Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters

Vincent Grenier,∥ Kayli N. Martinez,∥ Brittany R. Benlian,∥ Derek M. García-Almedina,∥ Benjamin K.Raliski, Steven C. Boggess, Johnathan C. Maza, Samantha J. Yang, Anneliese M. M. Gest, and Evan W. Miller*  

ABSTRACT: Voltage-sensitive fluorescent reporters can reveal fast changes in the membrane potential in neurons and cardiomyocytes. However, in many cases, illumination in the presence of the fluorescent reporters results in disruptions to the action potential shape that limits the length of recording sessions. We show here that a molecular prosthetic approach, previously limited to fluorophores, rather than indicators, can be used to substantially prolong imaging in neurons and cardiomyocytes.

INTRODUCTION

Fluorophores are indispensable in the investigation of living systems. The problem is they bleach and can perturb the very system they are meant to observe. Bleaching makes it difficult to make sustained measurements, and phototoxicity introduces artifacts by disrupting the underlying physiology of the biological system. Exogenous photoprotectant cocktails, often containing antioxidants or triplet-state quenchers (TSQs), are often added to imaging media to prolong imaging duration by either reducing photobleaching or phototoxicity. However, addition of exogenous photoprotectants often requires millimolar concentrations of hydrophobic compounds, which can modify lipid bilayer properties. Pioneering work showed that intramolecular tethering of a TSQ like the vitamin E derivative Trolox or cyclooctatetraene (COT), improves photostability and reduces the phototoxicity of common fluorophores in single molecule and cellular imaging, without the requirement for millimolar concentrations of lipophilic additives. In some live-cell contexts, cellular viability is improved without altering the rate of photobleaching.

However, this self-healing fluorophore strategy has never been applied in the context of fluorescent reporters, dyes which change their optical properties in response to biological cues, such as pH, Ca2+, or membrane potential. Additionally, because the molecular mechanisms behind self-healing fluorophores rely on tuning of electron and energy transfer rates between the fluorophore and TSQ, it was not clear whether the presence of a TSQ would interfere with the sensing mechanism of fluorescent reporters that utilize photoinduced electron transfer (PeT).

Voltage-sensitive dyes have been limited by toxicity induced by the presence of the dye and the intense illumination required for fast voltage imaging. We have been exploring new molecular wire scaffolds for fluorescent voltage indicators which utilize PeT as a voltage-sensitive trigger. These molecular wire-based voltage-sensitive fluorophores (VF dyes) show decreased phototoxicity, and we found that imaging of evoked neuronal action potentials under elevated illumination intensity (21 mW/mm²) altered the observed neuronal physiology, likely because plasma membranes are especially sensitive to dye-sensitized photodamage. The changed neuronal physiology manifested as non-evoked spikes, after-depolarizations, shifts in the baseline, and decreases in ΔF/F. We wondered whether the addition of exogenous photoprotectants, like Trolox or COT, might decrease the apparent disruptions to underlying neuronal physiology.

RESULTS

To test this, we added Trolox or COT (1 mM) to see if this reduced the number of artifacts when imaging with mVF-
sarcosine\textsuperscript{12} (Figure S1) or with VF2.1.Cl (Figure S6).\textsuperscript{13}
Addition of Trolox has only a modest effect on the proportion of neurons that fire normally during field stimulation (27% ± 17%, 95% Cl.I) compared to mVF-sarcosine alone (12% ± 11%), while COT significantly improves the fraction of normally firing neurons to 89% (±10%) (Figure S1). However, application of millimolar concentrations of lipophilic photoprotectants is often not practical, and we wondered whether a covalently tethered photoprotectant would reduce the impact on cellular physiology while retaining the voltage sensitivity of the native VF dye.

**Synthesis.** To test this hypothesis, we first synthesized a new VF dye, 1, with a cysteic acid residue that possesses a sulfonic acid group for water solubility and retention in cell membranes and a free amine for ready functionalization with Trolox or carboxy-COT (Scheme 1 and Scheme S1). From intermediate 1, we synthesized a VF dye with covalently tethered Trolox (1-Tro) or COT (1-COT) (Scheme 1).

**In Vitro and Cellular Characterization.** The new VF-conjugates show absorption and emission spectra nearly identical to the parent compound, with an absorbance maximum at 525 nm and an emission maximum near 540 nm (Figure 1a, Figure S2). In HEK293T cells, 1-COT is voltage sensitive, with a 17.9% \( \Delta F/F \) per 100 mV (Figure 1b, Figure S3). Both the parent 1 and conjugates, 1-Tro and 1-COT, localize to cell membranes in HEK293T cells, neurons, and human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) (Figure 1c–e, Figure S4).

**Imaging in Neurons.** We treated neurons with 1-Tro or 1-COT and imaged optical action potentials driven by extracellular stimulation. We compared the results to imaging with 1 alone or 1 plus the addition of exogenous photoprotective reagents Trolox or COT (Figure 2a,b). Similar to VF-sarcosine, imaging neuronal activity with 1 results in neurons without an artifact only 19% of the time (±10%, 95% confidence interval). Neurons treated with Trolox (1 mM) showed no improvement, but imaging with Trolox covalently conjugated to 1 (500 nM, 1-Tro) dramatically increased the proportion of neurons with artifact-free firing to 57% (±16%). COT had an even more dramatic effect. When 1 mM exogenous COT was added to neurons loaded with 1, the proportion of neurons without artifacts substantially increased, to 93% (±8%). However, millimolar concentrations of COT, a 2000X excess over 1, were required to observe this protective effect; at lower concentrations of 1 and 10 \( \mu \)M, still in stoichiometric excess over 1, a high percentage of cells still displayed artifacts (Figure S5). When COT is covalently attached to 1, 96% of neurons fire normally (±7%, Figure 2b).

**Imaging in Cardiomyocytes.** Because of the excellent performance of 1-COT in neurons, we investigated whether this protective effect could be observed in a distinct model system of electrically excitable cells. We loaded human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) with 1 alone (1 \( \mu \)M), 1 plus an equimolar amount of COT (1 \( \mu \)M), or the new 1-COT conjugate (1 \( \mu \)M) (Figure 3a–c). The amplitude of cardiac action potentials (AP) drops...
dramatically during imaging with 1 alone (Figure 3a, black). Addition of equimolar COT helps maintain AP height; however, the signal-to-noise (SNR) is substantially degraded at the end of a 60 s imaging session (Figure 3b, blue). In contrast, AP height remains nearly constant throughout the 60 s imaging bout with 1-COT (Figure 3c, green). In these experiments, the hiPSC-CMs are unpaced, which means that the beat rate, or number of APs, can vary from trial to trial. The protective effects of covalently tethered COT are even more profound after periods of extended imaging. Even after 10 min of continuous illumination, the AP duration (APD) and beat rate of hiPSC-CMs treated with 1-COT remains unchanged (Figure 3d, green; Figure S9). hi-PSC-CMs imaged with 1 alone show dramatic changes in APD even after 1 min of illumination, significant changes to APD, beat rate, and shape after 5 min, and stop beating shortly thereafter as evidenced by an arrest of beating and lack of APs after minute 6 (Figure 3d, black; Figure S9; Supplemental Videos 1, 2, 3, and 4). Examination of AP shape during the initial 10 s of imaging (Figure 3e, t = 0) and after 5 min of continuous illumination reveal profound changes to the shape of APs in hiPSC-CMs imaged with 1, while APs recorded after 5 min with 1-COT overlay closely with the initial APs (Figure 3f). Under maximum illumination, higher imaging speeds can be achieved using 1-COT, enabling high temporal resolution recording of AP rise times and upstroke velocity (Figure S7).

Under maximum illumination, higher imaging speeds can be achieved using 1-COT, enabling high temporal resolution recording of AP rise times and upstroke velocity (Figure S7). Similarly to the previously reported use of COT-conjugated fluorophores in live cells, 1-COT has a similar bleach rate to 1 (Figure S8) but substantially decreases phototoxicity.

**CONCLUSION**

In summary, we present a generalizable molecular prosthetic strategy for attaching TSQs to voltage-sensitive fluorophores in live cells, enabling high temporal resolution recording of AP rise times and upstroke velocity. This approach provides a generalizable method for overcoming limitations associated with the use of vigil dye in live-cell imaging, while avoiding disruptions to underlying cellular physiology. In contrast to previous studies in our lab, which altered the molecular wire of the voltage-sensitive fluorophore, this present strategy has the potential to be more universal and could likely be applied to any VF dye, such as the silicon-rhodamine fluorophore BeRST 1 or other indicators regardless of the molecular wire or fluorophore reporter.
Steven C. Boggess — Department of Chemistry, University of California, Berkeley, California 94720, United States
Johnathan C. Maza — Department of Chemistry, University of California, Berkeley, California 94720, United States; orcid.org/0000-0003-3898-8770
Samantha J. Yang — Department of Chemistry, University of California, Berkeley, California 94720, United States
Anneliese M. M. Gest — Department of Chemistry, University of California, Berkeley, California 94720, United States

Complete contact information is available at:
https://pubs.acs.org/10.1021/acscentsci.1c01153

Author Contributions
V.G., K.N.M., B.R.B., and D.M.G.-A. contributed equally.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

E.W.M. thanks the NIH (Grant R35GM119855) and the Camille Dreyfus Teacher-Scholar Program for support of this research. K.N.M., B.R.B., D.M.G.-A., B.K.R., S.C.B., J.C.M., and A.M.M.G. were supported, in part by the NIH (Grant T32GM066698). V.G. was supported, in part, by a graduate fellowship from NSERC. Confocal imaging was conducted at the CRL Molecular Imaging Center RRID:SCR_017852, supported by NIH Grant S10OD025063. We thank Holly Aaron and Feather Ives for instrumental training, support, and advice.

■ REFERENCES

(1) Dave, R.; Terry, D. S.; Munro, J. B.; Blanchard, S. C. Mitigating unwanted photophysical processes for improved single-molecule fluorescence imaging. Biophysical journal 2009, 96, 2371–2381.
(2) Alejo, J. L.; Blanchard, S. C.; Andersen, O. S. Small-molecule photostabilizing agents areeners of lipid bilayer properties. Biophysical journal 2013, 104, 2410–2418.
(3) Altman, R. B.; Terry, D. S.; Zhou, Z.; Zheng, Q.; Geggier, P.; Kolster, R. A.; Zhao, Y.; Javitch, J. A.; Warren, J. D.; Blanchard, S. C. Cyanine fluorophore derivatives with enhanced photostability. Nat. Methods 2012, 9, 68–71.
(4) Zheng, Q.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Zhao, H.; Asher, W.; Holsey, M.; Mathiasen, S.; Geggier, P.; Javitch, J. A.; et al. Electronic tuning of self-healing fluorophores for live-cell and single-molecule imaging. Chemical Science 2017, 8, 755–762.
(5) Yang, Z.; Li, L.; Ling, J.; Liu, T.; Huang, X.; Ying, Y.; Zhao, Y.; Zhao, Y.; Lei, K.; Chen, L.; et al. Cyclooctatetraene-conjugated cyanine mitochondrial probes minimize phototoxicity in fluorescence and nanoscopic imaging. Chemical Science 2020, 11, 8506–8516.
(6) Pati, A. K.; El Bakouri, O.; Jockusch, S.; Zhou, Z.; Altman, R. B.; Fitzgerald, G. A.; Asher, W. B.; Terry, D. S.; Borgia, A.; Holsey, M. D.; et al. Tuning the baird aromatic triplet-state energy of cyclooctatetraene to maximize the self-healing mechanism in organic fluorophores. Proc. Natl. Acad. Sci. U. S. A. 2020, 117, 24305–24315.
(7) Zheng, Q.; Juette, M. F.; Jockusch, S.; Wasserman, M. R.; Zhou, Z.; Altman, R. B.; Blanchard, S. C. Ultra-stable organic fluorophores for single-molecule research. Chem. Soc. Rev. 2014, 43, 1044–1056.
(8) Zheng, Q.; Jockusch, S.; Rodriguez-Calero, G. G.; Zhou, Z.; Zhao, H.; Altman, R. B.; Abruna, H. D.; Blanchard, S. C. Intramolecular triplet energy transfer is a general approach to improve organic fluorophore photostability. Photochemical & Photobiological Sciences 2016, 15, 196–203.
(9) Miller, E. W.; Lin, J. Y.; Frady, E. P.; Steinbach, P. A.; Kristan, W. B., Jr.; Tsien, R. Y. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. Proc. Natl. Acad. Sci. U.S.A. 2012, 109, 2114–2119.
(10) Liu, P.; Miller, E. W. Electrophysiology, unplugged: Imaging membrane potential with fluorescent indicators. Accounts of chemical research 2020, 53, 11–19.
(11) Sheetz, M. P.; Koppel, D. E. Membrane damage caused by irradiation of fluorescent concanavalin a. Proc. Natl. Acad. Sci. U. S. A. 1979, 76, 3314–3317.
(12) Grenier, V.; Daws, B. R.; Liu, P.; Miller, E. W. Splying on neuronal membrane potential with genetically targetable voltage indicators. J. Am. Chem. Soc. 2019, 141, 1349–1358.
(13) Miller, E. W.; Lin, J. Y.; Frady, E. P.; Steinbach, P. A.; Kristan, W. B.; Tsien, R. Y. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. Proc. Natl. Acad. Sci. U. S. A. 2012, 109, 2114–2119.
(14) Hoekstra, M.; Mummery, C.; Wilde, A.; Bezzina, C.; Verkerk, A. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. Frontiers in Physiology 2012, 3, 346.
(15) Boggess, S. C.; Gandhi, S. S.; Siemons, B. A.; Huebsch, N.; Healy, K. E.; Miller, E. W. New molecular scaffolds for fluorescent voltage indicators. ACS Chem. Biol. 2019, 14, 390–396.
(16) Boggess, S. C.; Gandhi, S. S.; Benlian, B. R.; Miller, E. W. Vinylfluorene molecular wires for voltage imaging with enhanced sensitivity and reduced phototoxicity. J. Am. Chem. Soc. 2021, 143, 11903.
(17) Huang, Y. L.; Walker, A. S.; Miller, E. W. A photostable silicon rhodamine platform for optical voltage sensing. J. Am. Chem. Soc. 2015, 137, 10767–10776.