Video Article

Biosensing Motor Neuron Membrane Potential in Live Zebrafish Embryos

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Abstract

The protocols described here are designed to allow researchers to study cell communication without altering the integrity of the environment in which the cells are located. Specifically, they have been developed to analyze the electrical activity of excitable cells, such as spinal neurons. In such a scenario, it is crucial to preserve the integrity of the spinal cell, but it is also important to preserve the anatomy and physiological shape of the systems involved. Indeed, the comprehension of the manner in which the nervous system-and other complex systems-works must be based on a systemic approach. For this reason, the live zebrafish embryo was chosen as a model system, and the spinal neuron membrane voltage changes were evaluated without interfering with the physiological conditions of the embryos.

Here, an approach combining the employment of zebrafish embryos with a FRET-based biosensor is described. Zebrafish embryos are characterized by a very simplified nervous system and are particularly suited for imaging applications thanks to their transparency, allowing for the employment of fluorescence-based voltage indicators at the plasma membrane during zebrafish development. The synergy between these two components makes it possible to analyze the electrical activity of the cells in intact living organisms, without perturbing the physiological state. Finally, this non-invasive approach can co-exist with other analyses (*e.g.,* spontaneous movement recordings, as shown here).

Video Link

The video component of this article can be found at https://www.jove.com/video/55297/

Introduction

In vivo systemic component analysis allows scientists to investigate cellular behavior in the most reliable way. This is particularly true when the activity under scrutiny is heavily influenced by cell-cell interactions (both contact- and non-contact-dependent), as in the nervous system, where membrane voltage changes drive the communication among excitable cells. The comprehension of the information encoded by these electrical signals is the key to understanding the way the nervous system works in both physiological and disease states.

In order to study cell electrical properties in the most non-invasive physiological conditions, several genetically encoded voltage indicators have been recently developed[1]. As opposed to the previous generations of optical voltage sensors (mainly voltage-sensitive dyes)[2], GEVIs allow for *in vivo* analyses of the intact neural system, and their expression can be limited to specific cell types or populations.

The zebrafish embryo is the *in vivo* "substrate" of choice to take advantage of the great potential attributed to GEVIs. In fact, thanks to its optical clarity and its simplified yet evolutionarily conserved nervous system, the zebrafish model allows for the straightforward identification and manipulation of every cellular component in a network. Indeed, the employment of the FRET-based GEVI Mermaid[3] led to the identification of pre-symptomatic alterations in spinal motor neuron behavior in a zebrafish model of amyotrophic lateral sclerosis (ALS)[4].

The following *in vivo* protocol describes how to monitor the electrical properties of spinal motor neurons in intact zebrafish embryos expressing Mermaid in a neuronal-specific manner. Moreover, it demonstrates how pharmacologically induced changes in such electrical properties can be associated with alterations in the frequency of embryonic spontaneous coilings, the stereotypic motor activity that characterizes the movement behavior of the zebrafish at very early stages of development.

Protocol

1. pHuC_Mermaid Plasmid Generation

NOTE: Mermaid is a biosensor developed by pairing the voltage-sensing domain (VSD) of the *Ciona intestinalis* (now *Ciona robusta*)[5] voltage sensor containing phosphatase (Ci-VSP) with the FRET partner fluorophores Umi-Kinoko Green (mUKG: donor) and a monomeric version of the orange-emitting fluorescent protein Kusabira Orange (mKOk: acceptor). For this biosensor, conformational changes of the VSD domain, induced by membrane depolarization, increase the proximity of the donor and acceptor fluorescent proteins, thus increasing the energy transfer between them (increasing the FRET Ratio)[3]. The VSD assures the efficient localization of the biosensor at the plasma membrane. The neuronal expression of the biosensor (in the spinal cord, the signal is detectable in both interneurons and motor neurons) is achieved by cloning the Mermaid open reading frame (ORF) under the zebrafish pan-neural promoter HuC[6].

1. **Amplify by polymerase chain reaction (PCR) the Mermaid ORF from the pCS4+ Mermaid plasmid[3] with Pfu (proofreading) DNA polymerase using the T3 Universal primer and the Mermaid *Sma*I primer (5′TATCCCGGGATTCGACGGTTCAGATTTTA) in order to insert a *Sma*I restriction site upstream of the Mermaid ORF.**
    1. Use the following PCR mixture: 0.5 µL of Pfu DNA polymerase, 7.5 µL of the specific 10x buffer, 1 µL of 10 mM dNTPs, 2.5 µL of dimethyl sulfoxide (DMSO), 0.5 µL (50 ng) of the plasmid preparation, and 1 µL of 20-µM stock solutions of each primer in a total volume of 50 µL.
    2. Heat the PCR mixture for 15 s at 95 °C, prime it for 15 s at 50 °C, and elongate it for 4 min at 72 °C for a total of 35 cycles.
2. Gel-purify the specific blunt PCR product using a commercial gel purification kit, following the manufacturer's protocol.
3. Clone the DNA fragment into the pCMV-SC blunt vector following the manufacturer's protocol.
4. **Linearize the Mermaid-positive plasmid (named pCMV-SC_Mermaid) with the *Sma*I restriction enzyme for the following insertion of the HuC promoter.**
    1. Set up the restriction reaction as follow: 0.5 µL (10 U) of *Sma*I restriction enzyme, 2 µL of the kit 10x buffer, and 5 µg of plasmid DNA in a total volume of 20 µL. Incubate the reaction at 25 °C for 1 h.
5. Gel-purify the digested plasmid using a commercial gel purification kit following the manufacturer's protocol.
6. **PCR-amplify the HuC promoter (pHuC), with Pfu DNA polymerase and zebrafish genomic DNA as template, using a pair of HuC-specific primers (HuCprom-forw1_SalI: 5′-GTAGTCGACCAGACTTGTCAAAAGGGTCCA and HuCprom-rev1: 5′-TCCATTCTTGACGTACAAAGATG) and spanning a 3,150-bp region upstream of the ATG.**
    1. Set up the PCR mixture using the following scheme: 0.5 µL of Pfu DNA polymerase, 7.5 µL of the specific 10x buffer, 1 µL of 10 mM dNTPs, 2.5 µL of DMSO, 200 ng of genomic DNA, and 1 µL of 20-µM stock solutions of each primer in a total volume of 50 µL.
    2. After an initial step of 2 min at 95 °C, heat the PCR mixture for 15 s at 95 °C, prime it for 15 s at 50°C, and elongate it for 4 min at 72 °C for a total of 35 cycles.
7. Gel-purify the specific blunt PCR product using a commercial gel purification kit following the manufacturer's protocol.
8. Ligate an equimolar amount of the purified pCMV-SC_Mermaid (step 1.5) and the pHuC DNA (step 1.7) using 1 µL of T4 DNA ligase and 1 µL of the specific 10X buffer in a total volume of 10 µL. Incubate the reaction for 16 h at 4 °C.
9. Transform an aliquot of competent cells with 5 µL of the ligation reaction (step 1.8) following the manufacturer's instructions.
10. **Select the pHuC-positive clones (pHuC_Mermaid) with the promoter inserted in the proper orientation by a *Sal*I-*Eco*RV double digestion (the *Sal*I restriction site has been inserted upstream of the promoter fragment in step 1.6, while the *Eco*RV restriction site is positioned downstream of the polyadenylation region, PolyA, of the pCMV-SC plasmid).**
    1. Set up the restriction reaction as follow: 0.5 µL (10 U) of both *Sal*I and *Eco*RV restriction enzymes, 2 µL of the kit10X buffer, and 1 µg of pHuC_Mermaid DNA in a total volume of 20 µL. Incubate the reaction at 37 °C for 1 h. Run the reactions onto an agarose gel.

2. Embryo Microinjection

1. Transfer the fertilized eggs obtained from wild-type (AB strain) or Sod1-G93R adult zebrafish[4] to a 10 mm Petri dish using a plastic pipette.
2. Rinse the embryos in cold (4 °C) fish water and immediately microinject them into the yolk with 200 pg of pHuC_Mermaid plasmid using a microinjector (for an overall and detailed description of the microinjection procedure, see References 7 and 8).
3. Using a plastic pipette, transfer the embryos to a Petri dish and incubate them in fish water at 28 °C until they reach the desired developmental stage (20-24 h post-fertilization, hpf) for the following analyses.

3. Spontaneous Tail Coiling Analysis

NOTE: Evaluate the spontaneous tail coiling behavior in 20-24 hpf embryos with or without the drug riluzole.

1. Transfer an embryo to a 90-mm round Petri dish filled with fish water containing 0.2% DMSO (riluzole vehicle) and manually dechorionate it using two jeweler's forceps with sharp tips. Incubate the embryo for 5 min.
2. Detect the tail coiling at RT during a 1 min video recording using a digital camera mounted on a stereomicroscope. Acquire time series at a time resolution of 30 frames/s.
3. Calculate the frequency of spontaneous tail coilings by counting the number of bends (both contralateral and ipsilateral) per time unit.
4. **To evaluate the effect of the drug riluzole, gently use a plastic Pasteur pipette to transfer the embryo to a new 90 mm Petri dish filled with fish water containing 5 µM riluzole.**

1. Incubate the embryo for 5 min before recording a 1-min video and performing the behavioral analysis as above.

4. Imaging Setup for Mermaid Biosensor Visualization in Living Embryos: Simultaneous Detection of Donor and Acceptor Signals

1. Mount the 20 - 24 hpf embryos in 1% low-melting-point agarose in fish water at 37 °C inside a 35 mm glass-bottomed imaging dish. Orient the embryos on their sides. Wait until the agarose solidifies at room temperature.
2. Transfer the imaging dish to the stage of a inverted confocal mounted on an inverted microscope. Identify motor neurons expressing the biosensor with a 20X objective (0.7 numerical aperture, NA) by exciting the mUKG with the 488 nm argon laser line and recording its emission between 495 and 525 nm.
3. For a FRET measurement, excite mUKG, the donor of the FRET pair, with the 488 nm laser line. Simultaneously detect, with a resonant scanner operating at 8,000 Hz in the bidirectional mode, the fluorescence emitted by the donor (between 495 and 525 nm) and the fluorescence emitted by the mKOk acceptor (between 550 and 650 nm, FRET channel). If available, use the 473 nm laser.
   NOTE: The excitation efficiency of the donor will be slightly reduced (85% instead of 93% with 488 nm), but the cross-excitation of the acceptor will be reduced as well (from 17% with the 488 nm to 9% with 473 nm laser line).
4. To reduce phototoxicity and fluorophore bleaching, minimize the illumination of the sample by reducing the power of the laser line (on the *beam path window* of the acquisition software).
5. Optimize the excitation to match gain and offset parameters that are set at the beginning of the experiment and kept constant throughout the session. To set the offset, change the color of the image to intensity values (by using the Q look-up table) and, while scanning with the laser off, turn the offset knob (smart offset) so that the background pixels have an intensity slightly higher than zero. With the same look-up table, by switching the laser on while scanning, turn the gain knob (smart gain) to maximize the signal-to-noise ratio, being careful to avoid saturated pixels.
6. Using an opened pinhole (2 airy units), acquire 16-bit images to provide a sufficient dynamic range for quantitative analyses. Avoid averaging to increase the acquisition speed and to minimize photobleaching.
7. In the software acquisition window, select an image field size of 512 x 64 pixels (pixel size: 605 nm) from the drop-down menu.
8. From the acquisition mode window, select *xyt* (time lapse on a single xy plane)*f*rom the drop-down menu and record the changes in embryo spinal neuron voltage by acquiring a single xy plane, setting the acquisition parameters to record one image every 30 ms for 1 min.
9. To evaluate the effect of riluzole administration on membrane depolarization in the same neuron, acquire a new dataset 5 min after the addition of fish water containing 5 µM riluzole.
10. For FRET analysis, use the ImageJ macro Biosensor_FRET (expressing single-chain FRET biosensors.
11. Evaluate the basal membrane FRET ratio of each neuron at t_1 as ((FRET mean - FRET background)/(Donor mean - Donor background)), where FRET and Donor mean intensity is the mean fluorescence intensity calculated in the same region of interest (ROI) drawn around the cell for each channel acquired and FRET and Donor background is the mean fluorescence intensity calculated in an ROI of the field of view without the fluorescent sample.
    NOTE: A detailed step-by-step description of the use of the plugin can be found at the www.med.unc.edu/microscopy/resources/imagej-plugins-and-macros/biosensor-fret website.
12. Use a graphing software to compare the frequency, amplitude, and duration of depolarization between different experimental paradigms. Compare two groups using an unpaired Student's *t*-test and consider mean values as statistically different when $P < 0.05$.

Representative Results

An expression vector carrying the FRET-based Mermaid biosensor coding sequence under the control of the pHuC pan-neuronal promoter, which drives the synthesis of the protein exclusively in the nervous system, was delivered into single-cell fertilized eggs by means of a microinjection in order to obtain transient transgenic embryos (**Figure 1**, left panel). After mastering the microinjection technique, the percentage of Mermaid-positive embryos was close to 100%. Among these, only the embryos expressing the proper amount of Mermaid biosensor at the plasma membrane were considered for FRET analysis/image acquisition.

Spontaneous coilings, motor responses that consist exclusively of a full body contraction bringing the tip of the tail to the head[9,10], were recorded at the stage of 20-24 hpf in regular fish medium (**Figure 1**, center panel and **Video 1**) and following incubation in 5 µM riluzole (**Video 2**). The statistical analyses of the changes in the frequency of the spontaneous tail coilings triggered by the drug riluzole are reported elsewhere[4].

To test whether alterations in the electrical activity of the spinal motor neurons were at the basis of the riluzole-induced changes in the spontaneous coiling frequency, the membrane potential in embryo spinal motor neurons were studied in a non-invasive fashion, measuring the fluorescence intensity ratio of the donor/acceptor FRET pair of the Mermaid biosensor (FRET ratio: **Figure 1**, right panel). Primary spinal motor neurons expressing the biosensor were identified (**Figure 2A**) and their basal spontaneous depolarization activities were recorded (**Figure 2B** and **Videos 3** and **4**). Together with the reduction in the frequency of tail coiling movements, riluzole administration reduced the frequency of spontaneous depolarization events (**Figure 2C**).

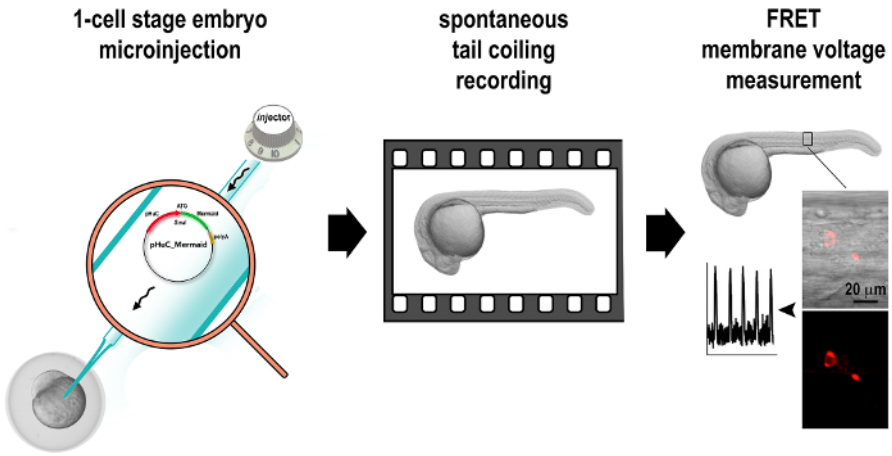


Figure 1: Flow Chart of the Experimental Procedure. Single-cell-stage embryos are collected and immediately microinjected with the pHuC_Mermaid plasmid to express the Mermaid FRET-voltage biosensor in a pan-neuronal fashion. At 20-24 hpf with incubation at 28 °C, single embryos are transferred under a stereomicroscope and their spontaneous tail coiling activity, with and without the drug riluzole present in the fish water, is video recorded and analyzed. The same embryos undergo *in vivo* FRET analysis in order to measure changes in motor neuron membrane potential. Please click here to view a larger version of this figure.

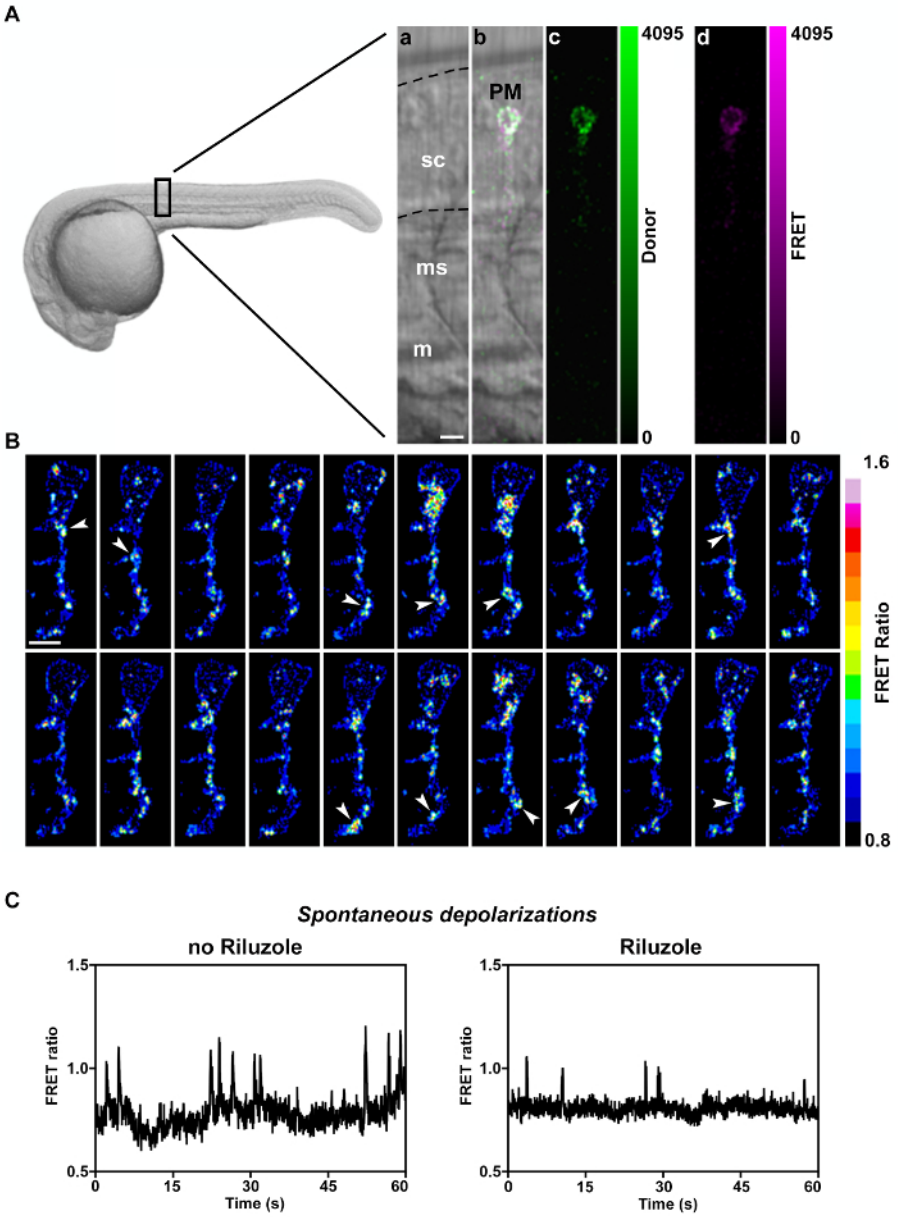
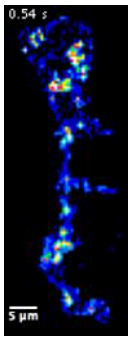


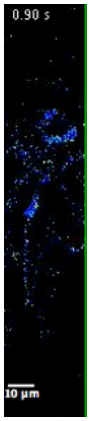
Figure 2: *In Vivo* FRET Analysis to Measure Changes in Motor Neuron Membrane Potential. (**A**) Wild-type zebrafish embryos with the mosaic expression of the FRET-based voltage biosensor Mermaid in the spinal neuron. (a) The bright field image shows the area of the zebrafish trunk identified by the black box (sc: spinal cord; ms: myoseptum; m: myotome). By superimposing on (a) the fluorescence signal detected (b), the efficient expression of the biosensor in a primary spinal motor neuron (PM) can be clearly visualized. The donor (c) and the FRET (d) channel detected are shown with the respective green and magenta look-up tables (LUT) on the right side. All the embryos are mounted in a cranial-to-caudal orientation. Scale bar = 10 µm. (**B**) The FRET ratio map displays the FRET ratio calculated every 0.03 s in the same motor neuron. It shows the basal spontaneous depolarization activity that the cell undergoes. For the Mermaid biosensor, the increase in the FRET ratio occurs when the membrane potential increases. Scale bar = 10 µm. (**C**) Representative example of spontaneous mean FRET ratio changes occurring in a spinal motor neuron of a Sod1-G93R zebrafish embryo during a 1 min recording. Riluzole administration reduces the frequency of the spontaneous depolarizations. Please click here to view a larger version of this figure.

Movie 1: Spontaneous Tail Coiling in 24-hpf Wild-typecontrol Embryos. Representative recording showing spontaneous tail coiling in a 24 hpf wild-type control embryo incubated in fish water. Please click here to view this video. (Right-click to download.)

Movie 2: Spontaneous Tail Coiling in 24-hpf Wild-typeriluzole-treated Embryos. Representative recording showing spontaneous tail coiling in a 24 hpf wild-type control embryo incubated in 5 µM riluzole (+R). Compared to controls, +R embryos showed a significant decrease in the frequency of spontaneous tail coiling behavior at 24 hpf. Please click here to view this video. (Right-click to download.)



Movie 3: Basal Spontaneous Depolarization Activity of a Primary Motor Neuron in the Spinal Cord of a Wild-type Zebrafish Embryo at 20 hpf. The Mermaid biosensor is so sensitive that it can detect the rapid changes in motor neuron membrane potential in the spinal cords of intact embryos. Please click here to view this video. (Right-click to download.)



Movie 4: Spontaneous Depolarization Activity is Coupled to Muscular Contraction. A secondary motor neuron in the spinal cord of a wild-type zebrafish embryo at 20 hpf undergoes spontaneous depolarization. In this case, each depolarization is associated to a contraction of the zebrafish muscles, organized in a functionally synchronized syncytium at this developmental stage. Please click here to view this video. (Right-click to download.)

Discussion

The protocol presented here allowed us to explore the association between the electrical properties of zebrafish embryo spinal motor neurons and the spontaneous coiling behavior, the earliest stereotypic motor activity, which appears around 17 hpf of embryonic development and lasts until 24 hpf[10].

Our approach provides researchers with a tool to study the neural system of intact embryos, fully preserving the complexity of the interactions between cells in a developing functional network. The *in vivo* imaging of zebrafish embryos has been performed by simply immobilizing them through immersion in low-melting agarose. The study of changes in cell membrane potential through the use of a biosensor represents one of the main advantages of this method. Canonical electrophysiological approaches, where neurons must be physically accessed by removing the enveloping tissues[11], are procedures that might alter the electrical properties of the cells under examination. Moreover, the FRET-based biosensor approach allows for the study of cell electrical properties without the use of anesthetics (*i.e.,* Tricaine, a sodium channel blocker), avoiding any potential disturbance associated with the administration of chemicals (*i.e.,* riluzole here), which might be crucial in the experimental plan. This method allowed us to focus on the modulation of both the electrical activity and the frequency of the spontaneous tail coilings, a behavioral response that prevents, in most cases, electrophysiological recordings without the administration of anesthetics. Finally, the employment of GEVIs offers the highest temporal experimental control because it allows work at a specific embryonic developmental stage. Our imaging/recording sessions spanned a very limited time window, allowing us to precisely compare the developmental stages of the different embryos in real time. All the conventional electrophysiological recordings, on the contrary, are usually conducted in larger and less precise time spans.

Measuring voltage changes by imaging techniques is, however, intrinsically difficult because of the nature of the signal itself, which can vary in speed, in frequency, and in the size of the changes in membrane potential. An ideal voltage sensor should combine fast responsiveness, high sensitivity, and high photon emission. For most FRET-based GEVIs, ratio changes vary as a function of membrane voltage variations, but the amplitude of the fluorescence ratio changes is tightly associated with the duration of the depolarization events. Recently, new GEVIs were developed and characterized, thus making it possible to choose the best probe in relation to the type of experimental issue, model, and imaging device.

Voltage biosensors are integral membrane proteins. When transfected into cultured cells or expressed in living organisms, the fluorescence of the plasma membrane-localized sensor will be, to a certain extent, associated with the fluorescence of the sensor in other intracellular compartments-where the protein can be mislocalized or aggregated-thus generating a background signal. The level of expression and the proper membrane localization of each construct should be monitored in simplified experimental models (*i.e.,* transfecting cell lines or primary cultures) before using the biosensors in living organisms.

In the present experimental setup, another potential critical step of the protocol might be represented by the efficiency of the transgenesis procedure. If necessary, the percentage of plasmid-positive cells can be drastically increased by employing the Tol2 transposon system[12]. An additional crucial aspect that needs to be considered is the loss of fluorescence emission in the donor fluorophore and the loss in FRET signal occurring during the imaging session because of the intense illumination. This should be avoided by minimizing the illumination of the sample, reducing the power of the laser line used. Similarly, the gain and offset of the photomultipliers must be carefully tuned at the beginning of every recording session to achieve the best signal-to-noise ratio in the acquired images as well as to avoid saturated pixels. All these parameters can vary depending on the type of cell examined, the characteristics of the biosensor, and the instruments used for image acquisition (wide field fluorescence microscopes can be also used, as recently described[13]). The researcher should always consider, however, that during *in vivo* imaging, the noise level might interfere with the specific signal of the sensor more than in *in vitro* recordings. Finally, if available, control measurements using mutant probes, which do not respond to voltage, would be a convenient way to evaluate the level of system-specific noise and/or artifacts.

The employment of zebrafish embryos represents a key feature of this approach thanks to their responsiveness to genetic manipulation and microscopic investigation. In fact, these characteristics make transient transgenesis and the FRET-based analysis of neuronal electrical

properties feasible. In our opinion, with the best-suited promoter and biosensor combinations, it would potentially be possible to selectively analyze the electrical activity of any cell type of interest and to parallel such activity with the embryonic phenotype.

Disclosures

The authors have nothing to disclose.

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