Bright monomeric near-infrared fluorescent proteins as protein fusion tags and biosensor building blocks for in vivo imaging

LEAD INVENTOR: Vladislav V. Verkhusha

TOOL / REAGENT

TECHNLOGY SUMMARY

Commonly used visible range fluorescent proteins (FPs), like GFP and RFP, have a limited range of fluorescence spectrum. Uses of FPs in this spectrum exhibit high background autofluorescence, high light-scattering, and potential cytotoxicity. Currently, conventional FPs in the blue-red optical region are not capable of producing efficient imaging in deep tissues, especially at the whole-body level. Dr. Verkhusha and his team were among the first to develop near-infrared (NIR) from bacterial phytochrome photoreceptors, BphPs. These NIR FPs exploit endogenously available biliverdin (catabolic product of heme) in all eukaryotic cells, including mammalian and human cells, to efficiently absorb and emit light in the NIR tissue transparency window of 650-900 nm. Having longer wavelengths, the NIR FPs are far less phototoxic to the cells and provide a high signal-to-background ratio due to low autofluorescence in the NIR. Using protein engineering, the team developed a series of NIR FP variants, miRFPs that are monomeric and with improved spectral properties. The latest in this evolution of NIR FPs is miRFP670nano, a monomeric FP providing several-fold higher brightness, faster maturation, and higher photostability than other NIR FPs. Moreover, miRFP670nano is the smallest NIR FP currently available (17 kDa); 1.6-fold smaller than GFP-like FPs. In addition, fixation with various reagents minimally affects the miRFP670nano brightness. The team successfully tested miRFP670nano in vitro and in vivo mouse model as shown in the Figure. Additional applications include using NIR FPs simultaneously with GFP-like FPs as biosensors or protein tags in a crosstalk-free state.

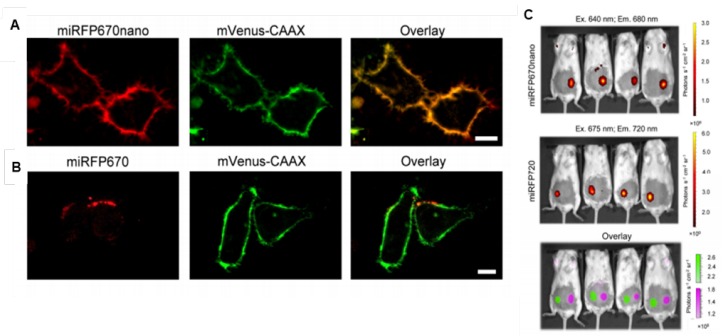

Figure: Internally inserted fluorescent tag between the helical and GTPase domains of the G protein alpha subunit. A. miRFP670nano and B. miRFP670 are shown with mVenus for membrane targeting CAAX motif. C. Characterization of two-color imaging of miRFP670nano and miRFP720 in vivo. Fluorescence images of living mice injected with HeLa cells expressing miRFP670nano and miRFP720 and its overlay are shown. The fluorescence images were obtained with excitation at 640 nm and emission at 680 nm for miRFP670nano and with excitation at 675 nm and emission at 720 nm for miRFP720.

APPLICATIONS

- Reporter for NIR live-cell microscopy and real-time whole-body fluorescence imaging
- Fusion tag for protein localization, including a unique internal protein tagging
- NIR reporter for protein-protein interaction (via fluorescence resonance energy transfer: FRET)
- Building block for activity measurement NIR biosensors Spectral multiplexing with blue-green-red GFP-like FPs, as well as opsin optogenetic tools
- NIR-light absorbing genetically encoded probe for photoacoustic tomography
- NIR live-cell fluorescence-activated cell sorting (FACS)

ADVANTAGES

- Less interference with protein folding, cellular functions and interactions due to small size
- High penetrance enables deep-tissue imaging
- Minimal autofluorescence
- Eliminates phototoxicity
- Superior brightness and protein stability in vivo and in vitro upon cell or tissue fixation
- Replicate coloring ability of existing visible range fluorescent proteins
- Due to closely located termini allows insertion into loops of tagged protein (internal tagging)

STAGE OF DEVELOPMENT

- Proof of concept, efficacy successfully tested *in vitro and in vivo on* live mice
- Developing additional fluorescence protein variants that are more NIR-shifted

RELEVANT LITERATURE

- Matlashov, M.E., et al, Nat Comm (2020)
- Oliinyk, O.S., et. al, Nat. Comm(2019)
- Shcherbakova, D.M. et al., Trends Biotechnol, (2018)

INTELLECTUAL PROPERTY

US Patent Application:

- 17/115,223

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Single component near-infrared optogenetic system for precise regulation of protein and gene function in whole live animal

LEAD INVENTOR: Vladislav V. Verkhusha

TOOL / REAGENT/ DIAGNOSTICS

BACKGROUND/UNMET NEED

Conventional optogenetic tools exploit blue-green light-controlled fluorophores that exhibit high background autofluorescence, phototoxicity, and scattering. Expressing these fluorophores within cells and tissues requires multidomain components. Packaging, transfecting, or transducing these multidomain components is highly cumbersome and challenging, severely limiting expression in vivo. Often these bulky fluorescent proteins interfere with protein folding, activity, and translocation intracellularly.

SOLUTION

Dr. Verkhusha and his team, with years of expertise in engineering near-infrared fluorescent proteins (NIR-FPs), have developed multi-generation optogenetic systems. The latest is a next-generation system, called iLight, a highly sophisticated first-in-class single-component system. iLight is an engineering marvel derived from wildtype IsPadC phytochrome through random mutagenesis and high-throughput screening. iLight is capable of self-oligomerization upon incidence of light, thereby drastically reducing components. In contrast, the 1st-generation (1st-gen) multi-component system uses BphRs and its natural binding partner, PpsR2. The two approaches differ in their respective activation wavelengths; iLight is activated at 630 -680 nm (far-red zone) whereas BphRs-PpsR2 at 740 - 780 nm (near-infrared).

Nevertheless, both systems enable precise regulation of a myriad of molecular functions; protein translocation, protein-protein interaction, regulating gene transcription, protein synthesis, and enzyme activation in vivo within mammalian cells and deep tissues of live animals. However, iLight supersedes the rests in the degree and efficiency of the regulation achieved. In addition, a clear advantage of iLight is its enhanced compatibility with the AAV vector system, which makes it amenable for gene and CAR-T therapy within deep tissues of whole live animals. As a proof of concept, the team successfully tested iLight in vitro and in vivo in live mouse models to accurately turn on and off gene transcription in a tissue-specific manner. In brief, iLight has tremendous potential as a next-generation optogenetic tool in basic research, R&D for drug discovery, and application in therapeutics

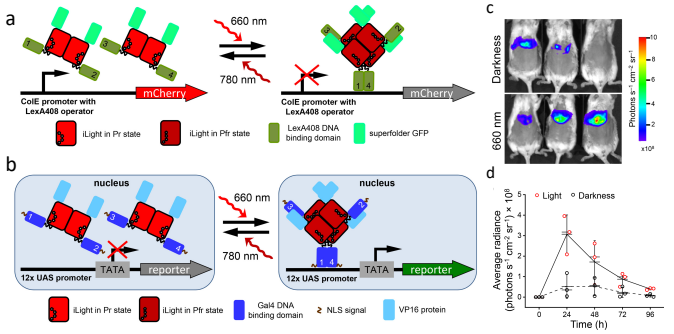

Figure: iLight-induced gene transcriptional activation. (a) Proposed model implemented in iLight system. (b) NLS-Gal4-DBD-iLight-VP16 and pG12-RLuc8 plasmids hydrodynamically co-transfected into mice liver cells. (c) RLuc8 luciferase reporter signals detected in mice kept in either darkness (top) or illuminated with 660 nm light for 48 h (bottom) are shown. (d) Kinetics of the RLuc8 reporter expression in mice shown in 'c', kept in darkness or illuminated for up to 96 h. Box plots show the median (center line), first and third quartiles (box edges), 1× the standard deviation (whiskers) and individual data points. n=3 individual animals.

APPLICATIONS

- Optogenetic applications including protein-protein interaction , protein localization
- Transcriptional activation/repression of gene in deep tissues of whole live animals
- Activation/inhibition of protein activity in deep tissues of whole live animals
- Spectral multiplexing with blue-green-red GFP-like FPs, as well as opsin optogenetic tools
- Light-controlled Induction of translocation and targeting of intracellular protein translocation
- iLight could be potentially use in gene therapy and CAR-T therapy
- 1st-gen system could be highly desirable in regulating gene function in plants
- Building block for NIR biosensors of various activity measurements using 1st-gen system

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ADVANTAGES

1st-generation optogenetic system

- Activation at near-infrared region ensures minimal background and phototoxicity
- Near-infrared enables high penetrance and regulation within deep tissue
- System of choice for manipulating or genetically altering plants

iLight system

- Supersedes in degree and efficiency of regulation achieved in vivo over other systems
- Activation at far-red ensures minimal background in the dark
- Enhanced efficiency of targeting cells and tissues due to reduced components
- Reduced components makes it compatible to AAV system

TABLE Comparison of the two systems

Properties	1st-gen (BphRs-PpsR2)	iLight
Monomer self-oligomerization	-	√
630-680 nm	-	√
740-780 nm	√	-
Deep tissue, cell imaging	√	√
Effective in presence of plant chromophores	√	-

STAGE OF DEVELOPMENT

- Proof of concept successfully tested in cell line and mouse model for:
  1. Protein activation
  2. Translocation
  3. Gene transcription

RELEVANT LITERATURE

- Kaberniuk et al, Nat Comm (2021)
- Redchuk et al., Nat Commun (2020)
- Redchuk et al., Nat Protoc (2018)
- Redchuk et al., Chembiochem (2018)
- Kaberniuk et al., Nat Methods (2016)

INTELLECTUAL PROPERTY

Issued US Patents:
- 10968256

Patent Pending:
- 63/190,540

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Near Infra-red light inducible nanobodies with diverse utilities

INVENTOR: Vladislav V. Verkhusha; Olena S. Oliinyk

TOOL / REAGENT/ DIAGNOSTICS

BACKGROUND/UNMET NEED

Nanobodies (Nbs) are touted as the next set of promising molecules because of their simplicity, high specificity, and small size (~15 kDa). Nanobodies when fused with fluorescent proteins (FP), form a formidable tool for optical imaging with myriad of potential applications, including as biosensors for detecting rare endogenous proteins, as markers for visualizing protein dynamics, and as regulators for manipulating signaling and gene expression. However, realization of full potential of fluorescent-Nb (FP-Nb) is severely limited by multiple factors including (1) high noise-to-signal ratio resulting from excess of unbound FP-Nbs, and (2) a limitation of potential uses for GFP or RFP as a result of poor tissue penetration, autofluorescence, and interference with protein folding and function.

SOLUTION

Dr. Verkhusha, an expert in the field of protein engineering, developed a new class of Nbs that are stable when bound and instable and rapidly degrading when unbound. To enable use of these Nbs as a robust tool for in vivo and in vitro manipulation of targets, they were genetically fused with next generation, highly optimized near infrared-FP (NIR-FP), called miRFP670nano. miRFP670nano is a powerful fluorescent protein, given it's small size (17 kDa), higher brightness, faster maturation, and enhanced photostability. The final products of such fusions are highly sophisticated NIR-Nbs that are highly specific, readily degradable, and allow deep tissue imaging at whole animal level with great resolution. In proof-of-concept studies, the team demonstrated that these NIR-Nbs efficiently enabled re-coloring of cellular structures and labelling and visualization of endogenous proteins in a background-free manner. In a further enhancement, the team also developed bi-specific NIR-Nbs. Together, these mono and bi-specific NIR-Nbs offer immense potential for use as tools in research, drug discovery and development and therapeutics. The table below summarizes the full spectrum of utilities and applications for these mono and bi-specific miRFP670-nanobodies.

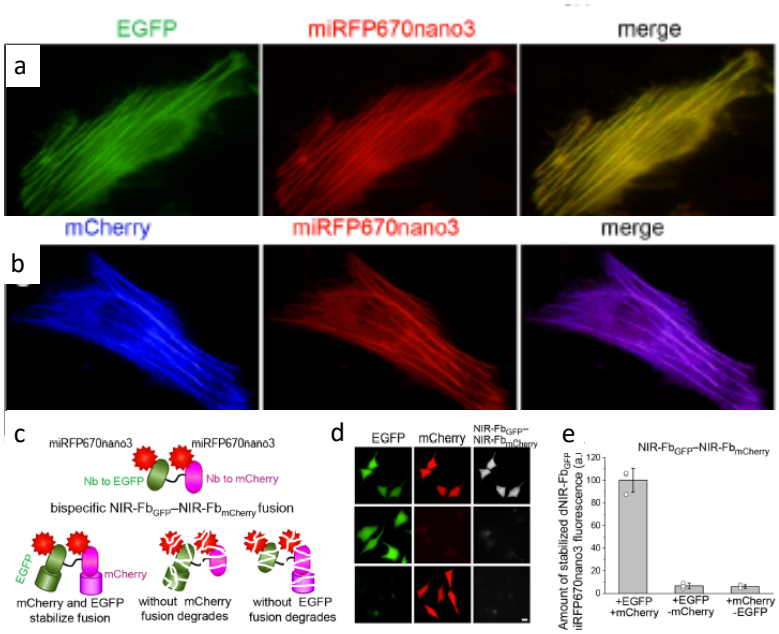

Figure: Demonstrates spectral multiplexing, where actin filaments already labelled with EGFP (a) or with mCherry (b) was recolored using NIR-Nb (NIR is miRFP670nano, red). Recoloring of same target organelle simultaneously at two different spectral ranges were possible due to use of Nbs that are specific to either EGFP (NIR-NbEGFP) or mCherry (NIR-NbmCherry) in panels (a) and (b) respectively. (c) Schematic showing the principle of destabilization and degradation of target antigen using bi-specific NIR-Nb that targets both EGFP and mCherry (NIR-FbGFP-NIR-FbmCherry). (d) In the proof of principle study, HeLa cells were transiently co-transfected with bothe EGFP and mCherry constructs (top row) or EGFP alone (middle row) and mCherry alone (bottom row). By controlling the availability of at least one cognate antigen (EGFP or mCherry) in the cell, the stability of the whole fusion NIR-FbGFP-NIR-FbmCherry can be degraded (middle and bottom row), but it is stabilized in presence of both cognate antigens (top row). (e) It is quantitative measure of the effect in (d).

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Near Infra-red light inducible nanobodies with diverse utilities

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TABLE **Comparing mono and bi-specific miRFP670-nanobodies**

Utilities/Applications	Mono-FP-Nbs	Bi-FP-Nbs
Number of antigens recognized simultaneously	1	2
High specificity and affinity	✓	✓
Allosterically unstable when unbound	✓	✓
Deep tissue penetration for optical manipulation	✓	✓
Whole animal imaging	✓	✓
Re-coloring of cellular structures	✓	✓
Labelling and visualization of endogenous proteins	✓	✓
Dual labelling of cellular structures and proteins	-	✓
Destabilizing, degrading target antigens or proteins	-	✓
Regulation of gene expression	✓	✓
Regulation of Kinase activities	-	✓
Use as biosensors	✓	✓
Protein dynamics	✓	✓
Targeting pathogenic antigens (oncogenic, viral etc)	-	✓
Allows spectral multiplexing with GFP, RFP, YFP etc	✓	✓

ADVANTAGES

- Small size (32 kDa): Nanobody (15 kDa) + miRFP670 (17 kDa)
- Small size reduces interference with target protein localization and function
- Universal design allows developing wide array of nanobodies against various intracellular antigens
- Activation at NIR region ensures minimal background and phototoxicity
- NIR light allow crosstalk free spectral multiplexing with violet-blue-green-orange light, which is widely used for optogenetic activation
- Compatible with AAV gene delivery vectors for targeted gene therapy
- High affinity and specificity reduce any off-target effects
- High signal to noise ratio with minimal background

STAGE OF DEVELOPMENT

- Proof of concept successfully tested in cell line and mouse model for:
  1. Protein activation
  2. Translocation
  3. Gene transcription

RELEVANT LITERATURE

- Matlashov, M.E., et al, Nat Comm (2020)
- Oliinyk, O.S., et. al, Nat. Comm (2019)
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Far red-light inducible chimeric receptor tyrosine kinases for precise regulation of intracellular function

LEAD INVENTOR: Vladislav V. Verkhusha; Anna V. Leopold

TOOL / REAGENT/ DIAGNOSTICS

BACKGROUND/UNMET NEED

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors that transmits signal across the cell membrane from its extracellular surface to interior. They regulate various cellular physiologies, including cell survival, growth, and proliferation. Consequently, RTKs are critical targets for therapeutic intervention due to their role in health and disease, namely, cancer, inflammation, diabetes and neurological disorders. Attempts to regulate RTKs by growth factor replacement are fraught with off-target effects. While currently engineered optogenic-RTK (opto-RTK) regulated by blue light fall far short of expectation due to issue with poor tissue penetrance and background noise.

TECHNOLOGY

Dr. Verkhusha, a pioneer in the field of engineering of fluorescent proteins and biosensors for super-resolution microscopy and deep-tissue imaging, and his group, have over the years discovered and developed near infra-red (NIR) and far-red (FR) fluorescent proteins with superior spectral properties. The latest addition is a series of chimeric opto-RTKs (opto-cRTKs) that remain completely inactive in darkness but activated upon stimulation by FR light. This feature makes them suited for all in vivo applications and manipulations. The optical part is made of photosensory core module (PCM) derived from Deinococcus radiodurans (DrBphP) bacterium. The team engineered a novel chimeric receptor that targeted DrBphP-PCM to the cell surface. Upon stimulation by FR light, the extracellular PCM-module underwent conformational changes that was transmitted via the EGFR transmembrane helices to the cytoplasmic RTK domains. The transmission of the optogenic signal from the cell exterior resulted in the re-orientation and trans-phosphorylation of the cytoplasmic RTK domains. Using the first generation of opto-cRTKs receptors, eDrEGFR and eDrHER2, the team successfully showed that both are capable of robust phosphorylation of RTK domains, components of the ERK 1/2 pathway, to drive ~30 and ~100-fold higher luciferase reporter expression. Remarkably, such activation is completely reversible attesting to the precise control. The second generation opto-cRTKs were modified by swapping the RTK domain of the chimeric opto-RTKs with fragments from FGFR1, TrkA, TrkB, c-Met, IIR-1, c-KIT or IR-1 receptors. Of these, eDrTrkB was utilized to demonstrate spatiotemporal regulation of cellular physiology in mouse cerebral cortex using AAV system. Significantly, the eDrTrkB expression enhanced wakeful non-REM (NREM) sleep following exposure to FR-light. These FR light inducible opto-cRTKs with their precise reversible regulation of cellular signaling, low background and high tissue penetrance are promising tools with myriad of applications in disease understanding, drug development and therapeutics.

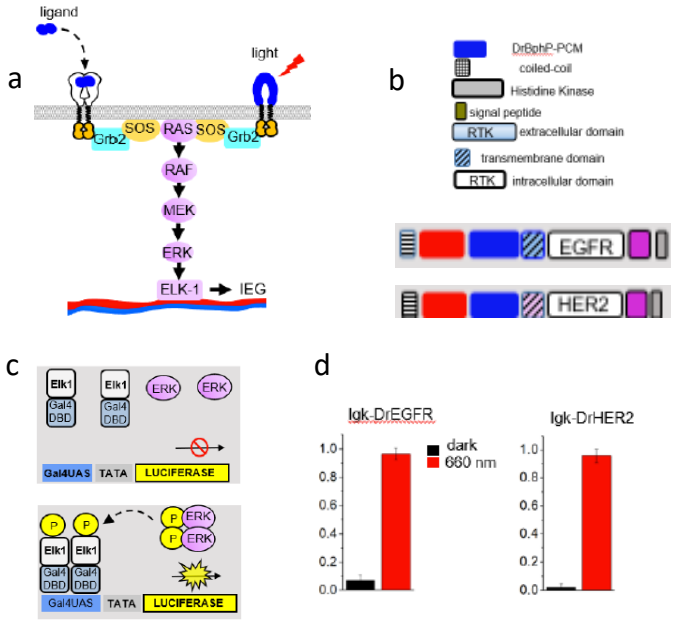

Figure: iLight-induced gene transcriptional activation. (a) Proposed model implemented in iLight system. (b) NLS-Gal4-DBD-iLight-VP16 and pG12-RLuc8 plasmids hydrodynamically co-transfected into mice liver cells. (c) RLuc8 luciferase reporter signals detected in mice kept in either darkness (top) or illuminated with 660 nm light for 48 h (bottom) are shown. (d) Kinetics of the RLuc8 reporter expression in mice shown in 'c', kept in darkness or illuminated for up to 96 h. Box plots show the median (center line), first and third quartiles (box edges), 1× the standard deviation (whiskers) and individual data points. n=3 individual animals.

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Far red-light inducible chimeric receptor tyrosine kinases for precise regulation of intracellular function

TOOL / REAGENT/ DIAGNOSTICS

APPLICATIONS

- Regulation of gene expression that are targets of RTK signaling cascade
- Regulation of protein activity in cell lines and in deep tissues of live animals
- Stably transfected cell lines for high-throughput screening of drugs that targets RTKs or its pathway components
- Develop transgenic mouse models of human diseases with spatiotemporal precision for drug development
- Ability to potentially replace growth factors in immunologically privileged tissues like tumor, brain, eyes
- Ability to potentially correct disease condition when delivered to target tissues using AAV system
- Design and functionality of opto-cRTKs can be potentially applied to other transmembrane receptors including TCR, Toll-like receptors, ILRs

ADVANTAGES

- Activation at far red region ensures minimal background and phototoxicity
- Far red activation enables high penetrance and regulation within deep tissue
- FR light allows crosstalk free spectral multiplexing with violet-blue-green-orange light, which is widely used for optogenetic activation of channel-rhodopsins
- Compatible with AAV gene delivery vectors for targeted gene therapy
- Exhibits excellent photoactivation contrast. Prolonged incubation in darkness (from 24 h to weeks in vivo) does not result in the non-specific activation
- Minimal non-specificity and devoid of any off-target effects as it is not activated by any growth factors
- Readily available, enhanced and fully optimized constructs for 9 different RTKs with roles in cancer, neurological disorders, inflammation and stemness

STAGE OF DEVELOPMENT

- Proof of concept successfully tested in cell line and mouse model for:
  1. Protein activation
  2. Translocation
  3. Gene transcription

RELEVANT LITERATURE

- Leopold et al., Chem Sci. 2020
- Leopold et al., J Mol Biol. 2020
- Redchuk et al., Nat Commun. 2020

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