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VoltageFluor dyes and fluorescence lifetime imaging for optical measurement of membrane potential

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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 to detect changes in membrane potential. In this chapter, we discuss the use of fluorescence lifetime imaging microscopy (FLIM) and voltage-sensitive fluorophores (VoltageFluors, or VF dyes) to estimate the millivolt value of membrane potentials in living cells. We discuss theory, application, protocols, and shortcomings of this approach.

1. Introduction

Membrane potential, Vm, is an important biophysical parameter in cellular physiology. Fast changes in Vm, 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 mitosis (Cone & Cone, 1976), cell cycle progression (Huang and Jan 2014), differentiation (Chen et al. 2019; Tsuchiya & Okada, 1982), and development (Levin, 2014).

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,

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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 clamp (Fertig, Blick, & Behrends, 2002), electrophysiology is still relatively limited in scale, making measurements of Vm 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 Vm are difficult to perform accurately. While they are useful for reporting changes in membrane potential, absolute measurements of actual millivolt values of Vm are difficult to obtain (Peterka, Takahashi, & Yuste, 2011), due to confounding factors such as dye concentration, and photobleaching, which complicate the creation of an accurate calibration (Lazzari-Dean, Gest, & Miller, 2021).

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) (Eq. 1) (Berezin & Achilefu, 2010). Because fluorescence lifetime depends only on the rates of radiative (k_r) and nonradiative decay (k_{nr}) pathways out of the excited state (Eq. 2) (Berezin & Achilefu, 2010), 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 concentration (Yellen & Mongeon, 2015). 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.

$$\tau = \frac{\varphi}{k_r} \tag{1}$$

$$\tau = \frac{1}{k_r + k_{nr}} \tag{2}$$

The utility of fluorescence lifetime as a way to read out membrane potential in particular has been demonstrated first with fluorescent proteins (Brinks, Klein, & Cohen, 2015), and then through the use of a photoinduced electron transfer (PeT)-based dye, VF2.1.Cl (Lazzari-Dean, Gest, & Miller, 2019), 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 (Fig. 1) (Miller et al., 2012; Lazzari-Dean et al., 2019). PeT is a non-radiative decay pathway out of the fluorophore's excited state; therefore, modulation of PeT also leads to modulation of the

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fluorescence lifetime. For VF2.1.Cl, negative membrane potentials lead to shorter lifetimes, and positive potentials to longer fluorescence lifetimes (Lazzari-Dean et al., 2019). These lifetimes of the probe in the cell membrane can be measured using FLIM and the intensity decay modeled mathematically using a multiexponential decay (Eq. 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) (Eq. 4).

$$F(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + \ldots + a_n e^{-t/\tau_n} \tag{3}$$

$$\tau_m = \frac{a_1\tau_1 + a_2\tau_2 + \ldots + a_n\tau_n}{a_1 + a_2 + \ldots + a_n} \tag{4}$$

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 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 Vm can therefore be measured across hundreds of cells or tracked longitudinally in particular cells in only a few hours of experiment time.

2. 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 (Brinks et al., 2015).

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2.1 Equipment

2.1.1 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

2.1.2 Electrophysiology

- P97 Pipette Puller (Sutter Instruments)
- Pipettes pulled from borosilicate glass with filament, resistances 4 to 7 MΩ (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)

2.2 Reagents

2.2.1 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 Na_3BO_4)

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- Cell dissociation reagent

2.2.2 Standards measurement

- 2 μM fluorescein in 0.1 N NaOH
- Quenched fluorescein for instrument response function (IRF) measurements [12.2 M NaI, 500 μM Fluorescein from 4 mM stock in 0.1 N NaOH, in H_2O]

2.2.3 Electrophysiology

- Internal solution for whole-cell patch-clamp electrophysiology [pH7.25, 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]

2.2.4 VF-FLIM in cells

- VoltageFluor VF2.1.Cl (solid or stock in DMSO)
- DMSO
- HEPES Buffer [1.26 mM $CaCl_2$*$2H_2O$, 0.49 mM $MgCl_2$*$6H_2O$, 0.41 mM $MgSO_4$*$7H_2O$, 5.33 mM KCl, 0.44 mM KH_2PO_4, 10 mM HEPES (pH7), 139.5 mM NaCl, 0.34 mM Na_2HPO_4, 5.56 mM D-Glucose]
- High-K^+ HEPES Buffer [1.26 mM $CaCl_2$*$2H_2O$, 0.49 mM $MgCl_2$* $6H_2O$, 0.41 mM $MgSO_4$*$7H_2O$, 119.6 mM KCl, 0.44 mM KH_2PO_4, 10 mM HEPES (pH7), 23.6 mM NaCl, 0.34 mM Na_2HPO_4, 5.56 mM D-Glucose]

3. 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 repetition 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 (Becker, 2019).

3.1 Measurement of standards and determination of fit parameters

3.1.1 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

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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 μM fluorescein quenched with iodide, the quenched fluorescein solution from the reagents above, in the center of the coverslip (around 20 μL is sufficient, only a droplet is needed).
2. Locate the droplet of solution and adjust the focal plane. Doing this using the LSM software and 488 laser is recommended
    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 s. 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

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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.

3.1.2 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 89 ps, is an example of a possible short-lifetime standard to use under these conditions in conjunction with fluorescein (Boens et al., 2007).

1. Insert a 25 mm coverslip into the attofluor chamber. Place a small drop of 2 μM fluorescein solution in the center of the coverslip (around 20 μL is sufficient, only a droplet is needed).

2. Follow steps 2–11 from Section 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

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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 (Tm) displayed is near the literature value for fluorescein (4000 ps) (Magde, Rojas, & Seybold, 1999) and the Chi^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 values (Becker, 2019). 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).

3.2 Determination of non-quenching concentrations

3.2.1 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 quenching (Chen & Knutson, 1988). This step is

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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 μL of poly-D-lysine (PDL). Place plate with coverslips in incubator at 37 °C for at least 1 hour, and up to 8 h (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 40–50% 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 μM 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 (Fig. 2A).
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 37C for 20 min. This allows time for the dye to selectively stain the plasma membrane
8. After 20 min, 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 Section 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

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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 (256 × 256 or 512 × 512). "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 toward 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 s. 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 collected, 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 alters the pulse shape and so will

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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

3.2.2 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 in Section 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.Cl in cultured cell lines, 2 components generally gives the best, most consistent fits (Lazzari-Dean et al., 2019).

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 256 × 256 and taken at zoom setting 2 in Zen on a 40 × 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. (Becker, 2019; Lazzari-Dean et al., 2019).

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 (Tm) 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 Tm), 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

3.2.3 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.

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This code is available as source code 2 in previous work. (Lazzari-Dean et al., 2019)

   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 (Fig. 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 another (Me e, Richard, & White, 2007), 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 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 (Fig. 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 (Fig. 2A).

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3.3 Generation of electrophysiological calibration

3.3.1 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 μL of poly-D-lysine (PDL). Place plate with coverslips in incubator at 37 °C for at least 1 hour, and up to 8 h (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 s
   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 Section 3.1.1 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 3.2.1, with a few key changes:
   a. Set the image size to 64 × 64 rather than 256 × 256 or 512 × 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: (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981) (Cummins, Rush, Estacion, Dib-Hajj, & Waxman, 2009) (Molleman, 2003) (Penner, 1995).
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

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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 (Fig. 2B) (Lazzari-Dean et al., 2019).

3.3.2 Analysis and generation of lifetime-voltage calibration

1. Follow the steps outlined in protocol in Section 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 in Section 3.2.3, recording mean Tm 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 (Tm) 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 Vm 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 follows (Lazzari-Dean et al., 2019):

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

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

$$RMSD = \sqrt{\sigma^2 + Bias^2}$$

6. Calculate an "inter-cell" error for the calibration

a. Calculate the optical determination of Vm 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 5 c, this time using the optical determination of the Vm 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

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3.4 Membrane potential measurements in large populations of cells

3.4.1 Population measurements of resting membrane potential

1. Plate cells and take FLIM images as in Section 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 Section 3.2.2
3. Follow steps 1–8 in Section 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 Section 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 (Fig. 2C).

3.5 Measuring membrane potential changes over time

3.5.1 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 2× 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 (Fig. 2D) (Lazzari-Dean et al., 2019). This 2× 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.

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3. Incubate sample with VoltageFluor dye as outlined in steps 6–9 of protocol in Section 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 Section 3.2.1, with the following changes

   a. Set collection and repeat time to 30 s, to acquire a 30 s exposure

   b. Adjust laser power such that the ADC reads 4–5*10^5 cps, to accommodate the shorter exposure times

   c. Set number of cycles to 6, for a 3-min 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-min 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 does not drift over the course of the recording period, and to make sure phototoxicity is minimized.

5. After the first frame has been acquired (30–35 s into the experiment) and just before the second begins, take 500 μL of the 2× drug solution, or the same amount of a 2× 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 Section 3.2.2

7. For this type of data, the code mentioned in Section 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 in Section 3.2.3 to refine the ROIs and collect the weighted average lifetime data

8. Apply the lifetime-voltage calibration generated in Section 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 Vm changes over time in each condition (Fig. 2D).

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4. Analysis

4.1 Representation of error in the lifetime-voltage calibration

In order to reliably and accurately utilize the lifetime-voltage calibration obtained in protocol in Section 3.3.2, 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" error (Lazzari-Dean et al., 2019).

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 Vm from it's 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 Vm 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 (Fig. 4A).

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 Vm 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 (Fig. 4B). 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 Section 3.5.1, than for determining the absolute membrane potential of a given cell, as outlined in Section 3.4.1. 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 Section 3.5.1 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.

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5. Summary

In this chapter, 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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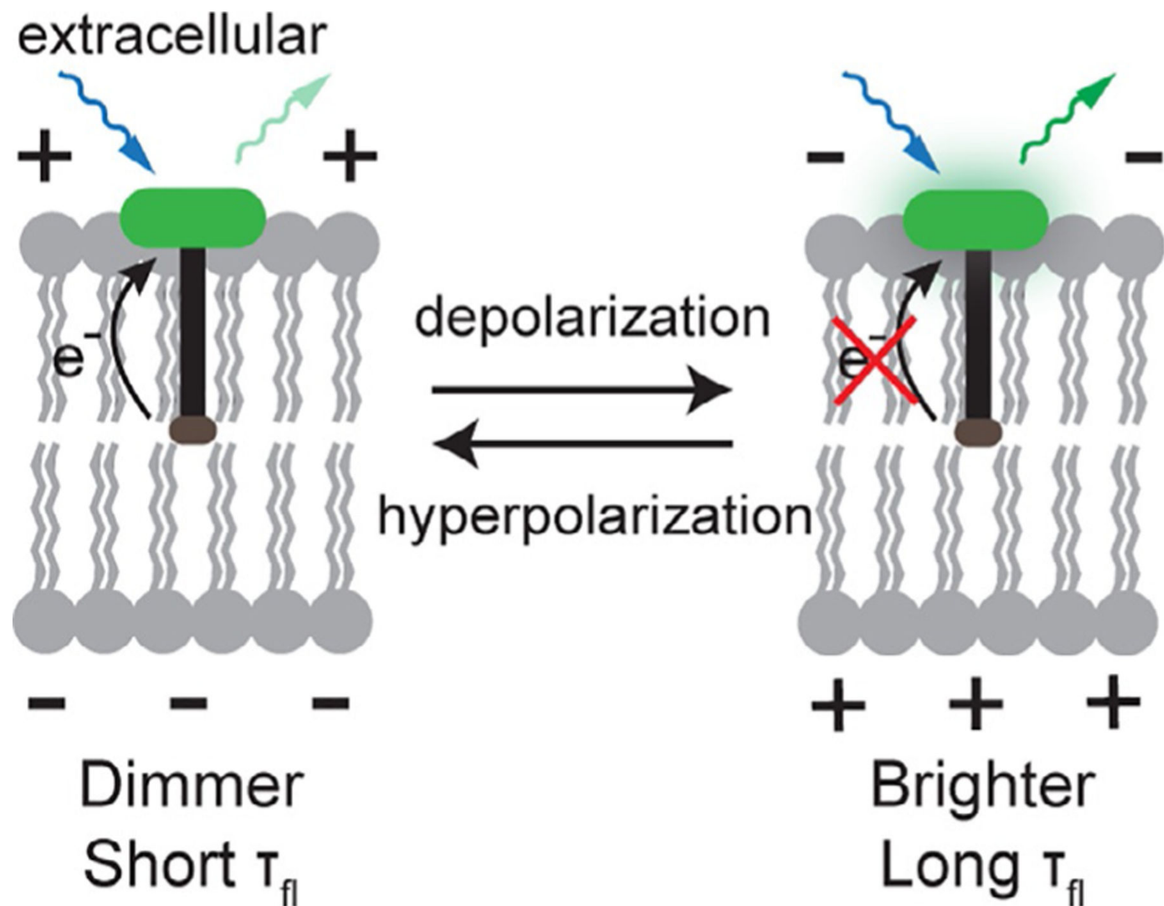

Fig. 1.

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, J.R., Gest, A.M.M., & Miller, E.W. (2019). Optical estimation of absolute membrane potential using fluorescence lifetime imaging*. ELife *8 (September). e44522*, https://doi.org/10.7554/eLife.44522 *under a CC-BY 4.0 license*.

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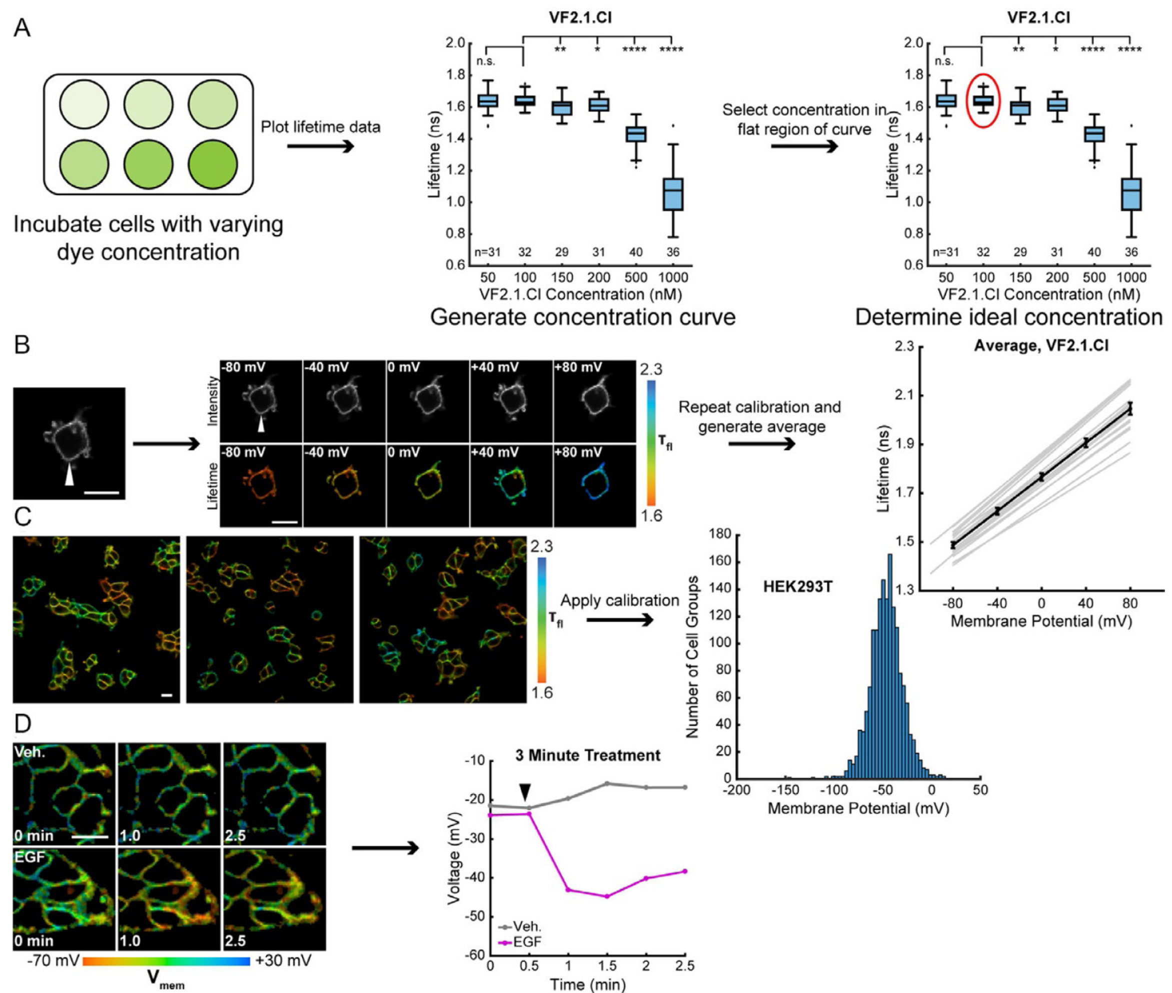

Fig. 2.

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, J.R., Gest, A.M.M., & Miller, E.W. (2019). Optical estimation of absolute membrane potential using fluorescence lifetime imaging*. ELife *8 (September). e44522*, https://doi.org/10.7554/eLife.44522 *under a CC-BY 4.0 license*.

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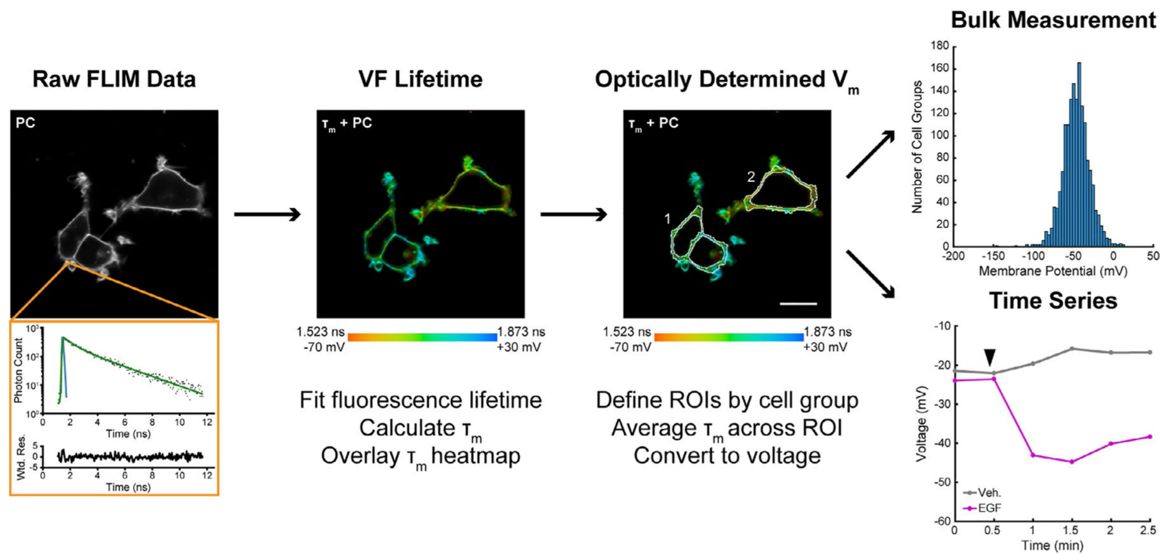

Fig. 3.

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 μm. *Figure adapted from Lazzari-Dean, J.R., Gest, A.M.M., & Miller, E.W. (2019). Optical estimation of absolute membrane potential using fluorescence lifetime imaging.* ELife *8 (September). e44522*, https://doi.org/10.7554/eLife.44522 *under a CC-BY 4.0 license under a CC-BY 4.0 license.*

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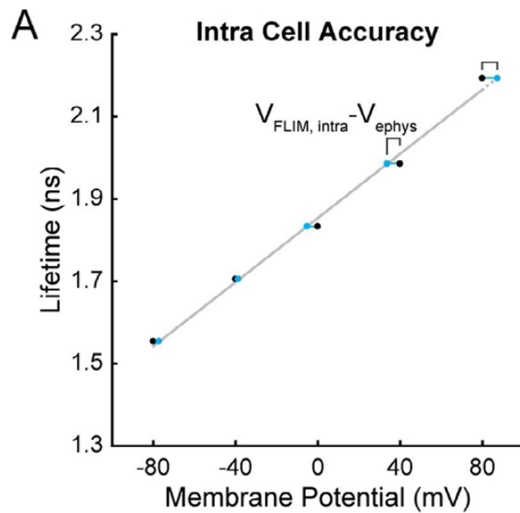

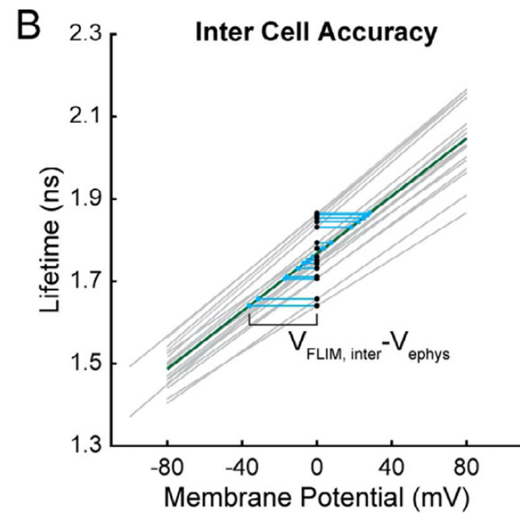

Fig. 4.

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 ± 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 τ_{fl}-V_{mem} relationship for a cell line. *Figure reproduced from Lazzari-Dean, J.R., Gest, A.M.M., & Miller, E.W. (2019). Optical estimation of absolute membrane potential using fluorescence lifetime imaging.* ELife *8 (September). e44522*, https://doi.org/10.7554/eLife.44522 *under a CC-BY 4.0 license under a CC-BY 4.0 license.*