De Novo Design Of Bright And Multi-Color Luciferases For Bioimaging

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BACKGROUND

Bioluminescence technology offers highly sensitive and non-invasive imaging in living organisms without the need for external excitation. Naturally occurring luciferases, the enzymes responsible for catalyzing light emission, constrained the full potential of luminescence technology for the past several decades due to their poor protein folding, large size, ATP dependency, and low efficiency.

Creation of the next generation of luciferases required breaking free of evolutionary constraints. This work describes the creation of novel bioluminescent enzymes that surpass qualities of native luciferase using AI-powered de novo protein design. These designer luciferase catalysts enable genetic labeling across molecular, cellular, and individual levels in a multiplexed manner, using the same underlying technology.

This advancement showcases the design of efficient enzymes from scratch in which our de novo luciferases will enable researchers to study complex biological phenomena effectively.

In the last three decades, the development of fluorescent protein families has brought a revolution in the way researchers study biological processes in living cells. However, the dependency on external excitation for FPs introduces inherent drawbacks, such as phototoxicity and autofluorescence background. These especially limit the applications for fluorescent proteins in vivo. Bioluminescence technologies, which rely on an enzyme-catalyzed chemiluminescent reaction of a chromophore substrate to emit photons without the need for external light sources, circumvent these limitations and offer several orders-of-magnitude-higher sensitivity than fluorescence for macro-scale imaging.

Practically implementing luciferases as general molecular proges has not progressed as far as fluroescent proteins due to a number of factors. Firefly luciferase (FLuc) is used widely for in vivo imaging, but it is dim, large (61 kDa), and ATP dependent. Gaussia luciferase (GLuc) is brighter than FLuc, but has five disulfide bonds and therefore cannot be used intracellularly. It is also prone to misfolding. Engineered variants of Renilla luciferase (RLuc) and Oplophorus Luciferase (NLuc) are brighter and more stable, but they emit blue light and have poor substrate specificity and therefore are difficult to used in multiplexed applications.

LuxSit luciferase (Monod Bio Inc.) is the first de novo designed luciferase and has superior folding fidelity and stability to natural luciferases, but more de novo luciferase species are necessary to meet the needs of researchers.

TECHNOLOGY DESCRIPTION

Andy Yeh's lab at UC Santa Cruz used deep-learning-based protein design methodologies were used to sample both the sequence and conformational spaces of de novo-designed luciferases to create a new class of luciferase catalysts, the neoLux series, with superior properties over native luciferases. These features include robust folding, extreme thermostability, compact size, ATP independence, higher catalytic efficiency, and unique substrate orthogonality. In addition, highly efficient neoLux-fluorescent protein FRET fusions for excitation-free multiplexed luminescence imaging in cells and mice were developed and have been demonstrated.

The neoLux series provides 10x greater brightness than LuxSit while maintaining compact size, stability to 100°C, ATP independance, and high substrate specificity. In addition, neoLux fusions with flourescent proteins were developed to expand the number of wavelengths of ligth emitted via Foerster resonance energy transfer (FRET). This allows for multiplexed imaging in cells and in vivo.

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OTHER INFORMATION

KEYWORDS

luciferase, bioluminescence, de novo protein design, multiplex luciferase, expression systems, FRET pairs

CATEGORIZED AS

- **Research Tools**
  - Expression System
  - Reagents
  - Screening Assays

RELATED CASES

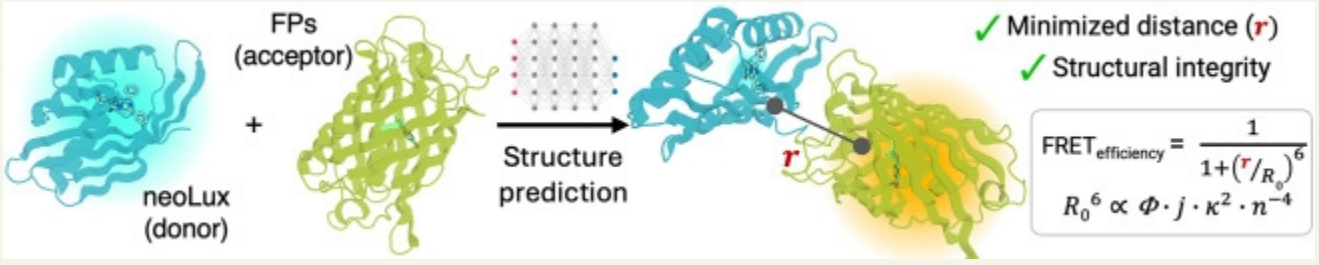
2024-799-0

Protein design resulted in the expression and testing of six designed sequences. Two of these displayed more than 10-fold higher activity than the commercially available designed luciferase LuxSit-i. One of these designs showed a monodisperse and monomeric SEC trace. This newly designed sequence was called neoLux1. A V83L was interoduced into the pocket of neoLux 1, resulting in an additional 47% increase in luciferase activity. This mutated form of neoLux1 was called neoLux1.2.

NeoLux1 and neoLux1.2 are 13.7 kDa each - smaller than any commonly used luciferase. They are highly soluble and well expressed in E.coli with monodispersed and monomeric folding. They have a 10- and 15-fold improvement in maximum brightness compared with LuxSit-i with neoLux 1.2 displaying excellent substrate specificity for the diphenylterazine (DTZ) luminescence substrate. NeoLux 1.2 remains structural integrity at 95ºC and can refold upon cooling to 25ºC. Other native or engineered luiferases are irreversibly unfolded upon exposure to high temperatures. NeoLux 1.2 also maintains its activity across a broad range of temperatures - with 83% activity even after exposure to 100°C for one hour.

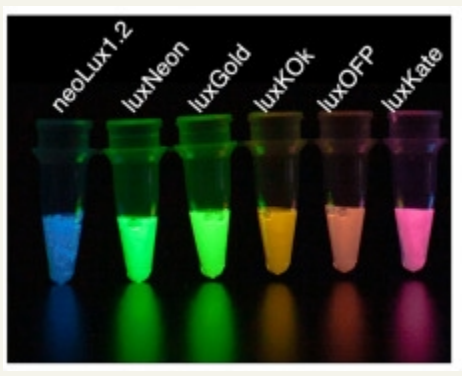
NeoLux 1.2 also offers favorable signal decay kinetics. RLuc, NLuc, and GLuc, have signal decay half lives that span ~ 5-15 minutes, neoLux 1.2 has an extended decay half life that lasts approximately 43 minutes in a standard buffered saline solution.

NeoLux 1 andNeoLux 1.2 are also efficient energy donors in FRET systems when paired with fluorescent proteins. These could result in a wider color palette of bioluminescent proteins than are currently available.



AlphaFold2 was used to predict the structure of putative neoLux1.2-fluorescent protein fusions as well as the optimal distances between the neoLux1.2 FRET donor and the fluorescent protein FRET acceptor to engineer linker lengths. The fluorescent proteins mNeonGreen, MGold, mKOk, CyOFP1, and mKate2 were selected as the optimal FRET acceptors. Linker libraries were screened to properly place the neoLux1.2 and fluorescent proteins for optimal distance and folding efficiency.

This screen resulted in five FRET pairs: lux-Neon, luxGold, luxKOk, luxOFP, and luxKate, each of which is capable of emitting more light than previously reported FRET pairs using native or engineered luciferases.



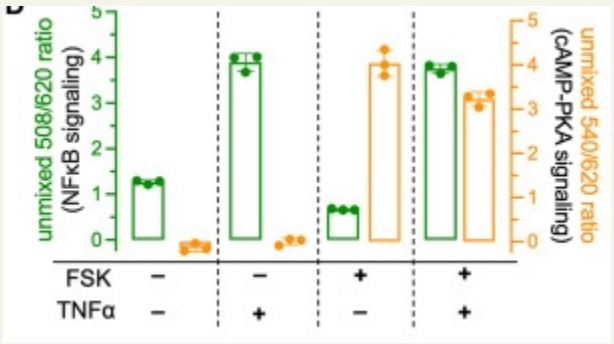
The above photo was taken with a standard smartphone. All FRET pairs are brighter than or equivalent to neoLux1.2 itself with similarl Km values and signal decary half lives compared with neoLux 1.2, indicating that the FRET fusions did not impact fluorescent protein folding or luciferase activity.

These FRET pairs can therefore be used in multiplexed cellular imaging - potentially more readily than with fluorescence due to the narrower emission spectra of the FRET pairs. All of these FRET pairs were expressed in HeLa cells and successfuly used to perform live cell imaing at single cell resolution. Each emission can be effectively separated using conventional filters.

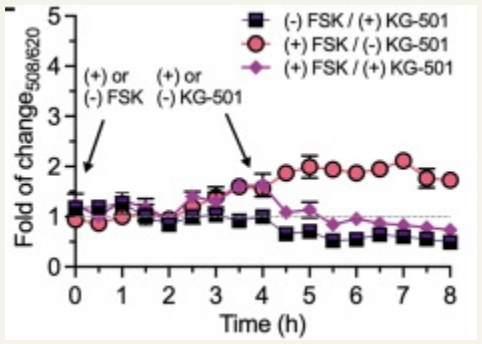
These new FRET pairs can also be used in dual luciferase assays. In a typical dual luciferase assay, FLuc and RLuc are coexpressed in cells and used as an experimental reporter and internal control. The assay requires the stepwise addition of two luciferin substrates and quenching procedures. In contrast, the newly described FRET pairs emit distinct signals that can be readily separated using optical filters. As a result, both reporter (e.g. luxNeon or LuxGold) and reference (luxOFP) signals can be acquired simultaneously with the same luciferin substrate. And since all FRET pairs are based on neoLux1.2, there is minimal difference in signal intensity, emission kinetics, buffer

conditions, etc.

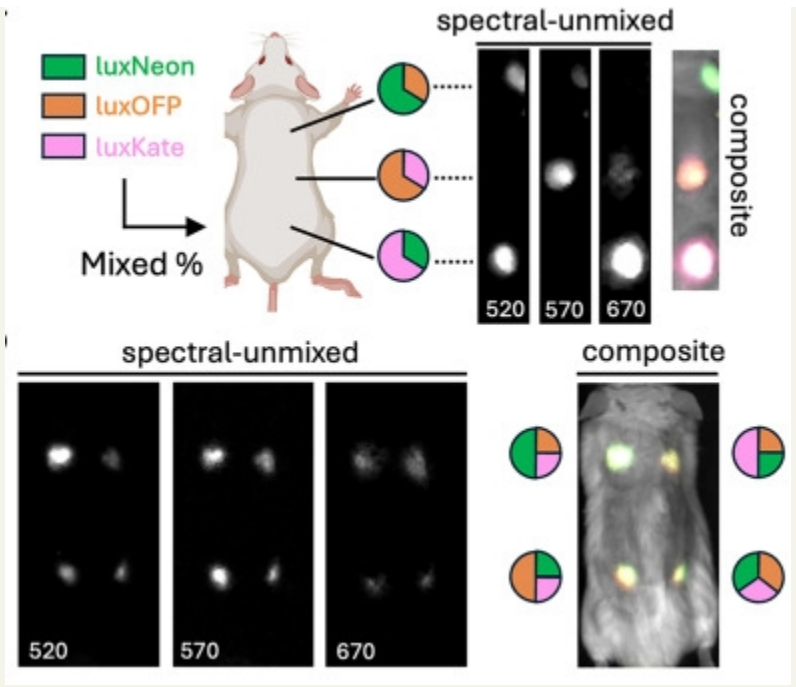
Because signals from e.g. luxNeon and luxGold can be spectrally unmixed using the correct filters, a triple luciferase format that allows concurrent quantification of at least two cellular pathways along with an internal reference is enabled.

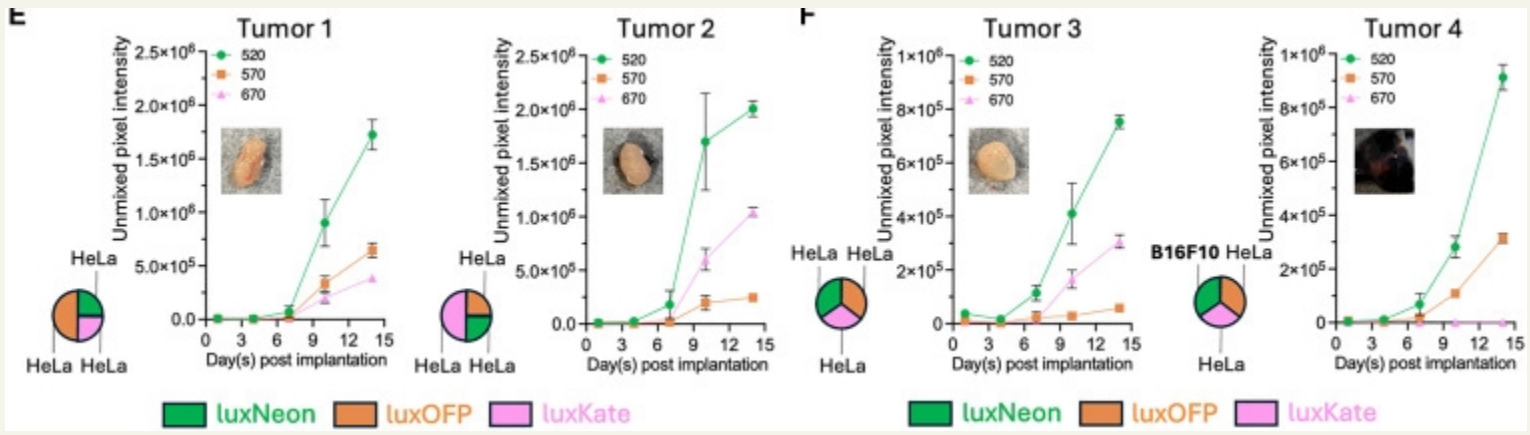


The cell permeable nature of the DTZ substrate allows continuous monitoring of expression in live cells. For instance, a recording of dynamic changes in the cAMP-PKA signaling with PEST-tagged luxNeon (as the reporter with increased degradation) and CMV-luxOFP (as the reference) was performed. The pathway activation was observed over extended durations after the addition of forskolin (FSK). The luciferase level dropped after treatment with KG-501, an inhibitor that disrupts the cAMP response element binding protein (CREB) and binding protein (CBP) complex formation.



Finally, the new FRET pairs enable superior multiplexed in vivo imaging. Unlike other commercially available luciferases, the new FRET pairs are specific only to the synthetic luciferin DTZ. So multiple ATP independent luciferases can be used in a single animal, mitigating imaging biases. To demonstrate this, two- and three- population mixed HeLa cells with different proportions of cells labeled with luxNeon, luxOFP, or luxKate were xenografted onto mice. Heterogenous tumors with varying cell populations were easily discerned after spectral unmixing. Gorowth of each population could be monitored over time and the mixtures could be simulatenously spectrally resolved at the same location during a single imaging event.





These brighter, more stable, and now multicolored luciferase enzymes that were all engineered to work on the same substrate, open up a wide range of possiblilities for cellular research. While this work shows their impact upon typical biological assays using conventional luciferase, more uses will be developed to meet current needs.

APPLICATIONS

- Improved, simpler to perform, dual luciferase assay
- Triple luciferase assay
- In vivo monitoring of tumor populations in vivo in real time
- Multiplexed cellular imaging
- Continuous monitoring of gene expression in live cells in real time
- Multiplexed monitoring of gene expression in live cells in real time.

ADVANTAGES

- NeoLux 1.2 is small, bright, stable, and heat resistant.
- It has an extended decay half life of (over 40 minutes) relative to other natural or engineered luciferases.
- It is highly specific for its substrate (DTX).
- Because of all these characteristics it was used to create a series of fusion proteins with fluorescent proteins and is a near perfect FRET partner. Five FRET pairs with neoLux 1.2 were generated, each glowing in a different color with the same intensity as neoLux1.2. All of them have the same characteristics as neoLux1.2 with regard to decay half life, ATP independence, specificity, for DTX, stability, etc.
- NeoLux 1.2 has been shown to work as well or better than other luciferases in common assays and enables other assays that were impossible before this (e.g. a triple luciferase assay).

RELATED MATERIALS

- De novo luciferases enable multiplexed bioluminescence imaging - 03/13/2025

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