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Publication Date

2022

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Time-resolved Fluorescence Microscopy Methods to Interrogate Chemistry and Biology

By

Anneliese MM Gest

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 Chris Chang

Professor Markita Landry

Fall 2022

Time-resolved Fluorescence Microscopy Methods to Interrogate Chemistry and Biology

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By Anneliese MM Gest

Abstract

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Membrane potential, V_{mem} , is the voltage across a cellular plasma membrane, produced by differences in ion concentration across the semipermeable membrane. Changes in V_{mem} over millisecond timescales transmit the electrical signals of action potentials in neurons and cardiomyocytes. Slower changes in V_{mem} over minutes, hours, or days affect processes such as differentiation, and set the resting membrane potential, which can affect neuronal and cardiac excitability. V_{mem} may also vary across substructures within cells, like organelles or dendritic spines, to enable localized electrical signaling. Forming a complete picture of the role of V_{mem} across these varied time and length scales requires techniques that can measure the value of V_{mem} in millivolts and reliably report both the dynamic changes in voltage and the absolute millivolt value of V_{mem} at rest.

In this dissertation, we develop fluorescence lifetime imaging microscopy (FLIM) of the VoltageFluor (VF) small-molecule voltage-sensitive dyes as a method for optically reporting absolute V_{mem} , and then expand the method to additional dye scaffolds and cellular targets. We review existing tools and methods for recording V_{mem} , highlighting the need for additional optical techniques alongside electrophysiology. Initially using the VoltageFluor VF2.1.Cl, we demonstrate that its fluorescence lifetime, τ_{fl} , can report V_{mem} in multiple cell lines with voltage resolution at a biologically relevant level (5 mV RMSD voltage changes, and 19 mV RMSD for V_{mem} in a single trial in HEK293T cells). We optically read out the V_{mem} of hundreds of cells and report voltage distributions consistent with the values measured electrophysiologically, with 100-fold improvement in throughput over electrophysiology.

To expand this FLIM technique to enable optical determination of V_{mem} in more complex cultures, such as neurons, we investigate two chemical-genetic targeting strategies. The first, VoltageSpy, has a 7-fold improvement in voltage resolution over the existing genetically encoded voltage indicator (GEVI) systems for reporting V_{mem} using FLIM. The second, VF-HaloTag, has a 10-fold improvement in resolution over the same GEVI FLIM indicator. VF-HaloTag also enables FLIM imaging of individual neurons and even individual dendrites with good spatial and lifetime resolution.

We also develop a red-shifted, carborhodamine-based voltage indicator with comparable sensitivity to VF2.1.Cl. This red-shifted indicator is robust to phototoxicity and photobleaching, while exhibiting a monoexponential fluorescence decay, requiring fewer photons for a reliable determination of τ_{fl} . This allows both rapid and extended FLIM imaging, allowing us to resolve cardiac action potentials in lifetime, and image sensitive cells for minutes at a time.

Finally, we utilize FLIM as a tool for examining structural modifications to the VF scaffold. We report a consistent trend across three dye series (six dyes), demonstrating the effect of altering the position of the molecular wire in the VF scaffold on τ_{fl} . This overall work showcases the utility of FLIM to optically read out voltage and shows how the technique can be expanded to measure voltage at various spatial and time scales, broadening the toolbox for optical readout of V_{mem} .

For My Family

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Acknowledgements

I want to thank everyone who has guided and supported me in this journey through graduate school, but to do that properly would take more words than are in the rest of this document. So, this will have to be a heavily abbreviated list, but I owe many more people than are listed here.

I have had the good fortune to have many excellent teachers over the years who have encouraged me to follow my love for science, and I want to thank just a few in specific. Ms. Rosinski, without you as my AP Chemistry teacher, I don't think I would have majored in chemistry. I need to thank all my chemistry professors at Macalester college, but special thanks go to two of them: Ronald Brisbois and Leah Witus. Professor Brisbois showed me that I should not, in fact, fear organic chemistry, and that I actually rather liked drawing all those curved arrows. It's all just another puzzle to solve. He also gave me my first opportunity to teach. It was a fantastic experience and set me on my current path. Leah brought me on as the second student ever in her lab, and I'm so glad she did. Leah introduced me to chemical biology as a field and got me started on a chemical biology project that was, in many ways, secretly an analytical project. Thank you for helping me discover I like the "fiddly nonsense" of quantitative analysis and spectroscopy – turns out it was the start of a trend. Leah has also continuously been a source of advice and support, even though I haven't been her student for years now – she is the epitome of a good mentor to her students.

Thank you to Matt Francis and Markita Landry for allowing me to rotate in their labs here at Berkeley. I learned so much from each of you and your lab members during my short time there. Thank you also to my committee, Chris Chang and Markita Landry.

I would like to give particular thanks to my advisor, Evan Miller. Thank you for allowing me to join your lab for my thesis research, a choice that I think surprised us both slightly at the time, but that I am constantly affirmed was the right one. Evan took a chance and allowed me to completely switch projects from my rotation and gave me the opportunity to explore the world of fluorescence lifetime imaging. I will always be grateful for Evan's guidance during the most stressful parts of my research, from suggestions on new control experiments, to talking me down when I was out of ideas for some particularly tricky troubleshooting. As I became a more advanced student, Evan was also always supportive of my career goals and interests, including teaching. He allowed me to develop a side project for an undergraduate and teach a high-demand course over my last summer in the lab. Perhaps it wasn't the most efficient for research, but it was important to me, and so it was important to Evan to make it work out. Evan was also incredibly supportive during a personally difficult time in my life – when I wasn't sure my health would enable me to finish my Ph.D. Without Evan's support at that time, I really might have left this dream of mine unfinished. Everyone says that your choice of advisor is critical for success and happiness in graduate school, and I couldn't agree more. I wouldn't do a Ph.D. any other way.

I can't go any further without thanking Holly Aaron of the Molecular Imaging Center for her advice, instruction, and incredible lenience as I learned FLIM and used the instruments she works so hard to provide to us all. Thank you for allowing me to learn on your beloved Deckard (RIP), with all its idiosyncrasies. From Holly I received so many connections to other scientists, brief discussions of light and physics, and so much help as an intermediary with vendors and microscope techs. Thanks to Holly, and Feather Ives as well, for putting on AIM every year, even throughout the COVID shutdowns. Thank you for the chance to speak at AIM, it meant a

lot to me to take that (virtual) stage and share what I've worked on with the scientists I've come to admire over the years. Thank you also, Holly, for allowing me to make semi-permanent changes to your beloved microscopes, especially in setting up electrophysiology on K. I learned so much in setting things up on K as it was installed and became ready for users. Your job is not an easy one, but we are all grateful for the work you do. More love to core facility managers, always.

I want to thank all the members of the Miller Lab, past and present, that I've been lucky enough to work alongside. As the last student in the lab to have known the very first students in the lab, I feel a particular bittersweetness in leaving. To the old guard, thank you for introducing me to the lab and showing me the ropes. Steven, thank you for welcoming me to Berkeley. Vince, thank you for attempting to teach me synthesis. I was nervous the whole time, but I really did learn so much from you. Josh, you beat me out of here by just a little bit, but I had a great time sharing two out of three rotations with you. To the younger generation, it's your world now. You'll do amazing work. Marisol, Susanna, and Julia Martin, I know you'll hold up the bio end of the lab – in truth you've been doing it for a while. I've had so much fun working with all of you, and I hope you've learned a little from me, because I've learned so much from you. Take good care of the microscopes.

Special thanks are due to Julia Lazzari-Dean, my mentor on the FLIM project. I know you think I give you too much credit when I say you taught me everything I know about FLIM, so I'll just say that you taught me a lot of what I know about FLIM, and set the groundwork for me figuring out the rest. Thank you for your long discussions with me after group meeting and for sending me whatever interesting papers you come across, which you somehow always found much faster than me. And thank you for always encouraging me to ask the tricky questions and consider the far off ideas.

Thank you to Pavel, for your friendship and support in and out of the lab. I enjoyed our extended chats about imaging, chemistry, Star Wars, and whatever else we got up to. I look forward to staying in touch, and hopefully more camping trips in big trees.

I am so glad I had the opportunity to work closely with Susanna Yaeger-Weiss for the last several years. I have been so glad to have you with me on Team FLIM, and I'm going to miss having you as my sounding board to make sure I'm not losing my mind when things go wrong. I've appreciated the chance to work with you, both in teaching you some things, and learning just as much from you. You're in the home stretch now yourself, and it's been great to watch you grow.

I've also been lucky enough to teach several times over. Thank you to all my students, and to Anne Baranger, for giving me the opportunity to have those teaching experiences. I've learned so much each time, but especially in teaching Chem 34 my last summer at Berkeley. Thanks to Abby Hinojosa for going on that journey with me. Thanks also to Lizy Choi, who jumped in on a side project that quite honestly was a lot for an undergraduate to handle on her own. We got through a lot despite the time constraints. I've learned so much from having you in the lab, and I wish you luck in the future.

I also want to thank those outside of Berkeley who've helped me to get here. Thank you to my friends from Macalester, and our HOUSE chat. Nothing gets you out of your head about your thesis like Jackbox games or movie marathons.

Dr. Marla DeVault, we've both done it! You got there a few months sooner, but it's not a race – I always look back fondly on when we would do chemistry homework together freshman

year, and now we've both finished grad school in chemistry. I'm glad we made it through and I'm glad to have shared it with you, albeit from afar.

To my long-running, long-suffering D&D group, our campaign has outlasted my Ph.D. And I'm sure our friendship will last longer still. Special thanks to Jem, for her hard work keeping that group in line, and accepting my rants about neurons, aka "the needy cells," that she had no context for. People say how important it is to have friends outside your field, and you all have demonstrated that for me. I think I would've had a much worse time without spending a couple hours a week in our little fantasy world.

Thank you to everyone who has ever donated blood. I don't know you, but I very genuinely might not have made it this far without you.

A big thank you to my cat, Professor Pumpkin. Your comments while writing this thesis were appreciated, though hopefully none of your edits have made it into the final draft.

Particularly strong thanks are necessary for my girlfriend and partner, Hilary Mogul. Thank you for getting excited about every talk, draft of a paper, or interview I had. Thanks for chats about how light and sound signal processing are similar, and for convincing me I can code even if it's mostly searching online resources for how to write the syntax. And most of all, thank you for your support and kindness through the past three years of this journey. You've been amazing while I've been a mess, and I wouldn't do it any other way.

Lastly, I want to give a huge thanks to my family, who have been supportive since day one, and even before. Thank you to my sister. You're my oldest best friend, Alessandra. Thank you to my parents, for always being there, for anything. They have supported me in every dream I've ever had, but especially my interest in science. From endless weekend trips to the zoo, the science museum, or sitting through the same state park ranger programs over and over again until I could've given them. Thank you so much, I couldn't have gotten here without you.

Chapter 1

Measuring Absolute Membrane Potential Across Space and Time

Portions of this review were published in the following scientific journal:
Lazzari-Dean, J.R.; Gest, A. M. M.; Miller, E. W. Measuring Absolute Membrane Potential Across Space and Time. *Annual Review of Biophysics* **2021**. 50, 1, 447-468.
DOI: 10.1146/annurev-biophys-062920-063555

Portions of this review were written in collaboration with Dr. Julia Lazzari-Dean

Abstract

Membrane potential (V_{mem}) is a fundamental biophysical signal present in all cells. V_{mem} signals range in time from milliseconds to days, and they span lengths from microns to centimeters. V_{mem} affects many cellular processes, ranging from neurotransmitter release to cell cycle control to tissue patterning. However, existing tools are not suitable for V_{mem} quantification in many of these areas. Here, we outline the diverse biology of V_{mem} , drafting a “wish list” for a V_{mem} sensing platform. We then use these guidelines to discuss electrode-based and optical platforms for interrogating V_{mem} . On one hand, electrode-based strategies exhibit excellent quantification but are most effective in short-term, cellular recordings. On the other hand, optical strategies provide easier access to diverse samples but generally only detect relative changes in V_{mem} . By combining the respective strengths of these technologies, recent advances in optical quantification of absolute V_{mem} enable new inquiries into V_{mem} biology.

Introduction

Membrane potential, or voltage across a lipid bilayer, is ubiquitous in biology. In electrically excitable cells such as neurons and cardiomyocytes, millisecond V_{mem} fluctuations cause release of neurotransmitters and contraction. Both excitable and non-excitable cells exhibit V_{mem} signals, which vary over time spans of seconds to days. Furthermore, an estimated 10 to 50% of the cellular energy budget goes to maintain V_{mem} , even in non-excitable cells¹. Given this large energy expenditure, the functions of V_{mem} span far beyond excitable electrical activity in the brain or heart²; recent work has linked V_{mem} to cell proliferation^{3,4}, differentiation^{5,6}, and tissue patterning⁷.

Membrane potentials result from differences in ion concentration across a semi-permeable membrane. Although this phenomenon is best studied with respect to the plasma membrane of mammalian cells, any selectively permeable membrane can maintain a voltage. For example, voltages in bacterial communities^{8–10} and across organelle membranes^{11,12} also have signaling roles. Unless otherwise indicated, we use the term membrane potential (V_{mem}) to indicate the plasma membrane potential between the cytosol and the extracellular space. In addition, we use absolute V_{mem} to indicate the value of the membrane potential in millivolts, rather than a relative measure of changes in V_{mem} .

V_{mem} is inherently a system-level property, determined by an array of ion channels and pumps and sensed by diverse cellular components. In mammalian cells, the primary ions involved in generating V_{mem} are Na^+ and K^+ (**Figure 1.1a**), but many other species (including Cl^- , Ca^{2+} , H^+ , and organic anions) play a role. Within the cellular V_{mem} response, the best documented class of voltage-sensitive proteins is voltage gated ion channels, which transduce V_{mem} changes into additional ionic currents or fluctuations in the second messenger Ca^{2+} . Nevertheless, all membrane-localized proteins experience V_{mem} , and more of these proteins may be sensitive to V_{mem} than previously thought. Evidence of V_{mem} sensitivity exists for various membrane components¹³, including phosphatases¹⁴, G protein coupled receptors^{15,16}, and the membrane organization itself¹⁷. For most systems, we have an incomplete understanding of both the determinants of V_{mem} and the factors which respond to V_{mem} .

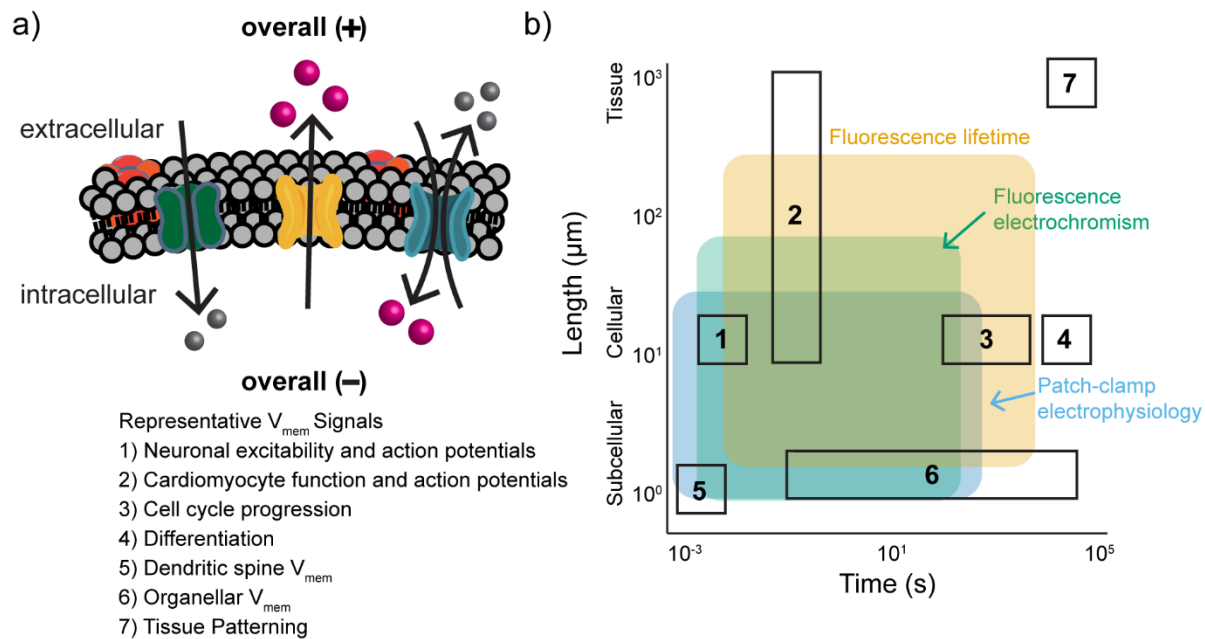


Figure 1.1 Membrane potential signals and measurement techniques across time and space. a) A schematic of cellular membrane potential, with a list of 7 sample biological processes where V_{mem} signaling plays a role. The resistance to ionic flow between compartments sets the length scale of V_{mem} signals; V_{mem} can scale across tissues or be compartmentalized within organelles or dendritic spines. The properties of ion channels and pumps that determine V_{mem} , as well as ion diffusion between compartments, dictate the time duration of a V_{mem} response. b) Biological space accessible to the three most common strategies for absolute V_{mem} measurement (shaded regions), overlaid with example biological process of interest (outlined boxes, numbered as in (a)). While many techniques can report cellular V_{mem} on the scale of milliseconds or seconds, longer recordings or recordings across large areas are difficult to access. Shaded areas indicate regions where single trial recordings retain voltage resolution on the scale of biological V_{mem} changes (≤ 20 mV) and minimally alter the biological sample. Recordings with fluorescence electrochromic dyes require re-calibration with an electrode on each cell, whereas fluorescence lifetime and patch-clamp electrophysiology can be applied as stand-alone measurements that are comparable between cells. Shaded areas are restricted to demonstrated application space for each technique and do not indicate the full extent of its possible use.

These gaps in our understanding are largely attributable to limitations in the existing V_{mem} recording toolkit, as most V_{mem} recording strategies are only tractable in a small subset of biological samples. In particular, strategies to record absolute V_{mem} and absolute V_{mem} changes (i.e. document that V_{mem} depolarized 20 mV) are unavailable in many systems (**Figure 1.1b**). To better understand necessary features of V_{mem} recording strategies, we first summarize known V_{mem} signaling pathways and build a “wish list” of features for an optimized V_{mem} reporter. We then turn our focus to the primary two approaches for recording membrane potential: electrodes and fluorescent sensors. We review recent progress in V_{mem} sensing platforms and highlight areas where development of new tools would facilitate discovery.

Membrane Potential Signaling across Time and Space

Membrane potential signaling is most extensively studied in excitable tissue, where rapid changes in cellular V_{mem} transmit information. However, important V_{mem} signals also occur across minutes, hours, and days². Similarly, as a spatial signal, V_{mem} is canonically treated as cell-autonomous and uniform in spherical model cells. Nevertheless, V_{mem} can be compartmentalized subcellularly^{11,12,18,19} and delocalized across tissues^{20–23}. Here, we break down V_{mem} signals into three length scales: cellular, subcellular, and tissue. From there, we enumerate the unique challenges each brings for V_{mem} measurement.

Cellular Membrane Potentials: Action Potentials, Cell Cycle Control, Etc.

The many ion gradients across the plasma membrane create a cellular V_{mem} , which is generally assumed to be uniform across a cell due to free diffusion of ions within the cytosol. Many cellular V_{mem} events are short-lived; their quantification requires both high **temporal resolution** and high **voltage resolution**. An important fast V_{mem} signal is the action potential (AP) in excitable cells. APs in mammalian neurons typically last a few milliseconds, involving a rapid depolarization of ~100 mV followed by rapid hyperpolarization back to the resting V_{mem} ²⁴. Many aspects of APs have important cellular effects: waveform, frequency, and V_{mem} (AP amplitude, as well as initial and final absolute V_{mem})²⁴. V_{mem} recording platforms have been engineered with the goal of recapitulating AP waveform and frequency, and modern versions of both electrode-based and optical strategies reflect excellent progress towards this end^{25–29}. On the other hand, options for recording AP amplitude or absolute V_{mem} are more limited (see below).

To extend recordings from milliseconds to minutes, hours, or days, two new requirements emerge. First, the V_{mem} recording technique must be **stable** over the relevant timeframe, displaying no artefactual, V_{mem} -independent changes in signal. Second, it must be **non-invasive**, with cellular processes continuing normally despite chronic observation. With existing tools, V_{mem} recordings on timescales longer than minutes are challenging and are infrequently attempted. As a result, we have an incomplete picture of what V_{mem} changes occur and little clarity regarding their effects. Nevertheless, it is evident that slower changes in V_{mem} are pleiotropic signals with important cellular roles. In neuroscience, for example, the resting V_{mem} of neurons influences the propensity to fire APs. Furthermore, neuronal resting V_{mem} changes in association with – and perhaps also regulates – diverse processes, including cellular metabolism³⁰ and circadian rhythm³¹. In addition, V_{mem} appears to hyperpolarize during neuronal development and differentiation, although the magnitude of the observed changes varies drastically with the measurement technique used^{32–34}.

Furthermore, non-excitable cells also show V_{mem} changes on long timescales, often broadly related to growth. For example, V_{mem} changes associated with progression from G1 to S phase of the cell cycle have been documented in various cell lines^{35–37}, and acute epidermal growth factor receptor activation can be accompanied by a V_{mem} signal^{38–40}. In a disease context, cancer cells may be generally more depolarized than their non-transformed counterparts, implying that V_{mem} modulation may offer selective growth advantages⁴¹. Nevertheless, because current techniques cannot monitor absolute V_{mem} on the same cell over hours to days, we lack a roadmap of V_{mem} throughout the cell cycle or in growth signaling.

Subcellular Membrane Potentials: Organelles and Membrane Compartmentalization

Because V_{mem} originates from ion concentration gradients, subcellular V_{mem} differences can only exist in areas of electrical compartmentalization, where diffusion is regionally restricted. Partial compartmentalization produces transient or metastable local differences in V_{mem} . To study subcellular V_{mem} differences, a technique requires exquisite **spatial resolution**, as well as **access**

to subcellular structures without damaging them. Isolating the V_{mem} of a subcellular compartment from the cellular V_{mem} (which may be larger in magnitude and certainly occupies a larger region of space) is particularly challenging. However, direct evidence for subcellular V_{mem} differences exists, along with a preponderance of indirect evidence that V_{mem} is locally regulated in membrane-bound compartments.

Electrical compartmentalization occurs where the plasma membrane forms compartmentalized processes, and the longevity of localized V_{mem} signals is determined by the resistance to ion flow in and out of the compartment. Transient subcellular V_{mem} signals occur in neurons, as voltage waveforms are transmitted and processed by dendrites⁴² and the axon initial segment⁴³. Because these electrical compartments have nearly free ionic flow with other parts of the cell, the signals are fleeting. Visualization of dendritic computation and action potential propagation requires exceptional temporal resolution, with recording rates often exceeding 10 kHz⁴⁴. Longer-lived subcellular differences likely exist in the micron-scale dendritic spines, insulated by high-resistance bottlenecks between the spine and the dendrite. Mounting evidence suggests that spine V_{mem} can be electrically distinct from the rest of the neuron, and its role in learning and memory is an area of active study^{18,45}. In non-excitable cells, V_{mem} compartmentalization at the plasma membrane has yet to be conclusively shown. Nevertheless, the subcellular enrichment of particular ion channels in primary cilia^{19,46} and filopodia⁴⁷ suggests that non-uniform plasma membrane V_{mem} is likely.

Organelle membranes also offer an opportunity for ionic, and therefore V_{mem} , compartmentalization. Organelle electrical recordings are both invasive and challenging, as they often involve rupturing the plasma membrane. Nevertheless, through heroic experimental efforts, we have some direct evidence for V_{mem} signals in organelles. For example, the mitochondrial membrane potential (and H^+ gradient) is required for successful oxidative phosphorylation¹², and depolarized mitochondrial membrane potential, as well as defective oxidative phosphorylation, is associated with neurodegenerative diseases⁴⁸. The lysosome contains excitable channels¹¹, and the endoplasmic reticulum membrane potential has been reported to respond to cellular V_{mem} ⁴⁹. Furthermore, where direct recordings have not been made, proteomic and transcriptomic studies of organelle channels and transporters imply complex physiology⁵⁰, and improved methods tailored for the unique constraints of these subcellular systems are of great interest.

Membrane Potentials in Tissue: Long-Range Interactions and Delocalization

At the other extreme of length scale, membrane potential carries information via long-range interactions in tissues. Direct experimental interrogation of V_{mem} in tissue requires **throughput and multiplexed measurement** across many cells simultaneously, as well as **the ability to measure in situ**. Electrical communication over distances is facilitated by synaptic transmission as well as by gap junctions. In the brain, neurons do not act in isolation but instead form circuits^{51,52}; understanding of behavior requires simultaneous V_{mem} mapping across many cells. Beyond neural circuits, V_{mem} can be delocalized across tissues via gap junctions, which allow free flow of ions and many small molecules between cells. Early studies of electrical coupling and gap junctions were performed in the *Drosophila* salivary gland, where resistance to current flow between cells is only slightly higher than the resistance to flow within cells²⁰. Since then, gap junctions have been found to be ubiquitous, with dysfunction connected to various diseases⁵³. Gap junctions also enable rapid exchange of V_{mem} information across tissues, such as the synchronous electrical signaling in the heart²¹.

Electrical coupling via gap junctions not only enables transmission of V_{mem} signals across a population of cells but also inextricably links V_{mem} between coupled cells. Despite this, many observations about cellular V_{mem} signaling are made on isolated cells in culture. For epithelial cells that exist in vivo as a tightly coupled tissue, this is an artificial electrical state. What happens to the surrounding tissue when an individual cell changes its V_{mem} throughout the cell cycle or as a response to growth factor signaling? It remains unclear how and to what extent cell-autonomous V_{mem} signals discovered in isolated cells will translate into tissues. Theoretical studies suggest that, in some conditions, stable and meta-stable electrical patterning is possible, but it is highly dependent on the electrical coupling in the tissue^{54,55}. These results imply that V_{mem} signals are accompanied – or at least modulated by – dynamic regulation of cell-cell coupling. Although some progress has been made toward direct visualization of V_{mem} distributions in tissue^{22,23}, such measurements are largely unexplored.

Taken together, diverse biological questions necessitate a non-invasive, multiplexed, in situ V_{mem} platform with excellent spatiotemporal and voltage resolution. Throughput and technical ease of use of the platform are also important, as they enable top-down profiling experiments that can help elucidate V_{mem} signaling networks. From the array of methods available today, no single approach shows all of these characteristics (**Table 1.1**, **Table 1.2**). Indeed, more realistically, a toolkit of recording strategies will be required to fully interrogate V_{mem} . Below, we discuss the performance of existing V_{mem} measurement techniques, with an eye toward their ability to report V_{mem} across space and time.

	Whole Cell Patch-Clamp ^a	Cell Attached ^b	Perforated Patch ^c	Nanopipettes ^d	Nanopillars ^e	Planar Patch Clamp ^f
Spatial Resolution	- (single point)	- (single point)	- (single point)	- (single point)	++ (grid of points)	- (single point)
Temporal Resolution	+++ (μ s-ms)	+++ (μ s-ms)	+++ (μ s-ms)	+++ (μ s-ms)	+++ (μ s-ms)	+++ (μ s-ms)
Voltage Resolution	+++ (1 mV)	+ (variable)	+++ (1 mV)	+++ (1 mV)	+++ (1 mV)	+++ (1 mV)
Stability	+ (min-1 hr)	+ (min-1 hr)	++ (~1 hr)	+ (min-1 hr)	+++ (days)	+ (min-1 hr)
Non-invasiveness	-	++	+	+	+	-
In situ capabilities	++	++	++	++	-	-
Access to subcellular structures	+	+	+	+++	++	-
Throughput/multiplexing	-	-	-	-	++	+++
Ease of use	-	-	-	-	+	++

Table 1.1 Comparison of Electrode-Based V_{mem} Recording Strategies

^aAs described in references ^{25,32,56–63}.

^bAs described in references ^{32,64,65}.

^cAs described in references ^{66–68}.

^dAs described in references ^{69,70}.

^eAs described in references ^{71,72}.

^fAs described in references ^{73,74}.

	Single-Color Fluorescence Intensity ^a	Single-Color Fluorescence Lifetime ^b	FRET- based ^c	Electro- chromic (Ratio- based) ^d	GEVI Temporal Dynamics ^e	Stimulated Raman Scattering ^f
Spatial Resolution ^g	+++ ($<1\ \mu\text{m}$)	++ ($\sim 1\ \mu\text{m}$) ^h	+++ ($<1\ \mu\text{m}$)	+++ ($<1\ \mu\text{m}$)	++ ($\sim 1\ \mu\text{m}$) ^h	+ ($>1\ \mu\text{m}$) ^h
Temporal Resolution	++ (1 ms)	+ (5 ms) ^h	+ (1 ms OR >10 ms) ⁱ	++ (1 ms)	- (s) ^h	++ (1 ms) ^h
Voltage Resolution ^j	- ($\gg 100\ \text{mV}$)	++ (VF: ^k 20 mV, GEVI >100 mV)	ND ^l	+ ($\sim 100\ \text{mV}$)	++ (10 mV)	ND
Stability	+ (minutes)	++ (hours)	++ (hours)	+ (minutes)	ND	ND
Non- invasiveness	+++	+++	+++	+++	+++	+++
In situ capabilities	+++	+++	+++	+++	+++	++
Access to subcellular structures ^m	++	++	++	++	++	++
Throughput/ multiplexing	+++	++	+++	+++	+	++
Ease of use	+++	+	++	++	-	-

Table 1.2 Comparison of Optical V_m Recording Strategies

^aMany single-color, fluorescence intensity V_{mem} sensors have been designed, although they are seldom used for absolute V_{mem} quantification. Data here are compiled from references ^{26–29}.

^bSingle-color fluorescence lifetime recordings have been shown for genetically encoded voltage indicators ⁷⁵ and for small molecule VoltageFluors ⁴⁰.

^cFRET-based systems may use either conformational changes of the indicator ⁷⁶, changes in the absorption spectrum of a rhodopsin derivative ^{77,78}, or translocation of charged groups in the membrane ^{79–81} to report V_{mem} . Many properties are similar across these architectures; metrics here represent all types of FRET-based strategies unless otherwise noted.

^dPerformance metrics tabulated here are aggregated from references ^{82–87}.

^eDemonstrated in reference ⁸⁸. Abbreviation: GEVI, genetically encoded voltage indicator.

^fDemonstrated in references ^{89,90}.

^gSpatial resolution is determined by the diffraction limit, as well as optics used in the microscope. Techniques rated ++ rather than +++ here generally require moderate spatial binning to obtain adequate signal.

^hFor fluorescence lifetime, GEVI temporal dynamics, and stimulated Raman scattering measurements, there is a trade-off between spatial and temporal resolution, and one may be sacrificed to improve the other. Here, we list the best values of either spatial or temporal resolution shown in the literature or that we have demonstrated in our laboratory.

ⁱFRET sensors based on protein conformational changes or dye translocation exhibit temporal resolution of >10 ms, whereas electrochromic FRET-based sensors can resolve millisecond-time V_{mem} events.

^jVoltage resolution listed here is for applying a calibration determined on one cell onto another individual cell; more precise V_{mem} measurements can be obtained if measurements from multiple cells are averaged. Most techniques have substantially better resolution for quantifying the magnitude of a V_{mem} change on an individual cell (e.g. VF-FLIM has 5 mV resolution for quantifying V_{mem} changes on a single cell versus 20 mV resolution for quantifying absolute V_{mem} alone).

^kAbbreviation: VF, VoltageFluor.

^lAbbreviation: ND, not determined.

^mLimited by delivery of sensor to intracellular structures and/or the ability to isolate signal from only the subcellular membrane structure from interest when all membrane structures are stained.

Electrode-Based Approaches for Recording Absolute Membrane Potential

Electrode-based techniques are the gold standard for recording membrane potentials, ion channel properties, and electrical currents in cells. Three primary configurations are used in cellular V_{mem} recordings: whole cell patch clamp, cell attached, and perforated patch (**Figure 1.2**). All three can quantify absolute V_{mem} with excellent temporal resolution, but they suffer from high invasiveness, instability over time, low throughput, and poor spatial resolution (**Table 1.1**).

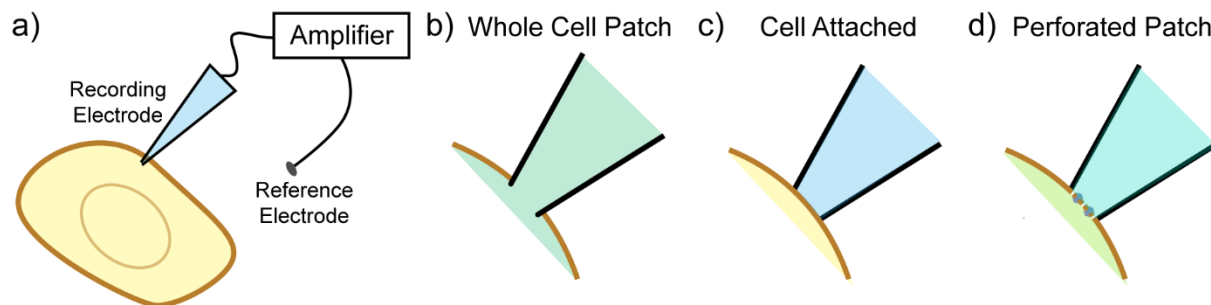


Figure 1.2 Electrophysiological configurations for V_{mem} recording. a) General schematic for electrode-based V_{mem} recordings, in which a cell comes into direct contact with an electrode. Voltage is measured as the difference between the recording electrode and a reference electrode in the bath solution. b-d) Close-up of the interface between the membrane and the electrode in different electrophysiology configurations. b) In whole cell patch-clamp electrophysiology, the plasma membrane is ruptured and the cytosol mixes with the recording electrode solution. c) In the cell attached configuration, the plasma membrane is left intact. d) In perforated patch, the membrane is not ruptured, but ionophores introduced into the recording solution allow ionic exchange across the membrane.

Intracellular Recordings of Absolute V_{mem}

In intracellular recording, V_{mem} is determined from the difference between a recording electrode in the cytosol and a reference electrode in the extracellular solution. More detailed treatment of the many capabilities of intracellular recording can be found elsewhere²⁵; we focus only on V_{mem} measurement. Two main types of intracellular recordings exist: sharp electrode and whole cell recordings. Sharp microelectrodes possess very fine tips and high tip resistances, which produces a variable tip potential and a V_{mem} artifact on the order of tens of millivolts^{25,91}. Furthermore, they create a leak in the cell of interest, producing artificially depolarized apparent V_{mem} and altered input resistance^{91,92}. Although sharp electrode recordings are still in use in certain *in vivo* preparations, they have largely been replaced by intracellular recordings in the whole-cell configuration. These “gigaseal” recordings use much larger patch electrodes with lower tip potentials; such electrodes can be sealed onto the cell with less damage than sharp electrodes⁵⁶. Whole cell recordings can also provide higher resolution information about the particular ions and types of channels involved in setting V_{mem} .

Intracellular recording provides a direct, *in situ* measurement of V_{mem} with sub-mV precision and excellent temporal resolution, but it suffers from a variety of drawbacks. First, the whole cell patch is highly invasive and disrupts the very processes under investigation. By dialyzing the cytosol with electrode internal solution, whole cell patch clamp washes out soluble signaling factors^{25,57,58}. In addition, physical contact with the electrode can activate mechanosensitive channels and signal transduction pathways^{93,94}, introducing further artifacts. With respect to the measurement itself, leaks in the seal between the recording electrode and the membrane can produce errors in recorded V_{mem} ³². More subtly, an electrode has poor spatial resolution, reporting V_{mem} at a point of contact that may or may not reflect other parts of the cell or tissue. For complex membrane structures or electrically coupled tissues, this space clamp error renders the effective recording area unknown^{62,63}. For subcellular recordings, electrode-based strategies struggle to physically access subcellular compartments, as the size of the electrode tip provides a lower bound on the size of structures that can be interrogated^{19,45}. Furthermore, intracellular recordings are challenging to execute. Attempting each whole cell recording takes an expert researcher or specialized robot 5-10 minutes, with a 10-30% success rate for these efforts⁵⁹⁻⁶¹. While *in situ* automated patch systems have reduced the need for human expertise and labor, they still do not achieve high throughput or multiplexing. Therefore, recording across many cells in the same tissue is nearly impossible, as is cataloguing V_{mem} subpopulations over many cells.

Reducing Invasiveness: Cell Attached and Perforated Patch Configurations

To mitigate the washout of soluble factors associated with whole cell recording, V_{mem} must be measured without disrupting the plasma membrane. This is possible in the “cell attached” configuration, in which an extracellular patch electrode is sealed onto the cell without rupturing the membrane. If the membrane resistance is approximately 100-fold lower than the seal resistance, the voltage across the small membrane region under the pipette will reflect cellular V_{mem} with reasonable accuracy⁶⁴. Alternatively, if the channel composition and electrical properties are known in the cells of interest, then the reversal potential of these channels (determined in the cell attached configuration) can also be used to infer V_{mem} . Such techniques have been demonstrated with the K^+ reversal potential in hippocampal interneurons⁶⁵, as well as the NMDA reversal potential in hippocampal CA3 neurons³². However, this strategy is difficult to extend, as it requires detailed knowledge of the inventory and behavior of channels in the specific system of interest.

Perforated patch recordings offer a more general solution to reduce invasiveness of whole cell recordings^{67,68}. In perforated patch, an ionophore placed in the pipette internal solution allows ions to cross the membrane without completely disrupting it, thereby electrically connecting the cytosol with the pipette solution. Because the membrane remains impermeable to the diffusion of larger molecules, perforated patch recording can last over an hour without run-down of relevant cytosolic factors^{66,67}.

Engineering Electrodes for Improved Performance

Planar Patch Clamp

Efforts to improve electrode-based recordings have also turned to the electrodes themselves. With the development of low-noise arrays of patch electrodes⁷³, commercial planar patch clamp systems can now perform simultaneous, automated electrophysiological experiments in 384 or 768 well arrayed format⁷⁴. These systems are increasingly used to screen compound libraries against ion channel targets, expediting the drug discovery process. However, the system requires a suspension of dissociated cells, which precludes recording in situ and over extended time courses. As a result, planar patch clamp is more suited for high throughput screening of ion channel pharmacology than analysis of cellular or tissue level voltage signaling.

Nanopipettes and Nanopillar Electrodes

The development of nanopipettes and nanopillar electrodes has enabled electrode-based access to broader length scales. First, nanopipettes are a smaller version of sharp microelectrodes; their reduced size brings flexibility and access to small cellular compartments. Nanopipettes have been used to record from micron-scale dendritic spines, providing some of the first direct evidence for functional voltage compartmentalization in these structures⁶⁹. In addition, the flexibility of the nanopipette can stabilize in vivo recordings during animal behavior⁷⁰. As with sharp electrodes, the high tip resistances of the nanopipettes filter the signal and create a shunt that changes the observed V_{mem} , so the output signal must be processed to obtain accurate results. Second, nanopillar electrode arrays enable simultaneous intracellular recordings from many cells in a culture via a grid of nano-scale electrodes^{71,72}. These platforms facilitate mapping of electrical activity across neuronal circuits or beating cardiomyocytes. However, the cells of interest must be cultured directly onto the electrode grid; nanopillar electrode arrays are not yet capable of in situ mapping of tissue V_{mem} . Overall, electrode engineering offers a promising avenue for increasing the spatial reach of electrophysiology.

Calibrated Optical Signals for Recording Absolute Membrane Potential

A variety of optical V_{mem} measurement strategies use fluorescence as a proxy for V_{mem} , employing small molecule or protein-based sensors engineered to display V_{mem} -sensitive fluorescence (**Table 1.2**). Optical platforms generally enjoy excellent spatial resolution, low invasiveness, and medium throughput. However, achieving the requisite temporal resolution, voltage resolution, and signal-to-noise has required more engineering. Optical strategies fall into four categories (single-color intensity, ratio-based, pump-probe, and single-color lifetime, **Figure 1.3**). In all cases, rigorous calibration must be performed to relate the optical signal to the underlying absolute V_{mem} , which is most effectively achieved by performing the optical recording while V_{mem} is tuned across a range of known values. As we will discuss, a key difference among the various fluorescence-based strategies is voltage resolution, which is a result of the reproducibility of this calibration over time and between cells.

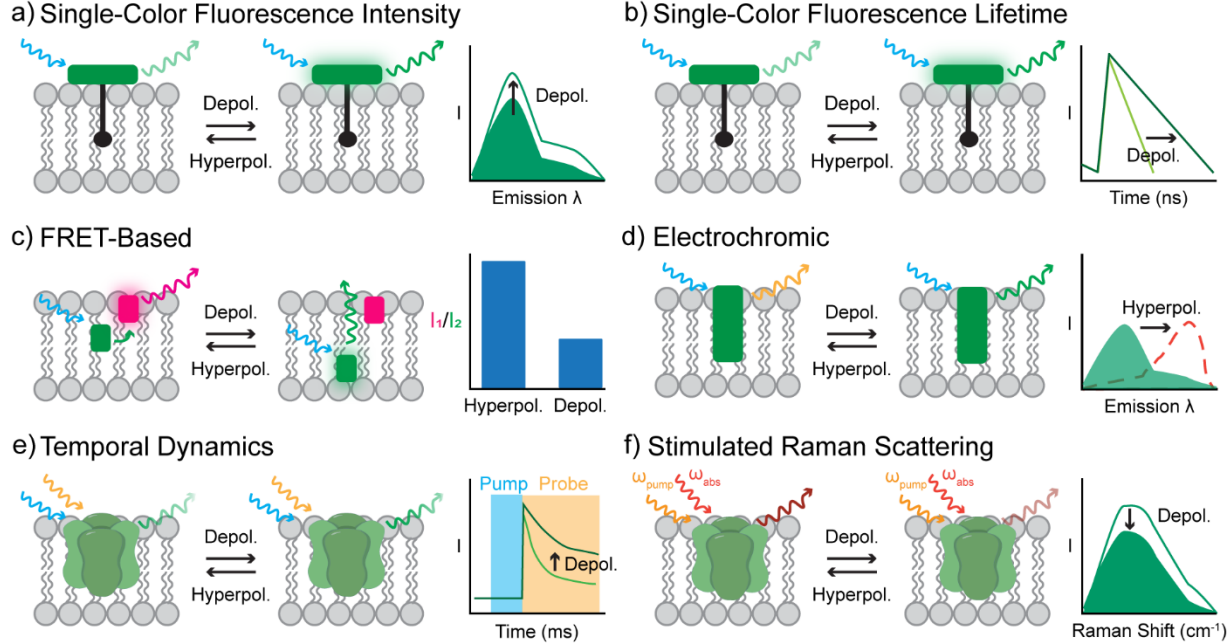


Figure 1.3 Optical strategies for reporting absolute V_{mem} . a) Single-color fluorescence intensity recordings detect changes in V_{mem} by changes in a sensor's fluorescence quantum yield (shown here), extinction coefficient, or cellular concentration. This technique generally cannot report absolute V_{mem} optically, although estimates can be made if calibrations are performed for every cell of interest. b) Single-color fluorescence lifetime can report absolute V_{mem} , particularly if the V_{mem} sensor operates via photoinduced electron transfer. c) FRET-based sensors show differences in FRET ratio of two fluorophores, often resulting from V_{mem} -dependent changes in the distance between the two. Diagram shows a FRET-oxonol system. d) The excitation and/or emission spectra of electrochromic dyes depends on the electric field in the plasma membrane. e) Certain GEVIs show V_{mem} -related differences in the temporal dynamics of the absorption of their excited state. f) Stimulated Raman scattering (SRS) imaging reports changes in V_{mem} -dependent vibrational frequencies. Depol.: depolarization; hyperpol.: hyperpolarization; λ : wavelength.

Strategies for Calibration of Optical V_{mem} Reporters

Electrode-based Calibration

The most accurate method for calibrating fluorescence with respect to V_{mem} is whole cell voltage clamp electrophysiology, which uses an intracellular recording configuration to inject current until the cell reaches the desired V_{mem} . The response across a range of V_{mem} can be evaluated, determining a calibration with both a slope and y-intercept. However, this approach is best when performed in isolated, single cells, and it suffers from the limitations of electrophysiology discussed above.

Pharmacological Calibration

If the system under study is inaccessible to electrode-based strategies, either pharmacology or intrinsic V_{mem} signals can be used to approximate the relationship between the optical signal and absolute V_{mem} . Reference values of V_{mem} have been established using ionophores for the primary ion establishing V_{mem} ⁹⁵⁻⁹⁸, high extracellular K^+ (120-150 mM)^{95,99}, and cell death/fixation with paraformaldehyde (PFA)⁹⁶. PFA and ionophores produce a more reliable 0 mV point than high K^+ treatments, but they interact with the plasma membrane itself, which can change the fluorescence properties of the voltage sensor⁸⁴. Furthermore, there is no reliable way

to access the sensitivity of the sensor (i.e. slope of signal versus V_{mem}) from a single pharmacological set point. On the other hand, in samples with well-described intrinsic electrical responses, a V_{mem} event of known magnitude can provide a standard for approximate determination of absolute V_{mem} sensitivity. Demonstrated reference V_{mem} signals include sustained hyperpolarization in Purkinje cells¹⁰⁰, depolarization resulting from glutamate uncaging¹⁰¹, and back-propagating action potentials in dendrites¹⁰². Such strategies are non-invasive but are limited to the small subset of samples where a useful and consistent V_{mem} signal can be identified.

Efforts Towards Optical Calibration

In many cases, this limited set of calibration options is inadequate. Recently developed optical strategies for V_{mem} control may eventually provide an alternative to the above approaches, although they cannot yet tune V_{mem} to defined voltages. Engineering efforts for optical V_{mem} actuators have focused on channelrhodopsins, proteins which pass current in response to light. For setting V_{mem} optically, step-function opsins may be particularly useful, as they continue to pass current for minutes after initial activation^{103,104}. Recently, light sensitivity was engineered into other ion channels via domain insertion, allowing diverse channels to serve as optogenetic actuators¹⁰⁵. These strategies could eventually allow precise setting of V_{mem} without an electrode.

Single Color, Fluorescence Intensity Recordings are Difficult to Relate to Absolute V_{mem}

In single-color fluorescence intensity-based recordings, V_{mem} affects the quantum yield or absorption coefficient of a sensor, producing changes in its fluorescence emission. Fluorescence intensity of a voltage sensor in a membrane is recorded over seconds or minutes, enabling detection of V_{mem} changes and characterization of V_{mem} waveforms. A variety of sensor architectures have been documented^{26–29}, including both small molecule dyes and genetically encoded voltage indicators (GEVIs). Many display sufficiently fast temporal resolution to follow millisecond-time action potentials. State-of-the-art systems can achieve cell-resolved recording of single action potentials in vivo from superficial brain regions, often with subcellular detail^{78,106,107}. However, single color fluorescence intensity is best suited for V_{mem} event detection and generally cannot report absolute V_{mem} . Methods that rely solely on fluorescence intensity to monitor V_{mem} display large differences in baseline signal between cells and over minutes to hours. These variations are V_{mem} -independent, arising from variable sensor loading/trafficking, cell morphology, sensor photobleaching, fluorescence quenching, and illumination intensity. As a result, calibrations made on one cell at a given time cannot be extended to other cells or even to the same cell hours later.

Instability in Calibrations of Single-Color Fluorescence Intensity

Some attempts have been made to calibrate fluorescence intensity to known V_{mem} at each cell or structure of interest, but caution must be employed in interpreting these results. Electrophysiological calibration of each individual cell is often challenging or impractical, so fluorescence intensity calibrations are generally performed with less-accurate pharmacological strategies. In some cases, this approach is the only viable way to interrogate V_{mem} – for example, gramicidin-based calibrations and the V_{mem} indicator Archaeorhodopsin (Arch) revealed subcellular differences in the action potential waveform⁹⁸. However, pharmacological calibration of a single color V_{mem} sensor is prone to artifacts. When calibrating from a single pharmacological set point rather than a range of V_{mem} set by an electrode, the researcher must assume that the fractional change in fluorescence ($\% \Delta F/F$) per mV of the indicator is the same for all samples. In reality, the $\Delta F/F$ response to a given ΔV_{mem} will depend on the ratio of productively engaged sensor to background sensor, which may vary in space with trafficking or loading of the sensor. Furthermore, even with electrode-based calibration, many voltage sensors display sensitivity to

membrane composition¹⁰⁸, which varies not only between cells but also within cells with complex membrane structures¹⁰⁹.

Ratio-Based Fluorescent Sensors Improve Reproducibility

Ratio-based voltage sensors show changes in the relative intensities of two fluorescence channels. The primary strategies for ratio-based fluorescent V_{mem} sensors rely on either FRET-based indicators or electrochromic dyes. If the stoichiometry between the two signals is known, the second channel can be used to correct for variability arising from cell morphology and dye loading. Ideally, the corrected signal would then be a stable proxy for absolute V_{mem} over time and across many cells without recalibration. In practice, ratio-based sensors achieve better absolute V_{mem} resolution than single-color intensity-based techniques, but they generally do not have sufficient resolution to quantify the biologically relevant V_{mem} differences between cells (~10-20 mV).

FRET-Based V_{mem} Sensors

Förster resonance energy transfer (FRET)-based V_{mem} sensors exhibit V_{mem} -dependent energy transfer from a donor to an acceptor fluorophore. Because the fluorescence intensities of the donor and acceptor are voltage sensitive in opposite directions, the ratio of the two can provide better fractional responses and higher signal to noise than a single color alone. Although a variety of FRET-based V_{mem} sensors exist, we are not aware of their use to document absolute V_{mem} , largely due to variable stoichiometry between the donor and acceptor. For genetically encoded systems⁷⁶⁻⁷⁸, different rates of photobleaching, folding, and productive trafficking to the plasma membrane lead to variability in FRET ratios between cells. For small molecule FRET-oxonol systems^{79,81}, differences in loading between the two lipophilic indicators leads to variability in the $\Delta F/F$ ⁸⁰. As a result, FRET ratio measurements are not extensible between cells. Furthermore, the FRET-based reporters that undergo conformational changes to sense V_{mem} suffer from reduced temporal resolution and toxicity from capacitive load. In practice, these probes are primarily used to detect action potentials, with the second color serving to improve signal to noise or reduce motion artifacts.

Electrochromic Ratio-Based V_{mem} sensors

Electrochromic dyes, such as the ANEPPS⁸² and ANNINE⁸³ classes, show V_{mem} -dependent excitation and/or emission spectra. A ratio signal is obtained by comparing the emission in a fixed band produced by excitation at two different wavelengths (or vice versa with a fixed excitation and two emission bands). Because electrochromic dyes are based on a charge shift mechanism, they have sub-millisecond temporal responses but display low sensitivity (~10% change per 100 mV)^{84,85}. Normalized ANEPPS fluorescence ratios are accurate to approximately 5 mV for quantifying V_{mem} **changes** on an individual cell^{86,87}. In combination with random access microscopy, di-8-ANEPPS ratios can report the action potential waveform in absolute V_{mem} optically with excellent spatiotemporal resolution (0.5 ms and 2 μm)⁸⁷. However, the V_{mem} calibration does not translate between cells and must be performed with an electrode on each cell of interest to achieve this accuracy. The origin of the variability of the absolute ratio between cells is unclear; it likely depends on a complex combination of temperature, membrane composition, and probe loading.

Molecular Beacons in Ratio-based V_{mem} Recording

A recent, promising architecture for ratio-based V_{mem} sensors uses a molecular beacon, where the sensor contains one fluorophore that is voltage-sensitive and a second fluorophore that is a voltage-independent reference. This strategy was tested with the GEVI Arch(D95N) fused to

GFP, but absolute V_{mem} quantification was not possible because of differential photobleaching of GFP and Arch(D95N)⁸⁸. More recently, the molecular beacon system was implemented with small molecule V_{mem} sensors in Voltair¹¹⁰, which comprises the VoltageFluor RVF5¹¹¹ and the reference fluorophore ATTO647N on a DNA chassis. By normalizing the RVF5 signal to ATTO647N, Voltair was able to estimate organellar V_{mem} optically based on a pharmacological calibration, but absolute V_{mem} resolution of this technique has not yet been determined.

Pump-probe Recordings: Accurate, with Complex Instrumentation

The temporal dynamics or vibrational signature of a probe do not depend on probe concentration, therefore offering a more reproducible optical parameter to report absolute V_{mem} . The temporal dynamics of the GEVI Arch(D95H)-eGFP can report absolute V_{mem} between cells with 10 mV resolution⁸⁸, the best yet reported for an optical system. However, access to and interpretation of such data is challenging for most laboratories, limiting its utility as a general V_{mem} sensing platform. Stimulated Raman Scattering (SRS) has also been investigated as a strategy for absolute V_{mem} recordings, but its V_{mem} resolution has yet to be determined. Recently, the SRS signal of near-infrared opsins was shown to be sensitive to bulk depolarization in *E. coli* membranes⁹⁰. Moreover, label-free detection of neuronal action potentials with SRS has been reported, although the mechanism of such V_{mem} sensitivity remains unclear⁸⁹. Further investigations of temporal dynamics and SRS as V_{mem} reporting platforms are of great interest, especially with an eye towards translating them into more widely available systems.

Fluorescence Lifetime Offers Accessible Absolute Optical V_{mem} Recordings

Fluorescence lifetime (τ_{fl}), or the amount of time that probe molecules remain in the fluorescent excited state, has recently garnered attention for its ability to report cellular properties (e.g. Ca^{2+} concentration or V_{mem}) absolutely using a single fluorescence channel¹¹². τ_{fl} is largely independent of fluorophore concentration, cellular morphology, and photobleaching, although it displays sensitivity to parameters such as temperature and viscosity. The primary downside to fluorescence lifetime imaging (FLIM) is its poor temporal resolution, with most acquisition times on the order of seconds.

Fluorescence Lifetime of GEVIs

Fluorescence lifetime-based V_{mem} recordings can be performed if τ_{fl} of a sensor changes reproducibly with V_{mem} . Many GEVIs sense V_{mem} through complex photocycles or multiple conformational states; this leads to low sensitivity in τ_{fl} or non-linear relationships between τ_{fl} and V_{mem} . For example, the rhodopsin derivative Arch(D95H) was first evaluated in FLIM for its absolute V_{mem} -reporting capabilities, but its τ_{fl} - V_{mem} relationship was difficult to interpret⁸⁸. Comparison of the V_{mem} sensitivity of three GEVIs in two-photon illuminated FLIM⁷⁵ revealed that only CAESR⁷⁷ was suitable for absolute V_{mem} recording. CAESR exhibits 20 mV accuracy for quantifying V_{mem} changes on a given cell in a one second bandwidth, but it displays too much variability to extend optical V_{mem} calibrations between cells⁷⁵. This between-cell variability perhaps arises from the complex dynamics of rhodopsin fluorescence, coupled with intracellular fluorescence signal from incorrectly trafficked protein. V_{mem} -sensitive FLIM has also been demonstrated for FRET-oxonol V_{mem} sensors¹¹³ and for accumulation-based sensors of mitochondrial potential¹¹⁴, but no estimation of the absolute V_{mem} resolution was made in these systems.

Fluorescence Lifetime of VoltageFluor Small Molecule Dyes

Fluorescence lifetime-based absolute V_{mem} imaging was developed further in our laboratory, achieving stable calibration between cells through the use of small-molecule VoltageFluors (VFs) as a voltage sensor. The voltage-sensing trigger for VoltageFluors is photoinduced electron transfer¹¹⁵, which translates to a rapid, linear V_{mem} response in both fluorescence intensity^{115,116} and fluorescence lifetime⁴⁰. The fluorescence lifetime of VF2.1.Cl (VF-FLIM) can be calibrated to report absolute V_{mem} with a resolution of 5 mV for quantifying voltage changes on individual cells. These calibrations can be extended from one cell to another while retaining a single trial V_{mem} resolution of 20 mV, which is sufficient to detect biologically relevant variation in resting V_{mem} , especially once multiple measurements are averaged. Because VF-FLIM calibrations are consistent for a given cell line, we were able to extend V_{mem} calibrations across thousands of cells after an initial electrode-based calibration on only a few cells. We applied VF-FLIM to study epidermal growth factor signaling and found a 15 mV hyperpolarizing response in carcinoma cells, showcasing its potential for elucidating diverse signaling roles of V_{mem} .

In addition to its improved V_{mem} resolution, VF-FLIM shows many promising attributes as a V_{mem} sensing platform. The increased stability of the calibration enhances throughput; an individual researcher using VF-FLIM could perform V_{mem} recordings at the speed of optical imaging (thousands of cells in an afternoon) rather than electrophysiology (~10 cells per day). Importantly, the temporal and spatial resolution of FLIM-based strategies are related: acquisition rates as fast as 200 Hz (exposures of 5 ms) can be obtained with reduced spatial resolution (**Chapter 4**), and spatial resolution can be improved from cellular to ~1 micron at the expense of imaging speed (**Chapter 3**). The VF-FLIM technology has been further improved through combination with red-shifted V_{mem} sensors (**Chapter 4**)^{117,118} and targeting of sensors to specific cell populations (**Chapter 3**)^{119–121}, both of which are discussed further in this dissertation.

In **Chapter 3** we discuss combining chemical and genetic strategies to create genetically targeted VF dyes compatible with VF-FLIM. Genetically targeting VF dyes enables optical monitoring of membrane potential in cells in more complex culture, such as primary neuronal culture, where high background from neuropil staining previously made imaging of individual neurons, let alone individual dendrites, difficult. The development of VF-FLIM measurements from genetically targeted cells (**Chapter 3**) paves the way for monitoring voltage in dendritic spines and other structures too small to be tractable for measurement by electrode. Expanding VF-FLIM to utilize red-shifted VoltageFluors, as discussed in **Chapter 4**, allows for longer-term imaging (>10 minutes of continuous imaging), allowing imaging of voltage changes that occur over longer times, as well as enabling faster imaging with less phototoxicity. VF-FLIM can additionally provide insight into the design of future generations of VF dyes, uncovering motifs that improve brightness and deliver longer-lifetime dyes (**Chapter 5**).¹²² Overall, FLIM-based absolute V_{mem} imaging extends the timescale and spatial scale over which V_{mem} can be recorded and displays some of the best V_{mem} resolution to date for optical systems, with the promise of improvement over both the time and spatial scales as the method is expanded further, including combining genetic targeting with red-shifted VoltageFluors.

Outlook

V_{mem} signals run the gamut from milliseconds to days and from subcellular to tissue-level (**Figure 1.1**). The existing V_{mem} toolkit can record most cellular V_{mem} signals and some subcellular ones, with time resolutions ranging from milliseconds to minutes. V_{mem} signals delocalized across tissues are still challenging to interrogate, especially those occurring over hours to days. On the other hand, V_{mem} signals at subcellular structures also remain challenging to access.

Electrode-based absolute V_{mem} recordings have defined the field for years and will remain pivotal, as engineering efforts continue to improve throughput and reduce invasiveness. On the other hand, progress in optical approaches for absolute membrane potential recording has already expanded the range of space and time over which V_{mem} can be recorded, especially at those structures that are difficult to access with electrode-based methods (**Chapter 3**). One of the key limitations to the scope of optical, absolute V_{mem} recording is the need for calibration in the cell or tissue of interest; we excitedly await improved optical actuators for V_{mem} . Further improvements in voltage-sensitive dyes and proteins stand to increase the absolute V_{mem} resolution of existing optical approaches. Improvements to photon counting FLIM hardware, as well as fast frequency-domain FLIM strategies ¹²³, are promising avenues for faster lifetime recordings, particularly when coupled with improvements in dyes or GEVIs that can either provide more photons with less toxicity, or the need for fewer photons to achieve accurate lifetime fits (**Chapter 4**). Optical V_{mem} recording in vivo can be multiplexed across more cells and larger areas by combination with lattice light-sheet imaging ¹²⁴ and other fast volumetric imaging strategies ¹²⁵.

With these technological advances, we are constructing a picture of V_{mem} across biological time and space scales. Nevertheless, our atlas remains far from complete. In many cases, the information carried by V_{mem} is unknown, and the molecular mechanisms of V_{mem} response remain obfuscated. Each newly observed V_{mem} signal raises additional, exciting questions: How does the cell bring about these V_{mem} changes? Which molecules respond to V_{mem} ? How are these signals transduced to downstream cellular processes? Uncovering the signaling networks surrounding V_{mem} will require platforms that interface with other cell profiling and “omics” techniques, as was recently pioneered by Patch-Seq technology ¹²⁶. With such efforts, we move ever closer to a systems-level understanding of membrane potential in all its diversity.

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Chapter 2

Optical Estimation of Absolute Membrane Potential with Fluorescence Lifetime Imaging

Portions of this work were published in the following scientific journal:
Lazzari-Dean, J. R.; Gest, A. M. M.; Miller, E. W. Optical Estimation of Absolute Membrane Potential Using Fluorescence Lifetime Imaging. *Elife* **2019**, 8, e44522.
<https://doi.org/10.7554/elifex.44522>.

Portions of this work were performed in collaboration with the following person:
This work was written in collaboration with Dr. Julia Lazzari-Dean

Abstract

All cells maintain ionic gradients across their plasma membranes, producing transmembrane potentials (V_{mem}). Mounting evidence suggests a relationship between resting V_{mem} and the physiology of non-excitable cells with implications in diverse areas, including cancer, cellular differentiation, and body patterning. A lack of non-invasive methods to record absolute V_{mem} limits our understanding of this fundamental signal. To address this need, we developed a fluorescence lifetime-based approach (VF-FLIM) to visualize and optically quantify V_{mem} with single-cell resolution in mammalian cell culture. Using VF-FLIM, we report V_{mem} distributions over thousands of cells, a 100-fold improvement relative to electrophysiological approaches. In human carcinoma cells, we visualize the voltage response to growth factor stimulation, stably recording a 10-15 mV hyperpolarization over minutes. Using pharmacological inhibitors, we identify the source of the hyperpolarization as the Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}3.1}$. The ability to optically quantify absolute V_{mem} with cellular resolution will allow a re-examination of its signaling roles.

Introduction

Membrane potential (V_{mem}) is an essential facet of cellular physiology. In electrically excitable cells, such as neurons and cardiomyocytes, voltage-gated ion channels enable rapid changes in membrane potential. These fast membrane potential changes, on the order of milliseconds to seconds, trigger release of neurotransmitters in neurons or contraction in myocytes. The resting membrane potentials of these cells, which change over longer timescales, affect their excitability. In non-electrically excitable cells, slower changes in V_{mem} —on the order of seconds to hours—are linked to a variety of fundamental cellular processes¹, including mitosis², cell cycle progression³, and differentiation⁴. Mounting lines of evidence point to the importance of electrochemical gradients in development, body patterning, and regeneration⁵.

Despite the importance of membrane potential to diverse processes over a range of time scales, the existing methods for recording V_{mem} are inadequate for characterizing distributions of V_{mem} states in a sample or studying gradual shifts in resting membrane potential (**Table 2.1**). Patch clamp electrophysiology remains the gold standard for recording cellular electrical parameters, but it is low throughput, highly invasive, and difficult to implement over extended time periods. Where reduced invasiveness or higher throughput analyses of V_{mem} are required, optical methods for detecting events involving V_{mem} changes (e.g. whether an action potential occurred) are often employed⁶⁻⁸. However, optical approaches generally use fluorescence intensity values as a readout, which cannot report either the value of V_{mem} in millivolts (“absolute V_{mem} ”) or the millivolt amount by which V_{mem} changed⁹. Variations in dye environment¹⁰, dye loading, illumination intensity, fluorophore bleaching, and/or cellular morphology complicate fluorescence intensity measurements, making calibration and determination of absolute membrane potential difficult or impossible. This limitation restricts optical analysis to detection of acute V_{mem} changes, which can be analyzed without comparisons of V_{mem} between cells or over long timescales.

	Patch-clamp electro- physiology	Single color fluorescence intensity imaging	Two- component ratiometric fluorescent sensors (FRET- oxonol)	Ratiometric, electrochromic fluorescent sensors (ANEPPS)	GEVI-based FLIM approaches	FLIM with VoltageF luors (VF- FLIM, <i>this work</i>)
Absolute V_{mem} resolution (between cell comparisons)	excellent	none ^a	very poor ^b	poor ^c	very poor ^d	good
Quantification of V_{mem} changes on a given cell	excellent	none ^a	poor ^b	poor ^c	poor ^d	excellent
Compatibility with long time scales	poor ^e	poor ^f	good ^g	good ^g	good	good
Temporal resolution	sub- millisecond	~1 ms ^h	2-500 ms ⁱ	~1 ms ^h	seconds ^j	seconds ^j
Minimal invasiveness, damage	very poor ^e	good	poor ^k	good	good	good
Throughput (cells/day)	10s	1000s	1000s	1000s	1000s	1000s
Spatial resolution	Single value per electrode ^l	Subcellular	Subcellular	Subcellular	Single value per laser path ^m	Single cell ⁿ

Table 2.1 Comparison of available approaches for measuring membrane potential in cells.

^aMeasurements vary too much to be converted to absolute voltage or interpreted across populations of cells. This variability is attributable to numerous confounding factors, including dye loading, photobleaching, and sample movement ⁹. ^bWhile in principle less variable than a single-color fluorescence intensity measurement, in practice, the signal depends strongly on the loading of two independent lipophilic indicators ^{13,80}, which can vary substantially. ^cANEPPS excitation ratios depend on a variety of non-voltage factors, in particular the membrane composition, leading to substantial artifacts in optical V_{mem} determinations ^{16,46}. ^dWith the GEVI CAESR in our hands, apparently poor protein trafficking produces large amounts of non-voltage-sensitive signal, which contaminates the FLIM recording and contributes to high cell to cell variability (**Fig. 2.5, Methods**). ^ePatch-clamp electrophysiology requires physical contact with the cell of interest, which causes damage to the cell and, in whole cell configurations, washout of intracellular factors. Slight movement of the cell or sample generally result in loss of the patch. ^fMovement of the cell and photobleaching of the dye both cause large changes to the signal over seconds to minutes. ^gRatio-calibrated imaging approaches use a second signal (usually another color of fluorescence) to correct for differences in dye concentration or changes in the region of interest that contaminate single-color intensity signals. If the rate of photobleaching is the same for both components, photobleaching artifacts can also be avoided. ^hLimited by photon count rates. ⁱLimited by probe movement in the membrane, which depends mostly on lipophilicity ¹². ^jPhoton counting based

lifetime imaging, like epifluorescence intensity imaging, is limited by photon count rates. Large numbers of photons per pixel must be collected to fit TCSPC FLIM data, often using a line scanning confocal approach, leading to slower acquisition speeds than epifluorescence-based intensity imaging.^kToxicity from capacitive load of the sensor¹².^lThe spatial resolution of electrophysiology is compromised by space clamp error, preventing interpretation of V_{mem} in regions far from the electrode (e.g. many neuronal processes)^{35,36}.^mAs demonstrated by Cohen and co-workers²⁷; in our hands with CAESR, we also experienced significant improvements in voltage resolution by fitting a single curve per FLIM image instead of processing the images pixel-wise (see **Methods**).ⁿIn this work, we calibrated VF-FLIM for V_{mem} measurements with single cell resolution. In principle, subcellular spatial resolution could be achieved with the VF-FLIM technique.

One strategy to address these fluorescence intensity artifacts and quantify cellular parameters optically is ratio-based imaging. For V_{mem} specifically, ratio-based signals can be accessed either with a two-component system or with an electrochromic voltage sensitive dye, but neither strategy has enabled accurate absolute V_{mem} recordings. Two-component FRET-oxonol systems, with independent chromophores for ratio-based calibration, have seen limited success¹¹, and they confer significant capacitive load on the cell¹². Further, their performance hinges on carefully tuned loading procedures of multiple lipophilic indicators¹³, which can be challenging to reproduce across different samples and days. On the other hand, electrochromic probes report voltage as changes in excitation and emission wavelengths of a single chromophore¹⁴. While they benefit from simpler loading procedures, signals from electrochromic styryl dyes require normalization with an electrode on each cell of interest to determine absolute V_{mem} accurately^{15–17}. As a result, ratiometric V_{mem} sensors cannot be used to optically quantify slow signals in the resting V_{mem} , which may be on the order of tens of millivolts. Indeed, ratiometric V_{mem} probes are most commonly applied to detect - rather than quantify - fast changes in V_{mem} ¹⁶, much like their single wavelength counterparts.

An alternative approach to improved quantification in optical measurements is fluorescence lifetime (τ_{fl}) imaging (FLIM), which measures the excited state lifetime of a population of fluorophores. Because fluorescence lifetime is an intrinsic property, FLIM can avoid many of the artifacts that confound extrinsic fluorescence intensity measurements, such as uneven dye loading, fluorophore bleaching, variations in illumination intensity, and detector sensitivity^{18,19}. If a fluorescent probe responds to the analyte of interest via changes in the lifetime of its excited state, there is the opportunity to use fluorescence lifetime to provide a more quantitative estimate of analyte parameters than can be achieved with fluorescence intensity alone. Although FLIM measurements can be affected by environmental factors such as temperature, ionic strength and local environment¹⁸, FLIM has been widely employed to record a number of biochemical and biophysical parameters, including intracellular Ca^{2+} concentration²⁰, viscosity²¹, GTPase activity²², kinase activity²³, and redox state (NADH/NAD^+ ratio)²⁴, among others¹⁹. Attempts to record absolute voltage with FLIM, however, have been limited in success^{25–27}. Previous work focused on genetically encoded voltage indicators (GEVIs), which either possess complex relationships between τ_{fl} and voltage²⁶ or show low sensitivity to voltage in lifetime²⁷ and require complex and technically challenging measurements of fast photochemical kinetic to estimate voltage²⁶. Because of this poor voltage resolution, the fluorescence lifetimes of GEVIs cannot be used to detect most biologically relevant voltage changes, which are on the order of tens of millivolts.

Fluorescent voltage indicators that use photoinduced electron transfer (PeT) as a voltage-sensing mechanism are promising candidates for a FLIM-based approach to optical V_{mem} quantification. Because PeT affects the nonradiative decay rate of the fluorophore excited state, it has been successfully translated from intensity to τ_{fl} imaging with a number of small molecule probes for Ca^{2+} ²⁸. We previously established that VoltageFluor (VF)-type dyes transduce changes in cellular membrane potential to changes in fluorescence intensity and that the voltage response of VF dyes is consistent with a photoinduced electron transfer (PeT)-based response mechanism^{29,30}. Changes in the transmembrane potential alter the rate of PeT^{31,32} from an electron-rich aniline donor to a fluorescent reporter, thereby modulating the fluorescence intensity of VF dyes²⁹ (**Fig. 2.1 A,B**). VoltageFluors also display low toxicity and rapid, linear responses to voltage.

Here, we develop fluorescence lifetime imaging of VoltageFluor dyes (VF-FLIM) as a quantitative, all-optical approach for recording absolute membrane potential with single cell resolution. Using patch-clamp electrophysiology as a standard, we demonstrate that VF-FLIM reports absolute membrane potential in single trials with 10 to 23 mV accuracy (root mean square deviation, RMSD; 15 second acquisition), depending on the cell line. In all cases tested, VF-FLIM tracks membrane potential *changes* with better than 5 mV accuracy (RMSD). We benchmark VF-FLIM against previously reported optical absolute V_{mem} recording approaches and demonstrate resolution improvements of 8-fold over ratiometric strategies and 19-fold over other lifetime-based strategies. To highlight the increased throughput relative to manual patch-clamp electrophysiology, we document resting membrane potentials of thousands of cells. To our knowledge, this work represents the first broad view of the distribution of resting membrane potentials present *in situ*. VF-FLIM is limited to acquisition speeds on the order of seconds, but it is well-suited for studying gradual V_{mem} dynamics. Using VF-FLIM, we quantify and track the evolution of a 10-15 mV V_{mem} hyperpolarization over minutes following epidermal growth factor (EGF) stimulation of human carcinoma cells. Through pharmacological perturbations, we conclude that the voltage changes following EGF stimulation arise from activation of the calcium-activated potassium channel $\text{K}_{\text{Ca}3.1}$. Our results show that fluorescence lifetime of VF dyes is a generalizable and effective approach for studying resting membrane potential in a range of cell lines.

Results

VoltageFluor Fluorescence Lifetime Varies Linearly with Membrane Potential

To characterize how the photoinduced electron transfer process affects fluorescence lifetime, we compared the τ_{fl} of the voltage-sensitive dye VF2.1.Cl with its voltage-insensitive counterpart VF2.0.Cl (**Fig. 2.1B**). We recorded the τ_{fl} of bath-applied VF dyes in HEK293T cells using time-correlated single-photon counting (TCSPC) FLIM (**Fig. 2.1C-E**). VF2.1.Cl is localized to the plasma membrane and exhibits a biexponential τ_{fl} decay with decay constants of approximately 0.9 and 2.6 ns (**Fig. 2.2**). For all subsequent analysis of VF2.1.Cl lifetime, we refer to the weighted average τ_{fl} , which is approximately 1.6 ns in HEK293T cell membranes at rest. VF2.0.Cl (**Fig. 2.1B**), which lacks the aniline substitution and is therefore voltage-insensitive³⁰, shows a τ_{fl} of 3.5 ns in cell membranes, which is similar to the lifetime of an unsubstituted fluorescein³³ (**Table 2.2**) We also examined VoltageFluor lifetimes at a variety of dye loading concentrations to test for concentration-dependent changes in dye lifetime, which have been reported for fluorescein derivatives³⁴. Shortened VF lifetimes were observed at high dye

concentrations (**Fig. 2.3**); all subsequent VF-FLIM studies were conducted at dye concentrations low enough to avoid this concentration-dependent change in lifetime.

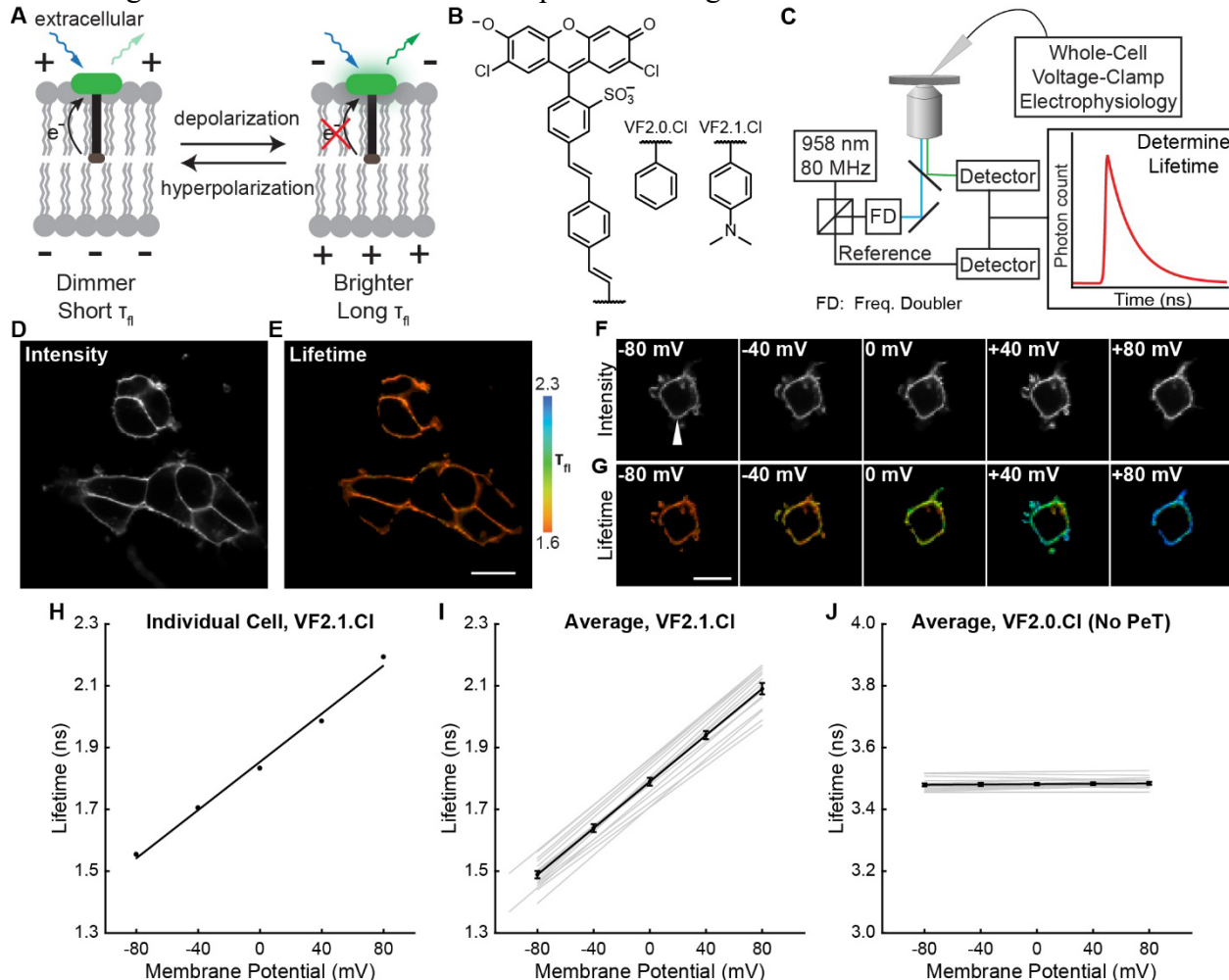


Figure 2.1 VoltageFluor FLIM linearly reports absolute membrane potential.

(A) Mechanism of VoltageFluor dyes, in which depolarization of the membrane potential attenuates the rate of photoinduced electron transfer. (B) Structures of the VF molecules used in this study. (C) Schematic of the TCSPC system used to measure fluorescence lifetime. Simultaneous electrophysiology was used to establish lifetime-voltage relationships. (D) Fluorescence intensity and (E) lifetime of HEK293T cells loaded with 100 nM VF2.1.Cl. (F) Intensity and (G) lifetime images of HEK293T cells voltage clamped at the indicated membrane potential. (H) Quantification of the single trial shown in (G), with a linear fit to the data. (I) Evaluation of VF2.1.Cl lifetime-voltage relationships in many individual HEK293T cells. Gray lines represent linear fits on individual cells. Black line is the average lifetime-voltage relationship across all cells (n=17). (J) VF2.0.Cl lifetime does not exhibit voltage-dependent changes. Gray lines represent linear fits on individual cells, and the black line is the average lifetime-voltage relationship across all cells (n=17). Scale bars represent 20 μ m. Error bars represent mean $\pm$ SEM.

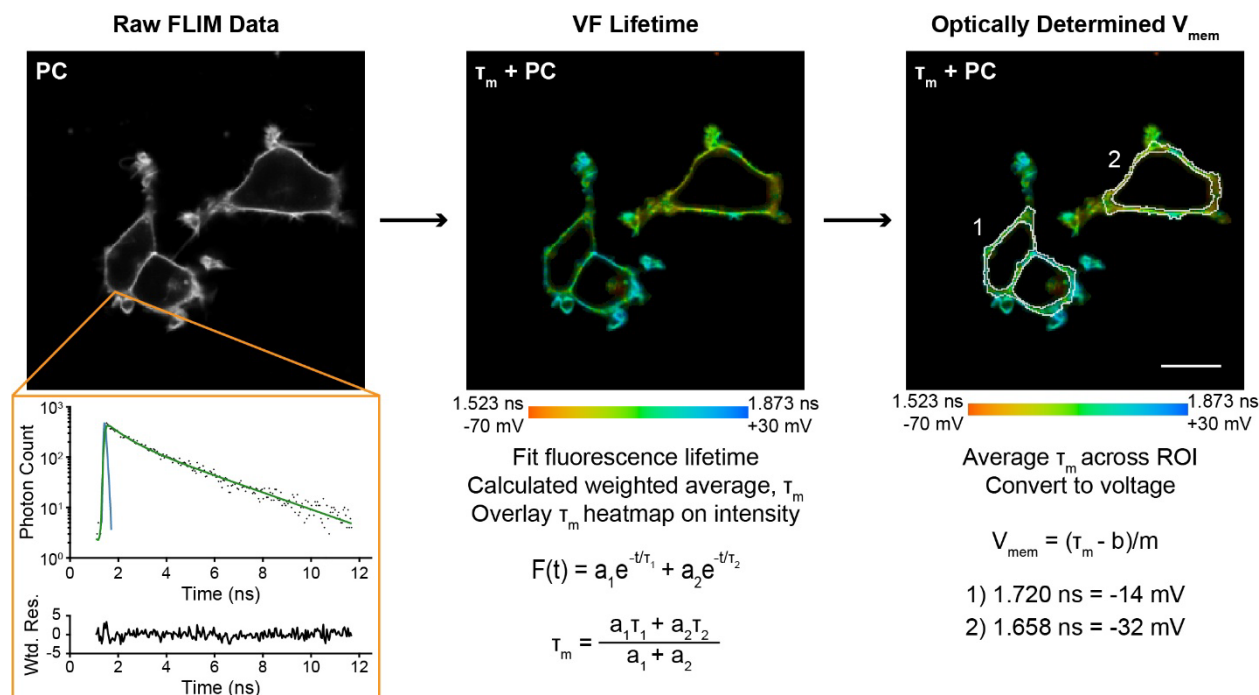


Figure 2.2 Overview of data processing to obtain membrane potential recordings from fluorescence lifetime.

Time-correlated photon data (black dots, first panel) collected at each pixel were fit to an exponential decay model (green) with iterative reconvolution of the instrument response function (IRF, blue). The two components of the fluorescence lifetimes were converted to a weighted average (middle panel). Cell membranes (white outlines) were identified, and τ_m was averaged within each of these regions of interest (ROIs, right panel). These lifetimes were then converted to voltage via a previously determined lifetime- V_{mem} standard curve with slope m and y-intercept b . Additional details of this process are provided in the **Methods**. Wtd. Res.: weighted residuals of the fit, τ_m : weighted average fluorescence lifetime, PC: photon count. $\tau_m + PC$ represents an overlay of the lifetime data (color heat map) onto the photon count image. Pixels that appear black in $\tau_m + PC$ images were below the required photon count threshold for fitting lifetime data; PC only images show photon counts without any such thresholds applied. Scale bar is 20 μm .

	% $\Delta F/F$	% $\Delta \tau/\tau$	Lifetime (ns)
Fluorescein	N/A	N/A	4.008 ± 0.009
Erythrosin B	N/A	N/A	0.083 ± 0.001
VF2.1.Cl	27	$22.4 \pm 0.4\%$	1.77 ± 0.02
VF2.0.Cl	0	$0.11 \pm 0.05\%$	3.482 ± 0.004

Table 2.2 Properties of lifetime standards and VoltageFluor dyes.

Fluorescein and erythrosin B standards were measured in drops of solution placed on a coverslip. For VF dyes, voltage sensitivities from intensity-based fluorescence imaging in HEK293T cells (% $\Delta F/F$, percent change in fluorescence intensity for a voltage step from -60 mV to +40 mV) are from previously published work³⁰. Lifetime data were obtained from voltage-clamp electrophysiology of HEK293T cells loaded with 100 nM VF. Lifetime listed here is the average 0 mV lifetime from the electrophysiology calibration. % $\Delta \tau/\tau$ is the percent change in lifetime

corresponding to a 100 mV step from -60 mV to +40 mV. Lifetime sample sizes: fluorescein 25, erythrosin B 25, VF2.1.Cl 17, VF2.0.Cl 17. For lifetime standards, each measurement was taken on a separate day. VF2.1.Cl data in HEK293T is duplicated in **Table 2.4**. Values are tabulated as mean $\pm$ SEM.

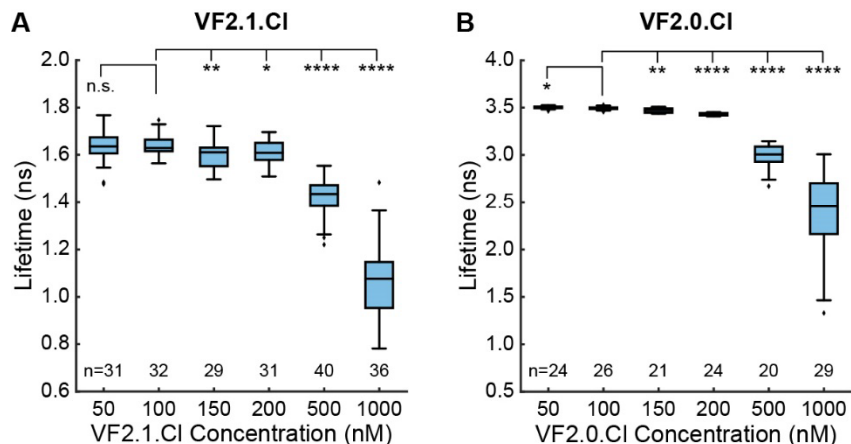


Figure 2.3 Concentration dependence of VoltageFluor lifetimes in HEK293T cells.

Changes in lifetime arising from addition of a range of concentrations of (A) VF2.1.Cl or (B) voltage-insensitive control VF2.0.Cl in HEK293T cells. Biexponential fit models were used for all VF2.1.Cl concentrations and 1 μ M VF2.0.Cl; a monoexponential model was used for all other VF2.0.Cl concentrations. Box plots represent the interquartile range, with whiskers and outliers determined with the Tukey method. Sample sizes indicate number of cell groups. Data were obtained over 2 to 4 different days from a total of 3 or 4 coverslips at each concentration. Asterisks indicate significant differences between the indicated concentration and the VF concentration used for electrophysiology experiments (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test).

To assess the voltage dependence of VoltageFluor τ_{fl} , we controlled the plasma membrane potential of HEK293T cells with whole-cell voltage-clamp electrophysiology while simultaneously measuring the τ_{fl} of VF2.1.Cl (**Figure 2.1C**). Single-cell recordings show a linear τ_{fl} response to applied voltage steps, and individual measurements deviate minimally from the linear fit (**Figure 2.1F-H**). VF2.1.Cl τ_{fl} is reproducible across different cells at the same resting membrane potential, allowing determination of V_{mem} from τ_{fl} images taken without concurrent electrophysiology (**Figure 2.1I**). Voltage-insensitive VF2.0.Cl shows no τ_{fl} change in response to voltage (**Figure 2.1J**, **Figure 2.4**), consistent with a τ_{fl} change in VF2.1.Cl arising from a voltage-dependent PeT process. In HEK293T cells, VF2.1.Cl exhibits a sensitivity of 3.50 ± 0.08 ps/mV and a 0 mV lifetime of 1.77 ± 0.02 ns, corresponding to a fractional change in τ_{fl} ($\Delta\tau/\tau$) of $22.4 \pm 0.4\%$ per 100 mV. These values are in good agreement with the 27% $\Delta F/F$ intensity change per 100 mV originally observed for VF2.1.Cl^{29,30}. Because $\% \Delta F/F$ is a fluorescence intensity-based metric, it cannot be used to measure absolute V_{mem} ; however, agreement between $\% \Delta F/F$ and $\% \Delta\tau/\tau$ is consistent with a PeT-based V_{mem} sensing mechanism in VFs. To estimate the voltage resolution of VF-FLIM, we analyzed the variability in successive measurements on the same cell (intra-cell resolution) and on different cells (inter-cell resolution, see **Methods**). We estimate that the resolution for tracking and quantifying voltage changes in a single HEK293T cell is 3.5 ± 0.4 mV (intra-cell resolution, average RMSD from each electrophysiological calibration, **Scheme 2.2**,

Methods), whereas the resolution for single-trial determination of a particular HEK293T cell's absolute V_{mem} is 19 mV (inter-cell resolution, RMSD of each calibration slope to the average calibration, **Scheme 2.2**) within a 15 second bandwidth.

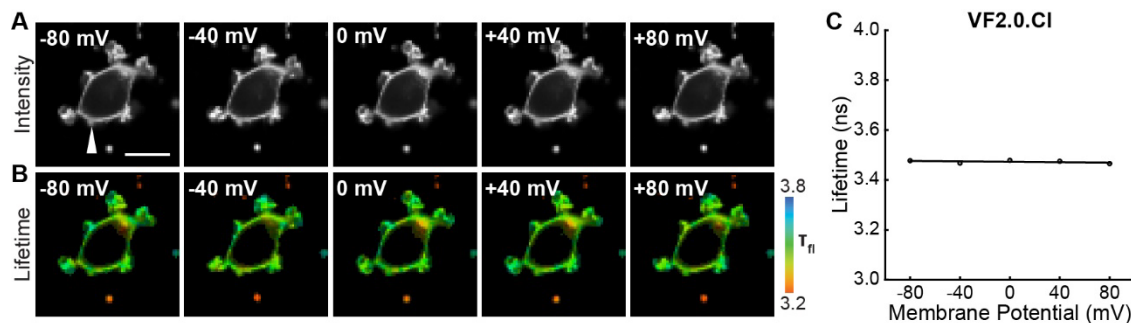


Figure 2.4 VF2.0.Cl lifetime does not depend on membrane potential.

(A) Photon count and (B) lifetime images of a single HEK293T cell loaded with 100 nM VF2.0.Cl, with the membrane potential held at the indicated value via whole-cell voltage clamp electrophysiology. White arrow indicates patch pipette. Scale bar is 20 μm . (C) Quantification of images shown in (B) for this individual cell. Black line is the line of best fit.

We compared the performance of VF-FLIM in HEK293T cells to that of two previously documented strategies for optical absolute V_{mem} determination. We first tested the voltage resolution of CAESR, the best previously reported GEVI for recording absolute V_{mem} with FLIM²⁷. Using simultaneous FLIM and voltage-clamp electrophysiology, we determined the relationship between τ_{fl} and V_{mem} for CAESR under 1 photon excitation (**Fig. 2.5**). We recorded a sensitivity of -1.2 ± 0.1 ps/mV and a 0 mV lifetime of 2.0 ± 0.2 ns, which corresponds to a $-6.1 \pm 0.8\%$ $\Delta\tau/\tau$ per 100 mV (mean $\pm$ SEM of 9 measurements), in agreement with the reported sensitivity of -0.9 ps/mV and 0 mV lifetime of 2.7 ns with 2 photon excitation²⁷. Relative to VF2.1.Cl, CAESR displays 3-fold lower sensitivity (-1.2 ps/mV vs 3.5 ps/mV in HEK293T cells) and 7-fold higher voltage-independent variability in lifetime (0.46 ns vs 0.07 ns, standard deviation of the 0 mV lifetime measurement). For CAESR in HEK293T cells, we calculate a voltage resolution of 33 ± 7 mV for quantifying voltage changes on an individual cell (intra-cell RMSD, compared to 3.5 mV for VF2.1.Cl, see **Methods**) and resolution of 370 mV for determination of a particular cell's absolute V_{mem} (inter-cell RMSD, compared to 19 mV for VF2.1.Cl).

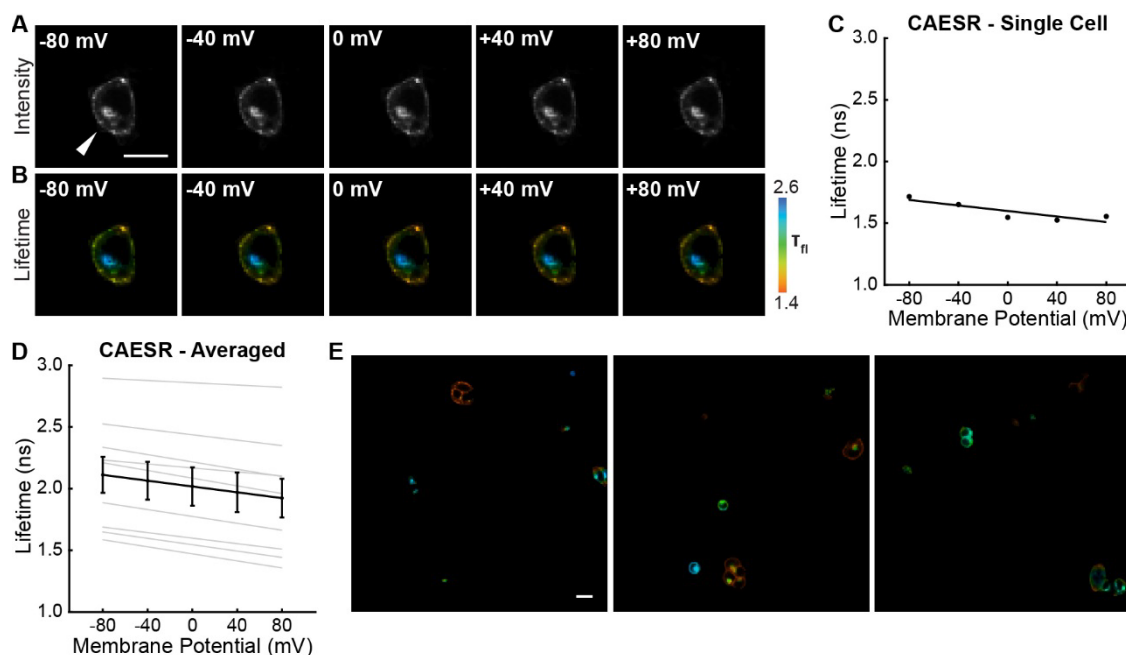


Figure 2.5 The GEVI CAESR shows variable lifetime-voltage relationships.

(A) Photon count and (B) lifetime images of a HEK293T cell expressing CAESR and held at the indicated V_{mem} with voltage-clamp electrophysiology. White arrow indicates voltage-clamped cell. (C) Lifetime- V_{mem} relationship from the cell in (B), based on a single fit from combined fluorescence decays of all pixels in the cell membrane at each potential (see **Methods**). Points indicate recordings at a given potential; solid line is line of best fit. (D) Evaluation of VF2.1.Cl lifetime-voltage relationships in many individual CAESR-expressing HEK293T cells. Gray lines represent linear fits on individual cells. Black line is the average fit across all cells ($n=9$). (E) Representative lifetime images of CAESR in HEK293T cells. Scale bars represent 20 μm .

We also measured the absolute voltage resolution of the ratio-based sensor di-8-ANEPPS, which reports membrane potential by the wavelength of its excitation and emission spectra¹⁴. Ratio-based imaging can be achieved by comparing the fluorescence emission at different excitation wavelengths¹⁶; here, we used the ratio, R , of the blue-excited emission to the green-excited emission (see **Methods**). Via simultaneous ratio imaging and whole cell voltage clamp electrophysiology, we record a sensitivity of 0.0039 ± 0.0004 R per mV, with a y-intercept (0 mV) R value of 1.8 ± 0.2 (**Fig. 2.6**; mean $\pm$ SEM of $n=16$ HEK293T cells). R depends on the excitation and emission conditions used but should be relatively reproducible on a given microscope rig. To compare R from our system with previous work, we normalized all R values to the R value at 0 mV for each cell. Using the above data, we obtain a sensitivity of 0.0022 ± 0.0002 normalized R per mV, with a 0 mV normalized R of 1.02 ± 0.02 , in good agreement with reported values (0.0015 normalized R per mV)¹⁶. For analysis of voltage resolution, we compare VF-FLIM to the non-normalized R , since normalization requires an electrode-based measurement for every recording and is thus not a truly optical strategy. From the non-normalized di-8-ANEPPS R , we obtain an intra-cell resolution (RMSD) of 18 ± 3 mV (5-fold less accurate than VF-FLIM) and an inter-cell resolution (RMSD) of 150 mV (8-fold less accurate than VF-FLIM). The sensitivities and resolutions of VF-FLIM, CAESR, and di-8-ANEPPS in HEK293T are tabulated in **Table 2.3**. Because cellular resting membrane potentials and voltage changes (e.g. action potentials) are on

the order of tens of millivolts, the resolution improvements achieved by VF-FLIM enable biologically relevant absolute V_{mem} recordings: impossible with previous approaches.

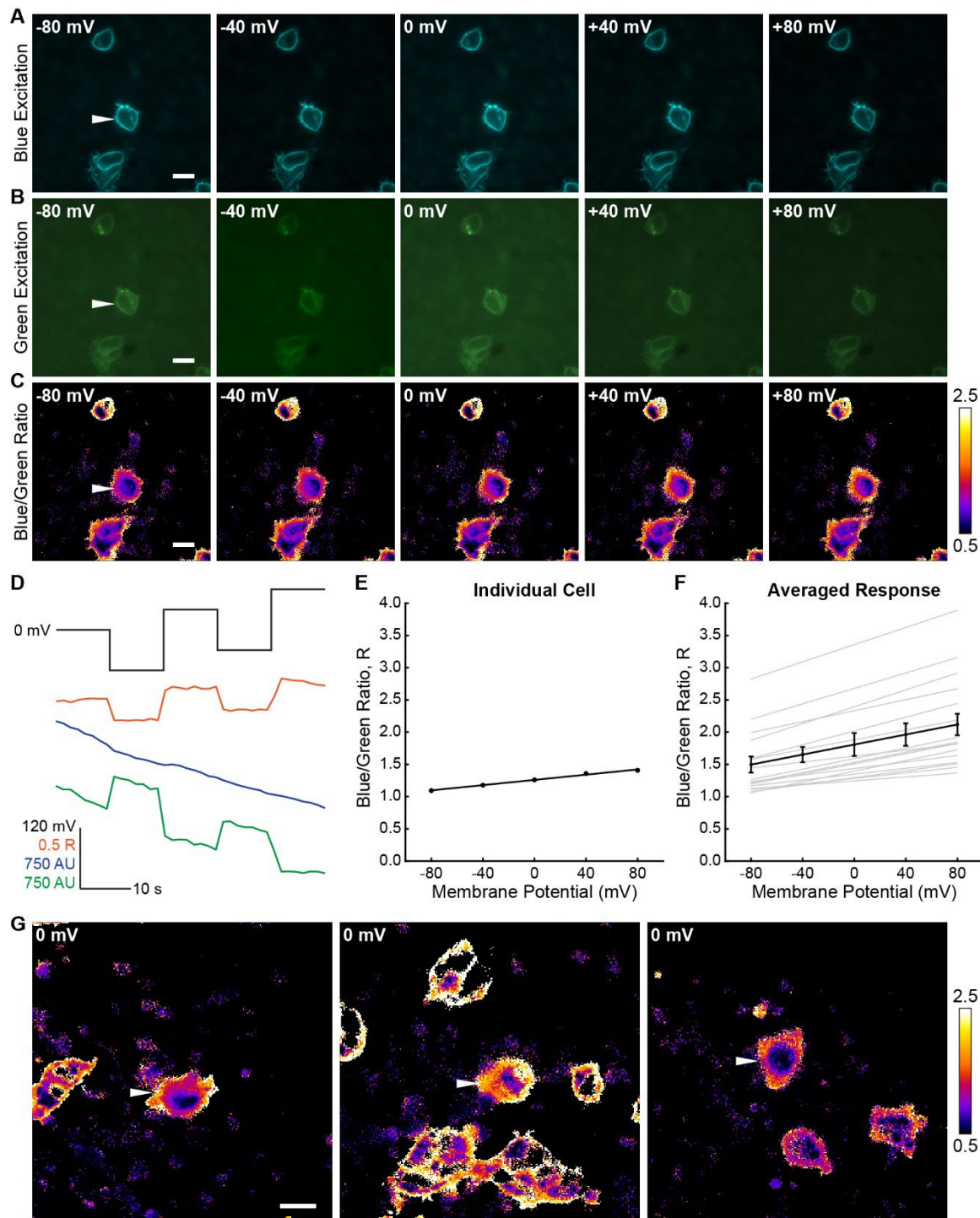


Figure 2.6 Ratiometric V_{mem} determinations with Di-8-ANEPPS in HEK293T cells.

Different fluorescence excitation wavelengths (blue and green) were used to generate ratiometric images; emission wavelengths were constant throughout the experiment. Details of epifluorescence ratiometric imaging and data processing are described in the **Methods**.

Simultaneous ratiometric imaging and whole cell patch clamp electrophysiology were used to determine the ratio between the blue and green excitation channels (blue/green ratio, R). Representative images of (A) blue-excited signal, (B) green-excited signal and (C) R of a HEK293T cell held at the indicated membrane potential with whole cell voltage clamp electrophysiology. White arrow indicates voltage clamped cell. Differences in background brightness between images are attributable to photobleaching of the probe; images were acquired in the order: 0, -80, +40, -40, +80 mV. (D) Time course of the voltage step protocol used for each cell (black line, top), along with a representative response in the fluorescence ratio (orange), blue excited fluorescence intensity (blue) and green excited fluorescence intensity (green). AU, arbitrary units. (E) R at various potentials for an individual HEK293T cell (black dots), along with an R- V_{mem} line of best fit (black line). Values shown are the average of all ratio images from a particular cell at a given potential, excluding the first and last ratio image taken for each V_{mem} value. Data shown in (D) and (E) are from the cell depicted in (A)-(C). (F) Aggregated lines of best fit (gray) for R- V_{mem} calibrations on multiple HEK293T cells. Average response is shown in black; ratios at each potential are depicted as mean $\pm$ SEM (n = 16 cells). (G) Additional representative R images of Di-8-ANEPPS in HEK293T cells. The cell indicated with the white arrow is held at 0 mV with electrophysiology; other cells are unperturbed. Images represent one frame at the given voltage and are not averaged. Regions of interest (ROIs) were drawn around the membrane only, avoiding both the internalized dye signal and the artificially high ratios just outside the cells (which appear as white edges) as much as possible. Scale bars represent 20 μm .

Strategy	Sensitivity	0 mV value	Intra-cell RMSD	Inter-cell RMSD
VF2.1.Cl fluorescence lifetime	3.50 ± 0.08 ps/mV	1.77 ± 0.02 ns	3.5 ± 0.4 mV	19 mV
CAESR fluorescence lifetime	-1.2 ± 0.1 ps/mV	2.0 ± 0.2 ns	33 ± 7 mV	370 mV
di-8-ANEPPS excitation ratio	0.0039 ± 0.0004 R/mV	1.8 ± 0.2	18 ± 3 mV	150 mV

Table 2.3 Comparison of optical approaches to absolute V_{mem} determination in HEK293T cells.

Data are compiled from **Fig. 2.1** (VF-FLIM, this work), **Fig. 2.5** (CAESR ²⁷), and **Fig. 2.6** (Di-8-ANEPPS ¹⁶). All data were obtained by simultaneous whole cell voltage clamp electrophysiology and optical recording in HEK293T (VF-FLIM n=17 cells, CAESR n=9, di-8-ANEPPS n=16). Calculation of intra and inter cell accuracies are performed via root-mean-square deviation (RMSD) and discussed in detail in the **Methods** (see **Resolution of VF-FLIM...**). Regions of interest were chosen at the plasma membrane in all cases. Di-8-ANEPPS data are presented as the ratio of signal obtained with blue excitation to signal obtained with green excitation (R, see **Methods**) and are not normalized to the 0 mV R.

Evaluation of VF-FLIM across Cell Lines and Culture Conditions

To test the generalizability of VF-FLIM, we determined $\tau_{\text{fl}}-V_{\text{mem}}$ calibrations in four additional commonly used cell lines: A431, CHO, MDA-MB-231, and MCF-7 (**Fig. 2.7**, **Fig. 2.8**,

Fig. 2.9). We observe a linear τ_{fl} response in all cell lines tested. The slope (voltage sensitivity) and y-intercept (0 mV lifetime) of the τ_{fl} - V_{mem} response varied slightly across cell lines, with average sensitivities of 3.1 to 3.7 ps/mV and average 0 mV lifetimes ranging from 1.74 to 1.87 ns. In all cell lines, we observed better voltage resolution for quantification of V_{mem} changes on a given cell versus comparisons of absolute V_{mem} between cells. Changes in voltage for a given cell could be quantified with resolutions at or better than 5 mV (intra-cell resolution, **Methods**). For absolute V_{mem} determination of a single cell, we observed voltage resolutions ranging from 10 to 23 mV (inter-cell resolution, 15 second acquisition time, **Table 2.4**). Statistically significant differences among the cell lines tested were observed for cellular τ_{fl} - V_{mem} calibrations in both the slope (One-way ANOVA with Welch's correction: $F(4, 23.07) = 18.12$, $p < 0.0001$) and average 0 mV lifetime (One-way ANOVA: $F(4, 67) = 14.43$, $p < 0.0001$). There were no statistically significant differences between A431, CHO, and HEK293T cells ($p > 0.05$, Games-Howell and Tukey-Kramer post hoc tests for the slope and 0 mV lifetime respectively). MDA-MB-231 and MCF-7 cells showed statistically significant variability from other cell lines in slope and/or 0 mV lifetime.

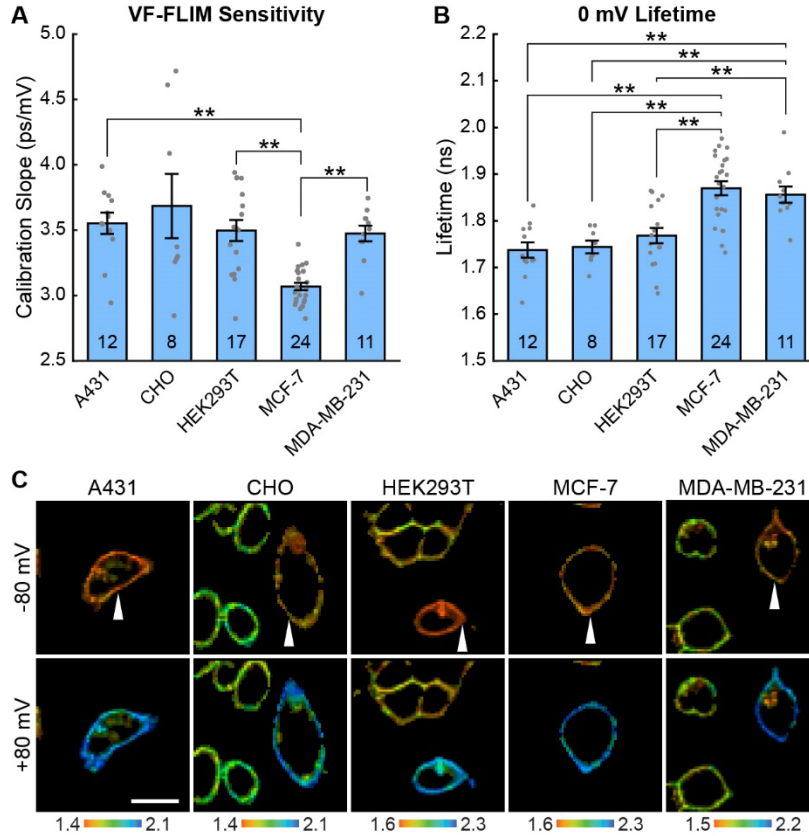


Figure 2.7 VF-FLIM is a general and portable method for optically determining membrane potential.

VF2.1.Cl lifetime-voltage relationships were determined with whole cell voltage clamp electrophysiology in five cell lines. (A) Slopes of the linear fits for single cell lifetime-voltage relationships, shown as mean $\pm$ S.E.M. Gray dots indicate results from individual cells. Statistically significant differences exist between groups (One-way ANOVA with Welch's correction: $F(4, 23.07) = 18.12$, $p < 0.0001$). Data were tested for normality (Shapiro-Wilk test, $p > 0.05$ for all cell lines) and homoscedasticity (Levene's test on the median, $F(4,67) = 5.07$, $p =$

0.0013). ** indicates $p < 0.01$; if significance is not indicated, $p > 0.05$ (Games-Howell post hoc test). (B) 0 mV reference point of linear fits for the lifetime-voltage relationship, shown as mean $\pm$ S.E.M. Gray dots indicate results from individual cells. Significant differences exist between groups (One-way ANOVA: $F(4, 67) = 14.43$, $p < 0.0001$). Data were tested for normality (Shapiro-Wilk test, $p > 0.05$ for all cell lines) and homoscedasticity (Levene's test on the median, $F(4,67) = 1.29$, $p = 0.28$). ** indicates $p < 0.01$; if significance is not indicated, $p > 0.05$ (Tukey-Kramer post hoc test). (C) Representative lifetime-intensity overlay images for each cell line with the indicated cells (white arrow) held at -80 mV (top) or +80 mV (bottom). Lifetime scales are in ns. Scale bar is 20 μ m.

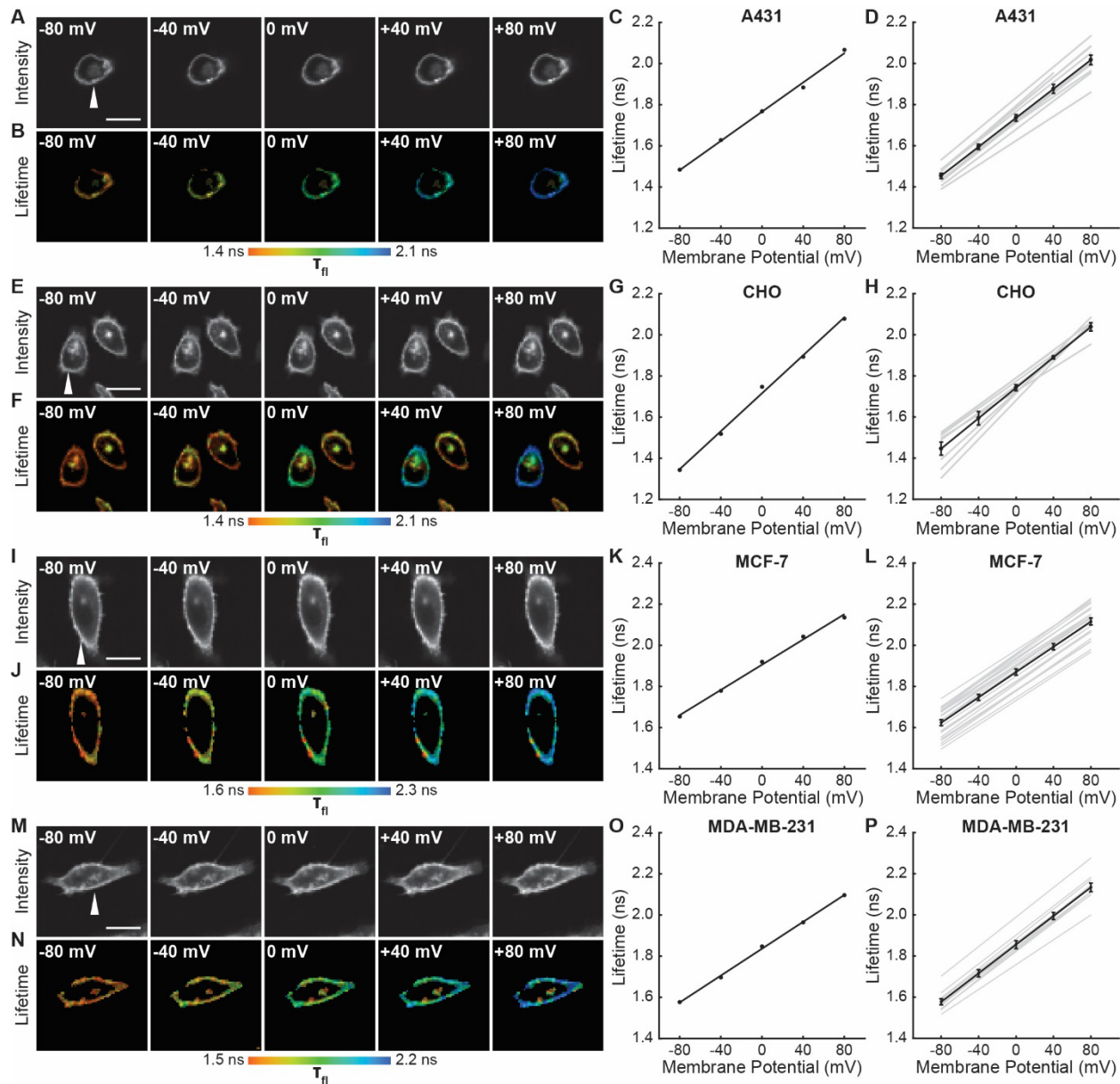


Figure 2.8 VoltageFluor lifetime reports voltage in diverse cell lines.

(A) Representative photon count and (B) lifetime images of a VF2.1.Cl in A431 cells with V_{mem} held at the indicated value with voltage-clamp electrophysiology. A431 cells were not serum

starved for these experiments. (C) Quantification of the images in (B), with the line of best fit for this single trial. (D) Lines of best fit for the lifetime- V_{mem} relationships of 12 A431 cells (gray lines). Average lifetime at each potential is shown as mean $\pm$ SEM, with the average line of best fit in black. (E)-(H) Lifetime- V_{mem} standard curve determination in CHO cells (n=8). (I)-(L) Lifetime- V_{mem} standard curve determination in MCF-7 cells (n=24). (M)-(P) Lifetime- V_{mem} standard curve determination in MDA-MB-231 cells (n=11). VF2.1.Cl concentration was 100 nM in all cases. White arrows indicates the voltage-clamped cell. Scale bars are 20 μm .

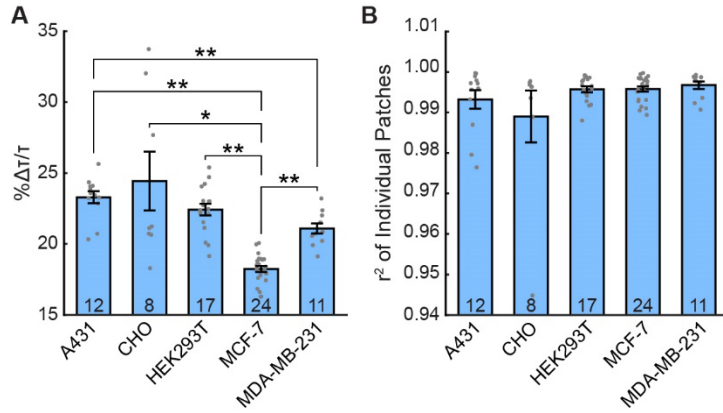


Figure 2.9 Additional parameters of linear lifetime-voltage standard curves.

(A) Percent change in VF2.1.Cl lifetime per 100 mV change in voltage, relative to the lifetime at -60 mV. Significant differences exist between cell lines (one-way ANOVA with Welch's correction, $F(4,24.08) = 41.75$, $p < 0.0001$). Data were tested for normality (Shapiro-Wilk test, $p > 0.05$ for all cell lines) and homoscedasticity (Levene's test on the median, $F(4,67) = 5.74$, $p = 0.00049$). Asterisks indicate statistically significant differences (* $p < 0.05$, ** $p < 0.01$, Games-Howell post hoc test). (B) Correlation coefficients (r^2) for the lines of best fit of VF2.1.Cl lifetime versus membrane potential. No significant differences exist in r^2 values between cell lines (Kruskal-Wallis test, $H = 3.20$, 4 degrees of freedom, $p = 0.53$). Data were tested for normality (Shapiro-Wilk test, $p < 0.05$ for 4 of 5 cell lines) and homoscedasticity (Levene's test on the median, $F(4,67) = 1.55$, $p = 0.20$). In both (A) and (B), data are shown as mean $\pm$ S.E.M., with gray dots indicating values from individual patches.

Cell Line	Slope (ps/mV)	0 mV lifetime (ns)	% $\Delta\tau/\tau$	RMSD, intra-cell (mV)	RMSD, inter-cell (mV)
A431	3.55 ± 0.08	1.74 ± 0.02	$23.3 \pm 0.4\%$	3.8 ± 0.7	15
CHO	3.68 ± 0.25	1.74 ± 0.01	$24 \pm 2\%$	5 ± 1	10
HEK293T	3.50 ± 0.08	1.77 ± 0.02	$22.4 \pm 0.4\%$	3.5 ± 0.4	19
MCF-7	3.07 ± 0.03	1.87 ± 0.01	$18.2 \pm 0.2\%$	3.4 ± 0.3	23
MDA-MB-231	3.47 ± 0.06	1.86 ± 0.02	$21.1 \pm 0.4\%$	3.0 ± 0.4	16

Table 2.4 Lifetime- V_{mem} standard curves for VF2.1.Cl lifetime in various cell lines.

Whole-cell voltage-clamp electrophysiology was used to determine the relationship between VF2.1.Cl lifetime and membrane potential in five different cell lines. Parameters of this linear model are listed above. The % $\Delta\tau/\tau$ is the percent change in the lifetime observed for a voltage step from -60 mV to +40 mV. The intra-cell RMSD represents the accuracy for quantifying voltage

changes in a particular cell (see **Methods**). The inter-cell RMSD represents the expected variability in single-trial absolute V_{mem} determinations. Sample sizes: A431 12, CHO 8, HEK293T 17, MCF-7 24, MDA-MB-231 11. All values are tabulated as mean $\pm$ SEM.

To verify that VF-FLIM was robust in groups of cells in addition to the isolated, single cells generally used for patch clamp electrophysiology, we determined lifetime-voltage relationships for small groups of A431 cells (**Fig. 2.10**). We found that calibrations made in small groups of cells are nearly identical to those obtained on individual cells, indicating that VF-FLIM only needs to be calibrated once for a given type of cell. For pairs or groups of three cells we recorded a sensitivity of 3.3 ± 0.2 ps/mV and a 0 mV lifetime of 1.78 ± 0.02 ns (mean $\pm$ SEM of 7 cells (5 pairs and 2 groups of 3); values are for the entire group, not just the cell in contact with the electrode), which is similar to the sensitivity of 3.55 ± 0.08 ps/mV and 0 mV lifetime of 1.74 ± 0.02 ns we observe in single A431 cells. The slight reduction in sensitivity seen in cell groups is likely attributable to space clamp error, which prevents complete voltage clamp of the cell group^{35,36}. Indeed, when we analyzed only the most responsive cell in the group (in contact with the electrode), we obtained a slope of 3.7 ± 0.1 ps/mV and 0 mV lifetime of 1.79 ± 0.02 ns, in good agreement with the single cell data. The space clamp error can be clearly visualized in **Fig. 2.10E**, where one cell in the group of 3 responded much less to the voltage command.

To test whether VF-FLIM is also extensible to cells maintained with different culture conditions, we recorded lifetime- V_{mem} relationship in serum-starved A431 cells (**Fig. 2.10F-K**), obtaining an average sensitivity of 3.6 ± 0.1 ps/mV and a 0 mV lifetime of 1.76 ± 0.01 ns ($n=7$; 2 single cells, 2 pairs, 3 groups of 3 cells; values are average lifetime across the whole cell group), in excellent agreement with the values obtained for non-serum starved cells. We also tested for concentration-dependent changes in VF lifetime in all five cell lines and in serum starvation conditions. Similar to VF2.1.Cl lifetime in HEK293T cells (**Fig. 2.3**), we observed shortening of VF2.1.Cl lifetimes beginning between 200 and 500 nM dye in all cases (**Fig. 2.11**). All subsequent experiments were carried out at VF2.1.Cl concentrations well below the regime where VF concentration-dependent lifetime changes were observed.

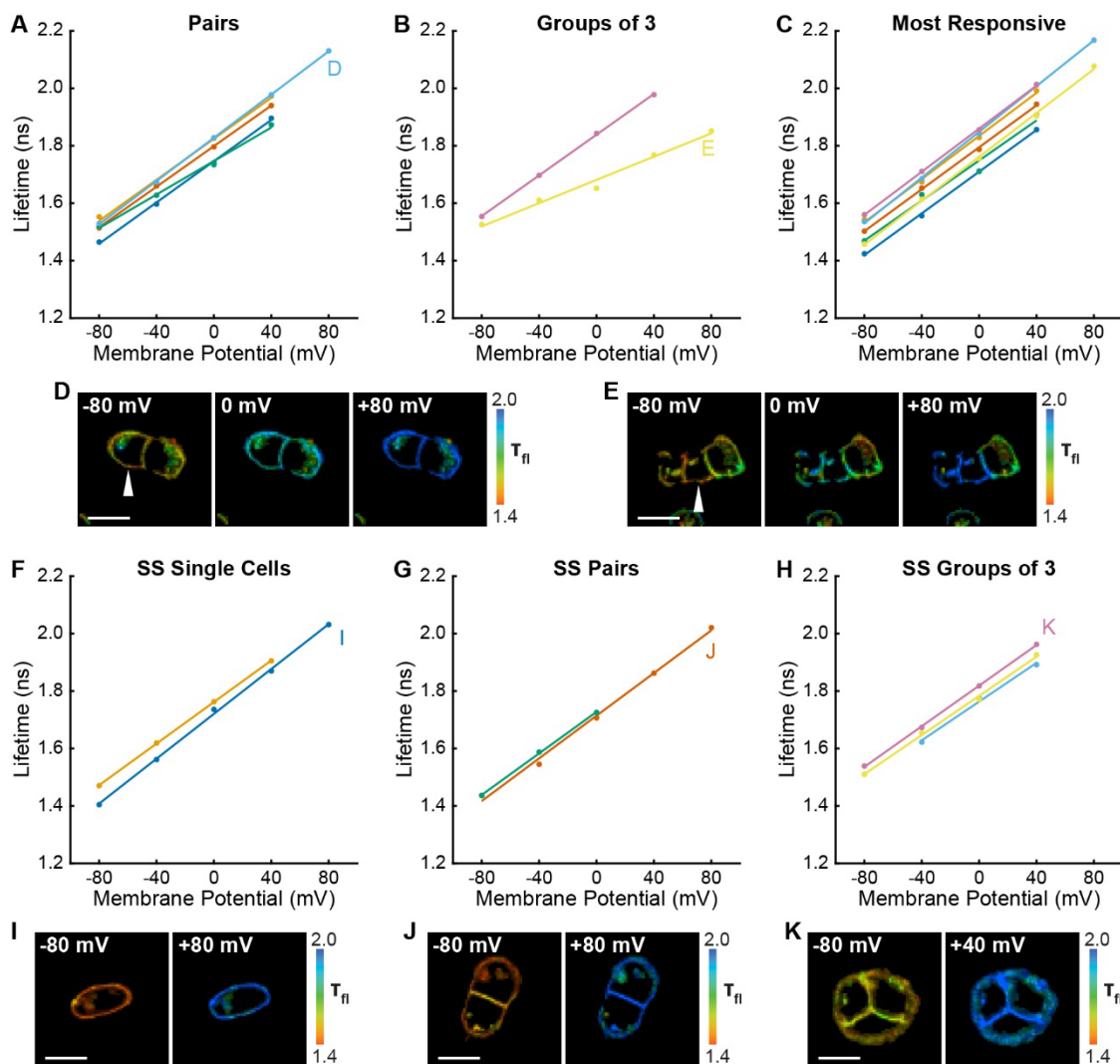


Fig. 2.10 Relationship between lifetime and membrane potential extends to groups of cells and across culture conditions.

Electrophysiological calibration of lifetime was performed on small groups of A431 cells and on serum starved (SS) A431 cells to verify that the V_{mem} -lifetime standard curves for a given cell line are generalizable across many cellular growth conditions. For all graphs, each line represents a group of cells. Letters on the graphs indicate the subfigure where images from that recording are shown. (A) Lifetime-voltage relationships in cell pairs, in which only one cell was directly controlled with voltage-clamp electrophysiology. (B) Lifetime-voltage relationships in groups of three cells, in which only one cell was directly controlled with voltage-clamp electrophysiology. (C) Lifetime for the most responsive cell from pairs and groups of three in (A) and (B). Line color codes are maintained from (A) and (B). (D, E) Representative lifetime images from (A) and (B) respectively. White arrow indicates cell directly controlled with electrophysiology. (F) Lifetime-voltage relationship in SS single cells, (G) pairs, and (H) groups of three cells. (I)-(K) Representative images from (F)-(H). Scale bars are 20 μm .

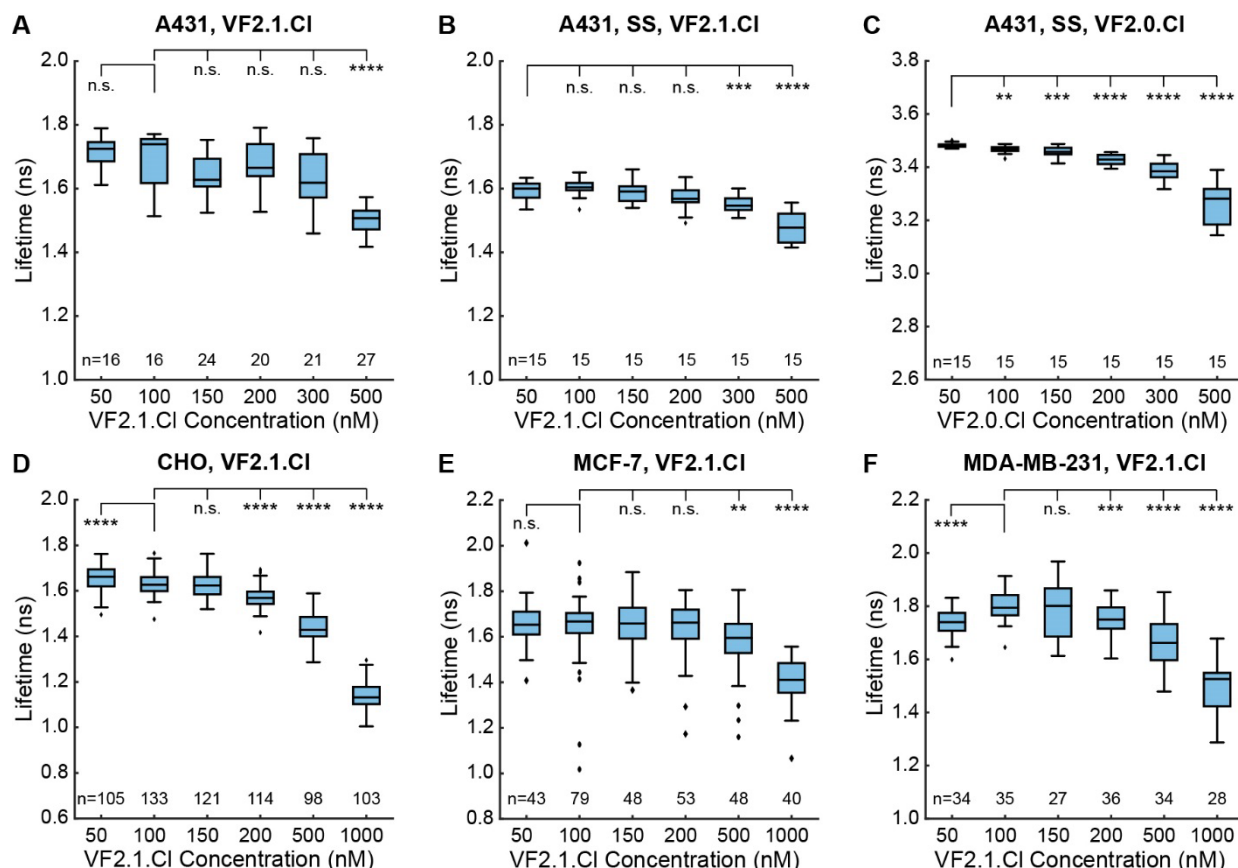


Figure 2.11 Concentration dependence of VoltageFluor lifetime in four cell lines.

A431 cells were analyzed with VF2.1.Cl both in (A) full serum and (B) serum-starved conditions. (C) VF2.0.Cl in serum-starved A431 cells. (D) VF2.1.Cl in CHO cells. (E) VF2.1.Cl in MCF-7 cells. (F) VF2.1.Cl in MDA-MB-231 cells. All VF2.1.Cl data were fit with a biexponential model, and all VF2.0.Cl data were fit with a monoexponential model. Box plots represent the interquartile range, with whiskers and outliers determined with the Tukey method. Sample sizes indicate number of cell groups. Data were acquired over 2 to 4 different days from a total of 3 or 4 coverslips at each concentration. Asterisks indicate significant differences between the indicated concentration and the VF concentration selected for additional experiments (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test).

Optical Determination of Resting Membrane Potential Distributions

The throughput of VF-FLIM enables cataloging of resting membrane potentials of thousands of cells in only a few hours of the experimenter's time. We optically recorded resting membrane potential distributions for A431, CHO, HEK293T, MCF-7, and MDA-MB-231 cells using VF-FLIM (Fig. 2.12, Fig. 2.13, Fig. 2.14). We report resting membrane potentials by cell group (Methods, Fig. 2.2) because adjacent cells in these cultures are electrically coupled to some degree via gap junctions³⁷. Each group of cells represents an independent sample for V_{mem} . In addition, the fluorescent signal originating from membranes of adjacent cells cannot be separated with a conventional optical microscope, so assignment of a region of membrane connecting multiple cells would be arbitrary. VF-FLIM images (Fig. 2.12, Fig. 2.13, Fig. 2.14) contain spatially resolved voltage information, but caution should be employed in interpreting pixel to

pixel differences in lifetime. Because VF-FLIM was calibrated here using the average plasma membrane τ_{fl} for each cell, optical V_{mem} should be interpreted per cell or cell group.

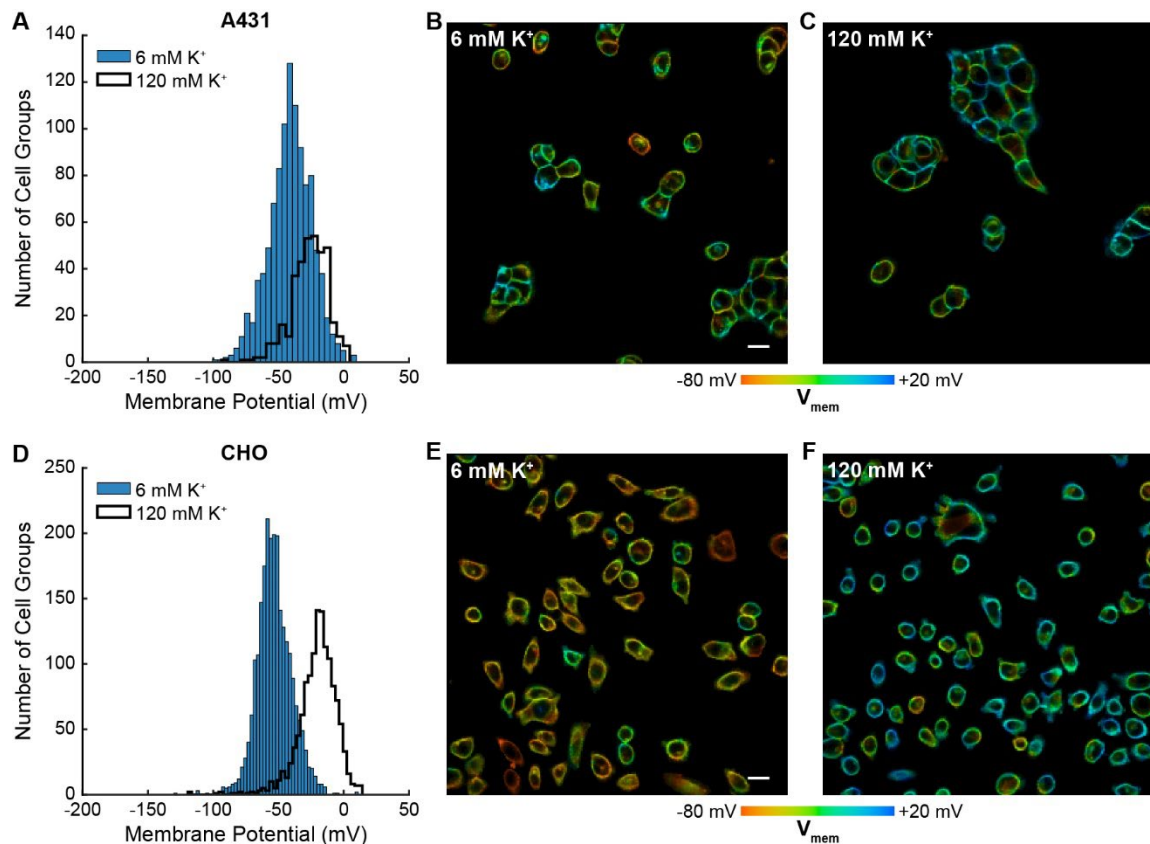


Figure 2.12 Rapid optical profiling of V_{mem} at rest and in high extracellular K^+ .

Fluorescence lifetime images of cells incubated with 100 nM VF2.1.Cl were used to determine V_{mem} from previously performed electrophysiological calibration (Fig. 2). (A) Histograms of V_{mem} values recorded in A431 cells incubated with 6 mM extracellular K^+ (commercial HBSS, $n=1056$) or 120 mM K^+ (high K^+ HBSS, $n=368$). (B) Representative lifetime image of A431 cells in 6 mM extracellular K^+ . (C) Representative lifetime image of A431 cells in 120 mM extracellular K^+ . (D) Histograms of V_{mem} values observed in CHO cells under normal ($n=2410$) and high K^+ ($n=1310$) conditions. Representative lifetime image of CHO cells in (E) 6 mM and (F) 120 mM extracellular K^+ . Histogram bin sizes were determined by the Freedman-Diaconis rule. Intensities in the lifetime-intensity overlay images are not scaled to each other. Scale bars, 20 μm .

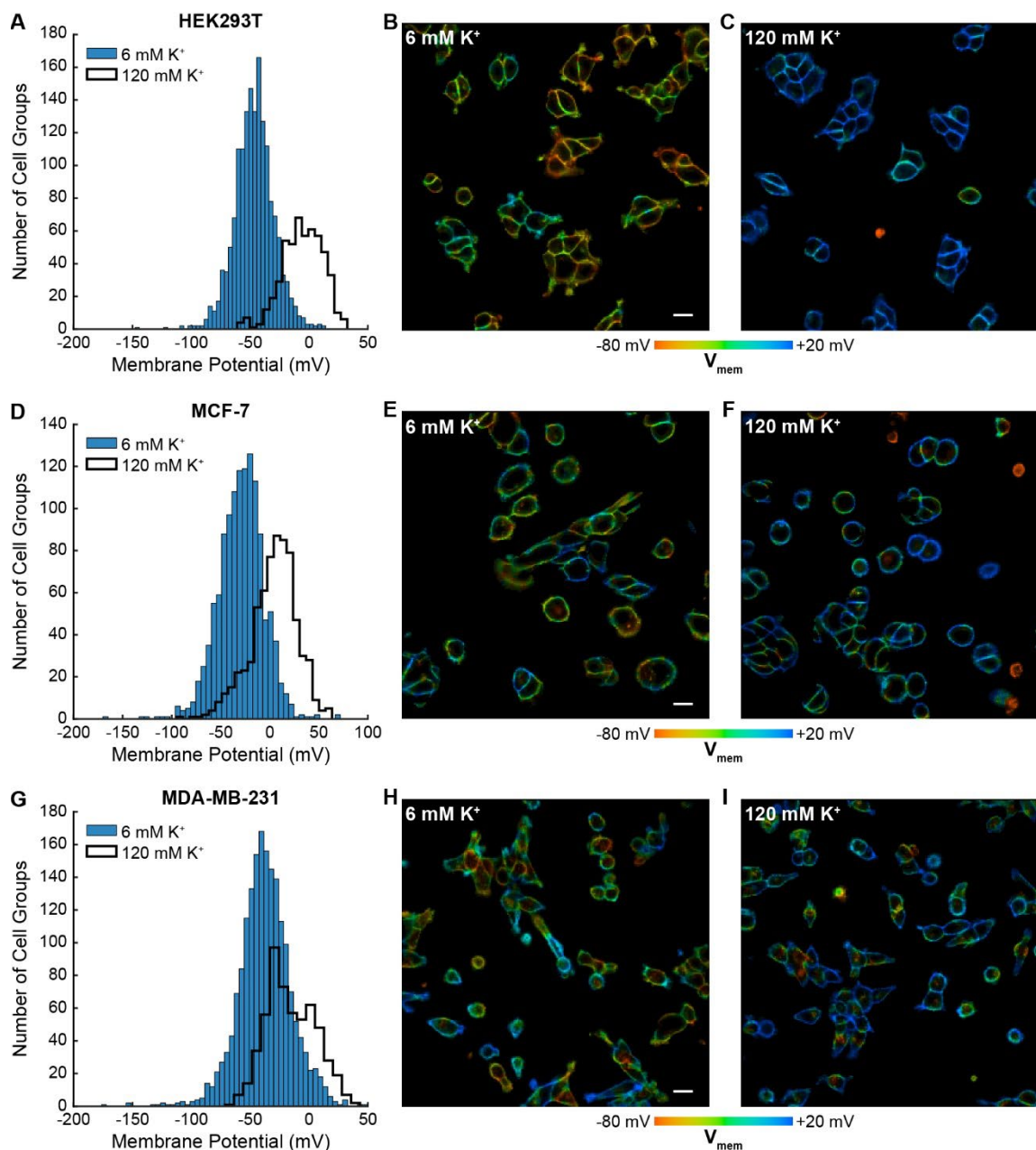


Figure 2.13 Optically recorded V_{mem} distributions in HEK293T, MCF-7 and MDA-MB-231 cells.

Fluorescence lifetime images of cells incubated with 100 nM VF2.1.Cl were used to determine V_{mem} from previously performed electrophysiological calibration (**Fig. 2**). (**A**) Histograms of V_{mem} values recorded in HEK293T cells incubated with 6 mM extracellular K^+ (commercial HBSS, $n=1613$) or 120 mM K^+ (high K^+ HBSS, $n=520$). (**B**) Representative lifetime image of HEK293T cells with 6 mM extracellular K^+ . (**C**) Representative lifetime image of HEK293T cells in 120 mM extracellular K^+ . (**D**) Histograms of V_{mem} values observed in MCF-7 cells under normal ($n=1259$) and high K^+ ($n=681$) conditions. Representative lifetime images of MCF-7 cells in (**E**) 6 mM and (**F**) 120 mM extracellular K^+ . (**G**) Histograms of V_{mem} values observed in MDA-MB-231 cells under normal ($n=1840$) and high K^+ ($n=558$) conditions. Representative lifetime images of MDA-MB-231 cells in (**H**) 6 mM and (**I**) 120 mM extracellular K^+ . Histogram bin sizes were determined

by the Freedman-Diaconis rule. Intensities in the lifetime-intensity overlay images are not scaled to each other. Scale bars, 20 μm .

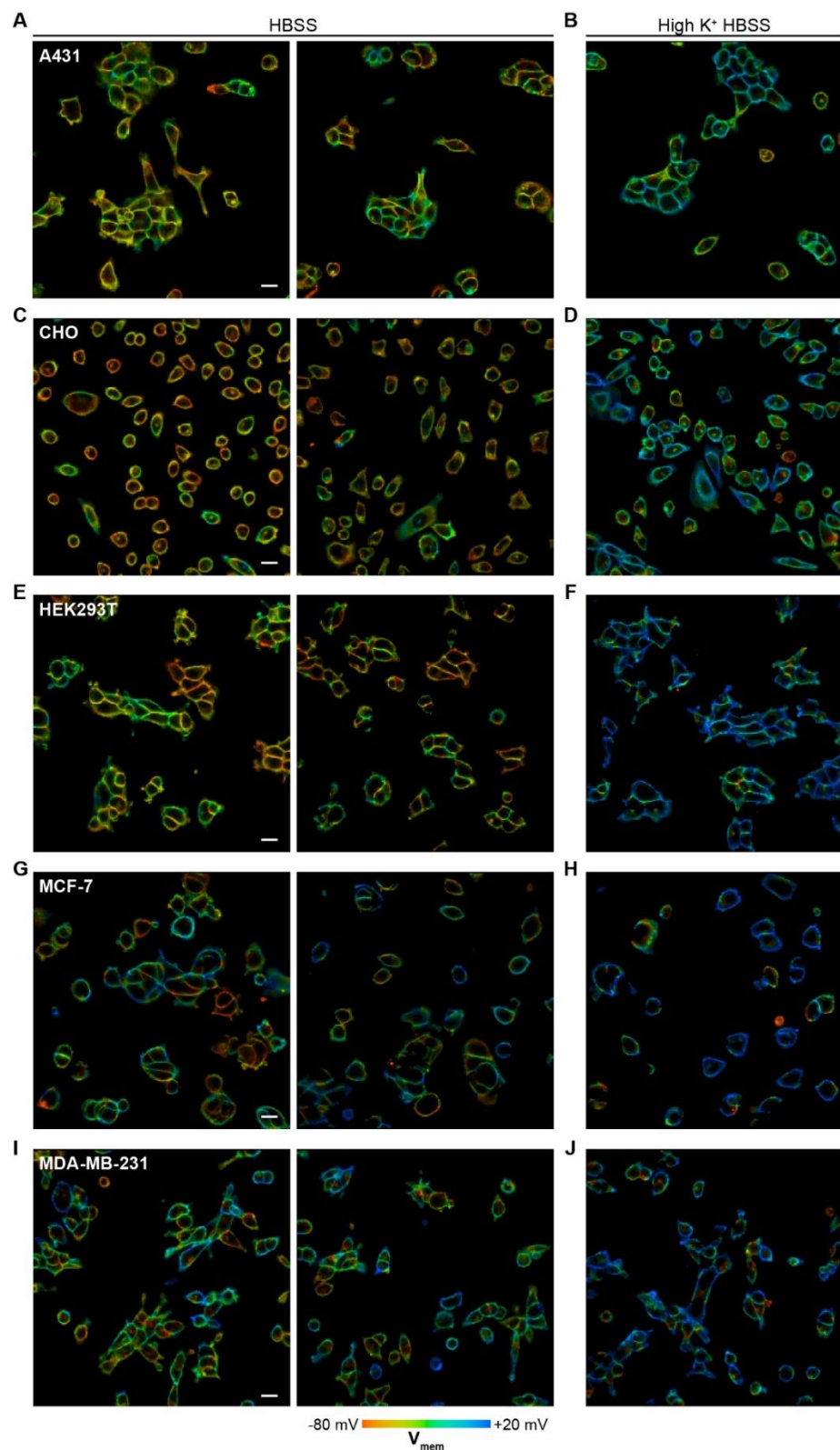


Figure 2.14 Representative images of cultured cell resting membrane potential. Representative VF-FLIM images of cells in standard imaging buffer (HBSS, 6 mM extracellular K^+) and high K^+ imaging buffer (high K^+ HBSS, 120 mM extracellular K^+). Membrane potential was calculated per cell group; analyses of pixel by pixel differences in lifetime fall beyond the resolution limit of the VF-FLIM calibrations in this work. Images depict A431 cells in (A) HBSS and (B) high K^+ HBSS; CHO cells in (C) HBSS and (D) high K^+ HBSS; HEK293T cells in (E) HBSS and (F) high K^+ HBSS; MCF-7 cells in (G) HBSS and (H) high K^+ HBSS, and MDA-MB-231 cells in (I) HBSS and (J) high K^+ HBSS.

Mean resting membrane potentials recorded by VF-FLIM range from -53 to -29 mV, depending on the cell line. These average V_{mem} values fall within the range reported in the literature for all of the cell lines we measured (**Table 2.5**). We also recorded resting membrane potentials in a high K^+ buffer (120 mM K^+ , “high K^+ HBSS”), where we observed a depolarization of 15 to 41 mV, bringing the mean V_{mem} up to -26 mV to +4 mV, again depending on the cell line. Although 120 mM extracellular K^+ should be strongly depolarizing, it will not necessarily produce a membrane potential of 0 mV. Because few literature reports of electrophysiological measurements in 120 mM K^+ exist as a point of comparison, we obtained a rough estimate of V_{mem} in 6 mM extracellular K^+ and 120 mM extracellular K^+ using the Goldman-Hodgkin-Katz (GHK) equation³⁸. Under our imaging conditions and with a broad range of possible ion permeabilities and intracellular ion concentrations, the GHK equation allows V_{mem} ranging from -91 to -27 mV in 6 mM extracellular K^+ and -25 to +2 mV in 120 mM extracellular K^+ (see **Methods**). Recorded VF-FLIM values fall well within this allowed range. Notably, although the GHK equation can determine ranges of reasonable V_{mem} values, GHK-based V_{mem} results are approximate at best because of the difficulty in obtaining accurate values of permeabilities and intracellular ion concentrations for specific cell lines. Direct measurement of V_{mem} , rather than theoretical calculation, is required to obtain accurate values.

	VF-FLIM		Patch-clamp electrophysiology	
Cell Line	Resting V_{mem} (mean $\pm$ SEM)	High K^+ V_{mem} (mean $\pm$ SEM)	Compiled reported average or median V_{mem} in cells at rest	Mean of ephys. values
A431	-41 ± 5	-26 ± 5	-64 ± 1 (mean $\pm$ SEM) ⁴²	-64
CHO	-53 ± 4	-20 ± 4	-21 ± 2 (mean $\pm$ SEM, 4 cells) ⁸¹ -31 ± 2.6 (mean $\pm$ SEM) ⁸² -35 (ranging -10 to -65 mV) ⁸³	-30
HEK293T	-47 ± 5	-6 ± 5	-45 (ranging -40 to -50 mV) ⁸⁴ -52 ± 1 (mean $\pm$ SEM) ⁸⁵ -35 ± 2 (mean $\pm$ SEM) ⁸⁶	-44
MCF-7	-29 ± 5	4 ± 5	-23 ± 1 (median $\pm$ SE of median) ⁵⁴ -36 ± 5 (mean $\pm$ SEM) ⁸⁷ -41 ± 20 (mean $\pm$ SD) ⁵³ -42 (no error given) ⁸⁸ -42 ± 5 (mean $\pm$ SEM) ⁸⁹	-36
MDA-MB-231	-38 ± 5	-15 ± 5	-19 ± 3 (mean $\pm$ SEM) ⁹⁰ -26 ± 8 (mean $\pm$ SEM) ⁹¹ -39 ± 5 (mean $\pm$ SEM) ⁸⁷	-28

Table 2.5 V_{mem} measurements made with VF-FLIM agree with previously reported values. Comparison of optically-determined resting membrane potential values (in millivolts) and previously reported values. This table summarizes data presented in **Fig. 2.12** and **Fig 2.13**. Optically determined membrane potentials were calculated from lifetime- V_{mem} standard curves (**Table 2.4**). For tabulated literature values, measures of error and central tendency were used from the original publication. In some cases, none were given or only ranges were discussed. The mean of the reported ephys values is the mean of the values listed here. Sample sizes for resting and elevated K^+ , respectively: A431 1056, 368; CHO 2410, 1310; HEK293T 1613, 520; MCF-7 1259, 681; MDA-MB-231 1840, 558.

Membrane potential dynamics in epidermal growth factor signaling

We thought VF-FLIM was a promising method for elucidating the roles of membrane potential in non-excitable cell signaling. Specifically, we wondered whether VF-FLIM might be well-suited to dissect conflicting reports surrounding changes in membrane potential during EGF/EGF receptor (EGFR)-mediated signaling. Receptor tyrosine kinase (RTK)-mediated signaling is a canonical signaling paradigm for eukaryotic cells, transducing extracellular signals into changes in cellular state. Although the involvement of second messengers like Ca^{2+} , cyclic nucleotides, and lipids are well characterized, membrane potential dynamics and their associated roles in non-excitable cell signaling remain less well-defined. In particular, the activation of EGFR

via EGF has variously been reported to be depolarizing³⁹, hyperpolarizing⁴⁰, or electrically silent^{41,42}.

We find that treatment of A431 cells with EGF results in a 15 mV hyperpolarization within 60-90 seconds in approximately 80% of cells (**Fig. 2.15A-C, Fig. 2.16, Fig. 2.17**), followed by a slow return to baseline within 15 minutes (**Fig. 2.15D-F, Fig. 2.18**), 30 second acquisitions). The voltage response to EGF is dose-dependent, with an EC_{50} of 90 ng/mL (14 nM) (**Fig. 2.19**). Vehicle-treated cells show very little τ_{fl} change (**Fig. 2.15A-F**). Identical experiments with voltage-insensitive VF2.0.Cl (**Fig. 2.15G-H, Fig. 2.16, 2.18, Fig. 2.20**) reveal little change in τ_{fl} upon EGF treatment, indicating the drop in τ_{fl} arises from membrane hyperpolarization. We observe the greatest hyperpolarization 1 to 3 minutes after treatment with EGF, which is abolished by inhibition of EGFR and ErbB2 tyrosine kinase activity with the covalent inhibitor canertinib (**Fig. 2.15I-J, Fig. 2.21**). Blockade of the EGFR kinase domain with gefitinib, a non-covalent inhibitor of EGFR, also results in a substantial decrease in the EGF-evoked hyperpolarization (**Fig. 2.15I-J, Fig. 2.21**). Together, these results indicate that A431 cells exhibit an EGF-induced hyperpolarization, which depends on the kinase activity of EGFR and persists on the timescale of minutes.

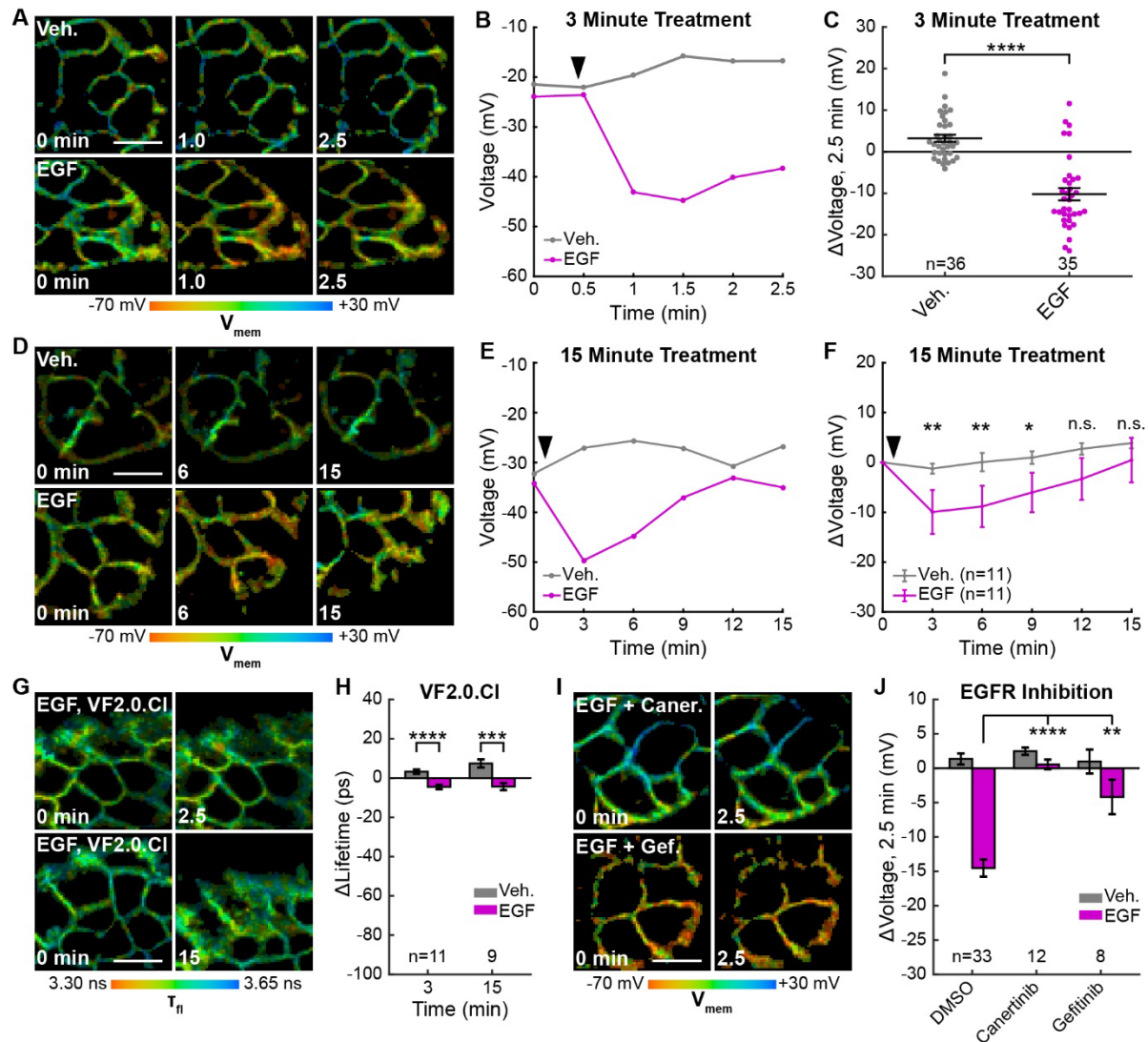


Figure 2.15 EGFR-mediated receptor tyrosine kinase activity produces a transient hyperpolarization in A431 cells.

(A) Representative VF-FLIM time series of A431 cells treated with imaging buffer vehicle (top) or 500 ng/mL EGF (80 nM, bottom). (B) Quantification of images in (A), with Vehicle (Veh.) / EGF added at black arrow. (C) Aggregated responses for various trials of cells treated with vehicle or EGF. (D) Lifetime images of longer-term effects of vehicle (top) or EGF (bottom) treatment. (E) Quantification of images in (D). (F) Average response of cells over the longer time course. (G) Images of VF2.0.Cl (voltage insensitive) lifetime before and after EGF treatment. No τ_{fl} change is observed 2.5 (top) or 15 minutes (bottom) following EGF treatment. (H) Average VF2.0.Cl lifetime changes following EGF treatment. VF2.0.Cl graphs and images are scaled across the same lifetime range (350 ps) as VF2.1.Cl plots and images. The small drift observed would correspond to 2-4 mV of voltage change in VF2.1.Cl lifetime. (I) Lifetime images of A431 cells before and after EGF addition, with 500 nM canertinib (top) or 10 μ M gefitinib (bottom). (J) Voltage changes 2.5 minutes after EGF addition in cells treated with DMSO (vehicle control) or an EGFR inhibitor. Scale bars are 20 μ m. (C,F,H): Asterisks indicate significant differences between vehicle and EGF at that time point. (J): Asterisks reflect significant differences between EGF-induced voltage

responses with DMSO vehicle or an EGFR inhibitor (n.s. $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, two-tailed, unpaired, unequal variances t-test).

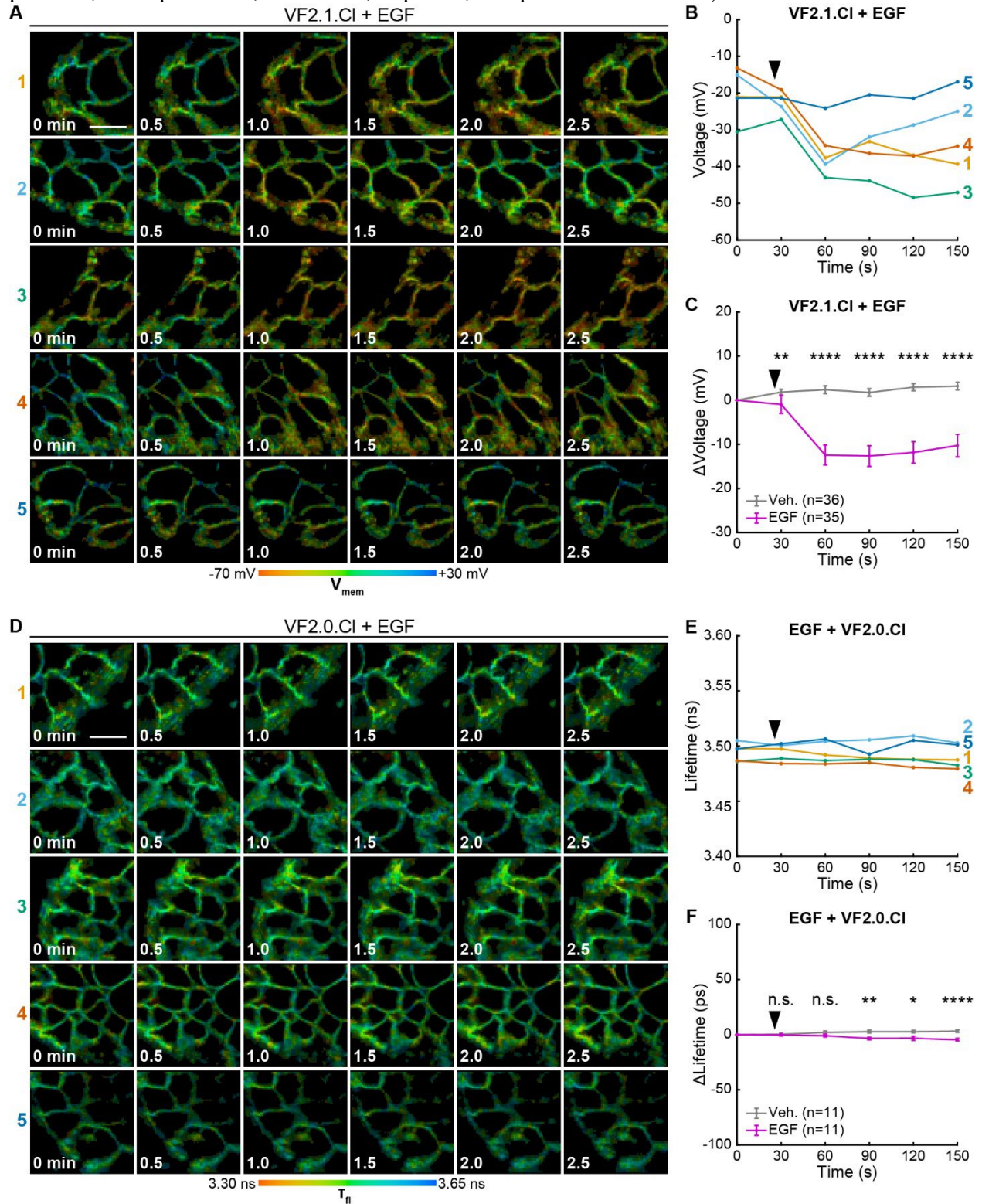


Figure 2.16 Individual VF-FLIM recordings of A431 EGF response.

(A) Representative 3 minute VF-FLIM recordings of A431 cells loaded with 50 nM VF2.1.Cl. 500 ng/mL EGF was added 30 seconds into the time series (black arrow). (B) Quantification of the images in (A), with a single trace per image series shown. (C) Average voltage change in A431 cells following the addition of imaging buffer vehicle (gray) or EGF (purple). (D) Control VF2.0.Cl (not voltage sensitive, 50 nM) images of A431 cells treated as in (A). Images are scaled across the same amount of lifetime space (350 ps) as the VF2.1.Cl images. (E) Quantification of the images in (D). (F) Average VF2.0.Cl lifetime change seen in A431 cells following the addition of imaging buffer vehicle (gray) or EGF (purple) in A431 cells. Graph is scaled across the same amount of lifetime space as the VF2.1.Cl data in (C). Asterisks indicate significant differences between vehicle and EGF treated cells at a given time point (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test). Scale bars are 20 μm .

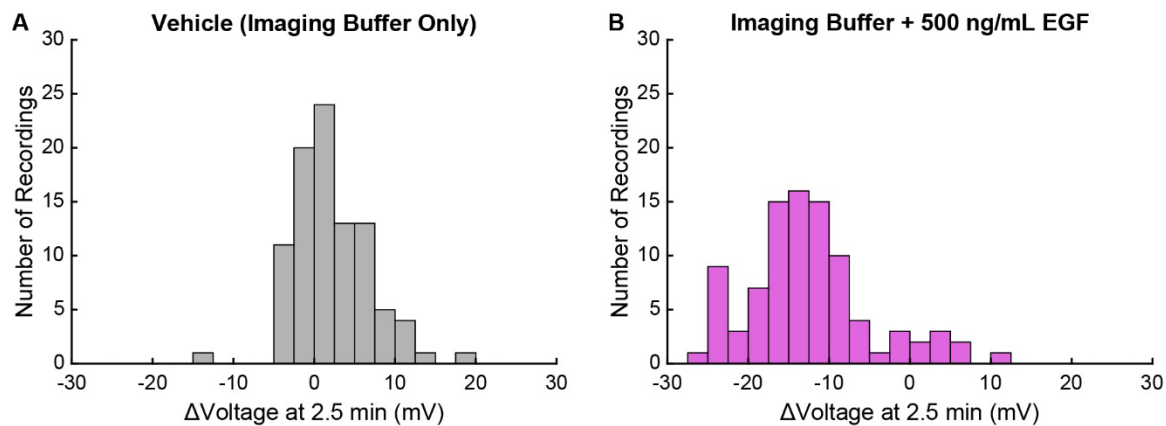


Figure 2.17 Membrane potential changes in A431 cells 2.5 minutes after EGF treatment. Comparison of V_{mem} changes observed in A431 cells 2.5 minutes after treatment with (A) imaging buffer vehicle or (B) 500 ng/mL EGF. Data shown here are compiled from Fig. 4C and Fig. 5A to provide a sense of overall distribution of the responses. Each recording contained a single group of approximately 5 to 10 cells. Sample sizes (number of recordings): Vehicle 93, EGF 92.

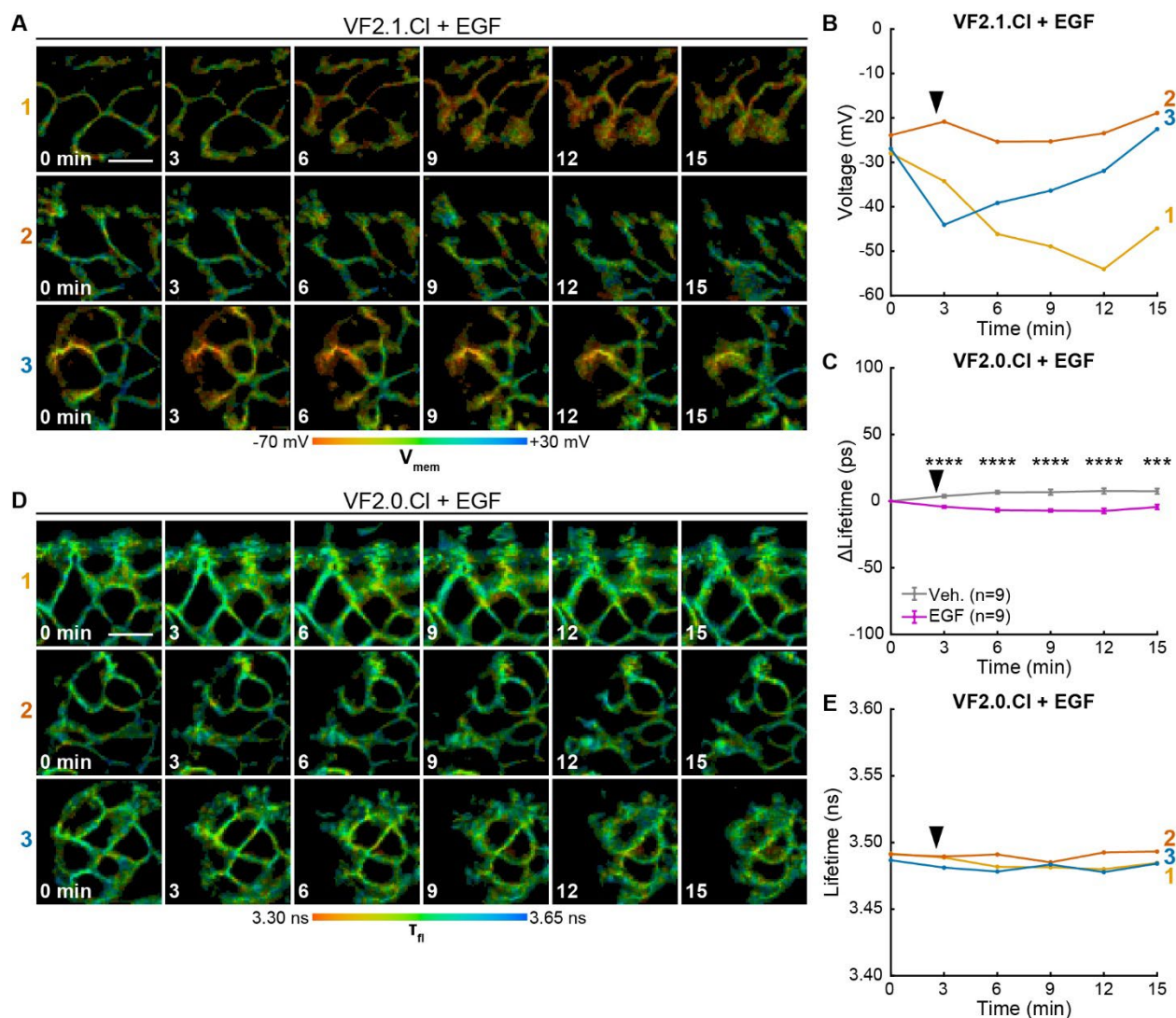


Figure 2.18 VF-FLIM reports A431 V_{mem} changes over 15 minutes.

(A) Representative longer term VF-FLIM recordings of A431 cells loaded with 50 nM VF2.1.Cl. 500 ng/mL EGF was added 30 seconds into the time series. (B) Quantification of the images in (A), with a single trace per image series shown. (C) Control VF2.0.Cl (not voltage sensitive, 50 nM) images of A431 cells treated as in (A). Images are scaled across the same total lifetime range (350 ps) as the VF2.1.Cl images. (D) Quantification of the recordings in (C). (E) Average VF2.0.Cl lifetime change seen in A431 cells following the addition of imaging buffer vehicle (gray) or EGF (purple). Asterisks indicate significant differences between vehicle and EGF treated cells at a given time point (** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test). Scale bars are 20 μ m.

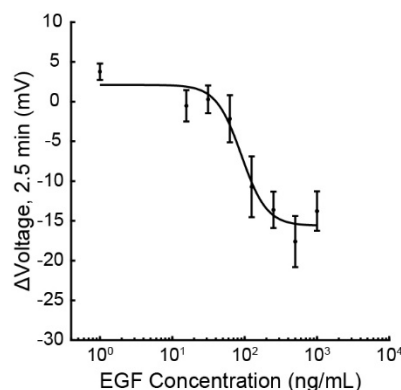


Figure 2.19 Dose-response relationship of A431 voltage response to EGF.

Data were fit to a four-parameter logistic function to obtain an EC_{50} of 90 ng/mL (95% CI: 47-130 ng/mL). Response to each EGF concentration is shown as mean $\pm$ SEM of 6 or 7 recordings (one group of 5-10 cells per recording).

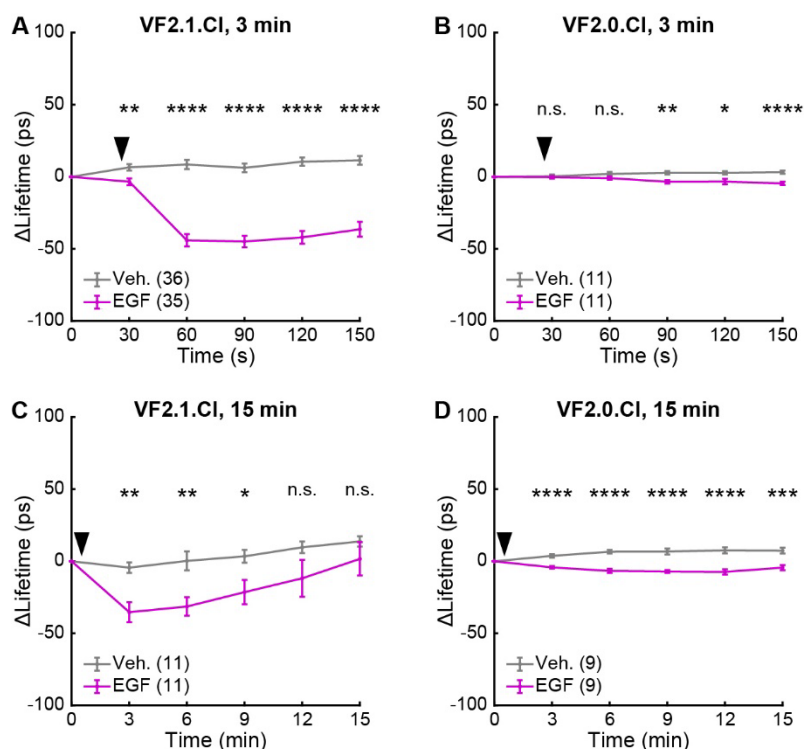


Figure 2.20 Effect sizes of VF2.1.Cl and VF2.0.Cl response to EGF treatment.

Average lifetime changes observed in A431 cells following the addition (black arrow) of imaging buffer vehicle (gray) or 500 ng/mL EGF (purple). (A) Cells incubated with 50 nM VF2.1.Cl and imaged for 3 minutes. (B) Cells incubated with 50 nM VF2.0.Cl (not voltage sensitive) and imaged for 3 minutes. (C) Cells incubated with 50 nM VF2.1.Cl and imaged intermittently for 15 minutes. (D) Cells incubated with 50 nM VF2.0.Cl (not voltage sensitive) and imaged intermittently for 15 minutes. Data are reproduced from Fig. 4, Fig. 4-supplement 1, and Fig. 4 - supplement 3, but here data are scaled in units of lifetime rather than voltage for facile comparison. Data are shown as mean $\pm$ SEM for the indicated number of recordings (one group of 5-10 cells per recording). Asterisks indicate significant differences between vehicle and EGF treated cells at a given time

point (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test).

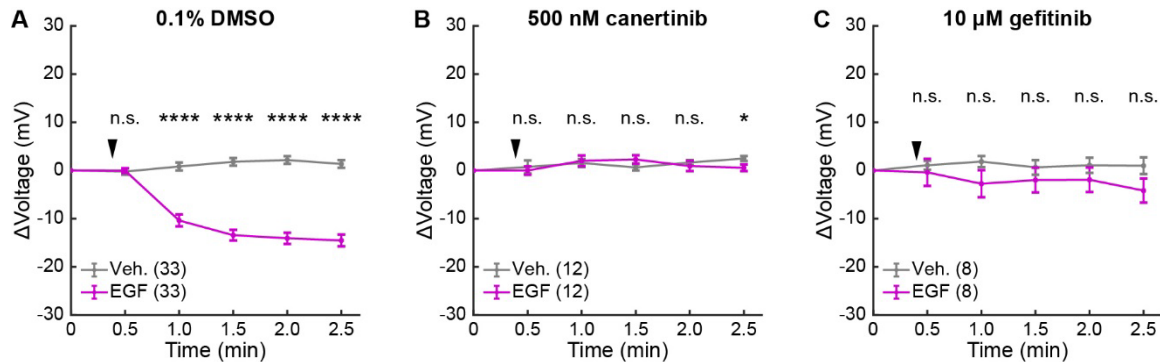


Figure 2.21 EGFR inhibitors abolish voltage response to EGF in A431 cells.

Average V_{mem} changes following the addition (black arrow) of imaging buffer vehicle (gray) or 500 ng/mL EGF (purple) to A431 cells pre-treated with the indicated drug or DMSO vehicle. 2.5 minute time points from this data are shown elsewhere (**Fig. 2.15J**); entire time series are shown here. Data are presented as mean $\pm$ SEM for the indicated number of recordings (one group of 5-10 cells per recording). Asterisks indicate significant differences between vehicle and EGF treated cells at a given time point (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test).

Outward K^+ currents could mediate EGF-induced hyperpolarization. Consistent with this hypothesis, dissipation of the K^+ driving force by raising extracellular $[K^+]$ completely abolishes the typical hyperpolarizing response to EGF and instead results in a small depolarizing potential of approximately 3 mV (**Fig. 2.22A**, **Fig. 2.23B**). Blockade of voltage-gated K^+ channels (K_v) with 4-aminopyridine (4-AP) prior to EGF treatment enhances the hyperpolarizing response to EGF (**Fig. 2.22A**, **2.22B**, **Fig. 2.23C**). In contrast, blockade of Ca^{2+} -activated K^+ channels (K_{Ca}) with charybdotoxin (CTX) results in a depolarizing potential of approximately 4 mV after exposure to EGF, similar to that observed with high extracellular $[K^+]$ (**Fig. 2.22A**, **2.22B**, **Fig. 2.23D**). TRAM-34, a specific inhibitor of the intermediate-conductance Ca^{2+} activated potassium channel $K_{Ca3.1}$ ⁴³, also abolishes EGF-induced hyperpolarization (**Fig. 2.22A**, **Fig. 2.23E**). CTX treatment has little effect on the resting membrane potential, while TRAM-34 or 4-AP depolarizes cells by approximately 5-10 mV (**Fig. 2.24**).

To explore the effects of other components of the EGFR pathway on EGF-induced hyperpolarization, we perturbed intra- and extracellular Ca^{2+} concentrations during EGF stimulation. Reduction of extracellular Ca^{2+} concentration did not substantially alter the EGF response (**Fig. 2.22A**, **Fig. 2.23F**). However, sequestration of intracellular Ca^{2+} with BAPTA-AM disrupts the hyperpolarization response. BAPTA-AM treated cells show a small, 4 mV depolarization in response to EGF treatment, similar to CTX-treated cells (**Fig. 2.22A**, **Fig. 2.23G**). Perturbation of Ca^{2+} levels had little effect on the resting membrane potential (**Fig. 2.24**). Introduction of wortmannin (1 μ M) to block downstream kinase activity has no effect on the membrane potential response to EGF, while orthovanadate addition (Na_3VO_4 , 100 μ M) to block phosphatase activity results in a small increase in the hyperpolarizing response (**Fig. 2.22A**, **Fig. 2.23H-I**). These results support a model for EGF-EGFR mediated hyperpolarization in which RTK

activity of EGFR causes release of internal Ca^{2+} stores to in turn open K_{Ca} channels and hyperpolarize the cell (**Fig. 2.22C**).

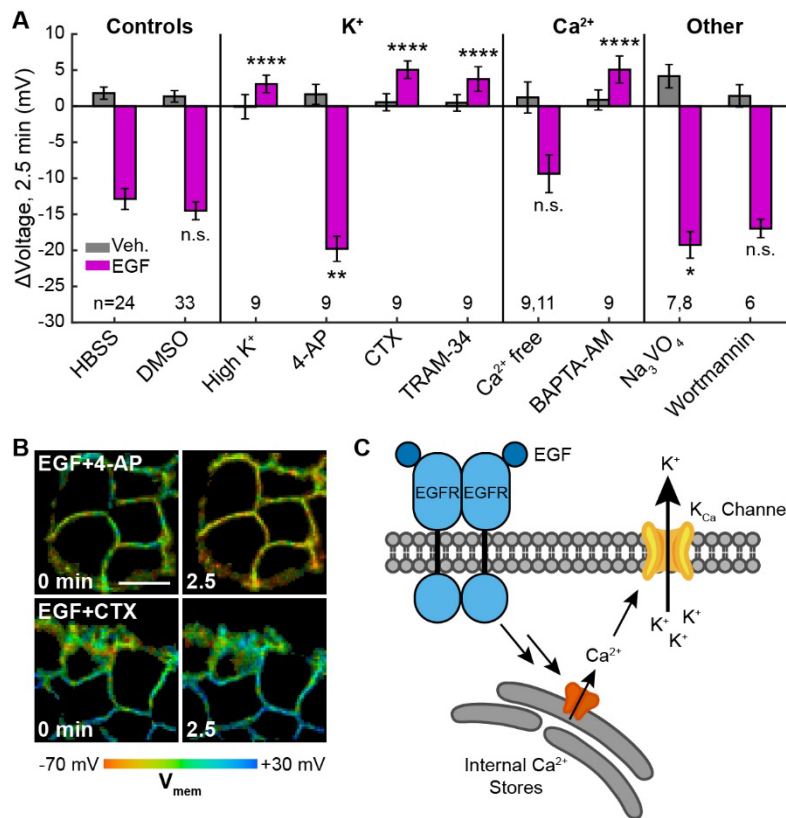


Figure 2.22 EGF-induced hyperpolarization is mediated by a Ca^{2+} activated K^+ channel. **(A)** Comparison of the V_{mem} change 2.5 minutes after EGF addition in cells incubated in unmodified imaging buffer (HBSS) or in modified solutions. **(B)** Lifetime images of A431 cells treated with 4-AP or CTX. **(C)** Model for membrane hyperpolarization following EGFR activation. Scale bar is 20 μm . Bars are mean $\pm$ SEM. Sample sizes listed are (Veh, EGF); where only one number is given, sample size was the same for both. Asterisks reflect significant differences in EGF-stimulated V_{mem} change between the unmodified control (HBSS or DMSO) and modified solutions (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-tailed, unpaired, unequal variances t-test). DMSO: 0.1% DMSO, high K^+ : 120 mM K^+ , 4-AP: 5 mM 4-aminopyridine, CTX: 100 nM charybdotoxin, TRAM-34: 200 nM TRAM-34, Ca^{2+} free: 0 mM Ca^{2+} and Mg^{2+} , BAPTA-AM: 10 μM bisaminophenoxyethanetetraacetic acid acetoxymethyl ester, Na_3VO_4 : 100 μM sodium orthovanadate, wortmannin: 1 μM wortmannin.

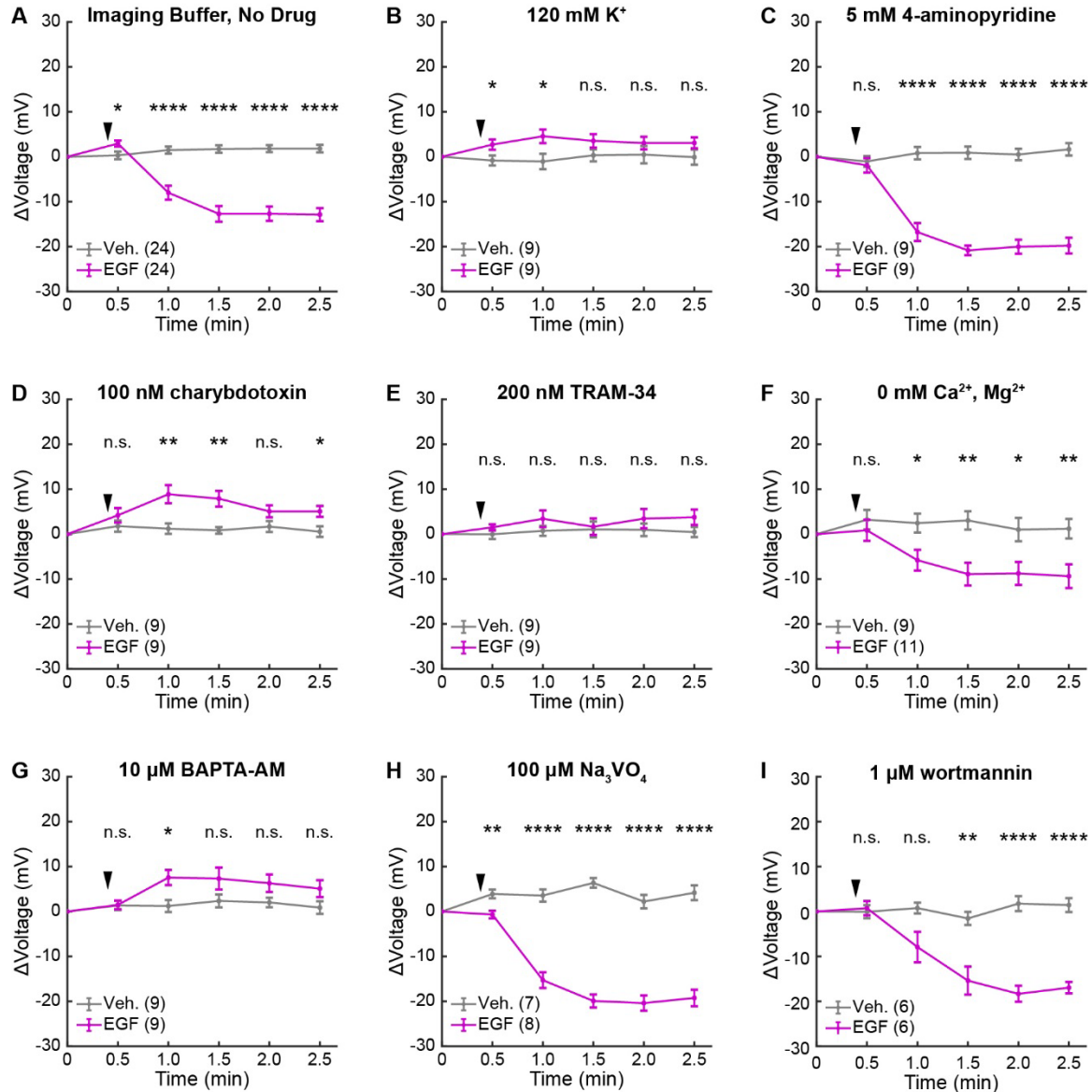


Figure 2.23 A431 voltage response to EGF with pharmacological intervention.

Average V_{mem} changes following the addition (black arrow) of imaging buffer vehicle (gray) or 500 ng/mL EGF (purple) to A431 cells pre-treated with the indicated drug or ionic composition change. 2.5 minute time points from this data are shown elsewhere (**Fig. 5**); entire time series are shown here to illustrate the time courses of the large hyperpolarizing current and small depolarizing current. Data are shown as mean $\pm$ SEM for the indicated number of recordings (one group of 5-10 cells per recording). Asterisks indicate significant differences between vehicle and EGF treated cells at a given time point (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test).

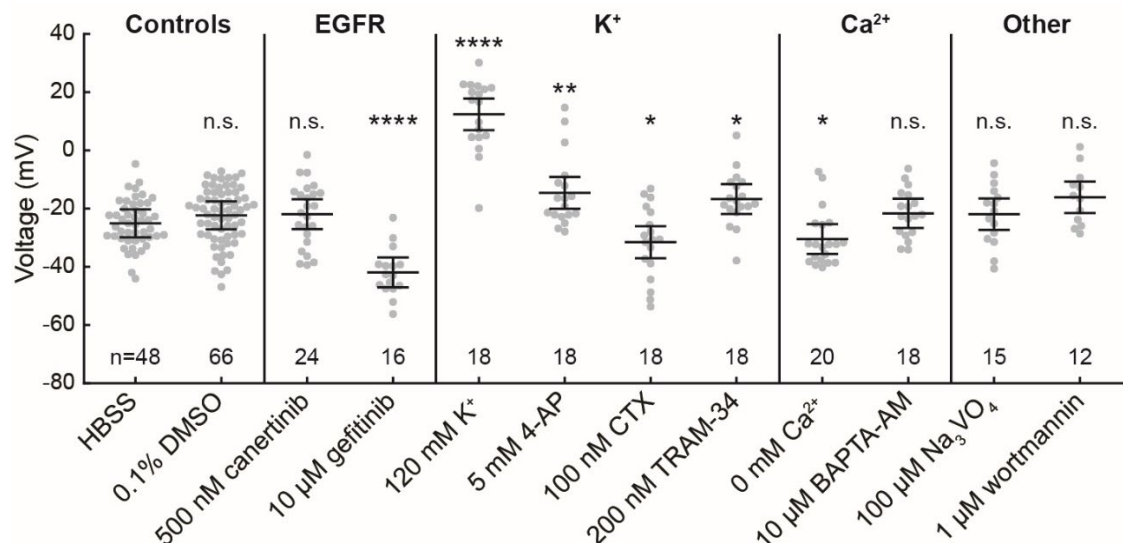


Figure 2.24 Effects of pharmacological and ionic perturbations on A431 resting membrane potential.

Data are the initial V_{mem} reference images for recordings used in EGF addition time series. Data are shown as mean $\pm$ SEM for the indicated number of images (one group of 5-10 cells per image), and gray dots represent individual images. Asterisks indicate significant differences between the appropriate vehicle (HBSS or 0.1% DMSO) and pharmacology treated cells (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-sided, unpaired, unequal variances t-test). CTX = charybdotoxin, 4-AP = 4-aminopyridine, BAPTA-AM = bisaminophenoxyethanetetraacetic acid acetoxymethyl ester.

Discussion

We report the design and implementation of a new method for optically quantifying absolute membrane potential in living cells. VF-FLIM enjoys 100-fold improved throughput over patch clamp electrophysiology, as well as improved spatial resolution. The performance of VF-FLIM hinges on a balance between resolution in three dimensions: membrane potential, space, and time. We discuss the advantages and disadvantages of VF-FLIM in this light, as well as the new application space that is made accessible by VF-FLIM.

Resolution of VF-FLIM: Voltage, Space, and Time

The key advantage of VF-FLIM over previously reported optical approaches is its superior V_{mem} resolution. Resolution can be interpreted as stability of the $\tau_{\text{fl}}-V_{\text{mem}}$ calibration over time and between cells. Any factors other than V_{mem} that change τ_{fl} decrease resolution. VF-FLIM exhibits a 19-fold improvement in inter-cell V_{mem} resolution over FLIM with the GEVI CAESR²⁷ and a 8-fold improvement over di-8-ANEPPS excitation ratios¹⁶. Although all optical strategies, including VF-FLIM, have worse V_{mem} resolution than modern electrophysiology, the greater throughput, improved spatial resolution, and reduced invasiveness of optical strategies make them a powerful complement to electrode-based recordings.

The sources of variability that reduce resolution of optical V_{mem} measurements are manifold, but two major contributors are membrane specificity of the stain and the complexity of the lipid environment. Nonspecific staining is fluorescence signal from anywhere other than the plasma membrane, such as contaminating intracellular staining from poorly trafficked (CAESR) or internalized (ANEPPS) sensor. In contrast, exogenously loaded VF2.1.Cl exhibits little fluorescence contribution from regions other than the plasma membrane. Secondly, membrane composition and dipole potential can vary between cells and cell lines, changing the local environment of the fluorescent indicator^{44,45}. Styryl dyes like di-8-ANEPPS can respond to changes in dipole potential^{16,46}, and VF dyes may be similarly sensitive to dipole potential. Additionally, fluorescence lifetime depends on certain environmental factors (e.g. temperature, viscosity, ionic strength)¹⁸, which may introduce variability. These parameters are usually determined by the biological system under study, and re-calibration is important if they change dramatically in an experiment.

VF-FLIM, like all optical approaches, improves upon the spatial resolution of patch clamp electrophysiology. While VF-FLIM records the V_{mem} of an optically defined region of interest (in this study a cell or cell group), electrophysiology records V_{mem} at an individual cell or part of a cell where the electrode makes contact, which may or may not reflect the V_{mem} of the entire cell or group. In this study, we interpret VF-FLIM at the whole cell level only, since that is the smallest unit in which the V_{mem} can be reliably calibrated by whole cell patch clamp electrophysiology. Intriguingly, there are differences in lifetime within some cells in VF-FLIM images at the pixel to pixel level. In small, mostly spherical cells under voltage clamp, one would expect uniform membrane potential³⁶, so these subcellular differences are most likely noise in the measurement. We speculate that most of this pixel-to-pixel noise comes from variability in fitting the biexponential lifetime model. Lifetime estimates at each pixel are calculated from 20 to 100-fold fewer photons than the lifetime value for the entire ROI. These lower photon counts at the single pixel level produce V_{mem} estimates that are less precise than the V_{mem} estimate for the entire ROI. Collection of more photons at each pixel could likely reduce this noise but would require longer acquisition times. We also cannot fully rule out an alternative explanation that the observed subcellular variability is the result of local differences in membrane composition⁴⁶.

V_{mem} recordings in systems too large or too small for electrophysiological study could be an important application of VF-FLIM. Despite the improbability of V_{mem} compartmentalization in individual HEK293T cells, other cells with complex morphology and processes may display real, subcellular V_{mem} differences. In addition, delocalized V_{mem} patterns across tissues could in theory be stable⁴⁷ and have been proposed to contribute to tissue development⁵. One remaining challenge in expanding VF-FLIM to these areas is the requirement for an initial calibration with voltage clamp electrophysiology. Alternative ways to control V_{mem} , such as ionophores or optogenetic actuators⁴⁸, may prove useful in these systems. When applying VF-FLIM to tissues, the cellular specificity of the VF stain becomes a consideration, as the VF2.1.Cl indicator used in this study labels all cell membranes efficiently. Looking ahead, recordings in tissue are an exciting area for future development of VF-FLIM, particularly in conjunction with cellular and sub-cellular strategies for targeting VF dyes^{49,50}.

To obtain absolute V_{mem} measurements with fluorescence lifetime, VF-FLIM sacrifices some of the temporal resolution of electrophysiology or intensity-based voltage imaging. VF-FLIM acquisition times are limited by the large numbers of photons needed per pixel in time-correlated single photon counting (see **Methods**). As a result, VF-FLIM in its current implementation can track V_{mem} events lasting longer than a few seconds. For “resting” membrane

potential or V_{mem} dynamics associated with cell growth or differentiation, this temporal resolution is likely sufficient. Nevertheless, in the future, we envision allying VF-FLIM with recently developed, faster lifetime imaging technology to enable optical quantification of more rapid V_{mem} responses^{51,52}.

Resting Membrane Potential Distributions in Cultured Cells

Using the improved V_{mem} resolution and throughput of VF-FLIM, we optically documented resting membrane potential distributions in cultured cells to characterize the membrane potential state(s) present. The presence and significance of distinct V_{mem} states in cell populations is mostly uncharacterized due to the throughput limitations of patch-clamp electrophysiology, but some reports suggest that distinct V_{mem} states arise during the various phases of the cell cycle^{53,54}. V_{mem} histograms presented in this work appear more or less unimodal, showing no clear sign of cell cycle-related V_{mem} states (**Fig. 2.12A,D; Fig. 2.13A,D,G**). We considered the possibility that VF-FLIM does not detect cell-cycle-related V_{mem} states because we report average V_{mem} across cell groups in cases where cells are in contact (**Fig. 2.2**). This explanation is unlikely for two reasons. First, V_{mem} distributions for CHO cells appear unimodal, even though CHO cultures were mostly comprised of isolated cells under the conditions tested (**Fig. 2.12D-F**). Second, theoretical work suggests that dramatically different V_{mem} states in adjacent cells are unlikely, as electrical coupling often leads to equilibration of V_{mem} across the cell group^{47,55}. Although we cannot rule out the possibility of poorly separated V_{mem} populations (i.e. with a mean difference in voltage below our resolution limit), VF-FLIM both prompts and enables a re-examination of the notion that bi- or multimodal V_{mem} distributions exist in cultured cells. Furthermore, VF-FLIM represents an exciting opportunity to experimentally visualize theorized V_{mem} patterns in culture and in more complex tissues. Studies towards this end are ongoing in our laboratory.

Epidermal Growth Factor Induces V_{mem} Signaling in A431 Cells

In the present study, we use VF-FLIM to provide the first cell-resolved, direct visualization of voltage changes induced by growth factor signaling. For long term V_{mem} recordings during growth-related processes, an optical approach is more attractive than an electrode-based one. Electrophysiology becomes increasingly challenging as time scale lengthens, especially if cells migrate, and washout of the cytosol with pipette solution can change the very signals under study^{56,57}. Previous attempts to electrophysiologically record V_{mem} in EGF-stimulated A431 cells were unsuccessful due to these technical challenges⁴⁰. Because whole cell voltage-clamp electrophysiology was intractable, the V_{mem} response in EGF-stimulated A431 cells was addressed indirectly through model cell lines expressing EGFR exogenously⁴⁰, bulk measurements on trypsinized cells in suspension⁵⁸, or cell-attached single channel recordings^{59–61}. By stably recording V_{mem} during EGF stimulation, VF-FLIM enables direct study of V_{mem} signaling in otherwise inaccessible pathways.

In conjunction with physiological manipulations and pharmacological perturbations, we explore the molecular mechanisms underlying EGF-induced hyperpolarization. We find that signaling along the EGF-EGFR axis results in a robust hyperpolarizing current carried by K^+ ions, passed by the Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}3.1}$, and mediated by intracellular Ca^{2+} (**Fig. 2.22C**). We achieve a complete loss of the hyperpolarizing response to EGF by altering the K^+ driving

force (“High K⁺” **Fig. 2.22A**, **Fig. 2.23B**), blocking calcium-activated K⁺ currents directly (“CTX” and “TRAM-34”, **Fig. 2.22A**, **Fig. 2.23D,E**), or intercepting cytosolic Ca²⁺ (“BAPTA-AM”, **Fig. 2.22A**, **Fig. 2.23G**). These results, combined with transcriptomic evidence that K_{Ca}3.1 is the major K_{Ca} channel in A431 cells ⁶², indicate that K_{Ca}3.1 mediates the observed hyperpolarization. Interestingly, under some conditions where K⁺-mediated hyperpolarization is blocked (“CTX,” “high K⁺,” “BAPTA-AM”), VF-FLIM reveals a small, secondary depolarizing current not visible during normal EGF stimulation. This current likely arises from initial Ca²⁺ entry into the cell, as previously observed during EGF signaling ^{63,64}. Although we have obtained direct and conclusive evidence of EGF-induced hyperpolarization in A431 cells, the interactions between this voltage change and downstream targets of EGFR remain incompletely characterized. Enhancing EGF signaling by blockade of cellular tyrosine phosphatases with orthovanadate ⁶⁵ correspondingly increases EGF-mediated hyperpolarization (“Na₃VO₄” **Fig. 2.22A**, **Fig. 2.23H**), but inhibition of downstream kinase activity appears to have little effect on hyperpolarization (“wortmannin” **Fig. 2.22A**, **Fig. 2.23I**).

In the context of RTK signaling, V_{mem} may serve to modulate the driving force for external Ca²⁺ entry ^{3,66} and thereby act as a regulator of this canonical signaling ion. Alternatively, V_{mem} may play a more subtle biophysical role, such as potentiating lipid reorganization in the plasma membrane ⁶⁷. Small changes in V_{mem} likely affect signaling pathways in ways that are currently completely unknown, but high throughput discovery of V_{mem} targets remains challenging. Combination of electrophysiology with single cell transcriptomics has begun to uncover relationships between V_{mem} and other cellular pathways in excitable cells ⁶⁸; such approaches could be coupled to higher throughput VF-FLIM methods to explore pathways that interact with V_{mem} in non-excitable contexts.

VF-FLIM represents a novel and general approach for interrogating the roles of membrane potential in fundamental cellular physiology. Future improvements to the voltage resolution could be made by use of more sensitive indicators, which may exhibit larger changes in fluorescence lifetime ³⁰. VF-FLIM can be further expanded to include the entire color palette of PeT-based voltage indicators ^{69,70}, allied with targeting methods to probe absolute membrane potential in heterogeneous cellular populations ^{49,50}, and coupled to high-speed imaging techniques for optical quantification of fast voltage events ^{51,52}.

Materials

VoltageFluor (VF) dyes VF2.1.Cl and VF2.0.Cl were synthesized in house according to previously described syntheses ³⁰. VFs were stored either as solids at room temperature or as 1000x DMSO stocks at -20°C. VF stock concentrations were normalized to the absorption of the dichlorofluorescein dye head via UV-Vis spectroscopy in Dulbecco’s phosphate buffered saline (dPBS, Thermo Fisher Scientific, Waltham, MA) pH 9 with 0.1% sodium dodecyl sulfate (w/v, SDS). Di-8-ANEPPS was purchased from Thermo Fisher Scientific. Di-8-ANEPPS was prepared as a 2 mM (2000x) stock solution in DMSO and stored at -20°C. Di-8-ANEPPS concentrations were determined via UV-Vis spectroscopy in methanol (ϵ at 498 nm: 41,000 cm⁻¹ M⁻¹ according to the manufacturer’s certificate of analysis).

All salts and buffers were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific. TRAM-34, 4-aminopyridine, and charybdotoxin were purchased from Sigma-Aldrich. Gefitinib, wortmannin, sodium orthovanadate, and BAPTA-AM were purchased from Fisher Scientific. Canertinib was a gift from the Kuriyan laboratory at UC Berkeley. Gefitinib,

wortmannin, canertinib, and TRAM-34 were made up as 1000x-10000x stock solutions in DMSO and stored at -20°C. Charybdotoxin was made up as a 1000x solution in water and stored at -80°C. 4-aminopyridine was made up as a 20x stock in imaging buffer (HBSS) and stored at 4°C. Recombinantly expressed epidermal growth factor was purchased from PeproTech (Rocky Hill, NJ) and aliquoted as a 1 mg/mL solution in water at -80°C.

Solid sodium orthovanadate was dissolved in water and activated before use ⁷¹. Briefly, orthovanadate solutions were repeatedly boiled and adjusted to pH 10 until the solution was clear and colorless. 200 mM activated orthovanadate stocks were aliquoted and stored at -20°C.

Unless otherwise noted, all imaging experiments were performed in Hank's Balanced Salt Solution (HBSS; Gibco/Thermo Fisher Scientific). HBSS composition in mM: 137.9 NaCl, 5.3 KCl, 5.6 D-glucose, 4.2 NaHCO₃, 1.3 CaCl₂, 0.49 MgCl₂, 0.44 KH₂PO₄, 0.41 MgSO₄, 0.34 Na₂HPO₄. High K⁺ HBSS was made in-house to 285 mOsmol and pH 7.3, containing (in mM): 120 KCl, 23.3 NaCl, 5.6 D-glucose, 4.2 NaHCO₃, 1.3 CaCl₂, 0.49 MgCl₂, 0.44 KH₂PO₄, 0.41 MgSO₄, 0.34 Na₂HPO₄. Nominally Ca²⁺/Mg²⁺ free HBSS (Gibco) contained, in mM: 137.9 NaCl, 5.3 KCl, 5.6 D-glucose, 4.2 NaHCO₃, 0.44 KH₂PO₄, 0.34 Na₂HPO₄.

Methods

Cell Culture

All cell lines were obtained from the UC Berkeley Cell Culture Facility and discarded after twenty-five passages. A431, HEK293T, MCF-7, and MDA-MB-231 cells were authenticated by short tandem repeat (STR) profiling. All cells were routinely tested for mycoplasma contamination. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose supplemented with 10% FBS (Seradigm (VWR); Radnor, PA) and 2 mM GlutaMAX (Gibco) in a 5% CO₂ incubator at 37°C. Media for MCF-7 cells was supplemented with 1 mM sodium pyruvate (Life Technologies/Thermo Fisher Scientific) and 1x non-essential amino acids (Thermo Fisher Scientific). Media for CHO.K1 (referred to as CHO throughout the text) cells was supplemented with 1x non-essential amino acids. HEK293T and MDA-MB-231 were dissociated with 0.05% Trypsin-EDTA with phenol red (Thermo Fisher Scientific) at 37°C, whereas A431, CHO, and MCF-7 cells were dissociated with 0.25% Trypsin-EDTA with phenol red at 37°C. To avoid potential toxicity of residual trypsin, all cells except for HEK293T were spun down at 250xg or 500xg for 5 minutes and re-suspended in fresh complete media during passaging.

For use in imaging experiments, cells were plated onto 25 mm diameter poly-D-lysine coated #1.5 glass coverslips (Electron Microscopy Sciences) in 6 well tissue culture plates (Corning; Corning, NY). To maximize cell attachment, coverslips were treated before use with 1-2 M HCl for 2-5 hours and washed overnight three times with 100% ethanol and three times with deionized water. Coverslips were sterilized by heating to 150°C for 2-3 hours. Before use, coverslips were incubated with poly-D-lysine (Sigma-Aldrich, made as a 0.1 mg/mL solution in phosphate-buffered saline with 10 mM Na₃BO₄) for 1-10 hours at 37°C and then washed twice with water and twice with Dulbecco's phosphate buffered saline (dPBS, Gibco).

A431, CHO, HEK293T, and MCF-7 were seeded onto glass coverslips 16-24 hours before microscopy experiments. MDA-MB-231 cells were seeded 48 hours before use because it facilitated formation of gigaseals during whole-cell voltage clamp electrophysiology. Cell densities used for optical resting membrane potential recordings (in 10³ cells per cm²) were: A431

42; CHO 42; HEK293T 42; MCF-7 63; MDA-MB-231 42. To ensure the presence of single cells for whole-cell voltage clamp electrophysiology, fast-growing cells were plated more sparsely (approximately 20% confluence) for electrophysiology experiments. Cell densities used for electrophysiology (in 10^3 cells per cm^2) were: A431 36-52; CHO 21; HEK293T 21; MCF-7 63; MDA-MB-231 42. To reduce their rapid growth rate, HEK293T cells were seeded onto glass coverslips in reduced glucose (1 g/L) DMEM with 10% FBS, 2 mM GlutaMAX, and 1 mM sodium pyruvate for electrophysiology experiments.

Cellular Loading of VoltageFluor Dyes

Cells were loaded with 1x VoltageFluor in HBSS for 20 minutes in a 37°C incubator with 5% CO₂. For most experiments, 100 nM VoltageFluor was used. Serum-starved A431 cells were loaded with 50 nM VoltageFluor. After VF loading, cells were washed once with HBSS and then placed in fresh HBSS for imaging. All imaging experiments were conducted at room temperature under ambient atmosphere. Cells were used immediately after loading the VF dye, and no cells were kept for longer than an hour at room temperature.

Whole-Cell Patch-Clamp Electrophysiology

Pipettes were pulled from borosilicate glass with filament (Sutter Instruments, Novato, CA) with resistances ranging from 4 to 7 M Ω with a P97 pipette puller (Sutter Instruments). Internal solution composition, in mM (pH 7.25, 285 mOsm/L): 125 potassium gluconate, 10 KCl, 5 NaCl, 1 EGTA, 10 HEPES, 2 ATP sodium salt, 0.3 GTP sodium salt. EGTA (tetraacid form) was prepared as a stock solution in either 1 M KOH or 10 M NaOH before addition to the internal solution. Pipettes were positioned with an MP-225 micromanipulator (Sutter Instruments). A liquid junction potential of -14 mV was determined by the Liquid Junction Potential Calculator in the pClamp software package⁷² (Molecular Devices, San Jose, CA), and all voltage step protocols were corrected for this offset.

For VF-FLIM and CAESR, electrophysiology recordings for VF-FLIM and CAESR were made with an Axopatch 200B amplifier and digitized with a Digidata 1440A (Molecular Devices). The software package used was pClamp 10.3. Signals were filtered with a 5 kHz low-pass Bessel filter. Correction for pipette capacitance was performed in the cell attached configuration. Voltage-lifetime calibrations were performed in V-clamp mode, with the cell held at the potential of interest for 15 or 30 seconds while lifetime was recorded. Potentials were applied in random order, and membrane test was conducted between each step to verify the quality of the patch. For single cell patching, recordings were only included if they maintained a 30:1 ratio of membrane resistance (R_m) to access resistance (R_a) and an R_a value below 30 M Ω throughout the recording. Due to the reduced health of HEK293T cells transfected with CAESR, recordings were used as long as they maintained a 10:1 R_m : R_a ratio, although most recordings were better than 30:1 R_m : R_a . Only recordings stable for at least 4 voltage steps (roughly 2 minutes) were included in the dataset.

For di-8-ANEPPS, electrophysiology recordings were made in the same manner as the above, with the following minor differences. Signals were digitized with a Digidata 1550B; the pClamp 10.6 software package was used (Molecular Devices). Potentials were applied in the order 0 mV, -80 mV, +40 mV, -40 mV, +80 mV for ten seconds at each step. Patch parameters were tested at the beginning and end of the patch program, rather than between each step. Only patches

that retained a 30:1 ratio of R_m to R_a and access resistance below 30 M Ω throughout the recording were included in the dataset.

For electrophysiology involving small groups of cells (**Fig. 2.10**), complete voltage clamp across the entire cell group was not possible. Recordings were used as long as R_a remained below 30 M Ω for at least three voltage steps. Most recordings also retained R_m : R_a ratios greater than 20:1.

Epidermal Growth Factor Treatment

A431 cells were serum starved prior to epidermal growth factor studies. Two days before the experiment, cells were trypsinized and suspended in complete media with 10% FBS. Cells were then spun down for 5 minutes at 500xg and re-suspended in reduced serum DMEM (2% FBS, 2 mM GlutaMAX, 4.5 g/L glucose). Cells were seeded onto 25 mm coverslips in 6 well plates at a density of 84×10^3 cells per cm². 4-5.5 hours before the experiment, the media was exchanged for serum-free DMEM (0% FBS, 2 mM GlutaMAX, 4.5 g/L glucose).

After 4-5.5 hours in serum-free media, cells were loaded with 50 nM VF dye as described above. In pharmacology experiments, the drug or vehicle was also added to the VF dye loading solution. All subsequent wash and imaging solutions also contained the drug or vehicle. For changes to buffer ionic composition, VoltageFluor dyes were loaded in unmodified HBSS to avoid toxicity from prolonged incubation with high K⁺ or without Ca²⁺. Immediately prior to use, cells were washed in the modified HBSS (120 mM K⁺ or 0 mM Ca²⁺) and recordings were made in the modified HBSS.

For analysis of short-term responses to EGF (3 minute time series), VF lifetime was recorded in 6 sequential 30 second exposures. Immediately after the conclusion of the first frame (30-35 seconds into the recording), EGF or vehicle (imaging buffer only) was added to the indicated final concentration from a 2x solution in HBSS imaging buffer. For analysis of long-term responses to EGF (15 minute time series), EGF addition occurred in the same way, but a gap of 150 seconds (without laser illumination) was allotted between each 30 second lifetime recording. Times given throughout the text correspond to the start of an exposure. Voltage changes at 2.5 minutes were calculated from the difference between an initial image (taken before imaging buffer vehicle or EGF addition) and a final image (a 30 second exposure starting 2.5 minutes into the time series).

Transfection and Imaging of CAESR in HEK293T

The CAESR plasmid was obtained as an agar stab (FCK-Quasar2-Citrine, Addgene #59172), cultured overnight in LB with 100 μ g/mL ampicillin, and isolated via a spin miniprep kit (Qiagen). HEK293T cells were plated at a density of 42,000 cells per cm² directly onto a 6 well tissue culture plate and incubated at 37°C in a humidified incubator for 24 hours prior to transfection. Transfections were performed with Lipofectamine 3000 according to the manufacturer's protocol (Thermo Fisher Scientific). Cells were allowed to grow an additional 24 hours after transfection before they were plated onto glass coverslips for microscopy experiments (as described above for electrophysiology of untransfected HEK293T cells).

Determination of EC₅₀ for EGF in A431 Cells

Average voltage changes 2.5 minutes after addition of EGF to serum deprived A431 cells were determined at different EGF concentrations, and these means were fit to a four parameter logistic function in MATLAB (MathWorks, Natick, MA).

Goldman-Hodgkin-Katz Estimation of V_{mem} Ranges in Different Imaging Buffers

If intracellular and extracellular concentrations, as well as relative permeabilities, of all ionic species are known, the Goldman-Hodgkin-Katz (GHK) equation (eqn. 1) can be used to calculate the resting membrane potential of a cell³⁸. In practice, the intracellular ion concentrations $[X]_{in}$ and relative permeabilities P_x are difficult to determine, so the GHK equation is not a substitute for direct measurement of V_{mem} . To obtain a range of reasonable V_{mem} values in systems where these concentrations and relative permeabilities are not known, we calculated possible V_{mem} using the “standard” parameters derived from the work of Hodgkin and Katz³⁸, as well as a value above and a value below each “standard” point. The values evaluated were the following: P_K 1; P_{Na} 0.01, 0.05, 0.2; P_{Cl} 0.2, 0.45, 0.9; $[K^+]_{in}$ 90, 150, 200 mM; $[Na^+]_{in}$ 5, 15, 50 mM; $[Cl^-]_{in}$ 2, 10, 35 mM. Extracellular ion concentrations $[X]_{out}$ were known (see **Materials**). In eqn. 1, R is the universal gas constant, T is the temperature (293 K for this experiment), and F is Faraday’s constant.

$$V_{mem} = \frac{RT}{F} \ln \frac{P_K[K^+]_{out} + P_{Na}[Na^+]_{out} + P_{Cl}[Cl^-]_{in}}{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{out}} \quad (1)$$

Fluorescence Lifetime Data Acquisition

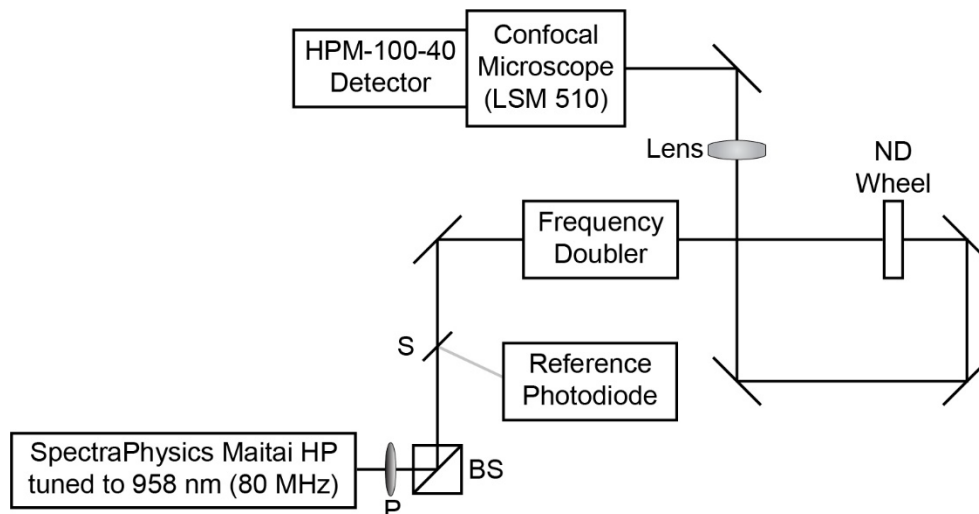
Fluorescence lifetime imaging was conducted on a LSM 510 inverted scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with an SPC-150 or SPC-150N single photon counting card (Becker & Hickl GmbH, Berlin, Germany) (**Scheme 2.1**). 80 MHz pulsed excitation was supplied by a Ti:Sapphire laser (MaiTai HP; SpectraPhysics, Santa Clara, CA) tuned to 958 nm and frequency-doubled to 479 nm. The laser was cooled by a recirculating water chiller (Neslab KMC100). Excitation light was directed into the microscope with a series of silver mirrors (Thorlabs, Newton, NJ or Newport Corporation, Irvine, CA).

Excitation light power at the sample was controlled with a neutral density (ND) wheel and a polarizer (P) followed by a polarizing beamsplitter (BS). Light was titrated such that VoltageFluor lifetime did not drift during the experiment, no phototoxicity was visible, and photon pile-up was not visible on the detector. For recordings at high VoltageFluor concentrations (**Fig. 2.3, Fig. 2.11**), reduced power was used to avoid saturating the detector. For optical voltage determinations using 50 or 100 nM VoltageFluor, typical average power at the sample was 5 μ W.

Fluorescence emission was collected through a 40x oil immersion objective (Zeiss) coated with immersion oil (Immersol 518F, Zeiss). Emitted photons were detected with a hybrid detector, HPM-100-40 (Becker & Hickl), based on a Hamamatsu R10467 GaAsP hybrid photomultiplier tube. Detector dark counts were kept below 1000 per second during acquisition. Emission light was collected through a 550/49 bandpass filter (Semrock, Rochester, NY) after passing through a 488 LP dichroic mirror (Zeiss). The reference photons for determination of photon arrival times were detected with a PHD-400-N high speed photodiode (Becker & Hickl). Data were acquired

with 256 time bins in the analog-to-digital-converter and either 64x64 or 256x256 pixels of spatial resolution (see discussion of pixel size below).

Routine evaluation of the proper functioning of the lifetime recording setup was performed by measurement of three standards (**Table 2.2**): 2 μM fluorescein in 0.1 N NaOH, 1 mg/mL erythrosin B in water (pH 7), and the instrument response function (IRF). The IRF was determined from a solution of 500 μM fluorescein and 12.2 M sodium iodide in 0.1 N NaOH. Because of the high concentration of iodide quencher, the IRF solution has a lifetime shorter than the detector response time, allowing approximation of the instrument response function under identical excitation and emission conditions as data acquisition⁷³.



Scheme 2.1 Optical diagram for time correlated single photon counting microscope.

Excitation light was supplied by a Ti:Sapphire laser tuned to 958 nm. A small amount of light was redirected by a beam sampler (S) to a reference photodiode. The remaining light was passed through a frequency doubler to obtain 479 nm excitation light, which entered the LSM510 confocal microscope. A polarizer (P) followed by a polarizing beamsplitter (BS), as well as a neutral density (ND) wheel, allowed control of the amount of light passed to the sample.

IRF Deconvolution

Signal from photons detected in a TCSPC apparatus are convolved with the instrument response (IRF). IRFs can be approximated by the SPCImage fitting software, but consistency of lifetime fits on VF-FLIM datasets was improved by using a measured IRF. Measured IRFs were incorporated by the iterative reconvolution method using SPCImage analysis software⁷⁴.

VoltageFluor Lifetime Fitting Model

All VoltageFluor lifetime data were fit using SPCImage (Becker & Hickl), which solves the nonlinear least squares problem using the Levenberg-Marquadt algorithm. VF2.1.C1 lifetime data were fit to a sum of two exponential decay components (eqn. 2). Attempts to fit the VF2.1.C1 data with a single exponential decay (eqn. 3) were unsatisfactory.

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \quad (2)$$

The fluorescence lifetime of VF2.0.Cl was adequately described by a single exponential decay for almost all data (eqn. 3). A second exponential component was necessary to fit data at VF2.0.Cl concentrations above 500 nM, likely attributable to the concentration-dependent decrease in lifetime that was observed high VF concentrations.

$$F(t) = a e^{-t/\tau} \quad (3)$$

For all data fit with the two component model, the weighted average of the two lifetimes, τ_m (eqn. 4), was used in subsequent analysis.

$$\tau_m = \frac{a_1 \tau_1 + a_2 \tau_2}{a_1 + a_2} \quad (4)$$

All lifetime images are represented as an overlay of photon count (pixel intensity) and weighted average lifetime (pixel color) throughout the text (τ_m + PC, **Scheme 2.2**). Pixels with insufficient signal to fit a fluorescence decay are shown in black. The photon counts, as well as the lifetimes, in image sequences on the same set of cells are scaled across the same range.

Additional Fit Parameters for VoltageFluor Lifetimes

Pixels with photon counts below 300 (VF2.1.Cl) or 150 (VF2.0.Cl) photons at the peak of the decay (time bin with the most signal) were omitted from analysis to ensure reproducible fits. Because the lifetime of VFs does not fully decay to baseline in a single 12.5 ns laser cycle, the incomplete multiexponentials fitting option was used, allowing the model to attribute some signal early in the decay to the previous laser cycle. Out of 256 time bins from the analog-to-digital converter (ADC), only data from time bins 23 to 240 were used in the final fit. The offset parameter (detector dark counts per ADC time bin per pixel) was set to zero. The number of iterations for the fit in SPCImage was increased to 20 to obtain converged fits. Shift between the IRF and the decay trace was fixed to 0.5 (in units of ADC time bins), which consistently gave lifetimes of standards erythrosin B (1 mg/mL in H₂O)⁷⁵ and fluorescein (2 μ M in 0.1 N NaOH, H₂O)³³ closest to reported values (**Table 2.2**).

Acquisition Time and Effective Pixel Size in Lifetime Data

To obtain sufficient photons but keep excitation light power minimal, binning between neighboring pixels was employed during fitting. This procedure effectively takes the lifetime as a spatial moving average across the image by including adjacent pixels in the decay for a given pixel. To obtain larger photon counts, the confocal pinhole was set between 2.5 and 3.5 airy units, which corresponds to optical section thickness of approximately 2.5 μ m.

Data Type	Acquired Pixel Width (μm)	Binned Pixel Width (μm)	Acquisition Time (s)	Img Size (pixels)
Concentration Curve (Fig. 2.3, Fig. 2.11)	0.44	3.08	75-90	256 x 256
V_{mem} Distributions (Fig. 2.12)	1.24	8.68	90-120	256 x 256
Electrophysiology Recording	1.00	3.01	15-30	64 x 64
EGF Time Series	0.88	2.64	30	64 x 64

Table 2.3 Pixel Sizes and Acquisition Times. All tabulated values are for an individual frame, although multiple sequential frames were recorded in both the electrophysiology and EGF experiments. For each recording type, the width of each pixel at acquisition is reported, as well as the width of the area included in the binned lifetime signal during fitting. All pixels are square. The acquisition time reflects the total time to collect the image, not the total time exposing each pixel. All FLIM images have 256 time bins in the ns regime, so a 256x256 spatial image size represents a 256x256x256 total dataset. Img = image.

Determination of Regions of Interest

Images were divided into cell groups, with each cell group as a single region of interest (ROI). ROIs were determined from photon count images, either manually from the cell morphology in FIJI⁷⁶ or automatically by sharpening and then thresholding the signal intensity with custom MATLAB code. Regions of images that were partially out of the optical section or contained punctate debris were omitted. Sample ROIs are shown in Fig. 2.2.

For cells that adjoin other cells, attribution of a membrane region to one cell versus the other is not possible. As such, we chose to interpret each cell group as an independent sample ('n') instead of extracting V_{mem} values for individual cells. Adjacent cells in a group are electrically coupled to varying degrees, and their resting membrane potentials are therefore not independent³⁷. While this approach did not fully utilize the spatial resolution of VF-FLIM, it prevented overestimation of biological sample size for the effect in question.

Conversion of Lifetime to Transmembrane Potential

The mean τ_m across all pixels in an ROI was used as the lifetime for that ROI. Lifetime values were mapped to transmembrane potential via the lifetime- V_{mem} standard curves determined with whole-cell voltage-clamp electrophysiology. For electrophysiology measurements, the relationship between the weighted average lifetime (eqn. 4) and membrane potential for each patched cell was determined by linear regression, yielding a sensitivity (m , ps/mV) and a 0 mV lifetime (b , ps) for each cell (eqn. 5). The average sensitivity and 0 mV point across all cells of a given type were used to convert subsequent lifetime measurements (τ) to V_{mem} (Table 2.1, eqn. 6). For quantifying changes in voltage (ΔV_{mem}) from changes in lifetime ($\Delta\tau$), only the average sensitivity is necessary (eqn. 7).

$$\tau = m * V_{\text{mem}} + b \quad (5)$$

$$V_{mem} = \frac{(\tau - b)}{m} \quad (6)$$

$$\Delta V_{mem} = \frac{(\Delta \tau)}{m} \quad (7)$$

Where standard error of the mean of a voltage determination (δV_{mem}) is given, error was propagated to include the standard errors of the slope (δm) and y-intercept (δb) of the voltage calibration, as well as the standard error of the lifetime measurements ($\delta \tau$) in the condition of interest (eqn. 8). For error in a voltage change ($\delta \Delta V_{mem}$), only error in the calibration slope was included in the propagated error (eqn. 9). Where standard deviation of VF-FLIM derived V_{mem} values is shown, a similar error propagation procedure was applied, using the standard deviation of the average sensitivity and 0 mV lifetime for that cell line.

$$\delta V_{mem} = |V_{mem}| \sqrt{\left(\frac{\sqrt{\delta \tau^2 + \delta b^2}}{\tau - b}\right)^2 + \left(\frac{\delta m}{m}\right)^2} \quad (8)$$

$$\delta \Delta V_{mem} = |\Delta V_{mem}| \sqrt{\left(\frac{\delta \Delta \tau}{\Delta \tau}\right)^2 + \left(\frac{\delta m}{m}\right)^2} \quad (9)$$

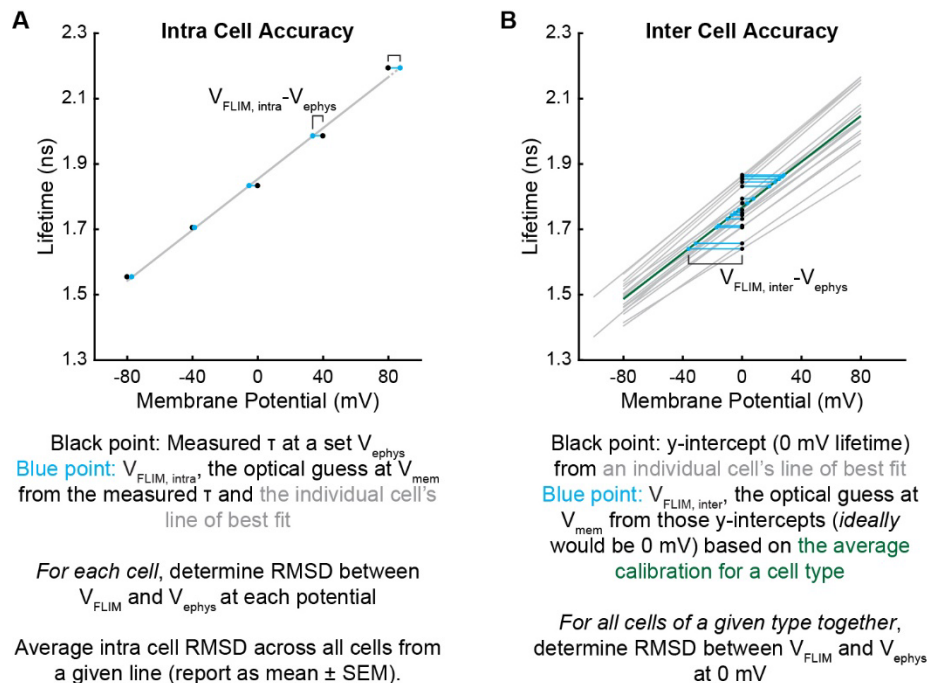
Resolution of VF-FLIM Voltage Determination

The intrinsic nature of fluorescence lifetime introduces a point of reference into the voltage measurement, from which a single lifetime image can be interpreted as resting membrane potential. Differences in this reference point (reported here as the 0 mV lifetime) over time and across cells provides an estimate of the voltage-independent noise in VF-FLIM. We report resolution as the root-mean-square deviation (RMSD) between the optically calculated voltage (V_{FLIM}) and the voltage set by whole-cell voltage clamp (V_{ephys}), which is analogous to the resolution calculations described previously by Cohen and co-workers²⁶. The RMSD of n measurements (eqn. 10) can be determined from the variance σ^2 (eqn. 11) and the bias (eqn. 12) of the estimator (in this case, VF-FLIM) relative to the “true” value (in this case, electrophysiology). These calculations are described graphically in **Scheme 2.2** below.

$$RMSD = \sqrt{\sigma^2 + Bias^2} \quad (10)$$

$$\sigma^2 = \frac{1}{n} \sum_{i=1}^n (V_{FLIM,i} - V_{ephys,i})^2 \quad (11)$$

$$Bias = \frac{1}{n} \sum_{i=1}^n V_{FLIM,i} - \frac{1}{n} \sum_{i=1}^n V_{ephys,i} \quad (12)$$



Scheme 2.2 Intra and inter-cell V_{mem} resolution calculations.

Data are taken directly from **Figure 2.1H,I** as an example. **(A)** Intra cell values are the RMSD between the voltage equivalent of the measured lifetime (V_{FLIM}) and voltage set by electrophysiology (V_{ophys}). V_{FLIM} values are calculated using that particular cell's line of best fit, so one value is obtained per cell. Here, we present intra cell error as the mean $\pm$ SEM of all cells from a given cell line. **(B)** Inter cell errors are the RMSD between the voltage-equivalent of the 0 mV lifetime for all cells tested from a cell line (V_{FLIM} , determined with the average slope and y-intercept for that cell line) and the ground truth value of 0 mV. Inter-cell accuracy is calculated from all of the calibration data for a cell line, so there is one value per cell line. Black points are experimental y-intercepts and blue points are the V_{FLIM} optical voltage determinations from those lifetimes. Gray lines are lines of best fit for individual cells. Green line in **(B)** represents the average $\tau_{fl}-V_{mem}$ relationship for a cell line.

The voltage-independent variations in lifetime are much larger between cells than within a cell. Therefore, the error in measuring absolute voltage *changes* on a given cell ("intra-cell" comparisons) is lower than the error in determining the absolute V_{mem} of that cell ("inter-cell" comparisons, since the calibration used is from another cell). We can therefore determine an "intra-cell" RMSD and an "inter-cell" RMSD to reflect the voltage resolution of these two types of measurements. To calculate "intra-cell" error, we look at the RMSD between V_{ophys} and V_{FLIM} using the $\tau_{fl}-V_{mem}$ relationship *for that specific cell*. Phrased another way, we are looking at the amount of error that would be expected in estimating V_{mem} of a cell if its exact $\tau_{fl}-V_{mem}$ relationship were known. This "intra cell" RMSD estimates the error expected in quantifying changes in V_{mem} on a given cell. We calculate an intra cell error for each cellular recording, so intra cell errors are reported throughout the text as a mean $\pm$ SEM of the intra cell errors for all individual cells of a given type. The average intra cell error was at or below 5 mV for all cell lines tested (**Table 2.4**).

The error in the absolute membrane potential determination ("inter-cell") is calculated here as the RMSD between the y-intercept (0 mV lifetime) of all of the individual cells' lifetime-voltage relationships and the 0 mV value for the averaged calibration *for all cells of a given type*. This

metric quantifies how well the lifetime- V_{mem} relationship for a given cell line is likely to represent an individual cell's lifetime- V_{mem} relationship. This “inter cell” RMSD ranged from 10 to 23 mV for the tested cell lines (**Table 2.4**). Much smaller errors for a population value of V_{mem} can be obtained by averaging V_{mem} recordings from multiple cells.

This method of calculating error assumes that the electrophysiology measurement is perfectly accurate and precise. Realistically, it is likely that some of the variation seen is due to the quality of the voltage clamp. As a result, these RMSD values provide a conservative upper bound for the voltage errors in VF-FLIM.

Analysis of CAESR Lifetimes

For sample images of CAESR in HEK293T (**Fig. 2.5**), fluorescence decays were fit using SPCImage to a biexponential decay model as described for VF2.1.CI above, using a peak photon threshold of 150 and a bin of 2 (binned pixel width of 5 μm). To better match the studies by Cohen and co-workers²⁷, which isolated the membrane fluorescence from cytosolic fluorescence by directing the laser path, the lifetime-voltage relationships were not determined with these square-binned images. Instead, membranes were manually identified, and the fluorescence decays from all membrane pixels were summed together before fitting once per cell. (This is in contrast to the processing of VoltageFluor data, where the superior signal to noise and localization enables fitting and analysis of the lifetime on a pixel by pixel basis). This “one fit per membrane” analysis of CAESR was performed in custom MATLAB code implementing a Nelder-Mead algorithm (adapted from Enderlein and Erdmann⁷⁷). CAESR data were fit to a biexponential model with the offset fixed to 0 and the color shift as a free parameter.

Di-8-ANEPPS Ratio-based Imaging

In preparation for imaging, HEK293T cells were plated as described above for electrophysiology. 1 μM di-8-ANEPPS was loaded for ten minutes in HBSS at room temperature and atmospheric CO_2 . Coverslips were washed twice in HBSS and transferred to fresh HBSS for imaging. No surfactants were used in the loading (e.g. Pluronic F-127) because their presence worsened cell robustness for whole-cell patch-clamp electrophysiology. All recordings were made with HBSS as an extracellular solution; no cells were kept for more than 30 minutes after dye loading due to the increasing presence of internalized dye.

Epifluorescence imaging was performed with an inverted Observer.Z1 (Carl Zeiss Microscopy) controlled with $\mu\text{Manager}$ 1.4 (Open Imaging)⁷⁸. Images were acquired with an Orca Flash 4 Digital CMOS camera (Hamamatsu Corporation; San Jose, CA). Excitation light was provided with a Spectra X light engine (Lumencor, Inc.; Beaverton, OR). Excitation wavelengths were selected with built-in filters in the Spectra X (440/20 bandpass filter for blue and 550/15 bandpass filter for green). Blue-excited images were obtained with an excitation power of 71 mW/mm^2 and an exposure time of 50 ms. Green-excited images were obtained with an excitation power of 136 mW/mm^2 and an exposure time of 500 ms. Emission light was collected with a 40x magnification oil immersion objective lens using Immersol 518F immersion oil (Zeiss). Fluorescence emission was selected with a 562 nm long pass dichroic mirror and further filtered by a 593/40 bandpass filter (Semrock). Excitation and emission wavelengths were selected to match previous work with this probe as closely as possible¹⁶ (current excitation[blue]: 440 ± 10 nm; reported excitation[blue]: 440 ± 15 nm; current excitation [green]: 550 ± 7.5 nm; reported

excitation: 530 ± 15 nm; current dichroic: 562 nm long-pass; reported dichroic: 565 nm; current emission: 593 ± 20 nm; reported emission: 570 nm long pass)

Di-8-ANEPPS Data Analysis

Single color (e.g. blue excited or green excited) fluorescence images were background subtracted at each pixel before ratios were calculated. The background value was determined from a region of interest near the center of the image that contained no cells and minimal fluorescent debris. Excitation ratios (“R”, blue signal divided by green signal, B/G) were then calculated pixelwise from the background subtracted fluorescence images. Pixels with less than 100 arbitrary units of signal in either the blue or the green channel were excluded from analysis and are depicted in black. Regions of interest (ROIs) were manually selected in ImageJ to include only area corresponding to the cell membrane. The ratio was averaged across all pixels in a given ROI (similar to the treatment for VF-FLIM, as described in **Fig. 2.1**). The ratio values per value of V_{mem} (set by whole cell patch clamp electrophysiology) in **Fig. 2.6E,F** are the average of these cell-averaged ratios obtained in 6 or 7 sequential images acquired while the V_{mem} was held at the indicated value.

Where normalized R values are discussed, these values were calculated by dividing the ratio at a given potential (averaged for an ROI as discussed above) by the ratio at 0 mV, as reported previously¹⁶. This normalization procedure requires electrode-based calibration for every individual recording and cannot be stably extended to all cells from a particular cell line. Therefore, it is not analogous to VF-FLIM and is not the point of comparison for voltage resolution.

Statistical Analysis

Mean $\pm$ standard error of the mean (S.E.M.) of data is reported throughout the text. Hypothesis testing was performed as indicated with either analysis of variance (ANOVA) followed by appropriate post hoc tests or two-sided, unpaired, unequal variances t-tests. Statistical tests were performed in Python 2 or 3 with the SciPy, pandas and Pingouin⁷⁹ packages. Unless otherwise noted, all data shown reflect at least three biological replicates (independent cultures measured on different days). Each of these biological replicates contained between 1 and 5 technical replicates (different samples of cells that were measured on the same day and had been prepared from the same cell stock). For tandem electrophysiology-FLIM measurements, each $\tau_{\text{fl}}-V_{\text{mem}}$ calibration includes three biological replicates to capture the variability expected during applications of VF-FLIM. No power analyses were performed before data were collected. Sample sizes throughout the text refer to the total number of cells or cell groups of a given type analyzed across all biological and technical replicates. Cell group identification is discussed in **Methods**. For experiments where resting membrane potential or resting membrane potential changes are compared to a baseline (Figures 3-5 and supplements), both control measurements and their physiologically or pharmacologically altered counterparts were recorded on each experimental day. Masking was not used during data collection or analysis.

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Chapter 3

Genetically Targeted VoltageFluor-FLIM for Cell Type Specific Optical Measurement of Membrane Potential

Portions of this work were done in collaboration with the following people:
VF-HaloTag was synthesized by Dr. Vincent Grenier
Plasmids were constructed by Dr. Pei Liu and Dr. Molly Kirk

Abstract

Membrane potential (V_m), the voltage across a cell membrane, is an important biophysical phenomenon, taking up much of the cell's energy to maintain. Changes in V_m are implicated in cellular signaling, from fast neuronal action potentials, to slower changes, such as those in long term potentiation and differentiation of neurons. Additionally, the millivolt value of V_m , the absolute voltage, has been hypothesized to be different in some subcellular structures, such as dendritic spines, which are known to be calcium compartmentalized with respect to the rest of the neuron. However, electrophysiological monitoring of voltage across long time scales or in small structures is difficult. The invasive nature of electrophysiology causes cell death over extended times, and can damage small structures. Additionally, the ability to access measurements of subcellular structures with electrophysiology is limited by the size of the electrode itself. The ability of existing optical methods to resolve this problem is limited due to the minimal ability of genetically encoded sensors to report absolute V_m with fidelity, and the fact that established chemical dyes that can report V_m are not cell-type or structure-specific in their staining. To begin to address these issues, we expand upon the VF-FLIM platform by adapting it for use with chemical-genetic targeting systems. We characterize and calibrate two different targeting protein/dye pairs (VoltageSpy and VF-HaloTag) in fluorescence lifetime and assess their performance in neurons for future applications towards making optical measurements of absolute membrane potential in primary neuronal culture.

Introduction

Membrane potential (V_m) arises due to difference in ionic concentration across the selectively permeable plasma membrane of cells. Many changes in membrane potential occur cell-wide, and the resting potential is set across the entire cell, in most cases. However, some cellular substructures are compartmentalized with regard to some ions and maintain an ionic gradient between that subcellular structure and the rest of the cell, resulting in subcellular membrane potential differences.¹⁻⁴ These include certain organelles, including mitochondria, which maintain a proton gradient and so their own membrane potential. Also of interest are subcellular structures like primary cilia and dendritic spines, which are compartmentalized with respect to calcium and may be electrically compartmentalized as well.⁵

Dendritic spines are small structures on neuronal dendrites that receive the majority of synaptic input, yet despite their importance in neuronal signaling, their geometry and small size has made it difficult to study their role in electrical signal conduction.⁶ It has been proposed that the high resistance of the spine neck could effectively compartmentalize dendritic spine voltage from the rest of the dendrite.⁷ In glutamate uncaging experiments, excitatory postsynaptic potentials were attenuated significantly between the dendritic spine and the parent dendrite.⁸ These results suggest that in addition to differences in voltage dynamics, spines may also have differences in resting membrane potential, which could affect excitability and profoundly alter synaptic signaling. However, direct investigation of V_m in small subcellular structures like dendritic spines has been limited by the tools available to measure V_m . The standard measurement technique, patch-clamp electrophysiology is not feasible at dendritic spines due to the small size of the spine head ($< 1 \mu\text{m}$), which is smaller than that of the electrode used for patch-clamp techniques.

Optical methods open an avenue to making measurements at these smaller dimensions, as they are limited by the optical diffraction limit, but not by the size of an electrode or glass pipette. However, many optical methods that can report voltage dynamics are poorly suited for

reporting measured values of resting membrane potential or reliably reporting the magnitude of the voltage changes observed (**Chapter 1**).⁹ VoltageFluor (VF) potentiometric dyes, however, report changes in V_m through a photoinduced electron transport mechanism (PeT). Because the voltage change affects the excited state of the dye, the voltage can be monitored through the fluorescence lifetime of the dye – this forms the basis for VoltageFluor-Fluorescence Lifetime Imaging Microscopy (VF-FLIM), the method outlined in **Chapter 2**.¹⁰ The first generation of VF-FLIM, however, utilized a VF dye that stains all membranes indiscriminately. This works well in the case of cultured cells where the cell density can be controlled through culture conditions, but makes resolving small structures in complex cultures, such as neuronal processes like axons and dendrites, let alone the smaller dendritic spines, difficult. Signal from dendritic spines becomes lost in the neuropil, and it becomes difficult to trace signal from a particular cell and track the lifetime throughout the dendritic arbor of that cell.

Genetically encoded voltage indicators (GEVIs) could resolve this issue, by providing fluorescent signal from only those genetically defined cells expressing the GEVI. However, many current GEVIs have mechanisms of voltage-sensing that do not involve changes in the excited state, and thus are not voltage-sensitive in lifetime. Those that are, such as the mCitrine-based CAESR have variable levels of voltage response, voltage-insensitive signal due to poor membrane trafficking, and CAESR has poor absolute voltage resolution, 19-fold poorer than the VF-FLIM technique.¹⁰ Combining the benefits of genetic sensors (cell-type specific signal, lack of background interference) with those of chemical dyes (increased sensitivity and resolution in lifetime, more predictable linear responses to voltage) would provide a platform for improved sensing of V_m and quantification of voltage changes in complex cultures and at subcellular structures.

To that end, we adapted two chemical-genetic voltage sensing systems, using a genetically encoded targeting protein and a synthetic VF dye that covalently tethers to the targeting protein. These two platforms, VoltageSpy¹¹ and VF-HaloTag, demonstrate cell-type specific staining, linear responses between voltage and lifetime, and improved resolution of V_m compared to FLIM-compatible GEVIs.

Results and Discussion

Fluorescence lifetime characterization of parent sarcosine-VF dye

Before pursuing lifetime measurements in genetically targeted dyes, we sought to characterize the resting lifetime and voltage sensitivity of the parent dye, mVF-Sarcosine in fluorescence lifetime. The characterization of mVF-Sarcosine provides a baseline for comparison to the targeted VoltageFluor (VF) behaviors in lifetime. This is a more apt comparison than the previously described VF2.1.Cl, as VF2.1.Cl has a different positioning of the phenylenevinylene molecular wire on the pendant ring relative to the fluorophore, and lacks the reactive handle that enables attachment of the genetic targeting moiety, while mVF-Sarcosine has its molecular wire in the meta position relative to the fluorophore (**Figure 3.2a**), and contains a sarcosine group, as does the genetically targeted dye VoltageSpy. The other targeted dye developed here, VF-HaloTag, uses a different anchoring and targeting group than VoltageSpy and mVF-Sarcosine, but still has the same meta positioning of the molecular wire, making mVF-Sarcosine a closer parent than VF2.1.Cl for comparison.

We measured the fluorescence lifetime of mVF-Sarcosine in HEK293T cells at rest at a range of dye concentrations. mVF-Sarcosine has a mean τ_{fl} of 1.91 ± 0.01 ns (mean $\pm$ SEM, 100 nM dye) in HEK293T cells (**Fig. 3.1**) The time-resolved fluorescence decay of mVF-Sarcosine in

HEK293T cells is well-described by a two-component exponential model. The lifetimes reported for this dye are thus the amplitude weighted mean of the two components, τ_{fl} . At higher concentrations, we observe concentration-dependent decreases in fluorescence lifetime (Chapter 2).¹⁰ Diminishing of the lifetime occurs between 500 nM and 1 μ M (Fig. 3.1, Figure 3.2b), and all subsequent usage of the dye was done at 100 nM, because this concentration was less variable than 150 nM or 200 nM dye, but still bright enough to visualize well.

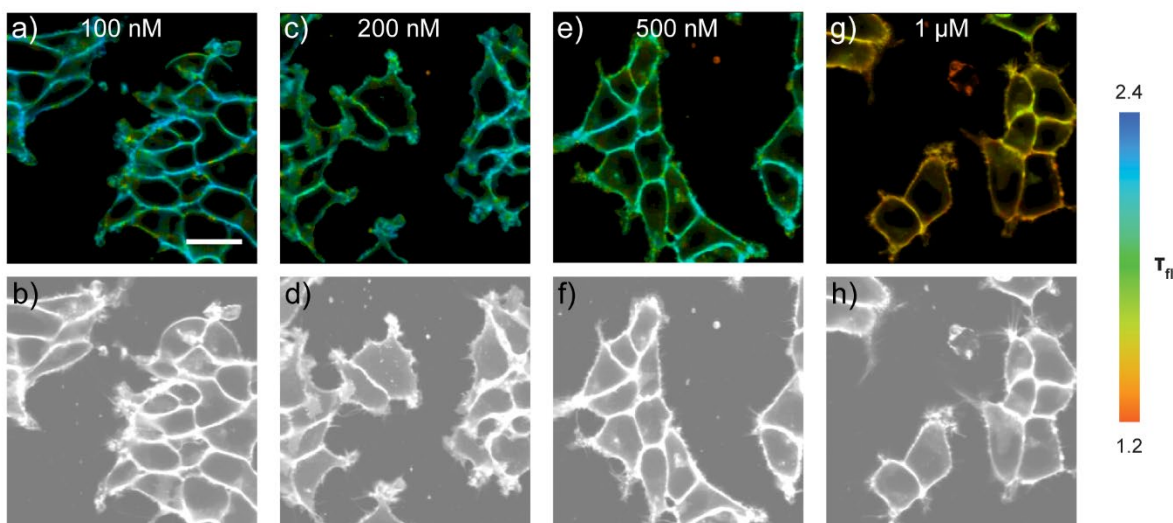


Figure 3.1 Fluorescence lifetime and photon counts of mVF-Sarcosine in HEK293T cells.

Fluorescence lifetime (a, c, e, g) and fluorescence intensity (photon count) (b, d, f, h) images of a),b) 100 nM, c),d) 200 nM, e),f) 500 nM, and g), h) 1 μ M mVF-Sarcosine in HEK293T cells. Fluorescence lifetime scaled from 1.2 to 2.4 ns. Fluorescence intensity / photon count images are not scaled to one another. Scale bar is 20 μ m.

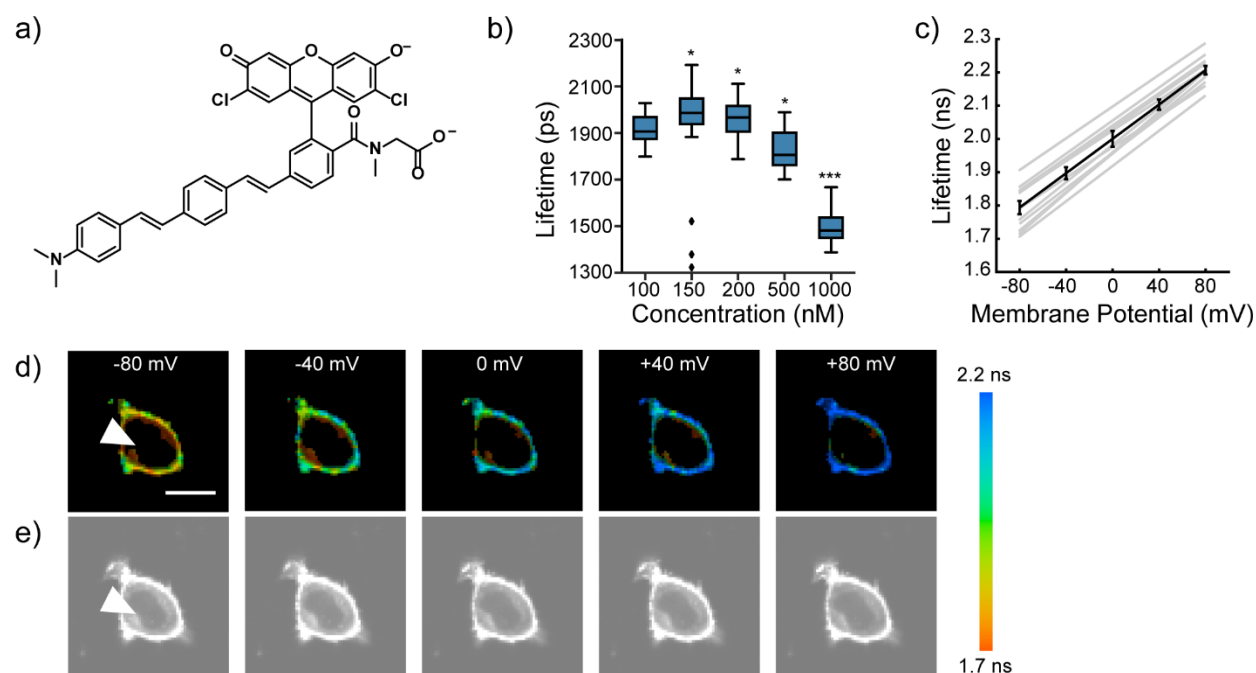


Figure 3.2 Structure and characterization of mVF-Sarcosine parent dye in FLIM.

a) Structure of mVF-Sarcosine, the parent dye for the VoltageSpy targeted dye. b) Concentration curve demonstrating concentration-dependent decrease in the fluorescence lifetime of mVF-Sarcosine, occurring around 500 nM nominal dye loading. * = $p < 0.05$. *** = $p < 0.001$, as compared to 100 nM dye. c) Graph of the linear relationship between τ_{fl} and voltage of mVF-Sarcosine. The gray lines represent individual patches of cells and their individual lines of best fit, and the black line represents the average calibration from the individual calibrations (error bars $\pm$ SEM; $n = 11$ cells). d) Rainbow lifetime heatmap overlaid on intensity image. Lifetime scaling from 1.7 ns to 2.2 ns. e) Photon count images at each potential. Photon count images are not scaled to one another. Scale bar 20 μ m.

mVF-Sarcosine shows V_m -sensitive τ_{fl} in voltage-clamped HEK293T cells. Voltages were applied to HEK293T cells stained with mVF-Sarcosine and lines of best fit were generated from the resulting τ_{fl} (**Figure 3.2c-d**). The sensitivity of the lifetime of mVF-Sarcosine is 2.58 ± 0.07 (mean $\pm$ SEM, $n = 11$ cells) ps/mV, with a y-intercept (or average lifetime at 0 mV) of 2.00 ± 0.02 ns (**Table 3.1**). Applying this calibration back to our measured lifetime of mVF-Sarcosine in HEK293T cells at rest reveals a resting V_m of -33.9 ± 4.7 mV (mean $\pm$ SEM), well within previously reported ranges for HEK293T cells, as reported both by other optical sensors and by electrophysiological measurements.^{10,12-15}

The voltage resolution, a measurement of the voltage-independent noise of this calibration, can be discussed in two ways: the intra-cell resolution and the inter-cell resolution. We use two different metrics of resolution for VF-FLIM calibrations because the lifetime-voltage relationship prediction is much more accurate for tracking changes in V_m values within a cell (intra-cell resolution) than for comparing values of V_m between cells (inter-cell resolution). For calculation of and explanation of inter- and intra-cell resolution, see **Methods**. The intra-cell resolution for mVF-Sarcosine in HEK293T cells is 10.4 ± 1.6 mV, and the inter-cell resolution is 20. mV. This intra-cell resolution is somewhat poorer than that previously reported for VF2.1.Cl. The inter-cell resolution of mVF-Sarcosine is similar to previously reported values of untargeted VF dyes.¹⁰

This calibration corresponds to a fractional change in τ_{fl} ($\Delta\tau/\tau$) of $14.0 \pm 0.5\%$ per 100 mV. Interestingly, this does not correspond particularly well with the fractional change observed in fluorescence intensity, $\Delta F/F$ per 100 mV, which was reported as $22.7 \pm 0.1\%$ per 100 mV, unlike previously reported VFs which had good agreement between their $\Delta\tau/\tau$ and $\Delta F/F$ values.¹¹ This discrepancy suggests the possibility that there may be a secondary voltage-sensitive mechanism other than PeT that does not affect the excited state of the dye, though this has not been confirmed experimentally. Nevertheless, these values of resting lifetime and sensitivity in HEK293T cells can be considered as a baseline upon which to compare the targeted dyes, VoltageSpy and VF-HaloTag in lifetime.

Lifetime measurements of VoltageSpy in HEK293T cells

The VoltageSpy chemical-genetic targeting system and dye was initially used in the Miller lab to selectively stain individual neurons within a crowded and complex culture of primary hippocampal-cortical neurons. It utilizes the SpyCatcher protein, expressed on the cell's plasma membrane, paired with a modified mVF-Sarcosine dye, with the sarcosine group appended to a polyethyleneglycol (PEG) linker attaching the VoltageFluor to the SpyTag

targeting peptide.¹¹ We thus sought to apply this successful system to extend the VF-FLIM method to allow us to optically read out membrane potential in genetically defined cells.

VoltageSpy in HEK293T cells also displays V_m -dependent τ_{fl} . In HEK293T cells expressing SpyCatcher and stained with 25 nM VoltageSpy, VoltageSpy has a lifetime-voltage sensitivity of 2.52 ± 0.16 ps/mV (mean $\pm$ SEM), and a y-intercept value for the calibration of 1.81 ± 0.04 ns (mean $\pm$ SEM) (**Figure 3.3a and b, Table 3.1**). This calibration corresponds to a fractional change of $15.1 \pm 0.9\%$ per 100 mV, in good agreement with both the results from the parent dye mVF-Sarcosine and the reported $\Delta F/F$, previously reported as $12.7\% \Delta F/F$ per 100 mV. HEK293T cells at rest treated with VoltageSpy have a mean τ_{fl} of 1760 ps, which corresponds to an average resting membrane potential of -18.8 ± 5.4 mV (mean $\pm$ SEM). This is more depolarized than the typically reported electrophysiological measurements of HEK293T cell V_m , which range from -24 mV¹⁵ to -52 mV.¹³ The depolarized values here may be a result of the variability in VoltageSpy calibration (Figure 3.3) or variability in cell health with transfected cells.

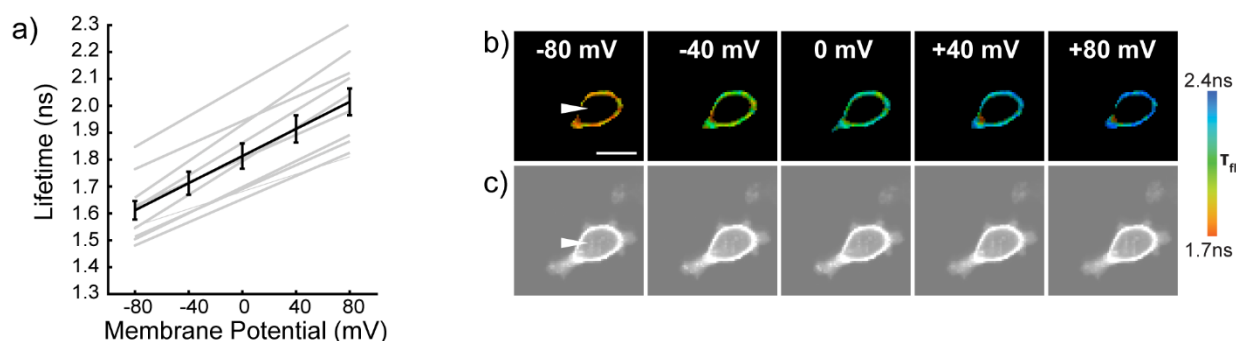


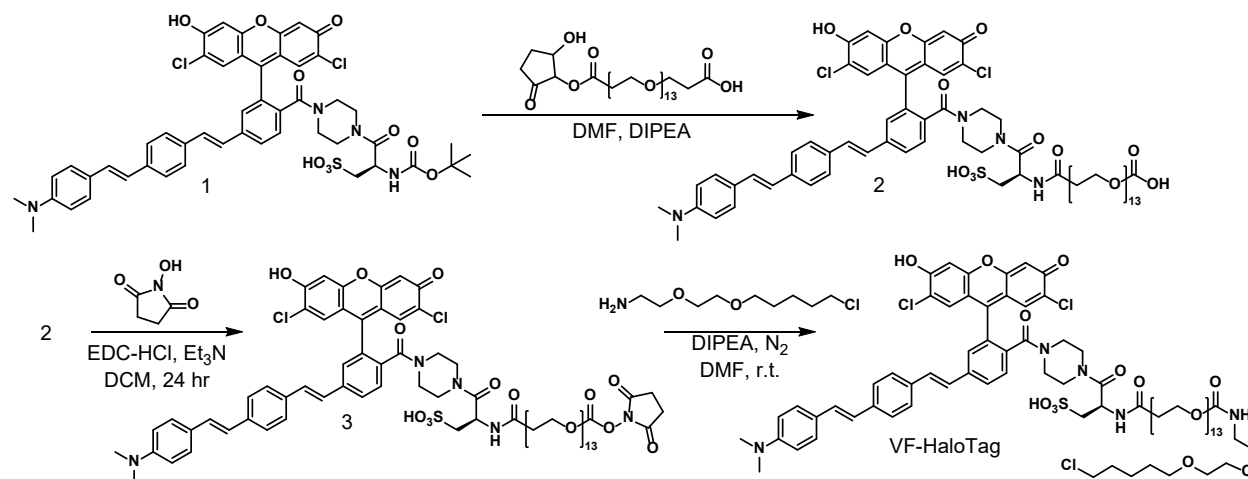
Figure 3.3 VoltageSpy shows V_m -dependent τ_{fl} .

a) Plot of the linear relationship between lifetime and voltage for VoltageSpy. Each gray line represents an individual cell's line of best fit. The black line represents the average calibration (error bars $\pm$ SEM). b) Lifetime and c) photon count images of an individual cell's changes in lifetime when voltage clamped at -80, -40, 0, +40, and +80 mV. Lifetime scaled from 1.7 to 2.4 ns. Photon count images are not scaled to one another. Scale bar 20 μ m.

The targeted VoltageSpy dye exhibits more uncertainty in its lifetime-voltage calibration than the parent mVF-Sarcosine. The intra-cell error of VoltageSpy in HEK293T cells is 12.2 ± 1.8 mV, similar to the parent dye mVF-Sarcosine. The inter-cell resolution, however, is much more variable, with an error of 52 mV. This is consistent with the increased variability in the y-intercept for the targeted dye as compared to the parent dye. These values of resolution suggest that VoltageSpy may find usage in VF-FLIM for quantitative measurements of membrane potential changes within a genetically defined cell over time, if the change is large enough in magnitude to be observed. However, the very large inter-cell resolution suggests that making comparisons between the lifetimes of two cells and trying to draw conclusions about their voltages is not feasible with VoltageSpy in these systems. This additional variability compared to the parent dye may be due to the restriction of the dye to an environment near the targeting protein. Alternatively, the long PEG linker may enable enough conformational flexibility in the dye that it could orient in the membrane in different ways across a cell and between cells.^{16,17}

Synthesis of VF-HaloTag

Due to the large inter-cell error exhibited by VoltageSpy in FLIM, we investigated an alternative chemical-genetic targeting pair, using the HaloTag system. The HaloTag protein reacts with a chloroalkane group, covalently tethering the payload attached to the other end of the linker to the protein, in this case, the VF-HaloTag dye.¹⁸ We previously showed that HaloTag targeting can be used for intensity-based voltage imaging with rhodamine and Si-rhodamine VF dyes.^{16,17}



Scheme 3.1 Synthesis of VF-HaloTag

Unlike VoltageSpy, VF-HaloTag does not use a sarcosine group as the membrane anchor and targeting group attachment site. Rather, it is based on a VoltageFluor scaffold with a piperazine-cysteic acid residue at the membrane anchoring site, as previously reported.¹⁹ Following the general strategy in the synthesis of VoltageSpy, a flexible PEG linker group was incorporated via an amide coupling of VF **1** (Scheme 3.1) to a heterobifunctional PEG₁₃ linker, with one end containing an ester and the other an acid. The free acid was then converted to an NHS group, in quantitative yield to produce **3**. This was then coupled to the haloalkane HaloTag ligand via amide coupling, and the final VF-HaloTag was purified by semi-preparative HPLC.

Fluorescence Intensity Characterization of VF-HaloTag

VF-HaloTag selectively labels HaloTag-expressing HEK293T cells. HEK293T cells were transfected with DNA encoding HaloTag, directed to the plasma membrane with either a pDisplay or DAF element, along with a nuclearly localized mCherry protein to confirm transfection. Cells were then treated with 100, 50, or 25 nM VF-HaloTag dye, and the relative fluorescence intensity recorded (Figure 3.4a, c, f). We then determined the contrast ratio between transfected cells and untransfected cells on the same image. Cells where the HaloTag protein was localized to the plasma membrane with pDisplay exhibit improved contrast ratio as compared to DAF, particularly at higher concentrations of dye: at 100 nM VF-HaloTag, HaloTag-DAF cells have a contrast ratio of transfected to untransfected cells of 4.8, while those with HaloTag-pDisplay have a ratio of 7.1 (Figure 3.4e). At lower concentrations (25 nM), the pDisplay construct performed even better, reaching a mean contrast ratio of 21.9, while DAF exhibits a mean contrast ratio of 12.6, similar to that reported for VoltageSpy in HEK293T cells, which was 12.9 at 25 nM dye.¹¹ Further work was performed at 25 nM dye and the pDisplay

construct was chosen for further work due to its improved contrast ratio, despite the DAF construct's slightly better brightness at low concentrations.

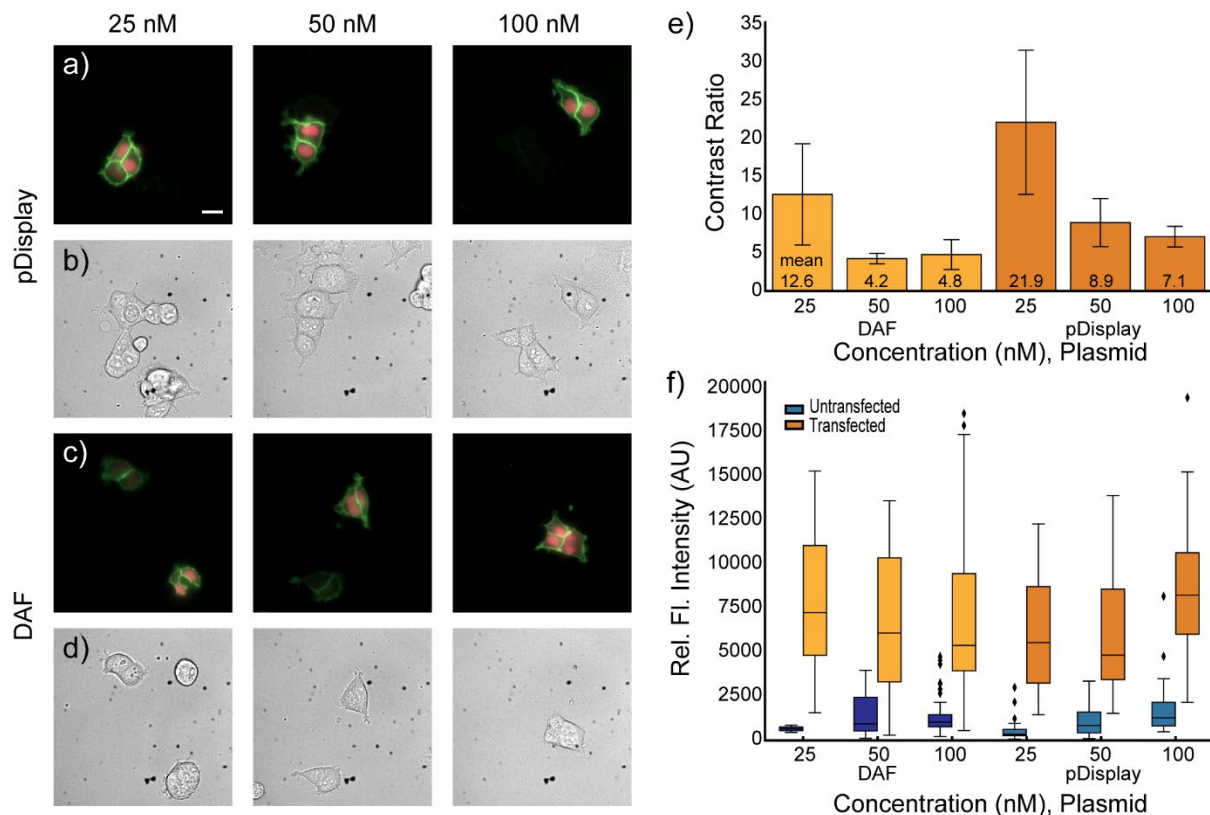


Figure 3.4 Comparison of DAF and pDisplay membrane targeting domains and cellular brightness.

a) Fluorescence images of VF-HaloTag stained cells expressing HaloTag with a pDisplay membrane secretion sequence, demonstrating the selectivity of VF-HaloTag staining (green) to only those cells transfected with mCherry (magenta). Scale bar 20 μ m. Fluorescence images all scaled to one another. b) Brightfield images of the cells transfected with the pDisplay construct. c) Fluorescence images of VF-HaloTag stained cells with a DAF construct. Fluorescence images scaled to one another and those in a). d) Brightfield images of the cells in c). e) Quantification of the contrast ratio between transfected and untransfected HEK293T cells. Contrast ratio was determined by normalizing the intensity of transfected cells by that of untransfected cells on the same image. Error bars for contrast ratio are mean $\pm$ SEM, from $n = 4$ images per condition. Annotations are mean contrast ratio at each condition. f) Quantification of relative fluorescence units for transfected and untransfected cells stained with VF-HaloTag on the same coverslip to determine contrast. Transfected cells were defined as those expressing nuclear mCherry. Relative intensities were background-subtracted.

VF-HaloTag shows V_m -dependent changes in fluorescence intensity. HEK293T cells transfected with the HaloTag-pDisplay plasmid and incubated with 25 nM VF-HaloTag were patched, and the fluorescence intensity measured at each membrane potential (**Figure 3.5a and c**). The cells were held at potentials from 100 to -100 mV, in 20 mV increments, in a whole-cell voltage-clamp configuration. VF-HaloTag exhibits a voltage sensitivity of $15.7 \pm 1.0\%$, slightly

improved sensitivity compared to VoltageSpy (**Figure 3.5b, Table 3.1**). This improved voltage-sensitivity in intensity suggests the potential for VF-HaloTag to be a more tractable dye in FLIM than VoltageSpy.

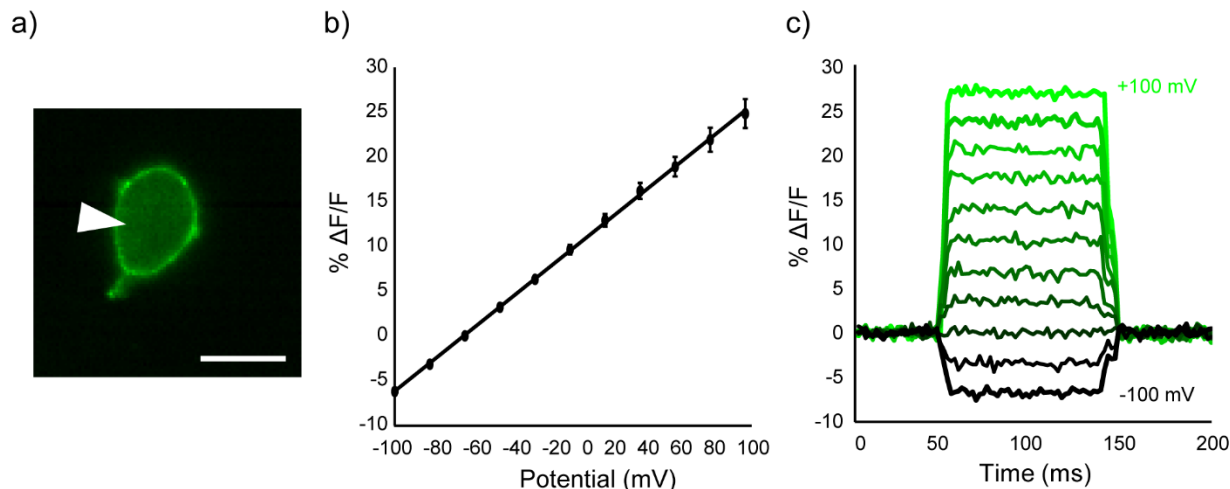


Figure 3.5 Electrophysiological characterization of VF-HaloTag in fluorescence intensity.

a) VF-HaloTag staining of a representative single HEK293T cell. Triangle indicates the position of the patch pipette. Scale bar 20 μm . b) Linear fluorescence response to voltage of VF-HaloTag to voltage, averaged over 7 cells. Scale bars are $\pm$ SEM. c) Representative concatenated trace of $\% \Delta F/F$ response to voltage, from +100 to -100 mV.

Lifetime measurements and calibration of VF-HaloTag in HEK293T Cells

VF-HaloTag shows V_m -dependent changes in τ_n . The fluorescence lifetime of VF-HaloTag is also not well described by a monoexponential or two-component exponential decay, and was instead fit with a three-component exponential decay model, and the lifetimes reported are the amplitude weighted average. The lifetime-voltage calibration of VF-HaloTag shows a sensitivity of 1.41 ± 0.10 ps/mV (mean $\pm$ SEM) with a y-intercept (0 mV lifetime) of 1.35 ± 0.02 ns (**Figure 3.6a and b**). The calculated fractional change in lifetime per 100 mV for this dye is $11.2 \pm 0.9\% \Delta\tau/\tau$, somewhat lower than the sensitivity in intensity, and slightly lower than that of VoltageSpy, which is the inverse of the relationship between the dyes in fluorescence intensity, where VF-HaloTag is more sensitive than VoltageSpy (**Table 3.1**). Applying this calibration to a measured lifetime of VF-HaloTag in HEK293T cells at rest converts the lifetime of 1307 ps to -31.2 mV, representing a more typical resting voltage for HEK293T cells than determined with VoltageSpy.

This improved measurement of resting voltage may be due in part to the differences in the error and resolution of this calibration for VF-HaloTag compared to VoltageSpy. For VF-HaloTag, the intra-cell error is 12.9 ± 2.3 mV, while the inter-cell error is 36.3 mV. While the intracellular voltage resolution is comparable to that of VoltageSpy, the intercellular resolution is improved (**Table 3.1**). This improved intercellular resolution may be why VF-HaloTag reports resting voltage in HEK293T cells at a more typical value than VoltageSpy. VF-HaloTag is superior to VoltageSpy for comparisons between cells, but still has reduced precision compared to the untargeted parent dyes. Its utility for monitoring resting voltage is thus still limited, but in cases where voltage changes are relatively large, VF-HaloTag may be a useful tool for quantifying voltage changes over time.

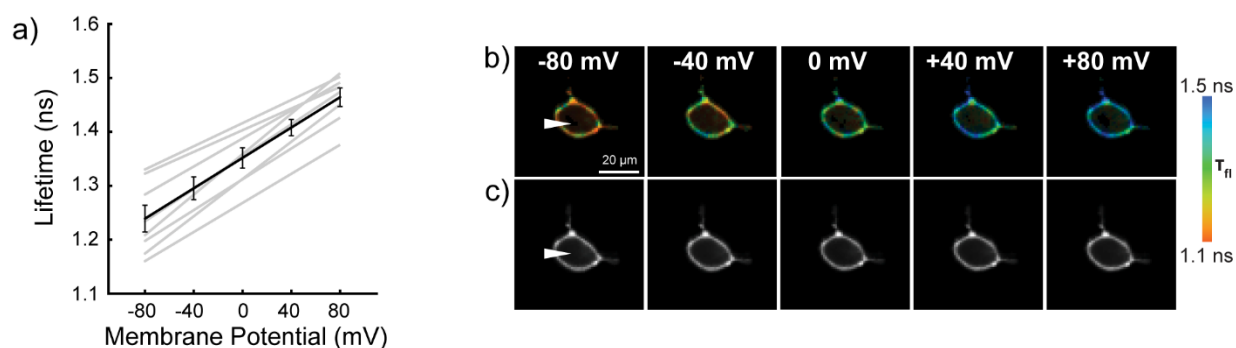


Figure 3.6 Lifetime-voltage relationship of VF-HaloTag.

a) Representation of the linear relationship between τ_n of VF-HaloTag and the membrane potential. Each gray line represents the lifetime-voltage relationship of an individual cell and its line of best fit. The black line is the average line of best fit (error bars $\pm$ SEM; $n = 8$ cells). b) Lifetime and c) photon count images of an individual cell's changes in lifetime at different voltages. Lifetime scaled from 1.1 ns to 1.5 ns. Photon counts are not scaled to one another. Scale bar 20 μ m.

Dye	Slope (ps/mV)	Y-Intercept (ns)	Intra-cell error (mV)	Inter-cell error (mV)	% $\Delta F/F$	% $\Delta \tau/\tau$
mVF-Sarcosine	2.58 ± 0.07	2.00 ± 0.02	10.4 ± 1.6	20.	22.7 ± 0.1^a	14.0 ± 0.5
VoltageSpy	2.52 ± 0.16	1.81 ± 0.04	12.2 ± 1.8	52	12.7 ± 0.1^a	15.1 ± 0.9
VF-HaloTag	1.41 ± 0.10	1.35 ± 0.02	12.9 ± 2.3	36	15.7 ± 1.0	11.2 ± 0.9

Table 3.1 Comparison of lifetime-voltage calibrations, % $\Delta F/F$, and % $\Delta \tau/\tau$.

All data determined from voltage-clamped HEK293T cells. [a] Reproduced from Grenier et. al., *J. Am. Chem. Soc.* **2019**, 141, 3, 1349-1358.

Evaluation of VF-HaloTag and VoltageSpy performance in Neurons

VF-HaloTag shows improvement in the inter-cell voltage resolution from lifetime measurements over VoltageSpy in HEK293T cells. The ultimate goal of these genetically targeted dyes, however, is for use in complicated cultures where equally staining all cells would be undesirable, such as in primary neuronal culture. To assess the performance of the two dyes in neuronal culture, we compared them in both fluorescence intensity-based imaging and FLIM (**Figure 3.7 a-h**). In epifluorescence microscopy, we compared the contrast ratio and brightness of VoltageSpy and VF-HaloTag in neurons (**Figure 3.7 i, j**). In a side-by-side comparison of VF-HaloTag and VoltageSpy, VoltageSpy has a contrast ratio of 7.3 (**Figure 3.7 i**), similar to the previously reported ratio of 5 under identical conditions.¹¹ At concentrations above 5 nM, VoltageSpy provides no contrast, due to non-specific labeling.

VF-HaloTag can be used at higher concentrations to achieve both high brightness and good contrast between HaloTag-expressing neurons and wild-type neurons. At 25 nM VF-HaloTag, neurons expressing HaloTag have a higher contrast ratio of 10.4 and a mean intensity three times brighter than the VoltageSpy-treated neurons. This suggests that while VoltageSpy is a baseline brighter dye, with a longer fluorescence lifetime, it exhibits more off-target staining at

higher concentrations, and VF-HaloTag can provide brighter staining while retaining the same or better contrast ratio (**Figure 3.7 i, j**). For this reason, VF-HaloTag is more amenable for use in neurons or other complex culture for photon-starved applications such as FLIM.

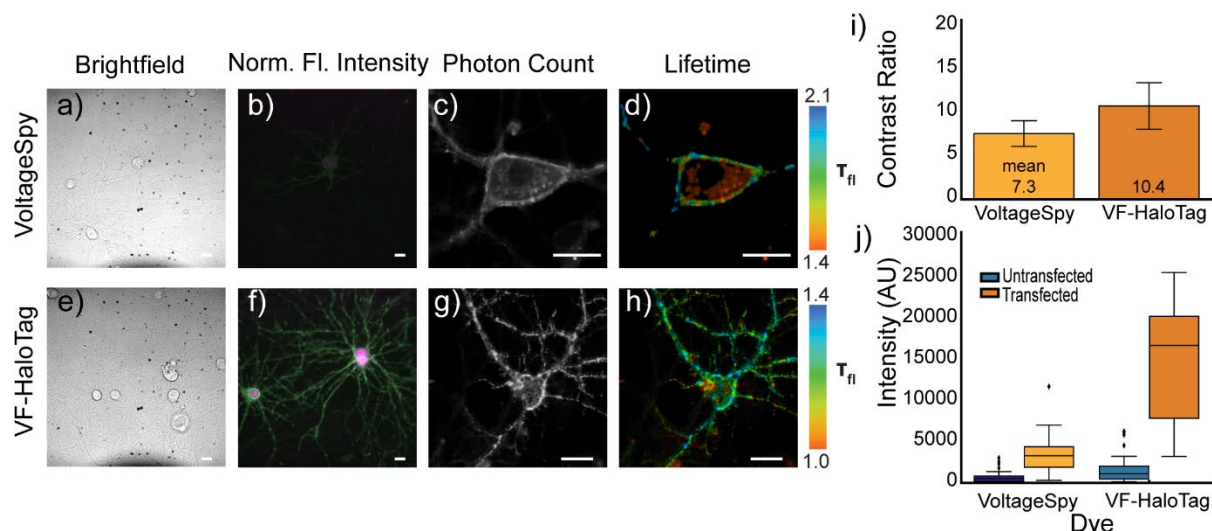


Figure 3.7 Comparison of the staining and contrast of VF-HaloTag and VoltageSpy in neurons, in both intensity and lifetime.

a) Brightfield image of VoltageSpy stained neurons. b) Fluorescence intensity image of VoltageSpy stained and transfected neuron. Normalized to f). Scalebars 20 μm . c) Photon count (fluorescence intensity) image of VoltageSpy stained neuron from FLIM acquisitions. Photon count images from FLIM are not normalized. d) Lifetime heatmap for VoltageSpy stained neuron. Scalebars 20 μm . Lifetime heatmap scaled from 1.4 ns to 2.1 ns. e) Brightfield image of VF-HaloTag stained neurons. f) Fluorescence intensity image of VF-HaloTag staining in a transfected neuron. Normalized to b). Scalebars are 20 μm . g) Photon count (fluorescence intensity) image of VF-HaloTag stained neuron from FLIM acquisition. Photon count images not normalized. h) Lifetime heatmap for a VF-HaloTag stained neuron. Scalebars 20 μm . Lifetime heatmap scaled from 1.0 ns to 1.4 ns. i) Quantification of contrast ratio (measured as the ratio of intensity of transfected neurons divided by the intensity of untransfected cells on the same coverslip) for both VoltageSpy and VF-HaloTag. Annotations are mean contrast ratio for each condition. Error bars are mean $\pm$ SEM for $n = 10$ images and j) fluorescence intensity for both VoltageSpy and VF-HaloTag. VoltageSpy was used at 5 nM dye, and VF-HaloTag was used at 25 nM dye.

This difference in brightness at similar contrast becomes very apparent in FLIM, as imaging of VoltageSpy without phototoxicity to the cells is limited to resolution of the neuronal somata, and portions of the larger dendritic processes proximal to the soma (**Figure 3.7c, d**). VF-HaloTag, however, enables imaging of the soma and many of the large and medium processes of the neurons, and allows visualization of the dendritic spines (**Figure 3.7g, h**). Individual spines are not quite resolvable in lifetime, and thus we are unable to determine the resting voltage reliably, due to the pixel binning (see discussion of effective pixel size in **Chapter 2**, and in **Methods**), which results in combining signal of the dendritic spine and neighboring dendrite. This pixel binning is necessary for VF-HaloTag, as many photons (at least 10,000 for a 3-component fit)²⁰ are needed per fit decay to obtain reliable and consistent lifetimes from the

decay. Future improvements in photon flux, either by developing a brighter dye or a red-shifted dye that enables longer image acquisition without phototoxicity could enable resolution of individual dendritic spines in lifetime, and the determination of spine V_m .

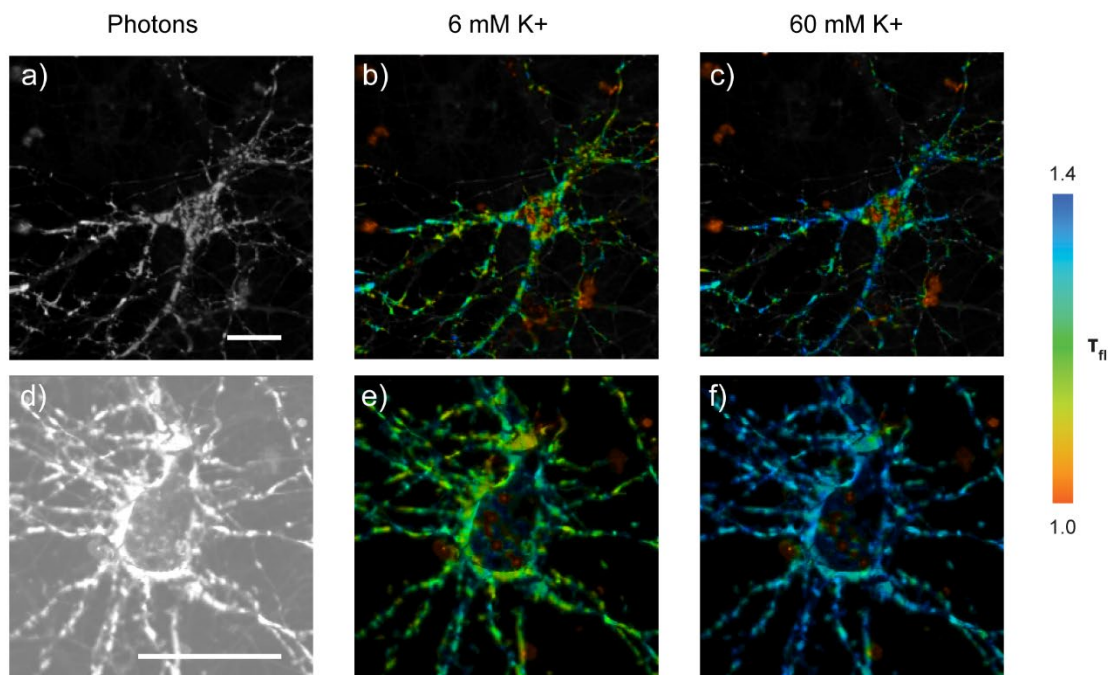


Figure 3.8 Change in fluorescence lifetime in neurons stained with VF-HaloTag upon depolarization with high K^+ buffer.

Images of primary hippocampal neurons stained with VF-HaloTag. a and d) Photon count, b and e) normal (6 mM K^+) buffer, and c and f) depolarized (60 mM K^+) due to exposure to high K^+ imaging buffer, demonstrating VF-HaloTag response to changes in resting voltage in neurons. a-c) and d-f) are images of different neurons, taken at different magnifications. Scale bars 20 μm .

While an electrophysiological calibration in neurons in FLIM was not tractable at this time, VF-HaloTag was able to report voltage changes in neurons induced by changes in ion concentrations. After acquiring a τ_{fl} image in low K^+ buffer (6 mM), the extracellular solution was replaced with a high K^+ solution (60 mM) to induce membrane depolarization (**Figure 3.8**). The τ_{fl} of VF-HaloTag increased by 60 to 120 ps, corresponding to changes in voltage (using the HEK293T cell calibration) of 43 to 86 mV. Attempts to utilize gramicidin for similar measurements were complicated by cellular toxicity and rapid cell death, and so it was not utilized as a method to characterize VF-HaloTag voltage response in neurons.

Conclusion

We extended the VF-FLIM technique of optical measurement of membrane potential from untargeted, broadly-applied dyes, to three new dye scaffolds, two of which are genetically targeted. One, VF-HaloTag, uses a novel VoltageFluor dye. These two different, orthogonal targeting techniques both enable staining of genetically defined cells. This enables fluorescence lifetime imaging in complex culture such as primary hippocampal neurons without the contribution of background fluorescence from neuropil, enabling the examination of the fluorescence lifetime signal from only a cell of interest, and the resolution of particular structures

in that cell. While at the moment VoltageSpy and VF-HaloTag have neither the brightness to supply sufficient photon count to look at dendritic spines nor the voltage resolution to make reliable comparisons between cells, both are a step forward in improving the spatial resolution of VF-FLIM by targeting specific cells.

Both dyes also represent a significant improvement in voltage resolution compared to previous genetically encoded sensors for reporting V_m in FLIM, representing a 7-fold improvement over CAESR (which has a 370 mV inter-cell voltage resolution in FLIM)^{10,21} for VoltageSpy, and a 10-fold improvement in the case of VF-HaloTag. Targeted VF-FLIM indicators could find use in looking at subcellular voltage in larger structures, such as throughout the dendritic arbor in neuronal culture. Modest improvements in brightness in the future could enable resolution of individual dendritic spines with VF-HaloTag. These dyes also set the groundwork for further expansion of targeted VF-FLIM, including using red-shifted VoltageFluors, which would allow for longer imaging times and resolution of dendritic spines with reduced cellular toxicity.

Materials and Methods

Immortalized cell line culture

HEK293T cells were obtained from the UC Berkeley Cell Culture Facility. The cell line was verified by STR profiling (short tandem repeat) and were tested routinely for mycoplasma. Cell lines were discarded after 25 passages. Cells were maintained in complete DMEM (Gibco, Thermo Fisher Scientific) supplemented with 4.5 g/L glucose, 2 mM GlutaMAX (Gibco), and 10% FBS (Seradigm) in a 37°C humidified incubator at 5% CO₂. Cells were passaged into fresh complete media every few days following dissociation with trypsin-EDTA (Gibco, 0.05%).

For imaging experiments, cells were plated onto prepared poly-D-lysine (PDL)-coated coverslips. Coverslips (#1.5, 25 mm diameter, Electron Microscopy Sciences) were acid washed for 2-5 hours in 1 M HCl. Coverslips were then washed three times overnight in 100% ethanol, followed by three times overnight in MilliQ (Millipore) purified water. Coverslips were sterilized by heating for 2-3 hours in a glassware oven to 150°C. Prior to seeding of cells, coverslips were incubated in 1x PDL (Sigma-Aldrich, made as a 0.1 mg/mL solution in phosphate-buffered saline with 10 mM Na₃BO₄) for 1-10 hours and then washed twice with sterile water and twice with 1x Dulbecco's Phosphate Buffered Saline (dPBS, Gibco).

For probe loading experiments, HEK293T were seeded onto prepared coverslips in complete DMEM at a density of 42-52 x10³ cells per cm² (in a 6 well tissue culture plate, Corning) and imaged approximately 24 hours after plating. For electrophysiology experiments, HEK293T were seeded at 26,000 cells/cm² in low glucose DMEM (Gibco; 1 g/L glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX, 10% FBS) and used 12-18 hours after plating.

Transfection of plasmids for resting membrane potential measurements was performed using Lipofectamine 3000 and P3000 reagent 2-3 hours after plating. Cells were imaged the next day. Transfection of plasmids for electrophysiology experiments were performed with the same reagents, applied to cells plated 24 hours previously at a density of 42,000 cells/cm² that had been plated directly onto a 6-well tissue culture plate. The cells were then allowed to grow for another 24 hours before being plated onto 25 mm glass coverslips at a density of 26,000 cells/cm² in low glucose DMEM as above.

Neuronal 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.

Hippocampi were dissected from embryonic day 19 Sprague Dawley rats (Charles River Laboratory) in cold, sterile HBSS (zero Ca^{2+} , zero Mg^{2+} , phenol red). 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% FBS, 2% B-27, 2% 1M dextrose (Fisher Scientific) and 1% GlutaMax. The dissociated cells were plated onto 25 mm diameter coverslips (Electron Microscopy Sciences) pre-treated with PDL (as above) at a density of 125,000 cells per coverslip 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. Imaging was performed on mature neurons 12-15 DIV.

Transfection of plasmids was performed using Lipofectamine 3000 (without P3000 reagent) at 6-7 DIV. Transfection media and DNA was allowed to incubate with the cells for 15 minutes to an hour, before the media was removed and replaced with fresh Neurobasal media.

Sample preparation and treatment for imaging

For all experiments in HEK293T or neurons, dyes were used at the determined loading concentrations (based on either concentration quenching or optimized contrast ratio), directly from a DMSO (or DMF, in the case of VoltageSpy) stock (final DMSO or DMF concentration in loading solution, 0.01). 1x loading solutions were prepared in imaging buffer (IB, made in-house; composition in mM: 139.5 NaCl, 10 HEPES, 5.6 D-glucose, 5.33 KCl, 1.26 CaCl_2 , 0.49 MgCl_2 , 0.44 KH_2PO_4 , 0.41 MgSO_4 , 0.34 NaH_2PO_4 ; 285 mOsm/L; pH 7.25). Osmolarity was determined with a $\mu\text{Osmette}$ (Precision Instruments). Cells were incubated at 37°C for 20-25 minutes in loading solution, washed once in IB, and transferred to fresh IB in an Attofluor cell imaging chamber (Invitrogen). All imaging experiments were performed in IB at room temperature.

For FLIM imaging in primary neuronal culture comparing lifetime at rest and after high potassium treatment, neurons were incubated in IB as above, and then imaged in 1 mL IB. After an initial baseline image was taken, 500 μL of IB was removed, and replaced with high K^+ IB (made in-house; composition in mM: 23.6 NaCl, 10 HEPES, 5.6 D-glucose, 119.6 KCl, 1.26 CaCl_2 , 0.49 MgCl_2 , 0.44 KH_2PO_4 , 0.41 MgSO_4 , 0.34 NaH_2PO_4 ; 285 mOsm/L; pH 7.25), and imaged again.

Widefield fluorescence microscopy

Epifluorescence imaging of HEK293T cells and neurons was performed on an AxioObserver Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor), controlled with $\mu\text{Manager}$ (V1.4, open-source, Open Imaging). Images were acquired using an EC-Plan-NEOFLUAR 40/1.3 oil immersion objective (40x; Zeiss). Images were focused onto an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu).

For electrophysiology imaging experiments, the functional imaging of VF-HaloTag 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 μm^2).

Epifluorescence image analysis.

For fluorescence intensity measurements, image analysis was performed in the FIJI installation of ImageJ (NIH).²² Regions of interest were drawn around cells or neuronal cell bodies and the mean fluorescence was calculated. Background fluorescence was subtracted by measuring the fluorescence where no cells grew. Contrast between transfected and untransfected cells was calculated by dividing the fluorescence intensity of transfected, VoltageSpy or VF-HaloTag labeled, cells by the median fluorescence intensity of untransfected cells on the same image, both background subtracted.

Analysis of voltage sensitivity in HEK293T cells was performed using ImageJ (FIJI). A region of interest (ROI) was selected and applied to all image frames. Fluorescence intensity values were calculated at known baseline and voltage step epochs. $\Delta F/F$ 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_0) 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_0 to give $\Delta F/F$ traces.

Whole cell patch clamp electrophysiology

Pipettes were pulled from borosilicate glass with filament (Sutter Instruments, Novato, CA) with resistances ranging from 4 to 7 M Ω with a P97 pipette puller (Sutter Instruments). Internal solution composition, in mM (pH 7.25, 285 mOsmol/L): 125 potassium gluconate, 10 KCl, 5 NaCl, 1 EGTA, 10 HEPES, 2 ATP sodium salt, 0.3 GTP sodium salt. EGTA (tetraacid form) was prepared as a stock solution in 1 M KOH before addition to the internal solution. Pipettes were positioned with an MP-225 micromanipulator (Sutter Instruments). For voltage clamp protocols, a liquid junction potential of -14 mV was determined by the Liquid Junction Potential Calculator in the pClamp software package (Molecular Devices, San Jose, CA), and voltage step protocols used in conjunction with fluorescence lifetime measurements were corrected for this offset.

Electrophysiology recordings were made with an Axopatch 200B amplifier and digitized with a Digidata 1440B (Molecular Devices) on the epifluorescence system. For epifluorescence voltage sensitivity measurements, cells were held at -60 mV and potentials from +100 to -100 mV were applied in descending order with 20 mV steps. Signals were sampled at 50 kHz and filtered with a 5 kHz low-pass Bessel filter. Correction for pipette capacitance was performed in the cell attached configuration. Recordings were only included if they maintained a 30:1 ratio of membrane resistance (R_m) to access resistance (R_a) and an R_a value below 30 M Ω throughout the recording.

For voltage sensitivity measurements on the fluorescence lifetime system, electrophysiology recordings were made with an Axopatch 200B amplifier and digitized with a Digidata 1440A (Molecular Devices). The cells were held at -60 mV and potentials from +80 to -80 mV were applied. Voltage steps were applied with a variable order of -80, -40, 0, and +40 mV steps, followed by a +80 mV step as the final voltage applied, with each voltage step holding for 15 seconds. Signals were sampled at 50 kHz and filtered with a 5 kHz low-pass Bessel filter. Correction for pipette capacitance was performed in the cell attached configuration. Recordings

were only included if they maintained a 30:1 ratio of membrane resistance (R_m) to access resistance (R_a) and an R_a value below 30 M Ω throughout the recording

Fluorescence Lifetime Imaging

Fluorescence lifetime imaging of HEK293T cells and some of the imaging of cultured hippocampal neurons was conducted on a LSM 510 inverted scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with an SPC-150 or SPC-150N single photon counting card (Becker & Hickl GmbH, Berlin, Germany), as outlined in **Chapter 2**. For optical voltage determinations using optimized concentrations of VoltageFluors (150 nM for mVF-Sarcosine, 25 nM for VoltageSpy, and 50 nM for VF-HaloTag), typical average power at the sample was 5 μ W.

Additional fluorescence lifetime imaging of cultured hippocampal neurons was conducted on an LSM 980 (Carl Zeiss AG, Oberkochen, Germany) inverted scanning confocal microscope. The 980 microscope was equipped with a SPC-150NX single photon counting card (Becker and Hickl). The LSM 980 was controlled with Zen Blue software (Zeiss), and the TCSPC unit was controlled with SPCM software (Becker-Hickl). Pulsed excitation at 50 MHz was supplied with a 488 nm diode laser (BDS-SM-488-FBC-101, Becker-Hickl) and controlled through an LHB-104 Laser Hub (Becker-Hickl). Power was adjusted with a neutral density filter wheel controlled through the software to maximize count rate without exceeding pile-up limit (5×10^6 photons/s at an individual pixel, 10% of the laser repetition rate).

On both systems, fluorescence emission was collected through a 40x/1.3 NA oil immersion objective (Zeiss) coated with immersion oil (Immersion 518F, Zeiss). On the 980 microscope some images of cultured neurons were collected with a 63x/1.4 NA PlanApochromat oil immersion objective (Zeiss). Emitted photons were detected with an HPM-100-40 (Becker & Hickl) hybrid detector, based on a Hamamatsu R10467 GaAsP hybrid photomultiplier tube. Detector dark counts were kept below 1000 per second during acquisition, and were generally lower than 200 per second on the 980 system. Emission light was collected through a 540/50 bandpass filter (Semrock, Rochester, NY) after passing through a 488 LP dichroic mirror (Zeiss). The reference photons for determination of photon arrival times were detected with a PHD-400-N high speed photodiode (Becker & Hickl). Data were acquired with 256 time bins in the analog-to-digital-converter and either 64x64 (510 only) or 256x256 (510 and 980 systems) pixels of spatial resolution (see discussion of pixel size in Chapter 2). The LSM 510 system had no means of temperature control, so all experiments were performed at room temperature. The LSM 980 had incubation, and so the XL incubation chamber was maintained at 25 °C for experiments with cultured neurons to assist in maintenance of cell viability during imaging without significant alteration of lifetime due to temperature.

Neurons were imaged both on the LSM 510 and the LSM 980 systems, using either the 40x or 63x objectives. For the 40x objective, cells were imaged for 90 seconds, and at image sizes of either 53 x 53 μ m or 70.7 x 70.7 μ m, resulting in unbinned pixel sizes of 0.21 μ m and 0.28 μ m. These correspond to binned pixel sizes of 1.47 μ m and 1.96 μ m using the “bin 3” setting in the SPCImage analysis software. For the images acquired using the 63x objective, the image sizes were 67.3 x 67.3 μ m, giving unbinned pixel sizes of 0.26 μ m, resulting in a binned pixel width of 1.84 μ m. All neuron images were 256 x 256 pixels.

The TCSPC detector parameters used for the LSM 980 system were the following: constant fraction discriminator (CFD) -50.98 mV, zero cross 5.29 mV. Sync parameters used

were the following: CFD -50.98 mV, zero cross 5.29 mV, offset 5.10 %, sync divider 1, dither range 1/16. Data were acquired with 256 TCSPC time channels.

Routine evaluation of the proper functioning of the lifetime recording setup was performed by measurement of three standards: 2 μM fluorescein in 0.1 N NaOH, 1 mg/mL erythrosin B in water (pH 7), and the instrument response function (IRF). The IRF was determined from a solution of 500 μM fluorescein and 12.2 M sodium iodide in 0.1 N NaOH, as discussed in **Chapter 2**.

Fluorescence lifetime fitting

All the VoltageFluor fluorescence lifetime data were fit using SPCImage (Becker-Hickl), which uses the Levenberg-Marquardt algorithm to solve the nonlinear least squares problem. Both VoltageSpy and mVF-Sarcosine were fit using the sum of two exponential decay components (equation 3.1), as using a single exponential decay did not produce a satisfactory fit, and fitting to three components resulted in overfitting. VF-HaloTag was fit using three exponential components (equation 3.2), as two components did not provide a satisfactory fit to the decay, as assessed for the voltage-clamped cells.

The amplitude-weighted average of the components (equation 3.3) was used in subsequent analysis, namely analysis of lifetime dependence on voltage and reports of resting membrane potential.

$$F = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} \quad [3.1]$$

$$F = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3} \quad [3.2]$$

$$\tau_m = \frac{a_1 \tau_1 + \dots + a_n \tau_n}{a_1 + \dots + a_n} \quad \text{for } n = 2 \text{ or } 3 \quad [3.3]$$

All lifetimes reported are the mean τ_n of a particular ROI, defined as the plasma membrane for an entire cell for HEK293T cells, or the plasma membrane of the soma and large processes of a neuron.

The experimentally measured instrument response was cropped, and reconvolved with the decay following the iterative reconvolution algorithm in SPCImage. Because the fluorescence lifetime of the VoltageFluors examined here did not completely decay in the 12.5 ns laser cycle from the 510 microscope, nor the 20 ns laser cycle from the LSM 980 microscope. The shift between the IRF and the decay trace was fixed to 0.5 for the analysis, as this was the value that gave reliable fit results for the fluorescein dye standard that most closely matched reported lifetime values. This shift value was consistent between the two systems.

Determination of relationship between lifetime and V_m

The relationship between weighted average lifetime (τ_n) and membrane potential was determined by linear regression, resulting in a sensitivity (slope (m), ps/mV) and a 0 mV lifetime (y-intercept (b), ps) for each cell (equation 3.4). The average sensitivity and 0 mV τ_n were determined from each of the cells. The means of these values were calculated to determine an “average calibration” which can be used to translate between measured τ_n and membrane voltage. For quantifying and discussing changes in voltage, only the sensitivity (slope) is necessary (equation 3.5).

$$\tau_{fl} = m * V_{mem} + b \quad [3.4]$$

$$\Delta V_{mem} = \frac{\Delta \tau_{fl}}{m} \quad [3.5]$$

Differences in the measured τ_{fl} at a defined reference point (for us, the 0 mV τ_{fl}) across measured cells provides an estimate of the voltage-independent noise for the lifetime-voltage calibration, and thus the resolution we can expect when using this calibration. We report this resolution as the root-mean-square deviation (RMSD, equation 3.6) between the optically determined voltage using the developed calibration (V_{FLIM}) and the voltage of the cell as set by voltage clamp (V_{ephys}). The RMSD of a set of n measurements can be determined from the variance (equation 3.7) and the bias (equation 3.8) of the estimator (V_{FLIM}) as compared to the assumed “true” value, in this case determined by electrophysiology (V_{ephys}).

$$RMSD = \sqrt{\sigma^2 + Bias^2} \quad [3.6]$$

$$\sigma^2 = \frac{1}{n} \sum_{i=1}^n (V_{FLIM,i} - V_{ephys,i})^2 \quad [3.7]$$

$$Bias = \frac{1}{n} \sum_{i=1}^n V_{FLIM,i} - \frac{1}{n} \sum_{i=1}^n V_{ephys,i} \quad [3.8]$$

The variations in τ_{fl} are larger between cells than within a cell, and thus the voltage resolution in HEK293T cells can be estimated with two values: the intra-cell resolution and the inter-cell resolution. The intra-cell resolution is a measure of the resolution expected for tracking and quantifying changes within an individual cell, determined by the RMSD between an individual cell’s “optical guess” at the lifetime for a particular voltage step, from its individual line of best fit, and the actual measured lifetime at that point. The inter-cell resolution is the single-trial resolution of estimating an individual HEK293T cell’s membrane potential from the measured τ_{fl} , determined from the RMSD between the 0 mV τ_{fl} value from each individual cell’s determined lifetime-voltage relation and the 0 mV τ_{fl} determined by the average calibration.

Statistical Analysis

Data are reported as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. For analysis of differences between groups, data were tested for normality (Shapiro-Wilk test and homoscedasticity (Levene’s test on the median)). Because some concentrations of mVF-Sarcosine did not satisfy the assumptions of normality for ANOVA, the Kruskal-Wallis rank sum test and Dunn’s post hoc test were used. All statistical analysis was performed in Python using the Scipy, Scikit-Posthocs, and Pingouin statistical analysis packages.²³

Plasmid construction

SpyCatcher encoding plasmids for both HEK293T cells and hippocampal neurons were utilized as previously reported. For expression in HEK293T cells, two plasmids were produced to compare for efficacy in HaloTag expression. HaloTag with an N-terminal IGK secretion sequence, C-terminal HA tag and decay accelerating factor (DAF) or PDGFR transmembrane domain (pDisplay, Invitrogen), along with downstream nuclear targeted mCherry (NLS-mCherry), separated by an internal ribosomal entry site (IRES) was subcloned into a pCDNA3 vector.

For expression in neurons, the HaloTag protein was subcloned into a pCDNA3 vector with human synapsin promoter (Syn) and a regulatory element from the woodchuck hepatitis virus (WPRE), and utilized a PAT3 signal peptide rather than IGK. Versions of this plasmid were produced both with and without the NLS-mCherry element. Plasmid without nuclear localized fluorescent protein were used for FLIM imaging of neurons to improve overall cell health.

The following sequences were used (5’ to 3’).

PAT3:

ATGCCACCTTCAACATCATTGCTGCTCCTCGCAGCACTTCTTCCATTCGCTTTACCAG
CAAGCGATTGGAAGACTGGAGAAGTCACTG

IGK:

ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACT
GGTGAC

HA: TATCCATATGATGTTCCAGATTATGCT

HaloTag:

GCAGAAATCGGTACTGGCTTTCCATTTCGACCCCCATTATGTGGAAGTCCTGGGCGAG
CGCATGCACTACGTTCGATGTTGGTCCGCGCGATGGCACCCCTGTGCTGTTCCCTGCAC
GGTAACCCGACCTCCTCCTACGTGTGGCGCAACATCATCCCGCATGTTGCACCGACC
CATCGCTGCATTGCTCCAGACCTGATCGGTATGGGCAAATCCGACAAACCAGACCTG
GGTTATTTCTTCGACGACCACGTCCGCTTCATGGATGCCTTCATCGAAGCCCTGGGTC
TGGAAGAGGTCGTCTCTGGTCATTACGACTGGGGCTCCGCTCTGGGTTTCCACTGGG
CCAAGCGCAATCCAGAGCGCGTCAAAGGTATTGCATTTATGGAGTTCATCCGCCCTA
TCCCGACCTGGGACGAATGGCCAGAATTTGCCCGCGAGACCTTCCAGGCCTTCCGCA
CCACCGACGTCGGCCGCAAGCTGATCATCGATCAGAACGTTTTTATCGAGGGGTACGC
TGCCGATGGGTGTCGTCCGCCCGCTGACTGAAGTCGAGATGGACCATTACCGCGAG
CCGTTCTGAATCCTGTTGACCGCGAGCCACTGTGGCGCTTCCCAAACGAGCTGCCA
ATCGCCGGTGAGCCAGCGAACATCGTCGCGCTGGTCGAAGAATACATGGACTGGCT
GCACCAGTCCCCTGTCCCGAAGCTGCTGTTCTGGGGCACCCAGGCGTTCTGATCCC
ACCGGCCGAAGCCGCTCGCCTGGCCAAAAGCCTGCCTAACTGCAAGGCTGTGGACA
TCGGCCCGGGTCTGAATCTGCTGCAAGAAGACAACCCGGACCTGATCGGCAGCGAG
ATCGCGCGCTGGCTGTCGACGCTCGAGATTTCCGGC

DAF:

CCAAATAAAGGAAGTGGAACCACTTCAGGTACTACCCGTCTTCTATCTGGGCGACACG
TGTTTCACGTTGACAGGTTTGCTTGGGACGCTAGTAACCATGGGCTTGCTGACT

pDisplay:

GCTGTGGGCCAGGACACGCAGGAGGTCATCGTGGTGCCACACTCCTTGCCCTTTAAG
GTGGTGGTGATCTCAGCCATCCTGGCCCTGGTGGTGCTCACCATCATCTCCCTTATCA
TCCTCATCATGCTTTGGCAGAAGAAGCCACGTTAG

IRES:

GCCCCTCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCC
GGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAG
GGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCTAGGGGTCTTTCCCTCTC
GCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGC
TTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCACC
TGCGGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGG
CGGCACAACCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGG
CTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGCACCCATTG
TATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTT
AAAAAACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTCTTTGAAAAACAC
GATGATAATATGGCCACA

NLS-mCherry:

ATGGTGCCCAAGAAGAAGAGGAAAGTCGTGAGCAAGGGCGAGGAGGACAACATGG
CCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGC

CACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGGCACCCAGA
 CCGCCAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGCCTGGGACATCCTGT
 CCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCG
 ACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCG
 AGGACGGCGGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTC
 ATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCA
 GAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCG
 CCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGA
 CGCCGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGACGCTGCCCCGGCGCCT
 ACAACGTCAACATCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATCGTG
 GAACAGTACGAGCGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTA
 CAAG

WPRE:

GCTTATCGATAATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCT
 TAACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCAT
 GCTATTGCTTCCCGTATGGCTTTCATTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTC
 TCTTTATGAGGAGTTGTGGCCCGTTGTGAGGCAACGTGGCGTGGTGTGCACTGTGTT
 TGCTGACGCAACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGG
 GACTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAACATCGCCGCCTGCCTTGCC
 CGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATTCCGTGGTGTGTCGGGG
 AAATCATCGTCCTTTCCTTGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGA
 CGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGCGGCCT
 GCTGCCGGCTCTGCGGCCTCTTCGCGCTTTCGCCTTCGCCCTCAGACGAGTCGGAT
 CTCCCTTTGGGCCGCTCCCCGCATCGATACCG

Synapsin Promoter:

GTGTCTAGACTGCAGAGGGGCCCTGCGTATGAGTGCAAGTGGGTTTTAGGACCAGGA
 TGAGGCGGGGTGGGGGTGCCTACCTGACGACCGACCCCGACCCACTGGACAAGCAC
 CCAACCCCCATTCCCCAAATTGCGCATCCCCTATCAGAGAGGGGGAGGGGAAACAG
 GATGCGGCGAGGCGCGTGCGCACTGCCAGCTTCAGCACCGCGGACAGTGCCTTCGC
 CCCCGCCTGGCGGCGCGCGCCACCGCCGCCTCAGCACTGAAGGCGCGCTGACGTCA
 CTCGCCGGTCCCCCGCAAACCTCCCCTTCCCGGCCACCTTGGTCGCGTCCGCGCCGCC
 GCCGGCCCAGCCGGACCGCACACGCGAGGCGCGAGATAGGGGGGCACGGGCGCG
 ACCATCTGCGCTGCGGCGCCGGCGACTCAGCGCTGCCTCAGTCTGCGGTGGGCAGC
 GGAGGAGTCGTGTCGTGCCTGAGAGCGCAGTCGAGA

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Chapter 4

A Red-Emitting Carborhodamine for Monitoring and Measuring Membrane Potential in FLIM

This work was conducted in collaboration with the following individuals:
Carborhodamine VoltageFluors were synthesized and characterized by Dr. Gloria Ortiz
Portions of the FLIM imaging on the LSM 880 microscope was done by Dr. Julia Lazzari-Dean

Abstract

Biological membrane potentials, or voltages, are a central facet of cellular life. Optical methods to visualize cellular membrane voltages with fluorescent indicators are an attractive complement to traditional electrode-based approaches, since imaging methods can be high throughput, less invasive, and provide more spatial resolution than electrodes.

Recently developed fluorescent indicators for voltage largely report changes in membrane voltage by monitoring voltage-dependent fluctuations in fluorescence intensity. However, it would be useful to be able monitor not only changes, but measure values of membrane potentials. This study discloses a new fluorescent indicator which can do both.

We synthesized a new sulfonated tetramethyl carborhodamine fluorophore. When this carborhodamine is conjugated with an electron-rich, methoxy (-OMe) containing phenylenevinylene molecular wire, the resulting molecule, CRhOMe, is a voltage-sensitive fluorophore with red/far-red fluorescence.

Using CRhOMe, changes in cellular membrane potential can be read out using fluorescence intensity or lifetime. In fluorescence intensity mode, CRhOMe tracks fast-spiking neuronal action potentials with greater signal-to-noise than state-of-the-art BeRST (another voltage-sensitive fluorophore). CRhOMe can also measure values of membrane potential. The fluorescence lifetime of CRhOMe follows a single exponential decay, unlike previously examined VoltageFluor dyes (including BeRST) substantially improving the quantification of membrane potential values using fluorescence lifetime imaging microscopy (FLIM). The combination of red-shifted excitation and emission, mono-exponential decay, and high voltage sensitivity enable fast FLIM recording of action potentials in cardiomyocytes. The ability to both monitor and measure membrane potentials with red light using CRhOMe makes it an important approach for studying biological voltages.

Introduction

Membrane potential plays central roles in the physiology of all living systems. Rapid changes in membrane potential initiates neurotransmitter release in neurons,¹ prompts muscle contraction in cardiomyocytes,^{2,3} and evokes insulin secretion in pancreatic β cells.⁴ In a related fashion, the value of the membrane potential – set largely by the difference in internal and external potassium ion concentration and the selective permeability of the plasma membrane to K^+ ions – plays an outsized role in the physiology of these cell types, controlling their excitability by setting the distance to action threshold. Further, values of membrane potentials in non-electrically excitable cells may play important roles in signaling, differentiation, and cell cycle progression. Befitting the importance of membrane potential, some 10 to 50% of oxygen consumption is directed towards setting the “resting” membrane potential through the action of the ATP-driven Na-K exchanger.⁵

The primary mode of monitoring changes in and measuring values of membrane potential is patch-clamp electrophysiology. Despite the centrality of membrane potential to cellular physiology across all organ systems, electrophysiology, the gold standard for membrane potential measurements, is nonetheless invasive, low throughput, and difficult to implement across spatial scales. Electrophysiological analysis of multiple cells requires highly specialized instrumentation,^{6,7} and interrogation of subcellular or intracellular membranes remain largely inaccessible except in limited cases.^{8–10}

To complement traditional electrode-based methods, we have been pursuing voltage-sensitive fluorophores (VF dyes) that sense voltage via a photoinduced electron transfer (PeT)-based mechanism.^{11–13} This approach can be adapted to a wide range of chemically-synthesized fluorophores.¹³ We find that dyes with excitation and emission in the red region of the spectrum are especially useful, since they are compatible with optical indicators and actuators that use blue or green light; well matched to commercially available powerful LEDs; and pair well with the wavelength-dependent quantum efficiency of typical sCMOS detectors, which falls off dramatically beyond 650 nm.^{14,15} These characteristics make VF dyes well suited for tracking fast membrane potential changes associated with action potentials in neurons (AP duration ~2 ms) or cardiomyocytes (AP duration ~100 ms).

We also showed that VF dyes can be used to estimate values of membrane potential. Using fluorescence lifetime imaging microscopy (FLIM) to measure the fluorescence lifetime of VF dyes in cell membranes, we can estimate millivolt values of membrane potential, since fluorescence lifetime is more resistant than fluorescence intensity to variations in dye loading, fluctuations in excitation power, or bleaching. Our initial efforts with fluorescein-based VF2.1.Cl showed that FLIM enables membrane potential value estimation with up to 19× improved resolution compared to existing methods.¹⁶ However, the long acquisition times required for FLIM, coupled with the blue-light excitation of VF2.1.Cl means that even low levels of phototoxicity becomes limiting in sensitive systems (like cardiomyocytes or neurons).¹⁷ Further, VF2.1.Cl has a two-component fluorescence lifetime decay, complicating analysis and requiring longer photon collection times.^{18,19}

Here, we describe a new VF dye based on a tetramethyl carborhodamine scaffold. The synthesis of these indicators is described in detail in the thesis of Gloria Ortiz, Ph.D. 2019. The new carborhodamine indicators have red excitation and emission profiles, show high voltage sensitivity, can detect rapid changes of membrane potential in hippocampal neurons, and possess long fluorescence lifetimes, making them useful for tracking membrane potential values and changes using FLIM.

Lifetime Studies in Cells

We recorded the time resolved fluorescence decay of the TMCRh fluorophore (1 μ M in water) with time correlated single photon counting (TCSPC) FLIM on a line scanning confocal microscope (Zeiss LSM 880 and 980). The time-resolved fluorescence decay was well described by a single exponential model and exhibited a lifetime of 3.06 ± 0.02 ns (mean $\pm$ SEM of 16 measurements, **Figure 4.1**). We then measured the fluorescence lifetime of CRhOMe in HEK293T cells at rest (**Figure 4.4a**), which was also best described by a single exponential decay. We observed a mean lifetime of 2.58 ± 0.09 ns (mean $\pm$ SEM of 71 cell groups).

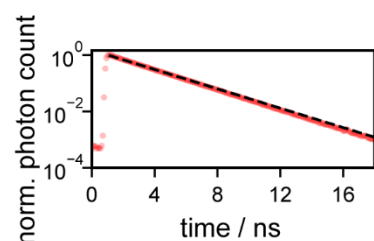


Figure 4.1 Lifetime decay of TMCRh fluorophore 7 in water.

Normalized fluorescence lifetime decay of the TMCRh fluorophore in water, fit with a monoexponential decay curve.

The resting lifetime data is consistent across time and cell lines. This resting lifetime data in HEK293T cells was acquired across three years and two microscopes with different FLIM systems. Values acquired on the LSM 880 have a lifetime of 2.61 ± 0.1 ns. Data from the LSM 980 have a mean lifetime of 2.55 ± 0.06 (mean $\pm$ SD for both). The values from both instruments are within one standard deviation of one another (**Figure 4.2**). In serum-starved A431 cells, the lifetime of CRhOMe was similar (2.6 ± 0.1 ns, mean $\pm$ SD of 18 cell groups). For the voltage-insensitive control compound TMCrhZero in HEK293T (**Figure 4.3d, 4.5**, we again observe a single exponential decay with a lifetime of 3.70 ± 0.01 (mean $\pm$ SD of 103 cell groups).

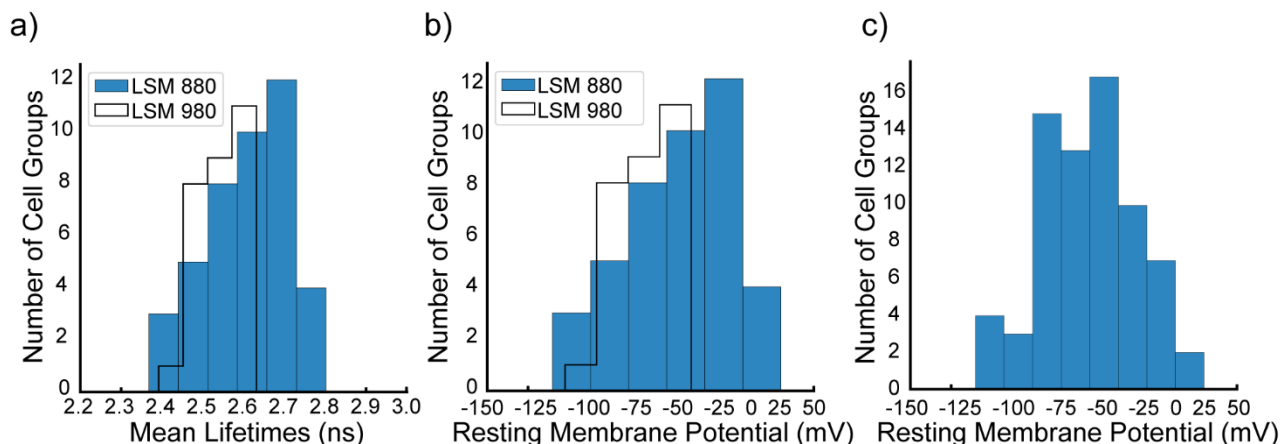


Figure 4.2 Distributions of mean lifetimes and resting potentials across FLIM systems.

Histograms representing the mean lifetimes and resting membrane potentials of HEK293T cells across both FLIM systems used. a) Mean lifetimes (ns) plotted against the frequency (in number of cell groups) for both the LSM 880 system (blue) and LSM 980 (outline). b) The resting membrane potentials determined by applying the calibration determined (Figure 4.4 in main text) to the lifetimes in a. c) Combined resting membrane potentials from both systems.

As a comparison, we also investigated the fluorescence lifetime in HEK293T cells of a previously reported far-red voltage indicator, BeRST1.²⁰ Unlike CRhOMe, BeRST1 is not well described by a single exponential decay (**Figure 4.4a**, second trace), and was instead fit with a two-component exponential decay. We report its lifetime as the amplitude-weighted average of the two lifetime components, as we have done for previous green VoltageFluors in FLIM.¹⁶ In HEK293T cells, BeRST1 exhibits a mean lifetime of 1.80 ± 0.07 ns (mean $\pm$ SD of 56 cell groups), significantly shorter and more complex than CRhOMe (**Figure 4.3e**).

We additionally measured lifetimes across a range of dye loading concentrations for both CRhOMe and BeRST1 to look for a concentration-dependent decrease in τ_{fl} (**Figure 4.3**), which we previously observed with green VoltageFluors.^{16,21} All subsequent experiments were conducted at concentrations below the point where concentration quenching was observed (approximately 500 nM in HEK293T culture, with most experiments done at 300 nM dye).

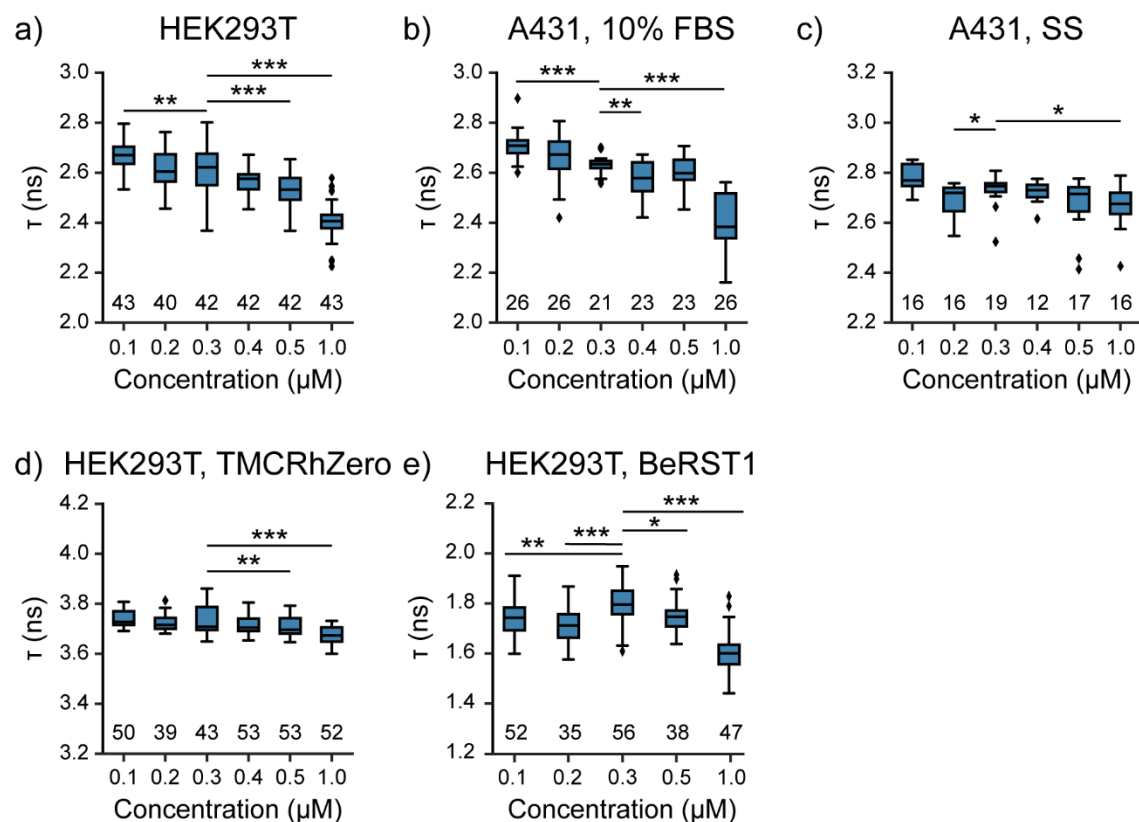


Figure 4.3 Fluorescence lifetime vs. concentration for CRhOMe and BeRST in different cell lines.

Plots of fluorescence lifetime of CRhOMe in HEK293T cells vs concentrations ranging from 100 nM dye to 1 μ M dye as applied to cells. b) CRhOMe in A431 cells. c) CRhOMe in serum starved A431 cells. d) TMCRhZero in HEK293T cells. e) BeRST1 dye in HEK 293T cells. Significance levels are reported as compared to 300 nM dye, and are the result of Kruskal-Wallis tests, and Dunn post-hoc analysis. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The y axis range for all plots is 1 ns.

Calibration of CRhOMe in cells with electrophysiology and gramicidin treatment

We generated a voltage- τ calibration using whole-cell, patch-clamp electrophysiology and simultaneous FLIM imaging to determine the voltage dependence of CRhOMe fluorescence lifetime. HEK 293T cells were treated with 300 nM CRhOMe, and then voltage-clamped at -80, -40, 0, +40, and +80 mV, and the fluorescence lifetime recorded. Individual measurements demonstrate a linear response of lifetime to changes in voltage (**Figure 4.4b-c**). Consistent responses among individual measurements allows for the development of an average calibration to represent the overall expected change in lifetime (in ps) per change in voltage (in mV). In HEK 293T cells, CRhOMe exhibits a sensitivity (slope of the calibration) of 3.09 ± 0.09 ps/mV, with a lifetime at 0 mV (the y-intercept of the calibration) of 2.71 ± 0.03 ns. Applying this calibration to our measured lifetime of HEK293T cells at rest, the mean resting membrane potential across all measurements is -41.0 ± 3.5 mV (mean $\pm$ SEM), consistent with previous optical and electrophysiological reports of HEK293T resting potential values. The voltage-insensitive control

compound, TMCrRhZero, does not exhibit a change in lifetime to an applied voltage, demonstrating a sensitivity of 4.5×10^{-5} ps/mV (**Figure 4.5**).

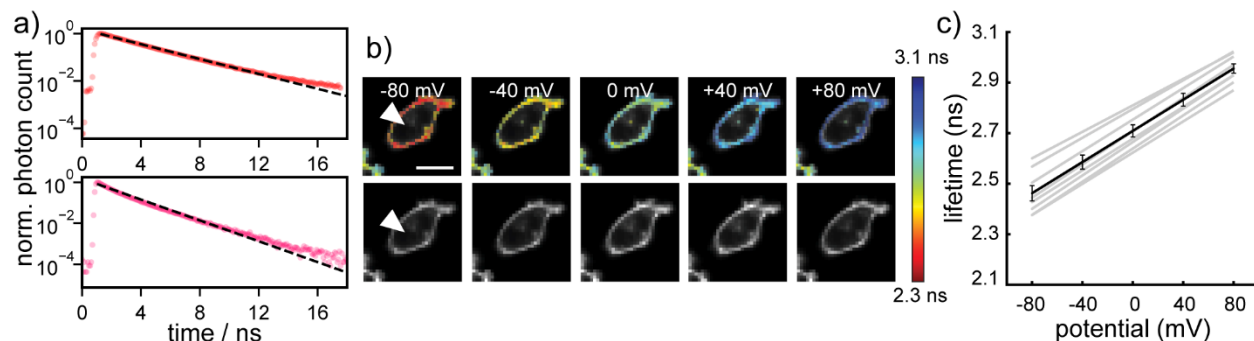


Figure 4.4. CRhOMe displays single-exponential fluorescence lifetime decays and a linear fluorescence lifetime-voltage relationship.

a) Plots of fluorescence lifetime decay expressed as normalized photon counts (log scale) vs time for CRhOMe (upper, red) BeRST (lower, magenta). Circles indicate photon count data; dashed line is a single exponential decay. **b)** Lifetime (colored) and intensity (grayscale) images of HEK 293T cells voltage-clamped at the indicated potentials. The lifetime heatmap is scaled from 2.3 to 3.1 ns. Scalebar is 20 μ m. Arrowhead indicates the voltage-clamped cell. **c)** Plot of CRhOMe fluorescence lifetime vs membrane potential in HEK 293T cells. Gray lines are individual cell calibrations (n = 17). The overall calibration is in black. Error bars are mean $\pm$ SEM.

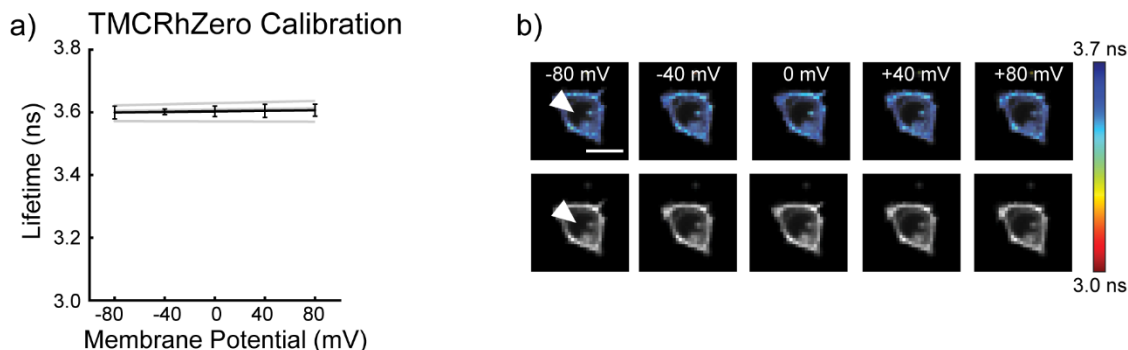


Figure 4.5 Comparison of voltage and lifetime response of TMCrRhZero dye in HEK293T cells.

a) Plot of lifetime vs. membrane potential for TMCrRhZero in HEK cells. Gray lines indicate individual cell responses (n = 3), and the black line indicates the overall average response, with a sensitivity (slope) of 4.5×10^{-5} ps/mV, and a lifetime at 0 mV of 3.6 ns. **b)** Fluorescence lifetime and intensity responses in HEK293T cells when voltage clamped at the indicated voltages. Lifetime heatmap scaled from 3.0 to 3.7 ns. Scalebar 20 μ m.

As a secondary calibration, and an attempt at providing a non-electrophysiological calibration for systems upon which patching may be impossible or difficult, we turned to the Na^+/K^+ ionophore gramicidin²² (**Figure 4.6-4.7**). Because it increases the permeability to both Na^+ and K^+ , we expect that gramicidin will depolarize cellular V_{mem} to approximately 0 mV. In HEK293T cells treated with 500 ng/mL gramicidin, the fluorescence lifetime of CRhOMe rose to 2.74 ± 0.09 ns (mean $\pm$ SD of 111 cell groups), which matches the 0 mV τ_{fl} of 2.71 ns determined

by patch clamp calibration. We observed a similar increase in fluorescence lifetime in serum-starved A431 cells treated with gramicidin (**Figure 4.6 d,e,f**). When we increased the gramicidin concentration to 1 $\mu\text{g/mL}$, there was no further increase in the fluorescence lifetime (2.75 ± 0.04 ns, mean $\pm$ SD of 50 cell groups) (**Figure 4.7**).

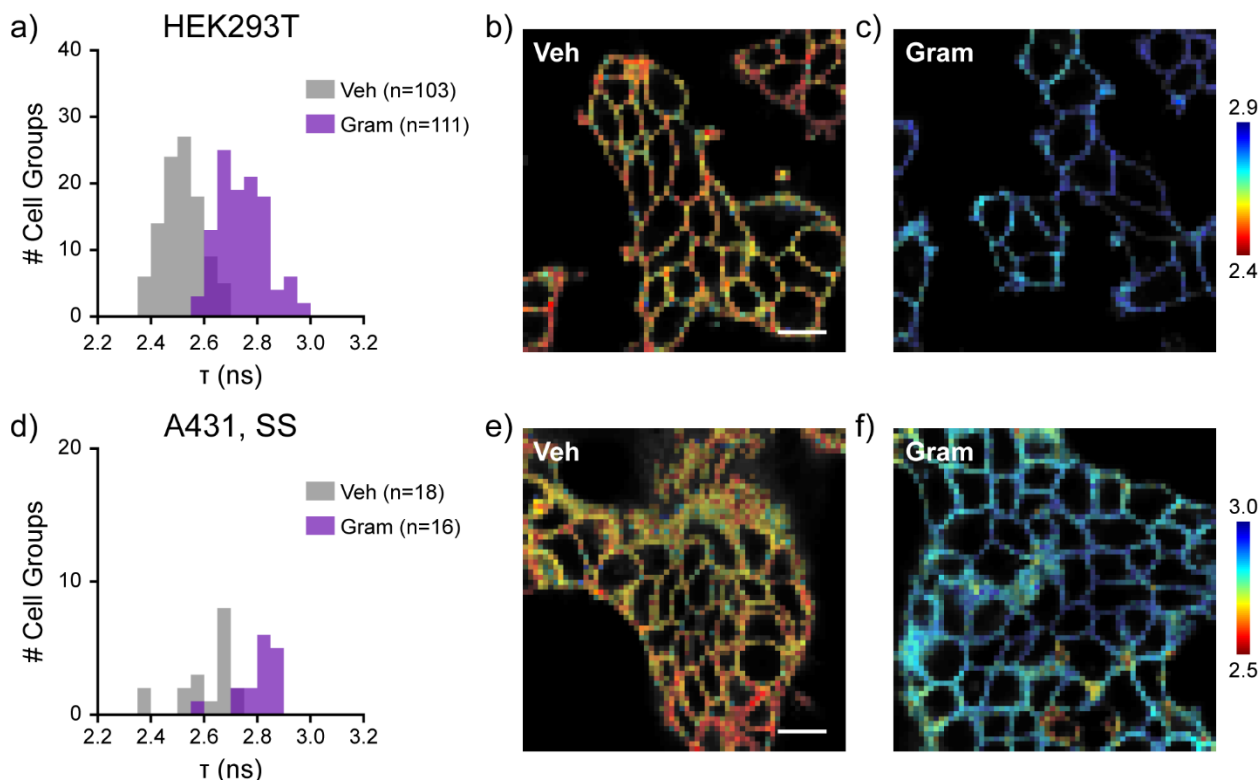


Figure 4.6 Lifetime of CRhOMe in cells at rest and depolarized with gramicidin.

a) Histogram of lifetimes observed for 300 nM CRhOMe in groups of HEK293T at rest (vehicle, Veh, 0.05-0.1% DMSO) and treated with 500 ng/mL gramicidin (Gram). b) Lifetime-photon count overlay image of HEK293T at rest and c) treated with 500 ng/mL gramicidin. d) Histogram of CRhOMe lifetimes observed in groups of serum starved (SS) A431 cells at rest and depolarized with gramicidin. e-f). Lifetime images of SS A431 at rest and gramicidin-treated, as in (b)-(c). Bin sizes were determined by the Freedman-Diaconis rule. Scale bars represent 20 μm . Photon counts of lifetime-photon count overlay images are not scaled to each other.

The spread in the lifetimes observed with and without 500 ng/mL gramicidin was similar. We would expect that a compound that moves all cells to 0 mV would decrease the variability in the measured lifetime. We did observe a slight decrease in the standard deviation at 1 $\mu\text{g/mL}$ gramicidin (**Figure 4.7a**), but we used 500 ng/mL gramicidin for further experiments to minimize nonspecific effects on the membrane and cell health.

We also recorded the fluorescence lifetime of TMCRhZero in HEK293T at rest and after incubation with 0.5 or 1 $\mu\text{g/mL}$ of gramicidin (**Figure 4.7**). We observed a slight change in the lifetime at 1 $\mu\text{g/mL}$ gramicidin. However, the effect was much smaller than that observed with the voltage-sensitive compound CRhOMe, suggesting that gramicidin does not directly modulate the fluorescence of the carborhodamine chromophore. Gramicidin may be an appropriate ionophore

for creating a reference point for quick calibrations in cell lines where a ground-truth patch clamping experiment is not feasible.

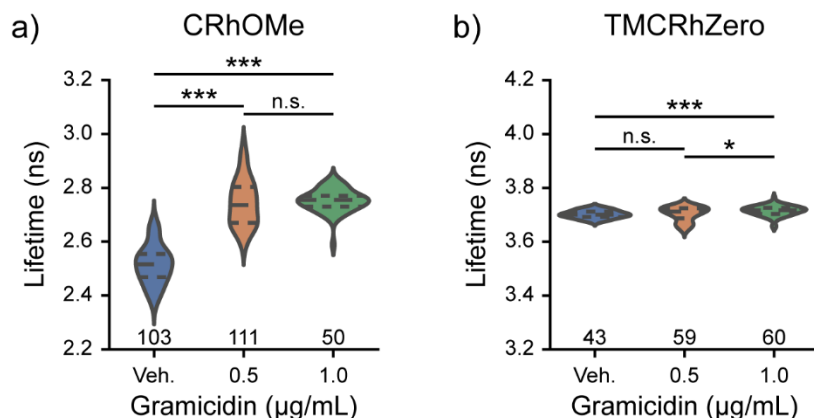


Figure 4.7 Gramicidin effects on CRhOMe and TMCRhZero lifetimes.

a) Violin plots of the fluorescence lifetime of CRhOMe in HEK293T incubated with varying concentrations of gramicidin or DMSO vehicle. Dashed lines within plots indicate the first quartile, median, and third quartile of the data. Significant differences between groups were observed (Welch's one-way ANOVA, $F(2, 166.3) = 352.86$, $p < 0.001$). Data for vehicle and 500 ng/mL are reproduced from **Figure 4.6**. b) Fluorescence lifetime of TMCRhZero in HEK293T plotted as in (a). Significant differences between groups were observed (Welch's one-way ANOVA, $F(2, 104.5) = 8.70$, $p < 0.001$). Asterisks indicate the results of Games-Howell post hoc tests (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Sample sizes (number of cell groups) are indicated on the plot for each category. Veh. = 0.05-0.1% DMSO.

Monitoring EGF-induced hyperpolarization in A431 cells

A key advantage of red-shifted voltage sensors is the ability to record for long periods of time with low phototoxicity. To evaluate CRhOMe FLIM in this context, we recorded the response of serum-starved A431 cells to re-introduction of EGF (500 ng/mL) for 15 minutes. We previously performed this experiment with the green VoltageFluor VF2.1.Cl.¹⁶ Using that system and an LSM 510 scanning confocal microscope, we were able to make six, intermittent V_{mem} measurements within a 15 minute time window, with each measurement lasting 30 seconds. These previous experiments revealed a hyperpolarizing response to EGF stimulation, facilitated by the kinase activity of EGFR, dependent on internal Ca^{2+} release, and mediated by the action of the Ca^{2+} -activated K^{+} channel, $\text{K}_{\text{Ca}3.1}$.¹⁶

Now, with CRhOMe as a voltage indicator and an improved confocal microscope (LSM 880), we were able to record continuously for 15 minutes, with each measurement lasting 5 seconds across a field of view 1.5 fold larger than the VF2.1.Cl study (**Figure 4.8** and **4.9**, total of ~180 V_{mem} measurements). In EGF-treated samples, we observe an immediate lifetime decrease of 75 ps on average with a steady return to baseline that did not complete within 15 minutes (**Figure 4.8**), consistent with the expected hyperpolarization. Samples treated with imaging buffer vehicle show no change in τ_{fl} of CRhOMe (**Figure 4.8a**). The control TMCRhZero displayed a steady, slight shortening of the lifetime (**Figure 4.8d**), which was identical when treated with either vehicle or EGF. We did not observe signs of phototoxicity in these recordings (increasing lifetime or changing cell morphology in the vehicle control).

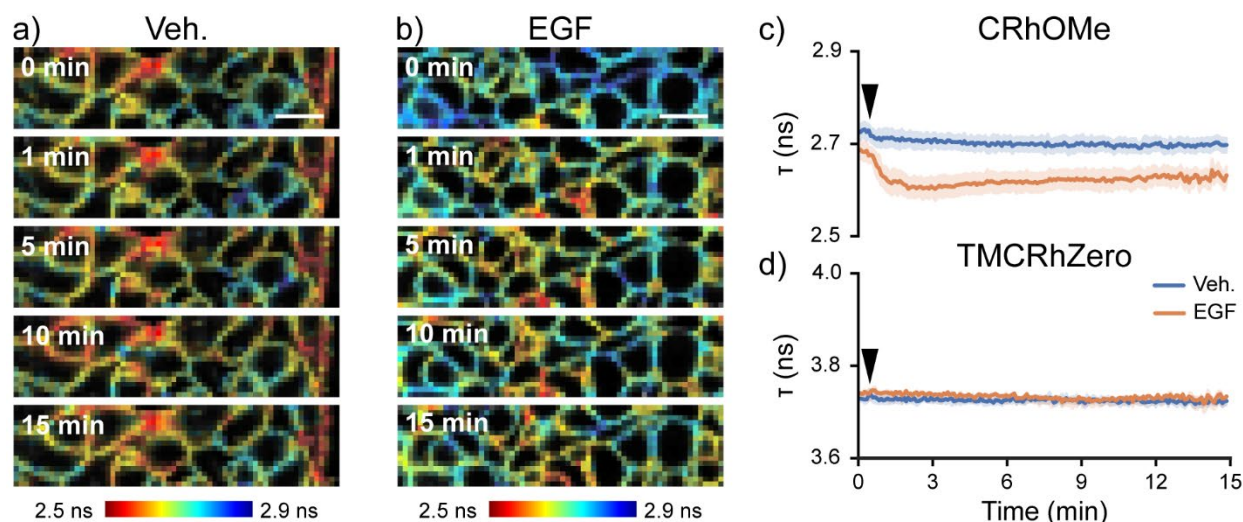


Figure 4.8. CRhOMe reports on EGF-induced hyperpolarization events in A431 cells with enhanced temporal and spatial resolution.

Snapshots from a τ time series of serum-starved A431 cells treated with either **a)** vehicle (imaging buffer) or **b)** EGF. Scale bar is 20 μm . Each snapshot is acquired at the indicated time after starting the experiment. Vehicle or EGF is added 20 s into the experiment. **c)** Mean lifetime of CRhOMe (top) or **d)** TMCRhZero (bottom) across the full recording. Shading represents standard error of the mean. Vehicle or 500 ng/mL EGF was added at the black arrow. Sample sizes: CRhOMe Veh 5, EGF 5; TMCRhZero Veh 3, EGF 3.

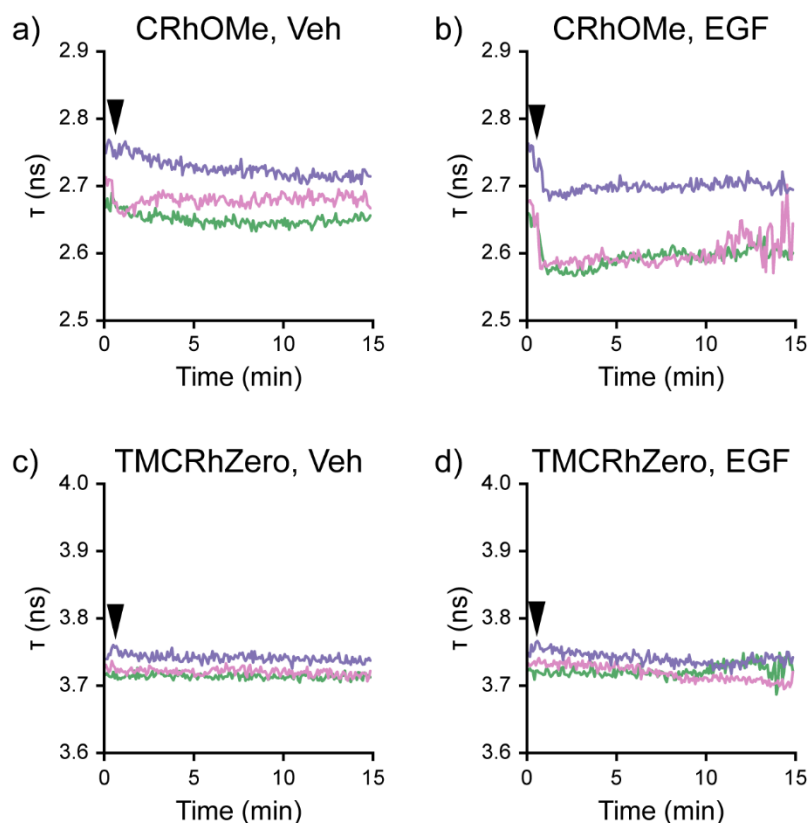


Figure 4.9 Individual recordings of the A431 V_{mem} response to EGF treatment.

Representative individual trials for CRhOMe stained A431 treated with a) vehicle (Veh) or b) 500 ng/mL EGF. Representative individual trials for TMCRhZero stained A431 treated with c) vehicle (Veh) or d) 500 ng/mL EGF. Vehicle or EGF was added at the black arrow. Recordings are quantified as the average lifetime per frame for all contiguous cells in the field of view. Colors represent distinct recordings; there is no relationship for colors between panels.

FLIM to monitor cardiac action potentials

To demonstrate the improved temporal resolution and stability to motion of our absolute V_{mem} sensing platform, we monitored the absolute V_{mem} of spontaneously beating human induced pluripotent stem cell derived cardiomyocytes (iCMs). Upon loading CRhOMe or TMCRhZero into iCM monolayers, we observed membrane localized fluorescence (**Figure 4.11, 4.10**). Because we are using a confocal pinhole larger than 1 airy unit to maximize photon count, some regions of the membrane appear as flat sheets where they traverse the optical section at a near-horizontal angle.

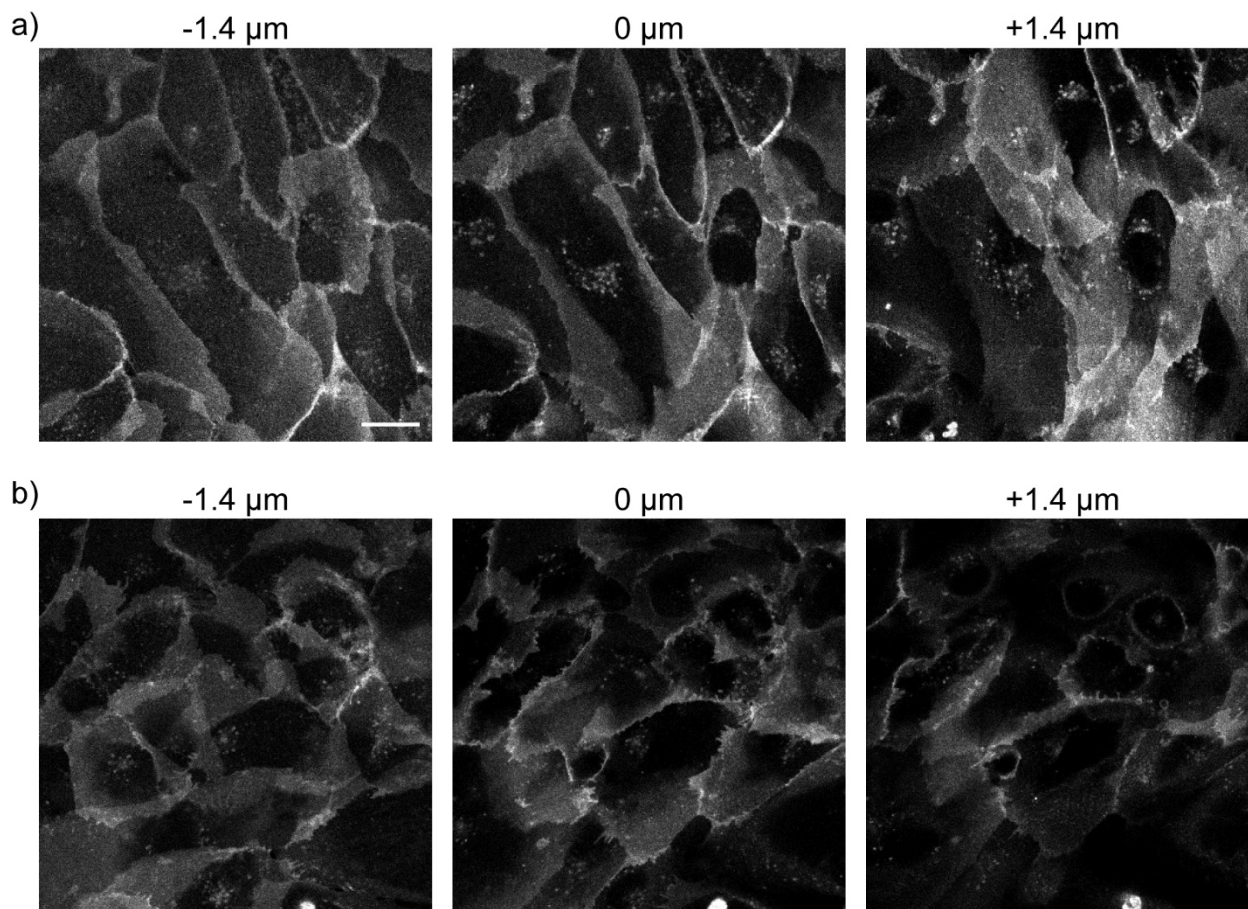


Figure 4.10 CRhOMe staining in iCMs is membrane-localized.

Confocal z stack images of two fields of view (a) and (b) of iCMs stained with 500 nM CRhOMe. Slices were recorded every 0.7 μm ; only a representative set are shown. Data were taken with the confocal pinhole set to the same settings as for FLIM (4.5 AU, 3.8 μm section). Scale bar is 20 μm .

The action potential (AP) in iCMs is typically on the order of 500 ms in duration. Intensity-based V_{mem} imaging techniques in cardiomyocytes generally use sampling rates between 50 and 500 Hz,^{23–25} but fluorescence lifetime imaging studies in other systems rarely exceed frame rates of 0.1 Hz. Using TCSPC FLIM, we acquired a raw lifetime data stream at 40 Hz (25 ms acquisition per frame). We binned successive pairs of frames to produce a final recording with 20 Hz frame rates and dramatically reduced noise in lifetime by increasing the photon count (**Figure 4.11d, Figure 4.12**). The limiting factor in the achievable frame rate was the speed at which the TCSPC electronics could process photons (the pile-up limit). We did not observe phototoxicity to the sample or drift in the lifetime; this is consistent with our ability to acquire continuously for 15 minutes in A431 cells (**Figure 4.8**).

We observe a stable lifetime baseline and consistent action potential morphology throughout all 10 second, 20 Hz frame rate recordings (**Figure 4.12**). We used TMCrRhZero to further verify that lifetime is insensitive to motion artifacts from the iCM contraction. Recordings with the voltage-insensitive derivative TMCrRhZero are stable throughout the 10 second acquisition, despite normal contraction of the iPSCs (**Figure 4.12e**).

In contrast, the photon count (fluorescence intensity) showed both a variable baseline and a variable AP waveform (**Figure 4.11c and 4.12b**), presumably from motion artifacts as the cardiomyocytes contract. Even though all wells and fields of view were loaded with the same concentration of dye (500 nM), the baseline intensity varied widely between recordings (**Figure 4.12b**). Differences in the amount of membrane in the field of view (**Figure 4.11a**), as well as differences in focal plane, are likely responsible for this. Normalizing the photon count into relative fluorescence units (**Figure 4.11c and 4.12c**), as is common with fluorescence intensity imaging, did not fully resolve these artifacts. Although the baseline was generally more consistent, gradual drifting was still visible. Furthermore, artifacts in the action potential morphology from cellular contraction (motion) were not resolved by this normalization.

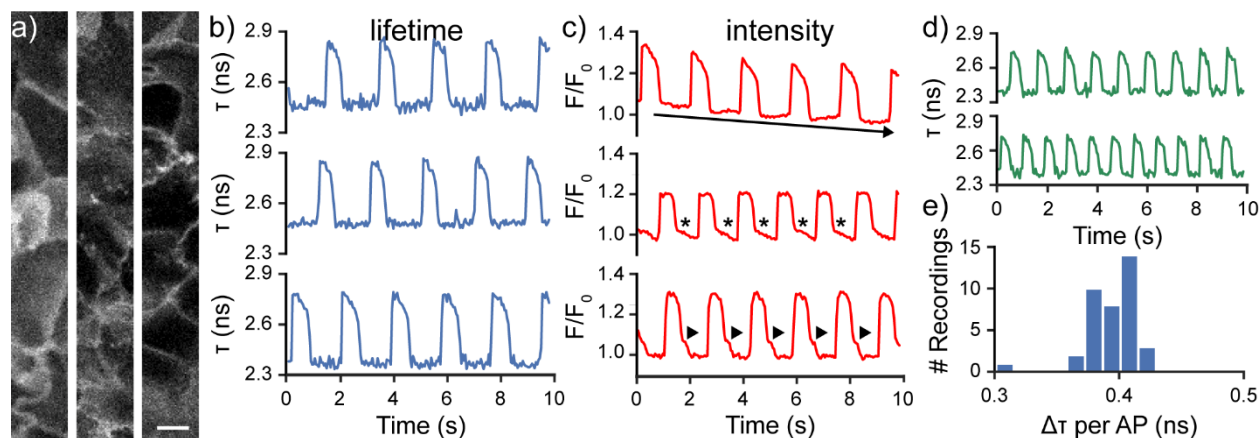


Figure 4.11. Fluorescence lifetime imaging of spontaneously beating cardiomyocytes using CRhOMe avoids artifacts associated with intensity-based imaging.

a) Representative confocal images of regions of iCMs used for 20 Hz lifetime imaging. Images here are fluorescence intensity only and were acquired for 400 ms (line averaged) to improve contrast. iCMs were stained with 500 nM CRhOMe. Scale bar is 10 μm . **b)** Plots of fluorescence **lifetime** of CRhOMe vs time in active hiPSC-CMs, quantified from the fields of view in (a). **c)** Plots of fluorescence **intensity** of CRhOMe vs time in active hiPSC-CMs from the same regions as in a and b. Top panel: arrow depicts photobleaching; middle panel: asterisks depict apparent AP morphological artifact; bottom panel: arrowheads show AP artifact. None of these artifacts appears

in the lifetime recording. **d)** Representative recordings of CRhOMe absolute V_{mem} imaging of iCMs treated with 1 μM isoproterenol, which accelerates beat rate. **e)** Amplitude of iCM action potentials, as measured by the change in τ of CRhOMe. Data represent 38 recordings from 8 iCM wells. Bin size for histogram was determined by the Freedman-Diaconis rule.

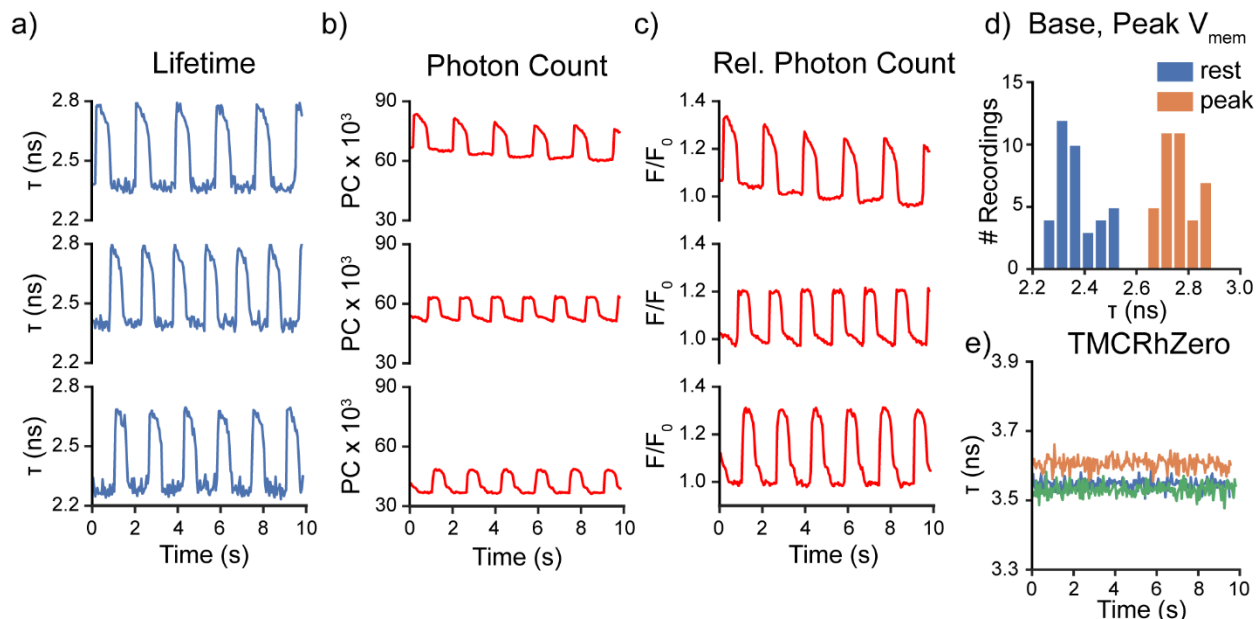


Figure 4.12 Comparison between lifetime, photon count, and relative photon count in hiPSC-CMs.

Data in panels (a) and (c) are from **Figure 4.11** and are reproduced here for comparison purposes with the data in panel b.

a) Fluorescence lifetime recordings from 3 representative fields of view. Data are not filtered or processed in any way after fitting. b) Photon count recordings from the same fields of view as in (a). c) Relative photon count (photon count divided by the average photon count baseline) for the same fields of view as in (a). d) Histograms representing the distributions of lifetimes observed at the base of an action potential (rest) and at the peak. e) Three individual traces of TMCRhZero in hiPSC-CMs, demonstrating that the lifetime of the voltage-insensitive dye doesn't change with cell contraction.

Using CRhOMe fluorescence lifetime as a proxy for absolute V_{mem} , we catalogued the action potential amplitude, resting τ , and peak τ across 40 iCM recordings (**Figure 4.12d**, **Figure 4.13**). We observe a consistent lifetime change of 0.394 ± 0.004 ns (mean $\pm$ SEM of 40 recordings across two differentiations). Resting τ (2.37 ± 0.01 ns) and peak τ (2.76 ± 0.01 ns) were somewhat more variable than the AP amplitude for these cells. We tested for concentration-dependent decrease in τ_{fl} by determining these AP parameters at CRhOMe concentrations of 300 nM, 500 nM, and 1 μM (**Figure 4.13c**). Minimal differences between concentrations were observed. Applying the previously determined sensitivity in HEK 293T cells (3.1 ps/mV) to cardiomyocytes suggests an AP amplitude of around 127 mV, based on an average lifetime change of 0.394 ns for each action potential. iCM action potentials are approximately 100 mV in amplitude, although they may range from 80-110 mV depending on differentiation conditions.^{26–29} The discrepancy between this value and the reported iCM AP amplitudes could be due to the previously observed slight dependence of VoltageFluor lifetime sensitivity on cell type.¹⁶ A future electrophysiological

calibration in iCMs would clarify the relationship between lifetime and voltage in this cell type specifically and enable a more quantitative analysis of these results.

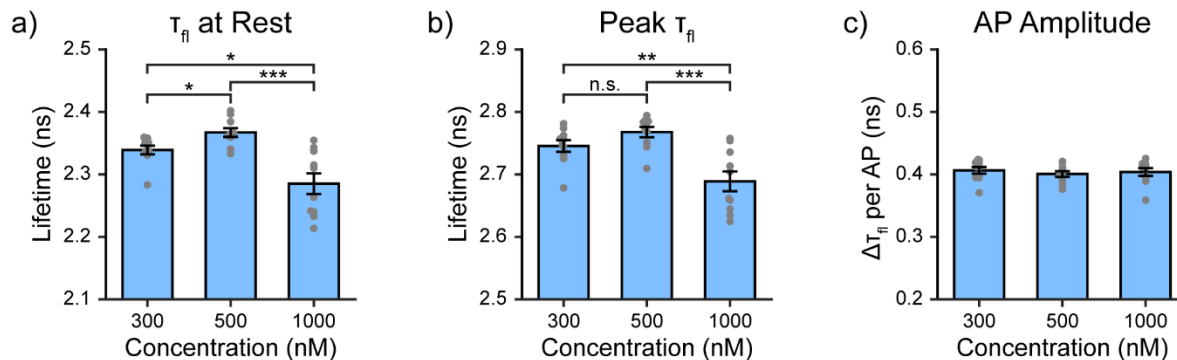


Figure 4.13 Effects of CRhOMe concentration on recorded iCM APs.

a) Resting lifetime of CRhOMe iCMs at different probe concentrations. Significant differences between concentrations were observed (Welch's ANOVA, $F(2,16.80) = 11.35$, $p = 0.0008$). b) Lifetime at the peak of iCM action potential (AP) at different CRhOMe concentrations. Significant differences between conditions were observed (Welch's ANOVA, $F(2, 17.13) = 9.48$, $p = 0.0017$). c) Action potential height (difference between resting and peak lifetime) at 300, 500, and 1000 nM CRhOMe. No significant differences between concentrations were observed (Fisher's ANOVA, $F(2,27) = 0.291$, $p = 0.75$). Data are shown as mean $\pm$ SEM of 10 recordings from two wells for each concentration. Asterisks reflect the significance level of Games-Howell post hoc tests (n.s. $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The 500 nM data shown here is a subset of the data shown in **Figure 4.11** in the main text and is reproduced here for comparison purposes.

To push the temporal resolution of CRhOMe FLIM further, we treated iCMs with the β adrenoreceptor agonist isoproterenol, which increases beat rate (**Figure 4.11d**).³⁰ Under these conditions, we observed spontaneous APs at approximately twice the frequency (~ 1.2 Hz versus ~ 0.6 Hz). Even with this more rapid activity, we were able to record the full AP waveform and a consistent baseline τ . As expected with an increase in beat rate, AP duration shortened in isoproterenol treated cells. AP amplitude appeared slightly smaller than in treated cells, and baseline τ remained unchanged (**Figure 4.11d**).

Conclusion

We developed a new VF dye, CRhOMe, which provides red to far-red excitation and emission, improved signal to noise ratios (SNR) compared to our previous BeRST indicator, and a monoexponential fluorescence lifetime decay, which substantially simplifies its use in FLIM, requiring an order of magnitude fewer photons for an accurate fit.^{31,32} These improvements allow us to image action potential dynamics in single trials in hippocampal neurons with improved SNR compared to BeRST, and measure hyperpolarization induced by epidermal growth factor-mediated (EGF) signaling in mammalian cells with 15 minutes of continuous illumination, a >5 -fold improvement over the first-generation VF-FLIM. CRhOMe is well tolerated in sensitive samples, and coupled with its brightness, fast response time, and mono-exponential fluorescence lifetime decay, this allows tracking of cardiac action potentials in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), eliminating artifacts associated with moving cells and opening the door to future calibration and determination of resting membrane potential values in excitable cells.

Materials and Methods

Materials

Dyes were stored either as solids at room temperature or as 1 mM stock solutions in DMSO at -20°C. All stock concentrations were determined via the absorbance of the carborhodamine chromophore using a 2501 Spectrophotometer (Shimadzu).

Epidermal growth factor (Peprotech) was made up as a 1 mg/mL stock solution in water and stored at -80°C. Gramicidin (Sigma-Aldrich) was purchased as a mixture of A, B, C, and D from *Bacillus brevis*. 1 µg/mL stocks of gramicidin were made up in DMSO and stored at -20°C. Isoproterenol was a gift from the Healy lab at UC Berkeley and was stored as a 10 mM stock in DMSO at -20°C.

Immortalized cell line culture

HEK293T and A431 cells were obtained from the UC Berkeley Cell Culture Facility. Both cell lines were verified by STR profiling (short tandem repeat) and were tested routinely for mycoplasma. Cell lines were discarded after 25 passages. Cells were maintained in complete DMEM (Gibco, Thermo Fisher Scientific) supplemented with 4.5 g/L glucose, 2 mM GlutaMAX (Gibco), and 10% FBS (Seradigm) in a 37°C humidified incubator at 5% CO₂. Cells were passaged into fresh complete media every few days following dissociation with trypsin-EDTA (Gibco, 0.05% for HEK293T, 0.25% for A431). Residual trypsin was removed from A431 cells by centrifugation for 5 minutes at 300xg.

For imaging experiments, cells were plated onto prepared poly-D-lysine (PDL)-coated coverslips. Coverslips (#1.5, either 12 mm or 25 mm diameter, Electron Microscopy Sciences) were acid washed for 2-5 hours in 1 M HCl. Coverslips were then washed three times overnight in 100% ethanol, followed by three times overnight in MilliQ (Millipore) purified water. Coverslips were sterilized by heating for 2-3 hours in a glassware oven to 150°C. Prior to seeding of cells, coverslips were incubated in 1x PDL (Sigma-Aldrich, made as a 0.1 mg/mL solution in phosphate-buffered saline with 10 mM Na₃BO₄) for 1-10 hours and then washed twice with sterile water and twice with 1x Dulbecco's Phosphate Buffered Saline (dPBS, Gibco).

For probe loading and gramicidin treatment experiments, HEK293T were seeded onto prepared coverslips in complete DMEM at a density of 42-52 x10³ cells per cm² (in a 6 well or 24 well tissue culture plate, Corning) and imaged approximately 24 hours after plating. For electrophysiology experiments, HEK293T were seeded at 26,000 cells/cm² in low glucose DMEM (Gibco; 1 g/L glucose, 1 mM sodium pyruvate, 2 mM GlutaMAX, 10% FBS) and used 12-24 hours after plating.

A431 cells were serum deprived prior to use.¹⁶ Two days before imaging experiments, cells were trypsinized and suspended in complete media with 10% FBS. The cells were then spun down for 5 minutes at 500xg and resuspended in low serum DMEM (4.5 g/L glucose, 2 mM GlutaMAX, 2% FBS). Cells were then seeded onto PDL-coated glass coverslips at a density of 83,000 cells/cm² in the low serum DMEM. 3.5-5.5 hours prior to imaging, media was exchanged for serum-free DMEM (4.5 g/L glucose, 2 mM GlutaMAX). Cells were loaded with dye after 4-5.5 hours in the serum-free media.

Culture of human induced pluripotent stem cell (hiPSC) derived cardiomyocytes

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

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 (Peprotech). 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 μ M IWP4 (Peprotech) 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. Imaging experiments were performed approximately two weeks after cells were seeded onto Matrigel-coated 24 well plates.

Whole cell patch clamp electrophysiology

Pipettes were pulled from borosilicate glass with filament (Sutter Instruments, Novato, CA) with resistances ranging from 4 to 7 M Ω with a P97 pipette puller (Sutter Instruments). Internal solution composition, in mM (pH 7.25, 285 mOsm/L): 125 potassium gluconate, 10 KCl, 5 NaCl, 1 EGTA, 10 HEPES, 2 ATP sodium salt, 0.3 GTP sodium salt. EGTA (tetraacid form) was prepared as a stock solution in 1 M KOH before addition to the internal solution. Pipettes were positioned with an MP-225 micromanipulator (Sutter Instruments). Voltage clamp protocols for epifluorescence experiments were not corrected for liquid junction potential. For voltage clamp protocols during fluorescence lifetime experiments, a liquid junction potential of -14 mV was determined by the Liquid Junction Potential Calculator in the pClamp software package (Molecular Devices, San Jose, CA), and voltage step protocols used in conjunction with fluorescence lifetime measurements were corrected for this offset.

Electrophysiology recordings were made with an Axopatch 200B amplifier and digitized with a Digidata 1440A (Molecular Devices). For epifluorescence voltage sensitivity measurements, cells were held at -60 mV and potentials from +100 to -100 mV were applied in descending order with 20 mV steps. Signals were sampled at 50 kHz and filtered with a 5 kHz low-pass Bessel filter. Correction for pipette capacitance was performed in the cell attached

configuration. Recordings were only included if they maintained a 30:1 ratio of membrane resistance (R_m) to access resistance (R_a) and an R_a value below 30 M Ω throughout the recording.

For voltage sensitivity measurements on the fluorescence lifetime system, cells were held at -60 mV and potentials from +80 to -80 mV were applied. Voltage steps were applied with a variable order of -80, -40, 0, and +40 mV steps, followed by a +80 mV step as the final voltage applied, with each voltage step holding for 15 seconds. Signals were sampled at 50 kHz and filtered with a 5 kHz low-pass Bessel filter. Correction for pipette capacitance was performed in the cell attached configuration. Recordings were only included if they maintained a 30:1 ratio of membrane resistance (R_m) to access resistance (R_a) and an R_a value below 30 M Ω throughout the recording.

Sample preparation and treatment for fluorescence lifetime imaging

For all experiments following concentration optimization (**Fig 4.3**) in HEK293T or A431, CRhOMe and TMCRhZero were used at 300 nM, directly from a 1 mM (3333x) DMSO stock (final DMSO concentration in loading solution, 0.03). 1x loading solutions were prepared in imaging buffer (IB, made in-house; composition in mM: 139.5 NaCl, 10 HEPES, 5.6 D-glucose, 5.33 KCl, 1.26 CaCl₂, 0.49 MgCl₂, 0.44 KH₂PO₄, 0.41 MgSO₄, 0.34 NaH₂PO₄; 290 mOsm/L; pH 7.25). Osmolarity was determined with a μ Osmette (Precision Instruments). Cells were incubated at 37°C for 20-25 minutes in loading solution, washed once in IB, and transferred to fresh IB in an Attotfluor cell imaging chamber (Invitrogen).

iCMs were loaded in RPMI with B27 supplement without phenol red containing 500 nM of CRhOMe or TMCRh Zero (0.05% final concentration of DMSO). Media was exchanged for fresh RPMI-B27 without phenol red before imaging.

Gramicidin solutions were prepared at either 0.5 μ g/mL or 1 μ g/mL in IB immediately before use. Immediately after dye loading, HEK293T and A431 were incubated for 5 minutes at room temperature IB containing either gramicidin or DMSO vehicle. Coverslips were then transferred to fresh IB with gramicidin or vehicle and imaged. To minimize media changes and time at room temperature for iCMs, iCMs were incubated for 5 minutes at 37°C in RPMI-B27 containing 0.5 μ g/mL gramicidin and then imaged in the same media.

For EGF treatment experiments in A431 cells, 2x EGF (1 μ g/mL) solutions were prepared in IB on the day of the experiment. Serum-starved A431 cells were loaded with 300 nM dye in IB after they had spent 4-5.5 hours in the serum-free media. Approximately twenty seconds into the FLIM recording, 500 μ L of IB or IB containing 2x EGF was gently pipetted into the imaging chamber (which contained 500 μ L IB). IB control recordings were made prior to EGF treatment recordings from a different field of view on the same sample.

Most imaging experiments except for those involving serum starved A431 cells were conducted under ambient atmosphere and at room temperature (18-22°C). To improve cell viability, some of the cardiomyocyte experiments were conducted with the XL incubation chamber on the LSM 880 set to 25°C. To minimize temperature fluctuations during extended recordings with serum-starved A431 cells, all work with these cells was conducted with the system incubation chamber set to 25°C and solutions pre-warmed to approximately 25°C. Samples were generally kept on the microscope for under 20 minutes; longer term EGF studies required samples to be kept on the microscope for up to 45 minutes.

Time Correlated Single Photon Counting (TCSPC)

Fluorescence lifetime imaging (FLIM) data were determined by TCSPC. Concentration curve data, A431 data, and cardiomyocyte data were acquired on an inverted LSM 880 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a FLIM upgrade kit (PicoQuant GmbH, Berlin, Germany). The LSM 880 was controlled with Zen Black software (Zeiss); the TCSPC unit was controlled with SymPhoTime 64 software (PicoQuant). Pulsed excitation at 40 MHz was supplied with a 640 nm diode laser (PicoQuant LDH-P-C-640B with a ZET635/20x cleanup filter, AHF/Chroma; average output wavelength 637 nm) controlled by a PDL-800-D laser controller (PicoQuant). Laser intensity from the PDL-800-D was kept at 24% to optimize instrument response function (IRF) shape; excitation power at the sample was controlled with a micrometer in the laser combining unit (PicoQuant). Power at the sample was adjusted to maximize count rate without exceeding the pile-up limit (a count rate of 4×10^6 photons/s at any individual pixel, 10% of the laser repetition rate). Because the dye was localized to membranes, large regions of the field of view were relatively dark, so average count rates varied from 1×10^5 - 2×10^6 counts per second depending on the sample and field of view. Average power at the sample ranged from 1-5 μ W.

Excitation light was coupled to the microscope with a polarization-maintaining single mode fiber and passed through a line pass dichroic (MBS 405/488/560/640) before reaching the sample. Emitted photons were collected with a 63x oil/1.4 NA Apochromat objective (Zeiss) immersed in Immersol 518F (Zeiss). The confocal pinhole was set to 300 (4.5 AU, optical slice thickness of approximately 3.9 μ m), increasing the size of the optical section but improving photon count rates for TCSPC. A 690/70 nm bandpass emission filter (AHF) was used. Single photons were detected with a PMA-Hybrid 40 detector (PicoQuant) and processed with a TH260 Pico Dual TCSPC Unit (T3 TCSPC mode). The detector parameters used were the following: constant fraction discriminator (CFD) -100 mV, zero cross -10 mV, offset -47000 ps. Sync parameters used were the following: CFD -150 mV, zero cross -10 mV, offset 0 ps, sync divider 8. Data were acquired with 1000 TCSPC time channels (0.025 ns/channel).

Electrophysiological lifetime calibration data were collected via TCSPC on an inverted LSM 980 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a SPC-150NX single photon counting card (Becker and Hickl). The LSM 880 was controlled with Zen Black software (Zeiss), and the TCSPC unit was controlled with SPCM software (Becker-Hickl). Pulsed excitation at 50 MHz was supplied with a 640 nm diode laser (BDS-SM-640-FBC-101, Becker-Hickl) and controlled through a LHB-104 Laser Hub (Becker-Hickl). Power was adjusted with a neutral density filter wheel to maximize count rate without exceeding pile-up limit (5×10^6 photons/s at an individual pixel, 10% of the laser repetition rate). Average power at the sample ranged from 1-3 μ W.

Excitation light was coupled to the microscope with a polarization-maintaining fiber and passed through a 405/485/560/640 beam splitter (Zeiss). Emitted photons were collected with a 40x oil/1.3 NA Apochromat objective (Zeiss), immersed in Immersol 518F (Zeiss). The confocal pinhole was set to 100 μ m (1.68 AU, 0.9 μ m optical section). The emitted photons were passed through a 676/37 emission filter (Semrock) and were detected with an HPM-100-40 hybrid detector (Becker-Hickl). The detector parameters used were the following: constant fraction discriminator (CFD) -50.98 mV, zero cross 5.29 mV. Sync parameters used were the following: CFD -50.98 mV, zero cross 5.29 mV, offset 5.10 %, sync divider 1, dither range 1/16. Data were acquired with 256 TCSPC time channels.

The IRF was measured each day from a sample of 1 mg/mL erythrosin B through the same imaging substrate (#1.5 coverglass or ibidi 24 well plate). 1 μ M TMCRh (small molecule dye head) in water was recorded daily as an additional standard to verify correct instrument function.

Monodirectional scanning was used for all applications except iCM spontaneous activity, where bidirectional scanning was used. Bidirectional scan correction is not possible in SymPhoTime, so the images of cardiomyocyte field of view (FOV) were acquired in Zen (same acquisition settings as FLIM, with the addition of 16x line averaging). Spatial and temporal resolution of the data acquisition for specific applications is enumerated in the table below.

Fluorescence lifetime data analysis

Time resolved fluorescence decays $I(t)$ were modeled as a monoexponential decay (equation 1) in custom Matlab code via a weighted least-squares approach. A represents the amplitude of the photon count signal and τ represents the fluorescence lifetime. TMCRhZero was well-modeled by a single exponential decay; CRhOMe contained slight structure in the residuals but two component decays resulted in overfitting. BeRST1 was not well-modeled by a single exponential decay, and for that reason not pursued for further characterization; lifetimes reported for BeRST1 are the amplitude-weighted average of a two-component decay fit, resulting from the same custom Matlab code.

$$I(t) = Ae^{-t/\tau} \quad [4.1]$$

Minimization of the reduced chi squared (χ^2 , equation 2) was used to determine the final parameter values, where n is the number of free parameters (number of fit time channels – number of free parameters in model), st is the first fit time channel, fi is the final fit time channel, z is the value of the fit model, and x is the value of the experimentally measured decay. Poisson weighting of the time channels was used.

$$\chi^2 = \frac{1}{n} \sum_{i=st}^{fi} \frac{(z_i - x_i)^2}{|z|} \quad [4.2]$$

The built-in minimization function `fmincon` with the interior-point algorithm was used, with the following settings: StepTolerance 1e-4, OptimalityTolerance 1e-4, ConstraintTolerance 1e-6. All fits converged in fewer than the maximum number of function evaluations (1e5). The fluorescence lifetime τ was constrained between 0 and 10 ns.

The experimentally measured instrument response function (IRF, erythrosin B fluorescence decay) was cropped time channels 40-60 out of 1000 channels (25 ns) for data from the LSM 880 microscope and cropped from time channels 29-48 out of 256 channels (20 ns) for data from the LSM 980 microscope, and reconvolved with the decay model before the reduced chi squared was determined. The time resolved signal was binned by a factor of 4 in time (to 250 channels representing 25 ns) for the data from the LSM 880 before fitting was performed to reduce fit noise with low photon counts, while the data from the LSM 980 was not binned in time. Time channels 20 through 980 (st through fi) of the unbinned signal from the LSM 880 data were used in the fit where photon counts were sufficient. Otherwise, the final time bin used was the first instance of zero photon counts in a time channel. The shift between the IRF and the measured time resolved decays was fixed to 0 for the data from the LSM 880; for the data from the LSM 980 microscope, the shift was fixed to -1, as this parameter can vary between TCSPC systems. The offset (time-independent background) was also fixed to 0, as the dark counts on both systems were generally negligible (20-100 counts per second during experiments). A threshold of 5000 total photons was used in pixelwise analysis. Pixels with photon counts below this threshold were excluded from analysis and appear black in images. Spatial binning was performed before fitting

for pixel-by-pixel analysis (**Table 4.1**). For global analysis of cardiomyocyte data, all photons in the field of view were used to fit one decay per time point.

Application	Raw Frame Acquisition Time (s)	Binned Frame Acquisition Time (s)	Number of Time Points (Total Time Series Duration)	Image Size (width x height, μm^2)	Pixel Size of Lifetime Image (binned, μm^2)
Single Image	0.94	75-90	1 (75-90 s)	135 x 135	2.1 x 2.1
Electrophysiology	0.24	15	5 (15 s)	53 x 53	1.7 x 1.7
EGF Treatment	0.63	5	36 (180 s), 177 (900 s)	135 x 33.7	6.3 x 6.3 *
iCM Spontaneous Activity	0.025	0.050	200 (10 s)	135 x 18	N/A (global analysis)

Table 4.1 Spatiotemporal resolution of fluorescence lifetime imaging data.

“Single image” data includes concentration curve loading tests and gramicidin treatment in cell lines (**Figure 4.3 and 4.6**). EGF time series are shown in **Figure 4.8 and Figure 4.9**. iCM spontaneous activity data are shown in **Figure 4.11 and Figures 4.10-4.12**. Raw frame acquisition time reflects the frame rate at which data were captured; successive frames were combined for later analysis to obtain good photon statistics. The number of time points is the number of analyzed, binned frames.

*For EGF treatment, pixel size after standard binning is the same as single images (2.1 x 2.1 μm^2). To improve photon statistics for pixelwise fitting, moving average binning was performed during analysis, as is common for Becker & Hickl FLIM products (e.g. SPCImage). Therefore, the lifetimes at each pixel were determined by photons from a 6.3 x 6.3 μm^2 area centered on the displayed pixel. The photon count image is displayed at the native resolution (2.1 μm per side of pixel).

Analysis of cardiomyocyte spontaneous action potential recordings

For action potential detection, lifetime recordings were transformed into a series of differences between successive frames (“difference trace”). This difference trace was filtered with a 6th order low pass Butterworth filter with the signal processing toolkit in SciPy (scipy.signal). A threshold that differentiated between action potentials and baseline was selected manually. The peak lifetime was defined as the value of the lifetime at the index identified by the above process. For each recording, the peak is reported as the mean of peaks of all action potentials in the recording. No filtering was used on lifetime traces shown or on lifetime data used for analysis; filtering was used exclusively in peak identification.

The baseline was defined from a series of 15 frames (750 ms) before the peak of each action potential (AP), beginning 20 frames before the peak and ending 5 frames before the peak. While this omits some areas of the baseline, it allows automatic processing at a variety of beat rates. The baseline reported for each recording is the average lifetime in all of these 15 frame windows except the first, which was often cut off by the start of the recording. For example, if a recording contained 6 APs, the baseline τ would represent the average of 75 frames of lifetime data (15 frames per AP from 5 APs).

The peak height was calculated as the average baseline subtracted from the average peak for each trace. For recordings containing gramicidin or made with TMCrhZero, no APs were visible, so the lifetime reported is the average across all frames in the 10 second recording.

For analysis of the photon count data (**Figure 4.12**), photon count data were taken from the same indices as defined by the lifetime trace (see above). The relative fluorescence intensity (F/F_0) was calculated by dividing each value in the photon count trace by the average photon count baseline.

FLIM electrophysiology calibration curves

Lifetime values were determined for membrane potentials using the lifetime-voltage standard curves determined with whole-cell voltage-clamp electrophysiology (**Fig 3**). The lifetime reported is the mean lifetime across all pixels in an ROI. ROIs were defined as the entire plasma membrane for the individual measured cell.

The relationship described by these curves between lifetime and membrane potential was determined by linear regression, resulting in a sensitivity (slope (m), ps/mV) and a 0 mV lifetime (y-intercept (b), ps) for each cell. The average sensitivity and 0 mV lifetime were determined from each of the cells. These means of these values were calculated to determine an “average calibration” which can be used to translate between measured lifetime and membrane voltage (equation 3). For quantifying and discussing changes in voltage, only the sensitivity (slope) is necessary (equation 4).

$$\tau = m * V_{mem} + b \quad [4.3]$$

$$\Delta V_{mem} = \frac{\Delta \tau}{m} \quad [4.4]$$

Differences in the measured lifetime at a defined reference point (for us, the 0 mV lifetime) across measured cells provides an estimate of the voltage-independent noise for the lifetime-voltage calibration of CRhOMe, and thus the resolution we can expect when using this calibration. We report this resolution as the root-mean-square deviation (RMSD) between the optically determined voltage using the developed calibration (V_{FLIM}) and the voltage of the cell as set by voltage clamp (V_{ephys}). The RMSD of a set of n measurements (equation 5) can be determined from the variance (equation 6) and the bias (equation 7) of the estimator (V_{FLIM}) as compared to the assumed “true” value, in this case determined by electrophysiology (V_{ephys}).

$$RMSD = \sqrt{\sigma^2 + Bias^2} \quad [4.5]$$

$$\sigma^2 = \frac{1}{n} \sum_{i=1}^n (V_{FLIM,i} - V_{ephys,i})^2 \quad [4.6]$$

$$Bias = \frac{1}{n} \sum_{i=1}^n V_{FLIM,i} - \frac{1}{n} \sum_{i=1}^n V_{ephys,i} \quad [4.7]$$

The variations in lifetime are larger between cells than within a cell, and thus the voltage resolution of CRhOMe lifetime in HEK 293T cells can be estimated with two values: the intra-cell resolution and the inter-cell resolution. The intra-cell resolution is a measure of the resolution expected for tracking and quantifying changes within an individual cell, determined by the RMSD between an individual cell’s “optical guess” at the lifetime for a particular voltage step, from its individual line of best fit, and the actual measured lifetime at that point. For CRhOMe, this intra-cell resolution is 6.36 ± 0.76 mV (RMSD, $\pm$ SEM). The inter-cell resolution is the single-trial resolution of estimating an individual HEK 293T cell’s membrane potential from the measured fluorescence lifetime, determined from the RMSD between the 0 mV lifetime value from each individual cell’s determined lifetime-voltage relation and the 0 mV lifetime determined by the average calibration. For CRhOMe in HEK 293T cells, this inter-cell resolution is 21 mV.

Statistical analysis

Data are reported as either mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM) throughout the text (as indicated). All aggregated data were acquired across at least two independent experimental days.

For analysis of differences between groups, all datasets were tested for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test on the median) before ANOVA was applied. For data that were both normal and homoscedastic, Fisher's one-way ANOVA with Tukey-Kramer post hoc tests was used ($p > 0.05$ on both the Shapiro-Wilk test and Levene's test). For data that were non-normally distributed and homoscedastic, the Kruskal-Wallis test with Dunn's test for pairwise post hoc tests was used. For data that were heteroscedastic ($p < 0.05$ on Levene's test), Welch's one-way ANOVA with Games-Howell post hoc tests was used, even if the data were significantly non-normal by Shapiro-Wilk. All statistical analysis was performed in Python using the SciPy, Scikit-Posthocs and Pingouin³³ statistical packages.

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Chapter 5

Evaluating the Effect of Molecular Wire Position on Voltage-Induced Fluorophore Lifetime and Sensitivity

Portions of this chapter are included in the following preprint:

Kulkarni, R. U.; Gest, A. M. M.; Lam, C. K.; Raliski, B. K.; James, F.; Adil, M. M.; Schaffer, D. V.; Wang, Y.; Miller, E. W. Computationally Assisted Design of High Signal-to-Noise Photoinduced Electron Transfer-Based Voltage-Sensitive Dyes. *ChemRxiv*. Preprint. DOI: 10.26434/chemrxiv.12401753.v1

Portions of this work were done in collaboration with the following people:

Iso, para, and disulfoVF2.2.OMeCl were synthesized by Dr. Rishi Kulkarni

Abstract

Fluorescence lifetime imaging microscopy (FLIM) can be used along with VoltageFluor potentiometric dyes to optically read out membrane potential in cells, due to the linear relationship between measured lifetime and applied voltage. However, fluorescence lifetime is also a useful proxy for quantum yield when *in vitro* quantum yield measurements are difficult or unreliable, as is the case for VoltageFluor (VF) dyes, which behave differently in cellular membranes than in solution. Because of this relationship and the properties of lifetime, fluorescence lifetime can be used to examine the relationship of VF structural modifications and the resulting effects on lifetime, photoinduced electron transfer (PeT), and voltage sensitivity. Here we utilize FLIM on eight dyes across three structural series and compare fluorescence lifetime, and so quantum yield, the efficiency of membrane loading of the dyes, and voltage-lifetime relationship.

Introduction

The ability to optically monitor biological phenomena with fluorescent sensors has revolutionized investigations in biochemistry, molecular biology, and neuroscience. Optical sensors for membrane potential, in particular, provide several benefits over the traditional electrophysiological approach, circumventing the issues of low throughput, low spatial resolution, and the invasiveness of electrophysiological measurement.¹ Our group is actively working to develop improved voltage sensors with improved sensitivity and signal-to-noise. However, further improvements require a better understanding of how the chemical properties of voltage sensitive dyes affect the voltage sensitivity exhibited. In this chapter, we explore how select structural changes affect the sensitivity and fluorescence lifetime of VoltageFluor (VF) potentiometric dyes.

VF dyes sense voltage changes through a photoinduced electron transfer (PeT) mechanism, in which the electric field across the cell membrane changes the rate of electron transfer from the donor to the acceptor in the dye molecule.² Thus, when the membrane is depolarized, the rate of PeT is decreased, resulting in less quenching of the acceptor, and an increase in acceptor fluorescence. When a cell hyperpolarizes, the rate of PeT increases, resulting in acceptor quenching, and diminishing of fluorescence. Because PeT is an excited state process, this change in fluorescence can be read out as a change in intensity, or in fluorescence lifetime.³ Reading out these effects via lifetime has several benefits, largely because fluorescence lifetime is largely insensitive to fluorophore concentration and excitation light power, both of which can confound intensity measurements. This improved resistance to experimental artifacts makes fluorescence lifetime a more robust and quantitative method for examining how structural changes affect VF photophysics.^{4,5}

We previously reported that a small change to the “anchoring group” of the molecule (**Figure 5.1a**) – an additional sulfonate group – led to an increase in the voltage sensitivity of the dye by 19% compared to the monosulfonated scaffold, due to improved alignment with the cell membrane and electric field.⁶ However, these disulfonated dyes were quite dim, and so we theorized that movement of the molecular wire group from its original position *para* to the xanthene fluorophore to a *meta* position could provide a similar improvement in electric field alignment, supported by molecular dynamics simulations of the dyes in a model membrane.⁷

However, the change in substitution pattern would likely change the electronics of the dye itself and affect the sensitivity and extent of PeT in the dye.

To examine the effect of wire position on dye electronics and inherent fluorescence properties more fully, in this chapter we compare three dye series: the VF2.2(OMe).Cl series, the VF2.1.Cl series, and the sarcosine dye series (**Figure 5.1 b,c,d**). We measure the fluorescence lifetime at rest in HEK293T cells to determine the effect of wire positioning on fluorescence lifetime, and by proxy, fluorescence quantum yield and brightness in these three dye series. We measure fluorescence lifetime at multiple concentrations for each dye, to look for the concentration at which shortening of the fluorescence lifetime occurs, to disentangle the effect of dye loading in the membrane from brightness, a measurement intensity-based measurements are not well-suited for due to factors such as photobleaching and light power that can affect apparent brightness separate from dye concentration.^{8,9} Additionally, for the sarcosine dye series, we measure the voltage sensitivity of the lifetime response for dyes with the wire in both the *meta* and the *para* positions, to examine the relationship between wire position and voltage-dependent rate of PeT, and begin to examine the possible effect of different anchoring groups on sensitivity.

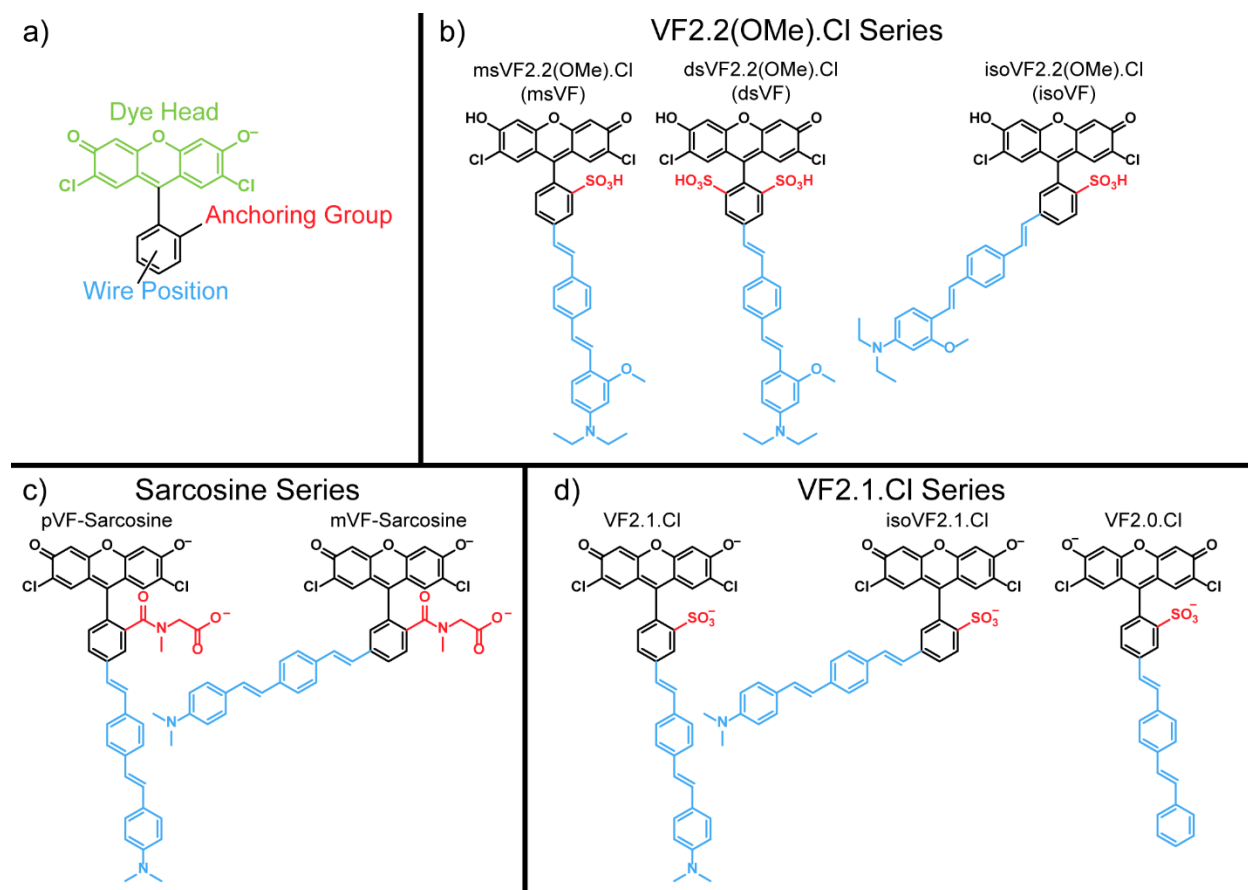


Figure 5.1 Dye series examined for structure-lifetime relationships.

a) General structure of a VoltageFluor (VF) dye with the structural motifs that can be changed for structure-function examinations. b) VF2.2(OMe).Cl dye series, with wire and anchoring groups highlighted. Includes msVF2.2(OMe).Cl, dsVF2.2(OMe).Cl, and isoVF2.2(OMe).Cl. c) Sarcosine dye series, with wire and anchoring groups highlighted. Includes pVF-Sarcosine and

mVF-Sarcosine. d) VF2.1.Cl series, with wire and anchoring groups highlighted. Includes VF2.1.Cl, isoVF2.1.Cl, and VF2.0.Cl.

Results and Discussion

Comparison of VF2.2(OMe).Cl Dyes

To investigate the hypothesis that changing the positioning of the molecular wire affects the extent of electron transfer three dyes were synthesized and tested in cells: msVF2.2(OMe).Cl (msVF), isoVF2.2(OMe).Cl (isoVF), dsVF2.2(OMe).Cl (dsVF) (**Figure 5.1**). In HEK293T cells, isoVF demonstrated increased cellular brightness compared to msVF and dsVF (**Fig. 5.2**), however, this increased cellular brightness does not necessitate an intrinsically brighter dye.

We hypothesize three potential mechanisms for the increased cellular fluorescence intensity of isoVF2.2(OMe).Cl compared to the msVF and dsVF dyes. The higher cellular brightness of isoVF could reflect differences in how efficiently the dye loads into the plasma membrane, affecting the local concentration of dye in the membrane, and thus brightness. Alternatively, the increased observed cellular brightness of isoVF could be due to the higher intrinsic quantum yield of the dye, consistent with the *in vitro* quantum yield data (**Table 5.1**).⁷ However, since the high pH of the *in vitro* measurements do not reflect conditions in and near cellular membranes, the actual, cellular quantum yield measured *in situ* might be quite different. Finally, in the cellular membrane environment, solvatochromism may alter the excitation and emission properties of the dyes to different degrees, resulting in a change in the apparent overall cellular brightness.

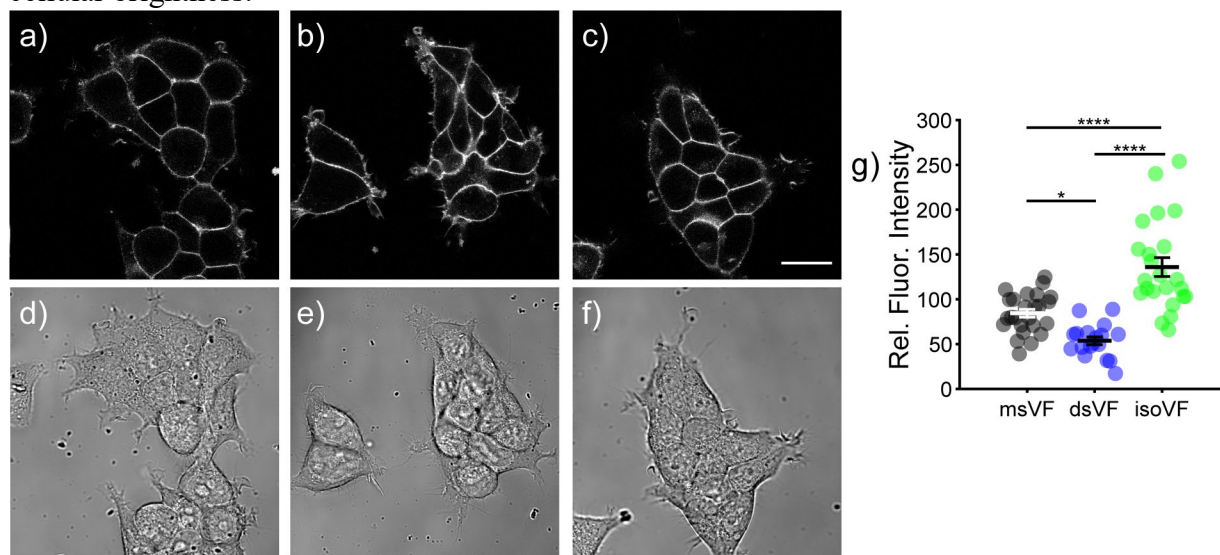


Figure 5.2. Imaging and quantification of VF dyes in HEK293T cells.

Laser scanning confocal images of HEK293T cells were stained with 300 nM of **a)** msVF2.2(OMe).Cl, **b)** dsVF2.2(OMe).Cl, or **c)** isoVF2.2(OMe).Cl and their corresponding **d-f)** differential interference contrast (DIC) images. All image acquisition settings parameters are identical. Scale bar is 20 μ m. **g)** Plot of fluorescence intensity measured by epifluorescence microscopy for each dye in HEK293T cells (at 500 nm). Data are mean fluorescence of cellular ROIs $\pm$ standard error of the mean for $n = 25, 18,$ or 23 cells (msVF, dsVF, or isoVF) across 3 separate coverslips. In epifluorescence imaging, ROIs were generated by circling the entire cell

outline. Statistical tests are one-way ANOVA Tukey's multiple comparisons post-hoc test. Adjusted p-values are * $p = 0.013$; **** $p < 0.0001$.

Entry	Φ_f^a	λ_{abs} nm ^a	λ_{em} nm ^a	$\Delta F/F$ (%) ^b	τ / ps ^c
msVF2.2(OMe).Cl	0.23	518	534	53 ± 1	312 ± 17
dsVF2.2(OMe).Cl	0.25	520	540	63 ± 2	393 ± 14
isoVF2.2(OMe).Cl	0.32	512	528	46 ± 1	714 ± 23

Table 5.1 Properties of ms-, ds-, and isoVF indicators.

[a] Acquired in phosphate buffered saline with 0.1 M NaOH (pH 12.5) and 0.1% Triton X-100.

[b] per 100 mV in HEK293Tcells. Data are mean $\pm$ S.E.M. for $n = 3$ patch-clamped

HEK293Tcells. [c] Acquired in resting HEK293Tcells. Error is 99% confidence interval determined via bootstrapping (100,000 shuffles).

Fluorescence lifetime imaging microscopy (FLIM) provides a way to assess the *in situ* quantum yield of the dyes. FLIM measures the fluorescence lifetime (τ_f) of dye molecules in living cells. The fluorescence lifetime is the average time required for a population of excited fluorophores to return to the ground state.¹⁰ The fluorescence lifetime is therefore a more intrinsic parameter compared to fluorescence intensity. While fluorescence intensity values can be confounded by changes in fluorophore concentration, variation in excitation power, and photobleaching, fluorescence lifetime is less dependent on these variables.⁴ A longer fluorescence lifetime directly correlates to a higher quantum yield of fluorescence.¹¹ Because VoltageFluors are lipophilic molecules, they may aggregate in solution, even in the presence of detergent. For this reason, *in vitro* quantum yields and molar absorptivities do not always reflect the observed brightness seen in cells – thus, the fluorescence lifetime in cells may provide a proxy for the fluorescence quantum yield in a lipid environment.

We measured the fluorescence lifetimes of the msVF, dsVF, and isoVF dyes in HEK293T cells using FLIM. We examined a range of loading concentrations for all three dyes in HEK293Tcells to find a concentration range where τ_f does not depend strongly on dye concentration (**Fig. 5.3**).³ At higher concentrations, the τ_f becomes shorter in a concentration-dependent manner, possibly from self-interaction between dye molecules in the membrane. Comparing the nominal concentration at which this shortening of τ_f occurs, isoVF τ_f decreases more dramatically and at lower concentrations than that of msVF or dsVF, both of which do not exhibit dramatic changes in lifetime at the concentrations examined. This suggests that isoVF loads more efficiently into the plasma membrane of cells, resulting in a higher local concentration in the membrane for isoVF than the other two dyes at the same nominal dye concentration. This may contribute, at least in part, to the higher cellular brightness observed for isoVF.

At concentrations below the point at which lifetime decreases, the fluorescence lifetime (τ_f) of isoVF2.2(OMe).Cl in HEK293Tcells is 714 ps (**Table 5.1, Fig. 5.4**). This is larger than the τ_f values of either msVF2.2(OMe).Cl (312 ps) or dsVF2.2(OMe).Cl (393 ps) (**Table 5.1, Fig.**

5.4). The nearly 2.3-fold larger τ_{fl} of isoVF2.2(OMe).Cl indicates a higher quantum yield of fluorescence in cellular membranes compared to msVF or dsVF.

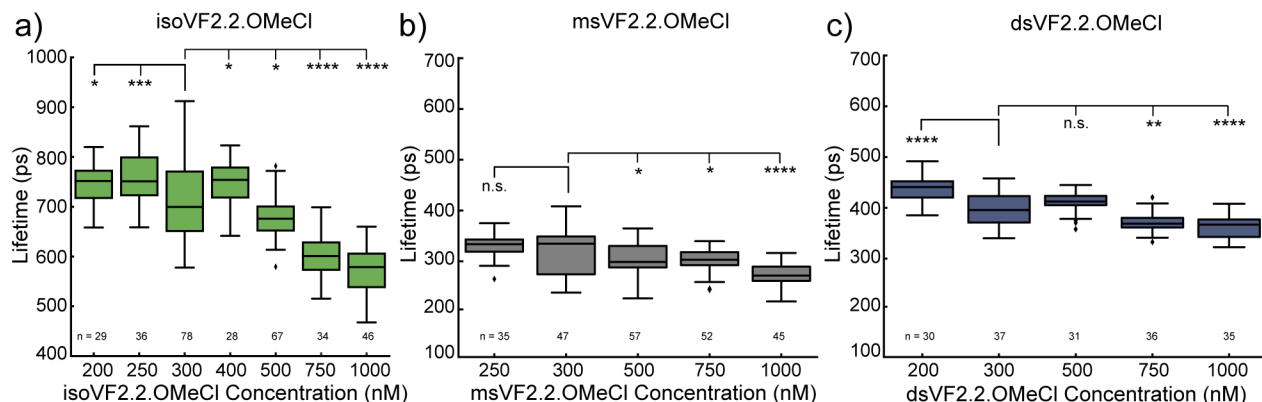


Figure 5.3 Fluorescence lifetime measurements at various concentrations in HEK293T cells.

Fluorescence lifetime measurements of a) isoVF2.2(OMe).Cl, b) msVF2.2(OMe).Cl, and c) dsVF2.2(OMe).Cl in HEK293T cells after loading at 200 – 1000 nM dye concentration. $n = 328$, 236, and 201 total cell groups containing a total of 1623, 1523, and 1754 cells, respectively. Solid lines indicate 25-50-75% quartiles. Values of n on the graphs are number of cell groups at each concentration. Kruskal-Wallis rank sum tests indicated significant differences in mean lifetimes between concentrations. Dunn post-hoc analysis identified significantly different concentrations. * = p -value < 0.05 , ** = p -value < 0.01 , *** = p -value < 0.001 , and **** = p -value < 0.0001 .

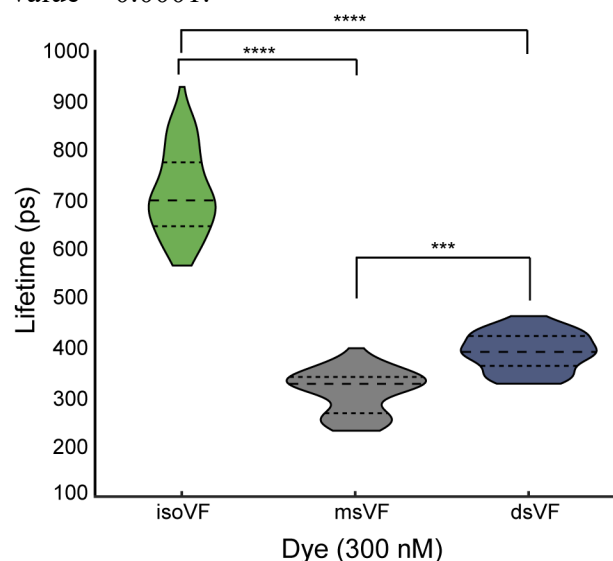


Figure 5.4. Fluorescence lifetime measurements of isoVF2.2(OMe).Cl, msVF2.2(OMe).Cl, and dsVF2.2(OMe).Cl in HEK293T cells.

All dyes were loaded at a concentration of 300 nM. $n = 78$, 47, and 37 cell groups containing a total of 378, 314, and 315 cells, respectively. Dashed lines indicate 25-50-75% quartiles.

Kruskal-Wallis rank sum tests indicated significant differences in mean lifetimes between concentrations. Dunn post-hoc analysis identified significantly different concentrations. P-values for all comparisons are less than 0.001.

We additionally used confocal laser scanning microscopy to look for changes in the excitation or emission profiles of the VF dyes in living HEK293T cells. Solvatochromism could alter the excitation or emission of the VF dyes, confounding a straightforward interpretation of fluorescence intensity. The emission spectra of all VF dyes in HEK293T cells are nearly superimposable (**Fig. 5.5a-b**). Additionally, excitation at either 488 nm or 514 nm has no effect on the shape of the emission spectra recorded in living HEK293T cells (**Fig. 5.5c-e**). Together, both fluorescence lifetime and emission spectral data in HEK293T cells suggest that the enhanced brightness of isoVF2.2(OMe).Cl in HEK293T cells is a result of higher fluorescence quantum yield, resulting in improved brightness, higher fluorescence quantum yield, all while maintaining 86% of the sensitivity of the msVF dye. This suggests the isoVF scaffold approaches a “sweet spot” of brightness and sensitivity.

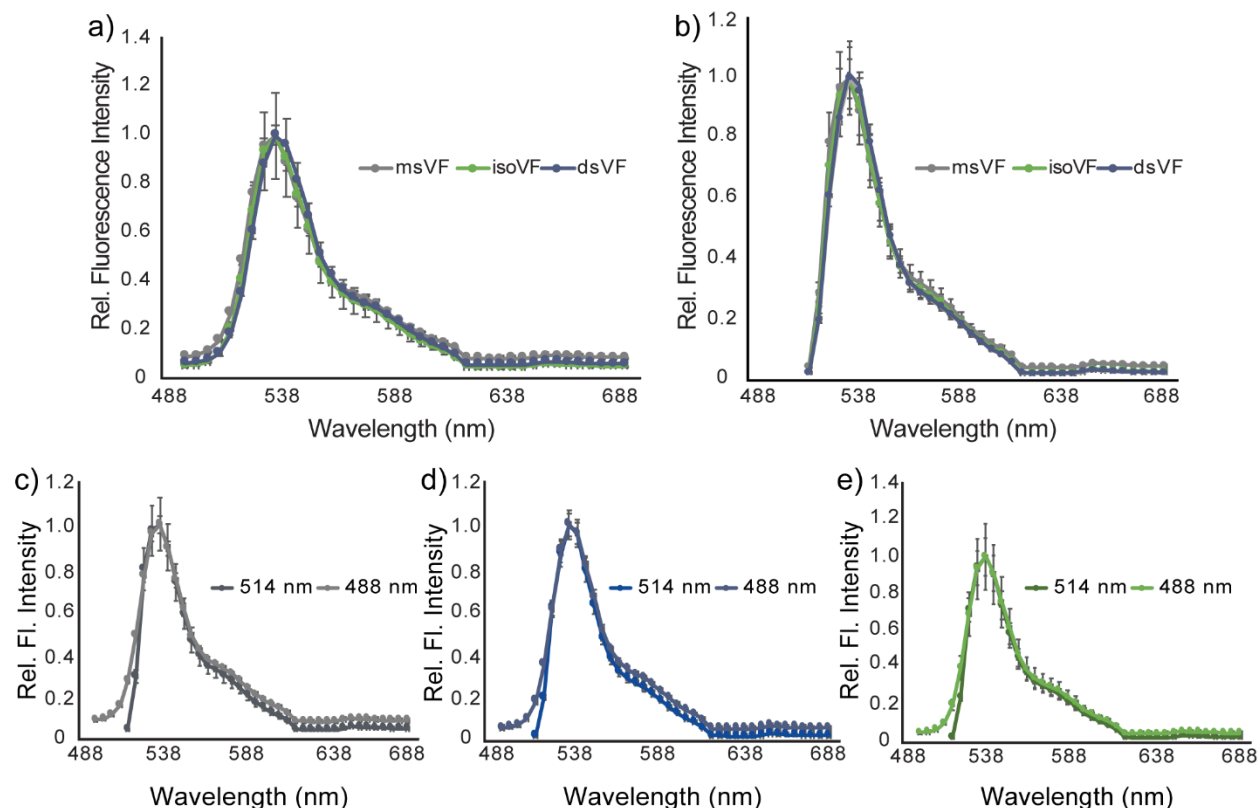


Figure 5.5. Emission scans of VF dyes in HEK293T cells with either 488 or 514 nm excitation. Plots of relative fluorescence intensity vs. wavelength for msVF2.2(OMe).Cl (grey), dsVF2.2(OMe).Cl (blue), and isoVF2.2(OMe).Cl (green). **a-b)** An overlay of relative fluorescence intensity vs wavelength for the three VF dyes with excitation provided at **a)** 488 nm or **b)** 514 nm. **d-e)** Overlays of relative fluorescence intensity vs wavelength for **c)** msVF2.2(OMe).Cl, **d)**

dsVF2.2(OMe).Cl, or e) isoVF2.2(OMe).Cl. Data points are average values $\pm$ standard error of the mean for membrane ROIs from $n = 3$ images.

Further comparisons on the VF2.1.Cl scaffold

Whether this trend of “iso” dyes with the wire at the meta position having longer fluorescence lifetimes (and thus higher fluorescence quantum yields) and τ_{fl} reduction at lower concentrations than corresponding dyes with a wire in the para position is a consistent trend requires further investigation. If the hypothesis that changing the wire position affects the favorability of PeT is true, the longer lifetime for meta-wire dyes should be consistent across VF dye scaffolds. To that end, we performed fluorescence lifetime imaging on a meta-wire analog of VF2.1.Cl, termed isoVF2.1.Cl.

We measured isoVF2.1.Cl lifetime in HEK293T cells at a variety of concentrations to identify the point of concentration-dependent lifetime decreases. A drop in fluorescence lifetime occurs around 500 nM isoVF2.1.Cl (**Fig. 5.6**). This is the same point at which this decrease in lifetime occurs for VF2.1.Cl and the voltage-insensitive VF2.0.Cl, which also has the molecular wire at the para position, though it lacks the dimethyl aniline at the base of the wire. This similar shape of the concentration curve and point of τ_{fl} -drop off suggests that the *meta* versus *para* wire positioning on its own does not necessarily affect the loading of the dye into the cell’s plasma membrane. Instead, the change in the wire position interacting with the other substituents in the VF2.2(OMe).Cl series may lead to the observed loading trend.

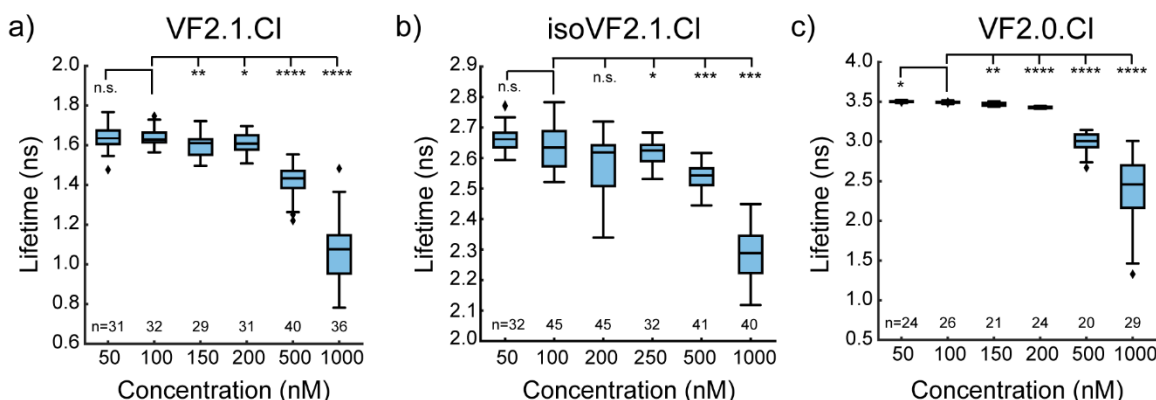


Figure 5.6 Concentration curves of VF2.1.Cl series dyes in HEK293T cells.

a) VF2.1.Cl dye with the wire in the para position relative to the fluorophore at various concentrations plotted against the fluorescence lifetime in ns. b) Lifetime plotted against concentration for isoVF2.1.Cl, with the wire in the meta position. c) Lifetime plotted against concentration for VF2.0.Cl, with no aniline donor on the molecular wire. a) and c) are repeated from **Chapter 2** for the sake of comparison. Solid lines indicate 25-50-75% quartiles. Values of n on the graphs are number of cell groups at each concentration. Welch’s ANOVA indicated significant differences in mean lifetimes between concentrations. Games-Howell post-hoc analysis identified significantly different concentrations. * = p -value < 0.05 , ** = p -value < 0.01 , *** = p -value < 0.001 , and **** = p -value < 0.0001 .

The lifetime of isoVF2.1.Cl, however, roughly follows the trend observed in the VF2.2(OMe).Cl dye series. At concentrations below the point where a decrease in lifetime is

observed, in HEK293T cells at rest isoVF2.1.Cl exhibits a fluorescence lifetime of 2.66 ± 0.01 ns (measured at 100 nM) (**Fig. 5.6**). This is approximately a nanosecond longer than VF2.1.Cl with the wire in the para position, which has a mean resting lifetime in HEK293T cells of 1.6 ns. VF2.0.Cl, which has no PeT contribution to the lifetime exhibits a mean lifetime of 3.5 ns in HEK293T cells. This is consistent with a hypothesis that changing the wire position relative to the fluorophore decreases the extent of PeT. We can thus look at this dye series in order of decreasing PeT, or increasing lifetime: VF2.1.Cl, isoVF2.1.Cl, and finally VF2.0.Cl.

Sarcosine scaffold lifetime and sensitivity

We then investigated the effect of the wire position on sensitivity in lifetime, using the sarcosine VoltageFluor scaffold, which replaces the sulfonate anchoring group with a sarcosine moiety.¹² This sarcosine group can be used as an alternative to the sulfonate that is more amenable to chemical modification, for instance as a means for appending groups to allow genetic targeting (**Chapter 3**).

mVF-Sarcosine demonstrates concentration-dependent changes in τ_{fl} of HEK293T cells starting at about 500 nM. This is a similar curve shape to that exhibited by pVF-Sarcosine, though the para wire dye may have a slightly shallower curve, lifetime changes begin between 500 and 750 nM nominal concentration of dye. This suggests that pVF-Sarcosine may load slightly differently than mVF-Sarcosine, but not with the dramatic differences seen in the VF2.2(OMe).Cl dye series. Like the other dyes examined in this chapter, mVF-Sarcosine exhibits an appreciably longer fluorescence lifetime, at 1.91 ± 0.01 ns, than pVF-Sarcosine, which has a fluorescence lifetime at 300 nM of 1.10 ± 0.02 ns (both mean $\pm$ SEM) (**Table 5.2**). These resting lifetimes are also notably shorter than those of isoVF2.1.Cl and VF2.1.Cl, which differ only in the anchoring group (sarcosine as opposed to the sulfonate of the VF2.1.Cl series). This may be due to the additional flexibility of the sarcosine group as compared to the sulfonate, providing additional non-radiative routes out of the excited state.¹⁰

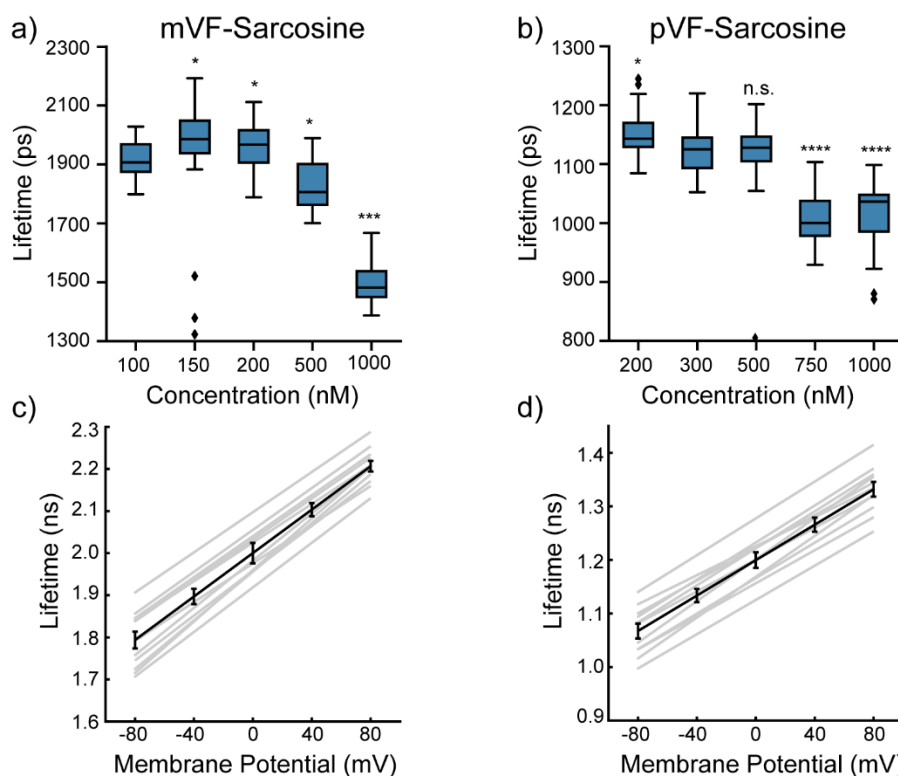


Figure 5.7 Sarcosine dye series concentration curves in HEK293T cells.

a) mVF-Sarcosine dye fluorescence lifetime at various concentrations in HEK293T cells.

Significance indicated as compared to 100 nM dye. Data repeated from **Chapter 3** for comparison. b) pVF-Sarcosine dye fluorescence lifetime at various concentrations in HEK293T cells. Significance indicated as compared to 300 nM dye. Kruskal-Wallis rank sum test indicated significantly different values of lifetimes, and Dunn's post-hoc analysis identified significantly different concentrations. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001, and **** = p-value < 0.0001. c) Lifetime-voltage relationship of mVF-Sarcosine (reproduced from **Chapter 3**). d) Lifetime-voltage relationship of pVF-Sarcosine. Error bars on both are $\pm$ SEM at each potential. Gray lines represent individual lines of best fit, black line represents average calibration.

Entry	$\Delta F/F$ (%) ^a	$\Delta\tau/\tau$ (%)	Lifetime Sensitivity (ps/mV)	τ / ns
pVF-Sarcosine	27	15.0	1.65	1.10 ± 0.02
mVF-Sarcosine	22	13.9	2.58	1.91 ± 0.01

Table 5.2 Properties of pVF- and mVF-Sarcosine VoltageFluors.

All data taken in HEK293T cells. [a] Reproduced from Grenier et. al., *J. Am. Chem. Soc.* **2019**, 141, 3, 1349-1358.

mVF-Sarcosine and pVF-Sarcosine have both been previously investigated in fluorescence intensity, and their reported voltage sensitivities are consistent with the hypothesis

that the meta wire position alters the electronics and favorability of PeT, as mVF-Sarcosine demonstrates slightly reduced voltage sensitivity (22% $\Delta F/F$) compared to pVF-Sarcosine (27% $\Delta F/F$) (**Table 5.2**).¹² We wished to further characterize the voltage sensitivity with a calibration between voltage and fluorescence lifetime, as this is a more absolute relationship than fluorescence intensity and can be more quantitatively calibrated for use as an optical readout of voltage in biological systems. For mVF-Sarcosine, the fractional change in lifetime per 100 mV is 13.9% $\Delta\tau/\tau$, and pVF-Sarcosine exhibits a fractional sensitivity of 15.0% $\Delta\tau/\tau$ per 100 mV (**Table 5.2, Figure 5.7 c, d**), consistent with the hypothesis that the molecular wire in the meta position results in decreased voltage sensitivity compared to the para position. Interestingly, this decrease in sensitivity is notable in the fractional change, but in terms of absolute lifetime change per millivolt of voltage change, mVF-Sarcosine is more sensitive.

Unexpectedly, these sarcosine-modified dyes exhibit different fractional changes in lifetime per 100 mV of voltage than their fractional change in intensity (**Table 5.2**). Other dyes, meanwhile, have shown more similar $\Delta F/F$ and $\Delta\tau/\tau$ values. For instance, VF2.1.Cl, which has a % $\Delta F/F$ of 27%, and % $\Delta\tau/\tau$ of 22% per 100 mV, in relatively good agreement with one another.³ This suggests, perhaps, something affecting the voltage sensitivity in intensity that does not affect PeT or the excited state of the dye, and so does not present as sensitivity in fluorescence lifetime. Further investigation into the effect of changes at the anchoring group on sensitivity and lifetime could provide a deeper understanding of how changes to the VoltageFluor scaffold affect the dye's function on a photophysical level.

Conclusion

In this chapter we utilize FLIM to examine the relationship between VF dye structure and photophysical property, and uncovered a consistent trend in VoltageFluor potentiometric probes: that changing the positioning of the molecular wire affects the length of the fluorescence lifetime, suggesting that molecular wire position affects the extent of photoinduced electron transfer occurring. This is reflected in the value of the fluorescence lifetime, which is longer for the dyes that have the molecular wire in the meta position than those with the wire in the para position (**Table 5.3**). Across three dichlorofluorescein-based dye series, the *meta* wire dye in the series has a longer lifetime in cells at rest than the *para* dyes, with increases in lifetime ranging from 61% to 129%. The effect is larger for the VF2.2(OMe).Cl series (129% increase) than the sarcosine or VF2.1.Cl series (74% and 61% respectively).

Entry	Sarcosine	VF2.1.Cl	VF2.2(OMe).Cl
<i>para</i> Wire Lifetime (ns)	1.10	1.65	0.31
<i>meta</i> Wire Lifetime (ns)	1.91	2.65	0.71
<i>para</i> Rel. Lifetime	1.00	1.00	1.00
<i>meta</i> Rel. Lifetime	1.74	1.61	2.29

Table 5.3 Comparison of *meta* and *para* dye lifetimes.

All data taken in HEK293T cells. Lifetimes reported in nanoseconds. Relative lifetimes normalized to the *para* wire dye lifetime for the series.

Fluorescence lifetime also reveals additional characteristics of the examined dye series. In the sarcosine dye series, mVF-Sarcosine demonstrates lower fractional voltage sensitivity in both intensity and lifetime than pVF-Sarcosine (**Table 5.2**). However, both dyes are much less sensitive in lifetime than would be assumed based on their sensitivity in intensity – this is not the case for VF2.1.Cl, nor some other dyes examined, including genetically targeted dyes (**Chapter 3**).³ This suggests that not only does the position and composition of the molecular wire affect the lifetime and sensitivity of VoltageFluors, but that the anchoring group on the pendant ring of the fluorophore may have effects as well, when until recently making changes at this group was considered relatively benign to the function of the VF.

Further understanding of the effect of structural changes at various positions of the VF could be obtained by examining the corresponding “zero” dye to the sarcosine VoltageFluor series (lacking the aniline at the base of the molecular wire). Additionally, the effect of loading that was approximated via the use of lifetime concentration curves and looking for concentration-dependent changes in lifetime could be validated and further explored with examinations of single molecule behavior and FCS determinations of fluorophore concentrations in cells.¹³ Finally, comparing various scaffolds with changes at the anchoring group to see its possible effects on lifetime and sensitivity, in conjunction with other photophysical measurements and molecular modeling. These efforts in computational modeling and development of different anchoring groups are both active areas of research in the lab and could be combined to great effect with fluorescence lifetime measurements to characterize the full spectrum of VF positional modification effects on sensitivity, lifetime (and quantum yield) and overall cellular brightness, to provide a more complete guide for chemists and biologists to design the most functional probe for a given application.

Methods

Spectroscopic studies

Stock solutions of VF dyes were prepared in DMSO (1.0–10 mM) and diluted with PBS (ThermoFisher #10010, 160 mM NaCl, pH 7.4; adjusted to pH 12.5 with 0.1 M NaOH). UV-Vis absorbance and fluorescence spectra were recorded using a Shimadzu 2501 Spectrophotometer (Shimadzu) and a Quantamaster 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

Human embryonic kidney 293T (HEK) cells were passaged and plated onto 25 mm glass coverslips pre-coated with Poly-D Lysine (PDL; 1 mg/ml; Sigma-Aldrich) to provide a confluency of ~ 50% for. HEK293T cells were plated and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% FBS and 1% Glutamax, detailed cell culture conditions are in (**Chapter 2, Chapter 3**).

Emission Scans in HEK293T Cells

Emission scans were collected on a Zeiss LSM 980 NLO Airyscan 2 equipped with 488 and 514 nm diode lasers (Zeiss). Images were acquired with a 40x/1.4 NA oil immersion objective (Zeiss) coated with immersion oil (Immersion Oil 518F, Zeiss) and focused onto a GaAsP detector (Zeiss). Emission scans were performed using the Zeiss lambda scan mode in the Zen Blue software, scanning emission using a diffraction grating and the main dichroic beamsplitter (MBS, Zeiss), which blocks excitation light, to sample from 518 to 698 nm when excited with 514 nm light, and from 498 to 698 nm when excited with 488 nm light, using 5 nm intervals. Excitation with 514 nm light was at 1.2% laser power, and 488 nm light was at 0.8% laser power. For data analysis, ROIs were drawn around cell membranes and the fluorescence intensity at each wavelength was recorded. For comparison across dyes, fluorescence intensity values were normalized to the average ($n = 3$ images) of the peak emission and plotted side-by-side (**Figure 5.5**).

Fluorescence Lifetime Imaging

Fluorescence lifetime imaging was performed on an LSM 510 scanning confocal microscope (Zeiss), equipped with an SPC-150N single photon counting card (Becker and Hickl GmbH). 80 MHz pulsed excitation light was supplied by a Ti:Sapphire laser (MaiTai HP; Spectraphysics) tuned to 958 nm and frequency-doubled to 479 nm. Images were acquired with a 40x/1.4 NA oil immersion objective (Zeiss) coated with immersion oil (Immersion Oil 518F, Zeiss). Emitted photons were detected with a hybrid detector, HPM-100-40 (Becker and Hickl), based on a Hamamatsu R10467 GaAsP hybrid photomultiplier tube. Reference photons for determining photon arrival times were detected with a PHD-400-N high speed photodiode (Becker and Hickl). Data were collected with 256 time bins and 256 x 256 pixels of spatial resolution, and 90 s acquisition time. Excitation light power was controlled with a neutral density wheel and a polarizer, followed by a polarizing beamsplitter. The light power was titrated so that lifetimes did not drift up over the course of the experiment, no phototoxicity was notable, and there was no photon pileup on the detector. Performance of the lifetime recording setup was assessed by regular measurements of three standards: 2 μ M fluorescein in 0.1 N NaOH, 1 mg/mL erythrosin B in water, and the measured instrument response function (IRF).¹⁵

The ideal concentration of VoltageFluor used to compare resting lifetimes was determined by measuring the lifetime of each dye at different concentrations and generating a concentration curve for each dye. The concentration selected to use for comparisons was a concentration at which the signal was bright, but in the “flat” region of the curve, at a concentration lower than the point at which shortening of the lifetime is observed. For isoVF2.2(OMe).Cl, msVF2.2(OMe).Cl, and dsVF2.2(OMe).Cl, 300 nM was selected (**Fig. 5.3, 5.4**). For VF2.1.Cl, isoVF2.1.Cl, and VF2.0.Cl, 100 nM was used. For mVF-Sarcosine, 100 nM was chosen, while 300 nM was used for the dimmer pVF-Sarcosine.

All lifetime data were fit with SPCImage (Becker and Hickl), which solves the nonlinear least squares problem using the Levenberg-Marquadt algorithm.¹⁴ isoVF2.2(OMe).Cl, msVF2.2(OMe).Cl, and dsVF2.2(OMe).Cl VoltageFluor lifetimes were fit to a sum of three exponential decay components (**Equation 5.1**); attempts to fit the data with a single or double exponential decay were not satisfactory for this dye series. VF2.1.Cl was fit with a biexponential

decay, as in **Chapter 2**, as was isoVF2.1.Cl. VF2.0Cl was fit with a monoexponential decay, except at high dye concentrations, where it was fit with a biexponential model. mVF-Sarcosine was fit with a biexponential decay, as in **Chapter 3**, and pVF-Sarcosine was fit with a biexponential model as well.

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3} \quad [5.1]$$

Fluorescence lifetimes fit to this model were reported as the amplitude-weighted average (**Equation 5.2**), and this value was used for subsequent analysis.

$$\tau_m = \frac{a_1 \tau_1 + a_2 \tau_2 + a_3 \tau_3}{a_1 + a_2 + a_3} \quad [5.2]$$

Pixels with less than 300 photon counts at the peak of the decay were omitted from lifetime analysis to ensure reproducible fits. As the lifetime of VoltageFluors does not fully decay within a single 12.5 ns laser cycle, the incomplete multiexponential fitting option in SPCImage, allowing the model to attribute some signal early in the decay to a previous laser cycle. Out of 256 time bins, only data from time bins 21 to 240 were used in the final fit. The offset parameter (the detector dark counts per time bin per pixel) was set to zero. The number of iterations for the fit in SPCImage was set to 20 to achieve converged fits. The shift between the IRF and the decay trace was fixed to 0.5 (time bins), which consistently gave lifetimes of the standards (fluorescein and erythrosin B).

After lifetime fitting, regions of interest (ROIs) were determined by dividing images into cell groups, with each cell group as a single ROI. ROIs were determined from photon count images by automatically sharpening and then thresholding the signal intensity with custom MATLAB code.³

Statistical analyses for concentration curves and dye comparisons were performed in python 3 using the pandas, SciPy, Pingouin, and scikit-posthocs packages.^{15,16} All fluorescence lifetime data shown reflects at least three biological replicates (independent cultures of HEK-293T cells on separate days), and each biological replicate reflects 3 to 5 technical replicates (different samples of cells on the same coverslip that were from the same cell stock).

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Appendix 1:
A Protocol for VoltageFluor Dyes and Fluorescence Lifetime Imaging for Optical Measurement of
Membrane Potential

Portions of this appendix were written in collaboration with the following people:
Susanna Yaeger-Weiss and Dr. Julia Lazzari-Dean

Portions of this appendix were published as a chapter in the following:
Gest, A. M. M.; Yaeger-Weiss, S. K.; Lazzari-Dean, J. R.; Miller, E. W. VoltageFluor Dyes
and Fluorescence Lifetime Imaging for Optical Measurement of Membrane Potential. In
Methods in Enzymology; Academic Press Inc., 2021; Vol. 653, pp 267–293.
<https://doi.org/10.1016/bs.mie.2021.02.009>.

Abstract

Membrane potential is a fundamental biophysical parameter common to all of cellular life. Traditional methods to measure membrane potential rely on electrodes, which are invasive and low-throughput. Optical methods to measure membrane potential are attractive because they have the potential to be less invasive and higher throughput than classic electrode based techniques. However, most optical measurements rely on changes in fluorescence intensity detect changes in membrane potential. In this chapter, we discuss these use of fluorescence lifetime imaging microscopy (FLIM) and Voltage-sensitive Fluorophores (VF dyes) to make estimate of the millivolt value of membrane potentials in living cells. We discuss theory, application, detail a protocol for making these measurements, and discuss shortcomings of the application.

Introduction

Membrane potential, V_m , is an important biophysical parameter in cellular physiology. Fast changes in V_m , such as action potentials on the millisecond timescale, drive the specialized physiology of neurons and cardiomyocytes. Changes over longer timescales, from seconds to minutes to hours, may also be important in cells traditionally considered not to be electrically excitable, and have been implicated in processes such as mitosis¹, cell cycle progression², differentiation^{3,4}, and development⁵.

The “gold standard” method for measuring membrane potential is patch-clamp electrophysiology. Electrophysiology involves direct electrical contact with the cell of interest, thereby providing an accurate and precise measure of the membrane potential of a cell. Additionally, because of this electrical contact, electrophysiology can make fast measurements of voltage changes, and for this reason, it has been useful in recording rapid signals such as action potentials. However, electrophysiology suffers in two main areas: its invasiveness and its lack of throughput. Because patch-clamp electrophysiology requires direct contact with the cell, and the whole-cell configuration ruptures the cell membrane, measurements taken with patch-clamp electrophysiology are inherently invasive and often terminal for the cell under investigation. Also because of the need for direct contact with the cell of interest, patch-clamp techniques are limited in the number of cells that can be measured at once. While there have been improvements in this area with specialized techniques designed for higher-throughput screens, such as planar patch clamp⁶, electrophysiology is still limited in scale, making measurements of V_m across large numbers of cells difficult.

Optical methods that use potentiometric fluorescent dyes address some of the shortcomings of invasiveness and throughput. Because these dyes can often be bath applied to many cells in a dish, or to a whole tissue, they have improved throughput compared to electrophysiology. Additionally, because they read out voltage optically, techniques involving these dyes are less invasive. However, intensity-based measurements of V_m are difficult to perform accurately. While they are useful for reporting changes in membrane potential, absolute measurements of actual millivolt values of V_m are difficult to obtain⁷, due to confounding factors such as dye concentration, and photobleaching, which complicate the creation of an accurate calibration⁸.

Fluorescence lifetime imaging microscopy (FLIM) offers an alternative to intensity-based methods and avoids many of these confounding factors. The fluorescence lifetime (τ) is a measure of the time a molecule spends in its excited state, and is intrinsic to the molecule

itself, being proportional to the fluorescence quantum yield (ϕ) (**Equation A1.1**)⁹. Because fluorescence lifetime depends only on the rates of radiative (k_r) and nonradiative decay (k_{nr}) pathways out of the excited state (**Equation A1.2**)⁹, the fluorescence lifetime of a fluorescent indicator can provide a useful readout based only on the factors that affect these decay pathways, as opposed to confounding experimental artifacts like photobleaching or indicator concentration¹⁰. Additionally, because lifetime is an optical technique, it can be minimally invasive and can be applied to many cells at once, and thus it is useful for making large-scale measurements.

$$\tau = \phi/k_r \quad (\text{A1.1})$$

$$\tau = \frac{1}{k_r + k_{nr}} \quad (\text{A1.2})$$

The utility of fluorescence lifetime as a way to read out membrane potential in particular has been demonstrated first with fluorescent proteins¹¹, and then through the use of a photoinduced electron transfer (PeT)-based dye, VF2.1.Cl¹², one of several dyes in a class known as VoltageFluors (VFs). In these dyes, the rate of PeT is modulated by the voltage across the cell's membrane, making the dye dimmer at more negative potentials and brighter at more positive potentials (**Figure A1.1**)^{13,12}. PeT is a non-radiative decay pathway out of the fluorophore's excited state; therefore, modulation of PeT also leads to modulation of the fluorescence lifetime. For VF2.1.Cl, negative membrane potentials lead to shorter lifetimes, and positive potentials to longer fluorescence lifetimes¹². These lifetimes of the probe in the cell membrane can be measured using FLIM and the intensity decay modeled mathematically using a multiexponential decay (**Equation A1.3**). If multiple exponential terms are present, the lifetime of the probe is then expressed as the amplitude-weighted average of the components (τ_m) where the lifetimes of each individual component (τ_n) are weighted by the amplitude of that component (a_n) (**Equation A1.4**).

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + \dots + a_n e^{-t/\tau_n} \quad (\text{A1.3})$$

$$\tau_m = \frac{a_1 \tau_1 + a_2 \tau_2 + \dots + a_n \tau_n}{a_1 + a_2 + \dots + a_n} \quad (\text{A1.4})$$

The relationship between membrane potential and lifetime of the voltage-sensitive fluorophore enables a lifetime-voltage calibration to be performed in cultured cells using patch-clamp electrophysiology. This calibration relates the weighted average lifetime (τ_m) to a particular membrane potential value. This is in contrast to $\Delta F/F$ relationships, which cannot easily report on actual membrane potential values, since the baseline F depends not only on membrane potential, but also on other confounding variables like dye concentration, variations in excitation intensity, and photobleaching. Once it has been determined, this lifetime-voltage calibration can then be applied relatively easily to lifetime measurements made on additional cells without re-calibration. Absolute values of V_m can therefore be measured across hundreds of cells or tracked longitudinally in particular cells in only a few hours of experiment time.

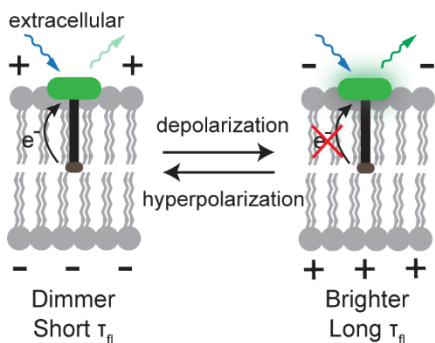


Figure A1.1. VoltageFluor dyes report membrane potential via photoinduced electron transfer.

At rest, electron transfer from the aniline (brown) to the fluorophore (green) quenches fluorescence, resulting in dimmer fluorescence intensity and shorter fluorescence lifetime (τ_{fl}). When the plasma membrane depolarizes, electron transfer is inhibited and fluorescence intensity is brighter. This also corresponds to longer fluorescence lifetime. Figure adapted from Lazzari-Dean, *et al. eLife*, **2019**, 8, e44522 under a CC-BY 4.0 license.

1. Rationale

Fluorescence lifetime, unlike fluorescence intensity, is a value that is intrinsic to the dye, modulated only by its environment and independent of factors such as photobleaching or the amount of dye present in the location of interest (outside of concentrations at which self-quenching occurs). For this reason, potentiometric dyes with mechanisms of voltage sensitivity which affect the dye's excited state can be used to optically read out absolute membrane potential, once they are calibrated in a given system. VoltageFluors, a class of dye which reports voltage through photoinduced electron transfer (PeT), are one variety of dye which may be calibrated and used to report bulk membrane potential measurements of many cells at once through the use of FLIM, in a technique referred to as VF-FLIM. VF2.1.Cl, a dichlorofluorescein-based VoltageFluor, is sufficiently bright and sensitive for use in FLIM experiments, and has been demonstrated a reliable reporter of membrane potential via VF-FLIM in multiple cultured cell lines, including HEK293T cells. However, this technique should be generalizable to many cultured cell types, other VoltageFluor dyes, and possibly other potentiometric indicators in which changes in membrane potential alter the excited-state lifetime, of suitable brightness and voltage sensitivity ¹¹.

Equipment

Image Acquisition and Analysis

- Glass coverslips (#1.5, 25 mm diameter) (Electron Microscopy Sciences)
- Attofluor imaging chamber (or similar dish that will allow use of a glass coverslip)
- SPC-150NX photon counting card (Becker-Hickl)
- HPM-100-40 GaAsP hybrid detector (Becker-Hickl)
- DCU-800 laser control unit (Becker-Hickl)
- Laser hub with 488 nm picosecond diode laser (Becker-Hickl)
- Zeiss 980 LSM Microscope (Zeiss)
- ZenBlue LSM software (Zeiss)

- SPCM FLIM acquisition software, version 9.84 (Becker-Hickl)
- SPCImage analysis software, version 8.3 (Becker-Hickl)
- ImageJ or other image processing software

Electrophysiology

- P97 Pipette Puller (Sutter Instruments)
- Pipettes pulled from borosilicate glass with filament, resistances 4 to 7 MOhms (Sutter Instruments)
- Patch-clamp electrophysiology rig
 - MP-225 Micromanipulator (Sutter Instruments)
 - Digitizer (e.g. Digidata 1550B) (Molecular Devices)
 - Amplifier (e.g. Axopatch 200B) (Molecular Devices)
 - Clampex software (Molecular Devices)

Reagents

Cell Culture

- Complete cell culture medium (e.g. DMEM with 4.5 g/L glucose, 2 mM GlutaMAX, 10% fetal bovine serum)
- Low glucose cell culture medium (e.g. DMEM with 1 g/L glucose, 2 mM GlutaMAX, 10% fetal bovine serum, 1 mM sodium pyruvate)
- Cultured cells (e.g. HEK293T)
- Dulbecco's Phosphate Buffered Saline (dPBS)
- Poly-D-lysine (Sigma-Aldrich, made as a 0.1 mg/mL solution in dPBS with 10 mM Na₃BO₄)
- Cell dissociation reagent

Standards Measurement

- 2 μ M fluorescein in 0.1 N NaOH
- Quenched fluorescein for IRF measurements [12.2 M NaI, 500 μ M Fluorescein from 4 mM stock in 0.1 N NaOH, in H₂O]

Electrophysiology

- Internal solution for whole-cell patch-clamp electrophysiology [pH 7.25, 285 mOsmol/L, 125 mM potassium gluconate, 10 mM KCl, 5 mM NaCl, 1 mM EGTA, 10 mM HEPES, 2 mM ATP sodium salt, 0.3 mM GTP sodium salt]

VF-FLIM in Cells

- VoltageFluor VF2.1.Cl (solid or stock in DMSO)
- DMSO
- HEPES Buffer [1.26 mM CaCl₂*2H₂O, 0.49 mM MgCl₂*6H₂O, 0.41 mM MgSO₄*7H₂O, 5.33 mM KCl, 0.44 mM KH₂PO₄, 10 mM HEPES (pH 7), 139.5 mM NaCl, 0.34 mM Na₂HPO₄, 5.56 mM D-Glucose]
- High-K⁺ HEPES Buffer [1.26 mM CaCl₂*2H₂O, 0.49 mM MgCl₂*6H₂O, 0.41 mM MgSO₄*7H₂O, 119.6 mM KCl, 0.44 mM KH₂PO₄, 10 mM HEPES (pH 7), 23.6 mM NaCl, 0.34 mM Na₂HPO₄, 5.56 mM D-Glucose]

2. Protocol

Note: While we specify a particular microscope and FLIM system and software, the protocol outlined below can be generalized to any point-scanning confocal microscope and any FLIM system. Software-related instructions in the protocol that follows are specific to the setup outlined above, but the theory and use of the VF2.1.Cl probe in FLIM applications should be generalizable to any system.

Critical: The protocol outlined below gives specific timings and photon counts per second for each data acquisition type. These are intended as examples of what worked on the specific system used in the design of VF-FLIM. Individual FLIM systems will vary, and the protocol is flexible to be used with any of them. Count rates up to 1% of the laser rep rate (80 MHz for the system this protocol is based on, though it has also been performed at 50 MHz) can be used, to avoid photon pileup, and consequent incorrect lifetime values

14.

Measurement of Standards and Determination of Fit Parameters

2.1.1 Measuring Instrument Response Function

Note: SPCM, along with other TCSPC software programs, allow for the use of an electronically-generated instrument response function (IRF) based on the laser pulse and system electronics. However, our protocol gives directions for a measured IRF, which we find gives more accurate lifetime determinations where short lifetime components are present.

1. Insert a 25 mm coverslip into the attofluor chamber. Place a small drop of 500 μ M fluorescein quenched with iodide, the quenched fluorescein solution from the reagents above, in the center of the coverslip (around 20 μ L is sufficient, only a droplet is needed), or other fluorophore with a very short lifetime to use as a measure of the instrument response function, depending on the wavelength at which you are working.
2. Locate the droplet of solution and adjust the focal plane. Doing this using the LSM software and 488 laser is recommended for fluorescein. Adjust as appropriate for other fluorophores and laser lines.
 - a) Using the Zen Smart Setup tool, select fluorescein from the drop-down, and select “Smartest” setting.
 - b) Enter the “live” imaging mode, and adjust the focus until fluorescence comes into view and reaches a maximum intensity value.
3. Switch the dichroic so that light is directed towards the photon counting detector for FLIM.
 - a) Be careful at this point not to saturate the detector. The quenched fluorescein solution outlined for use here is quite bright, and high laser power could cause saturation of the FLIM detector. Low laser power, around 5-10% and a small pinhole will help to avoid this.
4. In SPCM, first make sure that the relevant detector and laser line (488) are turned on.
5. In “Oscilloscope” imaging mode, set acquisition time and repetition time to 1 second. This is for optimizing the laser power and pinhole size for the standards.
6. In Zen, select the “Time Series” experiment option, and then set the time series to go for an arbitrarily long number of frames (around 10,000 is sufficient for most

- experiments; the purpose is just so that the scanning in Zen will not end prior to the completion of the FLIM acquisition). Set the zoom setting to 1 and the scan speed to 8.
7. Select start on the oscilloscope in SPCM, and then start the experiment in Zen.
 8. Adjust the pinhole in Zen (invisible light pinhole) and the optical laser power by selecting the “Laser Power Down” button, until the counts displayed for the analog to digital converter (ADC) in SPCM are around 3×10^3 counts per second (cps).
 - a) You can use a small pinhole to reduce the signal if necessary.
 9. Stop the acquisition in both SPCM and Zen. Switch to “Single” mode in SPCM, and set up autosave, if desired. Set the acquisition time to 10 s and the repetition time to 10 s.
 10. Start the experiment in Zen, and then in SPCM. Acquire two single decays in SPCM, so that an average can be taken, and to establish that the IRF remains consistent.
 11. Stop the experiment in both programs.
 12. Open the first decay trace in SPCImage via the “Import” function.
 13. The data should appear as a very short decay early in the time window. Adjust the time gates to clip closely on either side of the decay, where the peak meets baseline on either side.
 14. Select the IRF menu from the top tool bar, then select “Copy From Decay Data.” Then select “Copy to Clipboard.”
 15. Paste the data from the first decay into a program like Microsoft Excel, or other analysis program.
 16. Repeat steps 12-15 for the second trace, making sure to use the same time gates as for the first trace.
 17. Take the average for every time bin between the two traces, producing a third trace that represents the average of the two at each time bin.
 18. Copy the average trace from the spreadsheet, and import it for use in analysis in SPCImage by selecting the IRF menu from the tool bar, then selecting “paste from clipboard.”

Note: On a stable system, a single IRF measurement should suffice, but if using a measured IRF, taking a new one every hour of imaging time can aid in getting more reliable and consistent fits, especially if the temperature in the imaging room changes over time.

2.1.2 Measuring Fluorescein Standard and Setting Value of Shift for Analysis

Note: Use measurements of a standard (fluorescein in solution in this case) to determine that the FLIM system is working properly and to optimize fit parameters. Other fluorophores can be used as additional lifetime standards to confirm the system is working well. Erythrosin B, for example, with a lifetime of 85 ps, is an example of a possible short-lifetime standard to use under these conditions in conjunction with fluorescein¹⁵ Erythrosin B also works well as an IRF on this system for redder wavelengths than 488.

1. Insert a 25 mm coverslip into the attofluor chamber. Place a small drop of 2 μ M fluorescein solution in the center of the coverslip (around 20 μ L is sufficient, only a droplet is needed).
2. Follow steps 2-11 from 3.1.1, with the following changes:

- a. Adjust the pinhole and laser power until the counts displayed for the ADC in SPCM are around 5×10^4 cps.
 - b. You can use a small pinhole to reduce the signal if necessary.
3. Open the first decay trace in SPCImage via the “Import” function.
4. Import the instrument response function (IRF), either from a measured IRF taken previously or an electronically generated one.
5. Using the options tab, select the model window. Change the model to be an incomplete decay, and change the fit model to WLS (weighted least squares) if not already the default.

Note: SPCImage also has other fit models, including maximum-likelihood estimation (MLE) in the newer versions of the software, that may provide more reliable fits, as do other FLIM analysis software packages. The protocol outlined here uses WLS, however, the points are generalizable to use with other fitting algorithms.

6. In the window to the right of the decay, the fit parameters window, change the parameters as follows:
 - a. Single component decay.
 - b. Fix the offset to zero.
 - c. Fix the scatter to zero.
 - d. Fix the shift, adjusting the value in the box until the average lifetime (T_m) displayed is near the literature value for fluorescein (4000 ps)¹⁶ and the χ^2 value is near one.
 - i. This empirically derived value for the shift should be used in subsequent analyses of decays derived from cellular data. Fixing the shift makes analysis more accurate and faster, but an incorrect value for the shift can drastically change the calculated lifetime values¹⁴. Measure a known standard to determine the ideal shift, and then fix it for analysis of unknowns to ensure accurate fits.
 - ii. The shift should be close to 0 if the instrument response solution and the standard solution are spectrally similar.
 7. Repeat steps 12-15 for the second decay trace. The shift determined in step 15 for the first trace should replicate a good fit for the second trace.

Critical: Make sure that the ADC resolution of the measured IRF and the standards taken match that in the FIFO Image mode (the first-in-first-out time-tagged imaging mode that produces the FLIM image). For instance, if using an ADC resolution of 256 for your images, make sure that the ADC resolution is also 256 for any measured IRF or standards (Becker, 2019).

Determination of Non-Quenching Concentrations

2.2.1 Fluorescence Lifetime Imaging at Multiple Dye Concentrations

Note: Higher concentrations of fluorophore in the membrane can lead to shorter measured lifetimes due to concentration-dependent quenching¹⁷. This step is important to select a concentration for use in future experiments that avoids this quenching regime, but is still at high enough concentration to provide enough photons for analysis.

1. In a 6-well cell culture plate, place one 25 mm #1.5 coverslip in each well. Coat each coverslip with 500 μ L of poly-d-lysine (PDL). Place plate with coverslips in incubator at 37 °C for at least one hour, and up to 8 hours (overnight).
2. Pipette or aspirate off the PDL.
3. Wash each coverslip twice with 1 mL of water, and twice with 1 mL of dPBS.
4. Plate cells on each glass coverslip at the density appropriate for the chosen cell line, and using the cell culture protocol appropriate for the cell line. The cells should be around 40 to 50% confluent at the time of imaging, such that they have grown to form discrete groups of cells, but not a monolayer.
5. Determine a range of dye concentrations to test for concentration dependent quenching. For many cultured cell lines, a range from 50 nM to 1 μ M VF2.1.Cl showed a clear quenching curve, with a “flat” portion of the curve in which there was no concentration-dependent quenching and an inflection point at which the lifetimes became shorter, suggesting concentration-dependent quenching. This range allowed for determination of a concentration that was bright enough to utilize but avoided this concentration-dependent quenching (**Figure A1.2a**).

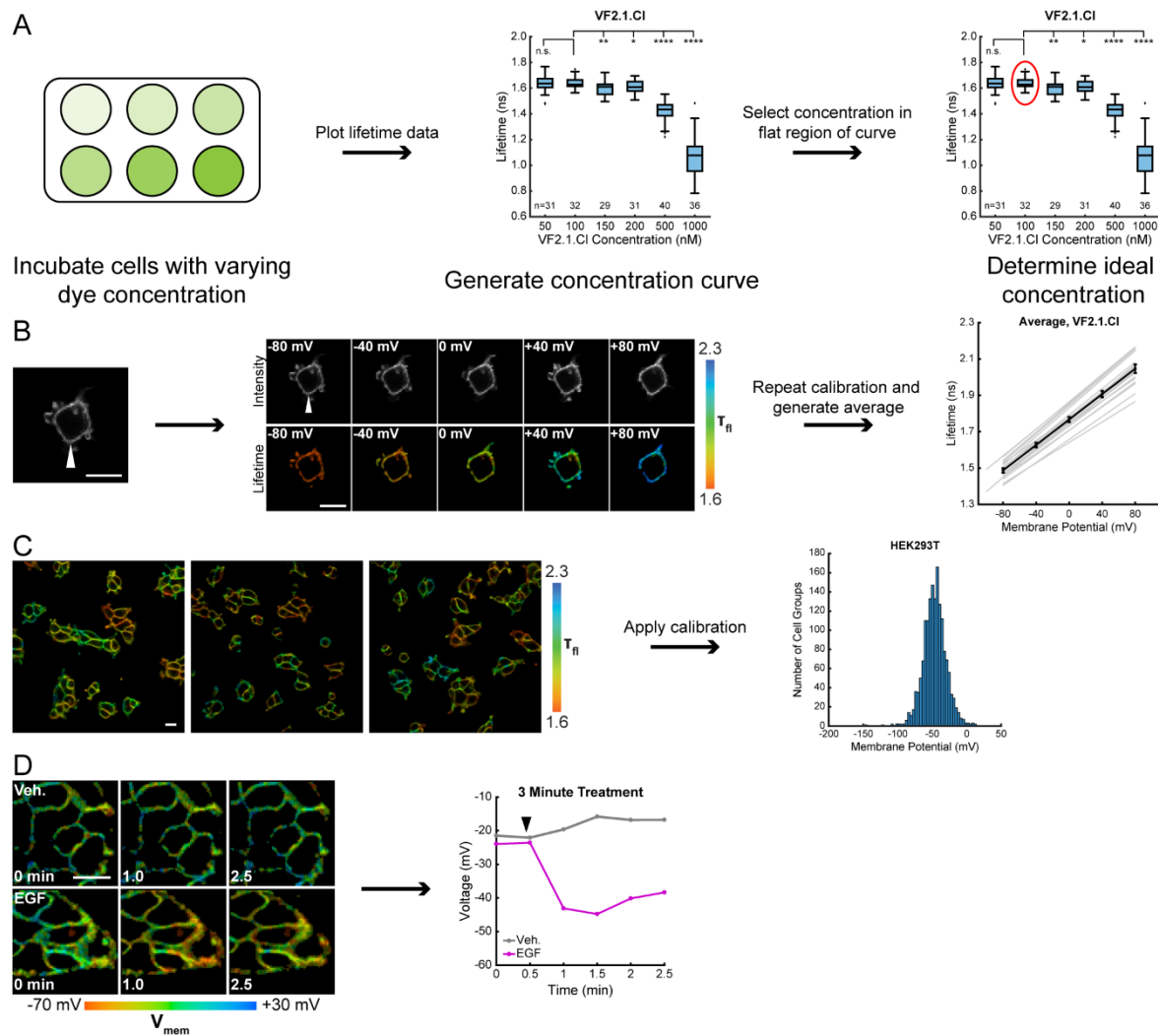


Figure A1.2. Overview of data acquisition. (A) Determination of optimal dye concentration. Incubate cells with a range of dye concentrations and plot the weighted average lifetimes. From the concentration curve, select a concentration in the flat region (red oval) to avoid concentration-dependent quenching at higher concentrations. Example data from HEK293T cells loaded with VF2.1.Cl. Scale bar is 20 μm . (B) Development of calibration between fluorescence lifetime and membrane potential. Voltage clamp single cells over a range of membrane potentials and acquire fluorescence lifetime data at each potential. Repeat this process for multiple single cells to generate an average calibration between fluorescence lifetime and membrane potential. Example intensity and lifetime images show HEK293T cells loaded with 100 nM VF2.1.Cl. Scale bar is 20 μm . (C) Bulk measurement of membrane potential. Take FLIM images of many cells. Use the calibration to convert fluorescence lifetime to voltage for each ROI and plot the data. The resulting histogram shows the distribution of membrane potential across the population of cells. Example FLIM images and data are for HEK293T cells loaded with 100 nM VF2.1.Cl. Scale bar is 20 μm . (D) Time series for tracking changes in membrane potential over time. Take FLIM images over the desired time scale. Apply the lifetime-voltage calibration and plot the voltage over time. Example FLIM images and data are for A431 cells loaded with 100 nM VF2.1.Cl and treated with imaging buffer vehicle or 500 ng/mL EGF. Scale bar is 20 μm . Data reproduced from Lazzari-Dean, *et al. eLife*, **2019**, 8, e44522 under a CC-BY 4.0 license.

6. To a 1.5 mL Eppendorf tube, add 999 μL of HEPES buffer, and the appropriate amount of concentrated VF2.1.Cl stock solution in DMSO, and additional DMSO such that the concentration is 0.1% DMSO by volume.
7. From one well of the 6-well culture plate, aspirate or pipette off culture media. Add in the dye solution in buffer, then return to the incubator at 37 C for 20 minutes. This allows time for the dye to selectively stain the plasma membrane.
8. After 20 minutes, remove the plate from the incubator, and pipette or aspirate off the dye solution. Replace the dye solution with 1 mL of buffer.
9. Using tweezers, gently lift the coverslip out of the well, and carefully place it in an Attofluor chamber, closing the chamber to form a tight seal, without cracking the glass. Add 1 mL of buffer to the chamber.
10. Using the same objective as was used in 3.1.1 and the eyepieces, locate the cells using transmitted light, and select a field of view.
11. Adjust the focal plane using the LSM software and 488 laser in Zen, as the fluorescence focal plane of the cells will be somewhat different from the transmitted light.
 - a. Using the Zen Smart Setup tool, select fluorescein or YFP from the drop-down, and select “Smartest” setting.
 - b. Enter the “Live” imaging mode and adjust the focus until fluorescence comes into view and the image displayed on the screen is bright and clear.
12. At this point, adjust the “Zoom” setting in Zen, and make sure that the image size in Zen matches the image size desired in SPCImage (256x256 or 512x512). “Zoom” setting 2 works well for investigating concentration-dependent quenching, as generally several cell groups can be located in one field of view.
13. Switch the dichroic so that light is directed towards the photon counting detector for FLIM.

14. In SPCM, first make sure that the relevant detector and laser line (488) are turned on.
15. In “Oscilloscope” imaging mode, set acquisition time and repetition time to 1 second. This is for optimizing the laser power and pinhole size.
16. In Zen, select the “Time Series” experiment option, and then set the time series to go for an arbitrarily long number of frames (around 10,000 is appropriate for most experiments; the purpose is just so that the scanning in Zen will not quit before the FLIM acquisition is done).
17. Select start on the oscilloscope in SPCM, and the start the experiment in Zen.
18. Adjust the pinhole in Zen (invisible light pinhole) and the laser power until the counts displayed for the ADC in SPCM are at least 1×10^5 counts per second (cps), and ideally closer to 3×10^5 cps, though this value will fluctuate as the laser scans across dark areas not stained with dye.
 - a. To achieve this count rate, it may be advantageous to increase the size of the pinhole above 1 AU. Values between 2 and 3 AU (e.g. 100 μm in Zen) often still give acceptable optical sectioning of membranes but allow for more photons to be collecting, enabling faster FLIM recordings. Longer recording times could also be used, at a lower count rate, for more light-sensitive samples, for instance.
 - b. The electronic laser power should be the same as used to measure any standards taken, and determined empirically for the samples used such that the pulse shape is good, meaning that the IRF is narrow and the decay traces are monotonic, with no bumps or aberrations, and enough counts are able to be generated by the sample of interest, without causing damage to the sample or photobleaching the dye.

Note: It is better to do coarse adjustments of laser power in the software by directly editing the laser power box, and typing the desired laser power. This adjusts the electronic power, and should be adjusted for what fits the sample and gives the best pulse shape, and then held constant across all experiments in a system (though this is generally best at medium power levels, and high laser power should be avoided). Fine adjustments should be done by clicking the L2 (or whichever is the appropriate laser line) UP or DOWN buttons, which adjusts a neutral density wheel to affect the optical laser power reaching the sample. Changing the electronic laser power changes the pulse shape and so will affect the IRF, while adjusting the optical power using the power UP and DOWN buttons will not (Becker, 2019).

19. Stop the acquisition in both SPCM and Zen. Switch to “FIFO Image” mode in SPCM, and set up autosave, if desired. Set the acquisition time to between 60 and 90 s, and the repetition time to match the acquisition time.
 - a. The exact time for a particular combination of dye and cell type may take some optimization.
20. Start the experiment in Zen, and then in SPCM.
21. Repeat steps 6-19 for all chosen concentrations of dye.

2.2.2 Analysis of FLIM Images in SPCImage

1. Import the first image file into SPCImage, using the “File” menu, and the “Import” feature.
2. Import the measured IRF into the analysis pane by first copying the measured IRF from the spreadsheet it was generated from to the clipboard, then selecting “IRF” and “Paste From Clipboard.”
3. Select the “Options” menu on the toolbar, then the “model” menu. Check the box marked “Incomplete Multiexponential” and make sure the default fit method is weighted least squares (WLS). Increase the number of iterations to twenty.
4. Adjust the T1 time gate to rest shortly before the peak of the decay, such that it catches some of the “tail” of the previous, incomplete decay. This should be a few time bins before the decay peak. T2 should end near the end of the measured decay, but not all the way at the end. Time bin 240 is appropriate for ADC resolution of 256.
5. Set the shift to the value determined in protocol 3.1.2 and check the box to fix it at that value. Fix the offset and scatter values to zero.
6. Change the number of components in the multiexponential decay. For VF2.1.C1 in cultured cell lines, 2 components generally gives the best, most consistent fits ¹².
7. Adjust the “Threshold” setting. This value should be adjusted empirically to a value that allows for consistent fits, and includes all of the bright membrane pixels, while excluding interior pixels. For the settings given in this protocol, a threshold of 300 at the peak of the decay has produced consistent fits.
8. Adjust the “Bin” setting. For images that are 256x256 and taken at zoom setting 2 in Zen on a 40x objective, setting that bin value to 3 allows for consistent fits across multiple images, and ensures there are sufficient photons in each binned pixel stained with dye.
 - a. The important point here is to bin such that there are sufficient photons for a consistent fit. There are enough photons with the bin settings used if the following are true:
 - i. Adding more photons (e.g. by binning further) doesn’t change the result significantly.
 - ii. Results are not notably different, beyond mild fluctuations, between successive measurements of the same sample.

Note: Binning in SPCImage is not “standard” binning, rather, it bins by essentially creating a moving average of binned pixels across the image, resulting in some pixels being double counted. Additionally, if using an analysis software other than SPCImage that uses a different definition of binning, 3 may not be sufficient to have enough photons at the pixels of interest. ^{12,14}

9. Select the “Calculate” menu from the toolbar, and then select “Decay Matrix”. This will apply the binning and the threshold to the image and produce a color-coded lifetime-intensity overlay image. The program will also plot a histogram of the weighted average lifetime values versus the number of pixels at each lifetime, and present an average value.

10. Check the quality of the fit by looking at the residuals displayed below the decay trace. These values are for the fit at a specific pixel, as is the decay trace, and will change slightly as the cursor is moved around the image.
11. Set the color to display the weighted average lifetime (T_m) as the color-coded value.
12. Once satisfied with the fits, the rest of the images that share a given measured IRF can be batch processed together. First, store the desired fit conditions as the default, and then select “Calculate” and then “Batch Processing,” and then selecting all the .sdt files that use a given IRF.
13. Copy over the next measured IRF for the next set of images, and repeat steps 1-13 for the remaining images from the experiment.
14. When all initial fits have been completed, export the lifetime and intensity images as .asc files by selecting the “File” menu, and selecting “Export.” In the export menu, check the boxes to export “Color Coded Value” (making sure that the color coded value selected from the “color” menu is T_m), and “Pixel Intensities.” Then, select “Batch Export” and allow the program to process the images.
 - a. Optionally, a bitmap image file can also be produced of the lifetime-intensity overlay image and the intensity image, with or without a color bar legend to display the color range used and the corresponding lifetime range.

2.2.3 Refinement of ROIs and Identification of Non-Quenching Concentrations

1. Use a published routine using custom Matlab code that automatically identifies regions of interest by first sharpening and then thresholding the intensity images. This code is available as source code 2 in previous work.¹²
 - a. This code will output a .csv file containing the weighted average lifetimes for each designated ROI, along with metadata including the concentrations and coverslip, as defined by the metadata file required by the script.
 - b. Instead of using this script, you may follow steps 2-7 below, which will allow you to run through the same steps accomplished in the code.
2. Convert the .asc files exported from SPCImage into TIFF files.
3. Open the intensity image in ImageJ or other visualization software.
4. Select regions of interest (ROIs). These should include the bright membranes of the cells, and exclude the background coverslip, as well as any debris, and the unstained interiors of cells (**Figure A1.3**). The ROIs should be whole cell groups, and should not be separated by individual cell. (Cells within a group are likely electrically coupled to one another¹⁸, depending on the chosen cell line; furthermore, the spatial resolution of a standard point-scanning confocal microscope makes separating abutting membranes impossible).
 - a. This can be done easily by using the threshold tool in ImageJ, and making a selection from that threshold, then saving that selection as an ROI. The ROI can then be further cleaned up from there.
5. Once all the ROIs for an image are defined from the intensity image, open the corresponding lifetime image. If binning was used during lifetime analysis, the lifetime image will have reduced spatial resolution compared to the intensity image.

6. Apply the ROIs to the lifetime image, and for each ROI on the lifetime image, take a measurement. The important value for in this case is the mean, which represents the mean weighted average lifetime across the ROI.
7. Copy these average values into a spreadsheet or other analysis program, such as Microsoft Excel, organizing them by dye concentration and sample.
8. Plot the weighted average lifetimes of each ROI broken down by category in plotting software of choice. Box and whisker plots or violin plots are convenient for this. There should appear to be a curve as in (**Figure A1.2a**), with a “flat” section where the mean lifetimes at different concentrations is fairly consistent, and a point at which the lifetime starts to decrease as concentration increases, revealing concentration-dependent quenching.
9. Select a concentration from the “flat” portion of the curve to use for future experiments (**Figure A1.2a**).

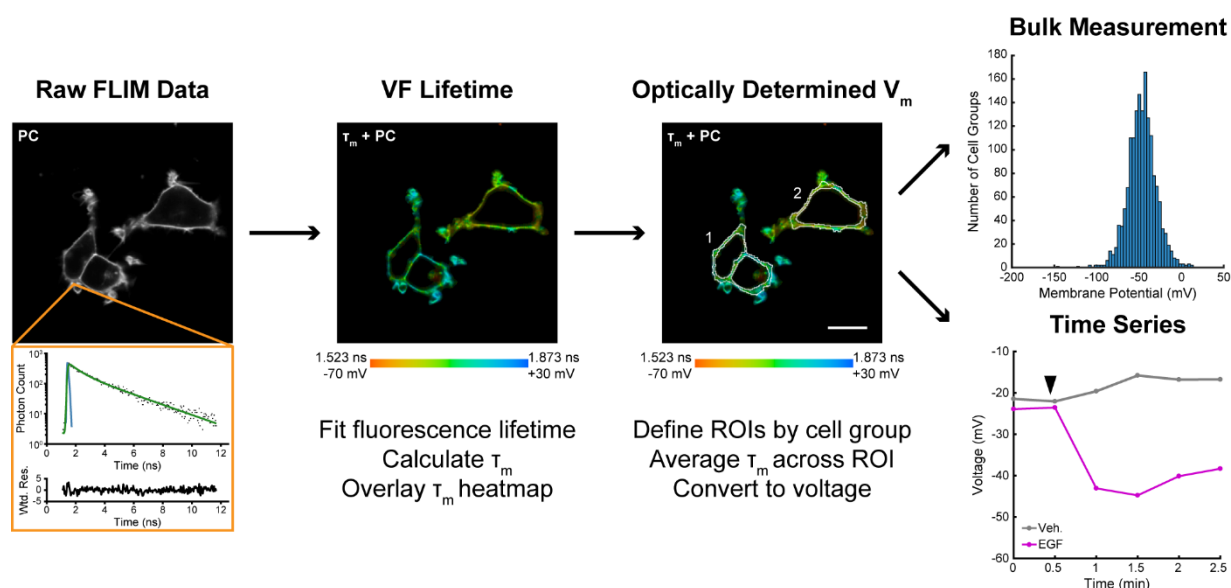


Figure A1.3. Overview of data analysis. Fit time-correlated photon data to an exponential decay model (first panel). Calculate the weighted average fluorescence lifetime (τ_m) and overlay heatmap onto the photon count (PC) image (second panel). Define regions of interest (ROIs, white outlines) by cell group and average τ_m across each ROI (third panel). Use the lifetime-voltage calibration to convert the averaged τ_m to membrane potential (V_m). Data can be plotted as a histogram showing the bulk membrane potential distribution or, if images were acquired in a time series, as a line graph showing the change in membrane potential over time. Example images and bulk measurement data are for HEK293T cells loaded with 100 nM VF2.1.Cl. Example time series data are for A431 cells loaded with 100 nM VF2.1.Cl and treated with imaging buffer vehicle or 500 ng/mL EGF. Scale bar is 20 μ m. Figure adapted from Lazzari-Dean, *et al. eLife*, **2019**, 8, e44522 under a CC-BY 4.0 license.

Generation of Electrophysiological Calibration

2.3.1 Simultaneous FLIM and Electrophysiology

1. In a 6-well cell culture plate, place one 25 mm #1.5 coverslip in each well. Coat each coverslip with 500 μ L of poly-D-lysine (PDL). Place plate with coverslips in incubator at 37 C for at least one hour, and up to 8 hours (overnight).
2. Pipette or aspirate off the PDL.
3. Wash each coverslip twice with 1 mL of water, and twice with 1 mL of dPBS.
4. Plate cells on each glass coverslip at an appropriate density, in low glucose media, such that at the time of imaging they will still be single cells.
5. Prepare five voltage-clamp protocols that hold the cell at a given potential for 15 seconds.
 - a. -80, -40, 0, +40 and +80 mV are potentials that worked well for HEK293T cells in the development of this protocol, but these values could be adjusted, and more values added.
6. Using the same objective as in 2.1.1 and in previous steps, and the eyepieces, identify a single cell to perform the calibration on. The cell should not be connected to any others, and should have a morphology typical of a healthy cell from the cell line.
7. Follow the imaging protocol in section 2.2.1, with a few key changes:
 - a. Set the image size to 64x64 rather than 256 x 256 or 512 x 512, in both Zen and SPCM.
 - b. Zoom in on the cell of interest. A setting in Zen of 3 to 4 works well for this.
 - c. FIFO Image acquisition times should be 15 s each.
 - d. Laser power and pinhole size should be adjusted such that each image is about 1×10^5 cps at a resting potential. Test that this is the case for a single image before running the full experiment.
8. Fill the glass pipette with internal solution and lower into the bath. Zero the voltage, and follow the steps for whole-cell patch-clamp electrophysiology, heavily abbreviated in steps 9-11. More detailed protocols and considerations can be found in the following references: ^{19,20,21,22}.
9. Locate the borosilicate glass pipette through the eyepieces, and position over the cell of interest.
10. Lower the pipette into contact with the cell's surface, entering cell-attached mode. Correct for pipette capacitance.
11. Enter whole-cell configuration, checking the quality of the patch using the "membrane test" feature. The ratio of membrane resistance to access resistance should be greater than 30.
12. Coordinate voltage clamp protocols and FLIM measurements such that the entire time the FLIM image is being acquired, the cell is being held at the desired voltage.
 - a. One way this can optionally be accomplished is by setting up a cable connecting the electrophysiology rig to the laser hub, and setting the voltage clamp protocol to trigger the laser, and setting the "Trigger" setting in SPCM to an external trigger.

13. Assess the quality and stability of the patch after running the voltage clamp protocol. If the patch has degraded or the cell has died, do not continue running the protocol, and start over with a new cell.
14. Repeat steps 11 and 12 with the subsequent potentials, assessing patch quality after each protocol. Apply the protocols in random order, to avoid artifacts such as changes to lifetime brought on by bleaching or cell death.
 - a. While random order is best, the +80 mV protocol is sometimes not tolerated as well by cells as the others when held for such a long time. For this reason, it may be useful to perform this one last, and if the patch has degraded or the cell dies during this step, it may be omitted from the calibration.
15. Repeat the above steps for subsequent cells, in order to have multiple cells from which to generate a general calibration (**Figure A1.2b**)¹².

2.3.2 Analysis and Generation of Lifetime-Voltage Calibration

1. Follow the steps outlined in protocol 3.2.2 for analysis in SPCImage, with the modification to step 8 that the “Bin” setting for these smaller, more zoomed-in calibration images should be set at 1 rather than 3.
2. Follow steps 1-6 in protocol 3.2.3, recording mean T_m at each potential, rather than at each concentration. For these images, identifying ROIs by hand, rather than using the matlab package mentioned in step 7 is recommended.
3. For each cell, plot the weighted average lifetime (T_m) versus the voltage, and generate a regression line for those points. Determine the equation of that regression line.
4. From all of the linear regressions, determine an average slope and an average y-intercept.
 - a. The average slope represents the sensitivity of the calibration, that is, the change in lifetime, in picoseconds, for each mV change in membrane potential.
 - b. The y-intercept represents the expected lifetime if the cell is at 0 mV, and is important for calculating membrane potential from a measured lifetime.
5. Calculate an “intra-cell” error for the calibration.
 - a. The “intra-cell” error is determined by the root-mean-square deviation (RMSD) between the voltage as defined by electrophysiology (assumed to be 100% accurate) and the voltage as determined using the calibration to convert the lifetime to the voltage for a specific cell, using that individual cell’s line of best fit. Perform this calculation for each cell, and report the mean ± SEM as the overall error. This value gives a sense of the resolution for measurements of voltage change in an individual cell, and is discussed in more detail in section 4.1.
 - b. Calculate the Bias, variance (σ^2), and RMSD, using the optical guess at V_m for each measured lifetime, using that individual cell’s line of best fit, as the values of V_{FLIM}
 - c. Equations for RMSD are as follows¹²:
 - i.
$$Bias = \frac{1}{n} \sum_{i=1}^n V_{FLIM,i} - \frac{1}{n} \sum_{i=1}^n V_{ephys,i}$$

- ii. $\sigma^2 = \frac{1}{n} \sum_{i=1}^n (V_{FLIM,i} - V_{ephys,i})^2$
 - iii. $RMSD = \sqrt{\sigma^2 + Bias^2}$
6. Calculate an “inter-cell” error for the calibration.
 - a. Calculate the optical determination of V_m for the 0 mV point on each individual calibration using the overall calibration. Ideally, this value should be 0 mV, however, there will likely be some spread in the individual 0 mV points, such that they give a calculated membrane potential that varies from 0 mV.
 - b. Calculate the RMSD using the equations in step 5c, this time using the optical determination of the V_m based on the lifetime, using the overall calibration for the cell line as V_{FLIM} , and the 0 mV value from the individual calibrations as V_{ephys} . This value gives a sense of the resolution for measurements of absolute voltage across many cells, or how well the average calibration predicts an individual cell’s membrane potential.

Membrane Potential Measurements in Large Populations of Cells

2.4.1 Population Measurements of Resting Membrane Potential

1. Plate cells and take FLIM images as in 3.2.1, with three changes.
 - a. In step 9, add either the regular HEPES buffer, for bulk measurements of resting membrane potential, or add 1 mL of high-K⁺ (120 mM) HEPES buffer to the imaging dish for a bulk perturbation that will shift the membrane potential of all cells close to zero.
 - b. In step 12, the zoom setting in Zen should be set to zoom 1, in order to image a larger number of cell groups at one time.
 - c. In step 19, the acquisition time should be 90 s.
2. Perform initial analysis in SPCImage as outlined in 3.2.2.
3. Follow steps 1-8 in 3.2.3 for ROI refinement and analysis, with the following changes.
 - a. Instead of recording the dye concentration used for that sample, list the condition for that sample, i.e. whether low or high-K⁺ buffer was used.
4. Use the lifetime-voltage calibration generated in 3.3.2 to convert the average lifetimes of each ROI for each condition into the corresponding membrane potential.
5. Plot the membrane potentials for each condition. Histograms are useful here to visualize the distribution of membrane potentials in the population of cells being investigated (**Figure A1.2c**).

Measuring Membrane Potential Changes Over Time

2.5.1 Tracking Membrane Potential Changes

1. Plate cells onto 25 mm #1.5 glass coverslips, culturing as appropriate for the cell line of interest.
2. Prepare a 2x stock solution of the drug intended for pharmacological perturbation of the membrane potential in buffer.
 - a. In serum-starved A431 cells, for instance, treatment with epidermal growth factor (EGF) results in a hyperpolarization that can be measured with VF-

FLIM (**Figure A1.2d**)¹². This 2x EGF solution was prepared at 1 µg/mL, for a final concentration of 500 ng/mL in the imaging dish.

- b. Keep this stock solution at the same temperature as your imaging buffer and sample to avoid temperature-dependent artifacts when you add it to the sample.

Note: This protocol is written for performing this experiment without a perfusion system. However, perfusion could be utilized instead for treating the system with drug/vehicle in these time series experiments.

3. Incubate sample with VoltageFluor dye as outlined in steps 6-9 of protocol 3.2.1, with the following change:
 - a. In step 9, when adding buffer back to the imaging chamber, add only 500 µL of imaging solution, rather than 1 mL.
4. Acquire FLIM images as in 3.2.1, with the following changes.
 - a. Set collection and repeat time to 30 seconds, to acquire a 30 second exposure.
 - b. Adjust laser power such that the ADC reads $4-5 \times 10^5$ cps, to accommodate the shorter exposure times.
 - c. Set number of cycles to 6, for a 3 minute time series. More cycles can be selected for imaging over a longer time. However, longer timescales can lead to phototoxicity, so it is recommended that there are pauses between image acquisition periods for longer timescales, rather than the sequential frames used for the 3 minute time series.
 - i. Make sure that autosave has been selected as on, and that images are saved after each cycle. This will save each image from each cycle as a separate file, appending to the end of each file name “c1” through “c6” or onward, depending on the number of cycles, to differentiate them by their place in the time series.

Critical: Whenever running a new sample or condition, especially those at higher light power or over longer timescales, it is important to first run a control solution in which vehicle, rather than drug, is added, to ensure that the lifetime doesn't drift over the course of the recording period, and to make sure phototoxicity is minimized.

5. After the first frame has been acquired (30 to 35 seconds into the experiment) and just before the second begins, take 500 µL of the 2x drug solution, or the same amount of a 2x vehicle solution, and add it to the imaging dish, being careful not to bump the imaging dish. Continue acquiring data.
6. Perform initial analysis in SPCImage for each frame as outlined in 3.2.2.
7. For this type of data, the code mentioned in 3.2.3 is not ideally set up for analyzing multiple frames of the same condition over time. Thus, it is better to perform the analysis by hand, using the steps outlined in steps 2-7 of 3.2.3 to refine the ROIs and collect the weighted average lifetime data.
8. Apply the lifetime-voltage calibration generated in 3.3.2 to each time point.
9. Plot Vm against time, for both drug-treated and vehicle-treated cells.

- a. A line graph is useful here, for tracking V_m changes over time in each condition (**Figure A1.2d**).

Generation of Electrophysiological calibration on Zeiss LSM 980 microscope, “K”

Note: This section of the protocol was not included in the original publication. It is included here to clarify specifics of the protocol above for use on the particular system at the UC Berkeley MIC, installed in 2020.

2.6.1 Simultaneous FLIM and electrophysiology on “K”

1. Follow steps 1-5 as in 2.3.1.
2. Set up a Faraday cage around the system, or make one from foil.
 - i. To assemble a foil Faraday cage, attach foil to the front of the LSM 980 microscope with tape, making sure the foil is long enough to stretch from the top of the microscope body above the incubation chamber to the bottom of the incubation chamber and make contact with the body again (**Figure A1.4**).
 - ii. Attach the foil to ground, e.g. by clamping the foil with an alligator clip and connecting to a brass grounding bar.
 - iii. Adjust the positioning and grounding of the foil such that noise from surrounding electronics, including controls and computers, is minimized.

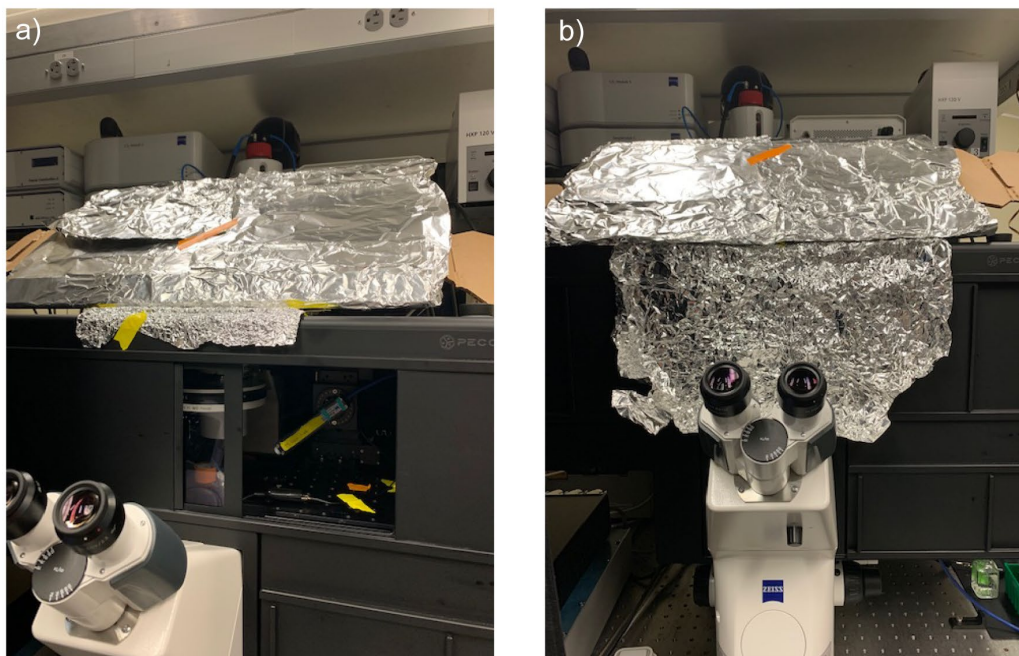


Figure A1.4 Foil setup on microscope “K” to reduce noise.

a) Foil setup to reduce noise when placing sample and locating pipette. There will still be noise with this setup. Make sure the foil is attached to ground (not pictured). b) Foil setup when the foil has been lowered to cover the front of the microscope and stage, as when going into whole-cell mode and applying voltage clamp protocols.

3. Follow step 6 and 7 as in 2.3.1, but change the image size in SPCM to 128x128 pixels, rather than 64x64 as in 2.3.1.
 - i. 64x64 pixels leads to artifacts and stretching of the image in a way that has so far not been able to be corrected by changing the “left border” or “pixel clock divider” settings.
Note: Ensure that there is enough signal in the desired acquisition time to get reliable fits in a 128x128 pixel image – more photons will be needed than for a 64x64 image.
4. Fill the glass pipette with internal solution and lower into the bath. Zero the voltage, and follow the steps for whole-cell patch-clamp electrophysiology.
 19,20,21,22
5. Locate the borosilicate glass pipette through the eyepieces, and position over the cell of interest, with foil set up as in **Fig. A1.4a**.
 - i. The readout in Clampex membrane test will be noisy at this point. Minimize noise as much as possible, but it is unlikely it can be eliminated.
6. Lower the pipette into contact with the cell’s surface, entering cell-attached mode. Correct for pipette capacitance.
7. Lower or unroll the foil on the front of the microscope to cover the front of the microscope, since the pipette will not be moved anymore, and looking at the microscope stage is no longer necessary (**Fig. 1.4b**).
 - i. This should reduce the noise significantly. If not, make sure the foil is touching the front body of the microscope, contacting the metal.
8. Enter whole-cell configuration, checking the quality of the patch using the “membrane test” feature. The ratio of membrane resistance to access resistance should be greater than 30.
9. Coordinate voltage clamp protocols and FLIM measurements such that the entire time the FLIM image is being acquired, the cell is being held at the desired voltage.
 - i. One way this can optionally be accomplished is by setting up a cable connecting the electrophysiology rig to the laser hub, and setting the voltage clamp protocol to trigger the laser, and setting the “Trigger” setting in SPCM to an external trigger.
 - ii. Ensure the Zen light path is set to send photons to the FLIM detector and start scanning. Ensure photons are being detected in SPCM. Keep the laser scanning throughout all the protocols of the patch.
 1. This could in the future be optimized further by adding an additional triggering cable to trigger laser scanning with the protocols in Clampex.
10. Assess the quality and stability of the patch after running the voltage clamp protocol. If the patch has degraded or the cell has died, do not continue running the protocol, and start over with a new cell.
11. Repeat steps 9 and 10 with the subsequent potentials, assessing patch quality after each protocol. Apply the protocols in random order, to avoid artifacts such as changes to lifetime brought on by bleaching or cell death.
 - i. While random order is best, the +80 mV protocol is sometimes not tolerated as well by cells as the others when held for such a long time. For this

reason, it may be useful to perform this one last, and if the patch has degraded or the cell dies during this step, it may be omitted from the calibration.

12. Repeat the above steps for subsequent cells, in order to have multiple cells from which to generate a general calibration (**Figure A1.2b**)¹².

3. Analysis

Representation of Error in the Lifetime-Voltage Calibration

In order to reliably and accurately utilize the lifetime-voltage calibration obtained in protocol 2.3.2, an understanding of the resolution of the calibration is necessary. The calibration tends to be much more accurate at tracking changes in a particular cell's voltage than identifying the absolute membrane potential for a given cell, based on the calibration. Thus, two different measurements of resolution or error are necessary in order to have a complete understanding of the calibration and the ways it can be used. We have termed these the “intra-cell” error and the “inter-cell” error¹².

The “intra-cell” error is the error in measuring voltage changes in a given cell over time. In other words, this value is the error expected in estimating a cell's V_m from its measured lifetime, given that we have the calibrated lifetime-voltage relationship for that particular cell. This value is determined by calculating the root-mean-square deviation (RMSD), based on $V_{FLIM, intra}$ and V_{ephys} for a given cell. In this context, $V_{FLIM, intra}$ is the V_m estimated from a given lifetime, using that particular cell's line of best fit, and V_{ephys} is the voltage set using electrophysiology at that same measured lifetime (**Figure A1.5a**).

The “inter-cell” error is the error in measuring the absolute voltage of a given cell, based on the overall calibration for that cell line. For the “inter-cell” error, the RMSD is calculated using $V_{FLIM, inter}$, defined as the optical guess at V_m for each 0 mV point on each individual calibration using the average overall calibration for that cell line (which would ideally give a value of zero) and V_{ephys} , which in this case is the y-intercept of each individual cell's calibration, or the 0 mV point on the linear regression for each cell (**Figure A1.5b**). Both of these metrics of error assume that the value set by voltage-clamp is completely accurate, but in practice there is probably some amount of error introduced via the electrophysiology as well.

The “intra-cell” error is much smaller than the “inter-cell” error, supporting that the VF-FLIM method is more accurate at reporting changes in lifetime in a particular cell, or group of cells, as outlined in 2.5.1, than for determining the absolute membrane potential of a given cell, as outlined in 2.4.1. This fact should be kept in mind for experiments using the calibration, and the limitations of the “inter-cell” resolution should especially be discussed when trying to identify absolute voltages and compare those voltages between different groups. If two experimental groups present a different measured voltage based on the lifetime-voltage calibration, but the difference is smaller than the “inter-cell” error, more validation to determine if the difference in measured voltage is meaningful will be necessary, using different techniques, for instance. The protocol outlined in 2.5.1 gives an example of the type of experiment that could be performed to track the membrane potential of a group of cells or a single cell over time, in response to a pharmacological treatment, using the smaller “intra-cell” error as the limit of resolution.

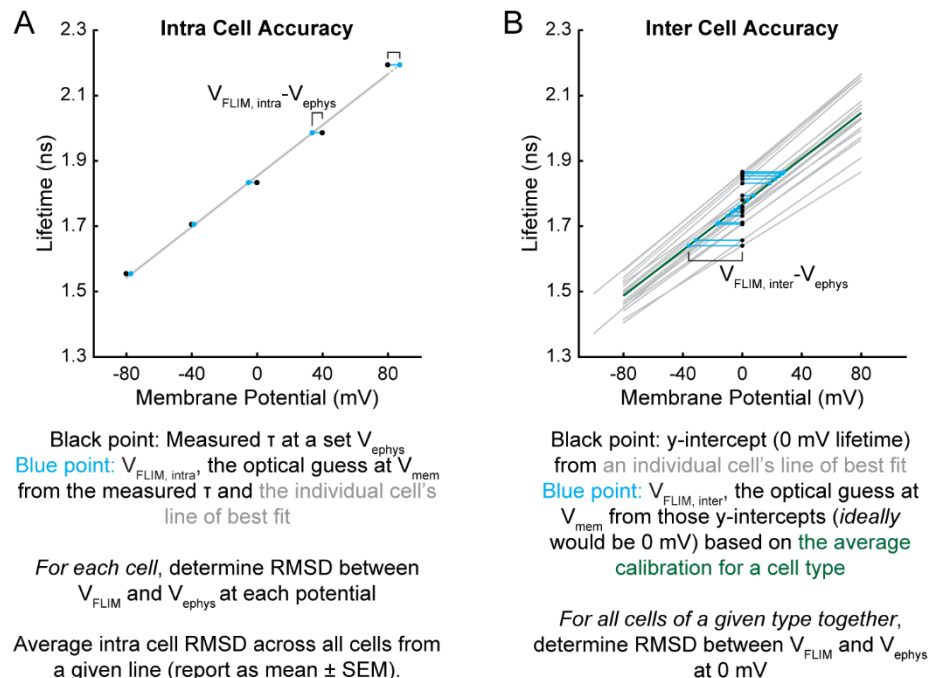


Figure A1.5. Intra and inter-cell V_{mem} resolution calculations. (A) Intra cell values are the RMSD between the voltage equivalent of the measured lifetime (V_{FLIM}) and voltage set by electrophysiology (V_{ephys}). V_{FLIM} values are calculated using that particular cell's line of best fit, so one value is obtained per cell. Here, we present intra cell error as the mean $\pm$ SEM of all cells from a given cell line. (B) Inter cell errors are the RMSD between the voltage-equivalent of the 0 mV lifetime for all cells tested from a cell line (V_{FLIM} , determined with the average slope and y-intercept for that cell line) and the ground truth value of 0 mV. Inter-cell accuracy is calculated from all of the calibration data for a cell line, so there is one value per cell line. Black points are experimental y-intercepts and blue points are the V_{FLIM} optical voltage determinations from those lifetimes. Gray lines are lines of best fit for individual cells. Green line in (B) represents the average $\tau_{\text{fl}}-V_{\text{mem}}$ relationship for a cell line. Figure reproduced from Lazzari-Dean, *et al. eLife*, **2019**, 8, e44522 under a CC-BY 4.0 license.

4. Summary

In this appendix, we outlined the use of the VoltageFluor-Fluorescence Lifetime Imaging Microscopy (VF-FLIM) method, using the potentiometric VoltageFluor dye VF2.1.Cl to perform bulk determination of membrane potential in large numbers of cells. This protocol takes readers through the steps of determining the proper concentration for use in their model system, generating a lifetime-voltage calibration using patch-clamp electrophysiology, and measuring bulk membrane potentials of hundreds of cells within a few hours. It is our hope that this method allows for the further investigation of membrane potential as a signal in populations of cells, not just in individual cells, and leads to further study of the role of membrane potential in non-electrically excitable systems.

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Appendix 2:

A Protocol for the Formation of Lipid Vesicles as a Model System to Study the Impact of Membrane Composition on VoltageFluor Fluorescence Lifetime

This protocol was developed and written in collaboration with the following people:

Jeong Yun (Lizy) Choi

Motivation

Fluorescence lifetime imaging microscopy (FLIM) in conjunction with VoltageFluor (VF) dyes enables optical determination of membrane potential values. This technique, VF-FLIM, enables membrane potential determination with greater than 100-fold throughput over electrophysiological methods, and with biologically relevant levels of voltage resolution.¹ However, the output lifetime sometimes varied between cultured cell types, even at the same potential. We hypothesize that this variability may be due in part to the fact that lifetime is sensitive to other factors than voltage that may differ between cell types, such as membrane tension² and membrane composition. However, due to the complexity of the membrane composition in even the simplest model cell types, performing controlled experiments to isolate the effect of lipid composition on lifetime is difficult in living cells. To that end, we turn to synthetic vesicles. Giant unilamellar vesicles (GUVs) and large unilamellar vesicles (LUVs) because they are amenable model systems to study cellular membranes, as they mimic important aspects of cellular membrane permeability and provide a simpler system that enables controlled experiments, in fluorescence intensity and lifetime.^{3,4} Here we determine the protocol for the formation of LUVs and GUVs, along with initial characterization via wide field microscopy in preparation to conduct comparative analysis of τ_n of VoltageFluor dyes in vesicles of different lipid composition using fluorescence lifetime imaging microscopy to determine the sensitivity of VoltageFluor potentiometric dyes' fluorescence lifetime to the lipid composition of their environment.

Methods

Materials and Instrumentation for Large Unilamellar Vesicles (LUVs)

10mg/ml POPC or other lipids in chloroform (Avanti Polar Lipids), stored as aliquots in chloroform at -80 °C

Rotovap

Pear-shaped 100mL flask

Chloroform

Extrusion Kit (Avanti Polar Lipids, SKU # 610000)

1x dPBS

Gas-tight syringe

Dynamic Light Scattering Instrument (e.g. Zetasizer, Malvern Panalytical)

Preparation of dry lipid film

1. Add 40 μ L of 10 mg/mL of lipid and 60 μ L of CHCl_3 into a 100 mL pear-shaped flask for a final lipid concentration of 4 mg/mL.
1. Concentrate the lipid solution using a rotovap, following the procedure below:
 - a. Turn on the water bath to 40 °C.
 - b. Clamp the sample onto the device.
 - c. Set the vacuum pressure and turn the vacuum on.
 - d. Close the rotovap from atmosphere.
 - e. Rotate the sample at ~10 rpm.
 - i. Slow rotation is necessary to get an evenly distributed film.
 - f. Make sure rotovap is inclined at a shallow angle, to make the lipid film thin and even as possible.
 - g. Lower the flask into the water.
 - h. Allow sample to concentrate under vacuum for 15 minutes.
 - i. Reduce the pressure and allow sample to dehydrate further for 5 minutes.
2. Place a septum on the lipid film flask.

3. Place the flask under vacuum on a Schlenk line, and allow it to dry further overnight.
 - a. The lipids should now be an opaque, even, thin white film around the bottom of the flask.

Hydration of lipid film

1. Pipette 500 μ L of 1X dPBS and gently add it onto the lipid film flask.
2. Set to rotate on rotovap at $\sim$ 10 rpm for 1 hour.
3. Assemble the extrusion kit following the manufacturer's instructions.
 - a. Damp the membrane and two filter supports completely in 1X dPBS
 - b. Assemble the extruder apparatus so that the membrane is in between the two filter supports.
 - c. Insert the one of the gas-tight syringes to one end of the apparatus.
 - d. Pull up all solution from the flask using a gas-tight syringe.
 - e. Connect the syringe to the extrusion kit and pump the syringe back and forth 21 times.
 - i. Minimize introducing air bubbles when pumping the vesicles.
 - ii. This will result in a relatively even opacity throughout the solution.
 - iii. The vesicle solution should end up in the opposite syringe to where it started.

Characterizing LUVs with Dynamic Light Scattering (DLS)

1. Allow the instrument 10 minutes to warm up.
2. Load 80 μ L of prepared vesicles into a cuvette.
3. Follow the sample preparation and use protocol for the DLS, abbreviated below.
4. Place the cuvette inside the device so that the arrow on the cuvette is facing outward, as indicated by the instrument.
5. Run the Zetasizer DLS with the following settings:
 - a. Lipid: Lysosome
 - b. Solvent: PBS
 - c. Time: 120 s
 - d. Temperature: 25.0 $^{\circ}$ C
6. Collect data to determine vesicle size
 - a. Ideal LUV samples should have:
 - i. Under 0.1 measured polydispersity index (PDI).
 - ii. Diameter of 100 -150 nm, as determined by the intensity mean readout of the system.

Outlook for LUV Method

LUVs produced using this method were uniform in size, and relatively monodisperse ($PDI < 0.1$). However, the size of LUVs can limit their applications. They are not suitable for visualization using optical microscopy, as they are 100nm in diameter, below the diffraction limit. While LUVs have successfully been used in lifetime characterization of VoltageFluors in the past, they do not produce lifetime images, only time-resolved decays.⁵ Additionally, they are unstable, aggregating over the course of an hour or so and thus difficult to work with for lifetime imaging. Therefore, GUVs, which are optically resolvable and thus should be able to provide spatial information about lipid composition and its effects on lifetime within a vesicle, may be better suited for this work.

Materials and Instrumentation for Giant Unilamellar Vesicles

10 mg/ml POPC or other lipids in chloroform (Avanti Polar Lipids), stored as aliquots in chloroform at -80 °C

Rotovap

Pear-shaped 100 mL flask

Round-bottom 100 mL flask

Chloroform

N₂ gas

100 mM sucrose solution and 100 mM glucose solution

Vacuum pump

Cannula

Needle

Attofluor imaging chamber

25 mm coverslips (#1.5, Electron Microscopy Sciences)

Inverted widefield microscope, for epifluorescence imaging.

Preparation of dry lipid film

4. Add 40 µL of 10 mg/mL of lipid and 60 µL of CHCl₃ into a 100mL pear-shaped flask for a final lipid concentration of 4 mg/mL.
5. Concentrate the lipid solution using a rotovap, following the procedure below:
 - j. Turn on the water bath to 40 °C.
 - k. Clamp the sample onto the device.
 - l. Set the vacuum pressure and turn the vacuum on.
 - m. Close the rotovap from atmosphere.
 - n. Rotate the sample at ~10 rpm.
 - i. Slow rotation is necessary to get an evenly distributed film.
 - o. Make sure rotovap is inclined at a shallow angle, to make the lipid film thin and even as possible.
 - p. Lower the flask into the water.
 - q. Allow sample to concentrate under vacuum for 15 minutes.
 - r. Reduce the pressure and allow sample to dehydrate further for 5 minutes.
6. Place a septum on the lipid film flask.
7. Place the flask under vacuum on a Schlenk line, and allow it to dry further overnight.
 - a. The lipids should now be an opaque, even, thin white film around the bottom of the flask.

Pre-hydration of lipid film

1. In a fume hood, heat an oil bath to 70 °C.
2. Secure a 100 mL round-bottom flask, and evacuate the flask under vacuum, then backfill with N₂. Repeat three times.
3. Take a 25 mL syringe and a short needle and draw up 15 mL of DI water.
4. Insert the water into the round bottom flask.
5. Adjust the N₂ flowing needle to touch the surface of the water, so that N₂ is bubbling through the solution.
6. When the oil bath has reached 70 °C, lower the flask down into the oil bath.
7. Take out the vent needle and replace it with a cannula. Make sure that the cannula is not touching the water.
8. Secure the flask containing the dried lipid film and make sure that it is placed higher than the round bottom flask.
 - A. This will reduce condensation of water forming in the cannula and dripping onto

the lipid film (**Figure A2.1**). Failing to do so may result in water droplets condensing in the cannula and dripping into the lipid film, resulting in uneven hydration, and less unilamellar vesicle formation.

9. Place the other end of the cannula into the lipid film flask.
10. Insert the vent needle onto the lipid film flask to ensure that pressure doesn't build up.
11. Allow lipid to pre-hydrate for 30 minutes.

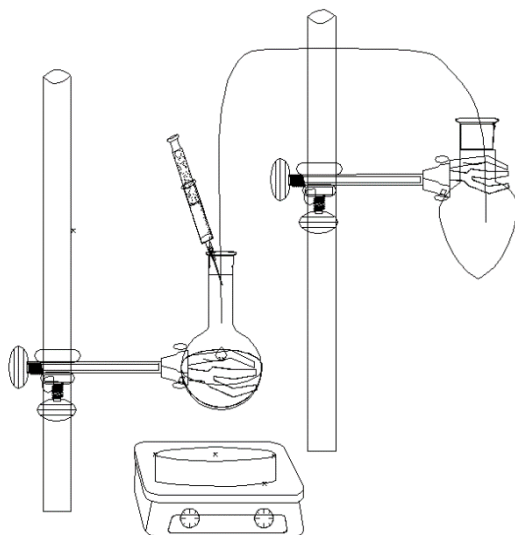


Figure A2.1 Schematic for the setup for pre-hydration of the lipid film.

Schematic for the gentle pre-hydration of the lipid film, demonstrating the relative positioning of the glassware, with the pear-shaped flask containing the lipid higher than the water and cannula to avoid condensation of water droplets onto the film.

Hydration of lipid film

1. Gently pipette the 100 mM sucrose solution down the side of the lipid-containing flask using a Pasteur pipette, until the edges of the lipid film are just covered with sucrose.
2. At this point, do not move the vesicle flask.
3. Allow the vesicles to hydrate for 18-24 hours.

Epifluorescence Microscopy of GUVs

1. Assemble the Attofluor imaging chamber and coverslip so that a water-tight seal is formed around the coverslip.
2. Gently pipette approximately 500 μ L of vesicle solution using a Pasteur pipette or P1000 micropipettor fitted with a 1 mL pipette tip onto the center of the coverslip
 - a. Vesicles can stick to plastic, so a Pasteur pipette can be used to avoid this. Otherwise, use a large pipette tip, as the small size of the openings on smaller tips can cause the vesicles to shear and break.
3. Pipette glucose solution on top so that the denser vesicle (sucrose) solution sinks towards the bottom of the coverslip.
 - a. This should allow the vesicles to settle towards the bottom and enable imaging of the vesicles.
4. **If imaging vesicles stained with dye only**, add dye from a 1000x DMSO stock of VoltageFluor dye in a 1:1000 ratio with vesicles in glucose/sucrose, directly onto coverslip in the imaging chamber. **Do not rinse and remove dye as you would with cells.**

- a. Gently pipette to mix with a glass Pasteur pipette or 1 mL micropipette tip.
 - b. Cover the chamber so it is not exposed to light. Allow the dye to incubate 10 minutes before imaging. Turn on the microscope and allow the instrument to warm up for 10 minutes.
5. Set the following imaging conditions on the microscope:
 - a. LED intensity: 25%
 - b. Bin: 4 x 4
 - c. Transmitted light power: 3.5%
 - d. Filter: VF-Cyan
 - e. Transmitted light exposure: 5 ms (adjust as needed, but keep consistent throughout imaging)
 - f. Fluorescent light exposure: 2 ms (adjust as needed, but keep consistent throughout imaging)
 - g. Objective Lens: 40x
6. Take images in both the transmitted light and fluorescence channels.
 - a. Images should look similar to those in **Fig. A2.2**. If vesicles have not fully settled to the bottom of the coverslip, they may be harder to see.
 - i. Vesicles will be in motion and drift across the coverslip unless additional anchoring strategies are employed. The difference in density between glucose and sucrose solutions was not enough to completely stabilize the vesicles for imaging.
 - ii. Unilamellar vesicles are less pronounced and more difficult to see, but are a better model for cellular membranes – select vesicles to study carefully.

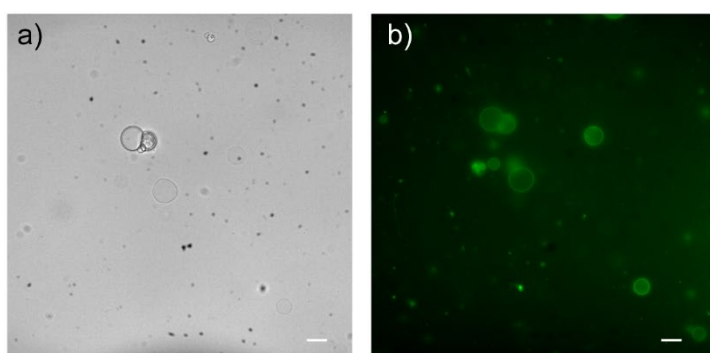


Figure A2.2 GUVs stained with 1 μ M VF2.1.Cl.

a) Brightfield and b) fluorescence images of GUVs stained with approximately 1 μ M VF2.1.Cl VoltageFluor Dye. Vesicles do not perfectly align between images as they were acquired consecutively, and vesicles, especially smaller ones, drifted slightly between image acquisitions. Scale bars are 20 μ m.

Outlook for GUV Method

This protocol using gentle swelling to produce GUVs produces vesicles that can be resolved optically in epifluorescence and confocal microscopy. However, gentle swelling is much less consistent than electroformation for vesicle production and leads to vesicles of variable sizes and lamellarities.⁶ Additionally, without additional methods to tether the vesicles to the base of the coverslip, the vesicles produced in this method are unsuitable for lifetime imaging, as during the long exposure times needed for FLIM, the vesicles diffuse out of the field of view. Vesicle tethering could be achieved relatively simply through coatings of the

coverslip, e.g. with casein.⁷ Alternatively, modified lipids containing biotin could be introduced to the vesicles, with the coverslips then coated with avidin to produce strong interactions and hold the vesicles in place on the coverslip surface. These additions to this protocol should keep the GUVs from moving long enough for the 60 second exposure times necessary for FLIM. At that point, additional changes could be made to vesicle membrane composition, and the effect of lipid composition on VF lifetime more systematically examined.

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