Far-red fluorescent proteins (FPs) are in a high demand for whole body imaging due to intrinsic optical properties of living tissues, which are almost opaque when illuminated with the wavelength shorter than 600 nm, due to light absorbing properties of hemoglobin and melanin. On the other hand, the wavelengths longer than 1100 nm are absorbed by water, which is always present in the living tissues as well. Thus, the optical window (also called near-infrared region) permitting a deep light penetration in the body is limited by the range of 650 – 900 nm{Konig, 2000 1993 /id}.

Up to date, a number of far-red FPs, approaching the 650 nm barrier, has been generated from various fluorescent and chromoproteins by directed evolution approach (Table 1). The first generation of recombinant far-red FPs includes HcRed{Gurskaya, 2001 152 /id}, mPlum{Wang, 2004 1003 /id}, and AQ143{Shkrob, 2005 143 /id} engineered from *Heteractis crispa* chromoprotein hcCP{Lukyanov, 2000 155 /id}, a monomeric DsRed-like protein mRFP1.2{Campbell, 2002 1034 /id}, and *Actinia equina* chromoprotein aeCP597{Shkrob, 2005 143 /id}, respectively (Table 1). These proteins were successfully demonstrated to be useful for numerous imaging applications{Chudakov, 2005 314 /id;Hoffman, 2005 1445 /id;Hoffman, 2005 1450 /id;Shcherbo, 2007 305 /id}; however, all of them have a relatively low brightness (about ten times lower than that of the enhanced green fluorescent protein (EGFP){Yang, 1996 2376 /id}) limiting their suitability for deep-tissue imaging.

The second generation of far-red FPs was substantially brighter (with the brightness only a half of that of EGFP{Yang, 1996 2376 /id}), but their emission maxima were at best reaching 640 nm (Table 1). Typical members of this group are dimeric far-red FP Katushka{Shcherbo, 2007 305 /id}, its monomeric version mKate{Shcherbo, 2007 305 /id}, and RFP639{Kredel, 2008 2352 /id}. Both Katushka and mKate were generated from *Entacmaea quadricolor* red fluorescent protein eqFP578{Merzlyak, 2007 307 /id}, whereas RFP639 was derived from eqFP578-related protein eqFP611{Wiedenmann, 2002 2025 /id}. The major drawback of the second generation of far-red FPs is their blue-shifted excitation band, impairing their efficient excitation in the living tissue.

The third generation of far-red FPs aptly combines the advantages of both previous far-red FP generations. Their excitation maxima demonstrate bathochromic shift as compared to the first

generation, whereas their brightness is usually higher than that of the second one (Table 1). Among these proteins there are four successors of mKate: mKate2{Shcherbo, 2009 2318 /id}, Neptune{Lin, 2009 2358 /id}, mNeptune{Lin, 2009 2358 /id}, and TagRFP657{Morozova, 2010 2369 /id}; one derivative of DsRed-Express2 – E2-Crimson{Strack, 2009 2359 /id}, as well as two Katushka variants - eqFP650 and eqFP670{Shcherbo, 2010 2374 /id}, ~~the latter~~ described here.

Crystallographic data available for wild type RFPs and their far-red successors indicate that all these proteins have chemically identical chromophores. Therefore, up to 90 nm bathochromic shift of their excitation and emission bands is caused solely by variation of the nearest chromophore environment. The most common reasons of red-shift of the excitation maxima in red FPs are: trans- to cis- isomerization of the chromophore; chromophore deprotonation; appearance of additional π-π stacking interactions between the chromophore and environment, and formation of new hydrogen bonds enabling proton transfer and increasing the chromophore polarization. For example, eqFP578 and eqFP611{Petersen, 2003 1855 /id} contain the trans-form of the chromophore, whereas their far-red successors Katushkaand RFP639{Nienhaus, 2008 2354 /id} contain the cis-form. In both cases, trans-cis isomerization of the chromophore provided by variation of its closest environment resulted in over 25 nm bathochromic shifts of the emission maxima.

The Ser197Tyr substitution in E2-Crimson, that presumably introduced additional π-π stacking interaction between Tyr197 and the chromophore, was reported to be one of the key mutations responsible for red shift of the fluorescence bands in this protein{Strack, 2009 2359 /id}. The crystal structure of Neptune revealed the presence of a new water-mediated hydrogen bond between acylimine oxygen of the chromophore and hydroxyl of Ser28, corresponding to a new way of red-shifting the emission maxima of FPs{Lin, 2009 2358 /id}. Other ways of red-shifting of FPs emission bands are also possible.

To further explore the reasons of bathochromic shift in FPs we have examined the crystal structures of two recently generated far-red fluorescent proteins, eqFP650 and eqFP670. These structures were determined at 1.8 Å and 1.6 Å resolution, respectively, providing detailed views of the environment of the chromophores.