



Lifetime imaging reveals long-distance non-neural bioelectric patterns on timescales from seconds to hours

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ABSTRACT

Translating the bioelectric code remains one of the core challenges to widespread biomedical translation of bioelectric interventions, as well as a better evolutionary understanding of how developmental ionic signaling evolved to become the basis of neural intelligence. To advance our understanding, it is crucial to develop model systems that allow simultaneous, quantitative study of diverse bioelectric parameters in living states, which must also be linked to cell- and tissue-level outcomes. Here, we apply quantitative Fluorescent Lifetime Imaging (FLIM) optical estimation of membrane potential (V_{mem}^{oe}) to map the bioelectric dynamics of spreading *Xenopus laevis* neural crest explants (NCEs) over > 17 h. We identify a slow hours-scale bioelectric component and distinct faster minutes-scale and seconds-scale components. These dynamics often span multiple cells, consistent with roles in the collective behavior of NCEs. We then use information theory to show that minutes-scale NCE V_{mem}^{oe} dynamics are largely distinct from calcium dynamics. Finally, we provide a survey of diverse bioelectric events revealing a deep complexity in collective bioelectric dynamics, likely involving tunneling nanotubes in their transmission, which suggests numerous avenues for further investigation. These combined data demonstrate FLIM as a powerful tool for observing subtle bioelectric signals at the large temporal and spatial scale needed for developmental biology studies.

1. Introduction

1.1. Bioelectricity as a primordial cellular language

There is a rapidly growing body of evidence that non-neural bioelectricity plays important roles in development, regeneration, cancer progression, and evolution (Bates, 2015; Harris, 2021; Levin, 2021). Voltage parameters regulate stem cell differentiation (Cao et al., 2018; Daley et al., 2020; König et al., 2004, 2006; van Vliet et al., 2010), proliferation (Bhavsar et al., 2020; Blackiston et al., 2009; Ding et al., 2012; Petsakou and Perrimon, 2022; Piggott et al., 2019), gene expression (Pai et al., 2016; Thomas et al., 2023), and cellular interpretation of canonical biochemical signaling factors (Belgacem and Borodinsky, 2015; Borodinsky and Belgacem, 2016; Borodinsky et al., 2015; Spann et al., 2021). Misexpression of ion channels induces ectopic eye formation in *Xenopus* embryos, even outside of areas competent for eye induction by the “master regulator” gene *pax 6* (Pai et al., 2012). Bioelectricity regulates wing development in *Drosophila*

(Dahal et al., 2012, 2017; George et al., 2019, 2023), the size of zebrafish fins (Perathoner et al., 2014), and the patterning of the vertebrate heart, brain, and face (Adams et al., 2016; Pai et al., 2015; Pitcairn et al., 2017; Vandenberg et al., 2011). It has also been implicated in the evolution of the highly specialized fins of flying fishes (Daane et al., 2021). Bioelectric intervention has been shown to rescue regeneration in non-regenerative contexts (Adams et al., 2013; Tseng et al., 2010), overcome chemical and genetic teratogenesis (Pai and Levin, 2022), and normalize tumor-like structures induced by human oncogenes (Chernet et al., 2014, 2016; Chernet and Levin, 2013; Lobikin et al., 2012).

Bioelectricity is also an appealing target for biomedical intervention due to the diverse array of known reagents that can induce complex, coordinated downstream responses in model systems *in vivo* – electropharmaceuticals — many of which are already FDA approved because of their role in neuromedicine and other indications (Balasubramanian et al., 2024; Kofman and Levin, 2024). And yet, important gaps remain with respect to predicting outcomes of bioelectric intervention, diagnosis of

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underlying bioelectric causes of pathology, and integration of bioelectricity with the greater cell, molecular and developmental biology knowledgebase. This is largely due to the daunting technical challenge of observing and interpreting the dynamics of bioelectric patterns at physiologically meaningful timescales. Bioelectricity is a complex computational medium (Levin and Martyniuk, 2018) that cannot be controlled by simple one-to-one input-to-output relations, and in order to control the bioelectric code, we must be able to read it. Here we report efforts to use the embryonic cell population known as the neural crest as a model system in which to probe bioelectrical encoding of developmentally relevant information (Cooper and Keller, 1984; Hotary and Robinson, 1990; Robinson, 1985; Stump and Robinson, 1983).

1.2. The neural crest is an intermediate between complex neural and subtle non-neural bioelectric archetypes

The neural crest is a multipotent stem cell population that sits at the interface between the neural and non-neural paradigms, ultimately differentiating into both neural and non-neural cell types (Bronner-Fraser, 1993; Fuchs and Sommer, 2007; Mayor and Aybar, 2001; Trainor, 2003). Neural crest cells (NCCs) collectively and intelligently navigate the forming embryo to adopt a diverse variety (Lo et al., 1997, 1999) of fates with cells becoming peripheral neurons, heart cells, melanocytes and cranial bone and cartilage among others. And, especially relevant to our study, they have repeatedly been shown to be bioelectrically active. Amphibian NCCs electrotax toward the cathode of exogenously generated electric fields (Gruler and Nuccitelli, 1991; Stump and Robinson, 1983) and along endogenous electric fields *in vivo* (Ferreira et al., 2025). Moreover, mutation of multiple key bioelectric effectors prevents normal NCC development, including K^+ channels (Adams et al., 2016; Belus et al., 2018), calcium channels (Melchionda et al., 2016), gap junctions (Lo et al., 1997, 1999), and the mechanosensitive cation non-specific channel Piezo1 (Canales Coutiño and Mayor, 2021; Ferreira et al., 2025). These functional data make the neural crest an appealing model for studying the bioelectric basis of collective cell behavior.

Beyond their appeal as an intermediary between neural and non-neural cell behavior, neural crest cells are an important model for collective cell behavior (Barriga and Theveneau, 2020; Hartmann and Mayor, 2023; Shellard and Mayor, 2020; Theveneau and Linker, 2017; Theveneau and Mayor, 2011a, 2011b; Theveneau et al., 2013). Developmental biology is, to a large extent, the study of systems in which large numbers of cells coordinate their activity to reliably achieve species-specific anatomical outcomes. Thus it is essential to understand the biophysical mechanisms that enable the functional cooperation of cell groups. NCC collectives chemotax toward the attractant Sdf1a, while individual cells are unable to do so (Theveneau et al., 2010), demonstrating an example in which this collective is “greater than the sum of its parts”. Cells expressing a dominant negative version of the Sdf1a receptor CXCR4 regain the ability to chemotax toward Sdf1a when mixed into a collective with wild type cells (Theveneau et al., 2010), further evidencing the collective nature of this tissue. NCC collectivity is structural as well as functional, as NCCs form supracellular actin cables that drive migrating collectives *in vitro* and *in vivo* (Shellard et al., 2018).

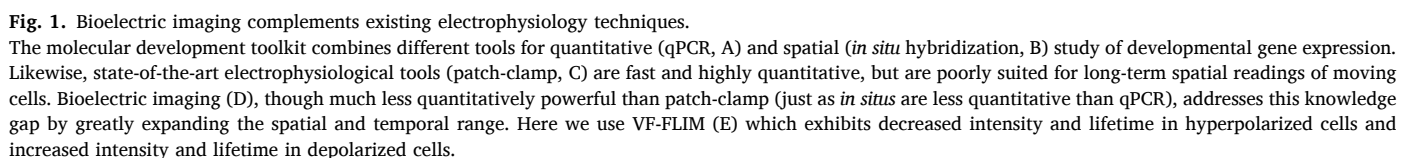
A key aspect of collective behavior is the alignment of components toward a consistent setpoint. NCCs show remarkable ability to do so: for example, in response to ablation they can migrate to deficient regions through non-canonical paths (Couly et al., 1996; Le Douarin et al., 2004) (as do neurons (Little et al., 2009)), and they can regulate their numbers either up or down, as needed, to achieve correct target morphology (Zarzosa et al., 2014). Perhaps most impressively, mouse neural crest cells grafted into chicken embryos can successfully navigate their new environment and successfully form teeth in the chicken's beak (Mitsiadis et al., 2003). This makes NCCs even more interesting as a nexus between developmental and neural bioelectricity, as they appear to be a minimal

model of collective behavior that is consistent with William James' definition of intelligence — the ability to achieve the same goal via different paths (Fields and Levin, 2022; James, 1890). This complex and highly plastic repertoire points to an underlying complexity and context-sensitive behavior that make the neural crest an appealing model for studying bioelectric communication and more generally, collective behavior in anatomical state space (McMillen and Levin, 2024a).

1.3. Fluorescent lifetime imaging as a tool for discovering bioelectric patterns

Studying developmental bioelectricity has many challenges, and the tradeoffs that are faced in many ways reflect the challenges of studying developmental gene expression. qPCR allows precise but destructive quantification of transcript levels at the expense of spatial and temporal resolution (Fig. 1A). Similarly, patch-clamp electrophysiology allows very precise measurement of subtle changes in membrane potential, but is generally restricted to a single cell at a time, and requires cells that are not moving. Molecular developmental biologists address the limitations of qPCR with an orthogonal technique: *in situ* hybridization (ISH) (Fig. 1B). ISH enables detection of spatial gene expression patterns, including dynamic (Palmeirim et al., 1997) and subtle (Mara et al., 2007) ones, but is poorly suited for quantification of gene expression changes between samples. Integration of complementary spatial and quantitative techniques allowed the field of molecular development to progress even in the absence of techniques directly integrating them, which have only become available relatively recently. Similarly, much of modern neuroscience is based on patch-clamp electrophysiology, which is highly sensitive to fast and subtle bioelectric signals but poorly suited for long-term study in moving cells (Lazzari-Dean et al., 2021) (Fig. 1C). Bioelectric imaging has the potential to do for bioelectricity what ISH has done for molecular developmental biology: semi-quantitatively detect spatial patterns that can be quantitatively investigated with its more sensitive but spatially weaker complementary technique (Fig. 1D). Further, high throughput molecular technologies like single-cell sequencing and spatial transcriptomics continue to expand the scale of cell and developmental biology studies. Bioelectric tools will need to scale up in parallel to effectively leverage these growing datasets, and the low-throughput nature of patch-clamp electrophysiology will become an increasingly large challenge. In this paper we explore the potential of FLIM to enable bioelectric analysis at a large scale in both time and space and identify novel dynamic collective bioelectric phenomena manifesting at these large scales that would be elusive at smaller spatial or temporal scales.

Evan Miller's group has developed a promising technology that combines VoltageFluor (VF) dyes with the highly quantitative imaging tool Fluorescent Lifetime Imaging (FLIM) which Miller's group has vetted using patch-clamp electrophysiology (Lazzari-Dean et al., 2019; Lazzari-Dean and Miller, 2021). VF dyes comprise a fluorophore, a molecular wire, and an electron donation quencher (Fig. 1E). They insert into the cell membrane like a thumb-tack, and once inserted their intensity and fluorescent lifetime change as the direction of the local electric field either pushes the electrons into the fluorophore (hyperpolarization), reducing its intensity and lifetime, or draws them out of it (depolarization), increasing intensity and lifetime. BeRST has been electrophysiologically validated in HEK cells and rat hippocampal neurons (Huang et al., 2015). VF2.1. Cl lifetime was first found to be V_{mem} sensitive via electrophysiology in HEK cells (Brinks et al., 2015), and later calibrated with A431, CHO, HEK293T, MCF-7 and MDA-MB-231 cells (Lazzari-Dean et al., 2019; Lazzari-Dean and Miller, 2021). VF-FLIM has even been used to estimate membrane potential in bacteria, which are not amenable to patch-clamping (Roy et al., 2025). Lazzari-Dean et al. report some variation in the sensitivity and 0 mV lifetime values between cell types, but similar general trends in all cases (Lazzari-Dean et al., 2019). We have observed similar lifetime



Because lifetime is an intrinsic property of a fluorophore that does not directly scale with dye concentration, FLIM largely eliminates confounds stemming from differential dye incorporation and cell shape changes. This is critical for reading non-neural bioelectric signals which tend to be very low magnitude and change over long time-periods, causing them to be easily obscured by even modest artifacts. After validating it in beating excised *Xenopus laevis* embryonic hearts, we use FLIM with the highly photostable far-red VF dye BeRST (Huang et al.,

Animal care was done in compliance with and under approval from the Institutional Animal Care and Use Committee (IACUC) under protocol number M2023-18 of Tufts University. Capped and tagged mRNA was generated using an mMessage Sp6 *in vitro* transcription kit

(ThermoFisher). Embryos were microinjected 4X at the 2-4 cell stage with a bolus of roughly 160 μm outer diameter containing 600 ng/ μL jGCaMP8s mRNA (Zhang et al., 2023), 10 ng/ μL mCherry-Erk2 mRNA (Toettcher et al., 2013) and 200 ng/ μL LifeAct-Turquoise 2 mRNA (Goedhart et al., 2012) in RNase-Free water (Ambion). pLifeAct-mTurquoise2 was a gift from Dorus Gadella (Addgene plasmid # 36,201; <http://n2t.net/addgene:36201>; RRID:Addgene_36,201). pHR SFFVp BFP-Erk2 was a gift from Wendell Lim (Addgene plasmid # 50,848; <http://n2t.net/addgene:50848>; RRID:Addgene_50,848).

2.2. Neural crest (NC) explanting onto fibronectin (FN) coated cover glass

NC explant procedure was modified from (Gougnard et al., 2021). NC removal was performed in Danilchick's For Amy (DFA) (Sater et al., 1993) media to minimize bioelectric effects from multiple media changes. Neural crest explants were removed during neurulation prior to closure of the anterior neural tube (NF stages 15-17). mRNA injection of multiple sensors coupled with rearing at 22 °C through gastrulation induces slight heterochrony, and while effort was made to take explants from similarly aged embryos, we report a conservatively large stage window. We attempted to mitigate effects of stage variation by cutting 4-5 explants per excised neural crest, pooling and thoroughly mixing the explants in DFA buffer and distributing explants evenly between conditions.

We performed experiments on three separate replicates from distinct clutches of eggs. For each replicate, we changed the spatial location of the three K^+ conditions to mitigate possible confounds from well location or explant imaging order. It is impractical to confirm the molecular identity of each explanted cell as such testing generally requires either genetically encodable reporters or fixation of the tissue, neither of which is compatible with our analysis. We therefore avoid referring to these explants as 'neural crest cells,' instead using the term *neural crest explants* (NCEs) to acknowledge the possibility of contamination with, or differentiation into, specific tissue types.

2.3. Optically estimated membrane potential ($V_{\text{mem}}^{\text{oe}}$)

While FLIM greatly improves the quantitative power of bioelectric imaging, as well as allowing monitoring of large numbers of cells, over developmental timescales, all without the disruption caused by penetrating them with electrodes, it is unreasonable to expect that bioelectric imaging maintains the same magnitude of accuracy as classic electrophysiological techniques in all cell types at all times. Dye internalization, artifacts from dye trapping, V_{mem} -independent effects on lifetime and other unforeseeable artifacts are necessary limitations of the current state of the technology. However, optical estimation does not suffer from the many confounds introduced by comparatively invasive electrophysiology techniques (Lazzari-Dean et al., 2021). We use the nomenclature "Optically Estimated V_{mem} " ($V_{\text{mem}}^{\text{oe}}$) throughout this paper to prevent confusion about what exactly we are reporting.

2.4. Dye staining

Neural crest explants were stained in a mixture of 445 μL standard DFA buffer containing 4.5 mM K^+ gluconate, 50 μL Nuc Blue reagent (Hoechst 33,342, Invitrogen R37605) and 5 μL of 0.5 mM BeRST DMSO stock (gift of Evan Miller, synthesized by Pharmaron, China) for 30 min in a 22 °C incubator while being protected from light. Staining media was gradually introduced to 8-well glass bottom chambered coverslips (Ibidi) to minimize disruption to explants and mixed between chambers to minimize uneven staining.

Heart explants were removed at stage 41 or 37/38 in 0.1X MMR containing 0.025% Tricaine (Fisher NC0872873) that was then replaced with 0.75X MMR. They were then stained for 30 min at 22 °C with either 50 μM (for stage 41) or 5 μM (for stage 37/38) BeRST, washed

thoroughly with fresh 0.75X MMR and compressed with a coverglass fragment using vacuum grease as a spacer. This procedure was conducted in the 27 mm glass bottom dishes (Nunc 150,682) in which the explants were imaged. Stage 37/38 hearts were imaged at roughly 18 °C while stage 41 hearts were imaged at roughly 15 °C to slow their beating rate to improve imaging.

2.5. Imaging

Imaging was performed using a Leica Stellaris 8 confocal microscope. Imaging conditions and directory of relevant metadata files (included as supplemental pdf files) are presented in Supplemental Table 1. Long-term imaging was performed at 20 °C. For the quantitative dataset discussed in Figs. 4–6 and Supplemental Figs. 1 and 3 independent replicates were performed with eggs collected from different clutches. 9 total explants were imaged per condition, 3 in each imaging session for the data in these figures. Slight variation due to stage travel rate was present between the three replicates, but because this amounted to only a few minutes over the >17 duration, timepoints between replicates were pooled. Images displayed may be from either 0.5 mM K^+ , 4.5 mM K^+ or 8.5 mM K^+ conditions unless specified. Some images were scaled up in Fiji without interpolation to make cleaner figures and movies, and some movies were exported with JPEG compression.

2.6. Image analysis

LasX FLIM quantification: Curve fitting is performed using the LasX FLIM/FCS module. While Lazzari-Dean et al. (2019) use a bi-exponential decay fit for VF2.1. Cl and a mono-exponential decay fit for VF2.0. Cl, we used a mono-exponential fit for all fluorophores due to the relatively low photon counts. For timeseries data reported in Figs. 4–6, BeRST lifetime images were binned in LasX prior to exporting to improve curve fitting by increasing photon values per fit, then scaled up in Fiji using bilinear interpolation or in CellProfiler. An intensity factor of 1 per count and a lifetime factor of 0.001 per count were applied so that 16-bit images scaled to picoseconds would be produced for analysis. Intensity data was exported from the "Lifetime" panel along with the mono-exponentially fit lifetime data to allow for the most direct comparison possible between intensity and lifetime measurements. As a result, in intensity images some background photons, and photons collected from multiple-detection-per-pulse events, were removed.

Whole-explant quantification: Explant timeseries were exported from LasX as separate intensity and lifetime channels. Unless processed by CellProfiler, binary masks were produced using either the BeRST intensity channel or NucBlue signal. For all Lifetime images (except for Fig. 2A, the top row of Fig. 2B and the bottom right image of Supplemental Movie 1), a binary threshold was arbitrarily set using the intensity image to distinguish cells from background. The images were converted to 32-bit and pixels below the threshold were set to NaN causing them to be displayed as 0 values in images and excluded from quantitative analysis. This thresholding was performed in Fiji (Schindelin et al., 2012).

Supplementary data related to this article can be found online at <http://doi.org/10.1016/j.ydbio.2026.08.001>

Kymographs: The kymograph in Fig. 5A was produced using the Dynamic Reslice tool on a line drawn at an arbitrary location on a masked lifetime timelapse. Kymographs in Fig. 6 were produced by using the NucBlue signal to mask the channels of interest then running the Radial Reslice tool in Fiji (Schindelin et al., 2012) and producing an average intensity projection. Images are formatted as 32-bit with background set to NaN to prevent below-threshold pixels from affecting the data. Positive derivative images were generated by subtracting an image from the image at the next timepoint and discarding any values below 0. For Fig. 6 panels H,I,L,M,P,Q,R,S and T derivative images were divided by the previous image to normalize for differential GCAMP8S protein levels to produce positive $\Delta\text{F}/\text{F}$.

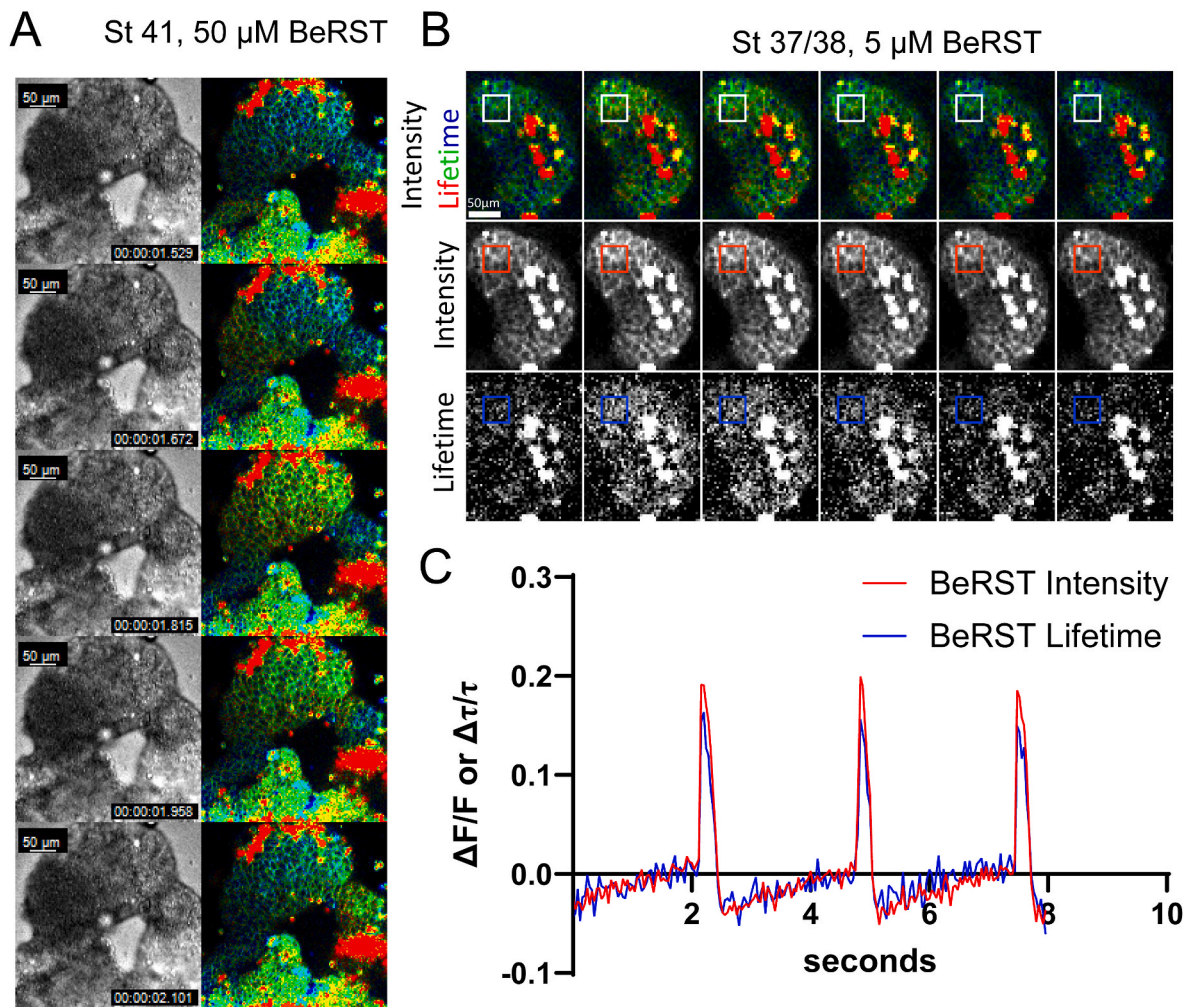


Fig. 2. BeRST lifetime correlates with BeRST intensity in *ex vivo* *X. laevis* embryonic heart tissue.

Clear waves of depolarization are visible in excised stage 41 heart tissue stained with 50 μM BeRST (A). In stage 37/38 heart tissue stained with 5 μM BeRST, intensity dynamics correlate with lifetime dynamics (B,C), indicating that BeRST lifetime changes with $V_{\text{mem}}^{\text{oe}}$ at this dye concentration in embryonic *X. laevis* tissue. Fluorescent lifetime heatmap range displayed in (A) is 1.2ns to 2ns and in (B) is 1.3ns to 1.9 ns? The lifetime row of (B) was arbitrarily masked using the intensity channel as described in the methods, and background pixels are represented as having a lifetime of 0.

Cell Profiler nucleus outlining: Our CellProfiler (Lamprecht et al., 2007; Stirling et al., 2021) pipeline is attached as [Supplemental Methods 1](#). In this study CellProfiler was only used to outline nuclei in Supplemental Movies 5 and 6.

Supplementary data related to this article can be found online at <http://doi.org/10.1016/j.ydbio.2026.08.001>

2.7. Informational analysis of $V_{\text{mem}}^{\text{oe}}$ and calcium kymographs

The kymographs were cropped to a height of 240 pixels, removing the outermost pixels, and binarized with a manually derived threshold. One threshold value was used for all $V_{\text{mem}}^{\text{oe}}$ kymographs and another for all calcium kymographs. The y-axis was then median-binned by a factor of 16 to produce an image 15 pixels high, with the pixels representing average values of a series of concentric circles 16 pixels thick. The x-axis of the resulting kymograph was replaced with time, converting the single kymograph image into a timeseries one pixel thick and 15 pixels high. Both timelapses were then spatially scaled up 100-fold and segmented to make each original pixel into a distinct ROI. A mosaic timelapse with calcium values on the left and $V_{\text{mem}}^{\text{oe}}$ values on the right was then created and saved as an .avi format file. The information theory software package CAIM (Moore and McMillen, 2019) was then used to extract timeseries data from each ROI and calculate the information

within each signaling paradigm at each ROI and the mutual information between each channel and each ROI. Data was processed in Excel and statistically analyzed and plotted in Prism. Statistical significance was determined using Friedman's test with Dunn's multiple comparison test.

2.8. Bioelectric event size estimation

Binarized data from the information analysis above was used to estimate the average size of bioelectric events within each explant. The average number of ROIs with signal was measured in each timepoint containing at least one active ROI in either the $V_{\text{mem}}^{\text{oe}}$ or calcium paradigm. Next, an average event size for each explant was determined. Explants that did not contain at least one $V_{\text{mem}}^{\text{oe}}$ event and one calcium event were excluded. Statistical significance was calculated in GraphPad Prism using a paired *t*-test.

2.9. Scan speed bioelectric event dynamic estimation

We observed linear signals perpendicular to the direction of scan. In order to estimate the dwell time for each scan line, we divided the frame capture duration of the image by the number of scan lines composing it. We then used ImageJ to draw a line along the masked lifetime image (background pixels being set as NaNs were not included in the analysis)

parallel to the scan direction and manually defined to be thick enough to encompass the bioelectric event with minimal contamination from nearby cells. We extracted the variation in lifetime over space using the Plot Profile tool in ImageJ and converted the spatial component to time using the scan line dwell time estimate and plotted the resulting lifetime vs. time plot.

2.10. Statistics

Lazic et al. (2018), Lord et al. (2020), and Motulsky (Motulsky) were used as guides for statistical analysis. Multiple neural crests from different animals were cut into smaller explants which were randomized and sorted into different K^+ conditions. Because randomization occurred at the explant level the sample size was calculated as the number of explants, and different explants were used as replicates for statistics. Explants were collected from 3 separate clutches and imaged in three separate sessions with the 3 conditions being imaged in parallel. Out of efficiency concerns, we did not explicitly account for possible clutch and imaging session level variability in our statistics, though in Fig. 4A we used different colored spots for each of the three replicates. Because long-term timelapses are time consuming, for both information and event size estimation we pool data from all three K^+ concentration conditions to increase statistical power and use color to denote to which condition each datapoint belongs. For the event size estimation, explants that do not have both V_{mem}^{oe} and calcium events were excluded. Statistical tests were conducted using GraphPad Prism 10.4.0 in accordance with its recommendations (Motulsky).

3. Results

3.1. Validating BeRST lifetime with embryonic cardiac action potential dynamics

We first sought to characterize BeRST due to its photostability and low-phototoxicity excitation wavelength for the study of long-term NCE bioelectric dynamics, choosing embryonic cardiac tissue as a validation target. While only the green VF2.1. Cl and VF2.0. Cl dyes have been characterized with FLIM (Lazzari-Dean et al., 2019; Lazzari-Dean and Miller, 2021), their comparatively toxic blue light excitation spectrum and poor photostability limit their usefulness for long-term studies. BeRST is more photostable and is excited by less phototoxic red light (Huang et al., 2015), making it an appealing candidate for FLIM studies which require high photon counts (Köllner and Wolfrum, 1992). Because the embryonic heart oscillates between hyperpolarization and depolarization as it beats, it is a powerful tool for validating V_{mem} dye sensitivity. To confirm that BeRST lifetime is V_{mem} sensitive, like its better-characterized green counterpart, we extracted heart tissue from stage 41 embryos and stained it with 50 μ M BeRST, revealing clear depolarization waves that migrated through the heart tissue (Supplemental Movie 1). To more closely align this validation with our NCE experiments we extracted younger beating heart tissue (stage 37/38) and stained it using the same staining conditions we used for neural crest explants. As with the published patch-clamp data for VF2.1. Cl (Lazzari-Dean et al., 2019; Lazzari-Dean and Miller, 2021), we found that the timing and magnitude of the $\Delta F/F$ of BeRST's intensity over time and the $\Delta\tau/\tau$ of its lifetime over time strongly agreed (Fig. 2A and B).

While patch-clamp electrophysiology data are unavailable for embryonic frog heart tissue, we can use data from other model organisms as a reference. In chickens, the magnitude of the cardiac action potential at day 3 post fertilization around the onset of beating is roughly 60 to 74.0 mV and increases to roughly 109.5 mV by day 14 of development (McDonald and DeHaan, 1973). In mice, action potential amplitude of E12.5 heart pacemaker cells has been reported at 74.9–86 mV (Rytkin et al., 2024). Extrapolating from the first peak and valley of Fig. 2C, we see a roughly 23% change in intensity that equates to a 96 mV change using the published BeRST sensitivity of 24% change/100 mV, and a

comparable change in BeRST lifetime. Thus, our readings agree relatively well with predicted embryonic cardiac behavior. Future work beyond the scope of this paper with patch-clamp electrophysiology will be needed to quantify and calibrate BeRST for specific cell types as has been done with VF2.1. Cl (Lazzari-Dean et al., 2019; Lazzari-Dean and Miller, 2021); here, we report optically estimated V_{mem} (V_{mem}^{oe}) instead of true V_{mem} to emphasize the current limitations of our approach.

3.2. Lifetime, but not intensity, analysis reveals physiologically realistic V_{mem}^{oe} patterns in NCEs

We hypothesized that there exist subtle bioelectric patterns in spreading neural crest explants that are not readily detectable by intensity-only fluorescent imaging. In order to test this, we compared the V_{mem}^{oe} patterns optically estimated by intensity and lifetime using a set of three bioelectric dyes.

We recently described a strategy (McMillen and Levin, 2024b) to validate specificity of bioelectric patterns detected with BeRST using the control dyes VF2.1. Cl, whose lifetime properties have been rigorously quantified with electrophysiology (Lazzari-Dean et al., 2019), and VF2.0. Cl, a negative control variant of VF2.1. Cl lacking the electron-donating quencher that confers V_{mem} sensitivity. We have found that co-staining NC explants with BeRST and VF2.0. Cl dramatically reduces the lifetime and variability of the VF2.0. Cl signal, suggesting a possible FRET interaction between the two dyes (data not shown). We therefore avoided co-staining BeRST with either VF2.1. Cl or VF2.0. Cl, instead staining separate explants in parallel. We observed 6 explants stained with VF2.0. Cl, 6 explants stained with VF2.1. Cl and 6 explants stained with BeRST. Imaging conditions were altered slightly between explants to generate comparable images. Explants presented in Fig. 3 were not obtained from injected embryos to prevent crosstalk between constructs and VF2.0. Cl and VF2.1. Cl.

To enable more rigorous quantification, we drew diagonal lines of 50 pixel-width in Fiji from the top left to the bottom of each image from Fig. 3A and measured the average value for each pixel above an arbitrary intensity threshold. We plotted both lifetime and intensity values in Fig. 3B with the x-axis showing pixel distance from the top left corner and estimated the approximate V_{mem}^{oe} deviation from mean recorded by each dye using published sensitivity values: 27%/100 mV for VF2.1. Cl intensity (Miller et al., 2012), 22.4%/100 mV for VF2.1. Cl lifetime (Lazzari-Dean et al., 2019) and 24%/100 mV for BeRST intensity (Huang et al., 2015). We used the VF2.1. Cl values for VF2.0. Cl as a 'false-positive' approximation, and the BeRST intensity sensitivity value for BeRST lifetime because they showed similar sensitivity in our cardiac action potential assay (Fig. 2C) and because VF2.1. Cl showed similar sensitivity with intensity and lifetime imaging.

We found that intensity and lifetime values correlated poorly for all three dyes. Further, we found that intensity values deviated beyond the expected physiological range (± 100 mV) even with VF2.0. Cl. Lifetime values, by contrast, remained within physiological ranges with a few notable exceptions (Fig. 3A denoted by arrows): a dead cell in the BeRST trace had a physiologically unrealistic low lifetime value (purple arrow), neurites in the VF2.1. Cl trace had an unexpectedly elevated lifetime value (white arrow), and a cell-free region in the VF2.0. Cl trace had unexpectedly low lifetime (red arrow). We also observed domains of reduced lifetime in the VF2.0. Cl image which, being V_{mem} insensitive, should be uniform. A likely cause of these depressions is local dye trapping causing concentration-dependent quenching, which has been described in both V_{mem} sensitive and insensitive dyes (Lazzari-Dean et al., 2019). The potential for this trapping is increased by the use of relatively high concentrations of the dyes (5 μ M), needed to facilitate penetration into our complex tissues.

In Fig. 3C we used the magnitude of cardiac action potentials measured in Fig. 2C as a benchmark for NCE variation with the expectation that NCE cell-to-cell V_{mem}^{oe} variation should be smaller in range than the cardiac action potential. This was not the case for the intensity

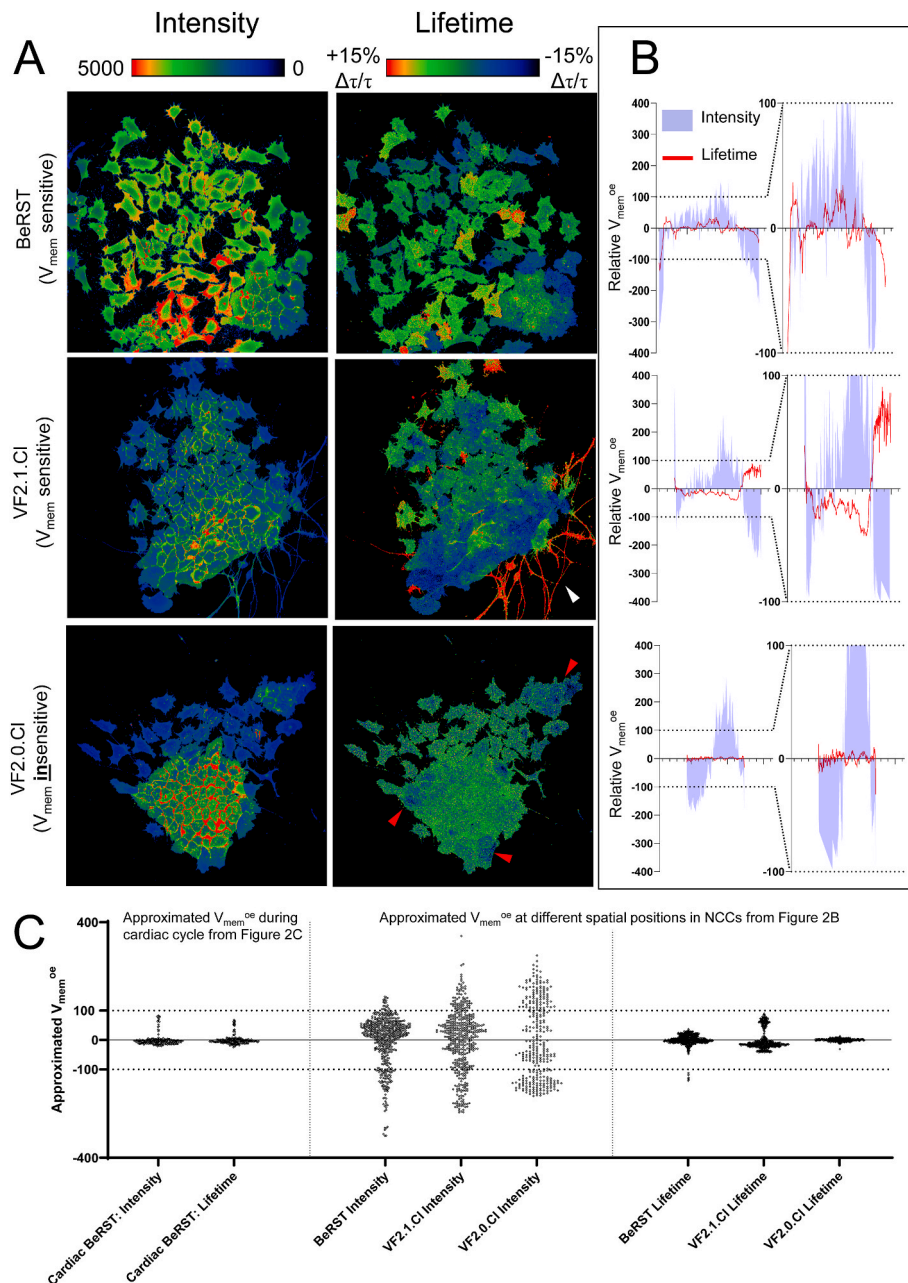


Fig. 3. Lifetime, but not intensity, shows physiologically feasible V_{mem}^{oe} variation

(A) Representative images of NCEs stained with BeRST, VF2.1. CI and VF2.0. CI pseudocolored to represent intensity values or $\Delta\tau/\tau$. Lifetime images were arbitrarily masked using a threshold from intensity image. Purple arrow = dead cell, white arrow = apparently artifactual strong depolarization in neurites, red arrow = apparent lifetime depressions suggesting dye trapping.

(B) Intensity values correlate poorly with lifetime values. Superimposed average intensity (blue, filled line) and lifetime (unfilled red line) along a 50 pixel-thick line drawn from the top left of each image to the bottom right with predicted V_{mem}^{oe} change vs. image average estimated based on published data. BeRST was assumed to have comparable lifetime sensitivity to its intensity sensitivity (see Fig. 2), and VF2.0. CI V_{mem}^{oe} was calculated with VF2.1. CI values for 'false-positive' detection.

(C) Comparison of the datapoints from Fig. 3B with the cardiac timeseries datapoints from Fig. 2C. Intensity values spread across an unrealistic >200 estimated mV range much greater than the cardiac action potential range, while aside from some artifacts, lifetime values are more consistent with realistic physiological values. Lifetime images were arbitrarily masked using the intensity channel as described in the methods, and background pixels are represented as having a lifetime of 0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

measurements, with even VF2.0. CI showing a much broader distribution than the cardiac action potential, strongly indicating that these values are artifactual. In contrast, the distribution of the lifetime values was narrower than the cardiac action potential values aside from the artifacts that we described above. Together, these data indicate that intensity-based bioelectric imaging does not reflect physiologically realistic bioelectric patterns in resting membrane potential. Lifetime imaging did show biologically realistic resting membrane potential

patterns, though some artifacts confound these patterns. Future work using patch-clamping electrophysiology will be needed to confirm that these patterns are truly V_{mem} , but given the low throughput and spatiotemporal limitations of that approach we report only V_{mem}^{oe} in this paper as described above. Also, because we only use BeRST for V_{mem}^{oe} for the rest of the paper we report lifetime directly instead of $\Delta\tau/\tau$ to most accurately represent our data.

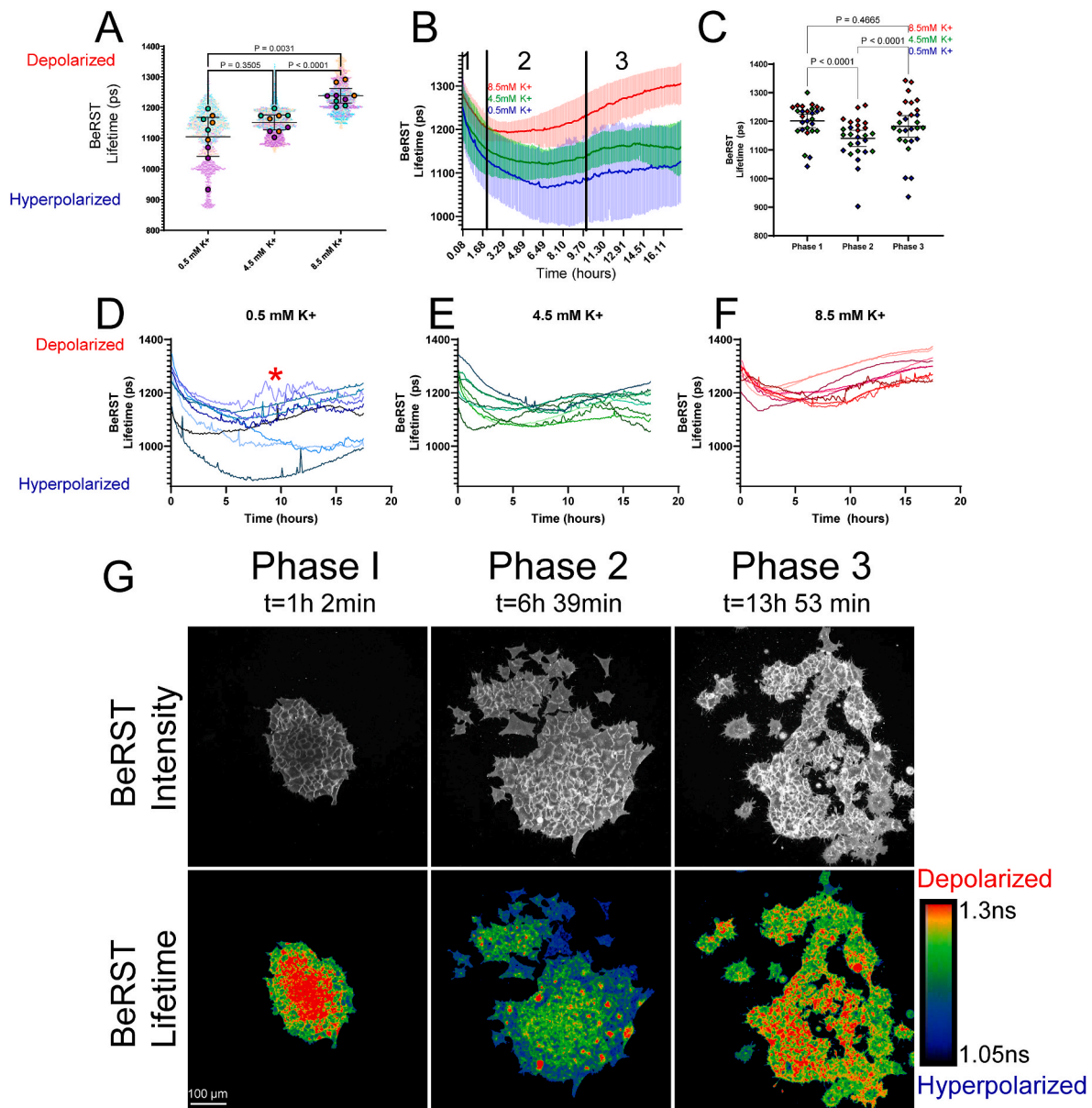


Fig. 4. NC explants exhibit a three-phase pattern of hours-scale bioelectric changes

(A) Increasing K^+ by 4 mM increases V_{mem}^{oe} , while decreasing K^+ by 4 mM does not decrease V_{mem}^{oe} . Significance determined using Browne-Forsythe and Welch ANOVA tests with Dunnett's T3 multiple comparison test in Prism. Large datapoints represent average values over the complete timescourse for a single explant (used to determine statistical significance), and small datapoints represent individual timepoints. Different colors represent different clutches. Plot produced following (Lord et al., 2020). Line at mean, error bar = 95% confidence interval.

(B) Mean whole-explant lifetime over time displayed with SD. Vertical lines demarcate Phases.

(C) V_{mem}^{oe} decreases from Phase 1 to Phase 2, then increases from Phase 2 to Phase 3 in timeseries pooled from all 3 K^+ conditions. Datapoints represent mean V_{mem}^{oe} values of a single explant during indicated Phase. Datapoint color indicates K^+ condition. Significance determined with repeated-measures one-way ANOVA with Tukey's multiple comparison test in Prism. Line at mean, error bar = 95% confidence interval.

(D-F) Traces of individual explants raised at different K^+ concentrations over time. Red asterisk in D indicates increased dynamics during Phase 2 in 0.5 mM K^+ condition.

(G) Representative images of NCE raised in 4.5 mM K^+ at the three different bioelectric phases. Lifetime images were bilinearly interpolated during upscaling to reflect analytical method.

Lifetime images were arbitrarily masked using the intensity channel as described in the methods, and background pixels are represented as having a lifetime of 0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Hours-scale bioelectric dynamics

Having identified spatial variation in bioelectric state, we next asked how bioelectric patterns evolve in time during neural crest cell migration and how these patterns respond to bioelectric intervention by altering media K^+ levels. Bioelectric imaging greatly improved the temporal and spatial range over which we could detect bioelectric

patterns. We leveraged this sensitivity window to explore V_{mem}^{oe} dynamics over a >17-h window as the neural crest explants spread out, migrate collectively, and begin to separate off from their collective to migrate individually. We performed a bulk lifetime analysis on the explant timelapses to estimate global V_{mem}^{oe} patterns over time by thresholding the Intensity component of the image to remove dim pixels from our analysis. Because this approach does not require image

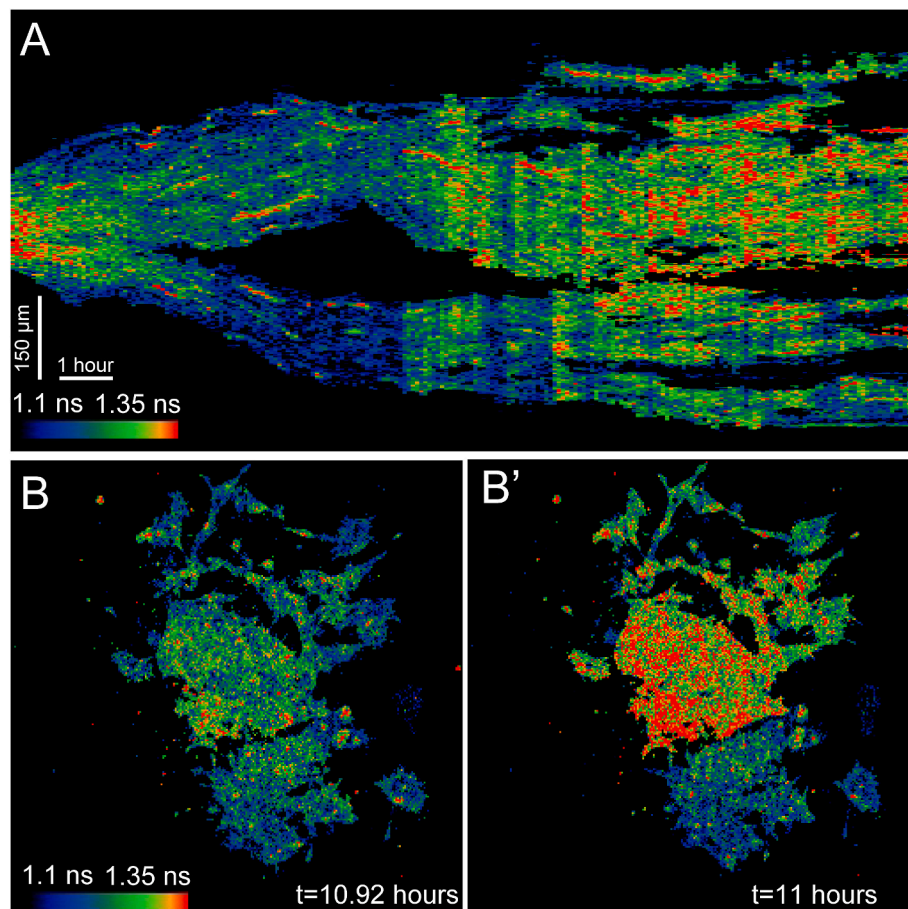


Fig. 5. NC explants exhibit multicellular signals on the scale of minutes

(A) Kymograph of masked lifetime values of a line drawn through NCE during spreading. The explant splits into two fragments that exhibit minutes-scale dynamics that spread within a section but not between fragments.

(B-B') Successive images of NCE shown in A showing apparent depolarization of upper fragment but not lower fragment.

Lifetime images were arbitrarily masked using the intensity channel as described in the methods, and background pixels are represented as having a lifetime of 0.

segmentation it is relatively unbiased, but is more sensitive to artifacts like those generated by cell death and dye internalization.

We first wanted to see whether we could detect effects of changing K^+ levels on BeRST lifetime. We determined the average value of each explant over the timecourse and found that, as expected (Bonzanni et al., 2020), increasing K^+ increased V_{mem}^{oe} (Fig. 4A). Unexpectedly, decreasing K^+ levels did not significantly decrease V_{mem}^{oe} (Fig. 4A). Plotting the mean value at each timepoint suggested that the standard deviation of the 0.5 mM K^+ condition is larger than the other two conditions.

In all three conditions, V_{mem}^{oe} followed a consistent three-phase pattern over time (Fig. 4B and C). When the data from all three conditions were pooled, each phase was statistically distinct from the preceding phase. Initially the explants appeared relatively depolarized. This depolarization generally manifested as a bioelectric gradient with the center of the explant appearing relatively depolarized with increased polarization toward the explant periphery, and was followed by a roughly 6.5-h period of gradual hyperpolarization. (Supplemental Movie 2). This central depolarization tended to flatten during this 6.5-h period as the explant hyperpolarized. The initial hyperpolarization window was followed by gradual depolarization over the next 10 or so hours, with Phase 3 not being significantly different from Phase 1.

Supplementary data related to this article can be found online at <http://doi.org/10.1016/j.ydbio.2026.08.001>

Phase 2 and 3 explants in 8.5 mM K^+ had significantly higher V_{mem}^{oe} than explants in 4.5 mM K^+ , while in Phase 1 none of the conditions

were significantly different (Supplemental Figure 1). This pattern was evident in Fig. 4B, as the gap between the mean of the 8.5 mM K^+ explants and the 4.5 mM K^+ explants started small in Phase 1, expanded in Phase 2 and remained large in Phase 3. These trends were consistent with an intrinsic variation in K^+ permeability as cells progressed through migration.

To better explore the unexpected lack of V_{mem}^{oe} reduction in the 0.5 mM K^+ condition, and informed by the relatively large standard deviation in this condition (Fig. 4B), we examined the long-term dynamics of each explant independently (Fig. 4D–F). These independent observations revealed relatively short, sharp depolarization events that were most prominent in the 0.5 mM condition (Fig. 4D, red asterisk) and seemed most evident during Phase 2. These signals confounded attempts to determine the resting membrane potential of cells in the 0.5 mM K^+ condition, explaining the high standard deviation in this condition and potentially explaining why it did not have significantly lower V_{mem}^{oe} than the 4.5 mM K^+ condition.

Taken together, these data revealed a slow, three-phase progression of V_{mem}^{oe} during NC explant spreading: an early, relatively depolarized phase characterized by a depolarized center that gradually becomes more hyperpolarized toward the periphery (Phase 1), a second more hyperpolarized phase in which this gradient weakens during which cells begin to separate from the explant (Phase 2), and a third phase of increasing depolarization (Phase 3). Together these data suggested that membrane K^+ permeability shifted as NCE migration progressed. Finally, we identified an unexpected increase in fast, short-duration

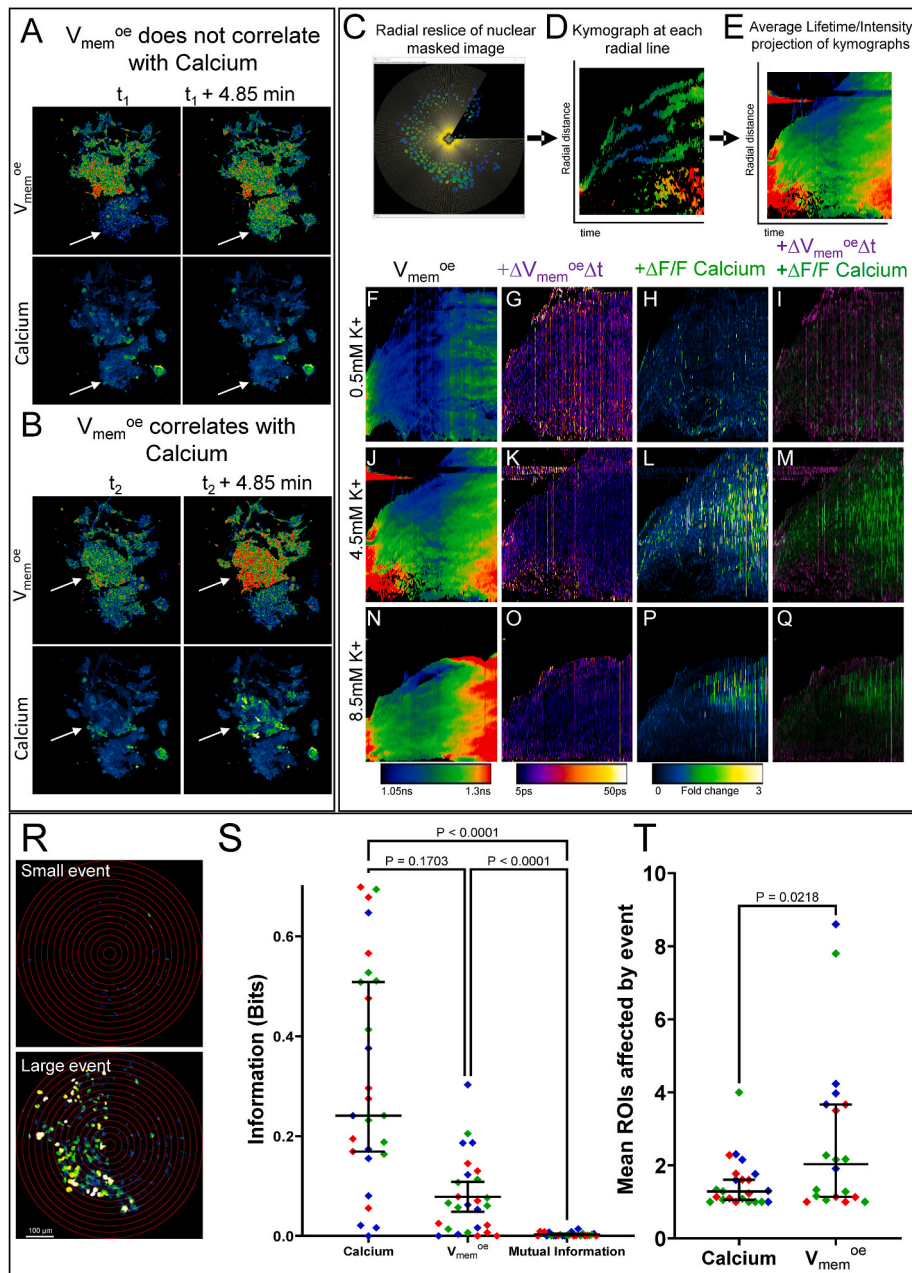


Fig. 6. Bioelectric signals poorly correlate with calcium signals during NCE spreading (A) A depolarization event in the lower portion of the explant (arrow) with no corresponding calcium signal. Fluorescent lifetime heatmap range displayed: 1.1ns to 1.35 ns (B) A depolarization event in the lower portion of the explant (arrow) with a corresponding calcium signal. Fluorescent lifetime heatmap range displayed: 1.1ns to 1.35 ns (C) Screenshot of radial reslicing of a lifetime image after application of a nuclear mask generated from Nuc Blue DNA stain signal. (D) Resultant kymograph of a single radial spoke. (E) Average intensity projection combining lifetime values at each radial spoke to produce a composite representation of bioelectric activity in kymograph space. (F,J,N) Representative kymographs of V_{mem}^{oe} at different K^+ concentrations. (G,K,O) Representative kymographs of the positive derivative of V_{mem}^{oe} at different K^+ concentrations. (H,L,P) Representative kymographs of calcium positive $\Delta F/F$ at different K^+ concentrations. (I,M,Q) Merge of V_{mem}^{oe} and calcium positive $\Delta F/F$ at different K^+ concentrations. (R) Movies are segmented into 15 concentric circles that are then used as ROIs for information (S) and object size analysis (T). Small events intersect a small number of circles producing low mutual information values, while larger events cross many circles to give high mutual information values. Images shown are calcium positive $\Delta F/F$. (S) The information contained in both the V_{mem}^{oe} and Calcium paradigms is greater than the mutual information between them (Friedman test with Dunn's multiple comparison post-test). Line at median, error bars = 95% confidence interval. (T) Bioelectric and calcium events were scored by the mean number of ROIs affected to estimate event size. Events without at least one bioelectric and calcium event were omitted. Significance determined via paired t -test to compare event size within each explant. Line at median, error bars = 95% confidence interval.

Values for S and T were obtained by pooling datasets with spot color signifying K^+ level: 0.5 mM K^+ (blue), 4.5 mM K^+ (green) and 8.5 mM K^+ (red). Lifetime images were arbitrarily masked using the intensity channel as described in the methods, and background pixels are represented as having a lifetime of 0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

depolarization events in the low K^+ condition, demonstrating that even very simple bioelectric interventions may have nonlinear phenotypic consequences.

3.4. NCEs exhibit minutes-scale multi-cell bioelectric dynamics

Closer qualitative analysis of these minutes-scale signals revealed that they often involved a large number of cells, but did not necessarily encompass the entire explant. In Fig. 5A and B, the example low K^+ treated explant has split into two fragments (see Supplemental Movie 2 for full video). In Fig. 5B, the bottom fragment depolarized as a collective while the upper fragment remained relatively constant. Fourteen minutes later, as seen in panel B', the upper fragment collectively depolarized while the lower fragment gradually hyperpolarized. This trend suggested that these two fragments contained separate dynamic bioelectric circuits. We have provided another example with combined V_{mem}^{oe} and intensity to show the diversity of minutes-scale bioelectric signals (Supplemental Movie 3).

Supplementary data related to this article can be found online at <http://doi.org/10.1016/j.ydbio.2026.08.001>

3.5. Minutes-scale bioelectric dynamics correlate poorly with calcium dynamics

In neurons, calcium dynamics are strongly correlated with depolarization (Smetters et al., 1999; Yuste and Katz, 1991). We compared V_{mem}^{oe} and calcium patterns in order to determine if this is true in the NC. Calcium signaling is one of the best-studied dynamic signaling paradigms in non-neural biology (Brodskiy et al., 2019; Brodskiy and Zartman, 2018), and it has regularly been used as a proxy for dynamic neural signals for decades (Alivisatos et al., 2012; Smetters et al., 1999; Yuste and Katz, 1991). It has further been found to be active during NCE migration and patterning (McKinney and Kulesa, 2011; McKinney et al., 2011). Disruption of the Ca^{2+}/H^+ channel AMO1 (Melchionda et al., 2016) and the nonspecific cation channel Piezo1 prevents normal NCE migration *in vivo* (Canales Coutino and Mayor, 2021). Given this correlation between dynamic neural signals and calcium, we hypothesized that the minutes-scale bioelectric signals we describe in the previous section are an undescribed component of the dynamic non-neural calcium paradigm.

We observed bioelectric signals with no correlated calcium signal (Fig. 6A) as well as bioelectric signals with correlated calcium signals (Fig. 6B). To quantify overlap between calcium and minutes-scale bioelectric dynamics we turned to kymography (Fig. 6C–Q). Because hours-scale and minutes-scale bioelectric signals differ by the relative rate of their signal changes, we deconvolved them by creating 'positive derivative' timeseries by subtracting the V_{mem}^{oe} lifetime (or calcium intensity) values at each timepoint from the following timepoint. We generated positive derivative timeseries by using the NucBlue nuclear signal to mask the timeseries and remove all datapoints outside of the nucleus ROI. This thresholding served two purposes: it removed much of the internalized dye that accrues within cells over time as the vesicles housing this dye tend to be occluded by the nucleus, and it removed noise from cell movement which is a substantial confound for this positive derivative approach. We also used nuclear thresholding with the original images. Calcium derivative images were divided by the previous timepoint to account for differential GCAMP8S protein levels to produce positive $\Delta F/F$. We then used the "Radial Reslice" tool in Fiji (Schindelin et al., 2012) to draw 360 kymographs (spaced 1° apart) spanning the center of the image to its periphery (Fig. 6C,D). Stacking these 360 kymographs into an average intensity projection produced a single image that conveyed dynamic information about the bioelectric evolution of the whole explant in space and time (Fig. 6E).

We show representative kymographs from our three conditions in Fig. 6F–Q. The hours-scale trends visualized by the undifferentiated kymographs aligned with the trends we described in our whole-explant

analysis (Fig. 4): Initial depolarization followed by hyperpolarization that eventually trended back to depolarization, a depolarization to hyperpolarization gradient from the center of the explant to the periphery that gradually faded, and a strong hyperpolarization in peripheral cells splitting off from the explant. The effects of external K^+ levels also aligned with the low K^+ condition demonstrating stronger hyperpolarization during Phase 2 than the other K^+ conditions, and the 8.5 mM K^+ condition demonstrating higher depolarization in Phase 3 than the other K^+ conditions.

Examination of the kymographic datasets led us to formulate two hypotheses: (1) that V_{mem}^{oe} and calcium dynamics were weakly coordinated in migrating NCEs, and (2) that V_{mem}^{oe} dynamics tended to be larger in scale than calcium dynamics. To quantitatively evaluate these hypotheses we applied well-established Information Theory formalism using the tool CAIM (Calcium Imaging) (Moore and McMillen, 2019), which has been previously used to quantify information flow in and between the calcium and cytoskeletal pathways in *Xenopus laevis* animal cap cells (McMillen et al., 2022). We segmented each timeseries into a series of concentric circles that were then used as ROIs for CAIM (Fig. 6R).

We pooled V_{mem}^{oe} and calcium data from the 0.5 mM K^+ , 4.5 mM K^+ and 8.5 mM K^+ conditions in an attempt to estimate the informational properties of these pathways throughout the spectrum of K^+ levels they may encounter during development and improve our statistical power. We determined an average information value for each explant. In this pooled data we detected an average of 0.318 bits of information in the V_{mem}^{oe} pathway and 0.062 bits in the calcium pathway (Fig. 6S), though these values are not significantly different.

To test our hypothesis that V_{mem}^{oe} and calcium are weakly correlated in migrating NCEs, we measured the mutual information between the two variables within each ROI and compared it to the amount of information measured in each pathway. Consistent with our hypothesis, mutual information between calcium and V_{mem}^{oe} was significantly lower than the mutual information measured within either variable alone (Fig. 6S), 3.7% of the information measured in V_{mem}^{oe} . These data suggest that the two pathways are largely, though not entirely, independent in migrating NCEs.

We observed that V_{mem}^{oe} events tended to incorporate more cells than calcium signaling events. To test this, we used the binarized ROI data generated for the CAIM analysis above and measured the average number of ROIs included in events of each signaling paradigm. Explants not exhibiting both calcium and bioelectric events were excluded from this analysis. The number of events was not considered, only the average size of the detected events. Each datapoint represents the average event size for the indicated paradigm for one explant, and the K^+ condition to which the explant belongs is indicated by the datapoint color (Fig. 6T).

From the image metadata we estimate that one line takes roughly 63.5 ms to collect (~ 32.511 s per image/512 lines per image). The published half-rise time in dissociated neuronal culture for jGCaMP8s is 10.2 ± 0.9 ms (Zhang et al., 2023), and the published half-decay time is 330.7 ± 32.0 ms (Zhang et al., 2023). Assuming the BeRST response is instantaneous, the lag between BeRST activation and jGCaMP8s activation should be well below the time between scanlines. Thus, if anything, the increased decay time of jGCaMP8s should inflate the mutual information between bioelectric and calcium dynamics, but does not explain the low mutual information that we detect.

3.6. Rare seconds-scale bioelectric patterns

The expanded spatial and temporal range of bioelectric imaging (Fig. 1) allows us to capture relatively rare events that would be extremely challenging to catch with classic needle-based electrophysiology. The relative rarity of spontaneous occurrence of these events has prevented us from drawing quantitative conclusions about them at this time, but should provide a foundation for future study while underscoring the fascinating complexity of the bioelectric world.

3.6.1. Scan artifacts reveal dynamic fast bioelectric signal kinetics

In explants raised for roughly 22 h at 18 °C, switching from 4.5 mM K^+ to 0.5 mM K^+ appears to stimulate bioelectric signals similar to those seen in Phase 2 of explants imaged over time at 20 °C. We leveraged this observation to survey for fast bioelectric events at increased magnification (Supplemental Movie 3). Under these conditions, we occasionally observed straight lines of depolarization perfectly parallel to the y-imaging axis. Because we are performing line-scanning imaging, we interpret these lines as seconds-scale bioelectric events that manifest between successive scan lines. Assuming that these bioelectric events are consistent across the cell, we can use the line-scan rate across an ROI parallel to the x-axis to estimate the kinetics of the bioelectric signal. We find that these signals can be repetitive and have relatively fast on-kinetics and slow off-kinetics (Fig. 7A). The frequency of these signals varies from cell-to-cell, suggesting a diversity in the underlying ion channel mechanism, though they tend to persist for on the order of seconds. Notably, the cell in Fig. 7A' was in direct contact with several neurite-like projections, presenting the possibility that neuronal activity may play a role in producing or modulating some of these seconds-scale bioelectric signals.

Strikingly, we found that these seconds-scale bioelectric events can span long ranges in cell collectives, and even propagate to cells outside of the core collective that are presumably linked only by tunneling nanotubes (Fig. 7A''). By assuming uniform activation across the collective in this case, we can estimate the collective seconds-scale bioelectric signal kinetics and find a third bioelectric pattern distinct from the ones in A' and A''. The diversity in on- and off-kinetics of these signals provides a tantalizing glimpse into the temporal encoding of non-neural bioelectric events, though the relative rarity of these events precludes deeper interpretation here.

3.6.2. Cells and cell collectives depolarize upon re-encountering their original collective

Supplemental Movie 4 reveals a fascinating phenomenon: it shows a cell hyperpolarize, break off from the collective, and migrate independently. It later contacts another location in the explant, depolarizes (Fig. 7B), and integrates into this new location. Contact appears to be mediated by tunneling nanotubes (McKinney et al., 2011; McMillen et al., 2021; Osswald et al., 2015, 2016; Teddy and Kulesa, 2004; Wang and Gerdes, 2012; Wang et al., 2010; Weil et al., 2017). We have also observed this phenomenon with cell collectives, which depolarize in unison when the most proximal member of the group re-contacts the greater collective (Supplemental Movies 5 and 6). This immediate, long-distance response points to a role for contact-mediated bioelectric signaling in balancing the rate and degree of spreading during NCE migration, though further study will be needed to evaluate its functionality.

Supplementary data related to this article can be found online at <http://doi.org/10.1016/j.ydbio.2026.08.001>

3.6.3. Bioelectric activity of NCE projections

Because it stains the cell membrane, BeRST effectively highlights tunneling nanotubes and other cellular projections that have a high ratio of cell membrane to cytoplasm. Such projections have been shown to mediate long-distance communication (McMillen et al., 2021), including bioelectric communication via gap junctions (Wang and Gerdes, 2012; Wang et al., 2010). In rare cases like that shown in Fig. 7C, we observed that depolarization of the cell body extended to its tunneling nanotubes. Notably, the tunneling nanotubes of multiple cells depolarized in unison, suggesting that the bioelectric reach of the cell collective is much greater than the space occupied by the cell bodies might suggest. This is consistent with our finding of relatively high

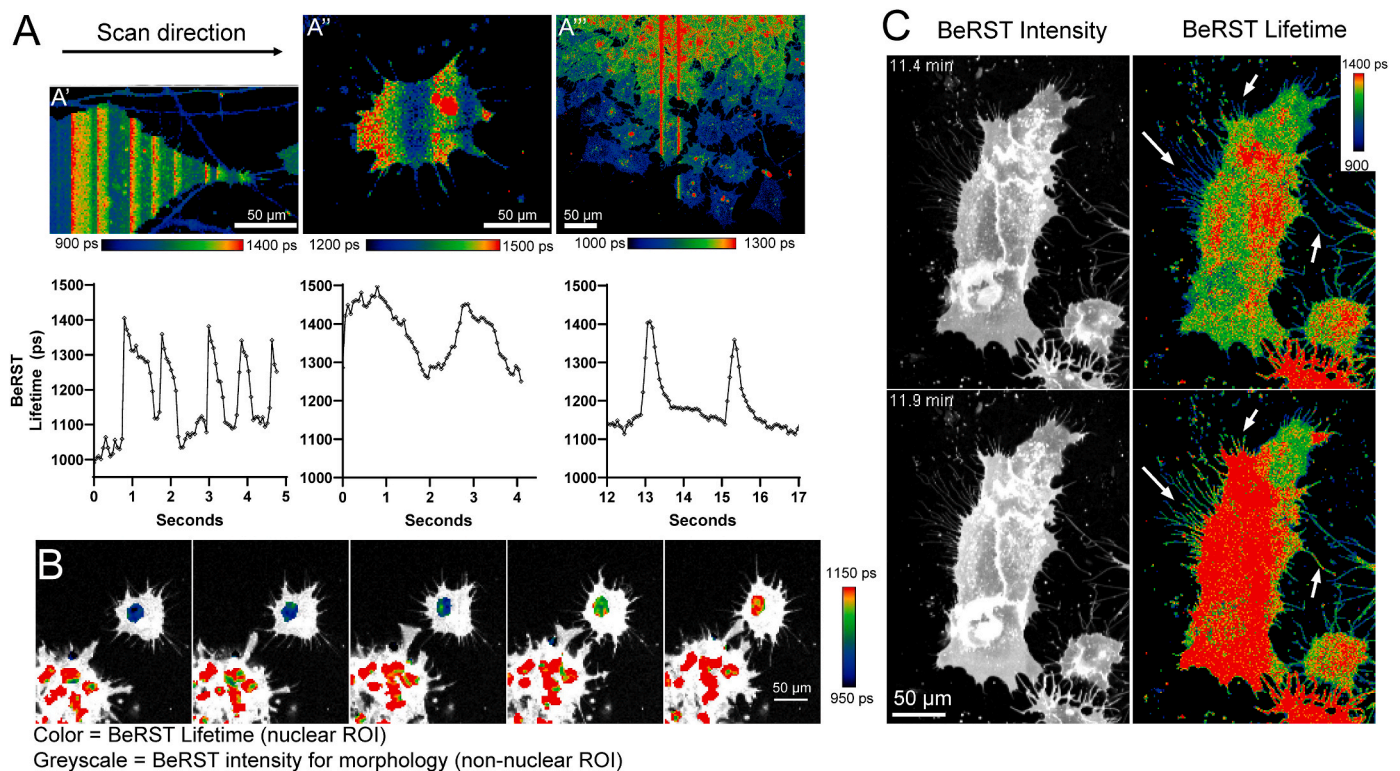


Fig. 7. Seconds-scale bioelectric patterns (A) Measurement of vertical scan-line artifacts facilitates estimation of the kinetics of bioelectric events. (A'-A'') depict different cells. (B) NucBlue signal was used to separate the nucleus ROI from the cell body. BeRST FLIM is shown in the nucleus ROI while BeRST intensity is shown in the cell body to show V_{mem}^{oe} and cell shape clearly at the same time. Depolarization of isolated cell upon re-contacting the collective. (C) Cellular projections appear to depolarize in concert with cell bodies.

Lifetime images were arbitrarily masked using the intensity channel as described in the methods, and background pixels are represented as having a lifetime of 0.

mutual information between cells in the $V_{\text{mem}}^{\text{oe}}$ pathway compared with the calcium pathway. More generally, the small size of tunneling nanotubes makes them exceedingly difficult to clamp with existing technology, and this finding demonstrates that bioelectric imaging may be a powerful tool for better understanding their dynamics.

4. Discussion

Developmental bioelectricity studies have long been hindered by a lack of tools to robustly observe bioelectric patterns at the large time and space scales over which development takes place. Moreover, significant questions exist about the relationship between the dynamics of neural and non-neural cells. We analyzed NCEs to reveal both similarities and differences between neural signaling and non-neural bioelectric signals. We demonstrate here that FLIM is an exciting possible solution to this challenge by imaging migration of NCEs over long distances for >17 h. Using FLIM we observe long-range dynamic bioelectric signals that would be elusive to smaller-scale approaches. We describe long-period bioelectric patterns that manifest over the course of hours and much faster bioelectric signals that manifest on the order of minutes to seconds. We then apply information theory to demonstrate that, unlike action potentials (Smetters et al., 1999; Yuste and Katz, 1991), these minutes-scale bioelectric signals are mostly distinct from calcium dynamics. We also describe some key pitfalls in bioelectric imaging and discuss how FLIM can be used to circumvent them.

4.1. The value of FLIM in revealing bioelectric patterns

A generally applicable finding is the danger of using intensity-only measurements to detect bioelectric patterns. While intensity and lifetime measurements generally agree in excitatory heart tissue, intensity imaging shows “false positive” patterns of non-physiological magnitude in NCEs. We thus strongly encourage researchers attempting intensity-only imaging with VF dyes to approximate the magnitude of the bioelectric changes that they observe, in order to determine their physiological feasibility.

4.2. Hours-scale bioelectric patterns

Migrating explanted cells follow a slow, three-phase bioelectric trajectory that manifests over a series of hours (Fig. 4, Supplemental Movie 2). In Phase 1, a strong gradient forms, with more depolarized cells on the inside and more hyperpolarized cells on the outside. In Phase 2, this gradient weakens as the whole explant hyperpolarizes and begins to break apart. Finally, in Phase 3, the explants again depolarize. Future work will be needed to determine the physiological function and *in vivo* relevance of these hours-scale signals, though recent work has identified other slow functional bioelectric patterns in chicken feather buds (Jiang et al., 2021) and frog neural crest cells (Ferreira et al., 2025) that may represent similar phenomena.

How do bioelectric interventions affect bioelectric signals? One of the biggest challenges in the field of bioelectricity is designing bioelectric interventions that reliably produce desired phenotypic outcomes (Pai et al., 2018; Pietak and Levin, 2017). Doing so is necessary for therapeutic intervention in regeneration and cancer treatment (Levin, 2024; Whited and Levin, 2019). Here, we took steps to bridge this gap by measuring the effects of a common bioelectric intervention, variation of extracellular K^+ levels, over an extended period of time. As predicted from previous work (Bonzanni et al., 2020), elevating extracellular K^+ appears to depolarize cells. Reducing K^+ does not significantly hyperpolarize cells, however. We further found that reduction of extracellular K^+ led to an unanticipated increase in minutes-scale bioelectric signals, demonstrating that even simple bioelectric interventions can have major nonlinear effects.

4.3. Minutes-scale and seconds-scale bioelectric dynamics

What role could minutes-scale and seconds-scale bioelectric signals play? There is a growing body of evidence that temporally encoded signaling dynamics are an underappreciated element of signal transduction, with pulsed and static inputs of the same growth factor having different effects (Goglia et al., 2020; Johnson and Toettcher, 2019; Purvis and Lahav, 2013; Wilson et al., 2017). Given that neural bioelectric signals are fundamentally temporally encoded, it follows that their more primitive non-neural counterparts would also have temporally encoded properties. The variable off- and on-kinetics we report in Fig. 7A provide evidence for the tantalizing possibility that there may exist a temporally encoded vocabulary of non-neural bioelectric signals. The rarity of these events concurrently limits our ability to study them. Future characterization work will address our key knowledge gap of designing bioelectric interventions to achieve specific outcomes.

There is also some evidence of this phenomenon in bioelectric signaling. Elegant work employing fluorescence-fluctuation correlation analysis of the genetically encoded voltage-indicator rEStus has shown that V_{mem} fluctuations can correlate between cells in immortalized A375, HEK293T and MCF cell lines, and the authors report reduced V_{mem} variation in confluent clusters (Rühl et al., 2024). The events that we describe are more discrete than the fluctuations described by Rühl et al. (2024), and while we see spatial variation increase in isolated cells, cells within collectives reliably produce robust minutes-scale signals. Further work will be needed to determine if the minutes-scale bioelectric signals that we report are alternative manifestations of the same phenomenon described by Rühl et al. or if they are separate pathways.

How do minutes-scale $V_{\text{mem}}^{\text{oe}}$ and calcium signaling compare in NCEs? Calcium and $V_{\text{mem}}^{\text{oe}}$ are largely distinct informational channels in NCEs, in a substantial departure from the neural paradigm. This disparity highlights the danger of using calcium as a proxy for V_{mem} in non-neural cells (Follmer et al., 2024) and underscores the importance of calcium-independent effectors of bioelectric signals. $V_{\text{mem}}^{\text{oe}}$ signals exhibit mutual information at greater distances than calcium signals. These findings add to a growing body of evidence that while definitely informative, calcium reporters may not entirely capture the entire diversity of bioelectric signals. Studies combining electrophysiology with V_{mem} and calcium reporters in neurons have repeatedly shown that V_{mem} reporter dynamics largely reflect subthreshold dynamics while calcium reporters report action potentials (Berger et al., 2007). In the brains of mice expressing genetically encoded calcium indicators the majority of action potentials are not detected under standard population imaging conditions, though detection becomes more faithful with optimized image acquisition (Huang et al., 2021). In cultured neurons, calcium imaging was found to preferentially miss action potentials without broad depolarization plateaus (Milicevic et al., 2024). Outside of neurons, preprinted work from Rühl et al. combining V_{mem} and calcium imaging in HEK293T cells shows that V_{mem} signals appear to travel long distances while calcium signals are much more restricted, similar to the pattern we describe in this paper (Rühl et al., 2026). Together, these data suggest a complex interplay between V_{mem} and calcium dynamics that will require extensive combined voltage image, calcium imaging and electrophysiology to fully unravel in various cell types and contexts that might be using diverse transduction machinery, in addition to calcium channels, to convert voltage change into downstream cellular responses.

This pattern aligns interestingly with the specialized function of bioelectricity in neurons, in which $V_{\text{mem}}^{\text{oe}}$ allows for fast long-distance communication while calcium mediates the relatively local process of vesicle release. Could the long-distance nature of the bioelectric signals that we see point to an intermediary evolutionary mechanism that was supplanted by axons in the brain? Better understanding the mechanistic origins of NCE signals may provide insights into the early evolution of neural bioelectricity.

What is the molecular basis of minutes- and seconds-scale bioelectric signals? Hyperpolarization-activated cyclic nucleotide-gated channels (HCN) increase membrane cation conductivity in response to hyperpolarization and have been shown to mediate long-range repair of chemical and genetic perturbation of *Xenopus* development (Pai et al., 2020; Pai and Levin, 2022; Pitcairn et al., 2017). These channels are known to function in stem cells (Bruzauskaitė et al., 2016; Johard et al., 2020; Lau et al., 2011), excitatory cells including neurons (Benarroch, 2013) and cardiomyocytes (Depuydt et al., 2022; Stevens and Pu, 2013), making them reasonable candidates for effectors of the minutes-scale bioelectric signals we describe here. However, HCN channels are calcium-permeable, and minutes-scale bioelectric signals only loosely correlate with calcium dynamics. Likewise, while the mechanosensitive nonspecific cation channel Piezo1 is necessary for proper NCC migration (Canales Coutiño and Mayor, 2021; Ferreira et al., 2025), it too allows calcium influx and thus it is an unlikely effector for minutes-scale bioelectric signals. Our data therefore point to alternative hyperpolarization-activated ion channels that can coordinate cell physiology over large distances making these appealing targets for future studies of repair and regeneration.

4.4. Limitations of this study

Due to the necessity of long-term imaging for our approach, we were limited in our ability to detect contamination from NCC-proximal tissues or differentiation of multipotent NCCs into any of the many types of cells to which they give rise. Additionally, while VF2.0. Cl is a powerful negative control for BeRST, its effectiveness is limited by the fact that the two dyes have different base fluorophores. VF2.0. Cl and BeRST may have different cellular uptake (potentially varying between cell types), penetration and internalization properties. Also, VF2.0. Cl is excited by relatively toxic blue light and bleaches rapidly, precluding its use as a control for long-term timeseries. These factors make it challenging to control for dye-specific artifacts. The recently produced VF dye, CRhOm (Gest et al., 2024), has been optimized for FLIM and has a V_{mem} -insensitive variant, making it a promising solution to these limitations. Furthermore, the genetically encoded voltage indicator rEstus (Rühl et al., 2024) has recently been validated to work with FLIM (Nair et al., 2025) and will act as a powerful semi-orthogonal approach for identifying dye-specific artifacts. Future work integrating dye-based, genetically encoded voltage indicator-based, and patch-clamp electrophysiology-based approaches, in the same cells, will ultimately be necessary for a robust and comprehensive understanding of the bioelectric code.

4.5. Future directions

Our work reveals a striking variety in bioelectric signals during NCE migration and provides a foundation for future studies to deconvolve the phenomenological and functional nature of contributions of inter-tissue interactions, cell differentiation and crosstalk between different NCE derivatives to the NCE bioelectric code. Immediate applications using bioelectric signals to predict and target NCEs in a way that alters its behavior or collectivity would impact many developmental (Boland, 1997), oncological (Gallik et al., 2017; Hu and Sauka-Spengler, 2022), and evolutionary (Jheon and Schneider, 2009) endpoints. More broadly, using NCEs to better understand the roles of ionic signaling for orchestrating cell behavior extends across evolutionary developmental biology (Keijzer and Arnellos, 2017; Keijzer et al., 2013), the biomedicine of birth defects and injury (Adams et al., 2016; Pai et al., 2020; Pai and Levin, 2022), and the use of diverse cell types in programmable biobotics (Ebrahimkhani and Levin, 2021).

CRedit authorship contribution statement

P. McMillen: Conceptualization, Data curation, Formal analysis,

Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **M. Levin:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of interest statement

The Levin lab receives sponsored research funding from two companies working to implement bioelectric-based interventions as biomedical applications. Co-author M.L. consults on the topic of developmental bioelectricity. The Levin lab also has patent applications on this topic.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2026.08.001>.

Data availability

Data will be made available on request.

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