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Communication

Bioluminescence Resonance Energy Transfer (BRET)-Mediated Protein Release from Self-Illuminating Photoresponsive Biomaterials

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ABSTRACT: Phototriggered release of various cargos, including soluble protein factors and small molecules, has the potential to correct aberrant biological events by offering spatiotemporal control over local therapeutic levels. However, the poor penetration depth of light historically limits implementation to subdermal regions, necessitating alternative methods of light delivery to achieve the full potential of photodynamic therapeutic release. Here, we introduce a strategy exploiting bioluminescence resonance energy transfer (BRET)—an energy transfer process between light-emitting Nanoluciferase (NLuc) and a photosensitive acceptor molecule—to drive biomolecule release from hydrogel biomaterials. Through a facile, one-pot, and high-yielding synthesis (60–70%), we synthesized a heterobifunctional ruthenium cross-linker bearing an aldehyde and an azide (CHO-Ru-N_3), a compound that we demonstrate undergoes predictable exchange of the azide-bearing ligand under blue-green light irradiation (>550 nm). Following site-specific conjugation to NLuc via sortase-tag enhanced protein ligation (STEPL), the modified protein was covalently attached to a poly(ethylene glycol) (PEG)-based hydrogel via strain-promoted azide–alkyne cycloaddition (SPAAC). Leveraging the high photosensitivity of Ru compounds, we demonstrate rapid and equivalent release of epidermal growth factor (EGF) via either direct illumination or via BRET-based bioluminolysis. As NLuc-originated luminescence can be controlled equivalently throughout the body, we anticipate that this unique protein release strategy will find use for locally triggered drug delivery following systemic administration of a small molecule.

Stimuli-responsive biomaterials offer substantial promise in controlled small molecule and protein therapeutic delivery, alleviating challenges of systemic drug administration through suppressed off-targeted interactions, immunoprotection, and prolonged cargo activity.[1] While engineered platforms responsive to endogenous cues (e.g., pH,[2,3] enzyme,[4] reactive oxygen species[5,6]) are useful for disease-directed delivery, those that respond to exogenous cues (e.g., light,[7] temperature,[8] ultrasound,[9,10] electromagnetic fields[11]) can afford user-defined release; the extent of active therapeutic release from these systems can be advantageously specified in time and space.

Light-responsive biomaterials have gained substantial interest for controlled drug delivery.[12] By molecularly fusing cargo to a stable biomaterial through a photolabile linker, bioactive therapeutic release can be near-instantaneously and bioorthogonally triggered via cytocompatible light exposure.[13−17] Though such systems have found substantial use for *in vitro* study, light's comparatively poor penetrance through tissue dramatically limits its *in vivo* utility; only optically accessible regions typically <1 cm from the light source can be manipulated. Ongoing efforts in the community seek to create materials that respond to low-energy light (e.g., visible, infrared) that better penetrates tissue.[18−20]

Complementing chemical efforts to make increasingly photoresponsive materials are technological advances in light delivery infrastructure, in the form of LED scopes, light guides, and implantable LED devices.[21] While these methods have brought significant advancement toward deploying light-sensitive technologies in the clinic, they require the development of sensitive chemistries alongside invention of new bioelectronics. An ideal system would eliminate the need for external illumination altogether, while retaining the powerful dose timing and magnitude control that light offers.

Seeking to develop a photoresponsive material platform that could be theoretically controlled anywhere in the body and without specialized equipment, we sought to employ bioluminescence resonance energy transfer (BRET)—an energy transfer process between a light-emitting luciferase and a photosensitive acceptor molecule—to drive biomolecule release from hydrogel biomaterials. Though BRET has not been previously utilized for material modulation, BRET-based photocleavage events have been previously reported via a mechanism termed *bioluminolysis* for the uncaging of bioactive molecular probes.[22−25] We hypothesized that bioluminolysis would be a powerful mechanism for chemo-optically triggered protein release from biomaterials.

Toward creation of self-illuminating photoresponsive biomaterials, we turned our initial focus toward the develop-

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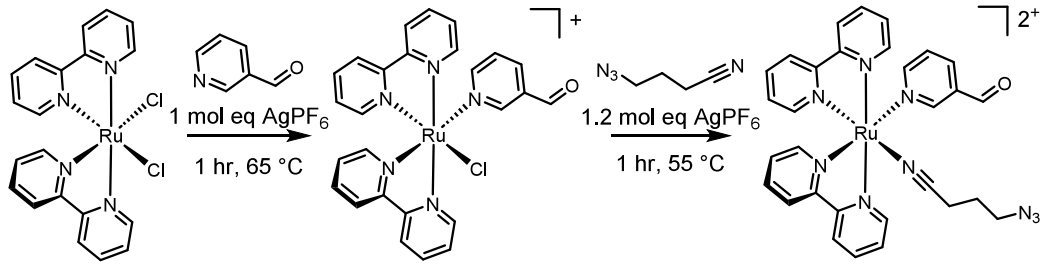
Scheme 1. One-Pot Synthesis of CHO-Ru-N_3, the Photocleavable Crosslinker Used in This Work



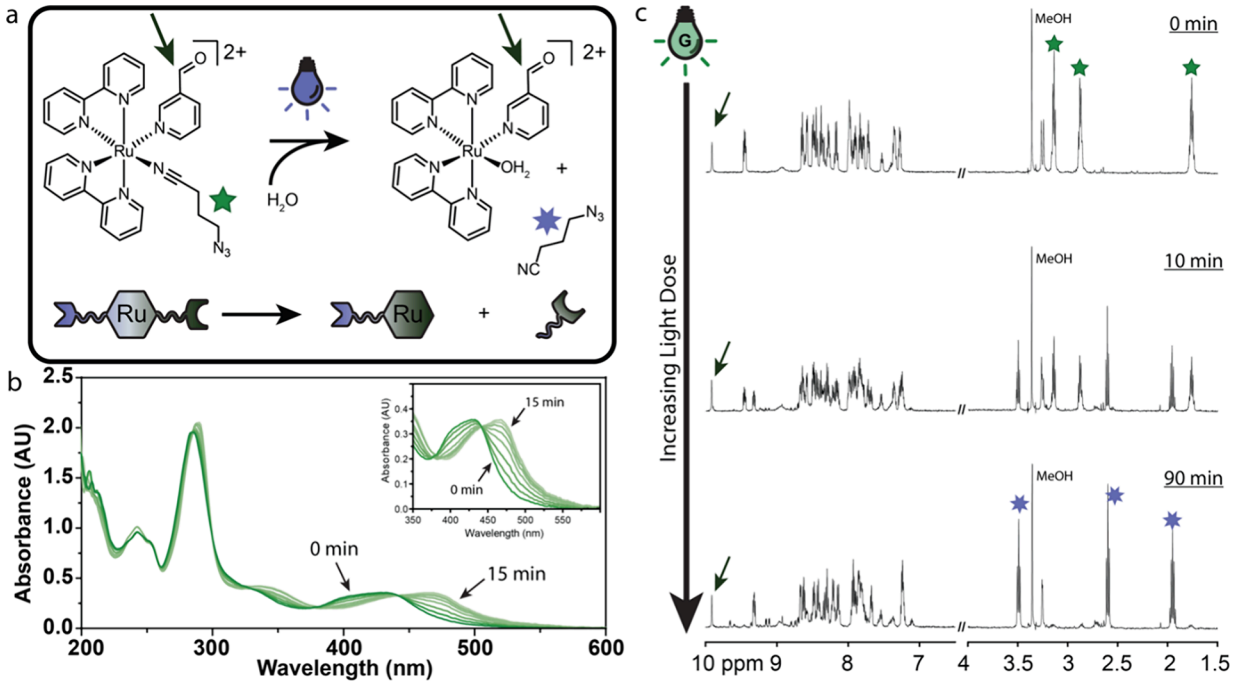


Figure 1. Photolysis of CHO-Ru-N_3 yields preferential exchange of nitrile-based ligand. (a) Scheme of photolysis of CHO-Ru-N_3. (b) UV−vis spectra of photolysis showing a single isosbestic point at 442 nm. (c) ^{1}H NMR tracking photolysis of CHO-Ru-N_3 shows clean exchange of coordinated 4-azidobutyronitrile ligand (green five-point stars) for soluble ligand (purple seven-point stars), while the aldehyde-associated proton (black arrow) remains stable. ^{1}H NMR was performed in D_2O, [CHO-Ru-N_3] = 3.7 mM. Sample was mixed thoroughly throughout photolysis (520 nm, 10 mW cm^{-2}).

ment of a heterobifunctional ruthenium polypyridyl complex to act as the photocleavable cross-linker. Critically, ruthenium polypyridyl complexes are uniquely highly sensitive to visible light exposure,[26−31] wavelengths that are readily accessed through bioluminescence. In aqueous environments, photolysis proceeds through a cytocompatible and radical-free ligand exchange with water. While photolabile homobifunctional linkers have been well-explored in the literature,[32−35] heterobifunctional linkers—those that can link species in a defined way and with a cleavage site that can be precisely engineered—are far less common. Seeking to eventually link protein to a hydrogel using reductive amination and strain-promoted azide−alkyne cycloaddition (SPAAC), we targeted a species bearing both aldehyde (CHO) and azide (N_3) functionalities.

[Ru(2,2′-bipyridine)$_2$(3-pyridinealdehyde)(4-azidobutyronitrile)][Cl]$_2$ (CHO-Ru-N_3) was synthesized by multiple steps in one reaction, followed by purification via silica column chromatography (Scheme 1, Figure S1−S2). In brief, Ru(2,2′-bipyridine)$_2$Cl$_2$ was prereacted in methanol with silver hexafluorophosphate ($AgPF_6$), following which 3-pyridinealdehyde was added and coordinated over 1 h while heating. Subsequently, another equivalent of $AgPF_6$ was added, along with 4-azidobutyronitrile, which was coordinated over 1 h of heating. The product was filtered to remove AgCl and purified by silica column chromatography in excellent yield (60−70%). To the best of our knowledge, this represents the first one-pot synthesis of a heterobifunctional photocleavable cross-linker.

Photochemical characterization of CHO-Ru-N_3 demonstrates rapid photolysis in response to visible light (455 nm, 3−5 mW cm^{-2}) and that 4-azidobutyronitrile is preferentially exchanged upon light exposure (Figure 1a). This is observed by UV−vis absorbance spectroscopy, wherein only one isosbestic point accompanies photolysis, suggesting the formation of a single photoproduct (Figure 1b). ^{1}H NMR confirmed the preferential exchange of the azide-bearing ligand with a clear shift of the aliphatic peaks corresponding to protons on 4-azidobutyronitrile, compared with a stable aldehyde proton (Figure 1c). This observed preferential ligand loss is confirmed by ESI-MS (Figure S3). The quantum yield of ligand exchange for CHO-Ru-N_3 at 455 nm is high, determined to be 0.16 ± 0.04 by kinetic analysis of the absorbance shift (Figure S4). Furthermore, the stability of

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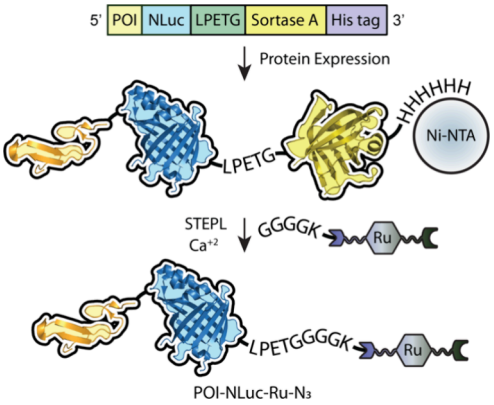
J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

CHO-Ru-N_3 was excellent when stored over 3 days in dark conditions both at room temperature and at 37 °C (Figure S5). CHO-Ru-N_3, being highly sensitive to visible light, was found to rapidly photocleave when exposed to ambient light conditions, with <50% intact CHO-Ru-N_3 remaining after 4 h (Figure S5).

Enthusiastic about CHO-Ru-N_3's ease of synthesis and response to visible light, we identified NanoLuciferase (NLuc) as a potential driver of BRET. Compared with conventional firefly and renilla luciferase, NLuc is far smaller (19 kDa) and brighter (100x more), both attractive features for BRET. To fuse CHO-Ru-N_3 site-specifically to NLuc (with an additional optionally fused protein-of-interest, POI), we employed sortase-mediated transpeptidation, whereby polyglycine probes are C-terminally appended to proteins bearing the LPETG sortase recognition sequence.[36,37] To drive single-step protein functionalization and purification, we implemented sortase-tag enhanced protein ligation (STEPL, Scheme 2).[16,38,39] Here,

Scheme 2. STEPL Modification of Proteins on the C-Terminus



NLuc is expressed as a fusion with the sortase recognition sequence and enzyme, as well as a 6xHis tag. Following immobilization on Ni-NTA, addition of a polyglycine probe in addition to Ca^{2+} drives intramolecular sortagging and concomitant release from the solid affinity matrix.

Toward creation of a sortaggable Ru-based probe, CHO-Ru-N_3 was conjugated with polypeptide H-GGGGK-NH_2 via reductive amination (see Supporting Information). Stabilization with sodium cyanoborohydride permitted direct functionalization of the lysine's ε-amino group without reaction with the N-terminus. The final product [H-GGGGK(Ru-N_3)-NH_2] was purified by HPLC (Figures 2, S6–S8). Upon light exposure, preferential exchange of the azide-bearing ligand was also observed in H-GGGGK(Ru-N_3)-NH_2 (Figure 2a). Time-course HPLC analysis demonstrated complete conversion of H-GGGGK(Ru-N_3)-NH_2 to a singular photoproduct H-GGGGK(Ru-H_2O)-NH_2 with the loss of the nitrile azide ligand, highlighted by the absence of a peak appearing only in the 220 nm channel, corresponding to the peptide alone (Figure 2b). This was confirmed by ESI-MS pre- (expected mass: 988.35/2 = 494.17) and post-photolysis (expected mass [+Na^+]: 918.28/2 = 459.14). Due to the +2 charge of CHO-Ru-N_3, mass hits for this compound appeared as m/2. ESI-MS showed a mass shift of −34.51 corresponding to the loss of one nitrile ligand in exchange for a water molecule (Figure 2c).

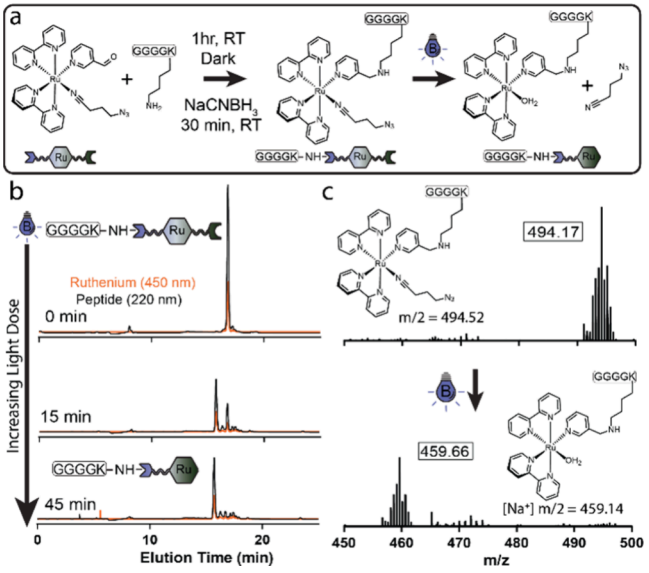


Figure 2. Synthesis and photolysis of H-GGGGK(Ru-N_3)-NH_2. (a) Scheme showing reductive amination reaction between CHO-Ru-N_3 and H-GGGGK-NH_2, followed by photolysis. (b) HPLC traces of H-GGGGK(Ru-N_3)-NH_2 show complete photolysis in situ after 45 min of irradiation (455 nm, 5 mW cm^{-2}). The final product has both peptide and ruthenium signal, indicating that the aldehyde ligand remains coordinated throughout photolysis. (c) ESI-MS of H-GGGGK(Ru-N_3)-NH_2 before and after light exposure (455 nm, 5 mW cm^{-2}, 5 min). A mass shift of −34.51 corresponds to the loss of the nitrile ligand 4-azidobutryonitrile and addition of one coordinated water molecule.

Following conjugation, H-GGGGK(Ru-N_3)-NH_2 was appended to NLuc via STEPL (see Supporting Information, Figure S9). The product (denoted NLuc-Ru-N_3) was confirmed by ESI-MS with an exact mass hit at 21,500 Da. After light exposure, the expected mass (21,407 Da) was observed with a −93 Da mass shift accompanying photolytic loss of the nitrile azide ligand (Figure 3a).

To assess the azide-reactivity and photolability of NLuc-Ru-N_3, the protein was reacted with a 20 kDa monomethyl-poly(ethylene glycol) singly modified with a strained alkyne, bicyclononyne (mPEG-BCN), via SPAAC. Reaction progression was monitored via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), whereby a mass upshift accompanied C-terminal PEGylation (Figure 3b). Following mild light exposure (455 nm, 5 mW cm^{-2}, 0–60 s), the PEG was photolytically released, reflected by a corresponding gel band downshift in SDS-PAGE.

Encouraged by the photosensitivity of NLuc-Ru-N_3, and towards demonstrating BRET-based delivery of a biologically relevant cargo from a material system, we expressed and modified an Epidermal Growth Factor (EGF)-NLuc-Ru-N_3 fusion species (Figure S10–11). To aid in protein release quantification, we fluorescently tagged the purified protein with BD630/680-NHS ester.[16] Fluorescent EGF-NLuc-Ru-N_3 was covalently tethered throughout PEG-based hydrogels formed through SPAAC; following incubation of EGF-NLuc-Ru-N_3 with PEG-tetraBCN for 3 h (final concentration of EGF-NLuc = 0.1 mM), hydrogels were formed upon addition of PEG-tetraazide. When kept in the dark, no background protein release was observed in the gel supernatant over several days both at room temperature and at 37 °C (Figure S12), highlighting the stability of the Ru linkage. As expected, some

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J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

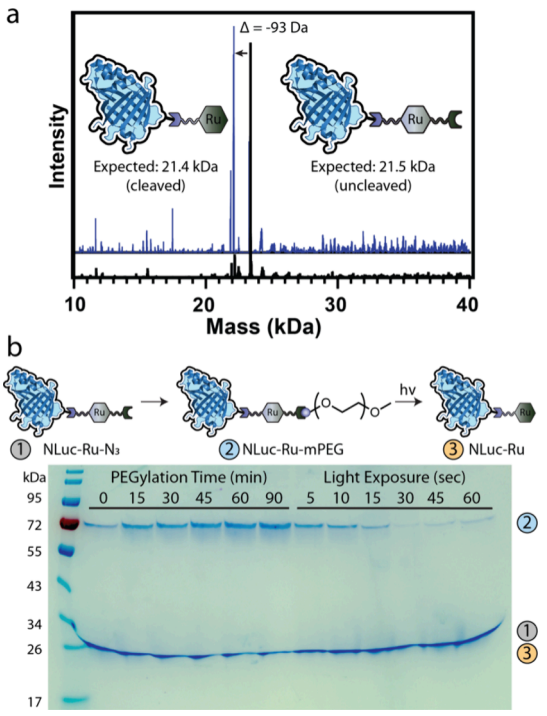

Figure 3. NLuc-Ru-N_3 photolysis. (a) ESI-MS of NLuc-Ru-N_3 pre- and postphotolysis shows a distinct mass shift of −93 da, corresponding to exchange of nitrile ligand for water (455 nm, 5 mW cm^{-2}, 5 min). (b) Gel shift assay tracking click reaction of NLuc-Ru-N_3 with mono-PEG-BCN. A shift in mass to heavier species corresponds to SPAAC reaction between azide-modified NLuc and alkyne-modified PEG polymer. This mass shift was reversed upon light (455 nm, 5 mW cm^{-2}, varying times).

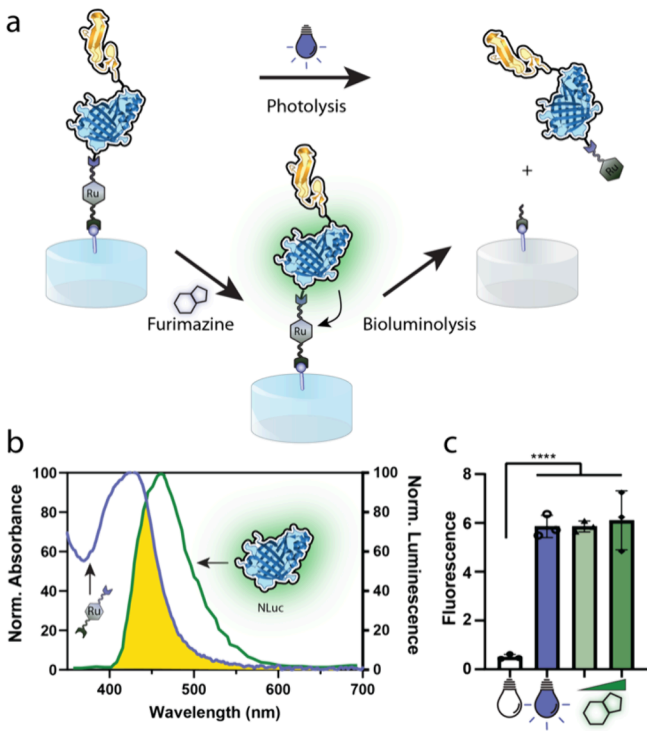

Figure 4. Bioluminolysis-based release of protein cargo from hydrogel biomaterials. (a) Coupling cargo to polymer matrices via a photocleavable linker permits release via either external light exposure or addition of luciferase substrate furimazine. (b) CHO-Ru-N_3 and NLuc exhibit overlapping absorbance and emission spectra respectively. (c) EGF-NLuc release from PEG-based hydrogel systems proceeds under light irradiation (450 nm, 2 min, 10 mW cm^{-2}) or furimazine (5 μM and 7 μM).

protein release accompanied gel storage under ambient light (Figure S13).

Owing to the strong spectral overlap between NLuc's luminescence and CHO-Ru-N_3's absorbance (Figure 4**a-b**), we expected that EGF-NLuc could be similarly released photochemically or via BRET. Protein release was quantified (via supernatant fluorescence) for gels exposed to visible light (455 nm, 2 min, 10 mW cm^{-2}) or NLuc's furimazine (Fz) substrate (5 and 7 μM). Each treatment yielded similar protein release amounts, well above that of the dark control ($p < 0.0001$), demonstrating BRET-based protein cargo release (Figure 4c, Figure S14).

Finally, we sought to quantify BRET efficiency between the NLuc and Ru linker via an SDS-PAGE fluorescent assay. After conjugating a Cy5.5-DBCO fluorophore to EGF-NLuc-Ru-N_3 via SPAAC, the EGF-NLuc-Ru-Cy5.5 species was exposed to Fz (0−60 mol equiv). After electrophoretic separation, a loss of Cy5.5 fluorescence corresponded to a BRET-based cleavage. Studies indicated an overall BRET efficiency of ∼1% (Figure S15). We anticipate that these values can be improved with molecular redesign and expect this platform to be easily transferable to other bioactive protein targets of interest in therapeutic and regenerative medicine.

CONCLUSIONS

The facile synthesis of ruthenium polypyridyl cross-linkers lends itself to the manufacturing of new, more complex heterobifunctional cross-linkers. We report the first one-pot synthesis of a heterobifunctional cross-linker useful for protein conjugation and drug delivery, CHO-Ru-N_3. This compound undergoes preferential ligand exchange of the nitrile-N_3 ligand, observed by ^{1}H NMR, ESI-MS, and UV−vis spectroscopy. CHO-Ru-N_3 was site-specifically conjugated to a polyglycine peptide via reductive amination with a Lys residue and used to modify a protein cargo NLuc via STEPL. Finally, we showed that bioluminescent energy can be used as an alternative light source for phototriggered protein cargo release out of hydrogel materials when used in concert with ruthenium-based cross-linkers. These data demonstrate the first use of BRET for biomaterial modulation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.4c03361.

Synthetic procedures, characterization data, plasmid cloning, and protein expression protocols (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EGF, Epidermal growth factor; NLuc, NanoLuc luciferase; PEG, poly(ethylene glycol); BCN, bicyclononyne; STEPL, sortase-tag enhanced protein ligation

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