Published in final edited form as:

Nat Methods. 2010 October ; 7(10): 827–829. doi:10.1038/nmeth.1501.

Near-infrared fluorescent proteins

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Abstract

Fluorescent proteins with emission wavelengths in the near-infrared and infrared range are in high demand for whole-body imaging techniques. We report near-infrared fluorescent proteins eqFP650 and eqFP670. To our knowledge, eqFP650 is the brightest fluorescent protein with emission maximum above 635 nm, and eqFP670 displays the most red-shifted emission maximum and high photostability.

Fluorescent proteins are reliable *in vivo* reporters for whole-body imaging techniques, and the development of far-red light-emitting proteins offered hope that a further red-shift of the emission is possible without dramatic loss of brightness^{1,2}. The next goal was to reach the near-infrared and infrared range, in which living tissues are even more transparent owing to low absorbance and light scattering³. Use of such fluorescent proteins for whole-body imaging would facilitate investigation of metastasis and tumor localization, cell migration, embryogenesis and other studies involving deep-tissue imaging. Although an infrared

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AUTHOR CONTRIBUTIONS

D.S., I.I.S. and L.S. developed fluorescent proteins. A.V.R. and V.B.L. performed experiments on Biospec imaging system. K.E.L., B.T.S. and G.D.L. performed experiments on a Caliper IVIS spectrum imaging system. A.G.Z. performed experiments on *Xenopus* embryos. T.V.G. and E.A.S. grew cells and performed microscopy experiments. K.M.S. generated transgenic zebrafish. O.V.B. performed flow cytometry analysis. K.A.L., G.D.L. and D.M.C. designed and planned the project, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemethods/>.

eqFP650 and eqFP670 are property of Evrogen JSC, Moscow, Russia. K.A.L. and D.M.C. have interest in Evrogen JSC. T.V.G. is employed by Evrogen JSC.

protein that requires biliverdin injection for fluorescence has been reported recently⁴, fully genetically encoded markers are preferable. We report recent progress in the development of near infrared fluorescent proteins based on the dimeric far-red fluorescent protein Katushka².

For whole-body imaging experiments, high reporter expression is usually required, and low cytotoxicity is crucial⁵. According to our experience, Katushka is not cytotoxic, as we have successfully generated stable lines of transgenic *Xenopus laevis*² and *Danio rerio* (Supplementary Fig. 1) expressing Katushka in muscle cells at high levels.

However, a recent report has suggested that more optimal low-toxicity fluorescent protein variants can be selected using a bacterial expression system⁵. We performed a similar screen (Online Methods) and identified a low-cytotoxicity Katushka variant named Katushka-9-5, which had spectral characteristics nearly identical to those of Katushka and had minimal negative impact on bacterial growth, in contrast to Katushka or monomeric (m)Neptune, a recently reported monomeric far-red fluorescent protein⁶ (Supplementary Fig. 2). Neither Katushka-9-5 nor Katushka demonstrated any visible cytotoxicity when expressed in mammalian HeLa cells (Supplementary Fig. 3). To compare cytotoxicity of Katushka-9-5 and Katushka *in vivo*, we microinjected plasmids in the animal poles of *X. laevis* embryos at the two-cell stage (20 pg per blastomere), and tracked the death rate by the tailbud stage (stage 25) and by the tadpole stage (stage 42). These experiments demonstrated lower toxicity of Katushka-9-5 compared to Katushka (Fig. 1a).

Because of its lower toxicity, we selected Katushka-9-5 for the development of fluorescent protein variants with further red-shifted emission. Based on the crystal structure of the related far-red fluorescent protein mKate⁷ (Protein Data Bank (PDB): 3BXXB), we selected amino acid positions in proximity to the chromophore (including Met14, Leu16, Met44, Thr62, Tyr121, Ser148, Ser165, Met167, Arg203 and Leu205) for targeted mutagenesis (numbering in accordance with *Aequorea victoria* GFP, avGFP; Supplementary Fig. 4). We performed semisaturated (2–5 amino acid variants) site-directed mutagenesis of these positions in several combinations and random mutagenesis. We manually screened bacterial libraries containing these mutants using a fluorescence stereomicroscope equipped with a 650LP emission filter and a built-in spectrofluorimeter. We screened more than a million individual colonies and selected two red-shifted variants, named eqFP650 and eqFP670, that have the substitutions N24G,M44A and M14T,N24G,M44C,S148N,S165N, respectively. Notably, Met44 is also substituted by a small amino acid in two recently published far-red fluorescent proteins, mNeptune⁶ and E2-Crimson⁸ (Supplementary Fig. 4). This mutation has been shown to create a cavity filled by a water molecule forming a hydrogen bond with the chromophore acylimine oxygen⁶ and appears to be a universal solution for providing a bathochromic shift of the fluorescence emission for DsRed-like chromophores.

Characteristics of eqFP650 and eqFP670 are summarized in Table 1 and Figure 1. Both proteins are characterized by fast maturation in *Escherichia coli* at 37 °C and demonstrate no residual short wavelength fluorescence of intermediate chromophore forms, in contrast to E2-Crimson, which exhibits a second bright blue emission peak, and mNeptune, which has a pronounced green peak (Supplementary Fig. 5). As evidence of complete chromophore

maturation, the absorbance spectra of freshly purified proteins were single peaks with minor absorption bands at 340–360 nm characteristic of red fluorescent proteins (Fig. 1d). In transiently transfected HeLa cells, eqFP650 produced the brightest signal in its spectral class (Fig. 1b). In HEK-293T cells that allow high expression, eqFP650 produced bright signal 14 h after transfection.

eqFP650 and eqFP670 have only one amino acid substitution in the outer surface of the beta-barrel (Gly24, characteristic for avGFP and E2-Crimson, Supplementary Fig. 4) and both inherited the dimeric nature of Katushka-9-5, as well as its low toxicity in bacterial tests (Supplementary Fig. 2). In eukaryotic cells, eqFP650 and eqFP670 were evenly distributed and demonstrated no aggregation or toxicity (Supplementary Fig. 6).

eqFP650 generally retained the high brightness of Katushka and was characterized by a strong bathochromic shift, with excitation and emission peaks at 592 nm and 650 nm, respectively (Fig. 1c and Supplementary Fig. 7). To date, eqFP650 is the brightest fluorescent protein with emission maxima above 635 nm and should be an optimal genetically encoded marker for *in vivo* imaging.

eqFP670 is characterized by lower brightness but stronger bathochromic shift, with excitation and emission peaks at 605 nm and 670 nm. eqFP670 is to our knowledge the first type of GFP-like fluorescent protein with such long-wavelength emission, approximately half of which falls in the infrared part of the spectrum. Compared to Katushka, eqFP670 had fourfold greater infrared brightness (Table 1). Furthermore, eqFP670 had high pH stability (Table 1) and extremely high photostability (Fig. 1e,f) and should allow for accumulation of the fluorescent signal over long exposure times. The combination of Asn148 and Asn165 in eqFP670 is unique and has not been encountered in other fluorescent proteins. This implies tight packing around the chromophore, which probably forms the basis of the high photostability and pH resistance.

To investigate detection of fluorescence in deep tissues by whole-animal imaging, we transiently transfected HEK 293T cells with a single plasmid encoding Katushka, eqFP650, eqFP670, mNeptune⁶ or E2-Crimson⁸ and injected cells intramuscularly into the gluteal region of mice. We co-transfected cells with firefly luciferase plasmid to normalize for transfection efficiency and total numbers of injected cells. Representative images of mice injected with cells transiently expressing E2-Crimson, mNeptune or eqFP650 showed that eqFP650 had the highest fluorescence (Fig. 2a), and quantification at various emission wavelengths showed higher fluorescence from eqFP650 at two excitation wavelengths (Fig. 2b,c). No cell implant was detectable with 640 nm excitation and infrared detection, probably owing to the high background signal.

To compare efficiency of infrared imaging, we used another imaging system. We subcutaneously (2-mm depth) injected equal portions of mature Katushka, eqFP650, eqFP670, E2-Crimson, Neptune and mNeptune protein. Injection of protein samples provided higher local concentration of a fluorescent protein than injection of transiently transfected cells, allowing for better signal-to-noise ratio. In this artificial system, all the proteins tested had comparable infrared signal (Fig. 3). This system, however, did not

account for multiple influences on fluorescent protein signal in living cells that were present in the *in vivo* experiment shown in Figure 2, such as protein maturation rate, protein turnover, mRNA stability and transcription rate. We did not attempt to quantify protein expression and do not know how it may have varied.

Although eqFP650 now may be the preferable near-infrared fluorescent protein for *in vivo* cell-labeling experiments, we believe that development of longer-wavelength fluorescent proteins such as eqFP670 and true monomeric variants for protein fusions will increase the sensitivity and capabilities of deep-tissue imaging techniques.

Online Methods

Cloning and gene construction

Cloning and gene construction were performed as described in ref. 2. Briefly, purification of PCR products and products of restriction digests was performed by gel electrophoresis and extraction using the QIAquick gel extraction kit (Qiagen). Plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen). Site-directed mutagenesis was performed by overlap-extension PCR¹¹. Random mutagenesis was performed using the Diversity PCR Random Mutagenesis kit (Clontech). For bacterial expression, a fluorescent protein gene was cloned into the pQE30 vector (Qiagen) using BamHI and HindIII restriction sites. For expression in eukaryotic cells, a fluorescent protein gene was cloned instead of *TurboGFP* into the pTurboGFP-N vector (Evrogen) using AgeI and NotI restriction sites.

Characterization of fluorescent proteins *in vitro*

Fluorescent proteins were characterized as described in ref. 12. Briefly, proteins were expressed in *E. coli* XL1 Blue strain (Invitrogen), purified using Talon metal-affinity resin (Clontech) and desalted using gel-filtration columns (Bio-Rad). Cary 100 UV/VIS Spectrophotometer and Varian Cary Eclipse Fluorescence spectrophotometer were used to measure absorption and excitation-emission spectra. All measurements were performed in 100 mM NaCl with 20 mM Tris-HCl (pH 7.5). Molar extinction coefficients were calculated based on the chromophore absorption in native proteins and proteins alkali-denatured with an equal volume of 2 M NaOH, considering that in alkaline conditions DsRed-like chromophore converts to the GFP-like one¹³ with an extinction coefficient of 44,000 M⁻¹ cm⁻¹ at 452 nm. Absorbance spectra were measured immediately after denaturation. Quantum yields were determined by direct comparison with mCherry. To measure pH stability, buffers in the pH range from 3 to 10 were used. An aliquot of purified protein was diluted in the corresponding buffer solution. After 1 h incubation at room temperature (25°C), the fluorescence brightness was measured. In each sample, actual final pH was measured using a microelectrode (Sartorius).

Photostability

Selected proteins underwent a photostability comparison test in LSM 510 confocal scanning microscope (Carl Zeiss) and epifluorescence Leica AFLX 6000 microscope. Proteins with 6His tags were bound to Talon metal affinity resin beads, placed on a glass slide and exposed to light. The following parameters were used for bleaching by a 561 nm laser line in

a confocal microscope: 63× oil-immersion objective, 1.5× zoom, 4 s per image scan rate and maximal power. For bleaching by a mercury arc lamp, a TexasRed filter set was used, which passes the 546 nm peak of a mercury lamp. Bleaching times were corrected on the molar extinction coefficients of the corresponding fluorescent protein at 546 nm or 561 nm.

Animal imaging and data analysis

HEK 293T cells were grown in DMEM (Invitrogen), 10% FBS, 1% glutamine and 0.1% penicillin-streptomycin-gentamicin. Cells were transfected with 8.7 µg of plasmid encoding various fluorescent proteins and 1 µg of plasmid encoding firefly luciferase by calcium phosphate precipitation as described previously¹³. Cells were used for experiments 2 d after transfection. The plasmid for E2-Crimson was provided courtesy of B. Glick (University of Chicago). All animal procedures were approved by the University of Michigan Committee on Use and Care of Animals. Mice were anesthetized with isoflurane and injected with 2.5×10^6 cells intramuscularly into the right and left gluteal musculature of each mouse. Cells were injected in 100 µl of a 1:1 mixture of F12 medium and matrigel (BD Biosciences). Imaging was performed approximately 1 h after injection. Bioluminescence and fluorescence imaging were performed on an IVIS Spectrum (Caliper) system. Fluorescence images were acquired with excitation and emission filters indicated in Figure 2, using 3-s acquisition, small binning and 12.5-cm field of view. After fluorescence imaging, bioluminescence imaging of firefly luciferase was performed as described previously¹⁴. Data for fluorescence and bioluminescence were quantified as efficiency and photons, respectively. To account for differences in transfection efficiency and actual numbers of injected cells, pseudocolor displays for fluorescence images were normalized for photons for firefly luciferase in each cell implant. Data were also graphed as ratios of fluorescence efficiency to firefly luciferase photons and presented as mean values $\pm$ s.e.m. ($n = 4-6$ samples per point).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank J.M. Steele for technical assistance with mouse experiments, and B.S. Glick (University of Chicago) for providing the plasmid encoding E2-Crimson. This work was supported by grants from Molecular and Cell Biology program RAS, the Howard Hughes Medical Institute (55005618), US National Institutes of Health (R01CA136553, R01CA136829 and P50CA093990), Rosnauka (02.512.12.2053) and RFBR (08-04-01702-a and 10-04-01042).

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Figure 1.

Cytotoxicity and spectral characteristics. **(a)** Katushka and Katushka-9-5 cytotoxicity in microinjected *X. laevis* embryos. Percentages of surviving embryos by the tailbud and tadpole stages are shown (means $\pm$ s.d.; $n = 5$ experiments each performed on 60 embryos). Significance was analyzed by *t*-test. **(b)** Flow cytometry analysis of HeLa cells 48 h after transient transfection with vectors encoding E2-Crimson, mNeptune, Neptune and eqFP650. Fluorescence brightness was normalized to the relative efficiency of excitation by the 488 nm laser line used. Emission was collected at 660–700 nm. Means $\pm$ s.d. are shown ($n = 3$

transfection experiments). **(c)** Normalized fluorescence excitation (solid lines) and emission (dashed lines) spectra for eqFP650 and eqFP670. **(d)** Normalized absorption spectra for eqFP650 and eqFP670. **(e,f)**. Normalized photobleaching curves for eqFP650, eqFP670, mNeptune and E2-Crimson using widefield fluorescence microscopy under metal halide illumination **(e)** or laser scanning confocal microscopy **(f)**. Error bars, s.d. ($n = 4$ experiments).

Figure 2. Whole-mouse imaging with IVIS Spectrum system (Caliper). **(a)** Representative fluorescence reflectance images (excitation filter, 605/30 nm and emission filter, 660/20 nm) of mice injected intramuscularly with HEK 293T cells expressing E2-Crimson, mNeptune or eqFP650. Asterisk denotes background fluorescence in mice injected with E2-Crimson cells. Scale bar shows pseudocolor scale for fluorescence. **(b,c)** Fluorescence efficiency from cell implants imaged with 570/30 nm **(b)** or 605/30 nm **(c)** excitation filters and a range of emission filters, normalized to photons from firefly luciferase to control for transfection efficiency and numbers of implanted cells. Means $\pm$ s.e.m. are shown ($n = 6-10$ per point). $*P < 0.05$ (t -test) for eqFP650 relative to other proteins.

Figure 3.

Whole-mouse infrared imaging with the Biospec system. Infrared fluorescence of subcutaneous injections of equal amounts of protein samples into a living mouse after normalization using the extinction coefficient at the absorption maximum wavelength of each protein. Excitation at 635 nm with photodiodes was used, and emission was measured over the integrated 700–900 nm range. Scale bar, 1 cm.

Table 1

Key characteristics of far-red fluorescent proteins

	mCherry	Katshka	RFP639	E2-Crimson	mNeptune	eqFP650	eqFP670
Excitation peak (nm)	587	588	588	605	599	592	605
Emission peak (nm)	610	635	639	646	649	650	670
Fluorescence quantum yield	0.22	0.34	0.18	0.12	0.18	0.24	0.06
Molar extinction coefficient ($M^{-1} cm^{-1}$) at excitation maximum	72,000	65,000	69,000	58,500	57,500	65,000	70,000
Brightness ^d	15,840	22,100	12,420	7,080	10,350	15,600	4,200
Molar extinction coefficient ($M^{-1} cm^{-1}$) at 635 nm	1,000	1,700	~2,000	12,640	7,900	4,300	15,700
Quantum yield in infrared (700–900 nm)	0.04	0.07	~0.05	0.03	0.05	0.07	0.03
Brightness in infrared ^b	40	119	~100	379	395	301	471
Photostability, widefield ^c	601	ND	ND	19	216	190	1,289
Photostability, confocal ^c	48	77	ND	14	29	67	>700
pKa	4.5	5.5	ND	ND	5.8	5.7	4.5
Reference	9	2	10	8	6	This work	This work

Proteins are ordered according to the position of emission maximum. ND, not determined.

^a Calculated as the product of the molar extinction coefficient and quantum yield.^b Calculated as the product of the extinction coefficient at 635 nm, quantum yield and emission fraction between 700 nm and 900 nm.^c Time to bleach 50% of fluorescence signal brightness.