origene; NM_006790.2); PCNA (19 aa), coding sequence cloned between AgeI and BspE1 (proliferating cell nuclear antigen; cDNA source: D. Gilbert, Florida State University, Tallahassee; NM_002592.2); plastin (10 aa), coding sequence cloned between BspE1 and XhoI (human plastin 1 (fimbrin); cDNA source: NM_002670.2); human Rab4a, coding sequence cloned between BglII and BamHI (Rab4a; cDNA source: V. Allen, University of Manchester; NM_0045783.3); LC3B (7 aa), coding sequence cloned between AgeI and BspE1 (rat LC3B; cDNA source: J.M. Tam, Harvard University; U05784.1); talin (22 aa), coding sequence cloned between AgeI and BspE1 (mouse talin 1; cDNA source: C. Waterman, US National Institutes of Health; NM_011602.5); β-tubulin (18 aa), coding sequence cloned between NheI and BglII (human β-tubulin; cDNA source: Clontech; NM_006082.2); and ZO-1 (14 aa), coding sequence cloned between AgeI and BspE1 (human tight junction protein 1; cDNA source: Blue Heron; NM_003257).
We performed the following restriction digests to prepare mNeonGreen fusion constructs in the mNeonGreen N1 plasmid (number of linker amino acids (aa) in parentheses): human non-muscle α-actinin, coding sequence cloned between EcoRI and NotI (cDNA source: T. Keller, Florida State University, Tallahassee; Genbank accession NM_001130005.1); human calnexin, coding sequence cloned between Agel and NotI (cDNA source: Origene; NM_001746.3); c-src (7 aa), coding sequence cloned between BamHI and EcoRI (chicken c-src; cDNA source: M. Resh, Sloan-Kettering, New York; NM_205457.2); connexin-43 (7 aa), coding sequence cloned between BamHI and NotI (rat Cx43; cDNA source: Matthias Falk, Lehigh University; NM_001004099.1); EB3 (7 aa), coding sequence cloned between BglII and BamHI (EB3; cDNA source: L. Cassimeris, Lehigh University; NM_012326.2); human keratin 18 (17 aa), coding sequence cloned between EcoRI and NotI (cDNA source: Open Biosystems; NM_008549.2); myosin IIA (14 aa) coding sequence cloned between NheI and BglII (mouse myosin IIA; cDNA source: Origene; NM_022410.2); human nucleoporin 50 kDa (10 aa), coding sequence cloned between BamHI and NotI (NUP50; cDNA source: Origene; NM_007172.3); human pyruvate dehydrogenase (10 aa), coding sequence cloned between Agel and NotI (human PDHA1; cDNA source: Origene; NM_000284.3); human peroxisomal membrane protein (10 aa), coding sequence cloned between NotI and Agel (PMP; cDNA source: Origene; NM_018666.1); human MAP Tau (10 aa), coding sequence cloned between Agel and NotI (MAP Tau; cDNA source: Origene; NM_016841.4); human TIR (20 aa), coding sequence cloned between BamHI and NotI (transferrin receptor; cDNA source: G. Patterson; NM_003234.2); human TPX2 (10 aa), coding sequence cloned between Agel and NotI (TPX2; cDNA source: P. Wadsworth, University of Massachusetts, Amherst; NM_012112.4); mouse VASP (10 aa), coding sequence cloned between NheI and BamHI (cDNA source: C. Waterman, US National Institutes of Health; NM_009499.2); vascular epithelial cadherin (10 aa), coding sequence cloned between Agel and NotI (human VE cadherin; cDNA source: Origene; vimentin (7 aa), coding sequence cloned between BamHI and NotI (human vimentin; cDNA source: R. Goldman, Northwestern University; NM_003380.3), zyxin (6 aa), coding sequence cloned between BamHI and NotI (human zyxin; cDNA source: Origene; NM_003461.4).

Expression plasmids were purified using the Qiagen Plasmid Maxi kit. Plasmids (~1 μg per dish) were transfected using Effectene (Qiagen) into cultures of HeLa (S3 or CCL) or MDCK (ATCC) cells. All cells were grown in a 1:1 mix of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 medium supplemented with 12.5% Cosmic calf serum (Thermo Scientific), unless otherwise noted. Transfections were performed using Effectene (Qiagen) following the manufacturer’s protocol. Cells were maintained under a humidified atmosphere of 5% CO₂ in air in Delta-T culture chambers (Biopetechs) during imaging. An Olympus IX71 equipped with Semrock Brightline filters and a Hamamatsu ImagEM camera, and a Nikon TE-2000 equipped with Omega QuantaMax filters and a Photometrics Cascade II camera were used for widefield imaging. Olympus FV1000 and Nikon C1Si confocal microscopes equipped with argon-ion (457 nm and 488 nm) and helium-neon (543 nm) or diode (561 nm) lasers were used to collect laser-scanning confocal images. After imaging, some cultures transfected with mNeonGreen fusions were fixed with 2% (w/v) paraformaldehyde (Electron Microscopy Sciences), washed several times in PBS supplemented with 0.05 M glycine, and mounted using a polyvinyl alcohol-based medium.

Superresolution imaging sample preparation. HeLa CCL-2 or S3 (ATCC) cells were seeded and grown in 35 mm glass-bottom dishes (MatTek Corporation) to ~80% confluency. The culture medium was a 1:1 mixture of DMEM and Ham’s F-12, supplemented with 12.5% Cosmic calf serum (Thermo Scientific). Cells were transiently transfected with plasmids coding for one of four fluorescent proteins (mNeonGreen, Clover, mEGFP or mEmerald) fused to actin, zyxin, keratin or myosin IIA using Effectene (Qiagen). At ~24 h after transfection, cells were fixed using 2% (w/v) paraformaldehyde for 15 min or, for keratin constructs, with cold methanol for 10 min. Live-cell imaging was conducted on selected cultures.

Stochastic single-molecule superresolution imaging. Imaging was performed on a Zeiss Elyra PS.1 inverted research microscope operated in total internal reflection fluorescence (TIRF) illumination mode using a Zeiss PlanApo 63× oil-immersion objective (NA = 1.4) with a 1.6 optivar placed in the light path, and a detection window of 495–575 nm. All images were collected over a 40.48 μm × 40.48 μm area of an electron-multiplying charge-coupled device (EMCCD) camera, and Semrock filters and a Nikon TE-2000 equipped with a Chroma FITC filter set to confirm proper localization.

Microscopy. Cell lines used for imaging were HeLa (CCL-2, ATCC), gray fox lung fibroblast (CCL-168, ATCC) and MDCK.
alcohol–PBS solution, whereas fixed keratin and myosin IIA constructs were imaged in 10 mM cysteamine in PBS.

Data analysis. SSMS imaging data sets were analyzed using the PALM analysis module available in the Zen 2010D software (Zeiss). The detection parameters for single emitters were kept constant across all experiments, with a peak mask size of 9 pixels and a minimum peak intensity to noise ratio of 6.0. Data from all overlapping emitters were discarded, and molecules were localized using a 2D Gaussian fit. After the initial analysis, model-based drift correction was applied, and single molecules emitting across multiple frames were grouped together. Grouping of molecules followed strict parameters, with a maximum on-time of 5 frames, a fluorescence off-gap of 0 frames and a capture radius of 1 pixel. To prevent confusion with possible fluorescence artifacts or fiducial markers, all data were restricted to single molecules emitting 150–1,200 photons for statistical analysis.

Organized smooth endoplasmic reticulum live-cell assay. To anchor the fluorescent protein to the cytosolic face of the endoplasmic reticulum (ER), sequence encoding the first 29 amino acids of rabbit Cytochrome p450 (CytERM) was inserted into a mEGFP-N1 (Clontech-style) vector as previously described27,28. All FP-CytERM fusions contained a 17 amino acid linker (RILQSTVPRARDPPVAT) between the fluorescent protein and CytERM signaling peptide sequence. Using AgeI and NotI sites, sequences encoding EGFP, mEGFP, mNeonGreen, Clover, mTurquoise and mRuby2 were inserted downstream of the sequence encoding the p450 transmembrane segment. HeLa (CCL-2, ATCC) cells were cultured in DMEM (Invitrogen) supplemented with 12.5% FBS (FBS; HyClone). The cells were then seeded onto 35-mm culture dishes containing 18 mm × 18 mm glass coverslips. Approximately 24 h after being seeded, cells were then transfected with 1 µg of DNA using Effectene (Qiagen) and maintained in a 5% CO2 incubator for at least 24 h before counting. Transient transfections were visually assayed on an Olympus IX71 inverted microscope equipped with an LCPPlanFluor 40× dry objective (NA = 0.60) and the appropriate filter sets. Cell counting was performed by counting the total number of cells in a field of view and then counting the number of cells with normal ER morphology; that is, containing no artifacts resembling organized smooth ER structures (for example, stacked cisternae or 'whorls'). Multiple viewfields were counted for several transfected dishes until a total of 10,000 cells had been counted. The number of 'normal morphology' cells was divided by the total number of cells to determine the percentage of 'normal morphology' cells for each fluorescent protein fusion.

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