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Generation and customization of biosynthetic excitable tissues for electrophysiological studies and cell-based therapies

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Abstract

We describe a two-stage protocol to generate electrically excitable and actively conducting cell networks with stable and customizable electrophysiological phenotypes. Using this method, we have engineered monoclally derived excitable tissues as a robust and reproducible platform to investigate how specific ion channels and mutations affect action potential (AP) shape and conduction. In the first stage of the protocol, we combine computational modeling, site-directed mutagenesis, and electrophysiological techniques to derive optimal sets of mammalian and/or prokaryotic ion channels that produce specific AP shape and conduction characteristics. In the second stage of the protocol, selected ion channels are stably expressed in unexcitable human cells by means of viral or nonviral delivery, followed by flow cytometry or antibiotic selection to purify the desired phenotype. This protocol can be used with traditional heterologous expression systems or primary excitable cells, and application of this method to primary fibroblasts may enable an alternative approach to cardiac cell therapy. Compared with existing methods, this protocol generates a well-defined, relatively homogeneous electrophysiological phenotype of excitable cells that facilitates experimental and computational studies of AP conduction and can decrease arrhythmogenic risk upon cell transplantation. Although basic cell culture and molecular biology techniques are sufficient to generate excitable tissues using the described protocol, experience with patch-clamp techniques is required to characterize and optimize derived cell populations.

INTRODUCTION

Cardiac arrhythmias are typically caused by inherited ion channel mutations (channelopathies) or acquired myocardial injuries (e.g., infarction) that result in substantial loss of cardiomyocytes and cause the formation of fibrous scar. Cardiac channelopathies and other excitable-tissue disorders have been traditionally studied *in vitro* using heterologous expression systems and, recently, human stem cell-derived cardiomyocytes^{1–3}. Stem cell-derived cardiomyocytes have been tested as a potential therapy for postmyocardial infarction

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS H.X.N., R.D.K., and N.B. jointly developed the protocol; H.X.N. and R.D.K. performed the experiments and generated the figures; and H.X.N. and N.B. co-wrote the manuscript.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

disease; however, in their current state, implantation into the heart may involve high arrhythmogenic risks^{4,5}.

In addition to stem cells, genetic engineering of unexcitable, terminally differentiated cells to attain a custom electrical phenotype, may offer an experimental platform to study the mechanisms of abnormal AP conduction and lead to the development of new therapies for excitable-tissue disorders. In particular, genetically engineered fibroblasts expressing different potassium channels (K_v1.3 and Kir2.1) injected into the right ventricle *in vivo* were shown to prolong the effective refractory period of cardio-myocytes and prevent induction of ventricular arrhythmias in a porcine model⁶. In addition, overexpression of the gap-junctional protein connexin-43 (Cx43) in engineered cells has been used to passively bridge short (<1 mm) conduction gaps between cardiomyocytes *in vitro*^{7,8} and enhance coupling between implanted cells and host myocytes *in vivo*^{9,10}. Bridging of larger conduction gaps, however, would require engineering of electrically excitable cells, which can support active AP propagation.

Development of the protocol

Although the shape of the cardiac AP is governed by the activity of various membrane channels, pumps, exchangers, and intracellular Ca²⁺-dependent mechanisms^{11–14}, we reasoned that engineered excitable cells must conceptually correspond to simplified computational models of cardiomyocytes (e.g., Noble¹⁵, Fenton-Karma^{16,17}), in which only a few currents are used to approximate complex cardiac membrane dynamics¹⁸. To illustrate this concept, in our recent studies^{19–22}, we used nonviral expression and selection to introduce critical components of AP generation and propagation into unexcitable human embryonic kidney 293 (HEK-293) cells by stable coexpression of only three membrane channels (Kir2.1, Na_v1.5, and Cx43). These channels were selected based on their critical roles in setting and maintaining the hyperpolarized cardiomyocyte resting membrane potential (Kir2.1), initiating the fast AP upstroke (Na_v1.5) and propagating excitation in the heart (Cx43)¹². We showed, in a stepwise manner, how the addition of each channel contributes to the development of electrical excitability and connectivity in these biosynthetic, monoclonally derived cells, named ‘Ex-293’. We have since demonstrated the utility of the Ex-293 line for cell- and tissue-level studies of basic electrophysiology^{19,21}, cardiac channelopathies²⁰, and pharmacology¹⁹, as well as for development of new computational models²². Furthermore, in our proof-of-concept studies, we showed that Ex-293 cells are capable of bridging cm-sized noncardiac gaps^{19,20} to substantially improve not only electrical but also mechanical function of cardiac tissues *in vitro*²⁰.

These promising results in Ex-293 cells prompted us to transfer this concept to more clinically relevant primary human cells for potential cardiac cell therapies. As nonviral gene delivery is mostly transient and largely inefficient in primary cells, the use of lentiviruses or adeno-associated viruses (AAVs) is required for stable overexpression of selected ion channel genes to confer electrical excitability in these cells. However, because the *SCN5A* gene encoding the mammalian Na_v1.5 channel is too large (~6.1 kb) to package into a high-titer lentivirus or AAV, this precludes its stable and efficient expression in primary cells. To circumvent this limitation, we explored the superfamily of prokaryotic sodium channels

(BacNa_v)^{23–25} as potential substitutes for the Na_v1.5 channel in biosynthetic excitable cells. BacNa_v channels are encoded by a smaller gene (<1 kb), making them ideal for efficient packaging into viral vectors for stable primary cell expression. We demonstrated that lentiviral co-delivery of the selected BacNa_v orthologs, Kir2.1, and Cx43, in primary fibroblasts successfully made these cells electrically excitable and actively conducting²⁶. Such engineered excitable fibroblasts (named ‘E-Fibs’) were shown to retain stable electrical properties upon extensive subculturing or conversion to a myofibroblast phenotype and were able to rescue cardiac conduction in an *in vitro* model of interstitial fibrosis²⁶, supporting the potential development of therapeutic applications.

We have also developed new computational models of biosynthetic cells^{22,26} that, in combination with mutagenesis and electrophysiological studies, enable more efficient electrical customization of the engineered cells for target applications.

Comparison with other methods

Heterologous expression systems have been traditionally used in conjunction with patch-clamp techniques to study the structure-function relationships of various ion channels and to elucidate the underlying mechanisms between specific channel mutations and associated channelopathies²⁷. However, results from these studies and observed tissue- and organ-level function, such as propagation of APs, are often linked only indirectly using tissue-specific computational models^{28–30}. AP conduction can also be studied in primary excitable cells and tissues; however, these studies are often limited by low throughput and reproducibility, heterogeneity or immaturity of cells, and the presence of diverse and often poorly characterized channels nonspecifically affected by drugs. Complementary to these traditional systems, expandable monoclonally derived biosynthetic cells and tissues, such as Ex-293, provide a well-defined and reproducible *in vitro* platform to dissect how specific ion channels and their mutations influence AP shape and conduction in a near-isogenic setting. However, use of such cell lines does not allow interrogation of how individual genetic variability may contribute to the development of disease from a given channel mutation. Furthermore, unlike native car-diomyocytes, in engineered cell lines derived from unexcitable cells, all dominant ionic currents are exogenously introduced and can thus be directly measured and used to construct realistic computational models with a quantitative correspondence to experimental studies.

Importantly, applications of engineered prokaryotic channels in primary fibroblasts, including transplantations of car-diomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs), could also provide a viable alternative to current cell-based therapies for cardiac diseases. Although they hold great promise for functional cardiac repair, use of hiPSC-CMs has several limitations, including a long and costly derivation procedure, limited proliferation, and an immature and heterogeneous electrophysiological phenotype leading to a high incidence of cardiac arrhythmias when these cells were transplanted into nonhuman primates^{4,5}. By contrast, AP-conducting fibroblasts can be produced and expanded in large quantities within a relatively short time and were shown to exhibit a stable, customizable, and relatively homogeneous electrical phenotype²⁶, which could potentially reduce arrhythmogenic risks upon transplantation.

Applications of the protocol

This protocol has been successfully used by our group and others to generate biosynthetic excitable cells and tissues starting from HEK-293 cells or primary fibroblasts from different organs and species^{19–21,26,31–34}. We have described the utility of such engineered cells for simultaneous single-cell- and tissue-level studies of basic ion channel physiology to examine the effects of channel antagonists, mutations, and electrical coupling on AP propagation^{19–21}. Furthermore, the simplified ion current makeup and well-characterized electrical properties of Ex-293 cells were used for the validation and tuning of new computational models of AP propagation²².

Using similar methods, Cohen and colleagues have generated a line of spontaneously spiking HEK-293 cells by stably expressing Kir2.1 and Na_v1.3 to enable high-throughput screening of genetically encoded voltage sensors³². Furthermore, combining components required for excitability (Na_v1.5 and Kir2.1) with a blue-light-excited ion channel and a red-light-activated voltage sensor allowed the generation of a stable excitable HEK-293 line with all-optical control and readout of AP propagation³³. This system allowed high-throughput pharmacological screening of ion channel agonists and antagonists, as illustrated in a recent pilot study involving the screening of Na_v1.7 inhibitors³⁴.

We have also shown that stable expression of functional T-type voltage-gated calcium channels (Ca_v3.3) could be used to generate Ca²⁺ transients and further tune the electrical properties of engineered excitable cells²⁰. This system could be used for high-throughput screening of genetically encoded calcium indicators. Importantly, the presence of calcium channels could be exploited to couple electrical excitability to a variety of intracellular signaling pathways to influence different cellular behaviors. This concept was elegantly demonstrated in a recent study by Xie *et al*, in which expression of the Ca_v1.3 channel in HEK-293 cells served to couple membrane depolarization to insulin expression³⁵. By further adding a glucose transporter and the K_{ATP} channel, the authors engineered β-cell-mimetic HEK-293 cells capable of correcting insulin deficiency in diabetic mice upon implantation³⁵.

Finally, we and others have also performed proof-of-concept *in vitro* studies demonstrating the application of this method to rescue and control electrical properties of primary unexcitable and excitable cells in simulated pathological conditions^{26,31}, thus creating the foundation for the development of novel cardiac cell- and gene-based therapies.

Limitations of the protocol

Despite several advantages, engineered excitable cell lines produced by this protocol lack features present in native excitable cells that may limit their use in certain *in vitro* applications. For example, HEK-293 cells lack auxiliary channel subunits³⁶ and certain interacting proteins that modulate channel biophysical properties, gene expression, and trafficking in cardiomyocytes³⁷, which may render the engineered Ex-293 line unsuitable as a model to investigate pathological and pharmacological responses involving these regulatory molecules. However, coexpression of SCN5A with relevant regulatory genes could overcome some of these limitations. Similarly, due to their homotetrameric structure,

BacNa_v channels lack the interdomain intracellular loops (e.g., DIII-DIV loop) that are essential for the function of eukaryotic Na_v channels (e.g., fast inactivation), thus limiting their activation frequency and pharmacological responses²³. BacNa_v also lacks essential transport motifs present in eukaryotic channels (e.g., Golgi export and endoplasmic reticulum export motifs), which may slow their trafficking in human cells. Incorporating these motifs into the channel could enhance its targeting to the mammalian cell membrane, as previously shown for microbial light-activated ion channels³⁸.

For potential cardiac cell therapy applications, one limitation of engineered excitable fibroblasts is the lack of active contractile properties. However, in co-cultures with actively conducting cardiomyocytes, fibroblasts improved both cardiac electrical conduction and contractile function by resynchronizing the activity of surrounding myocytes^{20,26}. Transplantation of engineered cells expressing proteins from microorganisms may also raise immunogenicity concerns³⁹, despite reports previously showing the absence of immune response upon *in vivo* delivery of microbial ion channels in rodents⁴⁰ and nonhuman primates⁴¹; therefore, animal tests must be conducted to examine the extent of immune and inflammatory responses during long-term expression of BacNa_v channels. To date, retroviral injection of a BacNa_v isoform (NaChBac) into the brains of immunocompetent mice has yielded continued expression of functional channels for at least 36 d⁴².

Experimental design of the protocol

The overall workflow of the protocol is described in **Figure 1**. We also provide detailed discussion of important aspects of the protocol below.

Cell sources.—The choice of unexcitable cells is dependent on their intended use. These cells are expected to have relatively weak endogenous ion channel expression and must be highly expandable *in vitro*. For nonclinical and nonanimal applications, immortalized cell lines are preferred due to their theoretically unlimited proliferative capacity and ability to generate clones with highly uniform properties. In particular, the widely studied HEK-293 immortalized cell line^{43,44} represents a generic model of electrically unexcitable cells with a small number of well-defined, low-amplitude endogenous currents^{45–8}. Their uniform morphology, simple adherent culturing conditions, suitability for patch clamping and genetic manipulation, and monoclonal selection capability make these cells ideal for engineering into biosynthetic excitable cell lines⁴⁹. Other sources of immortalized unexcitable cells commonly used in basic electrophysiology studies include CHO-K1 (ref. 50) and HeLa⁵¹ cells. *In vivo*-oriented applications, however, require the use of primary cells from an autologous or allogeneic source. Specifically, human dermal fibroblasts (HDFs) represent a good source of unexcitable primary cells because they have low expression levels of endogenous ion channels⁵², are readily obtainable from commercial sources or patients biopsies, are easy to culture and have a fast growth rate and stable phenotype, are highly expandable *in vitro* to therapeutically relevant quantities, and are amenable to exogenous gene expression via viral delivery^{26,53,54}. We suggest that users start with primary cells of the earliest available passage to maximize the final numbers of engineered excitable cells.

Channel selection and mutagenesis.—Unexcitable cells can be converted into electrically excitable and actively conducting cells via stable expression of a minimum set of two or three channel genes encoding a potassium channel to hyperpolarize membrane potential, a sodium channel to induce fast membrane depolarization upon application of an external stimulus, and a gap-junction channel to enhance intercellular electrical coupling. In Ex-293 cells, active AP conduction is achieved via expression of Kir2.1, Na_v1.5, and Cx43, respectively. In E-Fibs, BacNa_v channels are used in place of the mammalian Na_v1.5 channel.

It is worth noting, however, that the presence of all three channel components does not guarantee successful AP generation or conduction in engineered cells. The biophysical properties of the selected sodium channel—in particular, the kinetics and voltage dependency of activation and inactivation—must meet certain requirements. This is especially important for the selection of BacNa_v channels, as their properties have been adapted to serve other functional roles in bacteria rather than AP generation⁵⁵. When transfected in HEK-293 monolayers expressing Kir2.1 and Cx43, only two of seven BacNa_v orthologs successfully induced AP propagation (**Fig. 2**). Our patch-clamp recordings showed that failure to elicit APs in five tested orthologs was due to either highly hyperpolarized inactivation (Na_vBacL, Na_vMs, Na_vAb, and Na_vSheP) or highly depolarized activation (Na_vSilP). In such cases, modeling-guided mutagenesis could be performed to shift the activation and inactivation curves of the respective ortholog to more desired ranges, as we demonstrated for Na_vSheP²⁶. Computational modeling is also useful in providing initial predictions of the ranges of electrical properties (e.g., maximum upstroke velocity, AP duration (APD), and conduction velocity (CV)) produced with specific channel isoforms, given the typical range of channel conductance that can be achieved experimentally.

Based on these predictions, flow cytometry or monoclonal selection can be applied to derive a subset of engineered cells with a specific phenotype. If computational modeling predicts that a specific channel isoform will not yield the desired AP or conduction parameters, the user can consider other channel isoforms or the use of mutagenesis to adjust the channel gating kinetics. Generally, if the goal is to improve maximum upstroke velocity and CV, the use of Na_v channel isoforms or mutants with faster activation kinetics would be beneficial. If a longer APD is desired, the user can consider Na_v channels with slower inactivation kinetics; conversely, a shorter APD can be achieved using Na_v channels with faster inactivation or by introducing controlled expression of rectifier potassium channels such as hERG or K_v1.5 to engineer rapid (I_{Kr})⁵⁶ (**Fig. 3**) or ultrarapid (I_{Kur}) K⁺ current^{57,58}, yielding faster repolarization and a shorter APD. Regarding the small size of BacNa_v, there is also a possibility of co-expressing the isoforms with different kinetics to further adjust AP and conduction properties.

Delivery of genetic constructs and selection of engineered cells.—In this protocol, we provide two alternative routes for the delivery of channel genes into unexcitable cells and for the selection of engineered cells stably expressing these genes (Step 24). Step 24A can be used to derive monoclonal immortalized cell lines (such as Ex-293) and Step 24B should be followed to generate excitable primary cells (such as E-Fibs).

In Step 24A, we use nonviral delivery methods to transiently transfect cells with each channel gene in a stepwise manner. Each gene is subcloned into a mammalian expression vector of choice that also drives the expression of a unique fluorescent reporter gene. For the generation of the Ex-293 line, the bicistronic expression vector pCMV5(CuO)-IRES-GFP (qBiogene) was modified to create three genetic constructs: Na_v1.5-IRES-GFP, Kir2.1-IRES-mCherry, and Cx43-IRES-mOrange (**Fig.4a**). The internal ribosomal entry sequence (IRES) is located between the channel gene and the fluorescent reporter gene to provide a secondary translation site for the latter. In addition, it is strongly recommended that each genetic construct also includes a unique antibiotic resistance gene that will allow enrichment of positively and stably transfected cells before fluorescence-based selection with flow cytometry. Single cells are then isolated from each polyclonal selected line to create monoclonal lines exhibiting uniform expression of the transfected channel gene. This process is repeated for the remaining genetic constructs, resulting in the final cell line, which is monoclonal for all three channels. Recently, there have been several notable improvements in non-viral delivery systems that could further facilitate the derivation of monoclonal cell lines. Of note, the Sleeping Beauty transposon system^{59,60} provides a fast and efficient method to generate stably transfected cells, with a wide range of commercially available vector constructs containing different combinations of antibiotic-selection markers and fluorescent reporters⁶¹, enabling the expression and selection of multiple genes of interest.

In Step 24B, we use lentiviral transduction to enable stable expression of channel genes in primary cells (**Fig. 4b**). Each lentiviral construct contains a channel gene of interest linked with a unique fluorescent reporter gene via the viral 2A peptide⁶². The 2A peptides are preferred to IRES elements in lentiviral constructs for multicistronic gene expression because they are substantially smaller in size and are reported to produce nearly equimolar levels of the co-expressed proteins. Of note, however, is that different 2A peptides may vary in their ‘cleavage’ efficiencies⁶³, leaving extra amino acid residues at the C terminus of the upstream protein and the N terminus of the downstream protein, potentially exerting negative effects on protein function. For the engineering of E-Fibs, we generated two lentiviral constructs²⁶: pRRL-CMV-BacNa_v-P2A-Kir2.1-T2A-EGFP (to confer electrical excitability) and pRRL-CMV-Cx43-P2A-mCherry (to enhance electrical coupling). Lentiviral particles are then produced using the second-generation lentiviral packaging system (Box 1). Following lentiviral transductions, fibroblasts transduced with the two viruses are sorted for expression of both EGFP and mCherry.

Maintenance and assessment of phenotype stability.—Although the steps outlined in this protocol are sufficient to generate engineered cells with stable transgene expression, several procedures must be performed to ensure the maintenance of cell electrical properties at consistent levels. When performing the nonviral delivery method, it is strongly advised that transfected cells undergo antibiotic selection for 2–4 weeks before fluorescence sorting and that the derived cell line be cultured under the same antibiotic concentration for any long-term studies. The commonly used antibiotics and suggested ranges of concentrations are shown in **Table 1**. We suggest that the user make frozen stocks of the early-passage engineered cells with stable properties. If the engineered cells express fluorescent markers,

routine microscope monitoring is required to ensure that fluorescent expression is maintained throughout the cell population. Validation of electrical and conduction properties (Steps 25 and 26) must be performed every two to three passages, and if current densities are found to be reduced by >20%, it is recommended to discard the cells and thaw cells from an earlier passage.

MATERIALS

REAGENTS

Plasmids and DNAs

- Mammalian expression plasmid with a constitutive eukaryotic promoter (e.g., CMV, EF1 α , and CAG) driving the ion channel gene of interest, antibiotic resistance gene, and fluorescent reporter (e.g., Addgene, plasmid no. 45567 can serve as a vector for ion channel gene subcloning)
- Lentiviral transfer plasmid with a constitutive mammalian promoter (e.g., CMV, EF1 α , and CAG) driving the ion channel gene of interest and fluorescent reporter (Addgene, plasmid no. 17448 can serve as a vector for ion channel gene subcloning)
- Lentiviral VSV-G-expressing envelope plasmid pMD2.G (Addgene, plasmid no. 12259)
- Lentiviral packaging plasmid psPAX2 (Addgene, plasmid no. 12260)
- cDNAs encoding Kir2.1 (Addgene catalog no. 16518), Cx43 (Addgene catalog no. 2697) and Nav1.5 (donated by A. Grant³⁰) or prokaryotic Na_v channels (donated by K. Irie²⁵)

Molecular-biology reagents

- Quikchange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, cat. no. 200521)
- Plasmid Miniprep Kit (Qiagen, cat. no. 12123)
- Plasmid Midiprep Kit (Qiagen, cat. no. 12145)
- QIAquick Gel Extraction Kit (Qiagen, cat. no. 28704)
- One Shot Stbl3 chemically competent *Escherichia coli* (Thermo Fisher Scientific, cat. no. C737303)
- XLIO-Gold ultracompetent cells (Agilent Technologies, cat. no. 200315)
- SOC medium (Thermo Fisher Scientific, cat. no. 15544034)
- LB medium (Fisher Scientific, cat. no. BP9722–2)
- LB agar powder (Fisher Scientific, cat. no. BP 1425–500)
- Agarose (Bio-Rad, cat. no. 161–3101)
- Carbenicillin solution (50 mg/μl; Teknova, cat. no. 5646–25)

- DpnI (New England BioLabs, cat. no. ROI76S)

Immunostaining reagents and antibodies

- 16% (wt/vol) Paraformaldehyde (PFA; Electron Microscopy Sciences, cat. no. 15710) ! CAUTION PFA is harmful when swallowed or inhaled, and upon contact with eyes and skin. Wear protective clothing when handling and ensure adequate ventilation.
- Triton X-100 (Sigma-Aldrich, cat. no. 9002-93-1)
- Chicken serum (Sigma-Aldrich, cat. no. C5405)
- Rabbit anti-Cx43 primary antibody (Abeam, cat. no. abl 1370). Use at a 1:200 dilution
- Mouse anti-vimentin antibody (Santa. Cruz Biotechnology, cat. no. sc6260). Use at a 1:500 dilution
- Anti-rabbit secondary antibody (Alexa. Fluor 488; Thermo Fisher Scientific, cat. no. A-21441). Use at a 1:200 dilution
- Anti-mouse secondary antibody (Alexa. Fluor 594; Thermo Fisher Scientific, cat. no. A-21201). Use at a 1:200 dilution
- Hoechst 33342 nucleic acid stain (Thermo Fisher Scientific, cat. no. H1399). Use at a 1:200 dilution

Cells and culture media

- HEK-293 cells (ATCC, cat. no. CRL-1573)
- HEK-293FT cells (Thermo Fisher Scientific, cat. no. R700-07)
- Primary adult human dermal fibroblasts (HDFs; Gibco, cat. no. C-013-5C)
- Human ventricular fibroblasts (Lonza, cat. no. CC-2904)
- Human astrocytes (Lonza, cat. no. CC-2565) ! CAUTION All cell types used in the study must be checked regularly to ensure that they are authentic and not infected with mycoplasma.
- DMEM, low glucose (Sigma-Aldrich, cat. no. D6046)
- DMEM, high glucose (Thermo Fisher Scientific, cat. no. 11960-069)
- Fibroblast basal medium (ATCC, cat. no. PCS-201-030)
- Fibroblast Growth Kit-Low serum (ATCC, cat. no. PCS-201-041)
- Astrocyte basal medium (Lonza, cat. no. CC-3187)
- Astrocyte growth medium (AGM, SingleQuots supplements; Lonza, cat. no. CC-4123)
- FBS (Hyclone, cat. no. SH30071.03)

- Penicillin-streptomycin (10,000 U/ml; Thermo Fisher Scientific, cat.no. 15140163)
- Puromycin dihydrochloride (Thermo Fisher Scientific, cat. no. A1113802)

General reagents

- Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, cat.no. 11668027)
- Genjet *in vitro* DNA transfection reagent (SignaGen Laboratories, cat. no. SL100488)
- Opti-MEM I reduced serum medium (Thermo Fisher Scientific, cat. no. 31985062)
- Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific, cat. no. 14190250)
- Fibronectin (15 µg/ml, 30 min; Sigma-Aldrich, cat. no. F1 141–2MG)
- Calcein AM dye (Molecular Probes, 0.5 µM in DMEM; Thermo Fisher Scientific, cat. no. C3099)
- Voltage-sensitive dye (Di-4-ANEPPS, 10 µM; Biotium, cat. no. 61010)
- Lenti-X concentrator (Clontech, cat. no. 631231)
- Polybrene (Sigma-Aldrich, cat. no. H9268–5G)

EQUIPMENT

- Upright confocal microscope (Zeiss, model no. LSM 510)
- Inverted fluorescence microscope (Nikon, model no. TE2000)
- Multiclamp 700B amplifier (Axon Instruments)
- NIDAQ-MX computer interface (National Instruments)
- SD9 Grass stimulator (Grass Technologies)
- WinWCP software (J. Dempster, University of Strathclyde, http://spider.science.strath.ac.uk/sipbs/software_ses.htm)
- PCLAMP (Molecular Devices, <https://www.moleculardevices.com/systems/axon-conventional-patch-clamp/pclamp-11-software-suite>)
- MATLAB software (Mathworks, <https://www.mathworks.com/downloads/>)
- Lab VIEW software (National Instruments, <http://www.ni.com/en-us/shop/lab-view/download.html>)
- P-97 micropipette puller (Sutter Instrument)
- Patch-clamp glass pipette (OD 1.5 mm, ID 1.16 mm; Harvard Apparatus, cat. no. 30–0092)

- Thermal cycler (Bio-Rad, model no. T100)
- Gel electrophoresis system (Bio-Rad PowerPAC)
- NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)
- Digital heat blocks (VWR)
- Centrifuge (Eppendorf, model no. 581 0R)
- Microcentrifuge (Eppendorf, model no. 5424)
- MoFlo Astrios flow cytometer (Beckman Coulter) equipped with Summit 6.0 software
- 504-channel optical fiber array (Redshirt Imaging)
- Solid-state excitation light source (Lumencor, model no. SOLA SM)
- Optical filters (Chroma, cat. no. HQ520/40m)
- PSCRed photoresist (Brewer Science)
- 0.22- μ m Filter (VWR, cat. no. 28145–477)
- 0.45- μ m 50-ml Tube-top filter (Corning, cat. no. 28680–121)
- T-75 culture flask (Corning, cat. no. 430641U)
- Tissue culture plate (12 wells; Thermo Fisher Scientific, cat. no. 353043)
- Tissue culture plate (96-wells, flat bottom; Sigma-Aldrich, cat. no. CLS3595)
- 200- μ l PCR tube (VWR, cat. no. 89092–242)
- 1.7-ml Microtube (Genesee Scientific, cat. no. 22–282)
- FluoroDish (35-mm/100-mm, glass bottom; WPI, cat. no. FD3510–100)
- Falcon 5-ml round-bottom polystyrene test tube (Corning, model no. 352058)
- CellTrics 30- μ m disposable filter for FACS (Sysmex, cat. no. 04–004-2326)
- Aclar coverslip (Electron Microscopy Sciences, cat. no. 50425–25)
- Poly-L-lysine-coated glass coverslip (neuVibro, cat. no. H-18-pll)

REAGENT SETUP

HEK-293 growth medium—HEK-293 growth medium consists of DMEM (low glucose), 10% (vol/vol) FBS, and 1% (vol/vol) penicillin-streptomycin. The medium can be stored at 4 °C for up to 3 weeks.

HEK-293FT growth medium—HEK-293FT growth medium consists of DMEM (high glucose), 10% (vol/vol) FBS, and 1% (vol/vol) penicillin-streptomycin. The medium can be stored at 4 °C for up to 3 weeks.

HDF growth medium—HDF growth medium (also used as growth medium for human ventricular fibroblasts) consists of fibroblast basal medium supplemented with Fibroblast Growth Kit-Low serum. The medium can be stored at 4 °C for up to 3 weeks.

Human astrocyte growth medium—Human astrocyte growth medium consists of astrocyte basal medium supplemented with AGM SingleQuots supplements. The medium can be stored at 4 °C for up to 1 month.

Tyrode's solution—Tyrode's solution consists of 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.33 mM NaH₂PO₄, 5 mM HEPES, and 5 mM glucose. Adjust the pH to 7.4 with NaOH and adjust the osmolarity to 280 mOsm. The solution can be stored at 4 °C for up to 1 month. ! CAUTION NaOH is hazardous upon inhalation, ingestion, or direct contact with eyes and skin; wear protective clothing when handling.

Patch-clamp pipette solution—Patch-clamp pipette solution consists of 140 mM KCl, 10 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, 10 mM HEPES, and 5 mM MgATP. Adjust the pH to 7.3 with KOH and adjust the osmolarity to 270 mOsm. The solution can be divided into 1-ml aliquots, frozen, and stored at –20 °C for up to 6 months. Thaw a new aliquot before each patch-clamp session. ! CAUTION KOH is toxic upon inhalation, ingestion, or direct contact with eyes and skin; wear protective clothing when handling.

Dual voltage-clamp extracellular solution—Dual voltage-clamp extracellular solution consists of 135 mM NaCl, 5.4 mM CsCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 1 mM BaCl₂, and 5 mM glucose. Adjust the pH to 7.4 with NaOH. The solution can be stored at 4 °C for up to 1 month. ! CAUTION NaOH is hazardous upon inhalation, ingestion, or direct contact with eyes and skin; wear protective clothing when handling.

Dual voltage-clamp pipette solution—Dual voltage-clamp pipette solution consists of 140 mM CsCl, 10 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, 10 mM HEPES, and 5 mM MgATP. Adjust the pH to 7.3 with CsOH and adjust the osmolarity to 270 mOsm. The solution can be divided into 1-ml aliquots, frozen, and stored at –20 °C for up to 6 months. Thaw a new aliquot before each patch-clamp session. ! CAUTION CsOH is toxic upon inhalation, ingestion, or direct contact with eyes and skin; wear protective clothing when handling.

Immunostaining blocking solution—Immunostaining blocking solution consists of 5% (vol/vol) chicken serum and 0.1% (vol/vol) Triton X-100 (diluted in DPBS). The solution can be stored at 4 °C for up to 2 weeks.

EQUIPMENT SETUP

Optical mapping setup—The general setup for optical mapping experiments has been described in our previous publications^{64,65}. The photodiode recording system used has 504 optical fibers arranged in a 20-mm-diameter hexagonal bundle (Redshirt Imaging), time resolution as low as 50 μs, and a 750-μm spatial resolution (1x; macromapping contact

mode), as previously described⁶⁶. The cell monolayer coated on 21-mm Aclar coverslip is placed in a thermoregulated chamber after a 5-min room-temperature (20–22 °C) staining with the voltage-sensitive dye Di-4 ANEPPS, illuminated with excitation light from a solid-state excitation light source (Lumencor, SOLA SM) passed through a 520 ± 20 -nm band-pass filter (Chroma, cat. no. HQ520/40m). The emission filter consists of a glass coverslip spin-coated with three layers of PSCRed photoresist (Brewer Science) to allow recording of emitted fluorescence in the red spectrum (>590 nm)⁶⁵. The cell monolayer is paced using controlled stimulus application with the Grass stimulator, the recorded fluorescence is converted to voltage using photodiodes, and then signals are amplified, sampled at 2.4 kHz, and stored on a computer for MATLAB analysis. Light shutter control, data acquisition, and electrical stimulation are synchronized using Lab View. Data analysis is performed using custom MATLAB software as previously described⁶⁴.

Patch-clamp setup—The general instructions for the patch-clamp setup can be found in previously published protocols from our group⁶⁷ and others^{68,69}.

PROCEDURE

DNA construct selection and mutagenesis ● TIMING 1–2 weeks

- 1| Design the mutagenic primers using the QuikChange Primer Design Program (<http://www.genomics.agilent.com/primerDesignProgram.jsp>). Order forward and reverse primers from an oligo synthesis company such as Integrated DNA Technologies. Combine complementary forward and reverse primers in water into a mixture containing 50 ng/μl of each primer. As an example, we have previously used this method to introduce point substitutions into the Na_vSheP channel at residues D60 and E43 to alter the channel's gating kinetics. The list of mutagenic primers designed in that study was provided in Supplementary Table 2 of the corresponding publication²⁶.
- 2| In a 200-μl thin-walled PCR tube, prepare the mutagenesis reaction mixture containing 5 μl of 10× reaction buffer, 25 ng of DNA template of the wild-type Na_v channel subcloned into a mammalian expression plasmid or a lentiviral transfer plasmid (see 'Plasmids and DNAs' section), 2.5 μl of primer mix, 1 μl of dNTP mix, 40.5 μl of H₂O, and 1 μl of PfuUltra high-fidelity DNA polymerase (2.5 U/μl).
- 3| Place the PCR mixture into the thermocycler and cycle the PCR reaction using the following parameters:

Cycle number	Temperature	Duration
1	98 °C	30 s
2–19	98 °C	30 s
	60 °C	1 min
	72 °C	1 min per kb of template
20	72 °C	7 min

■ PAUSE POINT Samples can be stored overnight at 4 °C or up to 1 week at −20 °C.

- 4| Digest the parental template by adding 1 µl of DpnI restriction enzyme and 5 µl of NEB CutSmart buffer, and incubate at 37 °C for 1–4 h.
- 5| Gently add 5 µl of the reaction sample to 50-µl aliquots of XL10-Gold ultracompetent cells and incubate on ice for 30 min. Heat-shock at 42 °C for 45 s, and then place the sample on ice for 2 min.
- 6| Following the transformation reaction, add 500 µl of SOC medium and incubate the transformation mixture at 37 °C for 1 h with shaking at 250 r.p.m. Transfer 100 µl of the transformation mixture to an LB agar plate containing the appropriate antibiotic, spread the mixture evenly using glass beads, and then incubate the transformation plate overnight at 37 °C.

■ PAUSE POINT After overnight incubation, the plate can be stored at 4 °C for up to 1 week.

- 7| Pick two colonies from each plate and inoculate them in LB medium containing the appropriate antibiotic. Incubate overnight at 37 °C with shaking at 250 r.p.m.
- 8| Isolate the DNA using a commercial miniprep kit. Sequence the whole gene to make sure only the desired mutation is introduced. Store the DNA at −20 °C until you are ready to use it at Step 10.

? TROUBLESHOOTING

■ PAUSE POINT DNA can be stored for several months at −20 °C.

Characterization of channel activity in HEK-293 cells ● TIMING 1–2 weeks

- 9| Gently detach HEK-293 cells from a T-75 culture flask using 0.05% (vol/vol) trypsin-EDTA and seed them onto each well of a 12-well plate at a density of 5×10^4 cells per cm^2 . Incubate at 37 °C and 5% (vol/vol) CO_2 overnight until the cells grow to >90% confluency.
- 10| To characterize each channel (wild-type or mutant from Step 8), add 1 µg of plasmid DNA to 100 µl of prewarmed Opti-MEM media. In a separate microcentrifuge tube, add 3 µl of Lipofectamine 2000 to 100 µl of Opti-MEM. Gently mix the DNA and Lipofectamine 2000 solutions and incubate for 5 min at room temperature. Add the mixture to one well of the 12-well plate from Step 9 and incubate for 6–8 h at 37 °C and 5% (vol/vol) CO_2 before exchanging the solution with HEK-293 growth medium.
- 11| 24–36 h after the post-transfection medium change, gently trypsinize the transfected cells and plate them onto poly-L-lysine-coated glass coverslips (18 mm in diameter) at a density of 5×10^3 cells per cm^2 and incubate at room temperature for 5–8 h to facilitate attachment.
- 12| Using a micropipette puller, fabricate two recording glass pipettes via predefined cycles of heating and pulling per the manufacturer's instructions. Under a

microscope, fire-polish the tip of the pipette using a heating filament. Fill the pipette with patch-clamp pipette solution and mount it to the pipette holder, making sure that the silver-wire electrode is in contact with the pipette solution. For HEK-293 cells, a pipette tip resistance between 1 and 2 MΩ is recommended, whereas a slightly higher resistance (2–3 MΩ) is advised for primary cells. Transfer the coverslip with the transfected cells to a patch-clamp chamber perfused with Tyrode's solution.

- 13] Under a microscope, identify a single cell positively transfected based on its expression of a fluorescent reporter. Move the pipette toward the cell using the micromanipulator. Just before touching the cell surface, change the micromanipulator settings to slow mode and approach the cell slowly to facilitate good contact. Upon a slight increase in pipette resistance (~1.5 times), apply brief negative pressure to induce the formation of a GΩ seal between the pipette and cell membrane. To rupture the membrane patch between the pipette and the cytosol to achieve a whole-cell voltage-clamp configuration, apply a brief current pulse from the pipette.
- 14] In voltage-clamp mode, subject the cell to sequences of voltage pulses of predefined strength and duration to record the corresponding ion currents and enable analyses of channel properties. In general, to measure Na_v activation properties, step membrane voltage from a sufficiently hyperpolarized holding potential (whereby >90% of channels are available to activate) to varying test potentials (typically from –60 to 60 mV). Inactivation of Na_v can be derived from peak currents measured at a predefined voltage following varying prepulse potentials (typically from –160 to –20 mV). In our studies, Na_v activation is determined by stepping membrane voltage from a –100-mV holding potential to varying 500-ms voltage pulses (–50 to 60 mV in 10-mV increments); Na_v inactivation is derived from peak I_{Na} measured at 0 mV following 3 s of varying prepulse potentials (–160 to 50 mV in 10-mV increments).

Measure the current from Kir2.1 channels by varying 1-s test potentials in cells (–130 to 50 mV, increments of 10 mV) from a holding potential of –40 mV⁷⁰. Similar voltage-pulse sequences can be used to extract the endogenous background current in wild-type (nontransfected) cells.

For each channel, repeat Steps 13 and 14 for eight to ten cells. Dispose of glass pipettes and coverslips into a sharps disposal container after recording.

Computational modeling of engineered cells ● TIMING 1–2 weeks

- ▲ CRITICAL General guidelines in establishing mathematical models of individual ion channels, single excitable cells, and a continuous 1D cable of cells are described below. As an example, we have also provided our custom-made code and sample data (Supplementary Data) for predicting AP and conduction properties in an engineered cell model expressing Kir2.1 and different sodium channel variants.

- 15] From voltage-clamp recordings of Kir2.1 current in Kir2.1-transfected cells, extract the steady-state current value (I_{K1}) for each voltage step (V) to generate an I_{K1} - V curve. Fit this curve to the following equation, adapted from a time-dependent Nygren *et al*⁷¹ model:

$$I_{K1} = \bar{G}_{K1} \frac{V - E_K}{1 + e^{a(V - E_K - b)}}$$

where $\bar{G}_{K1}$ (the maximum conductance of the Kir2.1 channel), E_K (potassium Nernst potential), and a and b are fitting parameters to be determined. The typical ranges of $GK1$, E_K , and electrical values in our engineered cells are provided in **Table 2** as a general guide.

- 16] From voltage-clamp recordings of endogenous background current in wild-type cells, extract the steady-state current value (I_{endo}) for each voltage step (V) to generate an I_{endo} - V curve. Fit this curve to the following linear equation to determine the fitting parameters c and d : $I_{\text{endo}} = cV + d$
- 17] From voltage-clamp activation and inactivation recordings of each Na_v variant, extract $\bar{G}_{\text{Na}}$ (maximum conductance of the Na_v channel), E_{Na} (sodium Nernst potential), m_{∞} (the steady-state activation gating variable), and h_{∞} (the steady-state inactivation gating variable) for each voltage step. Fit m_{∞} and h_{∞} to the Boltzmann equation⁷² to derive the midpoint voltage ($V_{1/2}$) and slope factor (s):

$$m_{\infty} = \frac{1}{1 + \exp\left(\frac{V_{1/2} - V}{s}\right)} \quad h_{\infty} = \frac{1}{1 + \exp\left(\frac{V - V_{1/2}}{s}\right)}$$

- 18] Fit the initial and late phases of I_{Na} traces from the activation protocol to a single exponential function to derive the time constants of activation (τ_m) and inactivation (τ_h) for the corresponding voltage steps. Fit ($\tau_m(V)$) and ($\tau_h(V)$) to polynomial functions.
- 19] For each Na_v variant, construct the mathematical model of I_{Na} using the following sets of equations:

$$\begin{aligned} I_{\text{Na}}(V, t) &= \bar{G}_{\text{Na}} * m(V, t) * h(V, t) * (V - E_{\text{Na}}) \\ m(V, t) &= m_0 - (m_0 - m_{\infty}(1 - e^{-t/\tau_m})) \\ h(V, t) &= h_0 - (h_0 - h_{\infty}(1 - e^{-t/\tau_h})) \end{aligned}$$

where m_0 and h_0 are the corresponding gating variables at the resting membrane potential.

? TROUBLESHOOTING

- 20| Incorporate the models of ionic currents from the previous steps into the following Hodgkin-Huxley-based differential equation describing the change in membrane voltage of a single cell in response to combined effects from ionic currents through the membrane and external stimulus:

$$\frac{dV}{dt} = \frac{1}{C_m}(I_{Na}(V, t) + I_{K1}(V) + I_{endo}(V) - I_{stim}(V, t))$$

where C_m is the total membrane capacitance of the corresponding cell type (determined from patch-clamp experiments) and $I_{stim}(V, t)$ is the external stimulus current.

- 21| Run the simulation and export V as a function of time, using increasing I_{stim} values. The smallest stimulus amplitude required to elicit an AP is considered to be the threshold value ($I_{threshold}$). Repeat the simulation using $1.2 \times I_{threshold}$ and extract the following parameters from generated APs: maximum slope of AP upstroke phase ($(dV/dt)_{max}$), time of $(dV/dt)_{max}$ (activation time), and time difference between activation time and time of 80% repolarization (APD_{80}).
- 22| Based on this single-cell model, construct a connected multicellular model to simulate AP propagation. A simple AP propagation in a 1D cable is simulated using the core conductor model⁷³ described by the following equation:

$$\frac{1}{2\pi r R_i} \frac{\partial^2 V}{\partial x^2} = C_m \frac{\partial V}{\partial t} + I_{ion}$$

where r is the cable radius, R_i is the intracellular resistivity, and I_{ion} is the total ionic current ($I_{Na} + I_{K1} + I_{endo}$). R_i is chosen to fit the CV of a reference monoclonal line of engineered cells.

Run this multicellular propagation model and extract the CV, which is defined as the slope of the linear fit of the distance traveled plotted against the activation time along the cable.

▲ **CRITICAL STEP** With the exception of the parameters associated with Na_v gating variables, all parameters must remain constant in all simulations so that any differences in observed CVs can be attributed only to differences in the biophysical properties of tested Na_v variants. The typical ranges of electrical values in our engineered cells are provided in **Table 2** as a general guide. Users are also encouraged to refer to our previous publications^{19,22,26} for more detailed information on engineered cell models, including simulation parameters and more accurately defined electrical properties of our excitable cell lines.

- 23| From these simulation results, select the Na_v variants that generate the most desirable AP and conduction properties ($(dV/dt)_{max}$, APD_{80} , and CV) for subsequent *in vitro* testing. For our purpose of generating fast-conducting engineered cells, we selected the variants that yield the highest $(dV/dt)_{max}$ and CV values.

Generation of excitable cells ● TIMING 2 weeks–3 months

24| To generate a monoclonal line of excitable HEK-293 cells, follow option A. To generate excitable HDFs, follow option B.

(A) Generation of a monoclonal excitable HEK-293 cell line ● TIMING: 2–3 months

- (i)** Seed HEK-293 cells into one well of a six-well plate at a density of 6×10^4 cells per cm^2 in HEK-293 growth medium. Incubate overnight at 37 °C in a 5% (vol/vol) CO₂ humidified cell culture incubator.
- (ii)** After the cells reach 50–60% confluency the following day, wash them with DPBS to remove traces of antibiotic and serum, and exchange the medium for 2 ml of nonsupplemented high-glucose DMEM.
- (iii)** In a 1.5-ml microcentrifuge tube, add 2 µg of purified plasmid DNA of the selected ion channel (wild-type or a mutant generated from Step 8) to 100 µl of prewarmed high-glucose DMEM. In another 1.5-ml microcentrifuge tube, add 6 µl of GenJet transfection reagent to 100 µl of high-glucose DMEM. Add the GenJet solution to the plasmid DNA solution and incubate at room temperature for 15 min to enable the formation of the GenJet-DNA complexes.
- (iv)** Slowly pipette the transfection mixture dropwise onto the plated HEK-293 cells and return the cells to the incubator. Fluorescent reporter expression can be detected ~12 h after transfection.
- (v)** Eighteen hours after transfection, remove the medium containing the transfection mixture, wash the transfected HEK-293 cells with DPBS, and replace the medium with complete HEK-293 growth medium.
- (vi)** Forty-two hours after transfection, trypsinize the transfected HEK-293 cells and transfer them to a T-25 culture flask. Let the cells recover in HEK-293 growth medium at 37 °C in a 5% (vol/vol) CO₂ humidified cell culture incubator for 24 h.
- (vii)** Add an antibiotic of predetermined working concentration to the medium to begin selecting cells expressing the transfected plasmid.

▲ **CRITICAL STEP** The starting antibiotic concentration for selection will need to be determined empirically by subjecting nontransfected cells to a range of antibiotic concentrations and selecting the lowest dose that leads to complete cell death.

- (viii) Maintain continuous antibiotic selection for 2–3 weeks with frequent medium changes (every 3–5 d). Monitor the fluorescent expression in the cell population undergoing selection to ensure expansion of only transfected cells and generation of a polyclonal cell line.

? TROUBLESHOOTING.

- (ix) After the polyclonal cell line is created, perform FACS to isolate the cell population with the desired level of fluorescent expression. We select the top 10–30% of the population to ensure strong expression of the ion channel gene in the derived polyclonal line. Following FACS, allow the sorted cells to recover in complete growth medium at 37 °C and 5% (vol/vol) CO₂ for 5–7 d before proceeding to monoclonal selection.
- (x) Dissociate polyclonal cells by trypsin, resuspend in growth media, and filter through a 30-µm disposable filter. Dilute the cells with growth medium to a final concentration of five cells per ml. Transfer 200 µl of this cell solution to each well of a 96-well plate. This corresponds to an average seeding density of one cell per well. Leave the cells in the incubator for 2 weeks to allow them to grow into colonies.

▲ CRITICAL STEP Using this seeding density, monoclonal lines are frequently obtained in 20–30 wells of a 96-well plate. We also recommend preparing 96-well plates with lower (0.5 cells per well) and higher (two cells per well) seeding densities to account for variations in sample preparation and cell health.

- (xi) After 2 weeks of monoclonal selection, scan the 96-well plate under a fluorescence microscope and pick out wells containing single colonies with >60% confluency and high fluorescent expression. Trypsinize monoclonally derived cells from selected wells and expand them in larger culture settings (six-well plate, T-25 flask, or T-75 flask).

? TROUBLESHOOTING

■ PAUSE POINT Expanded monoclonal HEK-293 lines can be frozen in complete growth medium and 10% (vol/vol) DMSO and kept in liquid nitrogen for several years, and then thawed for experimental studies.

- (xii) Repeat Step 24A(i–xi) for expression and selection of the remaining channel genes.

(B) Generation of excitable human dermal fibroblasts ● TIMING 2–4 weeks

- (i)** Seed HDFs into one well of a six-well plate at a density of 10^4 cells per cm^2 in HDF growth medium. Incubate overnight at 37 °C in a 5% (vol/vol) CO₂ humidified cell culture incubator.
- (ii)** Replace the growth media with prewarmed DMEM low-glucose media, supplemented with 8 µg/ml polybrene, 1 h before transduction.
- (iii)** Add thawed lentiviral particles (see Box 1 for guidance) to the medium and incubate the cells with the virus for 72 h. Fluorescent reporter expression can be detected ~48 h after transduction.
- (iv)** Seventy-two hours after transduction, remove the medium containing the lentivirus, wash the transduced HDF monolayers with DPBS, and dissociate the cells using trypsin. Transfer the cells to a T-75 culture flask and let them recover and expand in HDF growth media.
- (v)** After transduced HDFs reach 70–90% confluency, dissociate them with trypsin and resuspend in 1 ml of DMEM low glucose. Filter the cells using a 30-µl cell filter into a 5-ml round-bottom tube. Keep the cells on ice until sorting.
- (vi)** Sort single cells into a new round-bottom tube according to the desired fluorescent expression. We recommend selecting a cell population with the top (10–30%) fluorescence intensity.
- (vii)** Transfer the sorted cells to a six-well plate and let them recover in HDF growth medium for 3–7 d at 37 °C and 5% (vol/vol) CO₂. Once the cells reach 70–90% confluency, they can be transferred to a T-75 flask for further expansion.
- (viii)** Repeat Step 24B(i-vii) for expression and selection of the remaining channel genes, if required.

Assessment and validation of the electrical properties of the engineered cells

● TIMING 1–3 d

- 25|** To assess and validate the electrical properties of the engineered cells, follow option A to quantify the AP properties in individual engineered cells via whole-cell patch clamp; follow option B to quantify the conduction properties via optical mapping of membrane potential in engineered cell monolayers; follow option C to visually examine the distribution of Cx43 gap-junctional proteins in engineered cell monolayers; follow option D to obtain qualitative comparison of

electrical coupling levels between monolayers from different cell populations; and follow option E to measure macroscopic gap-junction conductance between two neighboring cells.

- (A) Quantification of AP properties in engineered cells via whole-cell patch clamp ● TIMING 2–3 d
  - (i) Prepare cells for patch clamp and proceed to whole-cell configuration as described in Steps 11–13.
  - (ii) Once whole-cell configuration is achieved, switch to current-clamp mode with no holding current applied and wait for 1–2 min until the resting membrane potential (RMP) remains stable. For excitable cells expressing Kir2.1 channels, the RMP is expected to be between –75 and –80 mV.
  - (iii) Apply 1-ms depolarizing current steps (starting at 0.5 nA, with 0.25-nA increments) to elicit all-or-none AP response. The lowest current amplitude required to trigger AP is considered to be the threshold. Perform three to five AP recordings using stimulus amplitude at $1.2 \times$ threshold value.
  - (iv) Extract the RMP, maximum depolarization slope of the AP ($(dI/dt)_{\max}$), and the AP duration at 80% of repolarization (APD₈₀) using patch-clamp analysis software such as WinWCP (University of Strathclyde) or PCLAMP (Molecular Devices).
- (B) Quantification of conduction properties in engineered cell monolayers via optical mapping of membrane potential ● TIMING 2 d
  - (i) Seed 2×10^5 engineered cells onto a 21-mm fibronectin-coated Aclar coverslip and leave the cells in the incubator for 24–48 h to allow them to grow into fully confluent monolayers.
  - (ii) Stain confluent cell monolayers with a voltage-sensitive dye such as Di-4 ANEPPS for 5 min at room temperature. Wash the cells with Tyrode's solution for 5 min at room temperature to remove excessive staining and transfer them to an optical mapping chamber.
  - (iii) Illuminate the cell culture with green excitation light (520 + 20 nm) while applying stimulus pulses (10 ms, $1.2 \times$ threshold, 1 Hz) with a bipolar point electrode connected to a Grass stimulator. Record the emitted fluorescence signal in the red spectrum (>590 nm) to assess the AP propagation in the culture.
  - (iv) Using software custom-made for the user's optical mapping setup, calculate the CV as a measure of the average

propagation speed of the AP waveform across the cell culture. Users are encouraged to refer to our previous publications^{64,65} for a more detailed discussion of optical mapping data analysis.

- (C) Detection and assessment of gap-junction channel distribution via immunostaining ● TIMING 1–2 d
- (i) Fix a confluent monolayer of engineered cells (prepared as described in Step 25(B)(i)) with PFA (2% (vol/vol), diluted in DPBS) for 12 min at room temperature. Wash 3× in DPBS.
  - (ii) Block and permeabilize the cells in immunostaining blocking solution for 30 min at room temperature.
  - (iii) Incubate the cells with primary antibodies (mouse anti-vimentin 1:500 and rabbit anti-Cx43 1:200, diluted in blocking solution) overnight at 4 °C. Wash 3× in blocking solution.
  - (iv) Incubate the cells with secondary antibodies (anti-mouse Alexa Fluor 594, anti-rabbit Alexa Fluor 488, and Hoechst, diluted in blocking solution) overnight in the dark at 4 °C. Wash 3× in DPBS.
  - (v) Mount the coverslip onto the slide using mounting medium and let it dry for at least 2 h before imaging with a fluorescence microscope. Strong Cx43-based gap-junction formation can be observed as a punctated pattern at the cell-cell borders (**Fig. 5a,b**).
- (D) Assessment of functional electrical coupling via fluorescence recovery after photobleaching ● TIMING 2 d
- (i) Seed 2×10^5 engineered cells expressing Cx43 onto a 21-mm Aclar coverslip and incubate overnight at 37 °C, 5% (vol/vol) CO₂.
  - (ii) The following day, stain the cell monolayers with calcein AM dye (0.5 μM in DMEM low glucose) for 20 min at 37 °C. Wash three times with DMEM and exchange with fresh growth medium. Place the coverslip in a 35-mm glass-bottom dish and transfer to a confocal microscope.
  - (iii) Using an upright confocal microscope (e.g., Zeiss, model no. LSM 510), find a target cell and draw the photobleaching region around it. Photobleach the target cell with a 488-nm argon laser to remove calcein fluorescence signal. Acquire an image every 15 s until the fluorescence level in the target cell reaches a steady state.

? TROUBLESHOOTING

- (iv) At each time point, normalize the level of fluorescence in the target cell to the level in remote unbleached cells and the number of neighboring cells. Fit the fluorescence recovery curve (**Fig. 5c**) to a single exponential function using the MATLAB curve-fitting function (cftool) and extract the time constant of recovery.
- (E) Quantification of gap-junctional conductance via dual whole-cell patch-clamp recording ● TIMING 2–3 d

- (i) Seed the engineered cells onto 22-mm glass coverslips at low density (5,000 cells per cm²) and leave them in the incubator at 37 °C, 5% (vol/vol) CO₂ for 24–48 h to allow the formation of cell pairs.
- (ii) Transfer the coverslip to a patch-clamp chamber perfused with dual voltage-clamp extracellular solution. Prepare two recording glass pipettes as described in Step 12 and fill each pipette with dual voltage-clamp pipette solution. Mount each pipette to a pipette holder.
- (iii) Identify a target cell pair under a microscope at 40× magnification. If the engineered cells express a fluorescent reporter, make sure that both cells express the marker at relatively similar levels. In addition, it is recommended to find cell pairs with consistent cell-cell contact length.

? TROUBLESHOOTING

- (iv) Using the coarse mode of a micromanipulator, move each pipette to the vicinity of one cell.
- (v) Switch the micromanipulator settings to fine mode and approach one cell slowly to make gentle contact with the cell membrane at its center. Upon a slight increase in pipette resistance, apply gentle suction to induce the formation of a giga-seal between the pipette and cell membrane. Apply a brief (500 μs) current pulse using the ‘zap’ function of the amplifier to rupture the membrane patch, separating the cytosol and the dual voltage-clamp pipette solution to achieve whole-cell configuration.

Repeat this process with the other cell, using the second patch electrode (**Fig. 5d**).

▲ **CRITICAL STEP** Make sure that the seal resistance is at least 1 GΩ for each cell before proceeding to measurement. Otherwise, repeat with a new cell pair.

- (vi) In voltage-clamp mode, hold both cells at 0 mV. Apply 8-s voltage steps (from -100 mV to $+100$ mV in 20-mV increments) to one cell of the cell pair (cell 1) while holding the other (cell 2) at 0 mV. This creates a voltage gradient across the two cells, resulting in junctional current between them. For each voltage step in cell 1, record the resulting current response in cell 2. This current is equal in magnitude but opposite in sign to the junctional current between the two cells. A 20-s delay is recommended after each voltage step to allow the cells to recover.
- (vii) Calculate the gap-junctional conductance for each voltage step by dividing the measured junctional current by the applied voltage after correcting for membrane resistance and series resistance as previously described^{74,75}.

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? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**

•TIMING

Steps 1–8, DNA construct selection and mutagenesis: 1–2 weeks

Steps 9–14, characterization of channel activity in HEK-293 cells: 1–2 weeks

Steps 15–23, computational modeling of engineered cells: 1–2 weeks

Step 24A, generation of monoclonal excitable HEK-293 cell lines: 2–3 months

Step 24B, generation of excitable human dermal fibroblasts: 2–4 weeks

Step 25A, quantification of AP properties in engineered cells via whole-cell patch clamp: 2–3 d

Step 25B, quantification of conduction properties via optical mapping of membrane potential: 2 d

Step 25C, detection and assessment of gap-junction channel distribution via immunostaining: 1–2 d

Step 25D, assessment of functional electrical coupling via fluorescence recovery after photobleaching: 2 d

Step 25E, quantification of gap-junctional conductance via dual whole-cell patch-clamp recording: 2–3 d

Box 1, lentivirus production: 1 week

ANTICIPATED RESULTS—Engineered cells expressing Kir2.1 show robust inward-rectifying J_{K1} current and highly hyperpolarized RMP (between -75 and -80 mV, **Fig. 6a–d**). Strong Na_v channel expression in engineered cells produces voltage-dependent inward

currents with rapid activation and slower inactivation, characteristic of voltage-gated sodium channels (**Fig. 6e-h**).

Mammalian Na_v isoforms are expected to display faster kinetics than their prokaryotic counterparts, especially in inactivation. Engineered cells co-expressing Kir2.1 and Na_v channels with appropriate gating properties should elicit all-or-none APs upon application of external stimuli of increasing amplitude (**Fig. 6i-k**). AP characteristics of engineered cells can vary over wide ranges, depending on various factors. Specifically, we have observed maximum upstroke velocities in the range of 20–170 V per s, depending on channel expression levels, Na_v activation kinetics, and voltage dependencies of Na_v gating (**Table 4**). AP duration (APD₈₀) in engineered cells expressing Na_v1.5 (25–35 ms) is shorter than in those expressing BacNa_v (75–600 ms), due to the substantially faster inactivation kinetics of mammalian Na_v channels (**Table 4**). The large variation in APD in the latter group can be mainly attributed to differences in expression efficiency of exogenous channels, distinct inactivation kinetics of different BacNa_v orthologs²⁶, and variable levels of endogenous outward currents among the tested cell types (**Fig. 6j,k**).

Overexpression of Cx43 results in the robust formation of Cx43 gap junctions between neighboring engineered cells, as revealed by immunostaining (**Fig. 5a,b**). Functional studies with fluorescence recovery after photobleaching (FRAP) and dual patch clamp also show that Cx43 expression substantially enhances electrical coupling between engineered cells as compared with wild-type cells (**Fig. 5c**). Monolayers of engineered cells expressing all three channel genes support uniform AP propagation when electrically stimulated (**Fig. 7a-c**), with CVs observed in the range of 3–21 cm per s (**Table 4**). In general, faster CV is associated with higher Na_v and Cx43 expression levels, weaker Kir2.1 current, faster Na_v activation kinetics, and lower AP activation threshold. Flow cytometry can be used to fine-tune CV based on fluorescence expression levels (**Fig. 7d-g**) and to decrease variability in the engineered cell population (**Table 4**, HDF ShePm + Kir2.1 + Cx43, unsorted versus sorted). Similarly, monoclonal selection can be used to obtain cell lines with higher expression levels and lower variation as compared with a polyclonal population (**Table 4**, HEK ShePm + Kir2.1 +

Cx43, polyclonal versus monoclonal). Engineered cells can maintain stable electrical properties following continuous expansion and multiple freeze-thaw cycles. However, whereas cells derived from immortalized cell lines such as Ex-293s exhibit unlimited proliferative capacity, engineered primary cells such as E-Fibs progressively slow their proliferation and eventually become senescent

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Box 1 |

Lentivirus production ● TIMING 1 week

1. Thaw one vial of HEK-293FT cells ($\sim 2 \times 10^6$ cells in 1 ml) from stock stored in liquid nitrogen and let the cells recover in 10 ml of HEK-293FT growth medium in a 10-cm culture dish at 37 °C and 5% (vol/vol) CO₂.
2. Passage the cells every 2–3 d using a 1:5 dilution to maintain them at lower than 80% confluency.

▲CRITICAL STEP Earlier passages of HEK-293FT cells work best, but it is recommended to passage newly thawed cells at least three times before using them for transfection and lentivirus production. Avoid using cells that have undergone more than ten passages.

3. Dissociate the HEK-293FT cells from an 80% confluent 10-cm culture dish and replate into four 10-cm culture dishes. Leave in the incubator overnight at 37 °C and 5% (vol/vol) CO₂.
4. On the morning of transfection, exchange the medium for fresh prewarmed HEK-293FT growth medium. Continue monitoring cell confluency every hour until the cells become 85–90% confluent.

▲CRITICAL STEP The suggested confluency has been shown to minimize toxicity due to Lipofectamine while still ensuring high transfection efficiency.

5. Prepare the DNA and Lipofectamine mixtures in two separate 50-ml conical tubes as shown below and incubate for 5 min at room temperature.

DNA mixture	Lipofectamine mixture
6 ml of prewarmed Opti-MEM	6 ml of prewarmed Opti-MEM
40 µg of transfer plasmid	120 µl of Lipofectamine 2000
20 µg of packaging plasmid (psPAX2)	
8 µg of envelope plasmid (VSVg)	

6. Gently add the DNA mixture to the lipofectamine mixture. Incubate for 20 min at room temperature.
7. For each 10-cm dish of HEK-293FT cells, add 3 ml of DNA/Lipofectamine mix dropwise. Gently rock the dish to distribute the transfection complexes evenly without disturbing the cells. Incubate for 6–8 h at 37 °C.
8. Replace the medium with warm and fresh HEK-293FT growth medium and return the cells to the incubator.
9. 72 h after changing the medium, harvest the supernatant containing lentiviral particles. Spin-collect the supernatant at 500g for 5 min at 4 °C and then filter through a 0.45-µm filter flask to remove any cell debris.
10. Concentrate the lentiviral particles via ultracentrifugation or the use of a commercial lentivirus concentrator kit. For the latter method, gently mix one volume of Lenti-X concentrator with three volumes of filtered supernatant

and incubate the mixture overnight at 4 °C. Pellet lentiviral particles via centrifugation at 1,500g for 45 min at 4 °C, carefully remove the supernatant, and resuspend the pellet in 500 µl of DPBS. Fast-freeze viral particles on dry ice and store at –80 °C.

? TROUBLESHOOTING

■ **PAUSE POINT** Concentrated lentiviral particles can be kept at –80 °C for several months. We recommend storing them in small aliquots to avoid multiple thaw-freeze cycles.

Figure 1 |.

Overview of the protocol. A combination of three channels is typically required to convert unexcitable cells into excitable and actively conducting cells: a potassium channel (e.g., Kir2.1), a sodium channel (mammalian or prokaryotic Na_v), and a gap-junction channel (e.g., Cx43). Site-directed mutagenesis can be performed to induce desired changes in the properties of the selected Na_v channel (Steps 1–8). Here, a critical residue on the S2 transmembrane domain of a $BacNa_v$ channel is replaced by different amino acids, similar to what we have previously shown²⁶. Selected Na_v channels and Kir2.1 channels are then transiently transfected into HEK-293 cells, and patch-clamp recording is performed to extract channel properties (Steps 9–14). From the patch-clamp results, computational models of individual channels are created and combined to predict AP and conduction characteristics in engineered cells (Steps 15–23). Channels associated with the most desired properties are then delivered into unexcitable cells via nonviral or viral delivery methods, and positively transfected/transduced cells are selected using flow cytometry and/or antibiotic selection. These steps are repeated if necessary until all three-channel genes are stably expressed to generate electrically excitable cells (Step 24). Finally, the electrical

properties of the engineered cells are assessed for exogenous current densities, intercellular electrical coupling level, and speed of AP propagation (Steps 25 and 26). Parts of the figure are adapted from Nguyen *et al.*²⁶, Springer Nature. g , conductance; I , current density.

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Figure 2 | Screening of AP propagation in Kir2.1 + Cx43 HEK-293 cells transfected with BacNa_v variants. **(a,b)** AP propagation was supported in Kir2.1 + Cx43 HEK-293 monolayers expressing Na_vRosD G217A **(a)** or NaChBac **(b)** but not other tested BacNa_v variants. The G217A mutation of Na_vRosD was used because the wild-type channel produced a very small current (not shown). **(c,d)** The BacNa_v channels failed to support propagation of AP due to either hyperpolarized voltage-dependent inactivation **(c)** or a highly depolarized activation threshold **(d)**. Inactivation and activation curves are shown as Boltzmann fits based on the $V_{1/2}$ and slope factor values derived from patch-clamp results. Tested BacNa_v orthologs include channels from *Bacillus halodurans* (NaChBac), *Shewanella putrefaciens* (Na_vSheP), *Roseobacter denitrificans* (Na_vRosD), *Bacillus licheniformis* (Na_vBacL), *Silicibacter pomeroyi* (Na_vSilP), *Arcobacter butzleri* (Na_vAb), and *Magnetococcus marinus* (Na_vMs). G , channel conductance; V , membrane voltage.

Figure 3 |

Example of using hERG expression to vary the AP duration. **(a)** Three HEK-293 cells expressing I_{K1} , I_{Na} (Na_vRosD G217A), and varied levels of HERG-P2A-mCherry (low, medium, and high). **(b)** Corresponding AP traces from cells in **a**, showing that higher hERG (I_{Kr}) expression yields shorter APD. **(c)** HDFs transduced with hERG-P2A-mCherry lentivirus display robust mCherry expression. **(d,e)** Transduced HDFs show strong outward time-dependent current **(d)** and characteristic $I-V$ relationship of the hERG channel **(e, $n = 6$)**. Error bars indicate s.e.m.

Figure 4 |

Stepwise procedures for the generation of monoclonal Ex-293 line and E-HDFs (Step 24).

(a) Unexcitable wild-type HEK-293 cells were transfected with the Kir2.1-IRES-mCherry plasmid, and monoclonal lines were derived from cells that exhibited high mCherry fluorescence intensity. Subsequently, the Nav1.5-IRES-GFP plasmid was transfected into a Kir2.1 HEK-293 monoclonal cell line, and monoclonal lines were derived that expressed both mCherry and GFP. Finally, the Cx43-IRES-mOrange plasmid was transfected into a Kir2.1 + Nav1.5 HEK-293 monoclonal cell line to derive the Kir2.1 + Nav1.5 + Cx43 HEK-293 monoclonal line (Ex-293) with strong mOrange fluorescence in addition to mCherry and GFP fluorescence. Cells were selected by puromycin resistance, fluorescent cell sorting, and monoclonal isolation. Images of HEK-293 cells are reproduced from Kirkton *et al*¹⁹, Springer Nature, **(b)** Unexcitable wild-type HDF cells were transduced with pRRL-CMV-BacNav_v-P2A-Kir2.1-T2A-GFP and pRRL-CMV-Cx43-P2A-mCherry, and flow cytometry was performed to select E-HDF cells double-positive for GFP and mCherry. Transduction of the two viruses can be done either sequentially or simultaneously. Scale bars, 20 μ m **(a)**; 100 μ m **(b)**. *AMP-R*, ampicillin resistance gene; CMV5, pCMV5(CuO)-IRES-GFP; cPPT, central polypurine tract; CTS, central termination sequence; LTR, long terminal repeat; P2A, porcine teschovirus-1 2A peptide; *PUR-R*, puromycin resistance gene; RRE, Rev response element; T2A, *Thosea asigna* virus 2A peptide; WPRE, woodchuck hepatitis virus post-translational regulatory element.

Figure 5 |.

Characterization of electrical coupling in engineered cells (Step 25C-E). **(a)** Following stable Cx43-mCherry overexpression, the derived Cx43 HEK-293 cells formed abundant Cx43 gap junctions, which were not detected in wild-type (WT) HEK-293 cells (transfected with mCherry plasmid) (corresponding to Step 25C). **(b)** Similarly, robust staining of Cx43 at cell-cell borders was evident in HDFs transduced with Cx43 lentivirus but not in wild-type HDFs (corresponding to Step 25C). **(c)** FRAP was used to demonstrate substantially increased functional coupling in Cx43-expressing HEK-293s and HDFs as compared with WT cells ($n = 6-7$; error bars indicate s.e.m.) (corresponding to Step 25D). **(d)** Illustration of a dual patch-clamp experiment. Two abutting Ex-293 cells (labeled 1 and 2) were individually voltage-clamped in whole-cell mode. Initially, both cells were held at 0 mV. Cell 1 was then depolarized by a 20-mV voltage step ($V_m = 1$), whereas cell 2 remained clamped at 0 mV ($V_m = 2$), eliciting a current response, $I_m = 1$, (in cell 1) and a transjunctional current response, $I_m = 2$, (in cell 2). This enables the quantification of macroscopic gap-junctional conductance between the two cells. Representative dual-clamp current and voltage traces (corresponding to Step 25E) are reproduced from Kirkton *et al.*¹⁹, Springer Nature..

Figure 6 |.

Characterization of ion currents and APs in engineered cells (Step 25A). **(a,b)**

Representative voltage-clamp recordings show robust I_{K1} in HDFs transduced with Kir2.1

virus **(a)** but not in wild-type (WT) cells **(b)**. **(c)** Corresponding steady-state I - V curves in

Kir2.1-transduced and **WT** HDFs from **a** and **b**. **(d)** Kir2.1 expression substantially

hyperpolarizes the resting membrane potential of transduced HDFs as compared with WT

cells ($n = 6-8$; error bars indicate s.e.m.). * $P < 0.001$ versus **WT** HDFs. **(e,f)** Representative

recordings of I_{Na} activation **(e)** and inactivation **(f)** in HDFs transduced with NavheP D60N

lentivirus. **(g,h)** Corresponding peak I_{Na} - V curve **(g)** and voltage-dependent activation (m)

and inactivation (h) curves **(h)** of NavheP D60N-transduced cells. Boltzmann fits are shown

as solid lines, **(i)** Current pulses of increasing amplitude induce an all-or-none AP response

in Ex-293 cells, **(j)** Engineered cells derived from HEK-293 (E-HEK293), human dermal

fibroblast (E-HDF), human ventricular fibroblast (E-HVF), and human astrocyte (E-HA)

cells transduced with NavheP D60N-Kir2.1 lentivirus exhibit varying AP durations, **(k)**

Different BacNa_v variants, NavheP D60A (ShePm), Na_vRosD G217A (RosDm), and Na_vPz

(Pz), result in different APDs in Kir2.1-expressing HEK-293 cells.

Figure 7 |.

Characterization of AP propagation in engineered cells (Step 25B) and fine-tuning of conduction velocity with FACS. (a) Optical mapping snapshots of transmembrane voltage in a confluent isotropic monolayer of Ex-293 cells showing outward spread of AP propagation from a centrally located stimulus electrode. Times shown are times since stimulation; blue and red, rest and peak of AP; pulse symbol, electrode site. (b,c) Isochrone activation map (b, white circular lines; wavefront locations at denoted times in milliseconds) and conduction velocity (CV) map (c, length and direction of arrows indicate CV magnitude and wavefront direction, respectively) demonstrate spatially uniform AP propagation in a. (d,e) Fine-tuning of conduction velocity of E-HDF monolayers co-transduced with $\text{Na}_v\text{SheP D60N-Kir2.1-eGFP}$ and Cx43-mCherry lentiviruses using FACS. Sorting E-HDF population based on eGFP level (d) results in faster CVs for higher eGFP intensities (e). (f,g) Similarly, mCherry sorting (f) yields faster AP propagation in the high mCherry-expressing group (g). Error bars in e and g indicate s.e.m. * $P < 0.001$ versus low eGFP group in e or versus low or medium mCherry group in g. d-g adapted from Nguyen *et al.*²⁶, Springer Nature.

Table 1 |

Commonly used antibiotics for the generation of stable mammalian cell lines.

Antibiotic	Suggested concentration	Commercial source
Puromycin	1–5 µg/ml	Thermo Fisher Scientific, cat. no. A1113802
Geneticin	200–800 µg/ml	Thermo Fisher Scientific, cat. no. 10131035
Hygromycin B	100–600 µg/ml	Thermo Fisher Scientific, cat. no. 10687010
Zeocin	100–500 µg/ml	Thermo Fisher Scientific, cat. no. R25001
Blasticidin	1–10 µg/ml	Thermo Fisher Scientific, cat. no. R21001

Table 2 |

Typical ranges of engineered cell parameters

Parameter	Range of values
Total membrane surface area (A)	1,500–3,500 μm^2
Total membrane capacitance (C_m)	12–40 pF
Na_v maximum conductance (G_{Na})	20–120 nS
$\text{K}_{ir2.1}$ maximum total conductance (G_{K1})	20–100 nS
Resting membrane potential (V_{rest})	–75 to –80 mV
Sodium Nernst potential (E_{Na})	55–70 mV
Potassium Nernst potential (E_K)	–75 to –85 mV
Intracellular resistivity (R_i)	0.5–2 k Ω cm

Table 3 |

Troubleshooting table

Step	Problem	Possible reason	Solution
8	Low mutagenesis efficiency	Incomplete digestion of parental DNA template	Increase DpnI digestion time in Step 4 (to overnight if needed); use a smaller amount of DNA template for mutagenesis reaction in Step 3
19	The provided equations do not simulate mammalian Na _v characteristics well	Differences in gating mechanisms between mammalian and prokaryotic Na _v channels	The following equation has been shown to better describe current from the Na _v 1.5 channel ⁷⁶ : $I_{Na}(V, t) = \bar{G}_{Na} * m^3(V, t) * h(V, t) * j(V, t) * (V - E_{Na})$ where $j(V, t)$ is termed the slow inactivation gate
24A(viii)	Nontransfected cells still survive after 1 week of antibiotic selection	Initial antibiotic concentration is too low, or cells are too confluent	Increase antibiotic concentration in the medium. If colonies of nontransfected cells are detected, cell passaging at a 1:5 dilution is recommended as cell colonies tend to exhibit higher antibiotic resistance than single cells
24A(xi)	At 2 weeks of monoclonal selection, it is difficult to determine whether observed cells are derived from one or multiple colonies	Cells have become too confluent, and populations derived from multiple colonies might have merged into each other	Scan the 96-well plate at 1 week when individual clones are still distinguishable and discard wells with more than one colony
25D(iii)	Incomplete photobleaching of the target cell	Target cell is too large or very wellcoupled with neighboring cells	Increase laser intensity, pulse duration, and the number of pulses. If the cells are too well-coupled such that fluorescence recovery occurs too quickly with respect to photobleaching, try seeding the cells at a lower density and performing photobleaching with the target cell in a small group of cells or a cell pair
25E(iii)	Inconsistent frame of image acquisition	Coverslip shifting during recording	Seed the cells directly onto the fibronectin-coated glass-bottom dish
25E(vii)	High variation in measured gap-junctional conductance even in monoclonal lines	Inability to control different parameters of the cell pairs, such as cell size and cell-cell contact length	For a more reproducible and well-controlled method of generating cell pairs, we recommend that users use a micropatterning technique, if possible, as described in our previously published protocol ⁷⁷
Box 1, step 10	Low viral titer for constructs containing Kir2.1	Membrane hyperpolarization of HEK-293FT cells due to Kir2.1 expression could lead to high toxicity	Inhibit Kir2.1-induced membrane hyperpolarization by adding 5 mM BaCl ₂ to the growth media during lentivirus production

Table 4 |

Electrical and conduction properties of engineered cells

Cell type	APD ₈₀ (ms)	(dV/dt) _{max} (V/s)	CV (cm/s)
HEK Na _v 1.5 + Kir2.1 + Cx43 monoclonal Mean	20.9	150.6	23.3
s.d.	5.7	31.7	3.7
Coefficient of variation	0.27	0.21	0.16
HEK ShePm + Kir2.1 + Cx43 polyclonal			
Mean	81.2	117.5	9.7
s.d.	27.2	67	0.5
Coefficient of variation	0.33	0.57	0.05
HEK ShePm + Kir2.1 + Cx43 monoclonal			
Mean	62.35	151.65	19.3
s.d.	11.93	36.28	1.7
Coefficient of variation	0.19	0.24	0.09
HEK RosDm + Kir2.1 + Cx43 polyclonal			
Mean	286.4	40.5	3.6
s.d.	163	22	0.5
Coefficient of variation	0.57	0.54	0.14
HDF ShePm + Kir2.1 + Cx43 unsorted			
Mean	90.48	107.29	9.02
s.d.	33.82	51.15	1.43
Coefficient of variation	0.37	0.48	0.16
HDF ShePm + Kir2.1 + Cx43 sorted			
Mean	89.98	99.89	8.99
s.d.	28.20	25.46	0.85
Coefficient of variation	0.31	0.25	0.09