

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

The Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in the Characterization of Ultra-Stable Voltage Sensitive Fluorophores

Permalink

<https://escholarship.org/uc/item/4kn8q6zd>

Author

Martinez, Kayli Nicole

Publication Date

2022

Peer reviewed|Thesis/dissertation

The Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in the
Characterization of Ultra-Stable Voltage Sensitive Fluorophores

By

Kayli N. Martinez

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Evan W. Miller, Chair
Professor Michelle C.Y. Chang
Professor Stephen G. Brohawn

Fall 2022

The Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in the
Characterization of Ultra-Stable Voltage Sensitive Fluorophores

Copyright © 2022

By Kayli N. Martinez

Abstract

The Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in the Characterization of Ultra-Stable Voltage Sensitive Fluorophores

By

Kayli N. Martinez

Doctor of Philosophy, Chemistry

University of California, Berkeley

Professor Evan W. Miller, Chair

Voltage sensitive fluorescent indicators have been a useful method for monitoring cardiac electrophysiology for nearly 50 years, complementing traditional methods of electrophysiology such as patch clamp techniques. The impacts fluorophore phototoxicity on cell health have long been a concern in fluorescent imaging. VoltageFluors (VF dyes) are a class of voltage sensitive indicators that rely on photoinduced-electron transfer to sense changes in membrane potential. While our lab has made significant strides towards expanding the wavelengths of indicators offered and improving indicator voltage sensitivity, efforts to reduce fluorophore phototoxicity in the context of VF dyes was largely unexplored. The work presented in this dissertation explores a generalizable method for reducing the phototoxicity of our VF dyes, with the tethering of our probes to the triplet state quencher cyclooctatetraene (COT). Chapter 1 presents the development of VF-COT, a green, xanthene-based indicator intramolecularly tethered to COT. VF-COT can report cardiac action potentials for twice as long as its parent dye, without significant impacts on electrophysiology parameters. Chapter 2 presents BeRST-COT, a near-infrared, silicon rhodamine-based VF that demonstrates the generalizability of reducing phototoxicity with an intramolecularly tethered COT molecule. BeRST-COT reports cardiac electrophysiology for 2.7-times as long as its parent dye and reduces the relative production of singlet oxygen during imaging sessions. Chapters 1 and 2 present work utilizing cardiac cells towards efforts of better characterizing ultra-stable VF indicators. Appendix 1 aims to utilize VF dyes and fluorescence lifetime imaging to better quantify cardiac electrophysiology parameters and lays out the work completed and the rationale for future work in this project.

Table of Contents

Acknowledgements.....	ii
Introduction.....	1
Chapter 1: Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters.....	8
Supporting Information for Chapter 1.....	16
Chapter 2: Red-shifted fluorescent voltage sensitive indicator with molecular prosthetic for long-term cardiac imaging.....	47
Supporting Information for Chapter 2.....	58
Appendix 1: Optical Quantification of Absolute Voltage with Fluorescence Lifetime Imaging and VoltageFluor Indicators in hiPSC-CMs.....	67

Acknowledgements

To my mom, for without you this thesis would have never been possible. I've never been good at handling big life changes, and you've walked me through every single one of them. From my first research experience at Colorado State to moving across the country to Berkeley. You stood by me and every crazy decision I've made and supported me in every way imaginable. Thank you for being there for every big and small milestone, from driving cross country and moving me into my very first apartment to being here for my final presentation. Your constant love and encouragement for me and Lainey has allowed us to pursue our dreams. I couldn't have asked for anything more.

To my family, I love you all and your support from across the country means more than you know. I am who I am because of each and every one of you. Thank you for being my biggest fans.

To Evan, my PhD advisor, thank you for all your guidance from the time I was an undergrad in your lab until now. Thank you for answering every silly question, every email, and always lending a helping hand and giving advice. I also appreciate your support in all the other roles I took on during my graduate school experience, which gave me the chance to not only learn a variety of scientific skills but also gave me opportunities to learn so many transferrable skills for my future careers.

To the Miller lab, you all are the reason I enjoyed going to work every day. Our graduate school careers have been anything but typical between Covid and strikes, but I'm glad that we had each other to lean on through it all. Friday lunches were often my favorite parts of the week and I'll forever remember our lab shenanigans. Marisol and Susanna—what an honor it has been to go through graduate school with you. From learning to change an air tank (which I'd say we've gotten very good at over the years), going through qualifying exams and surviving the pandemic, and going from being mentored to mentoring others, we've been through it all. I couldn't have asked for better friends to do this with. Steven—thank you for passing your knowledge on to me. You were a great mentor and I'm thankful we got to overlap as lab mates. Samantha—some of the work presented here also belongs to you. Thank you for signing on to join me on these projects and being so wonderful to work with. You truly have become a vital part of my graduate school experience, and it has been so fun to mentor you and watch you grow into an independent scientist. I'm so excited to see where you go next.

To the CGLC members I've worked closely with over the years, thank you for being a part of what has easily been some of my favorite moments of graduate school. Joel, Mark, Nikki, and Jenna—recruitment has been one of my favorite things to work on for the last four years. Thank you, Joel and Mark, for teaching me so much and trusting my ideas and thank you to Nikki and Jenna for being so fun to work with and being willing to learn so much about recruitment and the CGLC. To the SURF program, thank you Jessica and Claire for being great advisors to work with and advocates for students. This program was so incredibly fun to work on for two years and I'll miss working with you both dearly.

To my mentors, I'm so grateful for the things you've taught me. Michelle Douskey—you have been a wonderful mentor to me. Teaching with you and for you was truly amazing, and I'm so grateful for the experiences you gave me. You truly trusted me as a partner in teaching and I

learned so much from you. You are an incredible teacher and have been an important part of my grad school experience. Jeanne Bundens—you truly provided me with some of the most important, foundational opportunities in science that got me to where I am today. I was just an 18-year-old who loved science and you helped me learn not only chemistry, but how to take my passion and do great things with it. I feel your support continually, and your mentorship and friendship over the last eight years has been so appreciated.

To Graham, you make life happier, lighter, funnier, and better. Thank you for being there for all the ups and downs, always lending a helping hand or an ear to listen, and for laughing with me. You've taught me how to better balance work and life and I'm so grateful for you and our life together. Thank you for being the best travel, dining, hosting, and life partner.

To all my friends near and far—Molly, Laura, Erika, Emily, Taylor, Nicole, Kerry, Susanna, Michelle, Marisol, Jamie, and everyone that has touched my life and supported me—you mean the world to me, and I couldn't have done grad school without all of you. You've all kept me sane with trips to visit me, vacations to new and fun places, girls' night dinners, check ins, and emotional support. To Nicole, you already know that I literally could not have finished this PhD without you. You know me better than I know myself most days and my life would not be the same without you. Knowing we get to leave graduate school and enter the world together has made this semester all the more special, and I can't wait to keep doing life together.

I once said I would never come to Berkeley for graduate school, and now I can't imagine what my life would look like without the friends, memories, and skills I've learned here. If you, the reader, have opened the pages of this dissertation, then you either know and love me and are here for that reason, or you're a really big fan of human induced pluripotent stem cell-derived cardiomyocytes. Either way, I thank you for your interest in learning more about my science. It's been a long, heartening, frustrating, joyful, and humbling experience to earn this PhD. So, thank you for taking time to read about it.

Introduction

Overview

Electrophysiology's inextricable link to overall human health and disease makes this an important area of study. Cardiac arrhythmias can occur when the highly concerted electrophysiology of the heart is disrupted by pharmaceutical, genetic, or other health factors.¹ Developments in fluorescent dyes, data processing software, charge coupled device (CCD) cameras, and light sources have advanced optical studies of electrophysiology such as whole-heart mapping.² These technologies have enhanced the ability to monitor specific regions (i.e. chambers) of the heart, develop assays for non-cardiotoxic drugs, and multiplex voltage sensitive fluorophores with calcium indicators, all of which have led to better understanding of action potential propagation, mechanisms of arrhythmias, and effects of physiological stimuli.³⁻⁸

Patch clamp electrophysiology methods have historically been the gold standard for electrode-based measurements of membrane potential, measuring electrophysiological changes with high temporal resolution and sensitivity.⁹ However, this is a technically challenging, low-throughput method that is destructive to cells and limits spatial resolution.¹⁰ Multielectrode arrays (MEAs) increase throughput, relative to patch clamp electrophysiology, by allowing for long-term recordings of extracellular field potentials of cells seeded in the array and are less invasive.^{11,12} However, MEAs do not allow for transmembrane potential recordings or monitoring of single cells and have limited spatial resolution.¹²

Voltage sensitive fluorophores can both complement and offer advantages over traditional methods of probing electrophysiology such as capabilities for single cell, monolayer, and tissue imaging and are relatively non-invasive. Voltage indicators localize to the plasma membrane and change their optical properties upon changes in the membrane potential of a cell.¹³

A class of voltage indicators considered “slow” dyes relies on the redistribution of a molecule upon changes in membrane potential. One example is merocyanine 540, which is shuttled into the nonpolar bilayer during membrane depolarization and shows an increase in fluorescence based on a solvatochromic response.³ A second example is a FRET-based coumarin-oxonol pair which includes a stationary fluorophore on the membrane and a lipophilic anion that partitions into the cell membrane, redistributing towards the inner or outer leaflet of the membrane based on membrane potential impacting the efficiency of FRET.¹⁴ As these dyes have slow kinetics, they are unable to monitor fast changes in membrane potential such as neuronal action potentials, and also add a significant capacitive load to the cell membrane.¹³

Additionally, there are “fast” dyes which are electrochromic voltage indicators responding to changes in membrane potential with fast kinetics, changing their spectral properties as a result of a molecular Stark effect.¹⁵⁻¹⁷ The spectral shift can be used for ratiometric voltage imaging, however this requires highly specific instrumental parameters. A commonly electrochromic indicator, di-4-ANNEPS, has been previously used in cardiac optical mapping.⁸ However, many electrochromic indicators have low voltage sensitivities and are relatively phototoxic to the cells they are aiming to study.^{18,19} The drawbacks of these voltage indicators demonstrate the need for the development and synthesis of new dyes for voltage imaging.

VoltageFluor Indicators

The Miller lab has developed a class of voltage sensitive fluorophores, or VoltageFluors (VFs), that rely on a photoinduced electron transfer (PeT) mechanism from an electron rich aniline donor near the inner leaflet of the cell membrane to a fluorophore anchored at the outer leaflet of the cell

membrane via a molecular wire (Fig. 1a,b).^{20–22} At resting or polarized membrane potentials, the rate of PeT is increased which in turn decreases fluorescence. At depolarized membrane potentials, the rate of PeT is decreased resulting in an increase in fluorescence (Fig. 1b). VF indicators respond to changes in membrane potential with fast kinetics.¹⁰ A focus of our lab is the development of new indicators that span the visible spectrum and increase voltage sensitivity, brightness, and signal to noise ratio, while reducing photobleaching and phototoxicity.

VoltageFluors developed with various fluorophore backbones, such as boron dipyrromethenes (BODIPYs), fluoresceins, and rhodamines, have provided a diverse library of voltage indicators at different excitation and emission wavelengths.¹⁰ This has benefits for microscopy by allowing for selection of probes to fit individual imaging needs, such as multiplexing a VoltageFluor with a calcium indicator, mitochondrial trackers, or other physiology probes that one may need or simply selecting a red-shifted probe with intent to reduce autofluorescence.^{23,24}

The Miller lab has also worked to better understand how structural design of indicators impacts voltage sensitivity. The conjugation of the aniline donor of VF indicators was thoroughly investigated in order to understand its impact on voltage sensitivity and fluorescence lifetime.²⁵ We've also investigated reverse-PeT dyes which “turn-off” with depolarizations in membrane potential, the ways in which the indicators orient in the cell membrane impact voltage sensitivity, and how changing the structure of the molecular wire impacts PeT.^{26–28}

Reducing Phototoxicity in Voltage Sensitive Indicators

Our lab is now also investigating the phototoxicity of our dyes and developing new, photostable voltage sensitive indicators. We initially showed that the use of the novel indicator fVF2 in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) allowed for long term imaging of cardiac action potentials. Other dyes caused an arrest of beating in the hiPSC-CMs, likely due to phototoxicity.²⁸ fVF2 has a modified, fluorene-based molecular wire, different from the majority of other VoltageFluors with a phenylene-vinylene wire. While we hypothesize the fluorene wire reduces phototoxicity, we have not yet done mechanistic studies to prove this. This observation sparked our interest in designing generalizable strategies to enhance photostability and reduce photobleaching and phototoxicity in VF dyes.

Phototoxicity primarily arises from the production of reactive oxygen species (ROS) during imaging.²⁹ The production of ROS can arise from the use of high energy wavelengths of light used to image. When these high energy photons hit endogenous chromophores such as flavins and NAD(P)H, it can cause autofluorescence and the possibility for the excited chromophores to crossover into the triplet state and sensitize molecular oxygen, generating reactive singlet oxygen. Most endogenous chromophores have excitation wavelengths between 250 and 500 nm.³⁰ A common method for reducing phototoxicity caused by excitation of these endogenous chromophores has been to simply image with red-shifted fluorescent indicators. However, this method is not always feasible if a fluorophore for a specific imaging need has not yet been

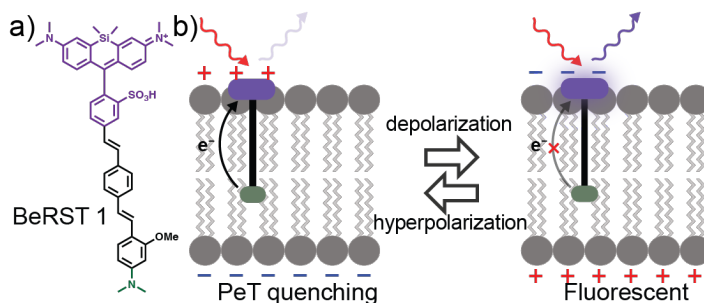


Figure 1. Mechanism of a VoltageFluor Indicator. **a)** Molecular structure of the VoltageFluor, BeRST 1, with an aniline donor (green), a molecular wire (black) and a fluorophore head (purple). **b)** Schematic of PeT in a VoltageFluor located in the membrane bilayer.

synthesized at a red-shifted/near-infrared (NIR) wavelength. Additionally, it can be difficult to rely only on red-shifted/NIR probes if an experiment requires multiplexing and imaging at multiple wavelengths of light to monitor more than one physiological parameter.

Additionally, phototoxicity can occur from the inherent likelihood of any given exogenous fluorophore to create ROS. For an ideal fluorophore, the molecule transitions from the S_0 to the S_1 state upon absorption of a photon and then transitions back to the S_0 state upon fluorescence and release of a photon. However, a chromophore can also undergo intersystem crossing into the nonradiative triplet excited state (T_1).³¹ While the quantum yield of the triplet state is relatively low, typically <0.01 , the lifetime of the triplet state is relatively long which can result in fluorophore blinking and bleaching. The triplet state fluorophore can interact with molecular oxygen via triplet energy transfer which further produces singlet state oxygen, an oxidizing agent with the ability to create other ROS.^{31,32} Singlet oxygen and other ROS can react with the indicators causing degradation of the fluorophore and photobleaching, or react with biomolecules causing phototoxicity.^{33,34}

The photostability of fluorophores can be enhanced by the addition of triplet state quenchers (TSQs) or conjugated photoprotectants.¹⁴ TSQs such as Trolox, 4-nitrobenzylalcohol (NBA), and 1,3,5,7-cyclooctatetraene (COT) reduce fluorophore photobleaching and blinking with the introduction of a reducing and oxidizing system that assists with a speedier recovery of the fluorophores singlet ground state.³⁵ However, these exogenous photostabilizing agents at experimentally relevant concentrations alter the properties of the cell bilayer, altering capacitance, and membrane protein functions.³⁶

The development of fluorophores with TSQs tethered to the fluorogenic center has led to a new class of ‘self-healing’ fluorophores and insights into the mechanisms of photostabilization in the presence of the TSQ.^{37,38} TSQ-tethered fluorophores show reduced photobleaching and blinking relative to their parent fluorophores by directly reducing the lifetime of the triplet state, showing the triplet state lifetime is related to instability of the fluorophore.^{31,38,39} The reduction of the triplet state lifetime via direct mitigation of the formation of triplet and radical states disables the production of ROS and should enable long-term use of the fluorophore. Pioneering work on TSQ-tethered fluorophores was previously performed on fluorophores in single-molecule imaging contexts.^{31,37,38}

Dissertation Work

Herein, I present dissertation work that focuses on the development and characterization of TSQ-tethered indicators of voltage sensitivity, the first examples of TSQ-stabilized indicators of cellular physiology. I demonstrate that this method is generalizable across variations of VoltageFluors and that these stabilized indicators allow for elongated imaging times and reduced phototoxicity as compared to their parent dyes.

In Chapter 1, I present a new VoltageFluor, VF-COT, a green, fluorescein-based dye functionalized with a covalently tethered COT molecule.⁴⁰ VF-COT boasts the ability to image cardiac action potentials for more than twice as long as its parent VF dye, without disrupting underlying cellular electrophysiology. VF-COT also reports neuronal electrophysiology without electrophysiological artifacts likely caused by phototoxicity.

In Chapter 2, I present currently unpublished work on a second TSQ-tethered VoltageFluor (TSQ-VF) named BeRST-COT, a silicon rhodamine-based indicator. BeRST-COT contains the intramolecular triplet state quencher and is an NIR indicator, qualities which both can help to mitigate phototoxicity of the fluorophore. BeRST-COT reduces photobleaching in HEK293T cells

and elongates imaging times during phototoxicity assays in hiPSC-CMs, relative to its parent dye. BeRST-COT produces less singlet oxygen than its parent dye, leading us to hypothesize that BeRST-COT has a shorter triplet state lifetime and is thus less phototoxic. The cellular dimness of BeRST-COT contrasted with the indicator's relatively long fluorescence lifetime leads us to hypothesize that BeRST-COT does not load well into the membrane bilayer, and future work with this dye would be synthetic efforts to improve loading capacity, solubility, and brightness.

The use of our stabilized TSQ-VFs in hiPSC-CMs is an effort to utilize biology to characterize the capabilities of our indicators. hiPSC-CMs have automaticity, or spontaneous contractions, as a result of their immature electrophysiological structure.⁴¹ While this immature electrophysiology creates other challenges for the use of hiPSC-CMs in studying cardiac biology, it aided our lab with an efficient method for determining the length of time our indicators could be functionally useful in cells with easily defined parameters of health such as action potential duration and beat rate.⁴² Previous VoltageFluor indicators such as BeRST 1 have also been used in the characterization of hiPSC-CMs by our collaborators, making hiPSC-CMs a good platform for demonstrating the usefulness of our new TSQ-VFs.^{23,43-46}

While much of the work herein presented uses biological samples to characterize voltage sensitive indicators, Appendix 1 of this dissertation is an effort to utilize our VoltageFluor indicators to characterize cardiac electrophysiology. As hiPSC-CMs are structurally and electrophysiologically immature, there is much focus on methods to mature these cells so they can make physiologically relevant statements about cardiac biology.⁴⁶⁻⁴⁸ Since traditional methods of determining absolute membrane potential values, such as patch-clamp electrophysiology, are technically challenging and low-throughput, this work aims to develop a high-throughput, optical method for measuring absolute values of membrane potential. This work lays out the rationale and methodology of using fluorescence lifetime imaging to characterize absolute membrane potentials in hiPSC-CMs.

In closing, the work presented in this dissertation has been interdisciplinary, using concepts in cell biology, microscopy, and chemical synthesis and physical organic chemistry. I lay out the work completed in each section, as well as rationale for future directions possible for each project presented.

References

1. Li, J. *et al.* Shining light on cardiac electrophysiology: From detection to intervention, from basic research to translational applications. *Life Sci.* **274**, 119357 (2021).
2. O'Shea, C. *et al.* Cardiac optical mapping – State-of-the-art and future challenges. *Int. J. Biochem. Cell Biol.* **126**, 105804 (2020).
3. Salama, G. & Morad, M. Merocyanine 540 as an Optical Probe of Transmembrane Electrical Activity in the Heart. *Science (80-.)*. **191**, 485–487 (1976).
4. Attin, M. & Clusin, W. T. Basic Concepts of Optical Mapping Techniques in Cardiac Electrophysiology. *Biol. Res. Nurs.* **11**, 195–207 (2009).
5. Lee, P. *et al.* In situ optical mapping of voltage and calcium in the heart. *PLoS One* **7**, (2012).
6. O'Shea, C. *et al.* High resolution optical mapping of cardiac electrophysiology in pre-clinical models. *Sci. Data* **9**, 1–12 (2022).
7. Sager, P. T., Gintant, G., Turner, J. R., Pettit, S. & Stockbridge, N. Rechanneling the cardiac proarrhythmia safety paradigm: A meeting report from the Cardiac Safety Research Consortium. *Am. Heart J.* **167**, 292–300 (2014).
8. Efimov, I. R., Nikolski, V. P. & Salama, G. Optical imaging of the heart. *Circ. Res.* **95**, 21–33 (2004).
9. Kornreich, B. G. The patch clamp technique: Principles and technical considerations. *J. Vet. Cardiol.* **9**, 25–37 (2007).
10. Liu, P. & Miller, E. W. Electrophysiology, Unplugged: Imaging Membrane Potential with Fluorescent Indicators. *Acc. Chem. Res.* **53**, 11–19 (2020).
11. Thomas, C. A., Springer, P. A., Loeb, G. E., Berwald-Netter, Y. & Okun, L. M. A miniature microelectrode array to monitor the bioelectric activity of cultured cells. *Exp. Cell Res.* **74**, 61–66 (1972).
12. Obien, M. E. J., Deligkaris, K., Bullmann, T., Bakkum, D. J. & Frey, U. Revealing neuronal function through microelectrode array recordings. *Front. Neurosci.* **9**, 423 (2015).
13. Miller, E. W. Small molecule fluorescent voltage indicators for studying membrane potential. *Curr. Opin. Chem. Biol.* **33**, 74–80 (2016).
14. González, J. E. & Tsien, R. Y. Improved indicators of cell membrane potential that use fluorescence resonance energy transfer. *Chem. Biol.* **4**, 269–277 (1997).
15. Loew, L. M., Scully, S., Simpson, L. & Waggoner, A. S. Evidence for a charge-shift electrochromic mechanism in a probe of membrane potential [21]. *Nature* **281**, 497–499 (1979).
16. Fluhler, E., Burnham, V. G. & Loew, L. M. Spectra, Membrane Binding, and Potentiometric Responses of New Charge Shift Probes†. *Biochemistry* **24**, 5749–5755 (1985).
17. Loew, L. M., Bonneville, G. W. & Surow, J. Charge Shift Optical Probes of Membrane Potential. Theory†. *Biochemistry* **17**, 4065–4071 (1978).
18. Schaffer, P., Ahammer, H., Müller, W., Koidl, B. & Windisch, H. Di-4-ANEPPS causes photodynamic damage to isolated cardiomyocytes. *Pflügers Arch. Eur. J. Physiol.* **426**, 548–551 (1994).
19. Skylaki, S., Hilsenbeck, O. & Schroeder, T. Challenges in long-term imaging and quantification of single-cell dynamics. *Nat. Biotechnol.* **34**, 1137–1144 (2016).
20. de Silva, A. P. *et al.* New Fluorescent Model Compounds for the Study of Photoinduced Electron Transfer: The Influence of a Molecular Electric Field in the Excited State. *Angew.*

- Chemie Int. Ed. English* **34**, 1728–1731 (1995).
21. Li, L. S. Fluorescence probes for membrane potentials based on mesoscopic electron transfer. *Nano Lett.* **7**, 2981–2986 (2007).
 22. Miller, E. W. *et al.* Optically monitoring voltage in neurons by photoinduced electron transfer through molecular wires. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2114–2119 (2012).
 23. Huang, Y. L., Walker, A. S. & Miller, E. W. A Photostable Silicon Rhodamine Platform for Optical Voltage Sensing. *J. Am. Chem. Soc.* **137**, 10767–10776 (2015).
 24. Klier, P. E. Z., Martin, J. G. & Miller, E. W. Imaging Reversible Mitochondrial Membrane Potential Dynamics with a Masked Rhodamine Voltage Reporter. *J. Am. Chem. Soc.* **143**, 4095–4099 (2021).
 25. Boggess, S. C. *et al.* Fluorescence lifetime predicts performance of voltage sensitive fluorophores in cardiomyocytes and neurons. *RSC Chem. Biol.* **2**, 248–258 (2021).
 26. McCann, J. T. *et al.* Flipping the Switch: Reverse-Demand Voltage-Sensitive Fluorophores. *J. Am. Chem. Soc.* **144**, 13050–13054 (2022).
 27. Kulkarni, R. U. *et al.* A Rationally Designed, General Strategy for Membrane Orientation of Photoinduced Electron Transfer-Based Voltage-Sensitive Dyes. *ACS Chem. Biol.* **12**, 407–413 (2017).
 28. Boggess, S. C. *et al.* New Molecular Scaffolds for Fluorescent Voltage Indicators. *ACS Chem. Biol.* **14**, 390–396 (2019).
 29. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* **39**, 1–15 (2017).
 30. Croce, A. C. & Bottiroli, G. Autofluorescence spectroscopy and imaging: A tool for biomedical research and diagnosis. *Eur. J. Histochem.* **58**, 320–337 (2014).
 31. Zheng, Q. *et al.* Ultra-stable organic fluorophores for single-molecule research. *Chem. Soc. Rev.* **43**, 1044–1056 (2014).
 32. Webb, J. P., McColgin, W. C., Peteeson, O. G., Stockman, D. L. & Eberly, J. H. Intersystem crossing rate and triplet state lifetime for a lasing dye. *J. Chem. Phys.* **53**, 4227–4229 (1970).
 33. Byers, G. W., Gross, S. & Henrichs, P. M. Direct and Sensitized Photooxidation of Cyanine Dyes. *Photochem. Photobiol.* **23**, 37–43 (1976).
 34. Davies, M. J. Reactive species formed on proteins exposed to singlet oxygen. *Photochem. Photobiol. Sci.* **3**, 17–25 (2004).
 35. Vogelsang, J. *et al.* A reducing and oxidizing system minimizes photobleaching and blinking of fluorescent dyes. *Angew. Chemie - Int. Ed.* **47**, 5465–5469 (2008).
 36. Alejo, J. L., Blanchard, S. C. & Andersen, O. S. Small-molecule photostabilizing agents are modifiers of lipid bilayer properties. *Biophys. J.* **104**, 2410–2418 (2013).
 37. Zheng, Q. *et al.* On the mechanisms of cyanine fluorophore photostabilization. *J. Phys. Chem. Lett.* **3**, 2200–2203 (2012).
 38. Altman, R. B. *et al.* Cyanine fluorophore derivatives with enhanced photostability. *Nat. Methods* **9**, 68–71 (2012).
 39. Tinnefeld, P. & Cordes, T. ‘Self-healing’ dyes: Intramolecular stabilization of organic fluorophores. *Nat. Methods* **9**, 426–427 (2012).
 40. Grenier, V. *et al.* Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters. *ACS Cent. Sci.* **8**, 118–121 (2022).
 41. Park, H. *et al.* Mechanism of automaticity in cardiomyocytes derived from human induced pluripotent stem cells. *J. Mol. Cell. Cardiol.* **16**, 461–470 (2015).
 42. Karakikes, I., Ameen, M., Termglinchan, V. & Wu, J. Human Induced Pluripotent Stem

- Cell-Derived Cardiomyocytes: Insights into Molecular, Cellular, and Functional Phenotypes. *Circ. Res.* **117**, 80–88 (2015).
43. McNamara, H. M. *et al.* Geometry-Dependent Arrhythmias in Electrically Excitable Tissues. *Cell Syst.* **7**, 359–370.e6 (2018).
 44. Charrez, B. *et al.* In Vitro Safety ‘Clinical Trial’ of the Cardiac Liability of Drug Polytherapy. *Clin. Transl. Sci.* (2021). doi:10.1111/cts.13038
 45. Charwat, V. *et al.* Validating the Arrhythmogenic Potential of High-, Intermediate-, and Low-Risk Drugs in a Human-Induced Pluripotent Stem Cell-Derived Cardiac Microphysiological System. *ACS Pharmacol. Transl. Sci.* **5**, 652–667 (2022).
 46. Huebsch, N. *et al.* Metabolically driven maturation of human-induced-pluripotent-stem-cell-derived cardiac microtissues on microfluidic chips. *Nat. Biomed. Eng.* **6**, 372–388 (2022).
 47. Bedada, F. B., Wheelwright, M. & Metzger, J. M. Maturation status of sarcomere structure and function in human iPSC-derived cardiac myocytes. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 1829–1838 (2016).
 48. Kolanowski, T. J., Antos, C. L. & Guan, K. Making human cardiomyocytes up to date: Derivation, maturation state and perspectives. *Int. J. Cardiol.* **241**, 379–386 (2017).

Chapter 1

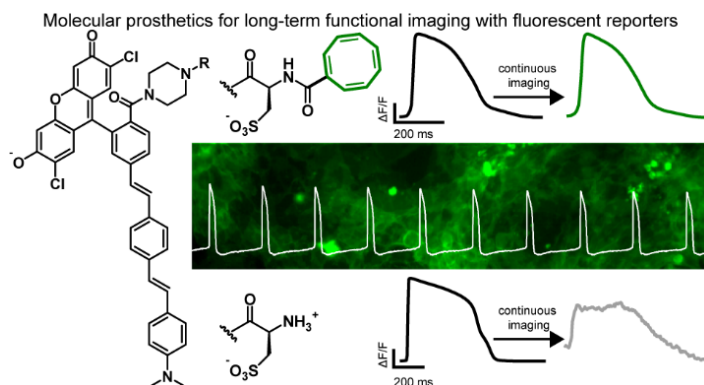
Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters

This work was published as:

Grenier, V.; Martinez, K.N.; Benlian, B.R.; García-Almedina, D.M.; Raliski, B.K.; Boggess, S.C.; Maza, J.C.; Yang, S.J.; Gest, A.M.M.; Miller, E.W. Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters. *ACS Cent. Sci.* **2022**, 8, 1, 118-121.

Abstract

Voltage-sensitive fluorescent reporters can reveal fast changes in membrane potential in neurons and cardiomyocytes. However, in many cases, illumination in the presence of the fluorescent reporters results in disruptions to 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.



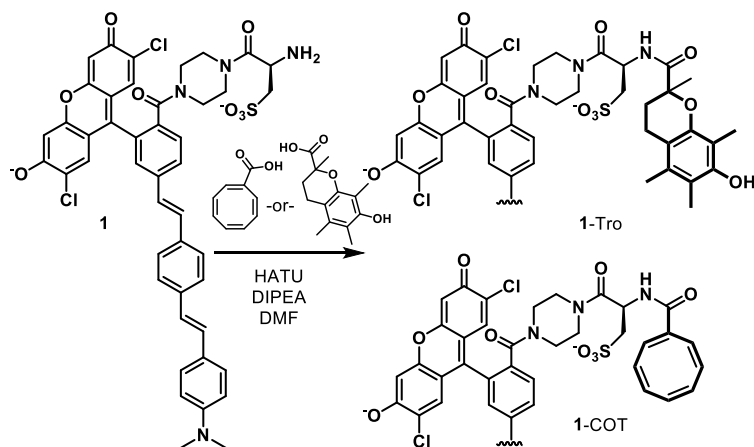
Introduction

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 required millimolar concentrations of hydrophobic compounds,¹ which can modify lipid bilayer properties.² Pioneering work showed that intramolecular tethering of a TSQ, like vitamin E derivative Trolox, or cyclooctatetraene (COT), improves photostability and reduces phototoxicity of common fluorophores in single molecule and cellular imaging,^{4, 5} without the requirement for mM concentrations of lipophilic additives.^{3, 4, 6} In some live-cell contexts, cellular viability is improved without altering the rate of photobleaching.⁵

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, Ca^{2+} , or membrane potential. Additionally, because the molecular mechanisms behind self-healing fluorophores rely on tuning of electron and energy transfer rates between fluorophore and TSQ,⁶⁻⁸ 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 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.^{9, 10} These molecular wire-based Voltage-sensitive Fluorophores (VF dyes) show decreased phototoxicity, we found that imaging of evoked neuronal action potentials under elevated illumination intensity (21 mW/mm^2) altered observed neuronal physiology, likely because plasma membranes are especially sensitive to dye-sensitized photodamage.¹¹ The changed neuronal physiology manifested as non-evoked spikes, after-depolarizations, shifts in the baseline, and decreases in $\Delta F/F$ (**Figure S1**). We wondered whether the addition of exogenous photoprotectants, like Trolox or COT, might decrease the apparent disruptions to underlying neuronal physiology.

Scheme 1. Synthesis of triple-state quencher VF dyes



Results

To test this, we added Trolox or COT (1 mM) to see if this reduced the number of artifacts when imaging with mVF-sarcosine¹² (**Figure S1**) or with VF2.1.Cl (**Figure S6**).¹³ Addition of Trolox has only a modest effect on the proportion of neurons that fire normally during field stimulation ($27\% \pm 17\%$, 95% C.I.) compared to mVF-sarcosine alone ($12\% \pm 11\%$), while COT significantly improves the fraction of normally firing neurons to $89\% (\pm 10\%)$ (**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.

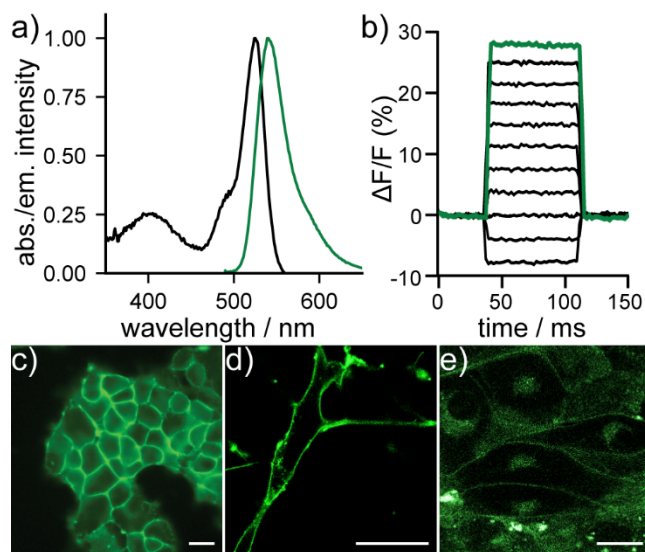


Figure 1. Characterization of TSQ-stabilized indicators. **a)** Plot of normalized absorbance and emission intensity for **1**-COT (200 nM) in phosphate-buffered saline (PBS, pH 7.2) with 0.1% Triton X-100. **b)** Plot of relative change in fluorescence of **1**-COT ($\Delta F/F$) vs. time in a patch-clamped HEK293T cell under whole-cell voltage-clamp conditions. **c)** Widefield, epifluorescence images of **1**-COT in HEK293T cells (1 μM). **d)** Confocal images of **1**-COT (0.5 μM) in **d)** cultured rat hippocampal neurons or **e)** hiPSC-derived cardiomyocytes. All scale bars are 20 μm .

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 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 ($\pm 10\%$, 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% ($\pm 16\%$). 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% ($\pm 8\%$). However, millimolar concentrations of COT—a 2000x excess over **1**—were required to observe this protective effect; at lower concentrations of 1 and 10 μM , 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 ($\pm 7\%$, **Figure 2b**).

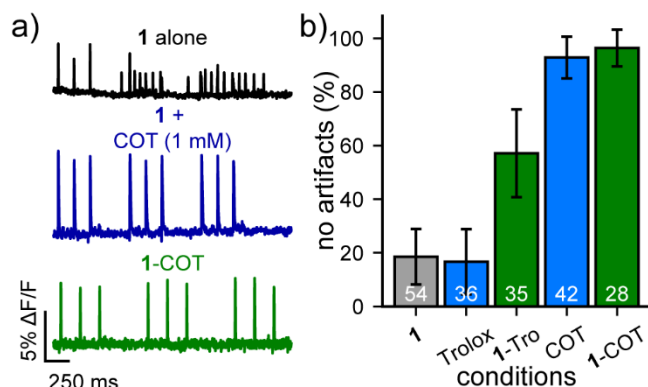


Figure 2. TSQ-stabilized indicators prevent unwanted physiological disruption in mammalian neurons. **a)** Plots of relative fluorescence ($\Delta F/F$) vs. time for neurons loaded with 500 nM of the indicated dye (**1**, black; **1** + 1 mM COT, blue; or **1**-COT, green) and stimulated with an extracellular electrode to evoke firing. Traces are single trials and are bleach corrected using a linear fit. **b)** Plot of fraction of neurons that exhibit no experimental artifacts during the neuronal stimulation protocol, including shifts in baseline, after depolarizations, or non-evoked spikes. Data are the proportion of cells without artifacts. Error bars are 95% confidence interval. White values indicate the number of analyzed neurons per condition.

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 μM), **1** plus an equimolar amount of COT (1 μM), or the new **1**-COT conjugate (1 μM) (**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**). hiPSC-CMs imaged with **1** alone show dramatic changes in APD even after 1 minute of illumination, significant changes to APD, beat rate, and shape after 5 minutes, 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 1-4**). Examination of AP shape during the initial 10 s of imaging (**Figure 3e**, $t = 0$) and after 5 minutes 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 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**).¹⁴ Similar to the previously reported use of COT-conjugated fluorophores in live cells,⁵ **1**-COT has a similar bleach rate to **1** (**Figure S8**), but substantially decreases phototoxicity.

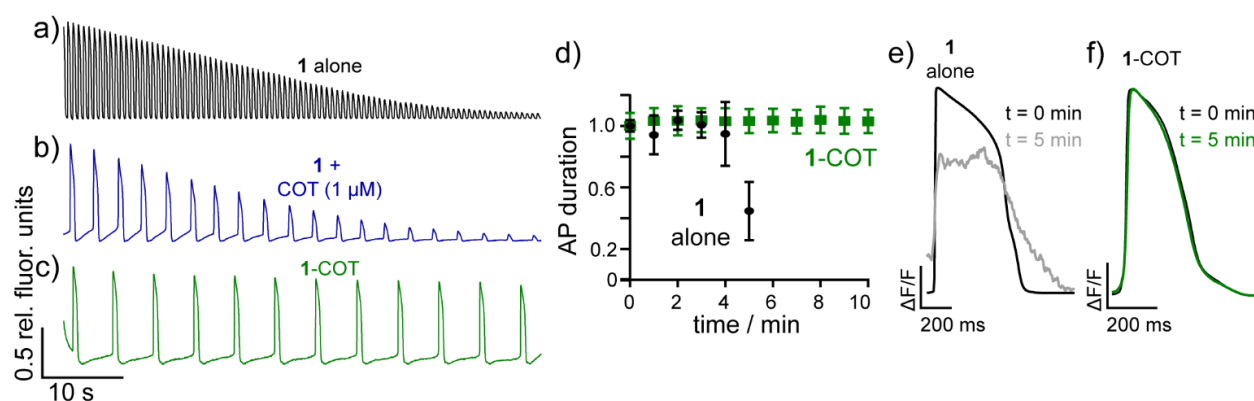


Figure 3. TSQ-stabilized indicators prevent unwanted physiological disruption in human induced pluripotent stem-cell derived cardiomyocytes (hiPSC-CMs). Plot of relative fluorescence vs. time for unpaced hiPSC-CMs loaded with 1 μ M of either **a**) **1** alone (black), **b**) **1** + COT (exogenously added, 1 μ M), or **c**) **1**-COT. Recordings are single trials and are bleach corrected using an asymmetric least squares fit. **d**) Plot of normalized action potential duration (APD) vs. time in spontaneously beating hiPSC-CMs loaded with **1** alone (black) or **1**-COT (green) and illuminated continuously for the indicated time. At 1 minute intervals, a 20 s movie was acquired and action potential duration was assessed. Data are mean $\pm$ S.E.M. for $n = 3$ separate experiments. Example optically-recorded action potentials for **e**) **1** alone (black/gray) or **f**) **1**-COT (black/green) at $t = 0$ min (black) and at $t = 5$ min (gray or green) of continuous illumination. AP traces are the average of 21 and 24 aligned APs for **1** alone (black/gray) and **1**-COT (black/green), respectively.

Conclusion

In summary, we present a generalizable molecular prosthetic that can be used to stabilize recordings made with fluorescent reporters. Previous studies elegantly showed that covalent attachment of TSQs could prolong the duty cycle of fluorophores, but to date, no study has highlighted a similar strategy for fluorescent molecules that report dynamically on their environment. Here, we show specifically that covalent attachment of TSQs like Trolox or COT

allow VF dyes to retain their performance in neurons or cardiomyocytes 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,^{15, 16} 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 fluorescent reporter.^{4,}

17

References

- [1] Dave, R., Terry, D. S., Munro, J. B., and 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., and Andersen, O. S. Small-molecule photostabilizing agents are modifiers 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., and Blanchard, S. C. Cyanine fluorophore derivatives with enhanced photostability *Nature methods* **2011**, *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 *Proceedings of the National Academy of Sciences* **2020**, *117*, 24305-24315.
- [7] Zheng, Q., Juette, M. F., Jockusch, S., Wasserman, M. R., Zhou, Z., Altman, R. B., and Blanchard, S. C. Ultra-stable organic fluorophores for single-molecule research *Chemical Society Reviews* **2014**, *43*, 1044-1056.
- [8] Zheng, Q., Jockusch, S., Rodríguez-Calero, G. G., Zhou, Z., Zhao, H., Altman, R. B., Abruña, H. D., and Blanchard, S. C. Intra-molecular 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., and Tsien, R. Y. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires *Proceedings of the National Academy of Sciences of the United States of America* **2012**, *109*, 2114-2119.
- [10] Liu, P., and Miller, E. W. Electrophysiology, unplugged: Imaging membrane potential with fluorescent indicators *Accounts of chemical research* **2020**, *53*, 11-19.
- [11] Sheetz, M. P., and Koppel, D. E. Membrane damage caused by irradiation of fluorescent concanavalin a *Proceedings of the National Academy of Sciences* **1979**, *76*, 3314-3317.
- [12] Grenier, V., Daws, B. R., Liu, P., and Miller, E. W. Spying on neuronal membrane potential with genetically targetable voltage indicators *Journal of the American Chemical Society* **2019**, *141*, 1349-1358.
- [13] Miller, E. W., Lin, J. Y., Frady, E. P., Steinbach, P. A., Kristan, W. B., and Tsien, R. Y. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires *Proceedings of the National Academy of Sciences* **2012**, *109*, 2114-2119.

- [14] Hoekstra, M., Mummery, C., Wilde, A., Bezzina, C., and Verkerk, A. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias *Frontiers in Physiology* **2012**, 3.
- [15] Boggess, S. C., Gandhi, S. S., Siemons, B. A., Huebsch, N., Healy, K. E., and Miller, E. W. New molecular scaffolds for fluorescent voltage indicators *ACS chemical biology* **2019**, 14, 390-396.
- [16] Boggess, S. C., Gandhi, S. S., Benlian, B. R., and Miller, E. W. Vinyl-fluorene molecular wires for voltage imaging with enhanced sensitivity and reduced phototoxicity *J Am Chem Soc* **2021**.
- [17] Huang, Y. L., Walker, A. S., and Miller, E. W. A photostable silicon rhodamine platform for optical voltage sensing *J Am Chem Soc* **2015**, 137, 10767-10776.

Chapter 1: Supplementary Information

Synthetic Methods

Palladium catalysts and phosphine ligands were purchased from Strem Chemical. Deuterated solvents used for NMR studies were purchased from Cambridge Isotope Laboratories. Anhydrous solvents and all other chemical reagents were purchased from Sigma Aldrich, Acros Chemical, or Oakwood Chemical (South Carolina, USA) and used without further purification. References to previously synthesized compounds are provided along with characterization data. Thin layer chromatography (TLC) (Silicycle, F254, 250 μm) and preparative thin layer chromatography (PTLC) (Silicycle, F254, 1000 μm) were performed on glass backed plates pre-coated with silica gel and visualized by fluorescence quenching under UV light. Flash column chromatography was performed on Silicycle Silica Flash F60 (230–400 Mesh) using a forced flow of air at 0.5–1.0 bar. NMR spectra were measured on Bruker AVB-400 MHz, 100 MHz, AVQ-400 MHz, 100 MHz, Bruker AV-600 MHz, 150 MHz. Chemical shifts are expressed in parts per million (ppm) and are referenced to CDCl_3 (7.26 ppm, 77.0 ppm), DMSO-d_6 (2.50 ppm, 40 ppm), or acetone- d_6 (2.04 ppm, 29.8 and 206.3 ppm). Coupling constants are reported as Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; m, multiplet. High-resolution mass spectra (HR-ESI-MS) were measured by the QB3/Chemistry mass spectrometry service at University of California, Berkeley. High performance liquid chromatography (HPLC) and low resolution ESI Mass Spectrometry were performed on an Agilent Infinity 1200 analytical instrument coupled to an Advion CMS-L ESI mass spectrometer. The column used for the analytical HPLC was Phenomenex Luna 5 μm C18(2) (4.6 mm I.D. $\times$ 75 mm) with a flow rate of 1.0 mL/min. The mobile phases were MQ-H₂O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade acetonitrile with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 254, 350 and 480 nm over 10 min with a gradient of 10-100% eluent B unless otherwise noted. Ultra-high performance liquid chromatography (UHPLC) for purification of final compounds was performed using a Waters Acquity Autopurification system equipped with a Phenomenex Luna 10 μm C18(2) column (21.2 mm I.D. $\times$ 250 mm with a flow rate of 30.0 mL/min, made available by the Catalysis Facility of Lawrence Berkeley National Laboratory (Berkeley, CA)). The mobile phases were MQ-H₂O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade acetonitrile with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 254 and 350 nm over 20 min with a gradient of 10-100% eluent B, unless otherwise noted.

Steady-state UV-vis and fluorescence spectroscopy

Stock solutions of VoltageFluors were prepared in DMSO and diluted with PBS (10 mM KH_2PO_4 , 30 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.55 M NaCl, pH 7.2) solution containing 0.10 % (w/w) SDS (1:500 dilution). UV-Vis absorbance and fluorescence spectra were recorded using a Shimadzu 2501 Spectrophotometer (Shimadzu) and a Quantmaster Master 4 L-format scanning spectrofluorometer (Photon Technologies International). The fluorometer is equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples were measured in 1-cm path length quartz cuvettes (Starna Cells).

Cell Culture

All animal procedures were approved by the UC Berkeley Animal Care and Use Committees and conformed to the NIH Guide for the Care and Use of Laboratory Animals and the Public Health Policy.

Human embryonic kidney cells (HEK293T)

HEK293T cells were acquired from the UC Berkeley Cell Culture Facility. Cells were passaged and plated onto 25 mm glass coverslips coated with Poly-D-Lysine (PDL; 1 mg/mL; Sigma-Aldrich) to a confluency of ~15% and 40% for electrophysiology and imaging, respectively. HEK293T cells for imaging were plated and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), and 1% Glutamax. To reduce rapid growth, HEK293T cells were maintained in DMEM supplemented with 1.0 g/L D-glucose, 10% FBS, 1% Glutamax, and 1 mM sodium pyruvate for electrophysiology experiments.

Rat hippocampal neurons

Hippocampi were dissected from embryonic day 19 Sprague Dawley rats (Charles River Laboratory) in cold sterile HBSS (zero Ca^{2+} , zero Mg^{2+}). All dissection products were supplied by Invitrogen, unless otherwise stated. Hippocampal tissue was treated with trypsin (2.5%) for 15 min at 37 °C. The tissue was triturated using fire polished Pasteur pipettes, in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS; Thermo Scientific), 2% B-27, 2% 1M D-glucose (Fisher Scientific) and 1% glutamax. The dissociated cells were plated onto 12 mm or 25 mm diameter coverslips (Fisher Scientific) pre-treated with PDL (as above) at a density of 27,000 cells or 125,000 cells per coverslip, respectively, in MEM supplemented media (as above). Neurons were maintained at 37 °C in a humidified incubator with 5 % CO_2 . At 1 day in vitro (DIV) half of the MEM-supplemented media was removed and replaced with Neurobasal media containing 2% B-27 supplement and 1% glutamax.

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)

hiPSCs (WTC11) were cultured on Matrigel (1:100 dilution; Corning)-coated 12 well-plates in StemFlex medium (Gibco). When the cell confluency reached 80–90%, which is referred to as day 0, the medium was switched to RPMI 1640 medium (Life Technologies) containing B27 minus insulin supplement (Life Technologies) and 10 μM CHIR99021 GSK3 inhibitor (Peprtech). At day 1, the medium was changed to RPMI 1640 medium containing B27 minus insulin supplement only. At day 3, medium was replaced to RPMI 1640 medium containing B27 supplement without insulin, and 5 μM IWP4 (Peprtech) for 2 days without medium change. On day 5, medium was replaced to RPMI 1640 medium containing B27 minus insulin supplement for 2 days without medium change. On day 7, medium was replaced with RPMI 1640 containing B27 with insulin supplement. After day 7, the medium was changed every two days. Confluent contracting sheets of beating cells appear between days 7 to 15.

Beating sheets were treated with collagenase II for 60-75 minutes. The collagenase solution was carefully transferred to cold DMEM, making sure cardiac sheets were not disturbed. Trypsin (0.25%) was added to dissociated sheets for 4-8 minutes and plated onto 6 well-plates coated with Matrigel (1:100 dilution) in RPMI 1640 medium containing B27 supplement plus ROCK inhibitor Y-27632. 24 hours later, the medium was replaced with fresh RPMI/B27 without ROCK inhibitor. Cardiomyocytes were maintained for 7 days, replacing media every other day, and then were

switched to RPMI 1640 medium (-glucose) supplemented with 4 mM sodium lactate (Sigma Aldrich). Cells were maintained in this media for 7 days, replacing every other day, then switched back to RPMI/B27 containing glucose. These purified cardiomyocytes were then used for imaging.

Lactate purified sheets were dissociated with 0.25% trypsin-EDTA (4-8 minutes, depending on density and quality of tissue) and plated onto Matrigel (1:100)-coated Ibidi® 24 well μ -plates (cat no. 82406) in RPMI 1640 medium containing B27 supplement (containing insulin). Medium was changed every 3 days until imaging. For loading hiPSC cardiomyocytes, voltage dyes were diluted 1 in 1000 in RPMI 1640 with B27 supplement minus Phenol Red to the desired final concentration. Cardiomyocytes were incubated in this solution for 20 minutes at 37 °C, then exchanged with dye-free RPMI 1640 with B27 supplement minus Phenol Red.

Fluorescence Imaging Conditions

For experiments with rat hippocampal neurons, Epifluorescence imaging was performed on an AxioExaminer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor), controlled with Slidebook (v6, Intelligent Imaging Innovations). Images were acquired with a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss) and focused onto either an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu).

Inverted epifluorescence imaging of HEK293T cells and hiPSC cardiomyocytes was performed on an AxioObserver Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor), controlled with μ Manager (V1.4, open-source, Open Imaging).^{2,3} were acquired using a Plan-Apochromat 20/0.8 air objective (20x, Zeiss) or an EC-Plan-NEOFLUAR 40/1.3 oil immersion objective (40x; Zeiss). Images were focused onto an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu).

More detailed imaging information for each experimental application is expanded below.

Membrane staining in HEK293T cells

HEK293T cells were incubated with an imaging buffer (IB) solution (composition in mM: 139.5 NaCl, 10 HEPES, 5.6 D-glucose, 5.3 KCl, 1.3 CaCl₂, 0.49 MgCl₂, 0.44 KH₂PO₄, 0.41 MgSO₄, 0.34 Na₂HPO₄; 290 mOsm/kg, pH 7.25) containing VoltageFluors (500 nM) at 37 °C for 20 min prior to transfer to fresh IB (no dye) for imaging. Microscopic images were acquired with a 40X/1.3NA oil objective (Zeiss) and OrcaFlash4.0 sCMOS camera (Hamamatsu). For fluorescence images, the excitation light was delivered from a LED (9.5 mW/mm²; 100 ms exposure time) at 475/34 (bandpass) nm and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP).

Membrane staining in rat hippocampal neurons

Neurons were incubated in HBSS containing VoltageFluors BeRST 1 and 1-COT (500 nM each) at 37 °C for 20 min prior to transfer to fresh HBSS (no dye) for imaging. Microscopic images were acquired with a 63x/1.4 PlanApo oil objective on a confocal Zeiss LSM 980 with Airyscan2 and BH FLIM. BeRST 1 fluorescence was delivered at 639 nm, 1-COT fluorescence was delivered at 488 nm, and transmitted images were taken at 405 nm (1.42 s exposure time per frame).

Membrane staining in hiPSC-CMs

hiPSC-CMs were incubated in RPMI/B27 (without phenol red) containing VoltageFluors BeRST 1 and 1-COT (500 nM each) at 37 °C for 20 min prior to media exchange with fresh RPMI/B27 (no phenol red, no dye) for imaging. Microscopic images were acquired with a 40x/1.3 oil objective on a confocal Zeiss LSM 980 with Airyscan2 and BH FLIM. BeRST 1 fluorescence was delivered at 639 nm, 1-COT fluorescence was delivered at 488 nm, and transmitted images were taken at 405 nm (1.89 s exposure time per frame).

Voltage sensitivity in HEK293T cells

Functional imaging of the VoltageFluors was performed using a 40x/1.3 NA oil immersion objective paired with image capture from the sCMOS camera at a sampling rate of 0.5 kHz, focused onto a 100x100 pixel² ROI (binned 4x4, 66.5x66.5 μm²). VoltageFluors were excited using the 475 nm LED with an intensity of 9.5 mW/mm². For initial voltage characterization, emission was collection with the emission filter and dichroic listed above.

Evoked activity in rat hippocampal neurons

Extracellular field stimulation was delivered by a SD9 Grass Stimulator connected to a recording chamber containing two platinum electrodes (Warner), with triggering provided through a Digidata 1440A digitizer and pCLAMP 10 software (Molecular Devices). Action potentials were triggered by 1 ms 80 V field potentials delivered at 5 Hz. To prevent recurrent activity the HBSS bath solution was supplemented with synaptic blockers 10 μM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; Santa Cruz Biotechnology) and 25 μM DL-2-Amino-5-phosphonopentanoic acid (APV; Sigma-Aldrich). Functional imaging was performed using the sCMOS camera and a 20x water objective. VF dyes (500 nM in HBSS) were excited with a 475/34 (bandpass) nm LED with an intensity of 21 mW/mm² and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP).

Voltage recordings of hiPSC cardiomyocytes and assessment of phototoxicity

Functional recordings of voltage indicators (0.5 μM) were performed using a 20x air objective paired with a sCMOS camera at a sampling rate of 0.2 kHz (4x4 binning and restricted to a 512x125 pixel frame for high-speed acquisition over long periods). Indicators were excited at 475/34 (bandpass) nm with an intensity of 11.1 mW/mm² and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP). Routine recordings were made for ten seconds.

Phototoxicity of VoltageFluor dyes was assessed in cardiomyocyte monolayers incubated with 0.5 μM of indicator. These were exposed to constant illumination from the excitation LED (475/34; bandpass) for up to 14 minutes, while typical ten second fluorescence recordings were made at the beginning of each minute.

For upstroke velocity and rise time estimations (**Figure S7**), imaging experiments were performed as described above, but at maximum illumination intensity (25.4 mW/mm²).

Image Analysis

HEK cell image analysis

Analysis of voltage sensitivity in HEK293T cells was performed using ImageJ (FIJI). Briefly, a region of interest (ROI) encompassing the cell body was selected and average fluorescence intensity was calculated for each frame. For background subtraction, a ROI encompassing a region without cells was selected and the average pixel intensity was calculated for each frame. A linear fit to the background trace was calculated and applied to the background, and this was used to subtract background signal from the fluorescence intensity trace. F/F_0 values were calculated by dividing the background subtracted trace by the median value of fluorescence when the cell is held at -60 mV. $\Delta F/F$ values were calculated by plotting the change in fluorescence (ΔF) vs the applied voltage step and finding the slope of a linear best-fit.

Rat hippocampal neuron image analysis

For analysis of voltage responses in neurons, regions of interest encompassing cell bodies (all of approximately the same size) were drawn in ImageJ and the mean fluorescence intensity for each frame extracted. $\Delta F/F$ values were calculated by first subtracting a mean background value from all raw fluorescence frames to give a background subtracted trace. A least squares linear regression was fit to the background subtracted trace and a bleaching curve, derived from the slope of the regression, was used to correct for photobleaching. A baseline fluorescence value (F_{base}) was calculated from the median of all the frames and was subtracted from each timepoint of the bleach corrected trace to yield a ΔF trace. The ΔF was then divided by F_{base} to give $\Delta F/F$ traces. No averaging was applied to any voltage traces.

Traces were then scored for the presence of artifacts (see **Figure S1c-g** for examples), including non-evoked spikes, after-depolarizations, and shifts in the baseline. Scoring of neurons was performed by 3 different experimenters. Experimenters were blind to the experimental conditions during scoring. The proportion of responding cells for each dye/condition was noted, and 95% confidence intervals were constructed according to the **Equation S1**.

$$C.I. = z^* \sqrt{\frac{p(1-p)}{n}} \quad \text{Equation S1}$$

Where C.I. is the confidence interval, p is the proportion of cells that show no artifacts during the trial, n is the number of cells analyzed, and z^* is critical value of z at 95% confidence ($z^* = 1.96$ for 95% confidence interval).

hiPSC-CM image analysis

Analysis of action potential (AP) data from hiPSC cardiomyocytes was performed using in-house MATLAB scripts based on previously developed software by the Efimov lab (Washington University, St. Louis, MO). Scripts are available upon request. Briefly, raw OME-tiffs recorded in μ Manager was read directly into MATLAB for batch-processing of large datasets (>30 Gb per experiment). The mean pixel intensity of the entire image (512x125 pixels) was calculated for each frame and a mean fluorescence trace was extracted for the entire stack. Photobleach correction was performed by subtracting an asymmetric least-squares fit of the data from the mean trace. No subtraction of background was possible due to staining of the entire monolayer. Individual AP events were identified through threshold detection based on a Schmidt trigger. Action potential duration (APD) values were calculated for each AP by finding the activation time (time of the maximum derivative of the AP upstroke) and the time the signal returns to 70, 50, and 10% of the maximum depolarization (APD30, APD50, APD90, respectively). APD values were corrected for variation due to spontaneous beat rate by Fridericia's formula (**Equation S2**). CL is the cycle

length, calculated as the time period from the beginning of one beat to the beginning of the succeeding beat.

$$APDc = \frac{APD}{\sqrt[3]{CL}} \quad \text{Equation S2}$$

Electrophysiology

For electrophysiological experiments in HEK293T, pipettes were pulled from borosilicate glass with filament (Sutter Instruments, BF150-86-10) with a P-97 pipette puller (Sutter Instruments) to a resistance of 4-7 MΩ. Pipettes were filled with an internal solution (composition, in mM): 125 potassium gluconate, 10 HEPES, 10 KCl, 5 NaCl, 2 ATP disodium salt, 1 EGTA, 0.3 GTP sodium salt (pH 7.25, 285 mOsm). EGTA (tetraacid form) was prepared as a stock solution in 1M KOH prior to addition to internal solution. Pipettes were positioned with an MP-225 micromanipulator (Sutter Instruments). Electrophysiological recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with a Digidata 1440B, sampled at 50 kHz, filtered at 5 kHz and recorded with pCLAMP 10 software (Molecular Devices).

Electrophysiology was performed in the whole cell voltage clamp configuration. After gigaseal formation and break-in, recordings were only pursued if series resistance in voltage clamp was less than 30 MΩ and the recording maintained a 30:1 ratio of membrane resistance to access resistance throughout all voltage steps. No series resistance compensation was applied. Fast capacitance was compensated in the cell attached configuration. All voltage clamp protocols were corrected for the calculated liquid junction (-14 mV, Liquid Junction Potential Calculator in pClamp). For tandem electrophysiology and fluorescence intensity recordings, cells were held at -60 mV and de- and hyper- polarizing steps were applied from +100 to -100 mV in 20 mV increments, with each step lasting 100 ms (for 1) or 75 ms (for 1-COT).

Supporting Figures

Figure S1. VF-sarcosine + photoprotectants

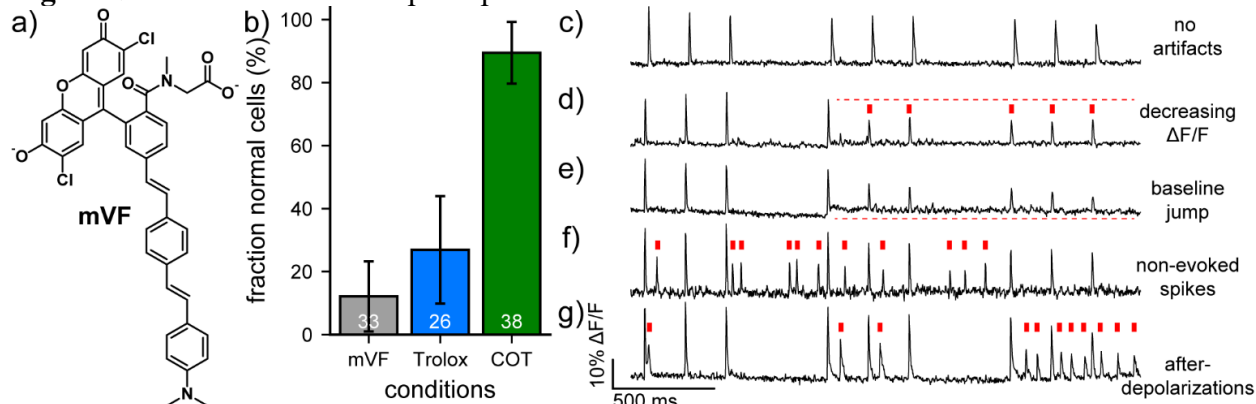


Figure S1. Effect of exogenous photoprotectants on neuronal activity imaging with mVF-sarcosine. **a)** Structure of mV used in these experiments (compound **13** from Grenier, *et al. JACS*, **2019**, *141*, 1359). **b)** Plot of fraction of normally-firing neurons (i.e. no shifts in baseline, after depolarization, or non-evoked spikes) vs. condition for rat hippocampal neurons imaged with **mVF** (500 nM) and subjected to the evoked activity protocol. Trolox and cyclooctatetraene (COT) were applied at a concentration of 1 mM. Error bars are the 95% confidence interval for each proportion. The number of neurons analyzed for each condition is indicated in white at the base of the bars. **c-g)** Example plots of neuronal activity traces used to determine normal firing. All plots are plots of fractional change in fluorescence ($\Delta F/F$) in rat hippocampal neurons stained with 500 nM mVF-sarc and stimulated with 3 sets of 3 pulses to evoke action potentials (APs). **c)** An example of a recording with no artifacts—this cell fires normally. **d)** An example of decreasing $\Delta F/F$ in the 2nd and 3rd round of stimulation. The dotted red line indicates the approximate $\Delta F/F$ for APs in the first round of recording. Red bars indicate APs with lower $\Delta F/F$. **e)** An example of a baseline jump. The dotted red line during the 2nd round of stimulation shows the off-set increase in baseline fluorescence. **f)** An example of a recording with non-evoked spikes. Red bars indicate spikes that arrived regardless of stimulation. **g)** An example of after-depolarizations, in which additional spikes are recorded after the stimulated spike. Red bars indicate after-depolarizations.

Figure S2. Absorption and Emission

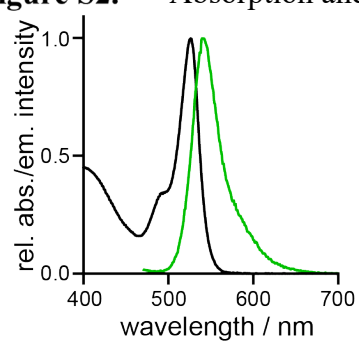


Figure S2. Absorption and emission spectra of **1**. Plots of normalized absorbance and emission intensity for **1** (200 nM) in phosphate-buffered saline (PBS, pH 7.2) with 0.1% Triton X-100.

Figure S3. Voltage sensitivity of **1** and **1-COT** (plot of $\Delta F/F$ vs mV)

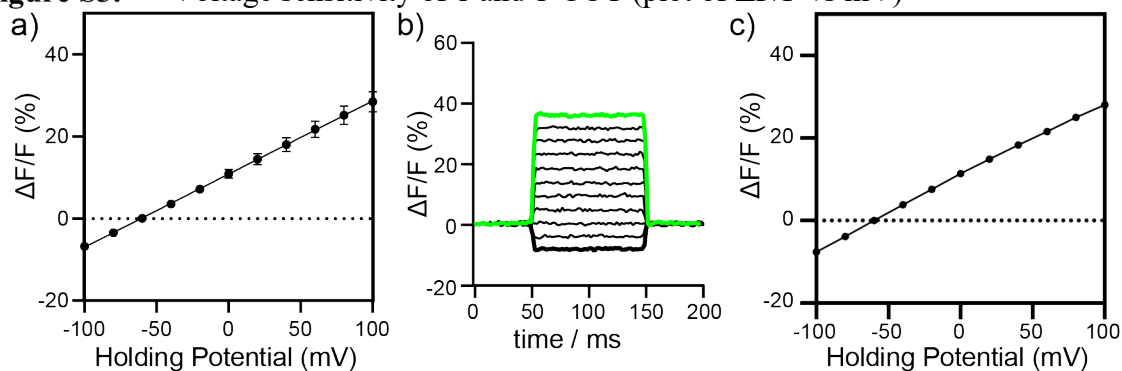


Figure S3. Voltage sensitivity of **1** and **1-COT**. **a)** Plot of fractional change in fluorescence ($\Delta F/F$) vs. membrane potential for **1**. Data are mean $\pm$ standard error of the mean for $n = 9$ determinations. Voltage sensitivity is $18\% \pm 0.6\%$ (S.E.M.) **b)** Plot of relative change in fluorescence of **1** ($\Delta F/F$) vs. time in a patch-clamped HEK293T cell under whole-cell voltage-clamp conditions. **c)** Plot of fractional change in fluorescence ($\Delta F/F$) vs. membrane potential for **1-COT**. Data are mean $\pm$ standard error of the mean for $n = 4$ determinations. Voltage sensitivity is $17.9\% \pm 0.6\%$ (S.E.M.)

Figure S4. Images of compounds in HEK cells, neurons, and hiPSC-CMs

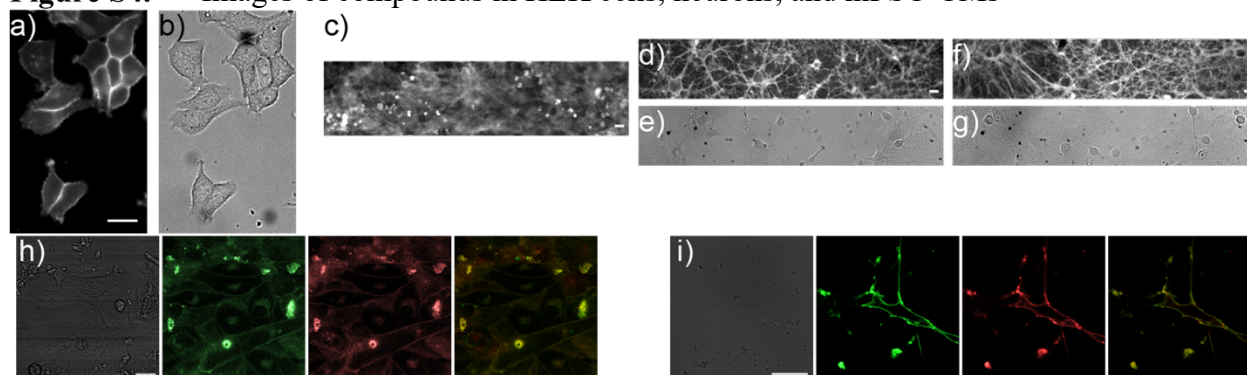


Figure S4. Imaging of **1**, **1-COT**, or **1-Tro** in HEK293T cells, neurons, and hiPSC-CMs. **a)** Widefield epifluorescence and **b)** transmitted light images of **1** in HEK cells. Widefield epifluorescence image of **1** in **c)** hiPSC-CMs, or **d)** dissociated rat hippocampal neurons. **e)** Transmitted light image of panel **d**. **f)** Widefield epifluorescence and **g)** transmitted light images of **1-Tro** in dissociated rat hippocampal neurons. **h)** (left to right) transmitted light image of **1-COT** in hiPSC-CMs, **1-COT** imaged at 488 nm, BeRST 1 co-stain imaged at 639 nm, composite image of **1-COT** and BeRST 1 staining the cell membranes – images are a zoomed-out version of **Figure 1e** in the main text. **i)** (left to right) transmitted light image of **1-COT** in neurons, **1-COT** imaged at 488 nm, BeRST 1 co-stain imaged at 639 nm, composite image of **1-COT** and BeRST 1 staining the cell membranes – images are a zoomed out version of **Figure 1d** in the main text. Scale bars are 20 μm for all images.

Figure S5. COT titration in neurons

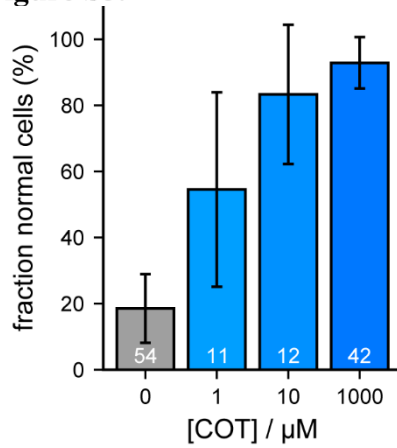


Figure S5. Titration of cylcooctatetraene (COT) in neurons. Plot of fraction of normally-firing neurons (i.e. no shifts in baseline, after depolarization, non-evoked spikes, or after-depolarizations) vs. concentration of COT (in μM) for rat hippocampal neurons imaged with **1** and subjected to the evoked activity protocol. Error bars are the 95% confidence interval for each proportion. The number of neurons analyzed for each condition is indicated in white at the base of the bars. Data for 0 μM and 1,000 μM COT are repeated from the main text, **Figure 2**, for ease of comparison.

Figure S6. Comparison of all dyes and conditions in neurons

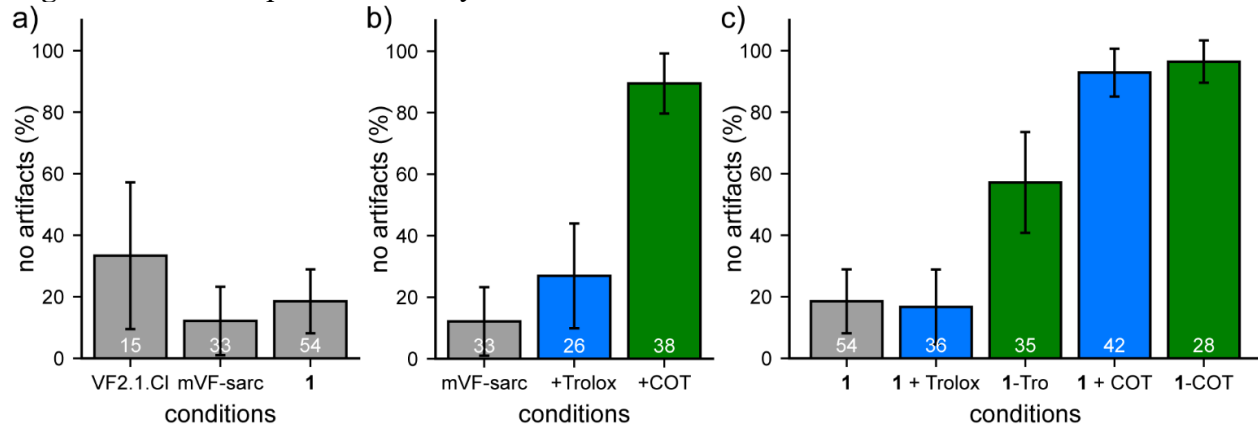


Figure S6. Comparison of all dyes and conditions in neurons. Plots of fraction of normally-firing neurons (i.e. no shifts in baseline, after depolarization, non-evoked spikes, or after-depolarizations) vs. all analyzed dye and additive conditions. **a)** Plot of normally-firing neurons vs. VF dye with no additives. VF2.1.Cl, mVF-sarc, or Compound **1**. Data for mVF-sarc and **1** are repeated from **Figure S1b** and **Figure 2b**, respectively, for comparison). **b)** Plot of normally-firing neurons vs. mVF-sarc with addition of exogenous Trolox (1 mM) and COT (1 mM). Data are repeated from **Figure S1b** for ease of comparison. **c)** Plot of normally-firing neurons vs. **1**, **1** + Trolox (1 mM), **1**-Tro, **1** + COT (1 mM), or **1**-COT. Data are repeated from **Figure 2b** in the main text, for ease of comparison. All error bars are $\pm$ 95% confidence interval. Numbers of neurons analyzed are indicated in white at the base of the respective bar.

Figure S7. Action potential kinetics estimation under high-speed, high-powered illumination

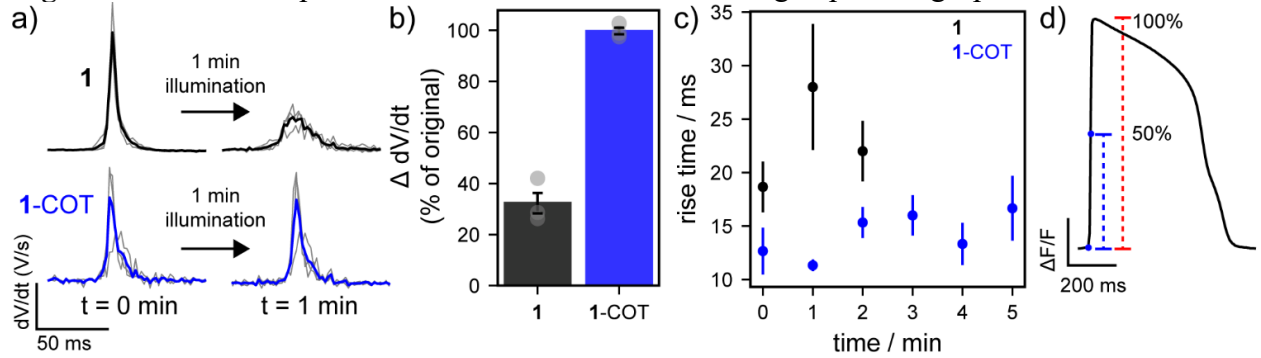


Figure S7. Action potential kinetics estimation under high-speed, high-powered illumination. **a)** Plots of maximum upstroke velocity (dV/dt , in units of V/s) vs. time for action potentials (AP) recorded with either **1** (black traces) or **1-COT** (blue traces). Thick colored line (black or blue) are the mean dV/dt values, individual dV/dt plots are in grey ($n = 3$ independent experiments). Plots show $t = 0$ min of illumination and after 1 min of continuous illumination. The mean dV/dt value was calculated by estimating an AP amplitude of 100 mV (Table 1 in *Front. Physiol.* **2012**, 3, 346; mean AP amplitude = 100 mV). Across all 6 experiments, including APs recorded with both **1** and **1-COT**, the mean upstroke velocity is 11.4 V/s (± 3.2 V/s, standard deviation, $n = 6$), which is within the range of recorded upstroke velocities for hiPSC-CMs reported in Hoekstra, *et al.* (Table 1 in *Front. Physiol.* **2012**, 3, 346; mean $dV/dt = 31$ V/s, min = 9 V/s, max = 115 V/s).¹ **b)** Plot of the change in dV/dt ($\Delta dV/dt$) as a percentage of original dV/dt after one minute of high powered illumination for **1** alone (black) or **1-COT** (blue). Data are mean $\Delta dV/dt$; error bars are S.E.M. for $n = 3$, and individual differences (paired before/after) are in grey circles. **c)** Plot of rise time vs. total illumination time for **1** (black) and **1-COT** (blue). Data are mean rise time (in ms); error bars are S.E.M. for $n = 3$ independent experiments. **d)** Rise times are calculated as the time difference between the initial rise from baseline (first blue circle) to the maximum slope at 50% (second blue dot, or blue dashed line) of maximum AP height (red dotted line). A schematized AP from **Figure 3e** in the main text is shown for illustrative purposes.

Figure S8. Photobleaching comparison in hiPSC-CMs

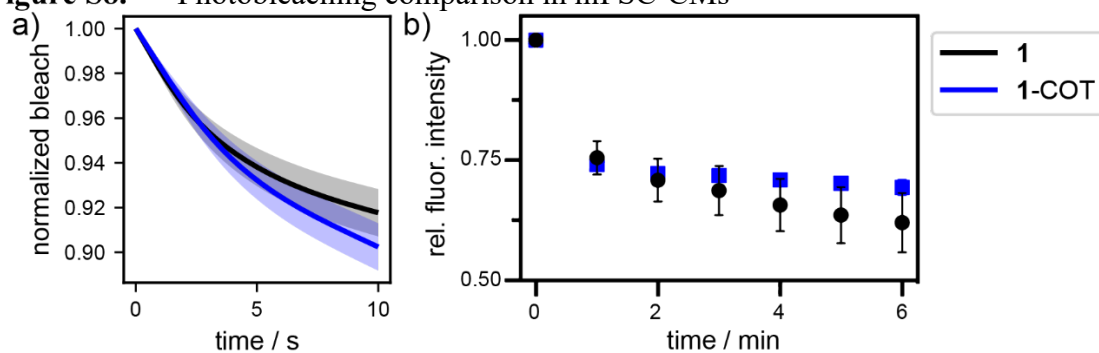


Figure S8. Photobleaching comparison in hiPSC-CMs. **a)** Plot of normalized photobleach rate for **1** or **1-COT** vs. time in hiPSC-CMs. Data are mean bleach rate for **1** (black line) or **1-COT** (blue line). Shaded area represents 95% confidence interval for n = 5 independent determinations. **b)** Plot of normalized fluorescence intensity values vs. time in hiPSC-CMs. S.E.M. are reported for n = 3 independent determinations.

Figure S9. Beats per minute comparison in hiPSC-CMs

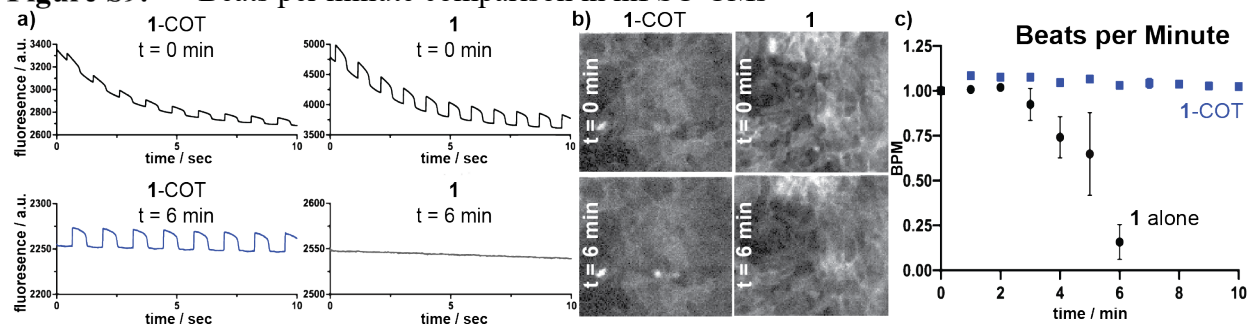


Figure S9. Beats per minute comparison in hiPSC-CMs. **a)** Plot of raw fluorescence values vs. time in hiPSC-CMs for **1** or **1-COT**. Data are representative of one sample. **b)** Representative images of hiPSC-CMs stained with **1** or **1-COT**. The cell membrane remains stained for **1** and **1-COT** at $t = 0$ and $t = 6$ minutes. Images are individual frames from **Supplemental Videos 1-4**, demonstrating beating or lack of beating in hiPSC-CMs. **c)** Plot of normalized beat rate vs. time in hiPSC-CMs. Data are a mean beat rate for **1** (black line) or **1-COT** (blue line). S.E.M. are reported for $n = 3$ independent determinations.

Supplemental Videos 1-4

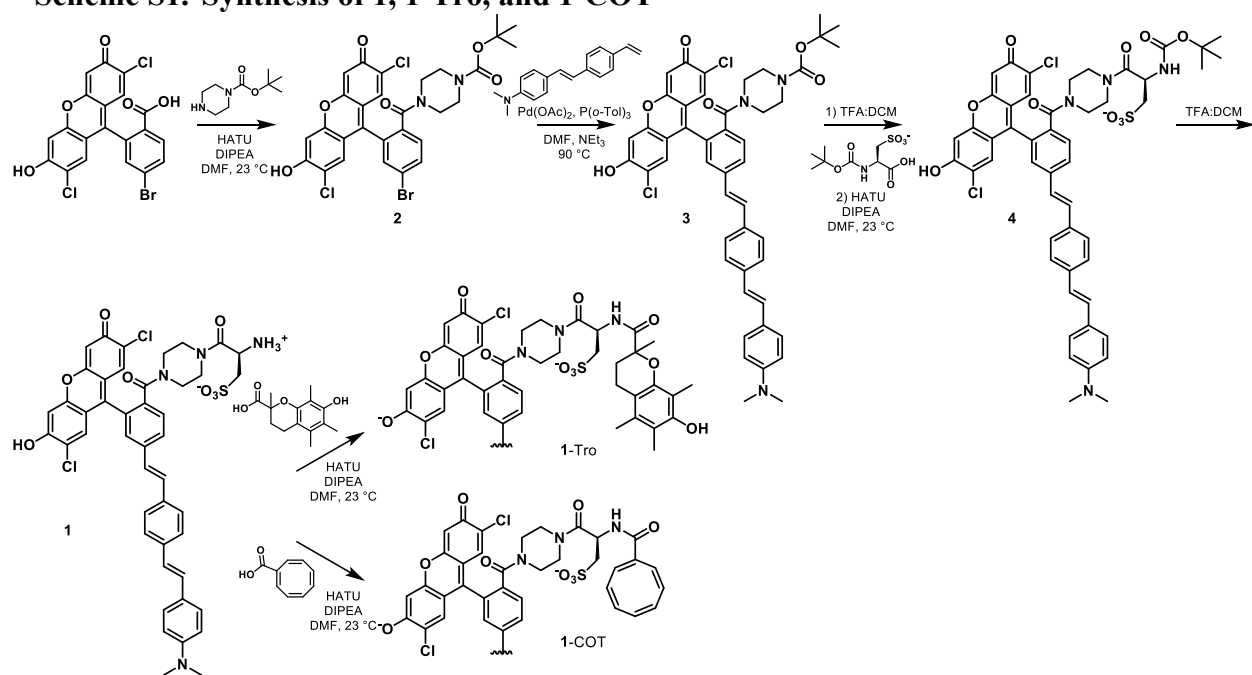
Visual comparison of contraction and healthy activity in hiPSC-CMs stained with **1** or **1-COT**. At $t = 0$ (**Video 1**) and $t = 6$ minutes (**Video 2**) for **1-COT**, cells are visually contracting, with healthy electrophysiology evidenced by the AP traces in **Figure S9**. **1-COT** is seen staining cell membranes over time. At $t = 0$ (**Video 3**) for compound **1** cells are visually contracting with healthy electrophysiology evidenced by the AP traces in **Figure S9**. At $t = 6$ minutes (**Video 4**), iPSC-CMs stained with compound **1** are no longer contracting and APs are not seen in the 10 s trace in **Figure S9**. Compound **1** remains fluorescent and in the cell membrane at minute 6 but is detrimental to cell health.

Table S1. Summary of neuron imaging data

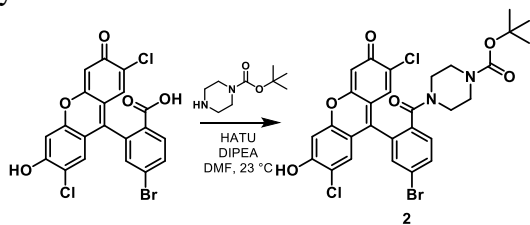
Dye	additive	[additive]	no artifacts (#)	no artifacts (%)	total cells	Figure
1	---	---	10	19%	54	2b / S6c
1	Trolox	1 mM	6	17%	35	2b / S6c
1	COT	1 mM	39	93%	42	2b / S6c
1 -Tro	---	---	20	57%	35	2b / S6c
1 -COT	---	---	27	96%	28	2b / S6c
1	COT	0.001 mM	6	55%	11	S5
1	COT	0.01 mM	10	83%	12	S5
mVF-sarc ^a	---	---	4	12%	33	S1b / S6b
mVF-sarc ^a	Trolox	1 mM	7	27%	26	S1b / S6b
mVF-sarc ^a	COT	1 mM	34	89%	38	S1b / S6b
VF2.1.Cl ^b	---	---	5	33%	15	S6a

^a Compound **13** from Grenier, *et al. JACS*, **2019**, *141*, 1359. ^b VF2.1.Cl from Miller, *et al. PNAS*, **2012**, *109*, 2114.

Scheme S1. Synthesis of 1, 1-TrO, and 1-COT



Synthesis of **2**.



Synthesis of **2**, or, *tert*-butyl 4-(4-bromo-2-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoyl)piperazine-1-carboxylate.

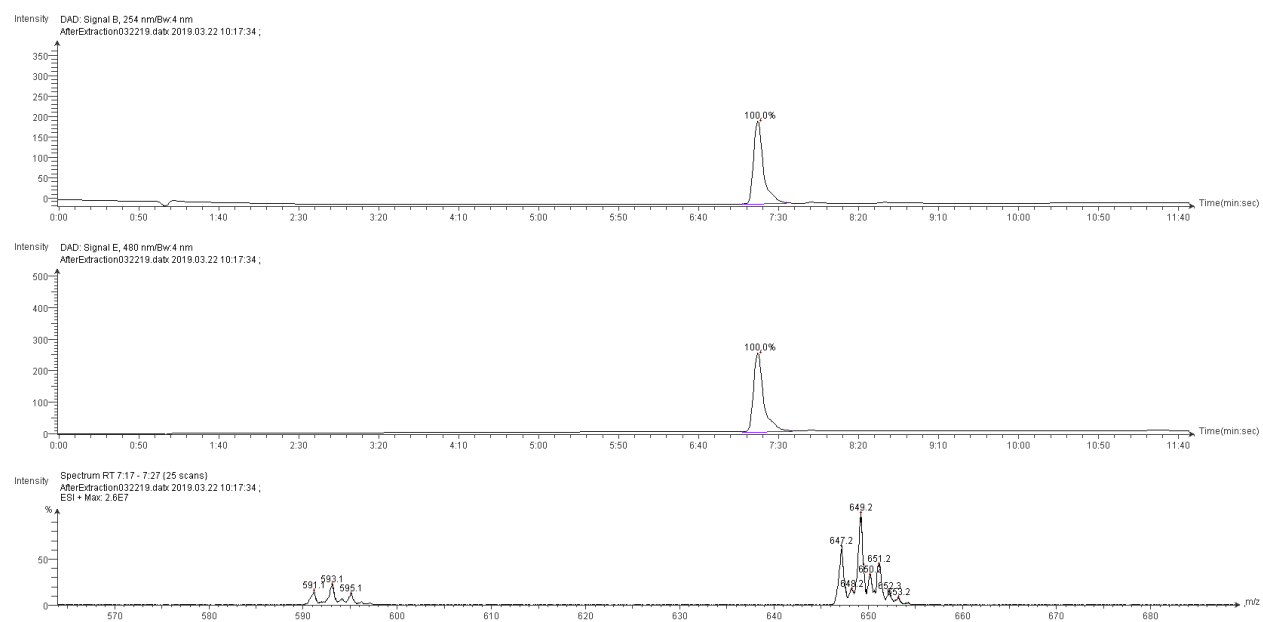
6-bromo-2',7'-dichlorofluorescein (317 mg, 660 μ mol), Boc-protected piperazine (127.6 mg, 685 μ mol) and HATU (695 μ mol) were dissolved in 2 mL anhydrous DMF, followed by addition of 575 μ L DIPEA (427 mg, 3.30 mmol). After stirring for 24 hours the reaction was diluted with 50 mL 20% iPrOH/DCM and washed five times with 20 mL water. The organic phase was dried over MgSO₄, decanted, and solvents removed under reduced pressure. Product was purified by flash column chromatography (10% MeOH/DCM) to obtain **2** (380 mg, 586 μ mol, 89%) as a red powder. Analytical HPLC retention time: 6.89 minutes.

¹H NMR (300 MHz, *d*₆-DMSO) δ 7.95 (dd, *J* = 8.3, 2.1 Hz, 1H), 7.85 (d, *J* = 2.0 Hz, 1H), 7.62 (d, *J* = 8.3 Hz, 1H), 7.17 (s, 2H), 6.77 (s, 2H), 3.38 (bm, 4H), 3.13 (bm, 4H), 1.4 (s, 9H).

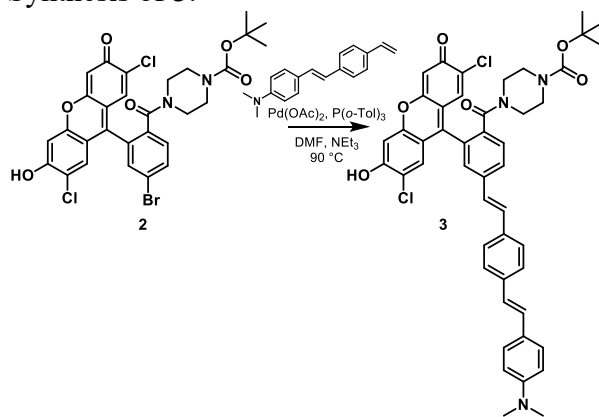
ESI-MS: [M+H]⁺ calculated 647.0, found 647.2.

[M-C₃H₈+H]⁺, calculated 591.0, found 591.1

Spectra S1. HPLC of 2.



Synthesis of **3**.



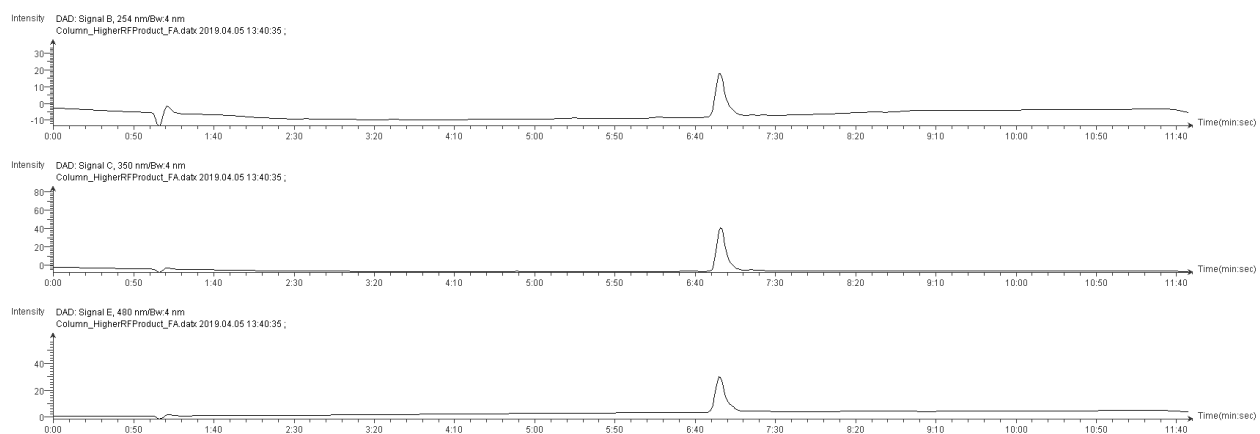
Synthesis of **3**, or, tert-butyl 4-(2-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthene-9-yl)-4-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzoyl)piperazine-1-carboxylate.

To an oven-dried Schlenk flask added **2** (350 mg, 540 μ mol), phenylene vinylene molecular wire (169 mg, 678 μ mol), Pd(OAc)₂ (1.80 mg, 8.01 μ mol) and P(*o*-Tol)₃ (15.5 μ mol). The flask was evacuated and backfilled with N₂ three times. Solid reagents were dissolved in 3 mL anhydrous DMF, 1.5 mL anhydrous Et₃N and the reaction stirred for 16 hours at 90 °C. After cooling to rt the reaction was diluted with 20 mL 20% iPrOH/DCM and filtered through a pad of celite. Solvents were removed under reduced pressure and product purified by flash column chromatography (10% MeOH/DCM) to obtain **3** (408 mg, 500 μ mol, 92%) as a red powder.

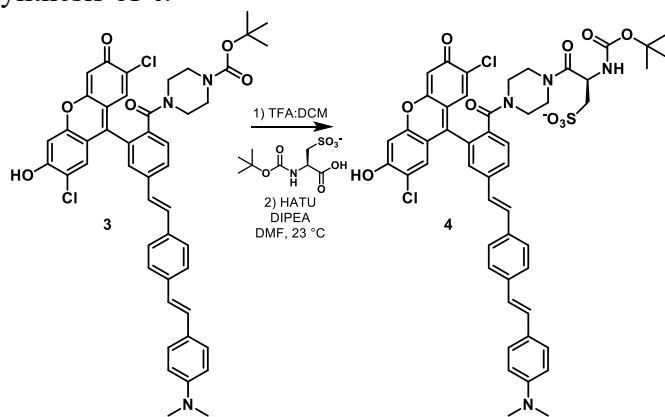
¹H NMR (300 MHz, *d*₆-DMSO) δ 7.91 (d, *J* = 2.1 Hz, 1H), 7.82 (d, *J* = 1.9 Hz, 1H), 7.76 (s, 1H), 7.65 (d, *J* = 8.1 Hz, 1H), 7.61-7.48 (m, 4H), 7.47-7.43 (m, 3H), 7.15 (d, *J* = 3.0 Hz, 3H), 7.00 (s, 1H), 6.72 (m, 4H), 3.12 (m, 8H), 2.93 (s, 6H), 1.40 (s, 9H).

ESI-MS [M+H]⁺ calculated 816.3 , found 816.4.

Spectra S2. HPLC of 3



Synthesis of 4.

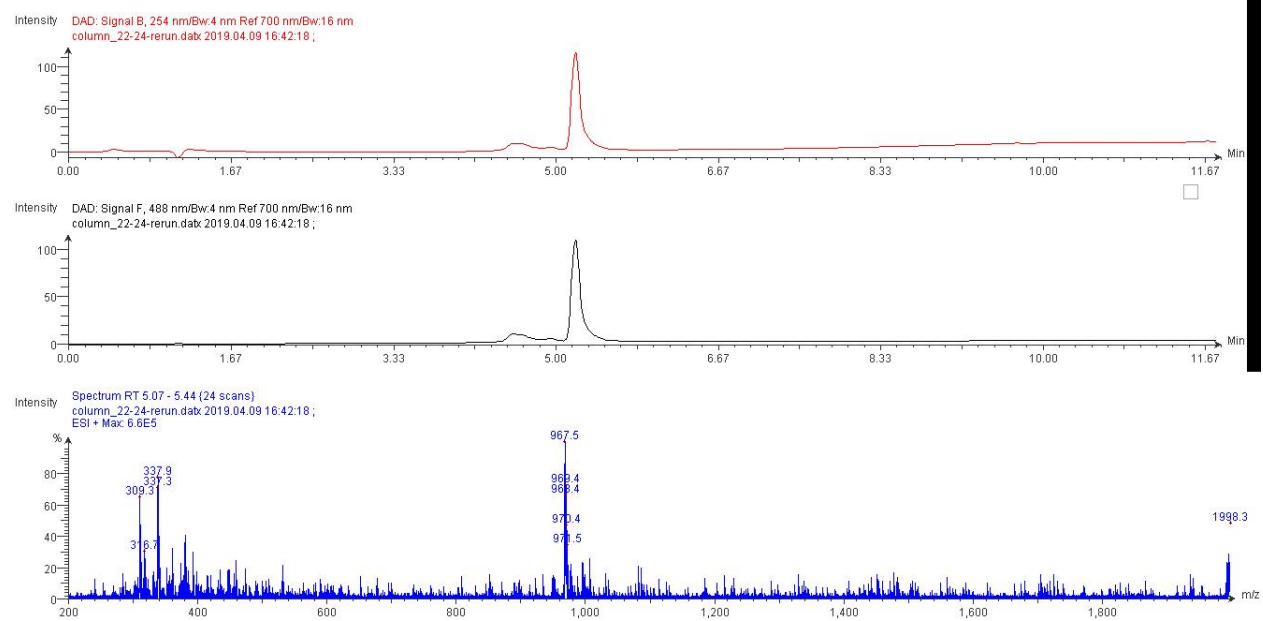


Synthesis of **4**, or (*R*)-2-((tert-butoxycarbonyl)amino)-3-(4-(2-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-4-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzoyl)piperazin-1-yl)-3-oxopropane-1-sulfonate

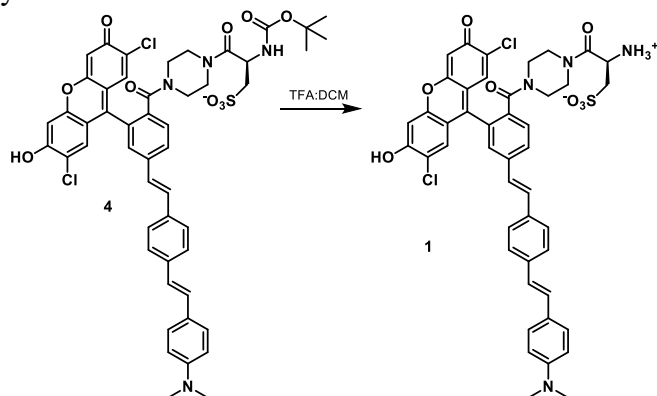
3 (154.3 mg, 188 μmol) was dissolved in 2 mL 1:1 TFA:DCM. After stirring for 30 minutes the reaction was concentrated under a stream of N_2 and residual solvents removed by toluene azeotrope. In the same round bottom, added Boc-protected cysteic acid (56.0 mg, 208 μmol) and HATU (82.5 mg, 217 μmol). Solid reagents were dissolved in 1 mL anhydrous DMF, followed by addition of 164 μL DIPEA (121 mg, 164 μmol). After stirring for 16 hours the reaction was neutralized by drop-wise addition of AcOH and solvents removed under reduced pressure. Crude material was purified by preparative TLC (15% MeOH, 5% AcOH in DCM, material eluted off silica with 10% MeOH/DCM) to obtain **4** as a red gum. Trituration with MeCN led to formation of a red powder, which could be isolated by vacuum filtration to obtain **4** (90.0 mg, 93.0 μmol , 50%).

ESI-MS $[\text{M}+\text{H}]^+$ calculated 967.3 , found 967.5.

Spectra S3. HPLC of 4



Synthesis of **1**.



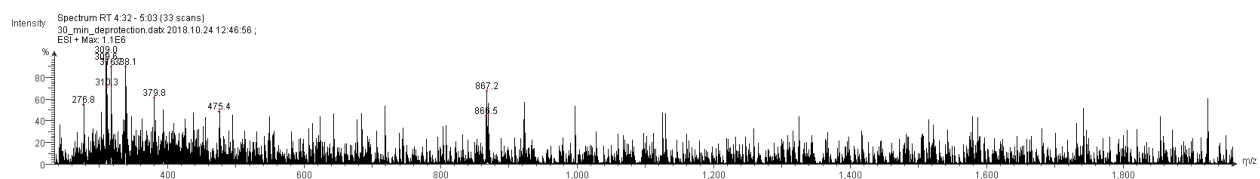
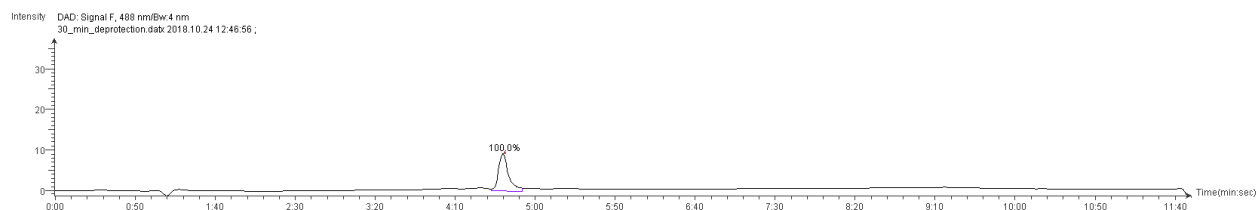
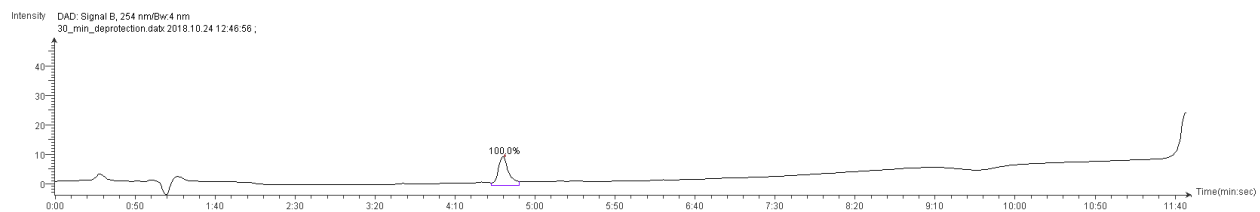
Synthesis of **1**, or, (*R*)-2-ammonio-3-(4-(2-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-4-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzoyl)piperazin-1-yl)-3-oxopropane-1-sulfonate.

4 (47.9 mg, 51.7 μ mol) was dissolved in 1 mL 1:1 TFA:DCM. After stirring for 30 minutes, the reaction was concentrated under a stream of N₂, after which residual solvents were removed by toluene azeotrope under reduced pressure. The resulting solid was suspended in MeCN and **1** (40.1 mg, 37.3 μ mol, 90%) was collected by vacuum filtration as an orange powder. Analytical HPLC retention time: 4.67 minutes.

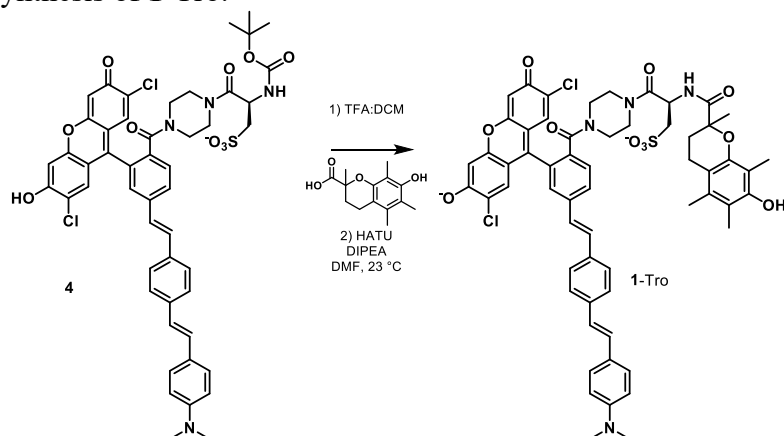
¹H NMR (600 MHz, *d*₆-DMSO) δ 8.03 (s, 3H), 7.93 (d, *J* = 8.2 Hz, 1H), 7.77 (s, 1H), 7.68 (d, *J* = 8.1 Hz, 1H), 7.61 – 7.50 (m, 3H), 7.50 – 7.30 (m, 4H), 7.18 (d, *J* = 16.3 Hz, 2H), 6.98 (d, *J* = 16.3 Hz, 1H), 6.73 (d, *J* = 8.5 Hz, 2H), 4.47 (s, 1H), 3.66 – 3.54 (m, 6H), 3.17 – 3.06 (m, 2H), 2.94 (s, 6H).

ESI-MS [M+H]⁺ calculated 867.2, found 867.2.

Spectra S4. HPLC of 1



Synthesis of 1-Tro.



Synthesis of **1-Tro**, or, (2*R*)-3-(4-(2-(2,7-dichloro-6-oxido-3-oxo-3*H*-xanthen-9-yl)-4-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzoyl)piperazin-1-yl)-2-(7-hydroxy-2,5,6,8-tetramethylchromane-2-carboxamido)-3-oxopropane-1-sulfonate

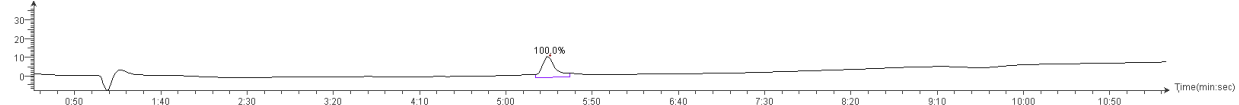
4 (42.1 mg, 43.5 μmol) was dissolved in 1 mL 1:1 TFA:DCM. After stirring for 1 hour the reaction was concentrated under a stream of N_2 and residual solvents removed by toluene azeotrope. In the same round bottom, added Trolox (12.2 mg, 48.6 μmol) and HATU (19.1 mg, 50.1 μmol). Solid reagents were dissolved in 1 mL anhydrous DMF, followed by addition of 37.9 μL DIPEA (28.1 mg, 217 μmol). After stirring for 16 hours the reaction was neutralized by addition of 50 μL AcOH and solvents removed under reduced pressure. The crude reaction was dissolved in MeOH, filtered through a 0.22 μm filter and purified by preparative HPLC to obtain **1-Tro** (9.49 mg, 8.63 μmol , 20%) as an orange powder.

^1H NMR (600 MHz, d_6 -DMSO) δ 7.93 (d, J = 8.3 Hz, 1H), 7.75 (s, 1H), 7.67 (dd, J = 8.1, 4.9 Hz, 1H), 7.57 (m, 4H), 7.51-7.31 (m, 4H), 7.17 (m, 3H), 7.00 (d, J = 16.3 Hz, 1H), 6.79 (m, 3H), 5.76 (s, 1H), 4.70 (m, 2H), 3.41 (s, 4H), 3.13-3.04 (m, 1H), 2.95 (s, 6H), 2.80-2.57 (m, 3H), 2.06 (m, 7H), 1.98 (m, 3H), 1.75-1.62 (m, 1H).

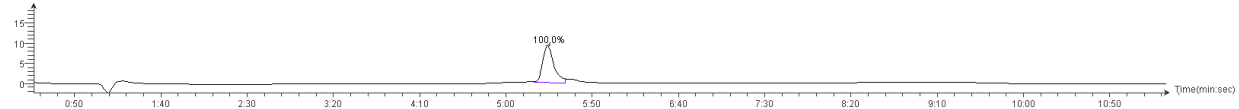
ESI-MS $[\text{M}+\text{H}]^+$ calculated 1099.3, found 1099.5.

Spectra S5. HPLC 1-Tro

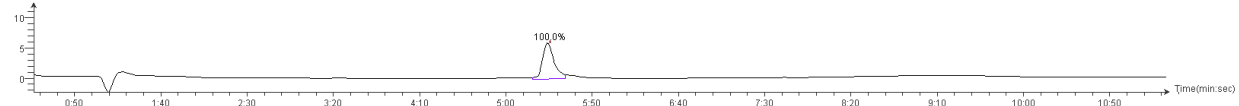
Intensity DAD: Signal B, 254 nm/Bw:4 nm
prep_F4.dab: 2018.10.04 17:32:57 ;



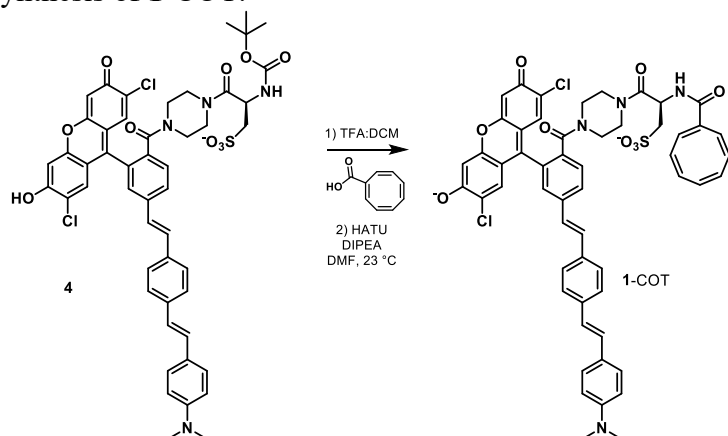
Intensity DAD: Signal F, 488 nm/Bw:4 nm
prep_F4.dab: 2018.10.04 17:32:57 ;



Intensity DAD: Signal D, 405 nm/Bw:4 nm
prep_F4.dab: 2018.10.04 17:32:57 ;



Synthesis of 1-COT.



Synthesis of 1-COT, or, (*R*)-2-((1*E*,3*Z*,5*Z*,7*Z*)-cycloocta-1,3,5,7-tetraene-1-carboxamido)-3-(4-(2-(2,7-dichloro-6-oxido-3-oxo-3*H*-xanthen-9-yl)-4-((*E*)-4-((*E*)-4-(dimethylamino)styryl)styryl)benzoyl)piperazin-1-yl)-3-oxopropane-1-sulfonate.

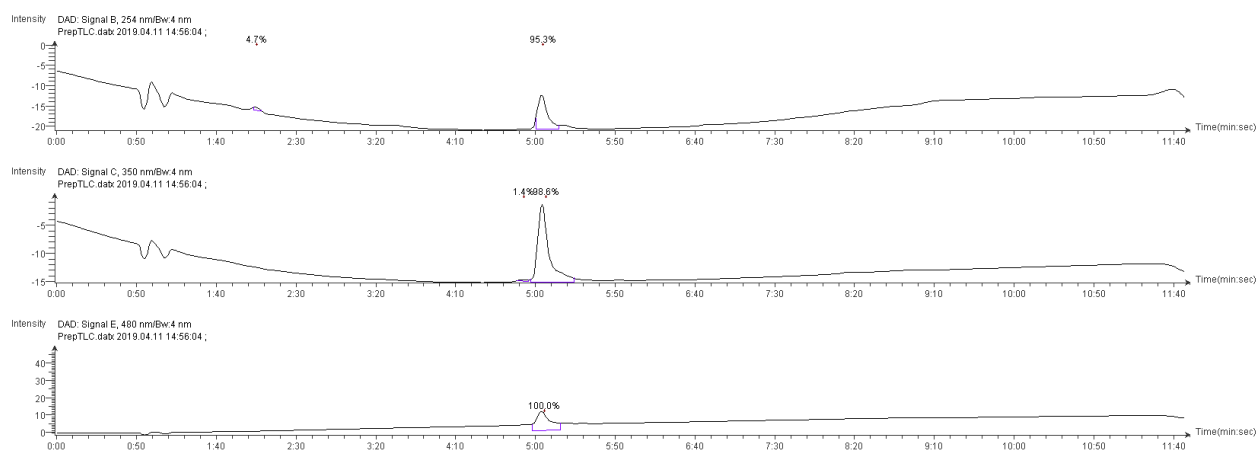
From DMGA summary (only source):

4 (23.6 mg, 24.4 μmol) was dissolved in 1 mL 1:1 TFA:DCM. After stirring for 30 minutes the reaction was concentrated under a stream of N_2 , after which residual solvents were removed by toluene azeotrope. To the same reaction vial, added COT- CO_2H (8.6 mg, 58.1 μmol) and HATU (18.4 mg, 48.4 μmol). Solid reagents were dissolved in 0.25 mL anhydrous DMF, followed by addition of 0.03 mL DIPEA (22.3 mg, 172 μmol). After stirring for 16 hours, the reaction was neutralized by drop-wise addition of AcOH and solvents removed under reduced pressure, azeotroping with toluene. Crude material was purified by preparative TLC (15% MeOH, 5% AcOH in DCM, material eluted off silica with 10% MeOH/DCM) to obtain 1-COT as a red gum (>95% pure by HPLC). Azeotroping with toluene led to formation of a red powder (14.5 mg, 60%).

ESI-MS $[\text{M}+\text{H}]^+$ calculated 997.2, found 997.3.

High resolution ESI-MS $[\text{M}-\text{H}]^-$ calculated 995.2290 ($^{12}\text{C}_{54}\text{H}_{45}\text{O}_9\text{N}_4^{35}\text{Cl}_2^{32}\text{S}_1$), found 995.2292.

Spectra S6. HPLC of 1-COT



References

- 1) Hoekstra, M., Mummery, C., Wilde, A., Bezzina, C. & Verkerk, A. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. *Frontiers in Physiology* **3**, doi:10.3389/fphys.2012.00346 (2012).

Chapter 2

Red-shifted fluorescent voltage sensitive indicator with molecular prosthetic for long-term cardiac imaging

This work was completed by:

Martinez, K.N.: Patch clamp electrophysiology, characterization and imaging of VFs in hiPSC-CMs, singlet oxygen sensor green experiments, FLIM VF loading experiments

Gerstner, N.C.: Synthesis of **1**-COT, spectroscopic characterization

Yang, S.J.: Relative brightness, photobleaching comparisons

Miller, E.W.: Principal Investigator

Abstract

Voltage sensitive indicators can report cardiac action potentials with fast kinetics but phototoxicity of the fluorophores can cause the production of reactive oxygen species, aberrant electrophysiology, and damage the health of the cells being studied. The use of an intramolecular triplet state quencher can promote photostability and self-healing of the fluorophore, which can increase the longevity of the indicator and elongate imaging sessions with healthy cells. Herein, we demonstrate a generalizable method for reducing phototoxicity and singlet oxygen production with a cyclooctatetraene molecular prosthetic tethered to a near infrared silicon rhodamine-based voltage indicator. This novel indicator, BeRST-COT, can image cardiac electrophysiology for 2.7x longer than its parent dye, and reduces the amount of singlet oxygen produced while imaging.

Introduction

Voltage sensitive fluorescent indicators have long been used in cardiac biology research for direct visualization of electrophysiology. These indicators complement traditional methods of monitoring cardiac electrophysiology such as whole-cell patch clamp electrophysiology.¹ The use of patch clamp electrophysiology has helped determine the electrophysiological effects of structural immaturity in hiPSC-CMs and provided insight into action potential (AP) parameters.² However, the patch clamp technique is difficult, lacks spatial resolution, and is a low throughput process. Voltage sensitive indicators offered a new method for studying electrophysiology and have led to progress in whole-heart optical mapping and understanding of cardiac arrhythmias.^{3,4} More recently, fluorescent indicators have been used in the characterization of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs),⁵⁻⁸ as well as in screening assays for arrhythmias and drug toxicity, including screening for COVID- therapeutics.⁹⁻¹²

Fluorescence imaging of cardiac electrophysiology dates back to 1976 with the study of frog hearts with merocyanine 540.¹³ However, merocyanine 540 has a low $\Delta F/F$ at 1% per 100 mV, photobleaches rapidly, and can be quite toxic.^{14,15} Electrochromic dyes, such as di-4-ANEPPS, have improved fractional fluorescence ($\Delta F/F$ of 10% per 100 mV), but are phototoxic, internalize from the outer leaflet of the cell membrane, and are photon limited due to the reliance on spectral changes of the dye.¹⁶⁻¹⁸ While phototoxicity and photobleaching can be somewhat reduced by altering experimental parameters such as light intensity and exposure times,¹⁹⁻²¹ there is also a need for the rational design of new, less toxic voltage sensitive indicators.^{22,23}

VoltageFluors (VFs) are a class of voltage sensitive indicators that anchor into the cell membrane and utilize a photoinduced electron transfer (PeT) mechanism to sense changes in membrane potential.²⁴ PeT rapidly occurs from an electron rich aniline donor located near the inner leaflet of the cell membrane, to a fluorophore anchored at the outer leaflet of the cell membrane, via a phenylenevinylene molecular wire.²⁴⁻²⁶ The result is a voltage sensitive fluorescent indicator reacting with fast kinetics to changes in membrane potential, demonstrating a linear correlation between fluorescence intensity and voltage.²⁷ VF indicators offer high temporal and spatial resolution, are high throughput, and allow the study of single cells, monolayers, and 3D cultures.

Previously we reported VF-COT, a green VoltageFluor indicator tethered to a cyclooctatetraene (COT) molecule via a piperazine-cysteic acid linker.²⁸ We utilized a method for reducing phototoxicity in fluorescence imaging by intramolecularly tethering the triplet state quencher (TSQ) cyclooctatetraene (COT) to the fluorophore with the goal of reducing the production of reactive oxygen species (ROS).²⁹⁻³¹ This method reduces the lifetime of the triplet state

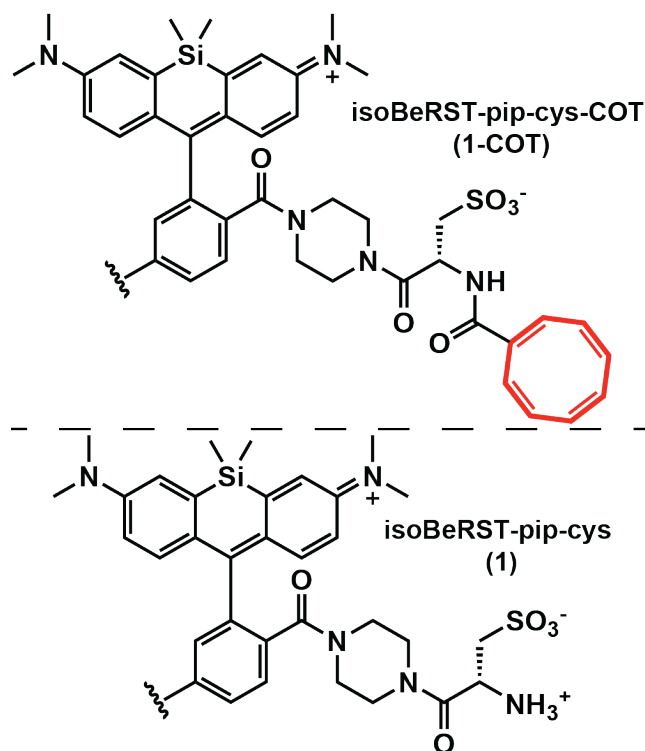
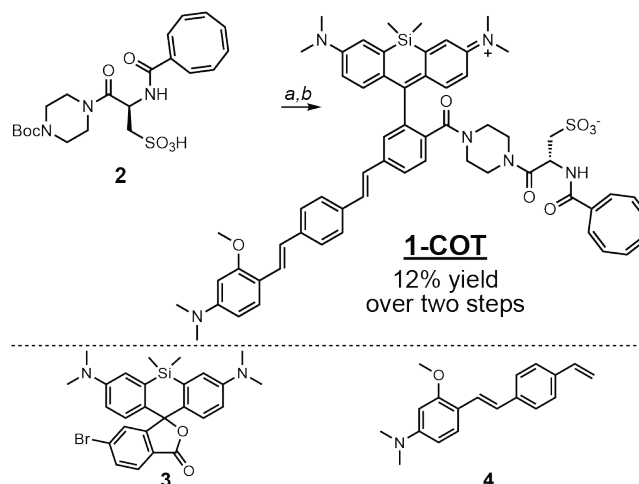


Figure 1. Molecular structures of VF probes. **top)** Structure of isoBeRST-pip-cys-COT (1-COT). **bottom)** Structure of isoBeRST-pip-cys (1).

fluorophore through intramolecular triplet energy transfer, reducing the amount of time that singlet oxygen can be produced and regenerating the ground state fluorophore in a process considered “self-healing.”^{29,32,33} VF-COT prolonged imaging time relative to its parent indicator lacking the triplet state quencher. VF-COT also reduced light-induced physiological disruptions in rat hippocampal neurons and hiPSC-CMs like spurious action potentials, runaway firing, or cessation of beating, likely by reducing singlet oxygen and overall ROS production.

Herein, we demonstrate the generalizability of the self-healing strategy of COT on voltage sensitive indicators with the silicon-rhodamine based fluorophore, **1-COT** (Fig. 1, top). **1-COT** is a near-infrared (NIR) VoltageFluor tethered to COT with a piperazine-cysteic acid linker. The indicator demonstrates reduced production of singlet oxygen, reduced photobleaching, and enables long-term imaging of cardiac action potentials relative to its parent dye. **1-COT** is preferable to its green counterpart as it minimizes effects of autofluorescence of endogenous chromophores, uses lower energy excitation wavelengths, and is better suited for potential multiplexing with green indicators. Additionally, BeRST 1, from which **1-COT** is derived, has already been used extensively for the study of iPSC-CMs.^{34–42}



Scheme 1. Synthesis of **1-COT**. **a**) i) TFA, CH₂Cl₂, rt, ii) **3**, (COCl)₂, CH₂Cl₂, rt, iii) Et₃N, DMF, rt; **b**) **4**, cat. Pd₂dba₃, QPhos, Et₃N, DMF, 50 °C.

Results and Discussion

Synthesis. The synthesis of isoBeRST-pip-cys-COT (**1-COT**) began by performing an amide coupling *N*-Boc-piperazine and protected cystine **S-X** (Figure SX). Our attempts to perform this amide coupling with various cysteine analogs were met with failure, necessitating the use of the cystine group. Oxidation of the cystine using hydrogen peroxide and formic acid, followed by re-installation of the Boc group resulted in the isolation of cysteine acid **S-X**. Deprotection of the primary amine followed by an amide coupling yielded the cyclooctatetraene amide **2**.

With the cyclooctatetraene amide **2** in hand, we tested different conditions in order to couple it to the dye with the molecular wire already installed. Unfortunately, these amide couplings were met with nothing but unreacted starting material; thus, we developed conditions in which the amide coupling occurred before the installation of the molecular wire. Deprotection of **2** followed by the coupling with the acid chloride derived from **X** resulted in the isolation of the silicon rhodamine **X** in 29% yield. The removal of all of the TFA from the deprotection proved to be essential for this reaction as trace amounts of TFA would decompose the DMF and result in small amounts of dimethylamine reacting with the acid chloride, generating a biproduct that was extremely challenging to separate from **X**. Finally, we developed conditions for the Heck coupling of **X** with the molecular wire **4**. While we initially performed the Heck coupling using tri-(*o*-tolyl)phosphine as a ligand at elevated temperatures, these conditions proved to be inconsistent due to the instability of **1-COT** to the elevated temperatures. Ultimately, we discovered that using QPhos as a ligand at milder temperatures resulted in the isolation of **1-COT** in 41% yield (Scheme 1).

In Vitro and Cellular Characterization. Spectroscopic characterization of **1-COT** shows that it has very similar absorbance and emission spectra compared to its parent fluorophore, **1** (Fig 1; Fig. 2a; Table S1).⁴³ **1-COT** has a quantum yield (Φ) of 0.013 (Table S1) and localizes to the cell membrane in both HEK293T cells and hiPSC-CMs (Fig. 2b,c), however it is only 13% as bright relative its parent dye (Fig. S1c). The cause for the reduction in relative brightness in cells could be related to the lower quantum yield and extinction coefficient of **1-COT** relative to its parent dye (**1**) (Table S1). **1-COT** is a functional voltage sensitive indicator with a $\Delta F/F$ of 11% $\pm$ 0.4% per 100 mV ($n = 5$) and is capable of resolving cardiac action potentials (Fig. 2d). However, **1-COT** is less voltage sensitive than parent dye (**1**) which has a previously reported $\Delta F/F$ of 24% $\pm$ 1.9% per 100 mV.⁴³ Voltage sensitivities are reported as the mean $\pm$ SEM.

Photostability of 1-COT

Reduced Photobleaching. **1-COT** reduces photobleaching compared to its parent dye, **1**. Where **1** sees an average of 10% ($n = 18$, SEM = 0.006) loss in fluorescence over three minutes of continuous imaging in HEK293T cells, **1-COT** maintains its fluorescence with a 0.2% ($n = 18$, SEM = 0.005) loss in fluorescence (Fig. 2e).

Long Term Recording in Cardiomyocytes. **1-COT** can report cardiac electrophysiology and allows for approximately 30 minutes of continuous imaging of hiPSC-CM action potentials. The ability to image for 30 minutes is a 2.7x improvement relative to its parent indicator, **1**, which can image continuously for up to 11 minutes but quickly demonstrates aberrant changes in action potential characterization (Fig. 3a). The use of **1** long-term gives rise to a dramatic 38% (SEM = 0.17, $n = 3$) increase in action potential duration at just 11 minutes (Fig. 3a,b,c), as well as a relatively rapid 32% (SEM = 0.11, $n = 3$) decrease in beats per minute (BPM) (Fig. S2) before cells show an arrest of beating, pointing to the phototoxicity of the indicator. Over the course of a 30-minute imaging session, **1-COT** reports stable cardiac action potentials with just a 19% (SEM = 0.031, $n = 3$) increase in action potential duration over the 30 minutes (Fig. 3a,d,e) and a 28% loss in BPM at $t = 30$ min (Fig. S2). These data point to relatively smaller phototoxicity effects with the use of the **1-COT** indicator. The ability of **1-COT** to image for 30 minutes shows its

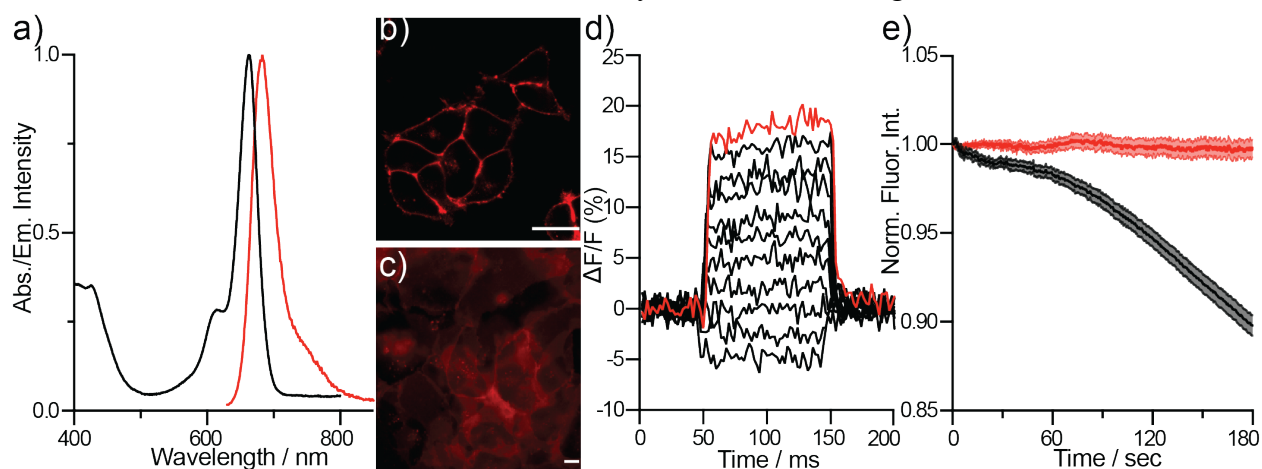


Figure 2. Characterization of **1-COT**. **a)** Absorbance (black) and emission (red) profile of **1-COT**. Confocal images of **1-COT** loaded at 0.5 μ M in **b)** HEK293T cells and **c)** hiPSC-CMs. Scale bars are 20 μ m. **d)** Plot of relative change in fluorescence of **1-COT** ($\Delta F/F$) vs. time in a patch-clamped HEK293T cell under whole-cell voltage-clamp conditions. **e)** Plot of relative fluorescence intensity vs time for **1** (black) or **1-COT** (red) in HEK293T cells. Shaded area represents the standard error of the mean for $n = 18$ individual cell rafts.

reduced phototoxicity as well as demonstrates its stability in functional imaging. As phototoxicity and photobleaching are a large concern in live-cell fluorescence imaging where cell health is very important, **1-COT** could reduce the risk of long-term imaging in cells. **1-COT** is likely allowing elongated imaging sessions due to reduced singlet oxygen production which is an effect of the intramolecularly tethered triplet state quencher which promotes self-healing and a reduction of the triplet state of the dye.³² However since **1-COT** shows reduced relative brightness in cells, the ability of **1-COT** to image for extended periods of time could be related to a smaller effective concentration of indicator loading into the cell membrane due to the increased hydrophobicity of the indicator with the addition of COT.

These data were collected via a phototoxicity assay which was performed by bath applying 500 nM of VoltageFluor in RPMI/B27 (minus phenol red) to hiPSC-CMs for 20 minutes, exchanging media for fresh RPMI/B27 (minus phenol red), and imaging under continuous illumination for 30 minutes or until an arrest of beating was seen, whichever came first. Ten-second recordings at 200 Hz were taken every minute for the duration of the imaging session. hiPSC-CMs are used for the phototoxicity experiments as action potential duration and beat rate are common parameters of cardiac cell health that we can measure with relative ease using an in-house MATLAB script.^{42,44} Additionally, voltage sensitive dyes are commonly used in cardiac biology thus making this a useful method to demonstrate the capabilities of our VoltageFluors.

Reduced Singlet Oxygen Production. **1-COT**'s elongated imaging capabilities could be a result of lower levels of ROS generation, lower levels of dye incorporation into the membrane, or a combination of the two. To investigate the former, we measured production of singlet oxygen by **1-COT** in solution using singlet oxygen sensor green (SOSG). SOSG is highly fluorescent at 525 nm upon interaction with singlet oxygen but is inherently dim in the absence of singlet oxygen due to intramolecular PeT quenching fluorescence.^{45,46}

1-COT produces a statistically insignificant amount of singlet oxygen as compared to the control,

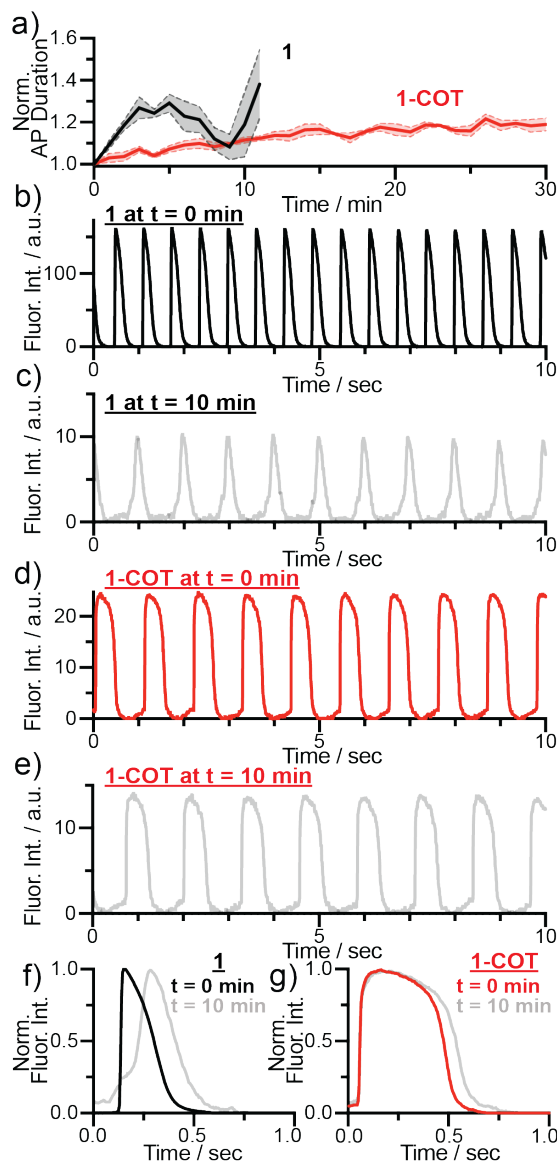


Figure 3. Characterization of action potential parameters. **a)** Plot of normalized action potential duration over time for **1** (black) and **1-COT** (red). At every minute, a 10 s acquisition was taken, and the action potential duration was measured. Data are a mean $\pm$ SEM of $n = 3$ trials. Plots of fluorescence intensity versus time demonstrating a representative action potential trace during a phototoxicity assay for **b)** **1** at $t = 0$ min, **c)** **1** at $t = 10$ min, **d)** **1-COT** at $t = 0$ min, and **e)** **1-COT** at $t = 10$ min. Average action potentials as optically recorded at $t = 0$ min and $t = 10$ min using **f)** **1** (black/grey) and **g)** **1-COT** (red/grey). Average traces are representative of the overall action potential traces shown in figure 3b-e.

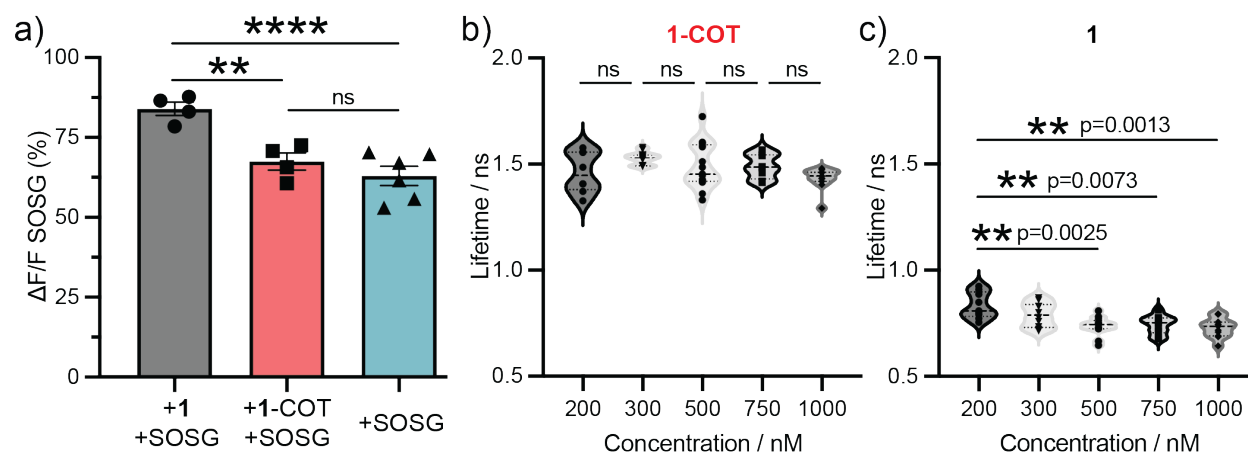


Figure 4. Characterization of singlet oxygen production and fluorescent lifetimes. **a)** Relative changes in SOSG emission in solution after continuous illumination of samples for 10 minutes. Concentrations for all probes are 1 μ M. Solutions represented are: **1** with SOSG present (grey); **1**-COT with SOSG present (red); and SOSG in solution (cyan). ANOVA test: $p < 0.0001$, ****; $p = 0.0015$, **. Concentration curve of fluorescence lifetime over increasing concentrations of **b)** **1**-COT and **c)** **1**. ANOVA test: lifetime variance over concentration increases in **1**-COT were not significant; p-values for **1** are listed.

while **1** produces 25% more singlet oxygen than **1**-COT on average ($n = 4$, $P < 0.0001$) (Fig. 4a). This leads us to conclude that **1**-COT is more photostable than its parent dye, and likely contains self-healing properties due to the reduction of the triplet state lifetime which leads to a lower production of singlet oxygen and reduced phototoxicity.^{29,32} We abstained from continuous monitoring of the green SOSG signal as SOSG itself can be a sensitizer of singlet oxygen, thus the SOSG fluorescence signal was collected only before and after illumination of the samples with the red LED for 10 minutes.⁴⁵ Negative controls tested included the illumination of **1** alone and **1**-COT alone. Neither showed an increase in green SOSG signal, demonstrating lack of spectral overlap between the VF dyes and SOSG (Fig. S3). These results suggest that reduced production of singlet oxygen contributes to the low phototoxicity of **1**-COT.

Reduced loading of 1-COT. While **1**-COT boasts the ability to image cardiac electrophysiology for about three times longer than **1**, the relative brightness of **1**-COT is only 13% that of its parent dye. While some of the dimness of this indicator may be attributed to the lower extinction coefficient and lower quantum yield, we also hypothesized that the addition of the hydrophobic COT molecule to the fluorophore may reduce overall loading of **1**-COT into the cell membrane. To help determine how **1**-COT is loading into the cell membrane compared to **1**, we performed a concentration curve using fluorescence lifetime imaging which allowed us to compare the lifetime of our VoltageFluors at various loading concentrations. Previous work provides rationale for the concentration curve and has shown that a decrease in fluorescence lifetime as concentration increases points to self-quenching as a result of high concentrations of excess fluorophore.^{47,48}

For **1**, we see a significant decrease in fluorescence lifetime at 500 nM concentrations, suggesting that self-quenching is occurring at 500 nM and above (Fig. 4c). For **1**-COT, there is no significant difference between the lifetimes at any of the tested concentrations, suggesting that the loading is not impacted by concentration (Fig. 4b). At 200 nM **1**, the average lifetime is 0.83 ns ($n = 9$, SEM = 0.020) while at the same concentration **1**-COT, the average lifetime is 1.46 ns ($n = 8$, SEM = 0.03382). **1**-COT has a longer fluorescence lifetime which would typically correlate to a brighter fluorophore, however **1**-COT is a functionally dimmer probe than its parent dye.

These data suggest that **1** loads well into membranes making it a functionally brighter indicator, but is inherently dimmer than **1-COT**. **1-COT** is an inherently brighter dye, but is likely loading inefficiently into cells, as seen in the insignificant changes in fluorescence lifetime over a concentration curve. We are possibly reducing the likelihood that **1-COT** enters the nonradiative triplet state with the addition of COT, increasing the fluorescence lifetime, while **1** is likely entering the triplet state with a higher frequency which reduces the fluorescence lifetime.⁴⁹ These results suggest that reduced loading of **1-COT** in the plasma membrane may also contribute to decreased toxicity, along with lower singlet oxygen production.

Conclusion

In this study, we demonstrate the generalizability of the addition of COT to a functional voltage sensitive fluorophore for increasing the photostability of the probe, elongating the amount of time an indicator can image, and reducing the production of singlet oxygen while imaging. **1-COT** is photostable showing minimal photobleaching over a 3-minute period, is capable of imaging and characterizing cardiac action potentials for at least 30 minutes and reduces the production of singlet oxygen compared to its parent dye by 25%.

1-COT fills a need in the library of voltage sensitive fluorophores for a near-infrared self-healing indicator, capable of performing long term imaging with minimal impact on cell health. The near-infrared spectrum of **1-COT** allowed for the relative determination of singlet oxygen production compared to **1**, an experiment which we were not able to with the previously reported green probe, **1-COT**, due to the spectral overlap. The NIR indicator also opens opportunities for experiments requiring multiplexing of voltage sensing with a different green indicator, such as calcium probes or mitochondrion trackers.

While **1-COT** provides many benefits during imaging, the indicator is only 13% as bright as its parent and is less voltage sensitive, leaving room for structural improvements of the indicator. Previous work by others has shown that close proximity of COT to the fluorogenic center of the indicator may further improve its photostability and may further decrease the lifetime of the triplet state of the molecule.³² We are also interested in rational structural design to improve the quantum yield and extinction coefficient of the probe to improve the overall brightness. Lastly, as we found the effective concentration of **1-COT** in the plasma membrane is likely lower than **1**, we are interested in increasing the solubility of future probes, likely with the addition of a sulfonate group.

Much work has been done by others to describe the mechanisms of self-healing fluorophores, and this study lays a foundation for the development, use, and future improvement of self-healing reporters of membrane potential and cellular physiology.

References

1. Garg, P. *et al.* Human induced pluripotent stem cell-derived cardiomyocytes as models for cardiac channelopathies: A primer for non-electrophysiologists. *Circ. Res.* **123**, 224–243 (2018).
2. Acker, C. D., Yan, P. & Loew, L. M. Recent progress in optical voltage-sensor technology and applications to cardiac research : from single cells to whole hearts. *Prog. Biophys. Mol. Biol.* (2019). doi:10.1016/j.pbiomolbio.2019.07.004
3. Berenfeld, O. *et al.* Spatially distributed dominant excitation frequencies reveal hidden organization in atrial fibrillation in the Langendorff-perfused sheep heart. *J. Cardiovasc. Electrophysiol.* **11**, 869–879 (2000).
4. Mironov, S., Jalife, J. & Tolkacheva, E. G. Role of conduction velocity restitution and short-

- term memory in the development of action potential duration alternans in isolated rabbit hearts. *Circulation* **118**, 17–25 (2008).
5. Herron, T. J. Calcium and voltage mapping in hiPSC-CM monolayers. *Cell Calcium* **59**, 84–90 (2016).
 6. Huebsch, N. *et al.* Metabolically driven maturation of human-induced-pluripotent-stem-cell-derived cardiac microtissues on microfluidic chips. *Nat. Biomed. Eng.* **6**, 372–388 (2022).
 7. McNamara, H. M. *et al.* Geometry-Dependent Arrhythmias in Electrically Excitable Tissues. *Cell Syst.* **7**, 359–370.e6 (2018).
 8. Muñoz, J. J. A. M. *et al.* Time-regulated transcripts with the potential to modulate human pluripotent stem cell-derived cardiomyocyte differentiation. *Stem Cell Res. Ther.* **13**, 1–27 (2022).
 9. Narkar, A., Willard, J. M. & Blinova, K. Chronic Cardiotoxicity Assays Using Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs). *Int. J. Mol. Sci.* **23**, (2022).
 10. Charrez, B. *et al.* Heart Muscle Microphysiological System for Cardiac Liability Prediction of Repurposed COVID-19 Therapeutics. *Front. Pharmacol.* **12**, 1–15 (2021).
 11. Charwat, V. *et al.* Validating the Arrhythmogenic Potential of High-, Intermediate-, and Low-Risk Drugs in a Human-Induced Pluripotent Stem Cell-Derived Cardiac Microphysiological System. *ACS Pharmacol. Transl. Sci.* **5**, 652–667 (2022).
 12. Arslanova, A. *et al.* Investigating inherited arrhythmias using hiPSC-derived cardiomyocytes. *Methods* **203**, 542–557 (2022).
 13. Salama, G. & Morad, M. Merocyanine 540 as an Optical Probe of Transmembrane Electrical Activity in the Heart. *Science (80-.)*. **191**, 485–487 (1976).
 14. Herron, T. J., Lee, P. & Jalife, J. Optical Imaging of Voltage and Calcium in Cardiac Cells & Tissues. *Circ. Res.* **110**, 609–623 (2012).
 15. Efimov, I. R., Nikolski, V. P. & Salama, G. Optical imaging of the heart. *Circ. Res.* **95**, 21–33 (2004).
 16. Loew, L. M., Scully, S., Simpson, L. & Waggoner, A. S. Evidence for a charge-shift electrochromic mechanism in a probe of membrane potential [21]. *Nature* **281**, 497–499 (1979).
 17. Schaffer, P., Ahammer, H., Müller, W., Koidl, B. & Windisch, H. Di-4-ANEPPS causes photodynamic damage to isolated cardiomyocytes. *Pflügers Arch. Eur. J. Physiol.* **426**, 548–551 (1994).
 18. Fluhler, E., Burnham, V. G. & Loew, L. M. Spectra, Membrane Binding, and Potentiometric Responses of New Charge Shift Probes†. *Biochemistry* **24**, 5749–5755 (1985).
 19. Icha, J., Weber, M., Waters, J. C. & Norden, C. Phototoxicity in live fluorescence microscopy, and how to avoid it. *BioEssays* **39**, 1–15 (2017).
 20. Tinevez, J. Y. *et al.* A quantitative method for measuring phototoxicity of a live cell imaging microscope. *Methods in Enzymology* **506**, (Elsevier Inc., 2012).
 21. Skylaki, S., Hilsenbeck, O. & Schroeder, T. Challenges in long-term imaging and quantification of single-cell dynamics. *Nat. Biotechnol.* **34**, 1137–1144 (2016).
 22. Rohr, S. & Salzberg, B. M. Multiple site optical recording of transmembrane voltage (MSORTV) in patterned growth heart cell cultures: assessing electrical behavior, with microsecond resolution, on a cellular and subcellular scale. *Biophys. J.* **67**, 1301–1315 (1994).

23. Acker, C. D., Yan, P. & Loew, L. M. Recent progress in optical voltage-sensor technology and applications to cardiac research: from single cells to whole hearts. *Prog. Biophys. Mol. Biol.* **154**, 3–10 (2019).
24. Miller, E. W. *et al.* Optically monitoring voltage in neurons by photoinduced electron transfer through molecular wires. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2114–2119 (2012).
25. Li, L. S. Fluorescence probes for membrane potentials based on mesoscopic electron transfer. *Nano Lett.* **7**, 2981–2986 (2007).
26. de Silva, A. P. *et al.* New Fluorescent Model Compounds for the Study of Photoinduced Electron Transfer: The Influence of a Molecular Electric Field in the Excited State. *Angew. Chemie Int. Ed. English* **34**, 1728–1731 (1995).
27. Liu, P. & Miller, E. W. Electrophysiology, Unplugged: Imaging Membrane Potential with Fluorescent Indicators. *Acc. Chem. Res.* **53**, 11–19 (2020).
28. Grenier, V. *et al.* Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters. *ACS Cent. Sci.* **8**, 118–121 (2022).
29. Zheng, Q. *et al.* Electronic tuning of self-healing fluorophores for live-cell and single-molecule imaging. *Chem. Sci.* **8**, 755–762 (2016).
30. Yang, Z. *et al.* Cyclooctatetraene-conjugated cyanine mitochondrial probes minimize phototoxicity in fluorescence and nanoscopic imaging. *Chem. Sci.* **11**, 8506–8516 (2020).
31. Zheng, Q., Jockusch, S., Zhou, Z. & Blanchard, S. C. The contribution of reactive oxygen species to the photobleaching of organic fluorophores. *Photochem. Photobiol.* **90**, 448–454 (2014).
32. Zheng, Q. *et al.* On the mechanisms of cyanine fluorophore photostabilization. *J. Phys. Chem. Lett.* **3**, 2200–2203 (2012).
33. Frutos, L. M., Castaño, O., Andrés, J. L., Merchán, M. & Acuña, A. U. A theory of nonvertical triplet energy transfer in terms of accurate potential energy surfaces: The transfer reaction from π , π^* triplet donors to 1,3,5,7-cyclooctatetraene. *J. Chem. Phys.* **120**, 1208–1216 (2004).
34. Huang, Y. L., Walker, A. S. & Miller, E. W. A Photostable Silicon Rhodamine Platform for Optical Voltage Sensing. *J. Am. Chem. Soc.* **137**, 10767–10776 (2015).
35. McNamara, H. M. *et al.* Bioelectrical domain walls in homogeneous tissues. *Nat. Phys.* **16**, 357–364 (2020).
36. Tveito, A. *et al.* Inversion and computational maturation of drug response using human stem cell derived cardiomyocytes in microphysiological systems. *Sci. Rep.* **8**, 1–14 (2018).
37. Jæger, K. H. *et al.* Improved Computational Identification of Drug Response Using Optical Measurements of Human Stem Cell Derived Cardiomyocytes in Microphysiological Systems. *Front. Pharmacol.* **10**, 1–24 (2020).
38. Huebsch, N. *et al.* Metabolically-Driven Maturation of hiPSC-Cell Derived Cardiac Chip. *bioRxiv* (2020).
39. Lee-Montiel, F. *et al.* Integrated hiPSC-based liver and heart microphysiological systems predict unsafe drug-drug interaction. *bioRxiv* (2020). doi:10.1101/2020.05.24.112771
40. Charrez, B. *et al.* In vitro safety “clinical trial” of the cardiac liability of hydroxychloroquine and azithromycin as COVID19 polytherapy. *bioRxiv* **10090**, (2020).
41. Klimas, A., Ortiz, G., Boggess, S. C., Miller, E. W. & Entcheva, E. Multimodal on-axis platform for all-optical electrophysiology with near-infrared probes in human stem-cell-derived cardiomyocytes. *Prog. Biophys. Mol. Biol.* **3**, 1–9 (2019).
42. Boggess, S. C. *et al.* New Molecular Scaffolds for Fluorescent Voltage Indicators. *ACS*

- Chem. Biol.* **14**, 390–396 (2019).
43. Ortiz, G. *et al.* A silicon-rhodamine chemical-genetic hybrid for far red voltage imaging from defined neurons in brain slice. *RSC Chem. Biol.* **2**, 1594–1599 (2021).
 44. Laughner, J. I., Ng, F. S., Sulkin, M. S., Martin Arthur, R. & Efimov, I. R. Processing and analysis of cardiac optical mapping data obtained with potentiometric dyes. *Am. J. Physiol. - Hear. Circ. Physiol.* **303**, (2012).
 45. Gollmer, A. *et al.* Singlet oxygen sensor green®: Photochemical behavior in solution and in a mammalian cell. *Photochem. Photobiol.* **87**, 671–679 (2011).
 46. Kim, S., Fujitsuka, M. & Majima, T. Photochemistry of singlet oxygen sensor green. *J. Phys. Chem. B* **117**, 13985–13992 (2013).
 47. Chen, W. *et al.* Fluorescence self-quenching from reporter dyes informs on the structural properties of amyloid clusters formed in vitro and in cells. *Nano Lett.* **17**, 143–149 (2017).
 48. Chen, R. F. & Knutson, J. R. Mechanism of fluorescence concentration quenching of carboxyfluorescein in liposomes: Energy transfer to nonfluorescent dimers. *Anal. Biochem.* **172**, 61–77 (1988).
 49. Berezin, M. Y. & Achilefu, S. Fluorescence lifetime measurements and biological imaging. *Chem. Rev.* **110**, 2641–2684 (2010).
 50. Lazzari-Dean, J. R., Gest, A. M. M. & Miller, E. W. Optical estimation of absolute membrane potential using fluorescence lifetime imaging. *Elife* **8**, 1–29 (2019).

Chapter 2: Supplementary Information

Chemical Synthesis and Spectroscopic Characterization

Performed by Nels C. Gerstner

Cell Culture

Human embryonic kidney (HEK) 293T cell culture

HEK 293T cells were acquired from the UC Berkeley Cell Culture Facility. Cells were passaged and plated onto 25 mm glass coverslips coated with Poly-D-Lysine (PDL; 1 mg/mL; Sigma-Aldrich) to a confluency of ~15% and 50% for electrophysiology and imaging, respectively. HEK293T cells were plated and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS), and 1% Glutamax.

Differentiation towards human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)

hiPSCs (WTC11) were cultured on Matrigel (1:100 dilution; Corning)-coated 12 well-plates in StemFlex medium (Gibco). When the cell confluency reached 80–90%, which is referred to as day 0, the medium was switched to RPMI 1640 medium (Life Technologies) containing B27 minus insulin supplement (Life Technologies) and 10 μ M CHIR99021 GSK3 inhibitor (Peprtech). At day 1, the medium was changed to RPMI 1640 medium containing B27 minus insulin supplement only. At day 2, medium was replaced to RPMI 1640 medium containing B27 supplement without insulin, and 5 μ M IWP4 (Peprtech) for 2 days without medium change. On day 4, medium was replaced to RPMI 1640 medium containing B27 minus insulin supplement for 2 days without medium change. On day 6 and 7, medium was replaced to a serum-free medium - RPMI 1640 containing B27 with insulin supplement. After day 7, the medium was changed every other day. Confluent contracting sheets of beating cells appear between days 7 to 15 and are ready for dissociation after this time.

Confluent sheets were treated with collagenase II for 60-74 minutes. The collagenase solution was carefully transferred to cold DMEM, making sure cardiac sheets were not disturbed. Cells were then dissociated with 0.25% trypsin-EDTA (4-10 minutes, depending on density and quality of tissue) and plated onto Matrigel (1:100)-coated 6 well-plates in RPMI 1640 medium containing B27 supplement plus ROCK inhibitor Y-27632. 24 hours later, the medium was replaced with fresh RPMI/B27 without the ROCK inhibitor. Cardiomyocytes were maintained for 7 days, replacing media every 48 hours, and then were switched to RPMI 1640 medium (minus glucose, supplemented with 4 mM sodium lactate (Sigma Aldrich)). Cells were maintained in this media for 7 days, replacing every 48 hours, then switched back to RPMI/B27 medium containing glucose. These purified cardiomyocytes were used for imaging.

The lactate purified sheets of cardiomyocytes were dissociated with 0.25% trypsin-EDTA (4-10 minutes, depending on density and quality of tissue) and plated onto Matrigel (1:100)-coated Ibidi® 24 well μ -plates (cat no. 82426) in RPMI 1640 medium containing B27 supplement (containing insulin). Medium was changed every 3 days until imaging. For loading hiPSC-cardiomyocytes, VoltageFluors were diluted in DMSO and then diluted in RPMI 1640 with B27 supplement minus

Phenol Red to a desired final concentration of 500 nM. Solutions were bath applied to incubating cardiomyocytes for 20 minutes at 37 °C, then exchanged with dye-free RPMI 1640 with B27 supplement minus Phenol Red.

Fluorescence Microscopy

Inverted epifluorescence imaging of HEK293T cells and hiPSC-CMs was performed on an AxioObserver Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor), controlled with μ Manager (V1.4, open-source, Open Imaging). Images were acquired using a Plan-Apochromat 20/0.8 air objective (20x; Zeiss) or an EC-Plan-NEOFLUAR 40/1.3 oil immersion objective (40x; Zeiss). Images were focused onto an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu). More detailed imaging information for each experimental application is expanded below.

Membrane staining in HEK293T cells

HEK293T cells were incubated with HBSS solution (Gibco) containing VoltageFluors (500 nM) at 37°C for 20 minutes and then exchanged for fresh HBSS (no dye) for imaging. Microscopic images were acquired with a 63x/1.4 PlanApo oil objective on a confocal Zeiss LSM 980 with Airyscan2 and BH FLIM. VoltageFluors were illuminated at 639 nm and transmitted images were taken at 405 nm (10.07 s exposure time per frame).

For photostability experiments, HEK cells were incubated separately with BeRST-COT (500 nM) or isoBeRST-pip-cys (500 nM) in HBSS at 37°C for 20 minutes and then exchanged for fresh HBSS (no dye) for imaging. Data were acquired with a 20x air objective (Zeiss) And OrcaFlash4.0 sCMOS camera (Hamamatsu). Images (2048x2048 pixels) were taken every 500 ms for 3 minutes with constant illumination of LED (2.88 W/cm²; 5 ms exposure time). The obtained fluorescence curves (background subtracted) were normalized with the fluorescence intensity at $t = 0$ seconds and averaged (18 different cell rafts for each dye).

Membrane staining in hiPSC-CMs

hiPSC-CMs were incubated in RPMI/B27 (without phenol red) containing VoltageFluors (500 nM) at 37°C for 20 minutes prior to a media exchange with fresh RPMI/B27 (no phenol red, no dye) for imaging. Microscopic images were acquired with a 40x/1.3NA oil objective (Zeiss) and OrcaFlash4.0 sCMOS camera (Hamamatsu). For fluorescence images, the excitation light was delivered from a LED (2.88 W/cm²; 100 ms exposure time) at 631/28 (bandpass) nm and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP).

Voltage Sensitivity in HEK293T cells

Functional imaging of BeRST-COT was performed using a 20x air objective (Zeiss) paired with image capture from a sCMOS camera at a 0.5 kHz sampling rate. Images were binned 4x4. VoltageFluors were excited using the 631 nm LED with an intensity of 2.88 W/cm². For initial voltage characterization, emission was collected with the emission filter and dichroic listed above.

Voltage Recordings of hiPSC-CMs and assessment of phototoxicity

Functional recordings of VoltageFluors (500 nM) were performed using a 20x air objective paired with a sCMOS camera at a sampling rate of 0.2 kHz (4x4 binning and restricted to a 512x125 pixel frame for high-speed acquisition over long periods). Indicators were excited at 631/28 (bandpass) nm with an intensity of 2.88 W/cm² and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). Routine recordings were made for ten seconds.

Phototoxicity of VoltageFluor dyes was assessed in hiPSC-CM monolayers incubated with 500 nM indicator. These were exposed to constant illumination from the excitation LED for up to 30 minutes, while ten second fluorescence recordings were made at the beginning of each minute.

Singlet Oxygen Sensor Green Measurements

Singlet oxygen sensor green (SOSG) (ThermoFisher Cat. No. S36002), isoBeRST-pip-cys, and BeRST-COT were used in HBSS (Gibco) at 1 μ M concentrations. 200 μ L stocks were made, and solutions were imaged by placing a 20 μ L drop on a glass coverslip. Initial SOSG fluorescence was captured by taking five images every two seconds with excitation light delivered from a LED (3.23 W/cm²; 5 ms exposure time) at 475/34 (bandpass) nm and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP). Fluorescence for the five initial snaps were averaged to obtain the initial SOSG fluorescence.

The solutions were exposed to constant illumination for ten minutes (image acquisition every 30 sec; 5 ms exposure) using the 631 nm LED with an intensity of 2.88 W/cm², and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). Final SOSF fluorescence was captured by taking five images every two seconds with excitation light delivered from a LED (3.23 W/cm²; 5 ms exposure time) at 475/34 (bandpass) nm and emission was collected with an emission filter (bandpass; 540/50 nm) after passing through a dichroic mirror (510 nm LP). Fluorescence for the five final snaps were averaged to obtain the final SOSG fluorescence.

Image Analysis

HEK cell image analysis

Analysis of voltage sensitivity in HEK293T cells was performed using ImageJ (FIJI). Briefly, a region of interest (ROI) encompassing the cell body was selected and average fluorescence intensity was calculated for each frame. For background subtraction, a ROI encompassing a region without cells was selected and the average pixel intensity was calculated for each frame. A linear fit to the background trace was calculated and applied to the background, and this was used to subtract background signal from the fluorescence intensity trace. F/F₀ values were calculated by dividing the background subtracted trace by the median value of fluorescence when the cell is held

at -60 mV. $\Delta F/F$ values were calculated by plotting the change in fluorescence (ΔF) vs the applied voltage step and finding the slope of a linear best-fit.

hiPSC-CM image analysis

Analysis of action potential (AP) data from hiPSC cardiomyocytes was performed using in-house MATLAB scripts based on previously developed software by the Efimov lab (Washington University, St. Louis, MO). Scripts are available upon request. Briefly, raw OME-tiffs recorded in μ Manager was read directly into MATLAB for batch-processing of large datasets (>30 Gb per experiment). The mean pixel intensity of the entire image (512x125 pixels) was calculated for each frame and a mean fluorescence trace was extracted for the entire stack. Photobleach correction was performed by subtracting an asymmetric least-squares fit of the data from the mean trace. No subtraction of background was possible due to staining of the entire monolayer. Individual AP events were identified through threshold detection based on a Schmidt trigger. Action potential duration (APD) values were calculated for each AP by finding the activation time (time of the maximum derivative of the AP upstroke) and the time the signal returns to 70, 50, and 10% of the maximum depolarization (APD30, APD50, APD90, respectively). APD values were corrected for variation due to spontaneous beat rate by Fridericia's formula (**Equation S2**). CL is the cycle length, calculated as the time period from the beginning of one beat to the beginning of the succeeding beat.

$$APD_c = \frac{APD}{\sqrt[3]{CL}} \quad \text{Equation S1}$$

Fluorescence Lifetime Imaging

FLIM Acquisition and Analysis

HEK293T cells were incubated with HBSS solution (Gibco) containing VoltageFluors (500 nM) at 37°C for 20 minutes and then exchanged for fresh HBSS (no dye) for imaging. A fluorescence lifetime imaging concentration curve in HEK293T cells was conducted performed with a 63x/1.4 PlanApo oil objective (coated with Zeiss Immersol Immersion Oil 518F 37C) on Zeiss AxioObserver inverted laser scanning confocal microscope, LSM 980 with Airyscan2, Spectra-Physics MaiTai HP laser, and Becker-Hickl TCSPC FLIM system. The LSM 980 was operated by Zen Blue software (Zeiss) and the TCSPC was operated by SPCM software (Becker-Hickl). Pulsed excitation at 50 MHz was applied to the cells with a 640 nm diode laser (Becker-Hickl), laser power was control with a neutral density filter wheel controlled by the software. Emission was detected with an HPM-100-40 (Becker-Hickl) hybrid detector, based on a Hamamatsu R10467 GasP hybrid photomultiplier tube.

Data were acquired with 256 time binds in the analog-to-digital-converter and 256x256 pixels of spatial resolution with a pinhole of 100 microns. The incubation chamber was maintained at 37°C. Cells were imaged for 75 seconds at an image size of 89.8x89.8 microns, and a pixel size of 0.35 microns. Routine evaluation of proper lifetime recordings were performed with the measurement of instrument response function (IRF) of erythrosine B in water (pH 7).

Lifetime decays were fit using in-house MATLAB scripts previously described.⁵⁰ BeRST-COT was fit using a 2-component model and isoBeRST-pip-cys was fit using a 3-component model. All lifetimes reported are the average lifetimes of an ROI.

Electrophysiology

For electrophysiological experiments in HEK293T, pipettes were pulled from borosilicate glass with filament (Sutter Instruments, BF150-86-10) with a P-97 pipette puller (Sutter Instruments) to a resistance of 4-7 M Ω . Pipettes were filled with an internal solution (composition, in mM): 125 potassium gluconate, 10 HEPES, 10 KCl, 5 NaCl, 2 ATP disodium salt, 1 EGTA, 0.3 GTP sodium salt (pH 7.25, 285 mOsm). EGTA (tetraacid form) was prepared as a stock solution in 1M KOH prior to addition to internal solution. Pipettes were positioned with an MP-225 micromanipulator (Sutter Instruments). Electrophysiological recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) at room temperature. The signals were digitized with a Digidata 1440B, sampled at 50 kHz, filtered at 5 kHz and recorded with pCLAMP 10 software (Molecular Devices).

Electrophysiology was performed in the whole cell voltage clamp configuration. After gigaseal formation and break-in, recordings were only pursued if series resistance in voltage clamp was less than 30 M Ω and the recording maintained a 30:1 ratio of membrane resistance to access resistance throughout all voltage steps. No series resistance compensation was applied. Fast capacitance was compensated in the cell attached configuration. All voltage clamp protocols were corrected for the calculated liquid junction (-14 mV, Liquid Junction Potential Calculator in pClamp). For tandem electrophysiology and fluorescence intensity recordings, cells were held at -60 mV and de- and hyper- polarizing steps were applied from +100 to -100 mV in 20 mV increments, with each step lasting 100 ms.

Supporting Tables

Table S1. Properties of **1 and **1-COT****

	1	1-COT
λ_{abs} / nm	662	663
λ_{em} / nm	681	683
Φ_{fl}	0.05	0.013
$\Delta F/F$	24% +/- 1.9%	11% +/- 0.4%
ϵ	172,000	95,000
Relative Brightness	1.0	0.13

Table S1. Characterization of absorption and emission maxima (λ), quantum yield (Φ), fractional change in fluorescence ($\Delta F/F$) extinction coefficient (ϵ) and relative brightness in HEK293T cells for **1** and **1-COT**.

Supporting Figures

Figure S1. Characterization of 1-COT in HEK293T Cells

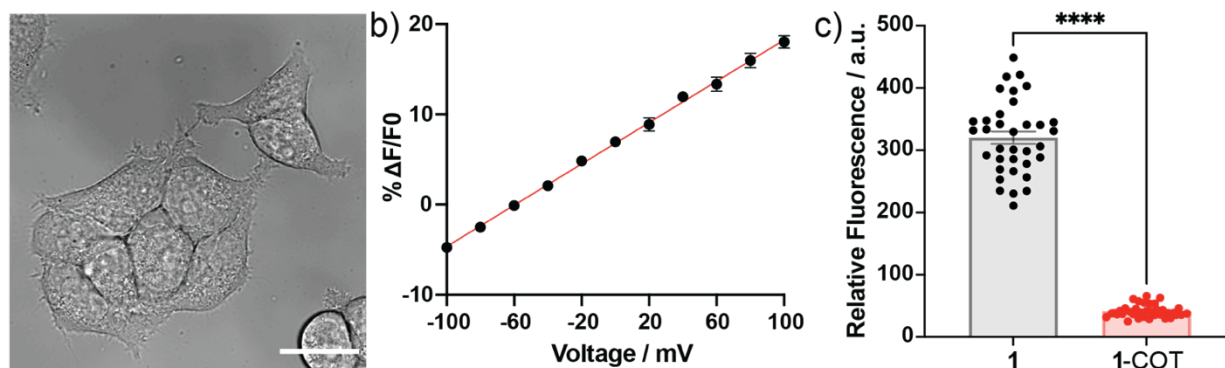


Figure S1. a) Transmitted light image of HEK293T cells stained with 1-COT, with corresponding image in Figure 2. Scale bar is 20 μm . **b)** Plot of fractional change in fluorescence ($\Delta F/F$) vs. membrane potential for 1-COT. Data are mean $\pm$ standard error of the mean for $n = 5$ determinations. Voltage sensitivity is $11\% \pm 0.4\%$ (S.E.M.). **c)** Plot of relative fluorescence of 1 vs. 1-COT in HEK293T cells. (1: $n = 35$; 1-COT: $n = 36$; $p < 0.0001$, ****).

Figure S2. Beats per Minute Comparison in hiPSC-CMs

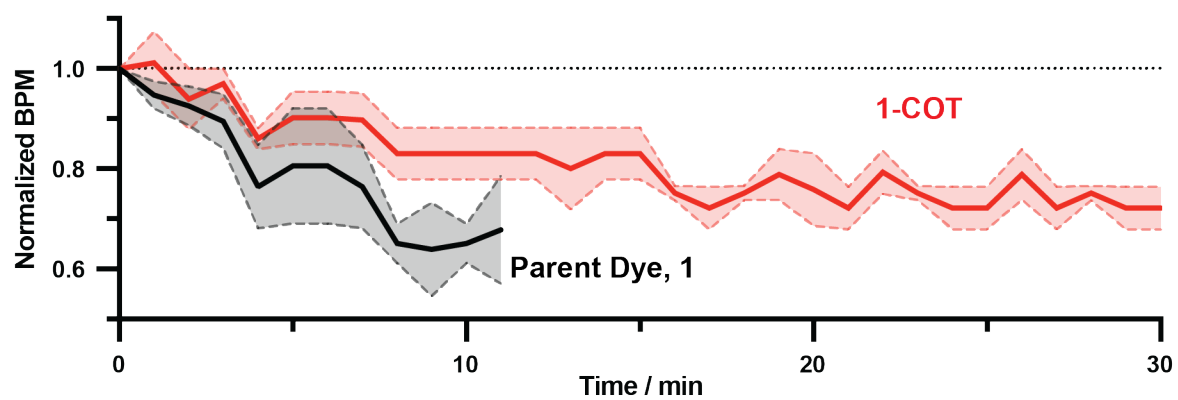


Figure S2. Plot of normalized beat rate vs. time in hiPSC-CMs. Data are a mean beat rate for **1** (black line) or **1-COT** (red line). S.E.M. are reported for n = 3 independent determinations.

Figure S3. Singlet Oxygen Sensor Green (SOSG) Controls

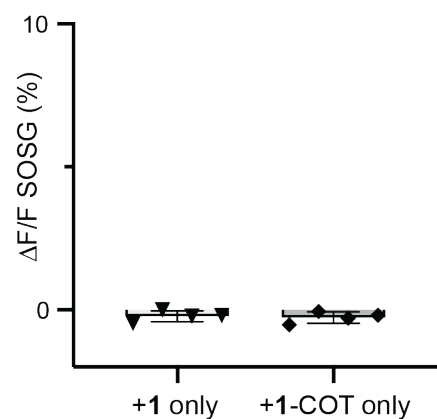


Figure S3. Control values for singlet oxygen sensor green. The fractional change in fluorescence of SOSG measured in the presence of **1** alone was -0.22% (SEM = 0.095; n = 4). The fractional change in fluorescence of SOSG measured in the presence of **1**-COT alone was -0.26% (SEM = 0.10; n = 4).

Appendix 1

Optical Quantification of Absolute Voltage with Fluorescence Lifetime Imaging and VoltageFluor Indicators in hiPSC-CMs

Abstract

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) hold promise to advance studies of human cardiac biology. However, iPSC-CMs are structurally and transcriptionally immature leading to spontaneously beating cells with a less negative resting membrane potential (RMP), resembling the electrophysiology of fetal cardiomyocytes. These immaturities hinder the relevance of hiPSC-CMs in drug development, specialized patient care, and basic cardiac research. Cell maturation through mechanical or metabolic manipulation or 3D-culture approaches is of active interest to the field. hiPSC-CM electrophysiology can be quantified via whole-cell patch clamp electrophysiology. However, patch clamp technique is difficult and low throughput, lacking spatial resolution. The Miller Lab turns to optical voltage imaging to complement traditional methods. Utilizing a photoinduced electron transfer (PeT) based mechanism to sense changes in voltage potential (V_{mem}), VoltageFluor (VF) probes are high throughput, reacting with fast kinetics to changes in V_{mem} . While intensity imaging using VF probes in hiPSC-CMs offers only relative changes in V_{mem} for cardiac action potentials (APs), fluorescence lifetime imaging (FLIM) has potential to provide absolute values of changes in voltage during an AP as well as the RMP. FLIM provides fluorescence lifetimes (τ_{fl}) of a fluorophore, providing τ_{fl} based on the exponential decay rate of a fluorophore, a property independent of dye concentration, loading, or photobleaching. Previous work has shown a correlation of V_{mem} and τ_{fl} for VoltageFluors in FLIM. The goal of this project was to develop a novel method to optically quantify absolute electrophysiology parameters in hiPSC-CM monolayers and tissues.

Introduction

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) hold great promise advance study of cardiac biology.¹ These cells are a renewable source of cardiomyocytes, are patient derived and physiologically relevant, and can replicate human disease phenotypes.² Yet, along with the great potential of hiPSC-CMs come drawbacks.³

hiPSC-CMs are structurally immature, and therefore more closely resemble fetal cardiomyocytes instead of adult cells. They have a smaller and rounder phenotype compared to adult cardiomyocytes, and have disorganized sarcomeric structure, lacking T-tubules.⁴ While hiPSC-CMs have similar transcription levels of most voltage-gated ion channels relative to adult cells, the inwardly rectifying K^+ current (I_{K1}) is significantly reduced and the funny current (I_f), typically only present in fetal cardiomyocytes, is present.¹ These transcriptional and structural differences lead to immature electrophysiology which results in automaticity, or spontaneously beating cells, with a less negative resting membrane potential (RMP) of ~ -60 mV versus the adult RMP of -85 mV.^{5,6} hiPSC-CMs also demonstrate slower conduction and upstroke velocities.⁷

These differences in electrophysiology are important when considering the relevance of research performed with hiPSC-CMs to drug development, specialized patient care, and basic cardiac science.¹ Maturation of hiPSC-CMs via metabolic manipulation, 3D-culture approaches, or mechanical stimulation is an area of development in the field.⁸

Patch clamp electrophysiology, considered a gold standard for studying electrophysiology, has aided in determining the electrophysiological effects of structural immaturity in hiPSC-CMs and has provided insight into action potential (AP) parameters.^{9,10} However, as previously discussed in the Introduction and Chapters 1 and 2 of this thesis, patch clamp electrophysiology is a technically challenging, low-throughput method lacking spatial resolution.

Our lab and others have historically used voltage sensitive indicators via intensity-based imaging, which can map relative changes in V_{mem} in contracting hiPSC-CMs over time.⁹ However, intensity imaging cannot provide absolute membrane potential values quantified in millivolts if not paired with patch clamp techniques. Intensity-based imaging is subject to variance due to differences in indicator loading between samples, photobleaching of the fluorophore, motion artifacts, and illumination intensity (Fig. 1c, left).^{11,12} This is a limitation of intensity imaging as the RMP and absolute change in voltage is necessary for phenotypic characterization of the

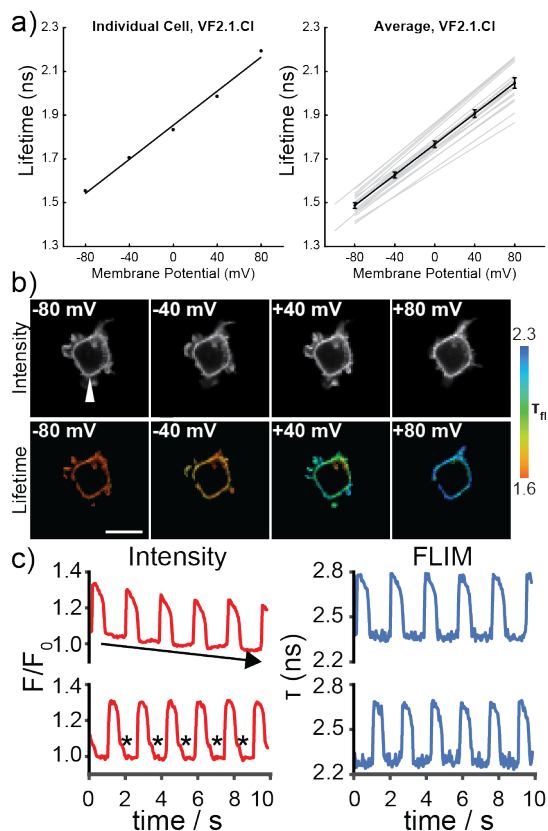


Figure 1. Introduction to FLIM **a)** Probe VF2.1.Cl demonstrates consistent and direct correlation of fluorescence lifetime and voltage potential. **b)** Intensity and lifetime images of VF2.1.Cl stained HEK293T cells at the indicated voltage potential. **c)** TMCRh.OMe can report cardiac APs, directly reading out fluorescence lifetime (right). The use of FLIM avoids photobleaching artifacts (top left) and movement artifacts reminiscent of early-after depolarizations (bottom left) seen in intensity-based imaging.

maturation of hiPSC-CMs, determining the strength of the I_{K1} , and chamber-specific information (atrial versus ventricular myocytes).^{13,14}

While ratiometric-based methods, such as electrochromic reporters and FRET-pairs have been used for determining absolute electrophysiology, these optical methods have drawbacks and are unable to reliably report accurate absolute voltage. Electrochromic dyes, which report membrane potential through changes in the excitation and emission wavelengths, require normalization via an electrode on the cell of interest to report accurate V_{mem} and FRET-oxonol systems can add significant capacitive load to cells.^{15–17}

A fast, high-throughput method for characterizing absolute membrane potentials using fluorescence lifetime imaging (FLIM) could advance the use and characterization of hiPSC-CMs for various applications. FLIM utilizes the fluorescence lifetime of a fluorophore, providing quantitative lifetime measurements based on the exponential decay rate of a fluorophore.¹¹ Fluorescent lifetime is an inherent property of a fluorophore, independent of indicator loading, concentration, and photobleaching.¹¹ VoltageFluor indicators demonstrate a linear correlation between V_{mem} (PeT).^{18–21} Changes in the rate of PeT affect the exponential decay rate of fluorescence, which correlates to a direct relationship between V_{mem} and fluorescence lifetime.^{11,22} Our lab has previously demonstrated the correlation between fluorescence lifetime and V_{mem} to develop the VF-FLIM method and make quantitative recordings of absolute membrane potential in cells (Fig. 1a,b).¹² Additionally, prior work has shown the successful usage of hiPSC-CMs in FLIM, as VF indicator TMCRh.OMe shows consistent absolute lifetime changes in hiPSC-CMs over the course of action potentials (Fig. 1c, right). When this preliminary data was taken by Julia Lazzari-Dean, the calibration of the dye (the subject of Aim 2) was not yet available due to microscope limitations that have been rectified with a new FLIM microscope. These prior data set the precedent for this project.

Thus, the goal of the work presented herein looks to develop a novel method for optically quantifying absolute membrane potentials (V_{mem}) in hiPSC-CM monolayers and tissues by utilizing optical voltage imaging with VoltageFluor indicators and fluorescence lifetime imaging.²³ The aim of this project was to utilize VoltageFluor indicators to measure electrophysiology in a high-throughput method. A high-throughput way of characterizing cardiac electrophysiology over long-term experiments has potential to benefit development of maturation protocols in hiPSC-CMs and drug development and drug screening in accordance with the Comprehensive In Vitro Pro Arrhythmia Assay Schema (CiPA) initiative.²⁴

This work would require completion of the following Aims for success of this project:

- 1) The development and troubleshooting of protocols for measuring fluctuating fluorescence lifetime of cardiac action potentials in FLIM. Previous work in the Miller lab has shown that hiPSC-CMs in FLIM show consistent fluctuations in lifetime, relating to changes in membrane potentials during action potentials. The main goal here is to develop this protocol on a new FLIM system and increase the speed of imaging acquisitions.
- 2) The development of a calibration curve in hiPSC-CMs to relate V_{mem} to τ_{fl} in FLIM for BeRST-COT (Thesis Chapter 2), and TMCRh.OMe (tetramethylcarborhodamine). This protocol has been described by Lazzari-Dean, *et al.* and would be followed to use voltage-clamp electrophysiology with FLIM to determine the correlation between τ_{fl} and V_{mem} and develop calibrations.¹² This calibration will allow us to optically quantify resting membrane potentials and absolute voltage changes in iPSC-CMs during an action potential.
- 3) Expand the scope of VF-FLIM to include both hiPSC-CM monolayers and “heart-on-a-chip” tissue mimetic devices to demonstrate broad usage of this method.^{25,26} The expansion of this

method to microphysiological systems can show the method is useful for validating maturation protocols for hiPSC-CMs or that it is useful in cardiotoxicity drug screening assays.

The work herein presented is work towards completion of the first aim. A new FLIM microscope, purchased and installed by the UC Berkeley Molecular Imaging Center (MIC), allowed for the feasibility of this project (NIH S10: 1S10OD025063-01A1). This microscope has the appropriate 640 nm wavelength laser appropriate for the excitation of BeRST-COT ($\lambda_{\text{ex}}=663$ nm, $\lambda_{\text{em}}=683$ nm, $\Delta F/F=11\%$, $\Phi=0.013$) and TMCRh.OMe ($\lambda_{\text{ex}}=623$ nm, $\lambda_{\text{em}}=643$ nm, $\Delta F/F=18\%$, $\Phi=0.038$). Delays in the installation of this microscope, brought about by Covid-19, meant this project was on hold until April 2021, with the ability to perform patch-clamp electrophysiology on this microscope only feasible in Summer 2022. In this chapter, I will describe the work completed toward the goal of completing Aim 1 and briefly present rationale for steps 2 and 3.

Aim 1 – Protocol Development for Fast FLIM Acquisitions in hiPSC-CMs

Determination of Data Collection Methods for Fast-FLIM

Method 1: Time Series / Cycle FLIM. According to the BH TCSPC Handbook, there are three methods for fast FLIM imaging with Becker-Hickl software.²⁷ The first of which is Time Series / Cycles FLIM (Fig. 2a). A time series can be created by using the “cycle” and “autosave” functions of the SPCM software. The main advantage of this method would be that the current mode of FLIM data analysis (FLIMFLAM)¹² would still be useable. Unfortunately for this method, the

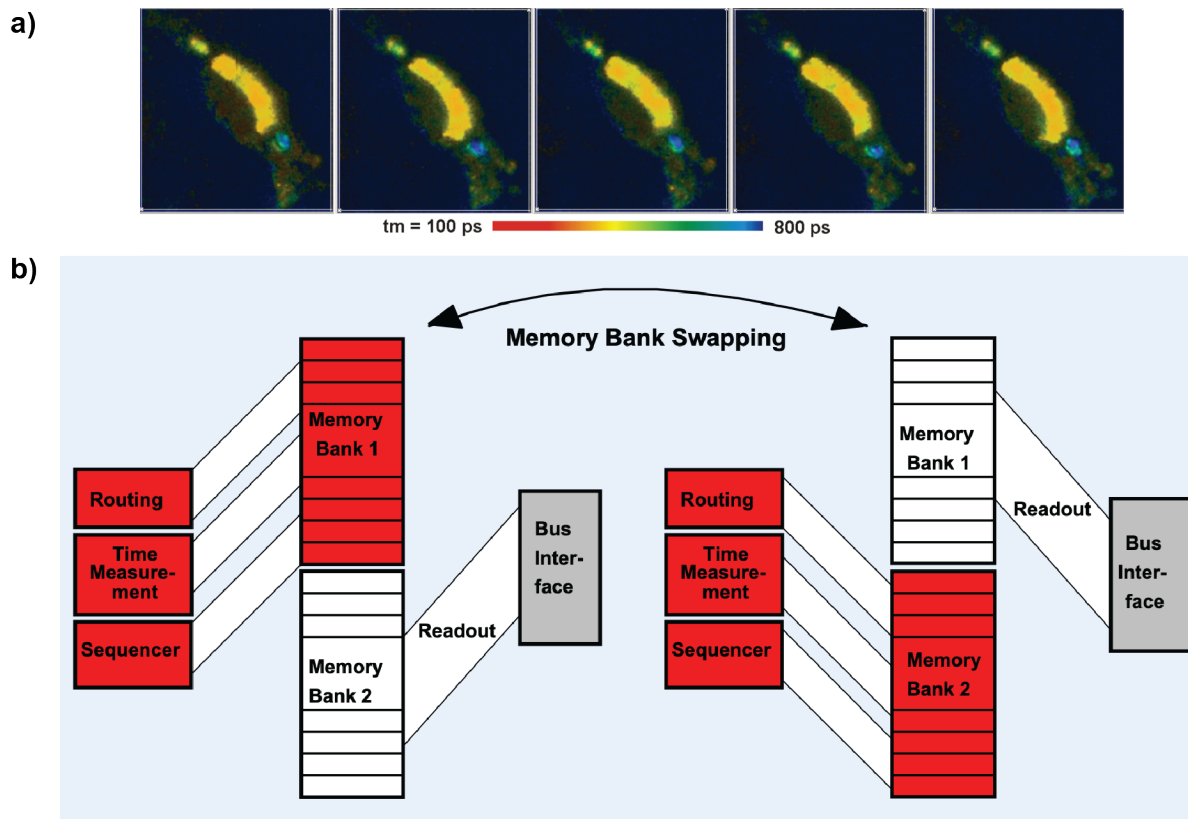


Figure 2. FLIM Methods for Fast Imaging. **a)** Representative image of Method 1 (Time Series / Cycle FLIM) used to image amoeba at one image per second. **b)** Schematic of memory bank swapping in Method 2 (Continuous Flow Imaging). Source: W. Becker, The bh TCSPC handbook. 9th edition (2021).

maximum acquisition speed according to the BH Handbook is 1-2 seconds. Since a cardiac action potential occurs on the order of about 200 to 500 ms, this temporal resolution would not be fast enough to resolve action potentials. The limitation of acquisition speed is likely contributed by the program saving each individual acquisition in between images. Additionally, relatively little is published on the use of this method. For these reasons, we briefly attempted this method, but the SPCM software could not keep up with the speeds that we need, crashing while imaging and lagging behind the speed we attempted to set the imaging at, which ranged from 20.5 to 40.94 ms. For these reasons, “Cycle FLIM” is not the best method for acquiring action potential data.

Method 2: Continuous Flow Imaging. The second method is Continuous Flow Imaging, where unlimited sequential recording can be made possible by memory bank swapping during acquisitions (Fig. 2b). When one memory bank is full, the sequencer swaps the banks. While the sequencer writes data into one bank, the other bank is read by the computer. Unfortunately, in initial testing of this method, we were unable to successfully get the method to work and would likely need to contact Becker-Hickl for assistance.

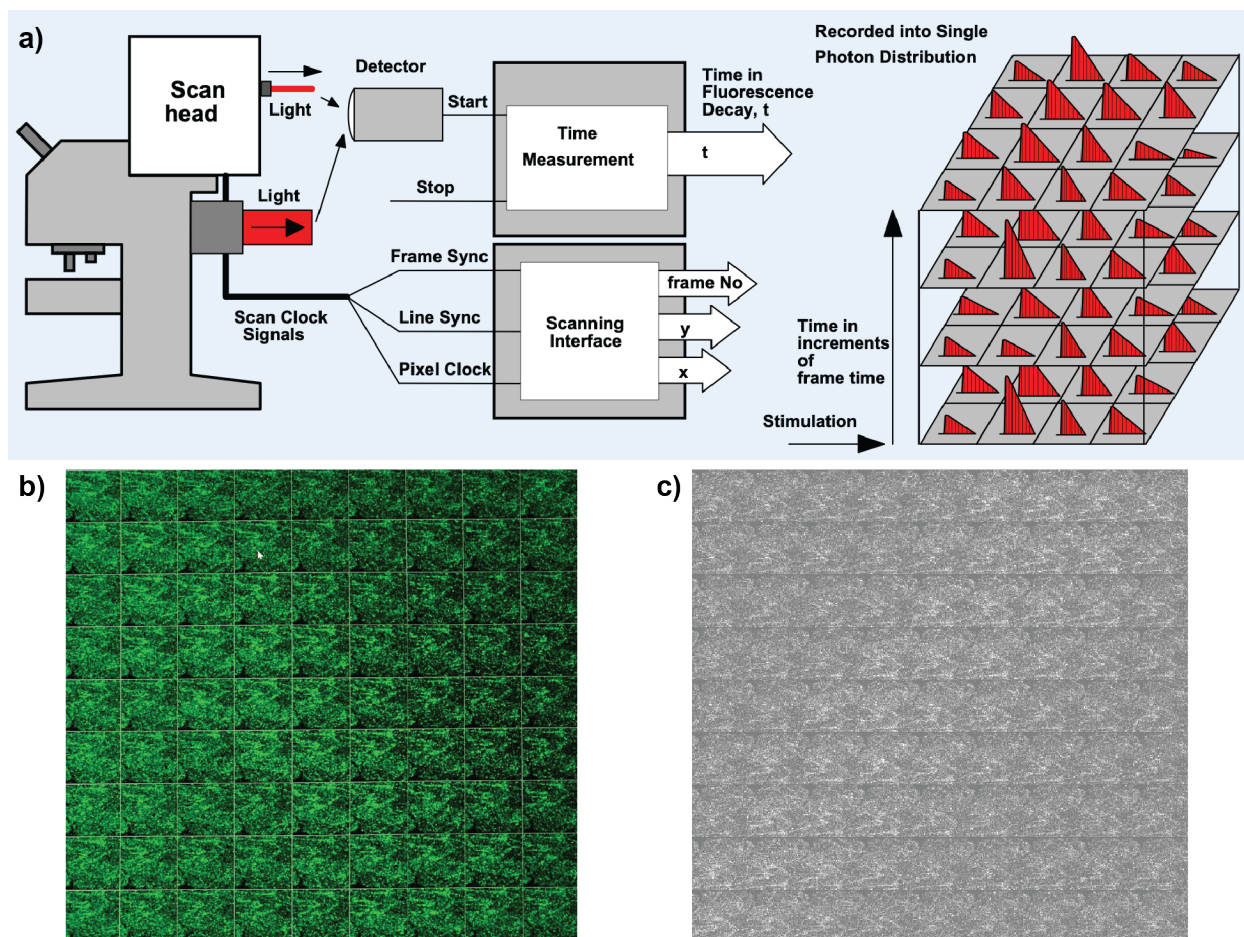


Figure 3. Temporal Mosaic FLIM. **a)** Schematic of temporal mosaic imaging in FLIM. Source: W. Becker, The bh TCSPC handbook. 9th edition (2021). **b)** Mosaic FLIM array of hiPSC-CMs from SPC Image. The array is 81 elements (9 by 9), each element is 64x64 pixels acquired at 65 ms. **c)** An exported bitmap file from SPC Image, with the total photon counts of each element stitched together and exported.

Method 3: Temporal Mosaic Imaging. The third method is Temporal Mosaic Imaging where a single region of interest of a sample is scanned at the same spatial position and images are recorded as an array of FLIM images contained within a single FLIM data file (Fig. 3a). Unfortunately, since the data file of the mosaic is a single, large data array, this mode required the development of a new method for data analysis. Advantages for this method are that no time is reserved for saving images while acquisitions are occurring, and that time series data can be accumulated. The time series accumulation would allow for a larger buildup of photons without having to trade accuracy for acquisition speed. Mosaic imaging may be the method most likely to succeed for this project, and likely the method most worth looking into further in the future.

In order to perform Mosaic imaging, we found that the mosaic must be a square array, such as what is shown in Fig. 3b composed of 9 by 9 images (for 81 “elements”). Other sized square arrays would also work, such as a 4 by 4 image mosaic (for 16 “elements”), however a rectangular array (such as 4 by 8 images) would not work. Additionally, the individual pixel size of each image must be a factor of 8 (i.e. 64x64 pixels as used in Fig. 3b, but not 100x100 pixels). While testing this method, acquisition speeds tested were 37 ms and 65 ms. Microscopic images were acquired with a 63x/1.4 PlanApo oil objective (coated with Zeiss Immersol Immersion Oil 518F 37C) on a confocal Zeiss LSM 980 with Airyscan2 and BH FLIM. TMCRh.Ome was bath-applied to hiPSC-CMs illuminated at 640 nm.

Data Analysis Methods for Temporal Mosaic Imaging

Data Analysis in hiPSC-CMs. The LSM 980 was operated by Zen Blue software (Zeiss) and the TCSPC was operated by SPCM software (Becker-Hickl). Pulsed excitation at 50 MHz was applied to the cells with a 640 nm diode laser (Becker-Hickl), laser power was control with a neutral density filter wheel controlled by the software. Emission was detected with an HPM-100-40 (Becker-Hickl) hybrid detector, based on a Hamamatsu R10467 GasP hybrid photomultiplier tube.

Data were acquired with 256 time bins in the analog-to-digital-converter. In testing of the Mosaic Imaging method, it was determined that the microscope could best maintain imaging speeds at 65 ms with parameters of 64x64 pixel images (2.10 μm pixel size) in a 9 by 9 mosaic with 81 elements (Fig. 3b). Using 32x32 pixels did allow us to attempt to image at 33 ms, however there was more lag time during the acquisition. The pinhole was tested at 51 μm and 100 μm . The incubation chamber was maintained at 37°C. Routine evaluation of proper lifetime recordings were performed with the measurement of instrument response function (IRF) of erythrosine B in water (pH 7).

Lifetime decays from the Mosaic Image are stored by SPCM as a single large array (Fig. 3b), meant for global processing of the lifetime data. Unfortunately, SPC Image is unable to process this data in a pixelwise manner, which would allow us to process the lifetimes of the individual elements which we expect to vary as action potentials occur. The SPC Image, it is possible to export the bitmap file of the array, with total photon counts of each “element” stitched together and exported (Fig. 3c), however this does not bring us closer to being able to export individual “element” or pixelwise lifetime decays.

Thus, lifetime decays had to be fit using a modified, in-house MATLAB script, developed with the help of Anneliese Gest. This MATLAB script, “mosaicParser,” is a function that takes .asc or .txt files of exported mosaic FLIM data, partitions out each frame, and then combines the decays at each pixel within a frame into a single decay for the frame by summing them. This method requires some pre-processing of the data in SPC Image by the user, requiring the .sdt files from SPCM be opened, the bin set to zero, and then exporting the mosaic array as an ascii file. The ascii

files must then be opened by a text editor, the header metadata deleted, and then files saved as a .asc or .txt file.

The mosaicParser MATLAB script requires three inputs: nDimensions, nFrames, and fBin. nDimensions is the number of pixels in x or y of each frame and nFrames is the number of frames acquired. Thus if your mosaic is made up of 81 frames of 64x64 pixel images as in Fig. 3b, nDimensions is 64 and nFrames is 81. fBin is the desired frame binning factor – if you want to combine three frames together into one, fBin is three. If you do not want to combine any frames, fBin is one. The data presented in Figure 4 has not been frame binned. In the current form of the MATLAB script, if nFrames is not divisible by fBin, the code will append columns filled with zeroes to the last frame until they are divisible. This can result in a final frame with much less signal than the previous frames. The user may want to discard this last frame manually in these situations. Data is output into the workspace and can be copied into an excel file or save as-is, or directly input into a fitting code such as ‘floptimize’. As this project is left with work to be completed, items to add to this script that could be beneficial include: the ability to export a .csv file, to make a version that parses out the frames without condensing all of the pixels into a single decay, and integrating this script into FLIMFLAM.¹²

This MATLAB script was used to produce the data in Figure 4. Mosaic FLIM was performed in hiPSC-CMs, which produced quite noisy lifetime fluctuations (Fig 4a,c, red). While these fluctuations are potentially representative of cardiac action potentials, we cannot delineate action potentials from noise in these lifetime traces. The lifetimes reported in hiPSC-CMs closely align with the data acquired previously by Julia Lazzari-Dean on the old FLIM microscope, in the range

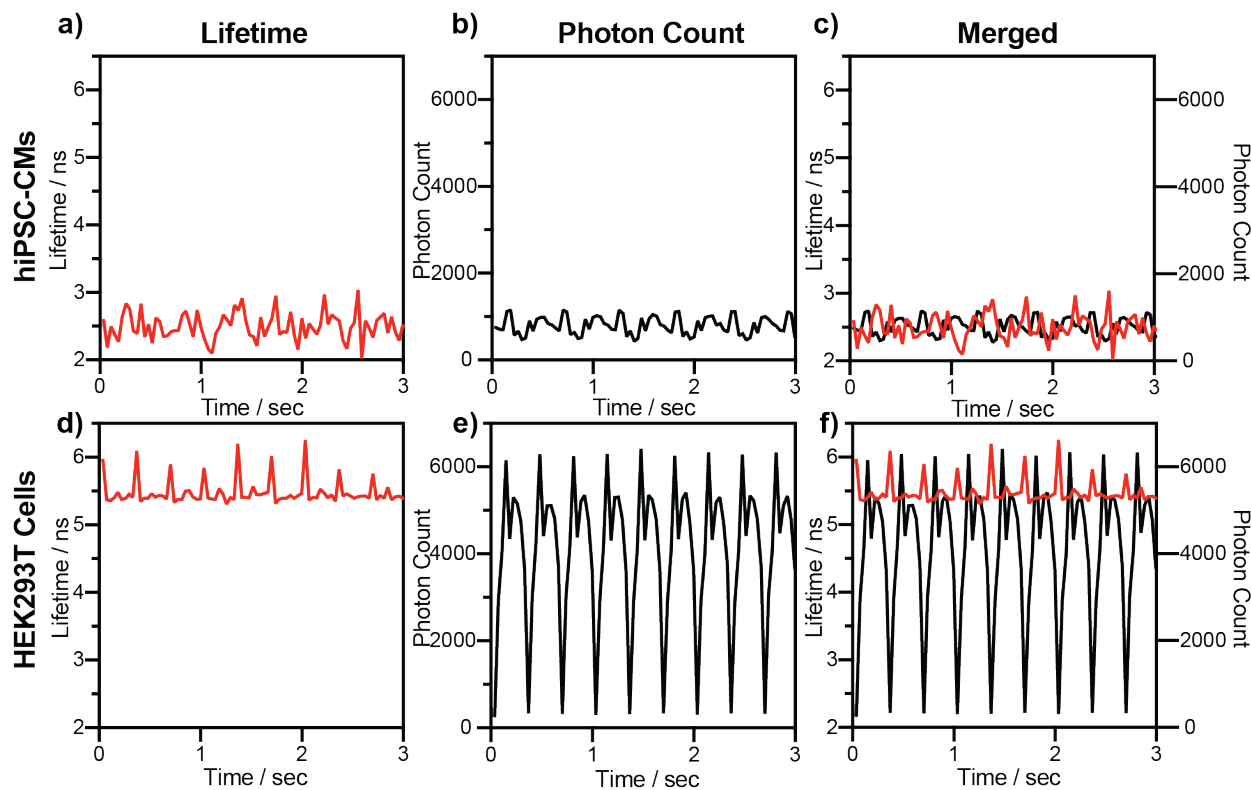


Figure 4. Mosaic FLIM results of fluorescence lifetime and photon count vs. time in cells stained with VoltageFluor TMCrH.OMe. **a)** Fluorescence lifetime (red), **b)** photon count (black), and **c)** merged graphs for hiPSC-CMs. **d)** Fluorescence lifetime (red), **e)** photon count (black), and **f)** merged graphs for HEK293T cells.

of 2 to 3 ns (Fig. 1c, right). Photon count is also represented in Figure 4 in hopes of tracking action potentials based on photon output, which should increase with depolarizations in membrane potential due to an action potential. However, the photon count trace is also quite noisy (Fig. 4b,c, black), and the photon count and lifetime peaks do not align, which is what we would expect during an action potential.

Mosaic FLIM in HEK293T cells was performed as a control. We expected to see a consistent photon count and consistent lifetime measurement across time, as the membrane potential in HEK293T cells does not change drastically over the course of three seconds. However, we saw photon count fluctuations of about 6000 photons (Fig. 4e,f, black), and lifetime fluctuations of about 0.6 ns (Fig. 4d,f, red). These lifetime fluctuations do appear to have some periodicity and fluctuate in a pattern, but do not align with an expectation of a consistent lifetime and consistent photon count. Not enough trials were completed to accurately gauge if these fluctuations are an artifact from using the mosaic FLIM method in SPCM, or if the MATLAB script is not properly reading the large mosaic arrays. A useful control in HEK293T cells would be to perform mosaic FLIM and acquire FLIM data in a standard imaging mode on the same cell raft to compare lifetime outputs. The comparison of the lifetimes acquired using the two different methods would inform us on the accuracy of lifetimes acquired with mosaic mode.

Future Work Towards the Use of Temporal Mosaic Imaging in hiPSC-CMs

Developing a Concentration Curve. Future work in this aim should include the development of a concentration curve in FLIM. To ensure the use of the most optimal concentrations of indicator, a concentration curve should be performed by measuring the lifetime of the VoltageFluor of interest at increasing concentrations between 200 nM and 1000 nM. Prior work has shown a decrease in fluorescence lifetime as concentrations increase is related to self-quenching of the fluorophore as a result of excess indicator present.^{28,29} The concentration that should be used would be the maximum concentration where the lifetime obtained in the concentration curve has not yet decreased. A concentration curve performed in HEK293T cells would minimize the amount of hiPSC-CMs that need to be differentiated, which could be beneficial for experimental timelines and ease. However, as hiPSC-CMs are plated in a monolayer, it would be best practice to develop the concentration curve in HEK293T cells plated for 80-90% confluency. The work presented herein was completed prior to the completion of a concentration curve as the focus was primarily on troubleshooting the instrument and software for fast FLIM acquisition and not yet focused on useful data acquisition.

hiPSC-CM Methods

Differentiation Towards Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs). hiPSCs (WTC11) were cultured on Matrigel (1:100 dilution; Corning)-coated 12 well-plates in StemFlex medium (Gibco). When the cell confluency reached 80–90%, which is referred to as day 0, the medium was switched to RPMI 1640 medium (Life Technologies) containing B27 minus insulin supplement (Life Technologies) and 10 μ M CHIR99021 GSK3 inhibitor (Peprtech). At day 1, the medium was changed to RPMI 1640 medium containing B27 minus insulin supplement only. At day 2, medium was replaced to RPMI 1640 medium containing B27 supplement without insulin, and 5 μ M IWP4 (Peprtech) for 2 days without medium change. On day 4, medium was replaced to RPMI 1640 medium containing B27 minus insulin supplement for 2 days without medium change. On day 6 and 7, medium was replaced to a serum-free medium - RPMI 1640 containing B27 with insulin supplement. After day 7, the medium was changed every

other day. Confluent contracting sheets of beating cells appear between days 7 to 15 and are ready for dissociation after this time.

Confluent sheets were treated with collagenase II for 60-74 minutes. The collagenase solution was carefully transferred to cold DMEM, making sure cardiac sheets were not disturbed. Cells were then dissociated with 0.25% trypsin-EDTA (4-10 minutes, depending on density and quality of tissue) and plated onto Matrigel (1:100)-coated 6 well-plates in RPMI 1640 medium containing B27 supplement plus ROCK inhibitor Y-27632. 24 hours later, the medium was replaced with fresh RPMI/B27 without the ROCK inhibitor. Cardiomyocytes were maintained for 7 days, replacing media every 48 hours, and then were switched to RPMI 1640 medium (minus glucose, supplemented with 4 mM sodium lactate (Sigma Aldrich)). Cells were maintained in this media for 7 days, replacing every 48 hours, then switched back to RPMI/B27 medium containing glucose. These purified cardiomyocytes were used for imaging.

The lactate purified sheets of cardiomyocytes were dissociated with 0.25% trypsin-EDTA (4-10 minutes, depending on density and quality of tissue) and plated onto Matrigel (1:100)-coated Ibidi® 24 well μ -plates (cat no. 82426) in RPMI 1640 medium containing B27 supplement (containing insulin). Medium was changed every 3 days until imaging. For loading hiPSC-cardiomyocytes, VoltageFluors were diluted in DMSO and then diluted in RPMI 1640 with B27 supplement minus Phenol Red to a desired final concentration of 500 nM. Solutions were bath applied to incubating cardiomyocytes for 20 minutes at 37 °C, the exchanged with dye-free RPMI 1640 with B27 supplement minus Phenol Red.

hiPSC-CM Preparation for Imaging. hiPSC-CMs in an Ibidi plate were incubated in RPMI/B27 (without phenol red) containing TMCRh.OMe (500 nM) at 37°C for 20 minutes prior to a media exchange with fresh RPMI/B27 (no phenol red, no dye) for imaging. Microscopic images were acquired with a 63x/1.4 PlanApo oil objective on a confocal Zeiss LSM 980 with Airyscan2 and BH FLIM. VoltageFluors were illuminated at 640 nm. The incubation chamber was maintained at 37°C

Aim 2 – Development of a Calibration Curve

In order to correlate τ_{fl} to V_{mem} for a given VoltageFluor, a calibration curve will be developed for each dye by performing patch clamp electrophysiology of hiPSC-CMs in FLIM. This method may be technically challenging because while our lab has patch clamp experience with neurons and HEK293T cells, we do not have in-house knowledge of how to patch hiPSC-CMs. The development of this method may require collaboration with a lab who knows how to patch hiPSC-CMs. This calibration curve will be used to then quantify cardiac action potential parameters such as resting membrane potential and absolute changes in V_{mem} during an action potential.

We were previously prevented from performing calibrations for red VF indicators due to a lack of the appropriate laser lines on the old FLIM microscope.

Aim 3 – Expanding FLIM for Quantitative Cardiac Measurements at the Tissue Level

As heart disease is a tissue-level disease, it is vital to study electrophysiology in tissue mimetic devices, thus the goal of this project would be to expand FLIM imaging from hiPSC-CM monolayers to engineered tissue constructs. The 3D microphysiological systems (MPS) developed in the Healy Lab at Berkeley, called “heart-on-a-chip” devices, mimic a tissue environment similar to human cardiac tissue and promote tissue level organization.²⁵ These devices also provide a platform for drug screening and cardiotoxicity assays and promote maturation of hiPSC-CMs.^{26,30}

BeRST 1, a parent derivative of BeRST-COT, has been previously used in MPS chips by the Healy lab, providing precedence for expanding this platform with FLIM imaging and quantitative electrophysiology analysis.^{26,30-32}

An all-optical imaging platform for MPS chips would combine multiple powerful technologies to quantify cardiac electrophysiology and better understand hiPSC-CMs. There would also be the possibility of combining this method with computational power to create a comprehensive comparison to adult cardiomyocytes or develop a more powerful drug screening platform.³³

References

1. Karakikes, I., Ameen, M., Termglinchan, V. & Wu, J. Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes: Insights into Molecular, Cellular, and Functional Phenotypes. *Circ. Res.* **117**, 80–88 (2015).
2. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions. *Nat. Protoc.* **8**, 162–175 (2013).
3. Kane, C., Couch, L. & Terracciano, C. M. N. Excitation-contraction coupling of human induced pluripotent stem cell-derived cardiomyocytes. *Front. Cell Dev. Biol.* **3**, 1–8 (2015).
4. Gherghiceanu, M. *et al.* Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: Comparative ultrastructure. *J. Cell. Mol. Med.* **15**, 2539–2551 (2011).
5. Goversen, B., van der Heyden, M. A. G., van Veen, T. A. B. & de Boer, T. P. The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: Special focus on IK1. *Pharmacol. Ther.* **183**, 127–136 (2018).
6. Hoekstra, M., Mummery, C. L., Wilde, A. A. M., Bezzina, C. R. & Verkerk, A. O. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. *Front. Physiol.* **3** AUG, 1–14 (2012).
7. Ma, J. *et al.* High purity human-induced pluripotent stem cell-derived cardiomyocytes: Electrophysiological properties of action potentials and ionic currents. *Am. J. Physiol. - Hear. Circ. Physiol.* **301**, 2006–2017 (2011).
8. Machiraju, P. & Greenway, S. C. Current methods for the maturation of induced pluripotent stem cell-derived cardiomyocytes. *World J. Stem Cells* **11**, 33–43 (2019).
9. Acker, C. D., Yan, P. & Loew, L. M. Recent progress in optical voltage-sensor technology and applications to cardiac research : from single cells to whole hearts. *Prog. Biophys. Mol. Biol.* (2019). doi:10.1016/j.pbiomolbio.2019.07.004
10. Garg, P. *et al.* Human induced pluripotent stem cell-derived cardiomyocytes as models for cardiac channelopathies: A primer for non-electrophysiologists. *Circ. Res.* **123**, 224–243 (2018).
11. Berezin, M. Y. & Achilefu, S. Fluorescence lifetime measurements and biological imaging. *Chem. Rev.* **110**, 2641–2684 (2010).
12. Lazzari-Dean, J. R., Gest, A. M. M. & Miller, E. W. Optical estimation of absolute membrane potential using fluorescence lifetime imaging. *Elife* **8**, 1–29 (2019).
13. Giles, W. R. & Noble, D. Rigorous Phenotyping of Cardiac iPSC Preparations Requires Knowledge of Their Resting Potential(s). *Biophys. J.* **110**, 278–280 (2016).
14. Du, D. T. M., Hellen, N., Kane, C. & Terracciano, C. M. N. Action potential morphology of human induced pluripotent stem cell-derived cardiomyocytes does not predict cardiac chamber specificity and is dependent on cell density. *Biophys. J.* **108**, 1–4 (2015).
15. Montana, V., Farkas, D. L. & Loew, L. M. Dual-Wavelength Ratiometric Fluorescence Measurements of Membrane Potential. *Biochemistry* **28**, 4536–4539 (1989).
16. González, J. E. & Tsien, R. Y. Improved indicators of cell membrane potential that use fluorescence resonance energy transfer. *Chem. Biol.* **4**, 269–277 (1997).
17. Loew, L. M., Scully, S., Simpson, L. & Waggoner, A. S. Evidence for a charge-shift electrochromic mechanism in a probe of membrane potential [21]. *Nature* **281**, 497–499 (1979).
18. Woodford, C. R. *et al.* Improved PeT molecules for optically sensing voltage in neurons. *J.*

- Am. Chem. Soc.* **137**, 1817–1824 (2015).
19. Miller, E. W. *et al.* Optically monitoring voltage in neurons by photoinduced electron transfer through molecular wires. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2114–2119 (2012).
 20. Li, L. S. Fluorescence probes for membrane potentials based on mesoscopic electron transfer. *Nano Lett.* **7**, 2981–2986 (2007).
 21. de Silva, A. P. *et al.* New Fluorescent Model Compounds for the Study of Photoinduced Electron Transfer: The Influence of a Molecular Electric Field in the Excited State. *Angew. Chemie Int. Ed. English* **34**, 1728–1731 (1995).
 22. Lakowicz, J. R., Szmacinski, H. & Johnson, M. L. Calcium imaging using fluorescence lifetimes and long-wavelength probes. *J. Fluoresc.* **2**, 47–62 (1992).
 23. Liu, P. & Miller, E. W. Electrophysiology, Unplugged: Imaging Membrane Potential with Fluorescent Indicators. *Acc. Chem. Res.* **53**, 11–19 (2020).
 24. Sager, P. T., Gintant, G., Turner, J. R., Pettit, S. & Stockbridge, N. Rechannelling the cardiac proarrhythmia safety paradigm: A meeting report from the Cardiac Safety Research Consortium. *Am. Heart J.* **167**, 292–300 (2014).
 25. Mathur, A. *et al.* Human iPSC-based cardiac microphysiological system for drug screening applications. *Sci. Rep.* **5**, 1–7 (2015).
 26. Huebsch, N. *et al.* Metabolically driven maturation of human-induced-pluripotent-stem-cell-derived cardiac microtissues on microfluidic chips. *Nat. Biomed. Eng.* **6**, 372–388 (2022).
 27. Becker, W. *The bh TCSPC Handbook.* (2021).
 28. Chen, W. *et al.* Fluorescence self-quenching from reporter dyes informs on the structural properties of amyloid clusters formed in vitro and in cells. *Nano Lett.* **17**, 143–149 (2017).
 29. Chen, R. F. & Knutson, J. R. Mechanism of fluorescence concentration quenching of carboxyfluorescein in liposomes: Energy transfer to nonfluorescent dimers. *Anal. Biochem.* **172**, 61–77 (1988).
 30. Charwat, V. *et al.* Validating the Arrhythmogenic Potential of High-, Intermediate-, and Low-Risk Drugs in a Human-Induced Pluripotent Stem Cell-Derived Cardiac Microphysiological System. *ACS Pharmacol. Transl. Sci.* **5**, 652–667 (2022).
 31. Jæger, K. H. *et al.* Improved Computational Identification of Drug Response Using Optical Measurements of Human Stem Cell Derived Cardiomyocytes in Microphysiological Systems. *Front. Pharmacol.* **10**, 1–24 (2020).
 32. Tveito, A. *et al.* Inversion and computational maturation of drug response using human stem cell derived cardiomyocytes in microphysiological systems. *Sci. Rep.* **8**, 1–14 (2018).
 33. Gaeta, S. A., Krogh-Madsen, T. & Christini, D. J. Feedback-control induced pattern formation in cardiac myocytes: a mathematical modeling study. *J. Theor. Biol.* **266**, 408–418 (2010).