

A wearable non-invasive sonogenetic pacemaker

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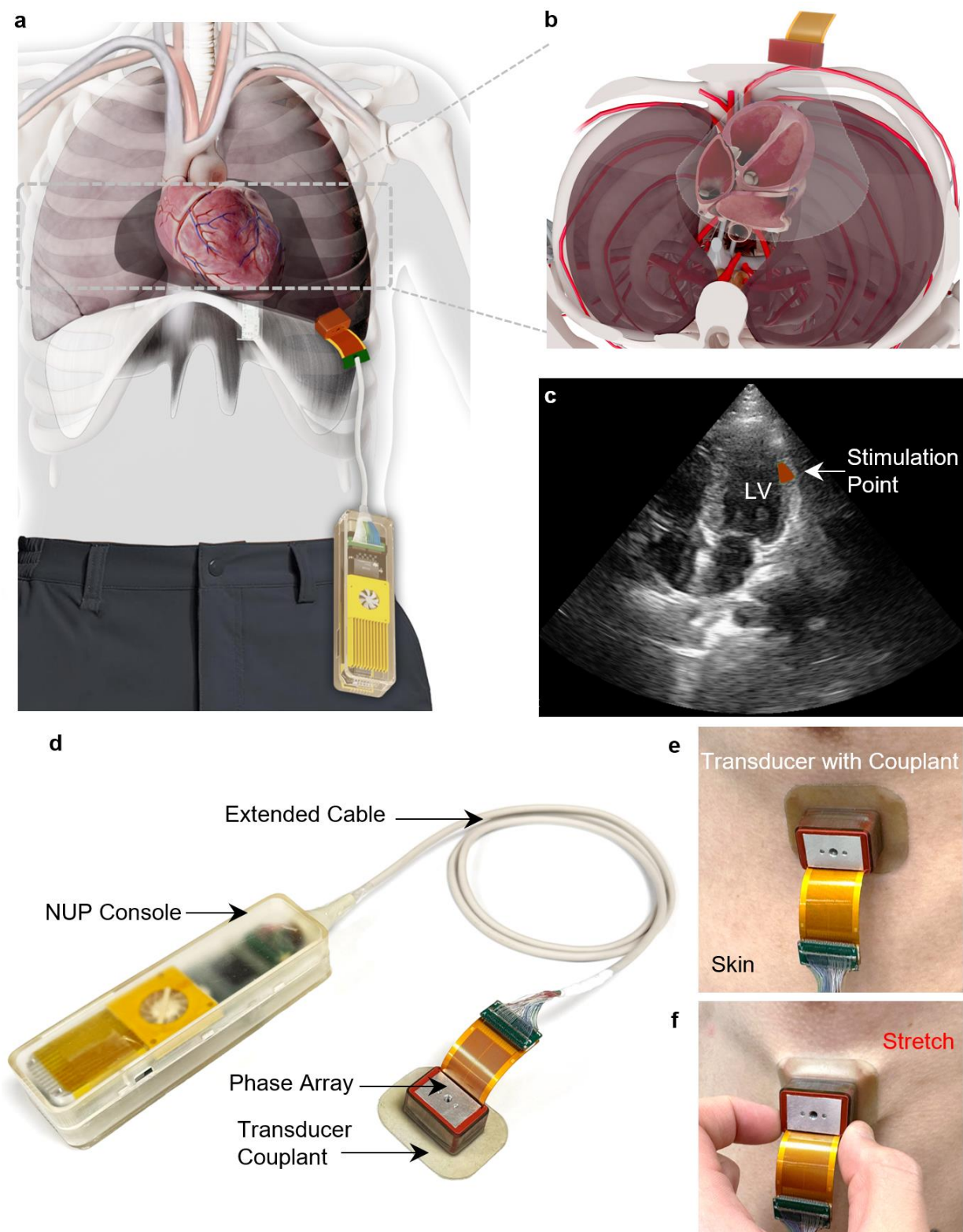
A wearable non-invasive sonogenetic pacemaker

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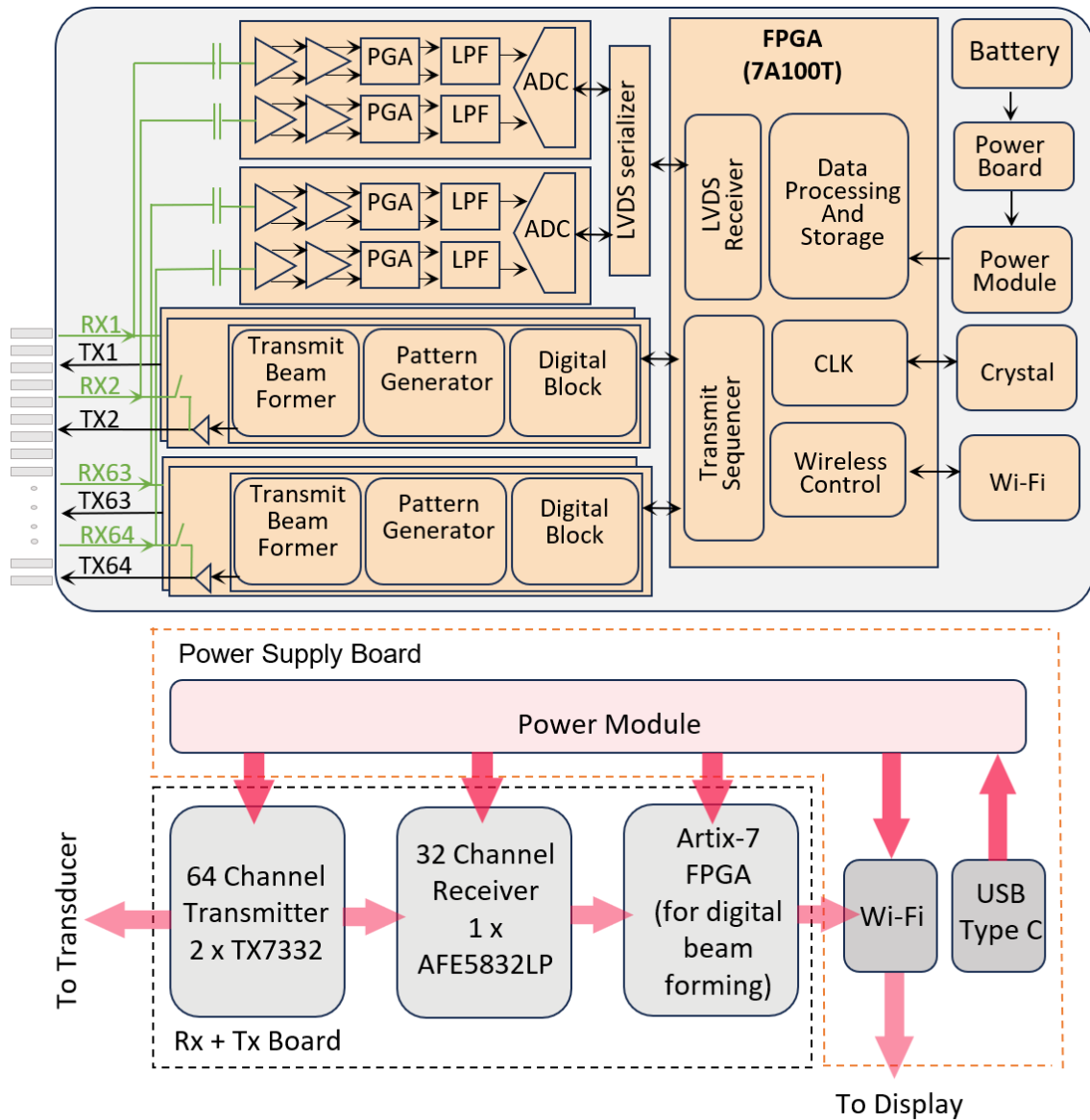
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1 Supplementary Figures



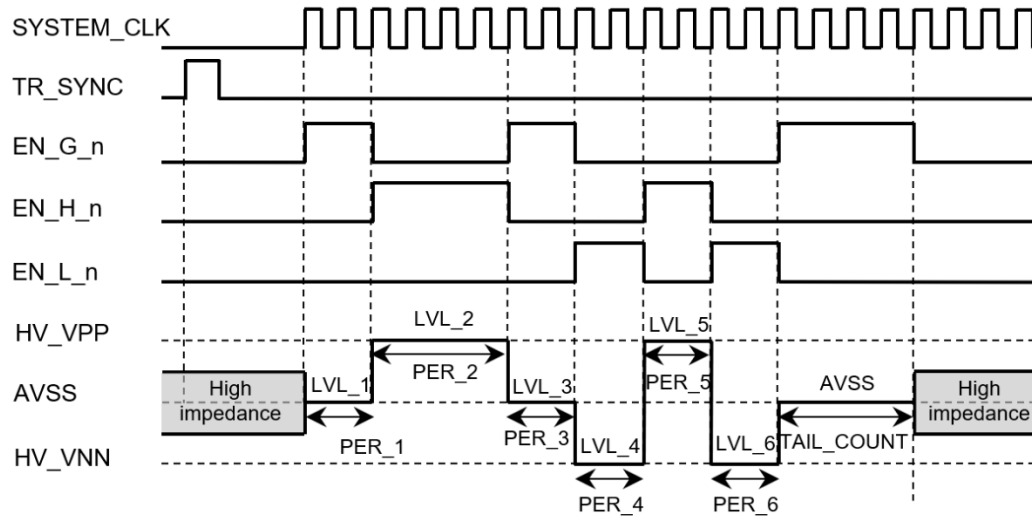
2 **Supplementary Fig. 1 | NUP system and its application for cardiac stimulation.** a, Schematic illustration
 3 of the NUP system applied for non-invasive cardiac pacing, showing its placement on the chest and connection
 4 to the portable control unit. b, Anatomical visualization of the heart in relation to the ultrasound stimulation
 5 site. c, B-mode ultrasound imaging of the left ventricle (LV), highlighting the targeted stimulation point. d,
 6 Illustration of the NUP system components, including the phased array transducer, coupling layer, and
 7 extended cable connecting to the control unit. e and f, Demonstration of the transducer attachment to the skin
 8 with a flexible couplant layer, showing its adaptability to skin deformation during movement.



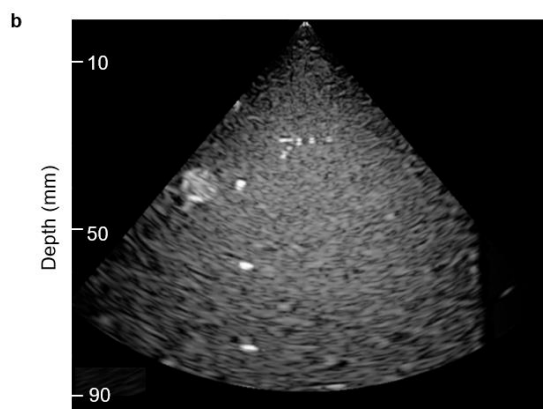
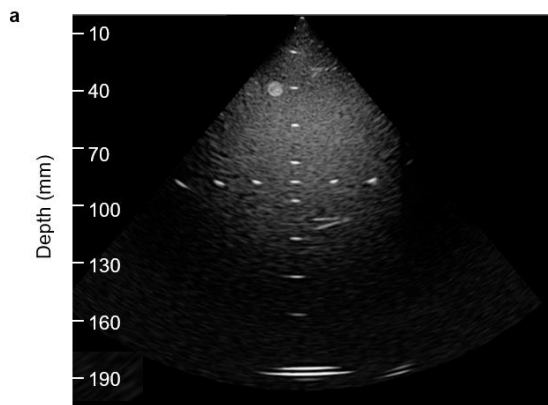
Supplementary Fig. 2 | Block diagram of the NUP system. Block diagram detailing the functional modules and signal transmission pathways within the ultrasound pacemaker, including components such as the analog front end (AFE), transmit beam former, pattern generator, and digital blocks, illustrating how the ultrasound signal is generated, controlled, and delivered to achieve effective cardiac pacing.

Pattern generator output for

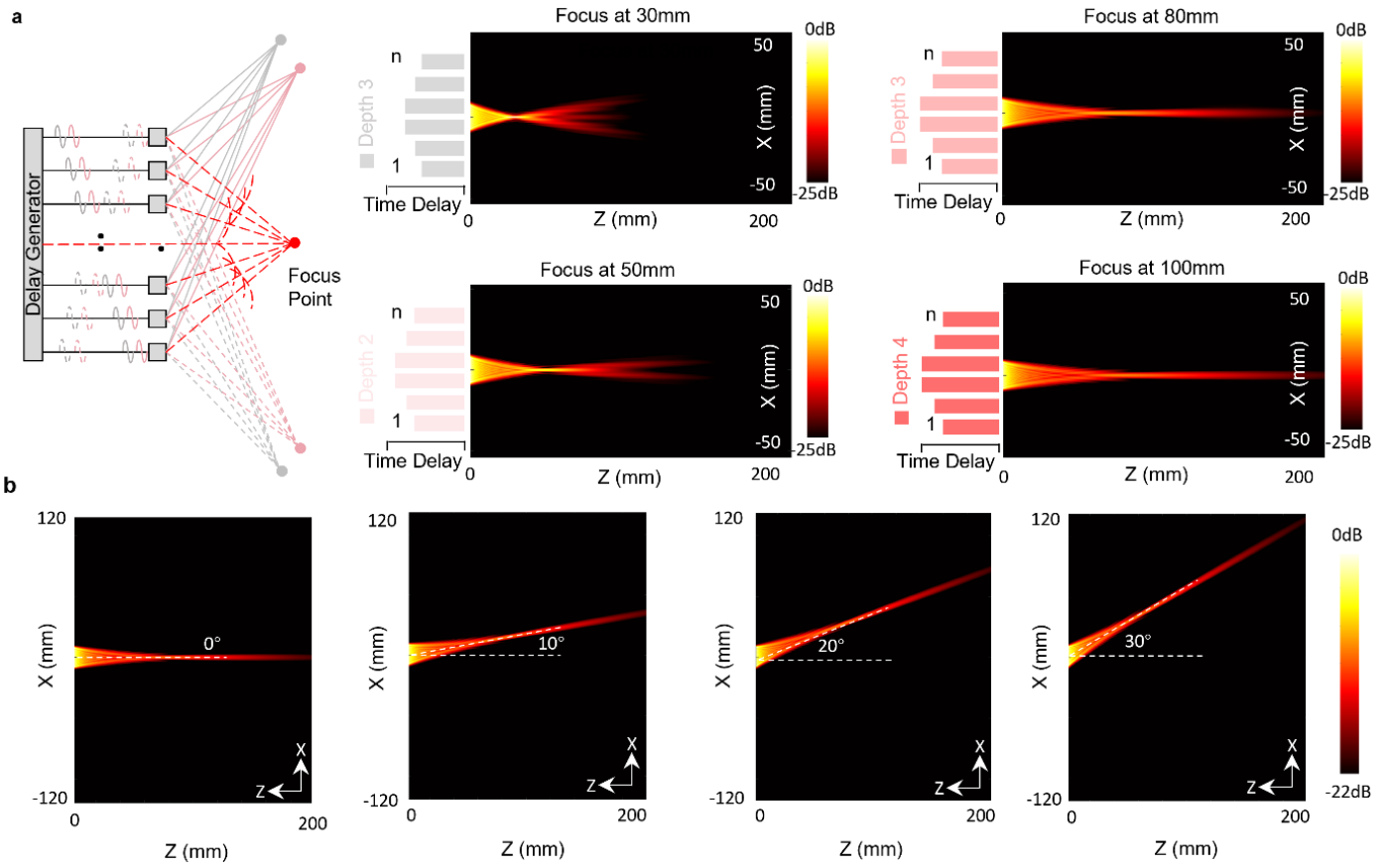
LVL_1 = 0b011, PER_1=0d0	LVL_5 = 0bx10, PER_5=0d0
LVL_2 = 0bx10, PER_2=0d2	LVL_6 = 0bx01, PER_6=0d0
LVL_3 = 0b011, PER_3=0d0	LVL_7 = 0b111, PER_2=0dx
LVL_4 = 0bx10, PER_4=0d0	TAIL_COUNT = 0d3, REPEAT_COUNT= 0



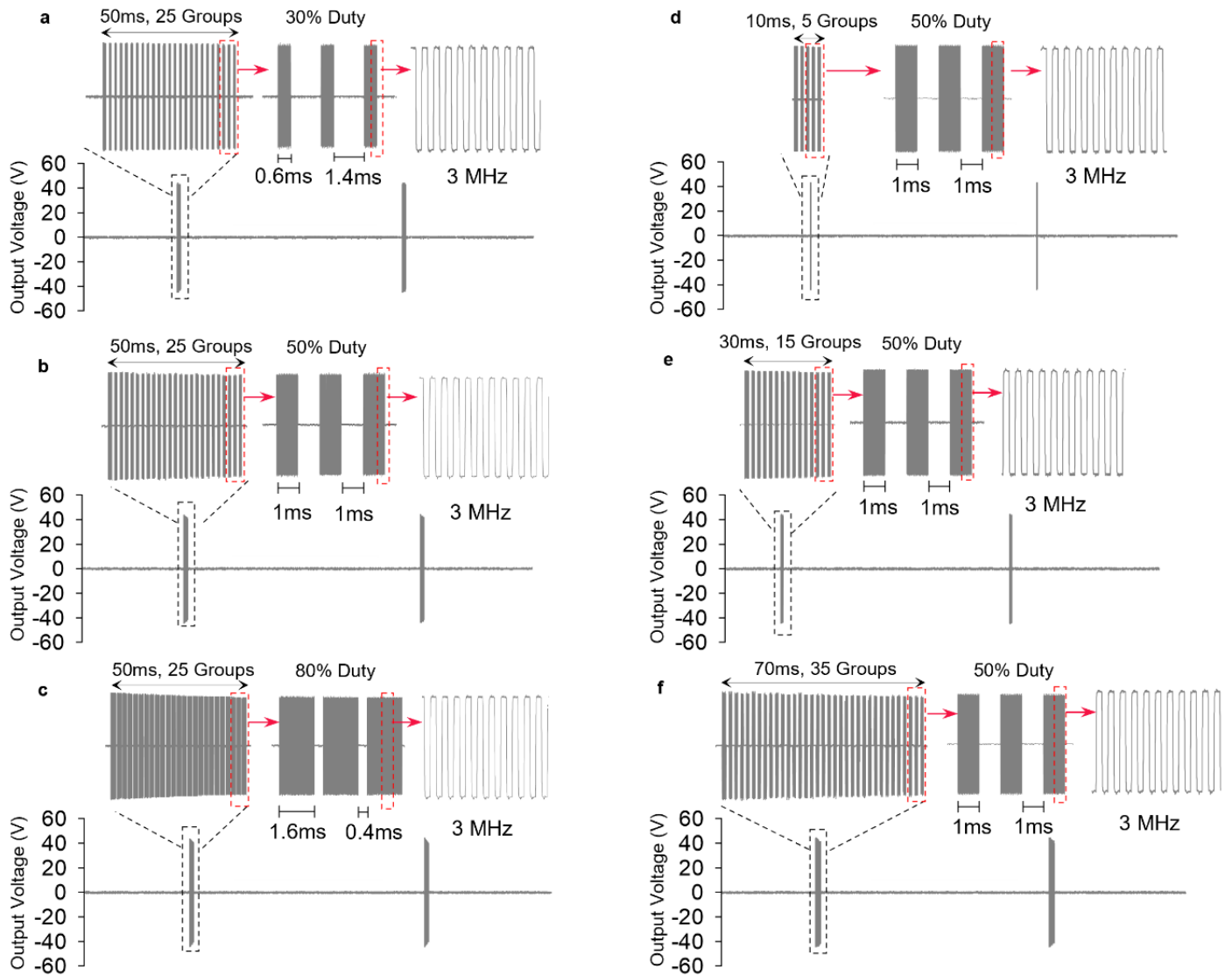
Supplementary Fig. 3 | Diagram of control sequence. Simulated control sequence of the main control TX 7332 for multiplexing and pulse-echo sensing.



Supplementary Fig. 4 | Resolution testing of the 64-channel phased array transducer. Resolution testing at focal depths of 100 mm **a**, and 40 mm **b**, using a phantom.



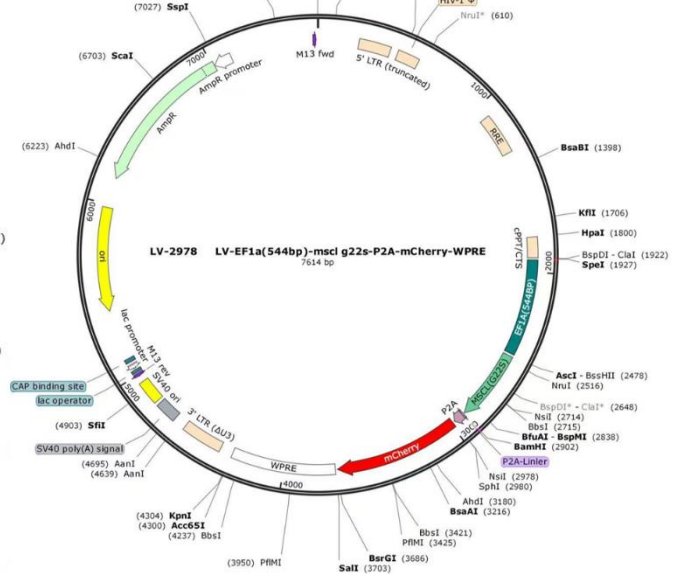
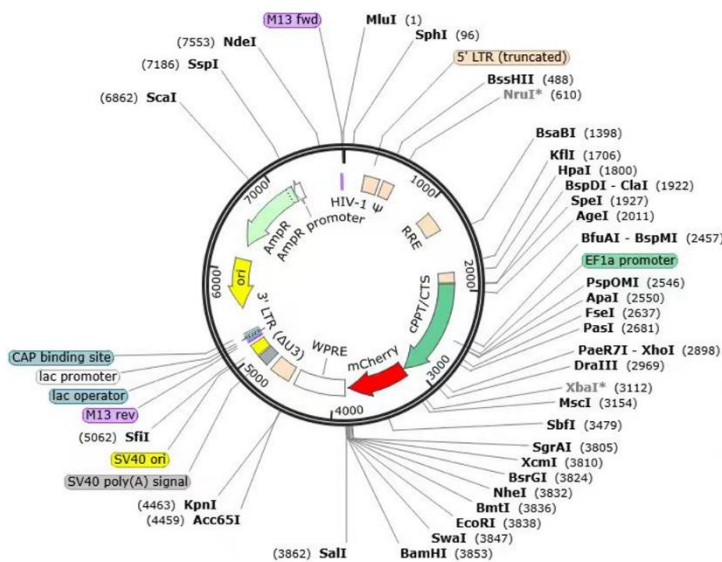
Supplementary Fig. 5 | Simulation focused acoustic fields of a 64-channel phased array transducer. a, Simulated beam patterns in the xz-plane at various focal depths: 30 mm, 50 mm, 80 mm, and 100 mm. **b,** Beam shapes in the xz-plane for a focal depth of 100 mm with beam steering angles of 0°, 10°, -20°, and 30°, along with the corresponding xy-plane views at $z = 100$ mm. Scale bar = 50 mm. (Transducer frequency: 3.0 MHz, Kerf: 0.02 mm, single element width: 0.254 mm, split into two sub-elements of 0.127 mm width; element length: 13 mm. Simulations were performed using Field II.). SPL (a.u.), SPL: Sound pressure level.



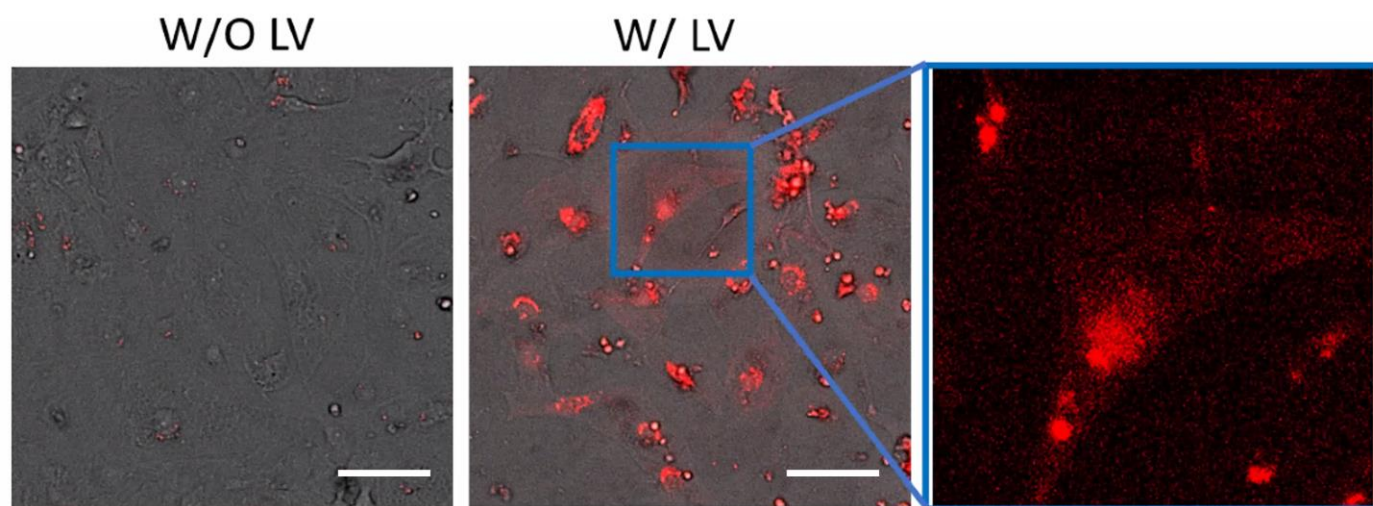
1 **Supplementary Fig. 6 | Waveform outputs of the NUP system.** Figure A–F shows the output waveforms in
2 pure stimulation mode. **a**, A single 50 ms output, comprising 25 groups, each lasting 2 ms. Each group consists
3 of a continuous 3 MHz square wave emission for 0.6 ms, followed by a 1.4 ms rest period, resulting in a total
4 duty cycle of 30%. **b**, A single 50 ms output with a duty cycle of 50%, where each group includes a continuous
5 3 MHz square wave emission for 1 ms and a 1 ms rest period. **c**, A single 50 ms output with an 80% duty cycle,
6 where each group comprises a 1.6 ms continuous 3 MHz square wave emission followed by a 0.4 ms rest.
7 Outputs with a 50% duty cycle and varied emission durations: **d**, 10 ms (5 groups of 2 ms each), **e**, 30 ms, and
8 **f**, 70 ms. upplementary Fig. 7. Schematic diagram of the viral plasmid construction.

Plasmid Map of LV-EF1a(544bp)-mCherry-WPRE

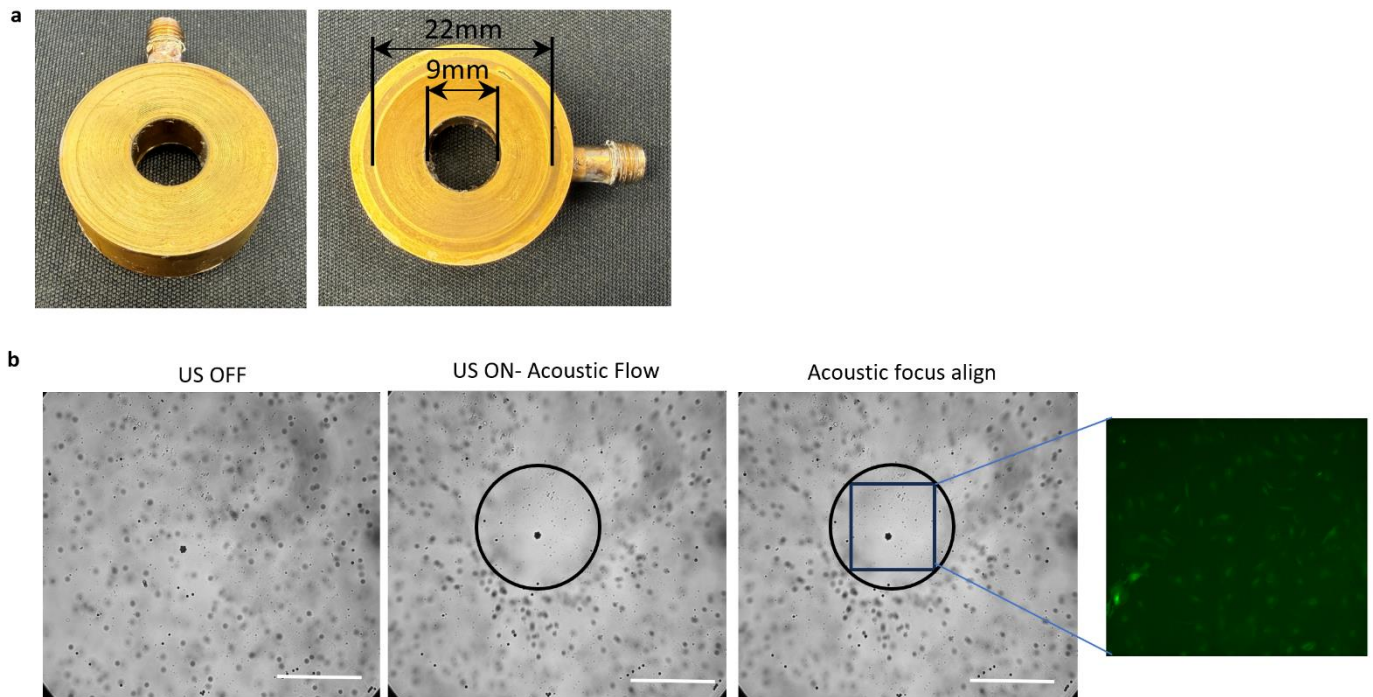
Plasmid Map of LV-EF1a(544bp)-MscL G22S-P2A-mCherry-WPRE



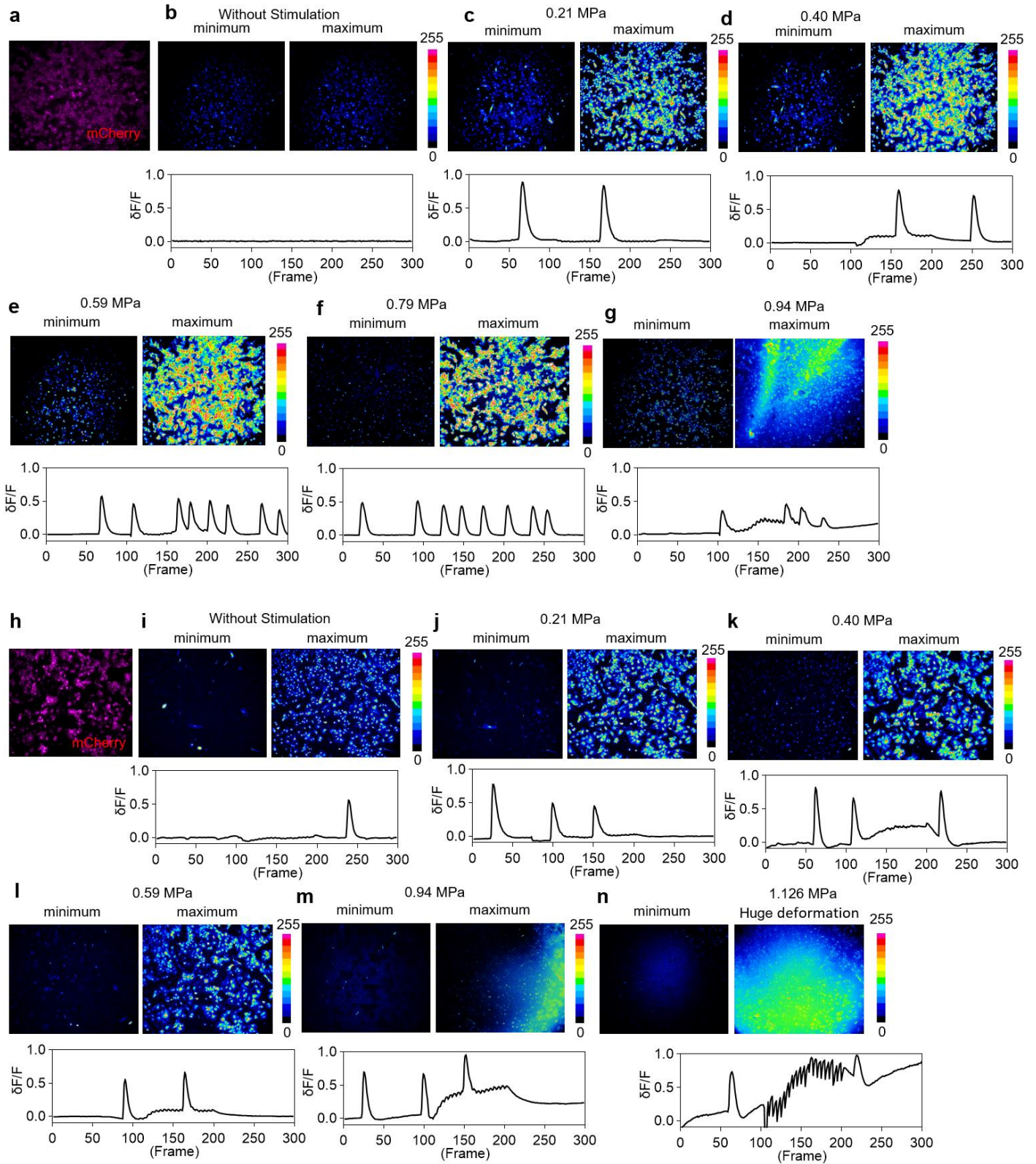
Supplementary Fig. 7 | Schematic diagram of the viral plasmid construction. This figure displays plasmid maps for the experimental and control lentiviral vectors used in this study. (Right) Utilizes P2A to connect MscL-G22S, GCaMP8m and mCherry for non-fusion expression, which allow MscL-G22S, mCherry to be expressed separately. P2A generally exhibits the highest cleavage efficiency (nearly 100% in some cases), the cleavage site is located between Gly-/-Pro. mCherry serves as a reporter for transduction/expression and provides a proxy for construct expression. (Left) The plasmid LV-EF1a-mCherry-WPRE served as the negative control.



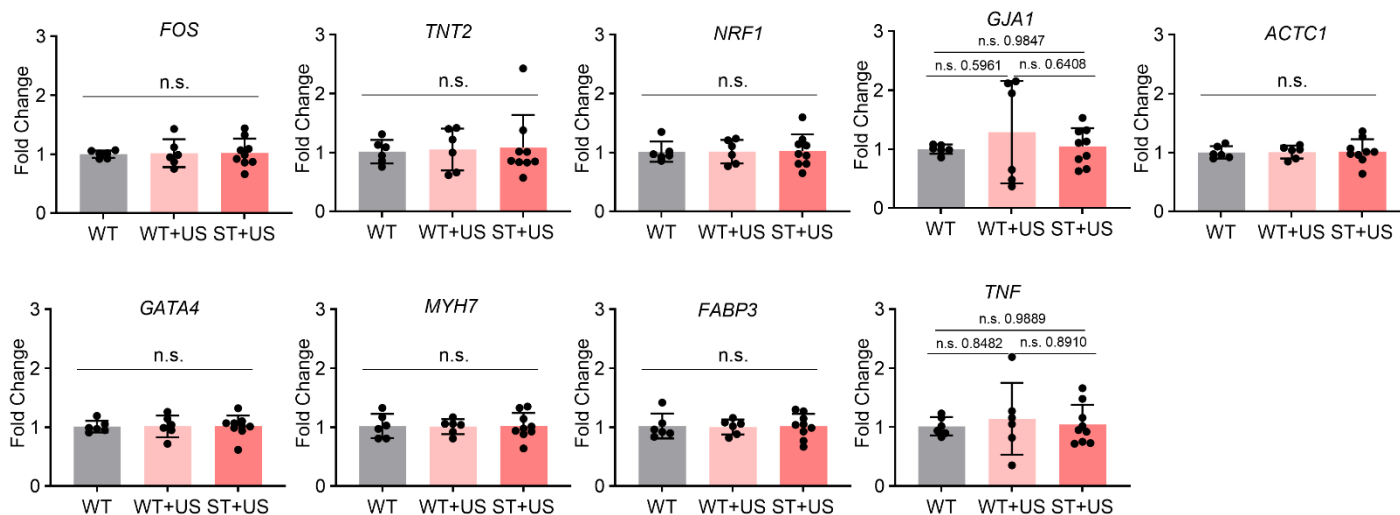
Supplementary Fig. 8 | Cell preparation for the stimulation experiment. Fluorescence microscopy images showing the transfection efficiency of cardiomyocytes, with red fluorescence indicating successful expression of the mCherry reporter gene. Cells without lentiviral transfection (W/O LV) are shown on the left, while transfected cells (W/ LV) are depicted on the right, with a magnified inset highlighting mCherry expression in individual cells. Using 4× objective lens. Scale bar = 100 μ m.



Supplementary Fig. 9 | Instrumentation setup for the stimulation experiment. **a**, Front and top views of the custom-made ultrasound probe used for stimulation, with dimensions of 22 mm outer diameter and 9 mm inner diameter. Pulse-echo testing was conducted to assess probe performance, and the corresponding frequency response was analyzed using Fast Fourier Transform to determine the center frequency and bandwidth. **b**, Distribution of microbeads within the field of view under Ultrasound OFF and ON conditions, as observed through a 4× objective lens. The activation of ultrasound resulted in a noticeable redistribution of microbeads, the black circle indicates acoustic flow induced by the ultrasound. The blue square indicates the view of the 20× objective lens. Scale bar: 1mm.

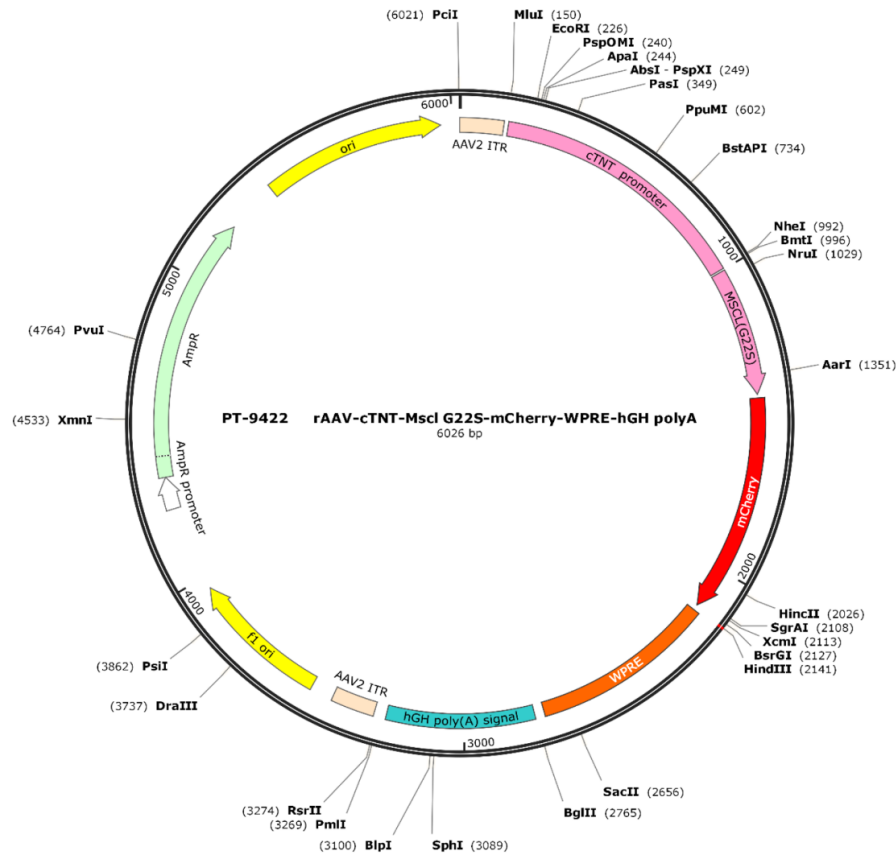


Supplementary Fig. 10 | Calcium imaging of MscL-G22S-mediated sonogenetic activation in cardiomyocytes at varying acoustic pressures. Each condition includes minimum and maximum fluorescence intensity images and a corresponding $\Delta F/F$ trace. **a** is a representative mCherry fluorescence image. **b** shows the baseline recording without ultrasound stimulation. **c-g** show recordings during the application of increasing acoustic pressures, specifically 0.21 MPa in **c**, 0.40 MPa in **d**, 0.59 MPa in panel **e**, 0.79 MPa in **f**, and 0.94 MPa in **g**. **h-n** show calcium imaging of control cardiomyocytes transduced with mCherry only. **h** is a representative mCherry fluorescence image for the control group. **i** shows the baseline recording without ultrasound stimulation. **j-n** show recordings during the application of increasing acoustic pressures, specifically 0.21 MPa in **j**, 0.40 MPa in **k**, 0.59 MPa in **l**, 0.94 MPa in **m**, and 1.126 MPa in **n**.

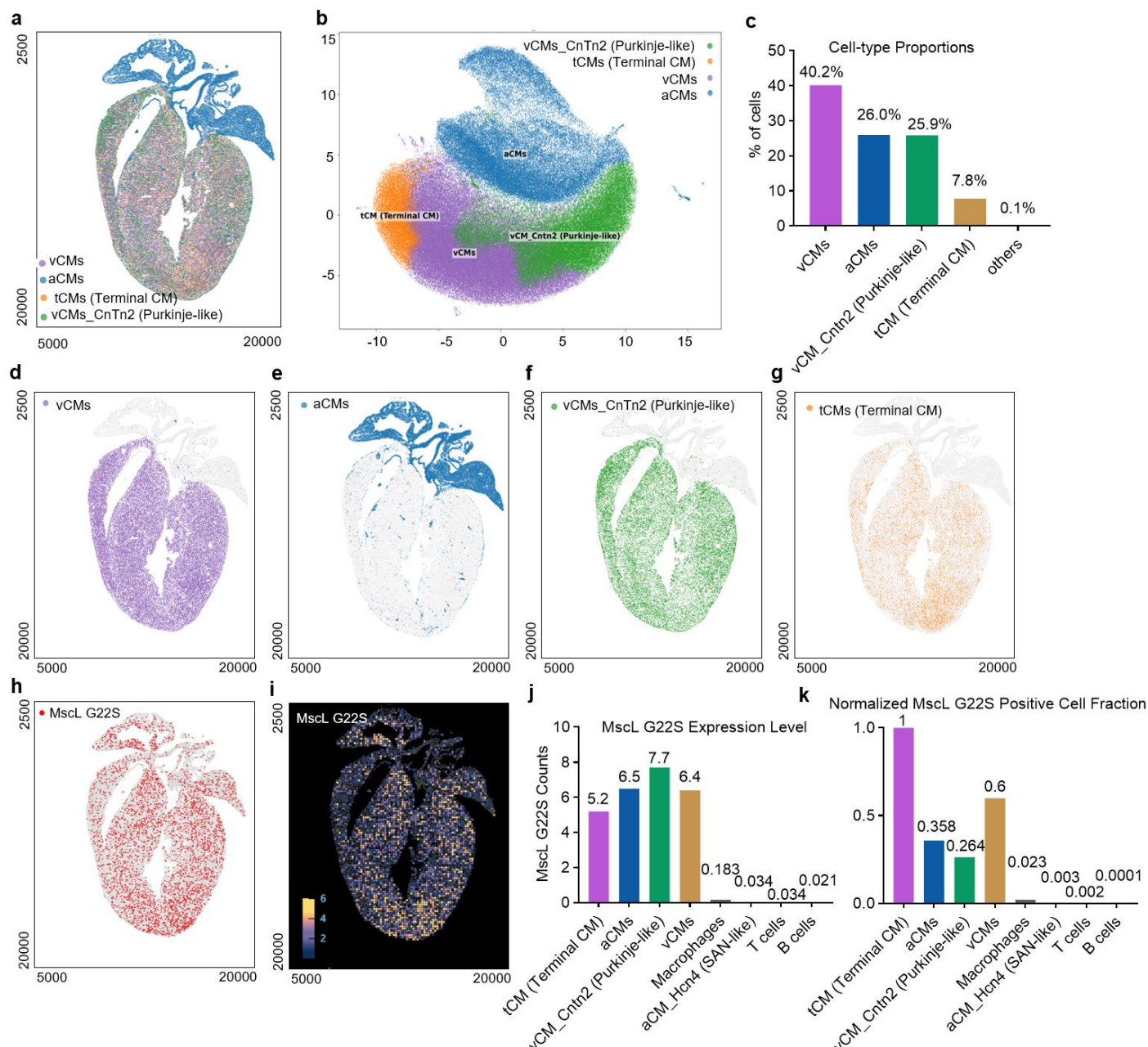


Supplementary Fig. 11 | Gene expression analysis following ultrasound pacing in cardiomyocytes. RT-qPCR was performed 1 day after the ultrasound stimulation protocol to quantify mRNA levels of *FOS*, *TNNT2* (cardiac troponin T), *NRF1*, *GJA1*, *ACTC1*, *GATA4*, *MYH7*, *FABP3*, and *TNF*. Transcript levels were normalized to *GAPDH* and plotted as fold change relative to the WT (unstimulated) group. No significant differences were detected among groups for the genes tested. Dots represent individual samples; bars show mean $\pm$ s.e.m. Sample sizes were: WT (no US), $n = 6$; WT + US, $n = 6$; ST + US, $n = 9$, where ST indicates sonogenetics-transfected cardiomyocytes. Statistics: one-way ANOVA for each gene followed by two-sided Tukey's multiple-comparisons test; n.s., $P > 0.05$ (exact P values shown where indicated).

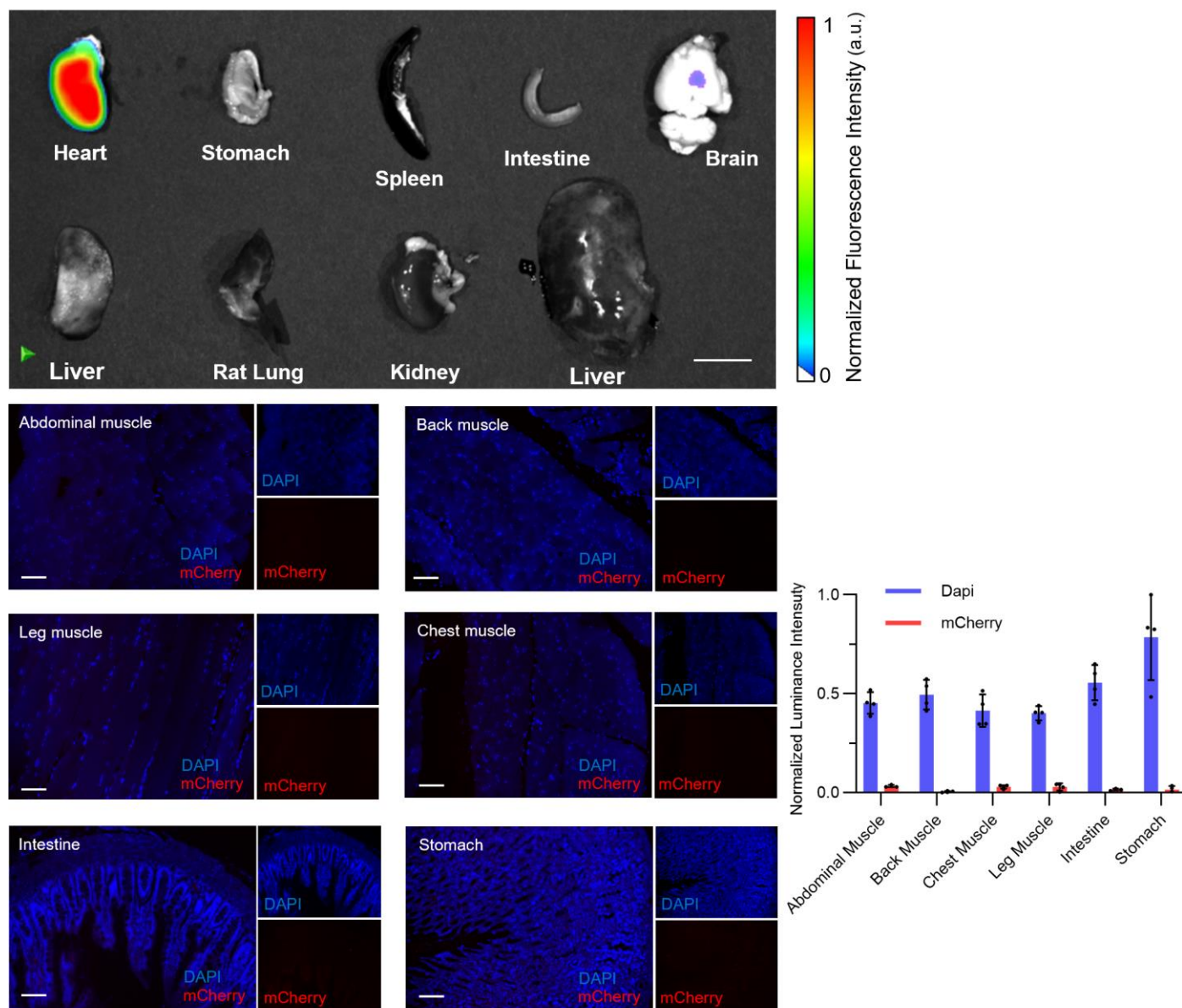
Plasmid Map of AAV2-cTNT-MscI G22S-mCherry-WPRE-polyA



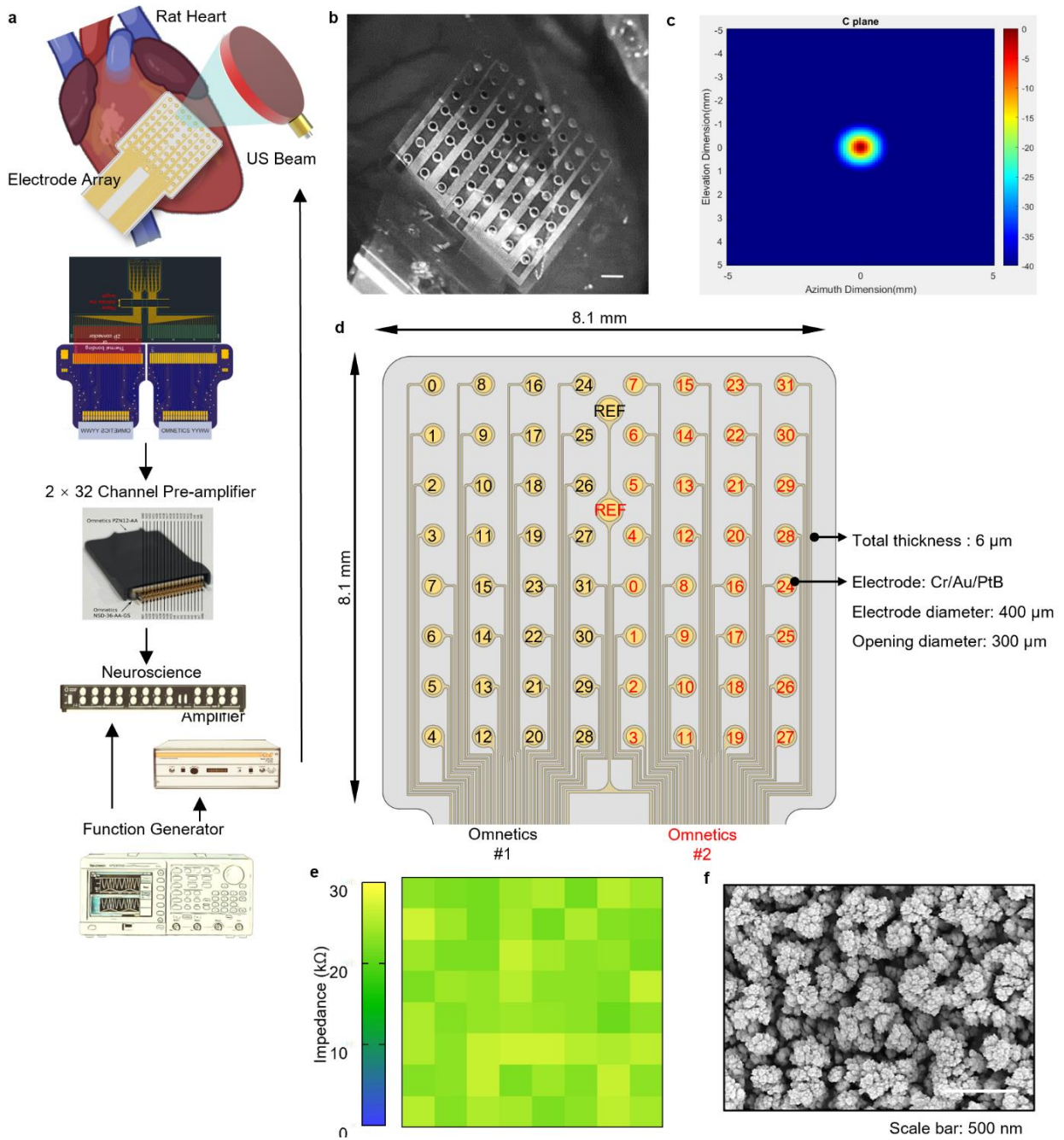
Supplementary Fig. 12 | Viral plasmid design for cardiomyocyte-targeted expression of MscL-G22S. Schematic of the expression cassette in which the mechanosensitive channel MscL-G22S is expressed as a C-terminal fusion with mCherry, joined by a GSAT linker. Expression is driven by the cardiomyocyte-enriched cTnT (*TNNT2*) promoter. The mCherry tag enables visualization of the spatial distribution of the fusion protein and provides a qualitative readout of expression. WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) was included to enhance transgene expression, and hGH poly(A) provides a transcription termination and polyadenylation signal



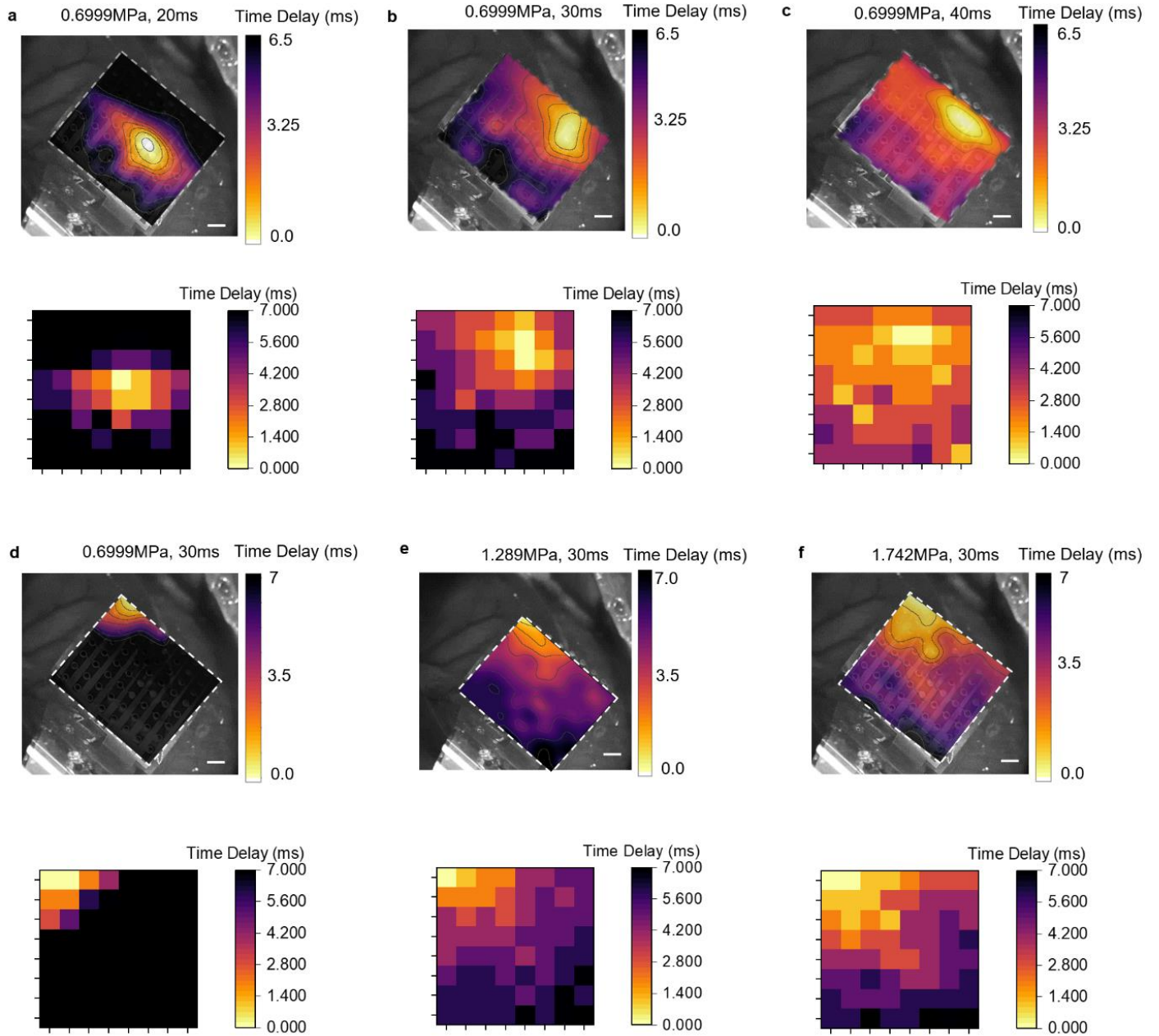
Supplementary Fig. 13 | Spatial transcriptomic analysis of cell populations and MscL G22S expression in the mice transduced heart. **a**, Spatial plot of annotated cell types overlaid on the cardiac tissue section. **b**, UMAP plot identifying distinct cell type clusters. **c**, Bar chart showing the cell-type proportions of the identified populations. **d-g**, Spatial distribution maps for the four major cardiomyocyte subtypes identified: ventricular (vCMs), atrial (aCMs), Purkinje-like (vCMs_cnTn2), and terminal (tCMs) cardiomyocytes. **h-i**, Spatial plot (**h**) and intensity map (**i**) showing the location and expression level of the MscL G22S transgene. **j**, Quantification of the average MscL-G22S expression level (counts) across different cell types. **k**, Normalized fraction of MscL G22S-positive cells within each annotated cell type.



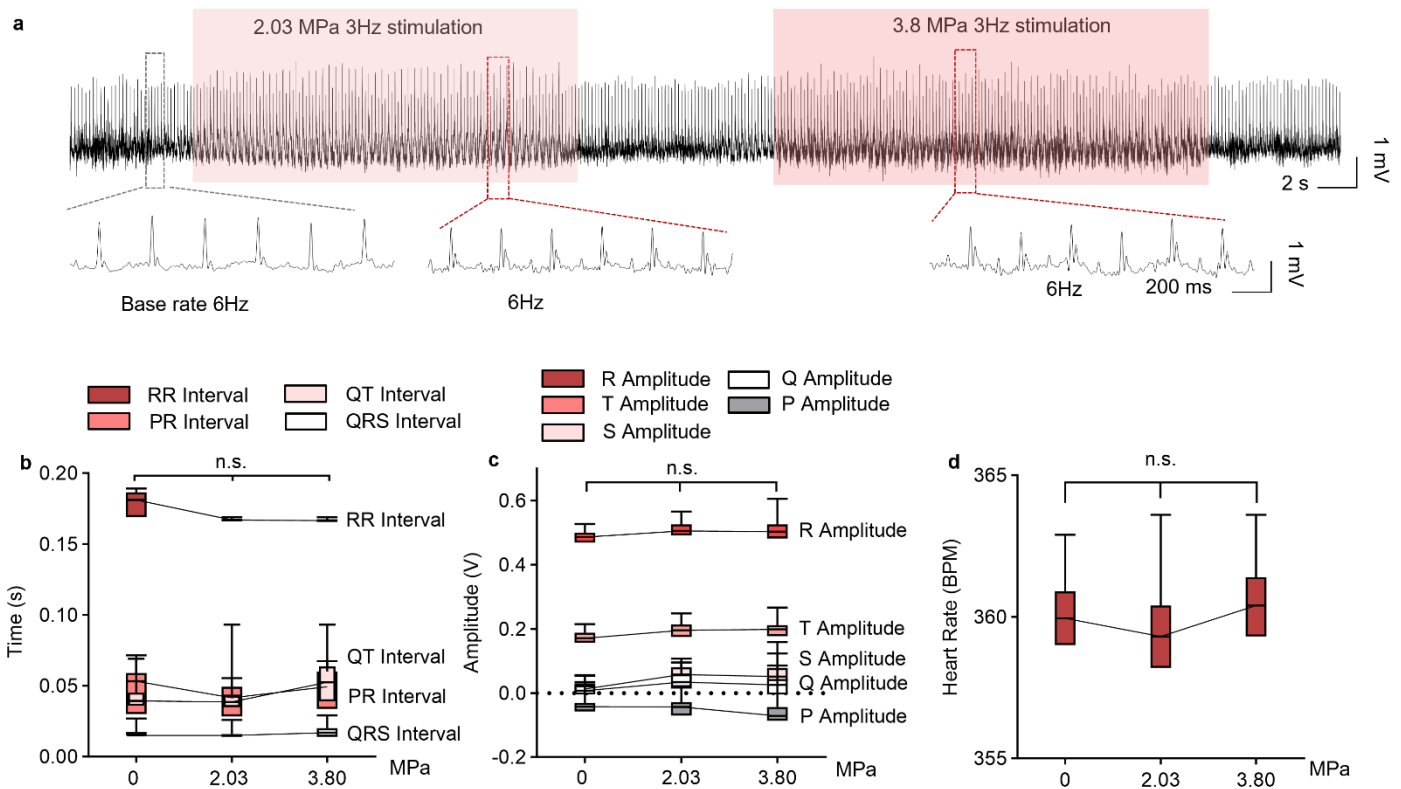
Supplementary Fig. 14 | Systemic and tissue-level distribution of mCherry reporter expression. Top, representative ex vivo IVIS fluorescence imaging of major organs collected from an ST rat at 3 months post-transduction. Scale bar, 10 mm. Bottom left, representative fluorescence microscopy images of non-cardiac tissues (abdominal, back, chest and leg muscle, intestine, and stomach). Insets show DAPI-stained nuclei (blue) and mCherry signal (red). Scale bar, 200 μ m. Bottom right, quantification of normalized fluorescence intensity for DAPI and mCherry across tissue types (n = 4 biologically independent rats). Bars indicate mean $\pm$ s.e.m.



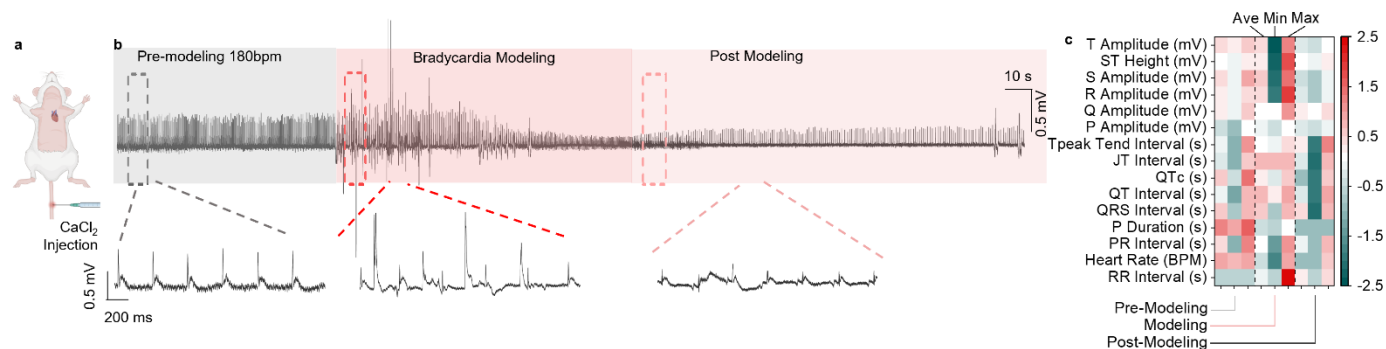
Supplementary Fig. 15 | Ultrasound stimulation for electrophysiology acquisition using a flexible microelectrode arrays (MEA). **a**, Schematic representation of the experimental setup showing the flexible MEA positioned on the surface of the rat heart, with simultaneous ultrasound beam exposure for sonogenetic stimulation. The MEA records electrophysiological signals from the heart while exposed to ultrasound energy. **(b)**, Actual photograph of the MEA positioned on the rat heart (scale bar: 1 mm), demonstrating the precise placement and contact of electrodes on the cardiac surface. **c**, Simulated ultrasound intensity distribution in the C-plane view at the focal point, illustrating the concentration of ultrasound energy at the MEA-targeted region, which is crucial for effective stimulation. **d**, Detailed design of the MEA, showing electrode arrangement, specifications including electrode diameter (400 μm), opening diameter (300 μm), electrode thickness (20 nm Cr / 200 nm Au), and total thickness (6 μm polyimide). The MEA is designed to ensure flexibility and conformal contact with cardiac tissue. The signal acquisition flow is depicted, showing the connection of the MEA to a 2 $\times$ 32 channel pre-amplifier, followed by neuroscience data acquisition hardware and a function generator with an amplifier for controlled ultrasound stimulation and accurate data collection from cardiac tissue during the experiment. **e**, Spatial map of the impedances of the electrode array at 1 kHz in in PBS (pH 7.4) at room temperature. **f**, SEM image of the nanoporous platinum black (PtB) coating on the electrodes. Parts of this figure (**a**) was created with BioRender.com.



Supplementary Fig. 16 | Heatmaps of ultrasound-provoked electrophysiological signals across the MEA-attached heart. a-c, Heatmaps displaying time delay (ms) of the ultrasound-induced signal at a constant acoustic pressure (0.6999 MPa) with varying stimulation durations (20 ms, 30 ms, 40 ms). **d-f,** Heatmaps showing time delay (ms) of the ultrasound-induced signal at a constant stimulation duration (30 ms) with different acoustic pressure (0.699MPa, 1.289MPa and 1.742 MPa). 3D surface plots are generated for each heatmap to visually represent the spatial distribution of signal propagation time delay, with lower time delay values in the center appearing at a lower depth. Scale bar: 1 mm. n=3 for each condition.

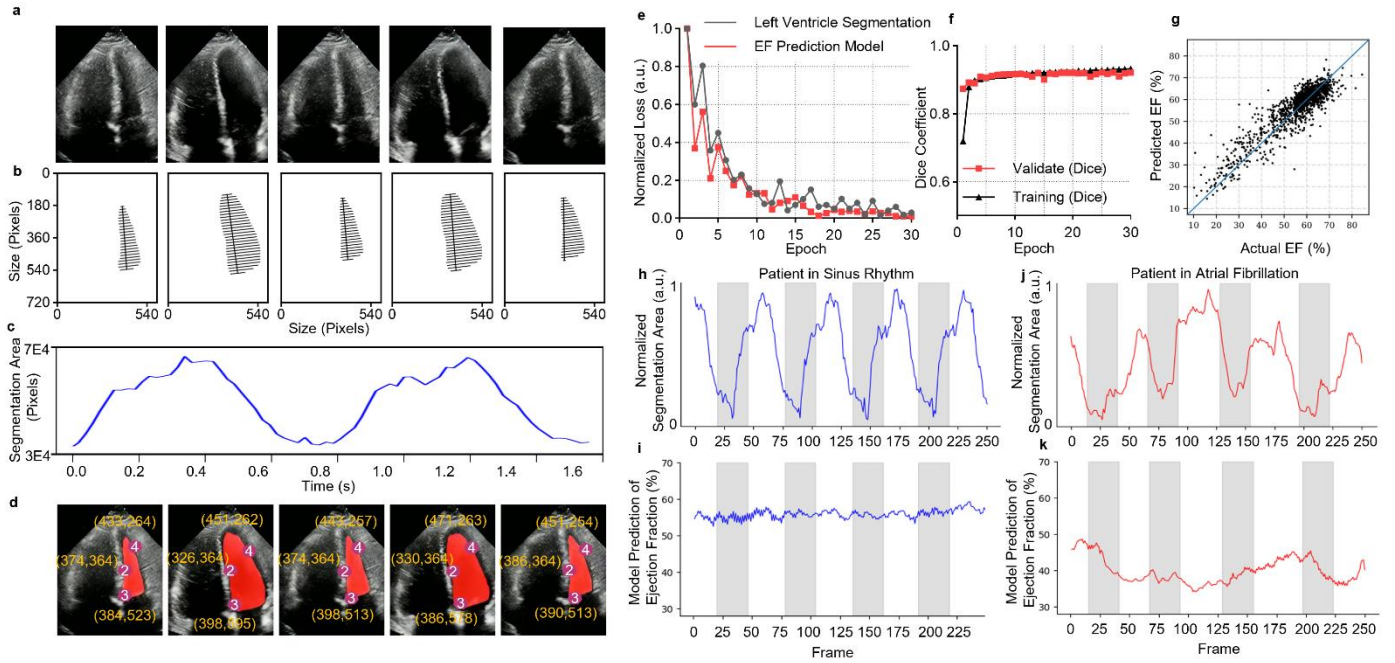


Supplementary Fig. 17 | Effects of NUP at pulse repetition frequency (PRF) lower than cardiac pacing rates on ST rat. **a**, Electrocardiogram (ECG) waveforms showing the baseline heart rate at a frequency of 6 Hz. The red-highlighted sections represent periods of ultrasound stimulation at 3 Hz, applied at two different acoustic pressures: 2.03 MPa and 3.80 MPa. Enlarged sections below the main waveform trace highlight the detailed structure of individual ECG cycles during baseline and stimulation phases, showing the changes in cardiac rhythm with reduced ultrasound frequency. **b**, Analysis of time intervals for key ECG components, including RR, QT, PR, and QRS intervals, under ultrasound stimulation at 3 Hz with increasing pressure. These data illustrate the effects of ultrasound stimulation on the temporal aspects of the cardiac cycle, showing that no statistically significant changes were observed. $n=5$. **c**, Changes in the amplitude of PQRST components (P, Q, R, S, and T amplitudes) at different pressures during the 3 Hz ultrasound stimulation, providing insight into how the reduced frequency impacts the electrical signals of myocardial depolarization and repolarization. **d**, Heart rate variations (in beats per minute, bpm) under baseline and ultrasound stimulation at 2.03 MPa and 3.80 MPa, showing that there were no significant differences in heart rate during these conditions. Data are presented as mean $\pm$ standard deviation, with "n.s." indicating no statistically significant differences. **b-d**, Box plots show the median (centre line), the interquartile range (box; 25th–75th percentiles), and whiskers extending to the minimum and maximum values.

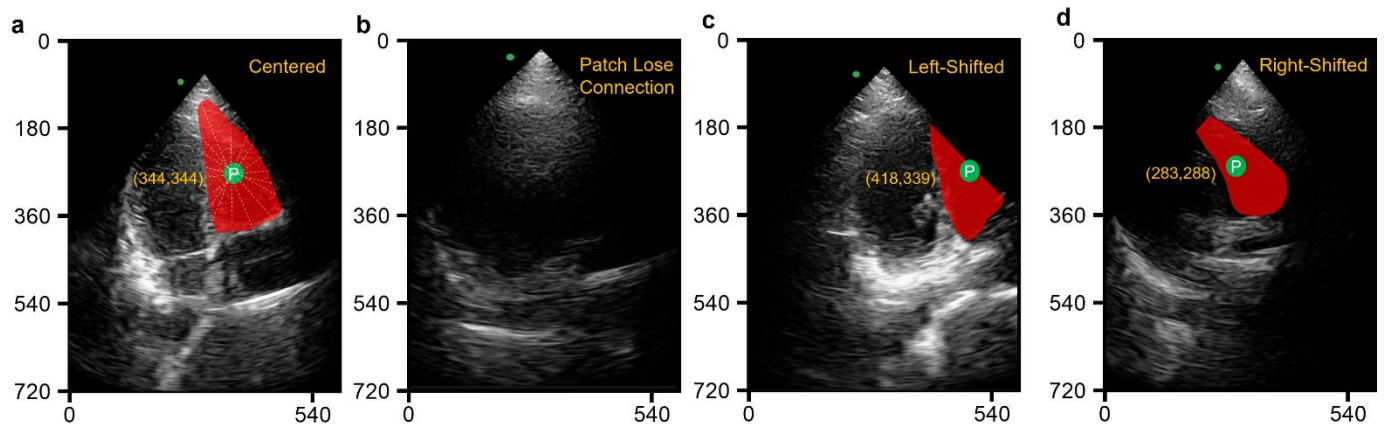


Supplementary Fig. 18 | ECG recordings and analysis of ST arrhythmia rat without ultrasound pacing.

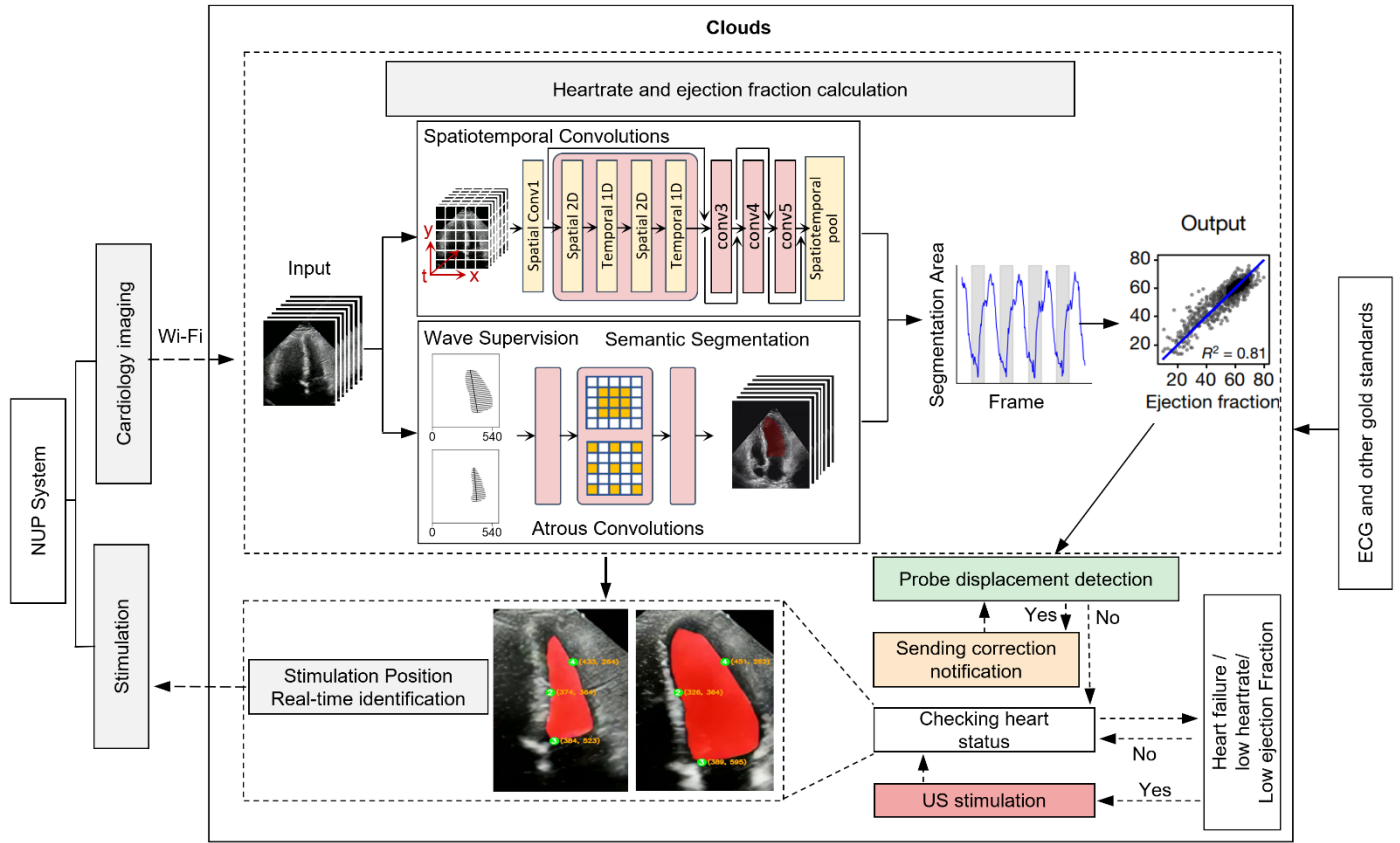
This figure shows control experiments for a ST rat after a chemically-induced arrhythmia, with no ultrasound pacing applied. The ECG traces in panels **b** show the progression from a stable baseline (Pre-modeling, ~180 bpm), through the onset of arrhythmia (Bradycardia Modeling), and into a subsequent observation period (Post Modeling). Insets below each trace provide magnified views of the ECG waveform for each respective stage. The heatmaps in panels **c** and **f** display a statistical analysis of key ECG parameters, normalized by Z-score. n=5. Parts of this figure (**a**) was created with BioRender.com.



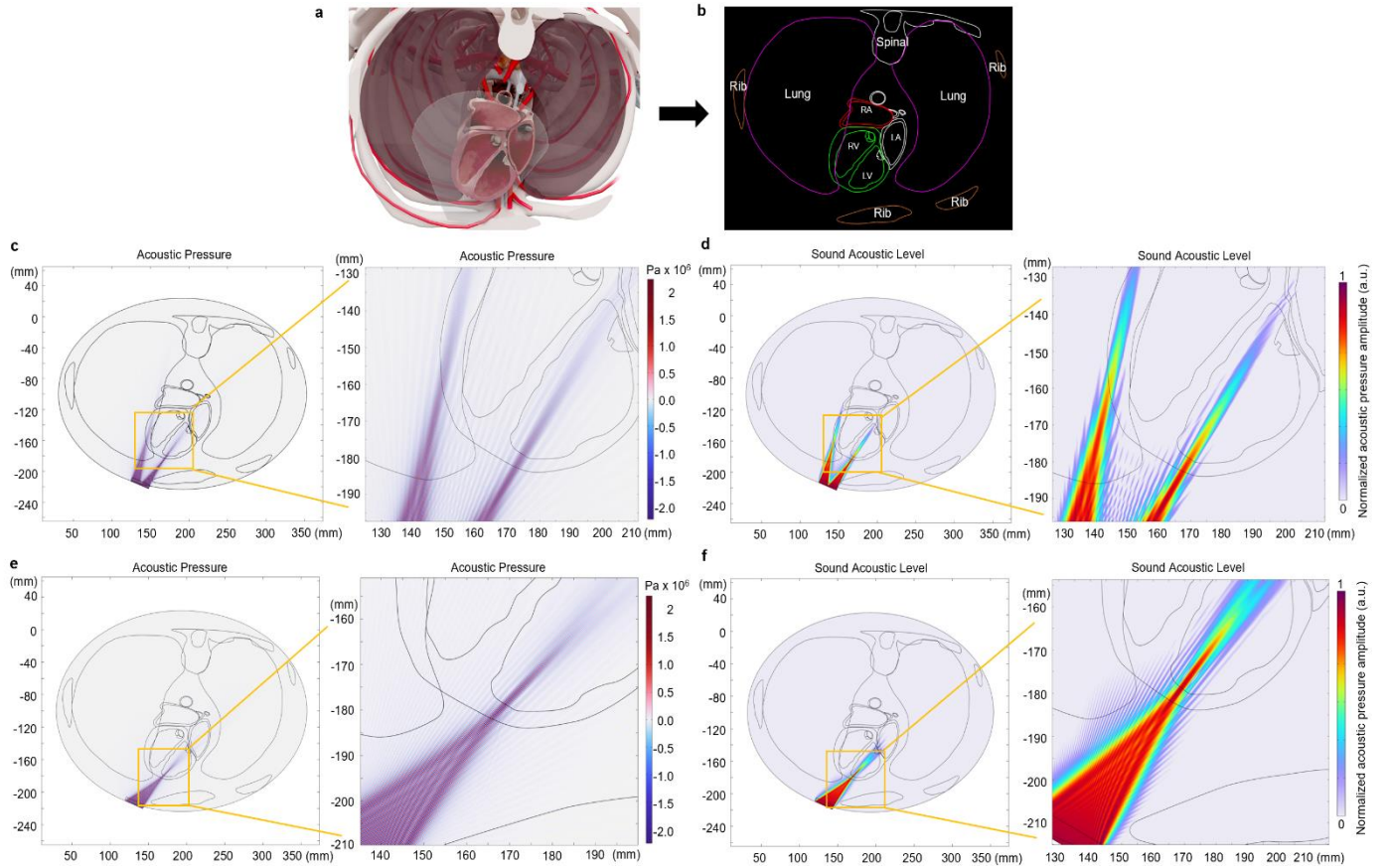
Supplementary Fig. 19 | Machine learning model (left ventricle segmentation and ejection fraction prediction) performance. **a**, Representative transthoracic echocardiography B-mode frames of the human heart (apical four-chamber view). **b,c**, Automated LV cross-sectional area tracking across the cardiac cycle, with example frames (**b**) and the corresponding LV area–time trace (**c**, area in pixels). **d**, Example visualization of three stimulation-point positions overlaid on the ultrasound image (pixel coordinates). **e**, Training loss curves for the LV segmentation model (DeepLabV3) and EF prediction model (VideoResNet). Loss is plotted as normalized values (a.u.) over training epochs. **f**, Dice similarity coefficient (unitless, 0–1) for the LV segmentation model on training and validation datasets across epochs. **g**, Predicted EF (%) versus reference EF (%) for the test set; the diagonal line indicates identity. **h**, LV segmentation area over time for a representative subject in sinus rhythm (area in pixels; time in seconds). **i**, Frame-by-frame EF predictions for the same subject in sinus rhythm (EF in %; frame index, a.u.). **j**, LV segmentation area over time for a representative subject with atrial fibrillation (area in pixels; time in seconds). **k**, Frame-by-frame EF predictions for the atrial-fibrillation example (EF in %; frame index, a.u.).



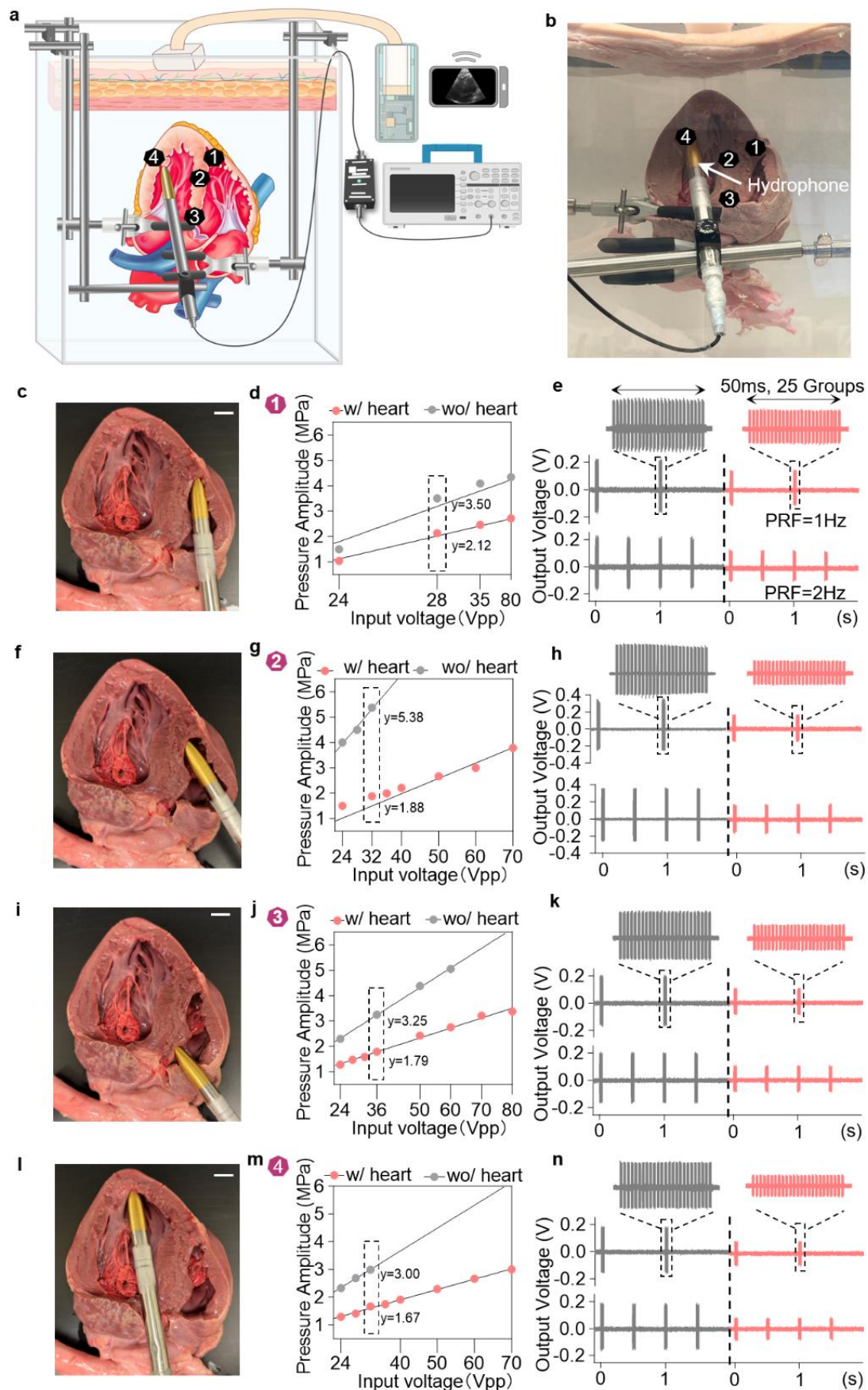
Supplementary Fig. 20 | Real-time AI-guided feedback for probe displacement. Representative ultrasound images showing the automated feedback system for maintaining correct probe alignment. **a**, Centered probe with full visualization of the left ventricle (red overlay). **b**, Connection lost due to ineffective coupling or the heart moving outside the imaging field. **c** Left-shifted and **d** right-shifted probes, with directional correction alerts generated to guide realignment.



Supplementary Fig. 21 | The flowchart of the NUP system with cloud-based AI model. The NUP system has two primary functions: imaging and stimulation. Imaging data is continuously transmitted via Wi-Fi to a cloud server for processing. The cloud-based model includes two key functions: predicting ejection fraction and segmenting the left ventricle, while providing real-time coordinates of three points. The left ventricle was automatically segmented using the DeepLabV3 model. To address the vanishing gradient problem, ResNet-50 was chosen as the backbone network. The model employs dilated convolutions and introduces the Atrous Spatial Pyramid Pooling (ASPP) module with different dilation rates to enhance segmentation capability for targets of varying scales. We also made modifications to the original model to enable real-time output of the spatial coordinates of fixed stimulation points on the left ventricle after segmentation, to support stimulation needs. In addition, EchoNet-Dynamic, which features residual connections and cross-frame spatiotemporal convolution, was used as the convolutional neural network (CNN) model to predict ejection fraction. The network integrates both spatial and temporal information during convolution to perform video-level ejection fraction prediction, enabling sequential evaluation of cardiac function. Before determining the need for stimulation, the system first performs a critical probe displacement detection safety check. This step verifies that the coordinates of the segmented LV are within a pre-calibrated safe range. If any positional deviation is detected, the system withholds stimulation and sends a correction notification. Only after confirming correct probe placement does the system proceed to evaluate the cardiac status (e.g., heart failure, low heart rate, or low EF) to decide whether to initiate ultrasound stimulation. Our system configuration also allows for the integration of gold-standard methods, such as ECG, to assist in cardiac function prediction.

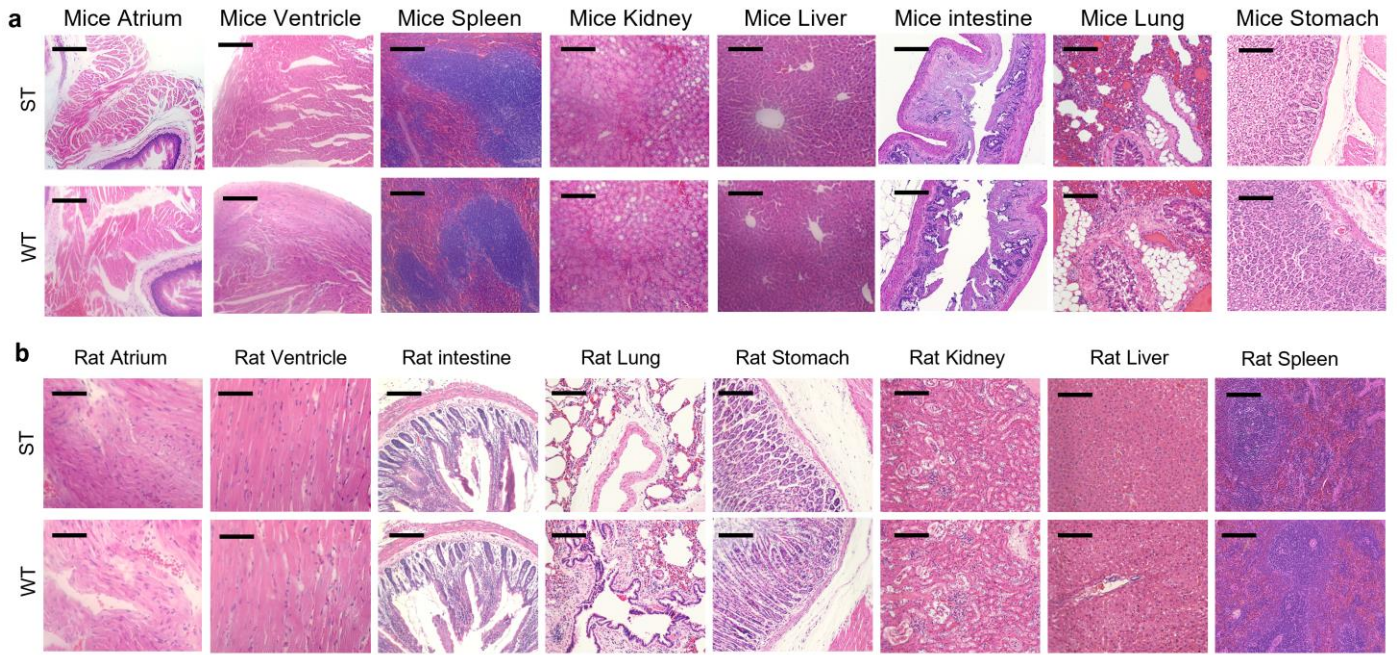


Supplementary Fig. 22 | Ultrasound multi-site pacing simulation in a human-thorax model. (a, b) A 3D thoracic rendering (a) and the corresponding axial segmentation (b) used for finite-element simulations. **(c, d)** Simulation of a dual-site steering solution that places two foci in distinct cardiac regions. The results are shown as both instantaneous acoustic pressure (c) and normalized sound acoustic level (SAL) (d). **(e, f)** Simulation of a single-site steering solution targeting one cardiac region, also shown as acoustic pressure (e) and SAL (f). COMSOL Multiphysics 6.2 was used for simulation.

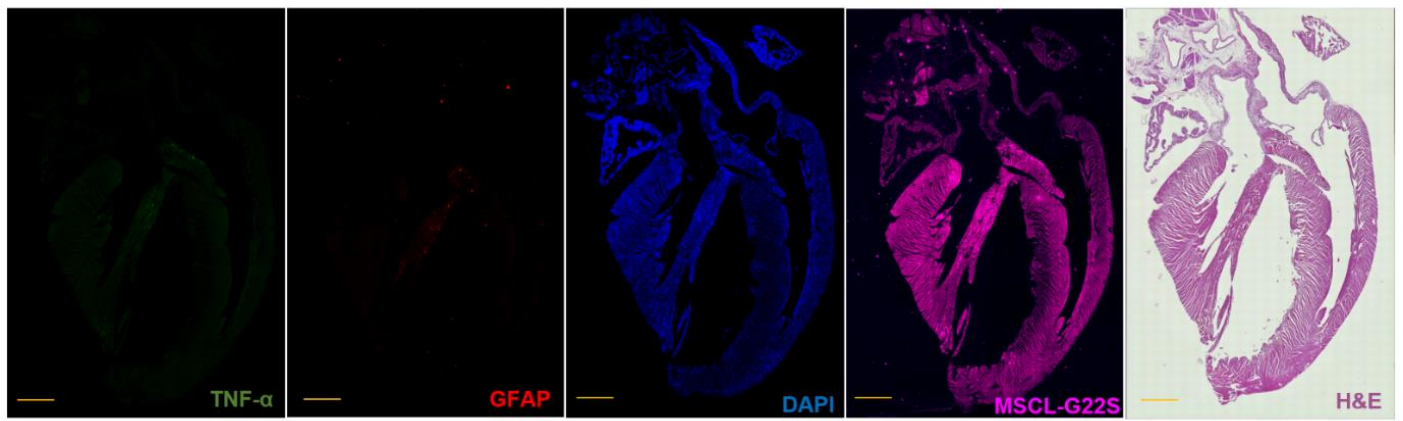


Supplementary Fig. 23 | Ultrasound energy attenuation analysis across different cardiac stimulation sites using hydrophone testing. **a-b**, Schematic of hydrophone placement at different location of the porcine heart, including His-bundle pacing (HBP), right ventricular apical pacing (RVA), left bundle branch area pacing (LBBAP), and cardiac resynchronization therapy (CRT). **c**, Hydrophone placement at the His Bundle Pacing (HBP) site. **d**, Corresponding ultrasound pressure amplitude measurements at HBP with and without

heart tissue (w/ heart, wo/ heart) as a function of input voltage (V_{pp}). **e**, Output voltage waveforms at HBP under 1 Hz and 2 Hz pulse repetition frequency. **f**, Hydrophone placement at the Cardiac Resynchronization Therapy (CRT) site. **g**, Ultrasound pressure amplitude measurements at CRT with and without heart tissue. **h**, Output voltage waveforms at CRT under 1 Hz and 2 Hz PRF. **i**, Hydrophone placement at the Left Bundle Branch Area Pacing (LBBAP) site. **j**, Ultrasound pressure amplitude measurements at LBBAP with and without heart tissue. **k**, Output voltage waveforms at LBBAP under 1 Hz and 2 Hz PRF. **l**, Hydrophone placement at the Right Ventricular Apex (RVA) site. **m**, Ultrasound pressure amplitude measurements at RVA with and without heart tissue. **n**, Output voltage waveforms at RVA under 1 Hz and 2 Hz PRF. Scale bar: 10 mm. Parts of this figure (**a**) was created with BioRender.com.

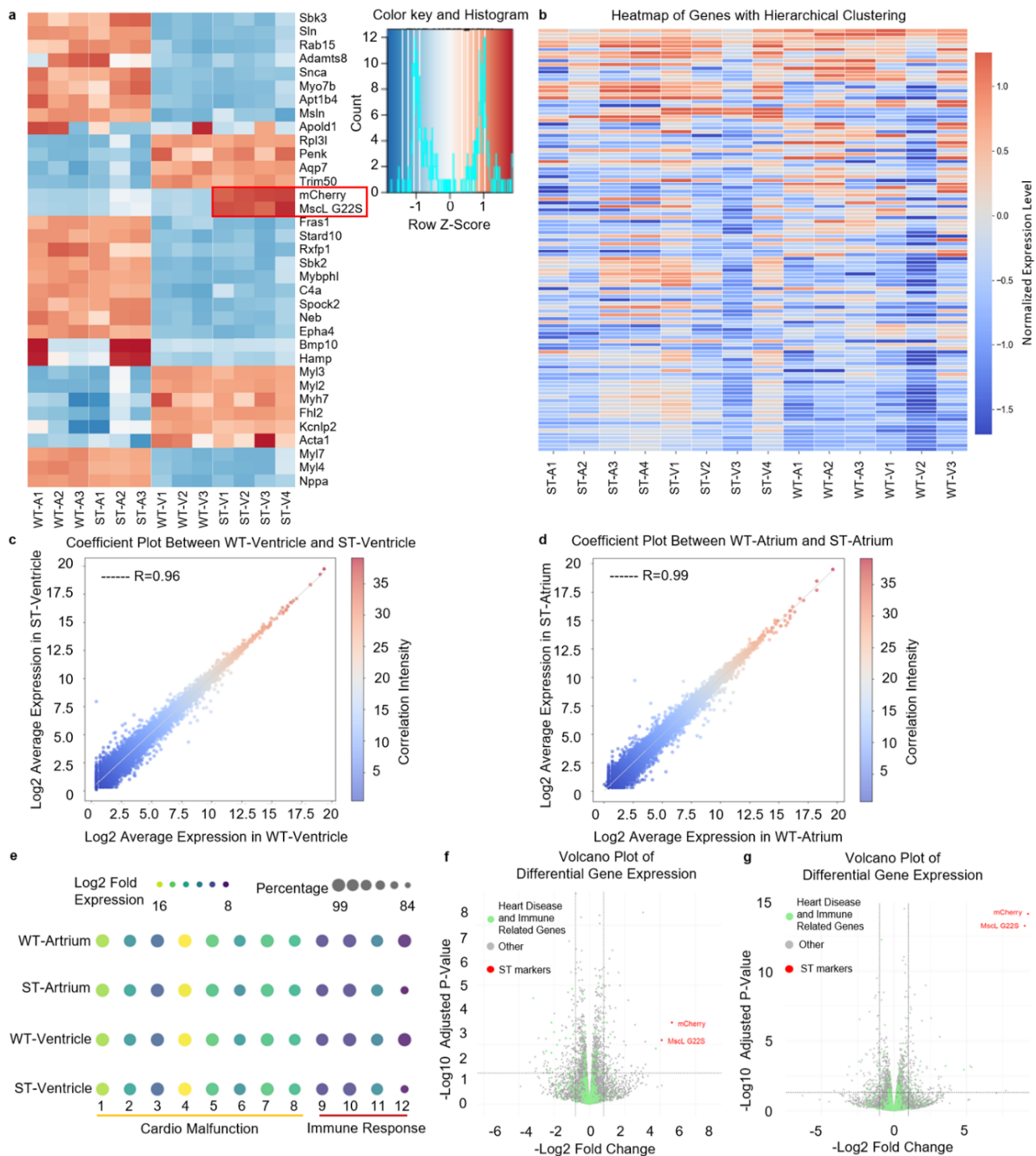


Supplementary Fig. 24 | Histological analysis of heart and organ tissues from ST and WT rats. a, Representative H&E-stained sections from mice, including atrium, ventricle, spleen, kidney, liver, intestine, lung, and stomach, shown for ST (top) and WT (bottom). **b,** Representative H&E-stained sections from rats, including atrium, ventricle, intestine, lung, stomach, kidney, liver, and spleen, shown for ST (top) and WT (bottom). Representative images are from $n = 5$ biologically independent animals per group, and similar histological findings were observed across all animals. Scale bars, 200 μm .

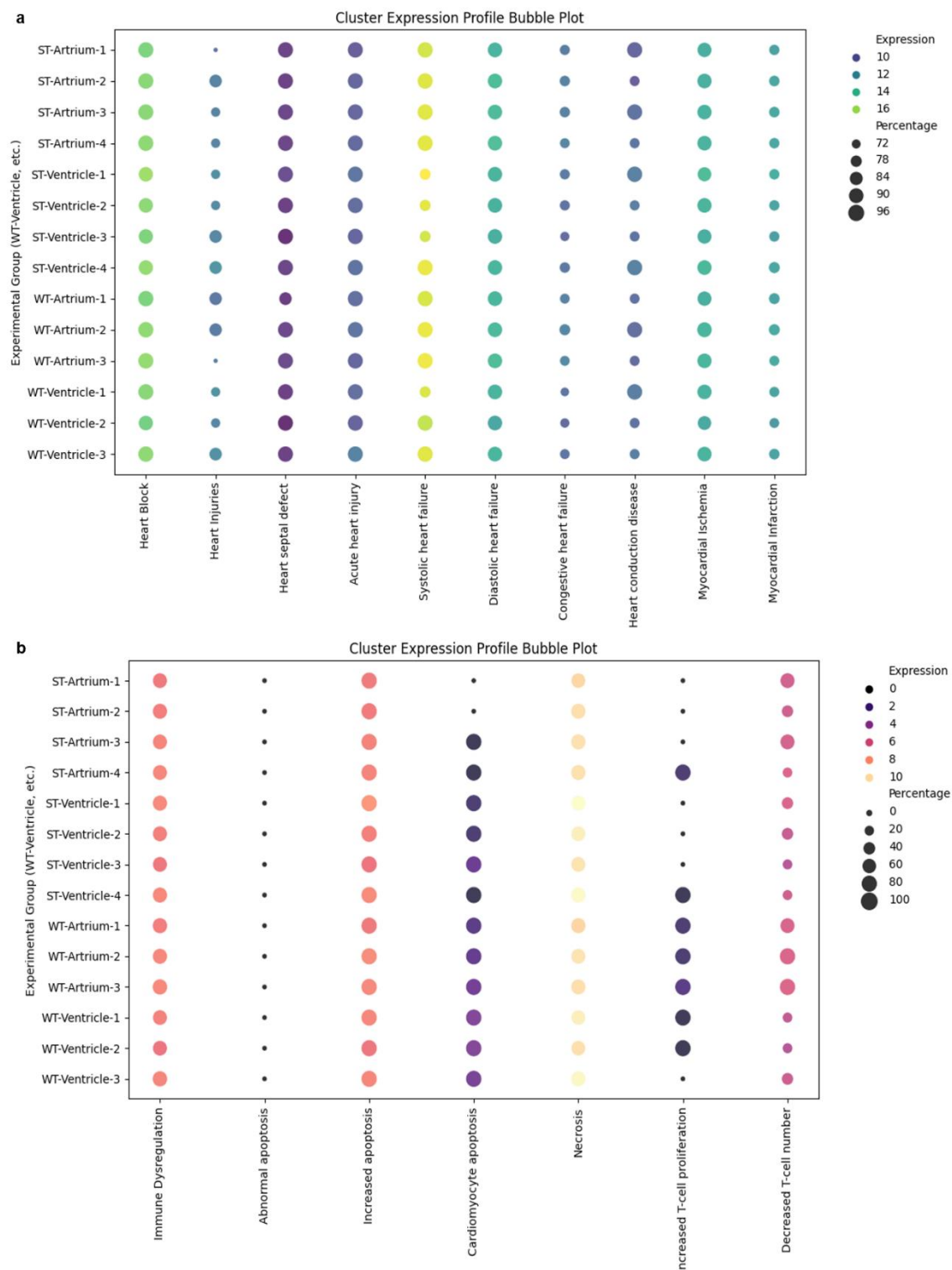


1 **Supplementary Fig. 25 | Histological staining of cardiac tissue post-sonogenetic transduction.** TNF- α
 2 (green) and GFAP (red) staining reveal the absence of significant inflammation or gliosis, respectively,
 3 following sonogenetic transduction. Nuclear counterstaining with DAPI (blue) and MscL G22S (magenta)
 4 demonstrates successful transduction. Hematoxylin and eosin (H&E) staining highlights preserved tissue
 5 architecture. Scale bar = 1 mm for full heart images.

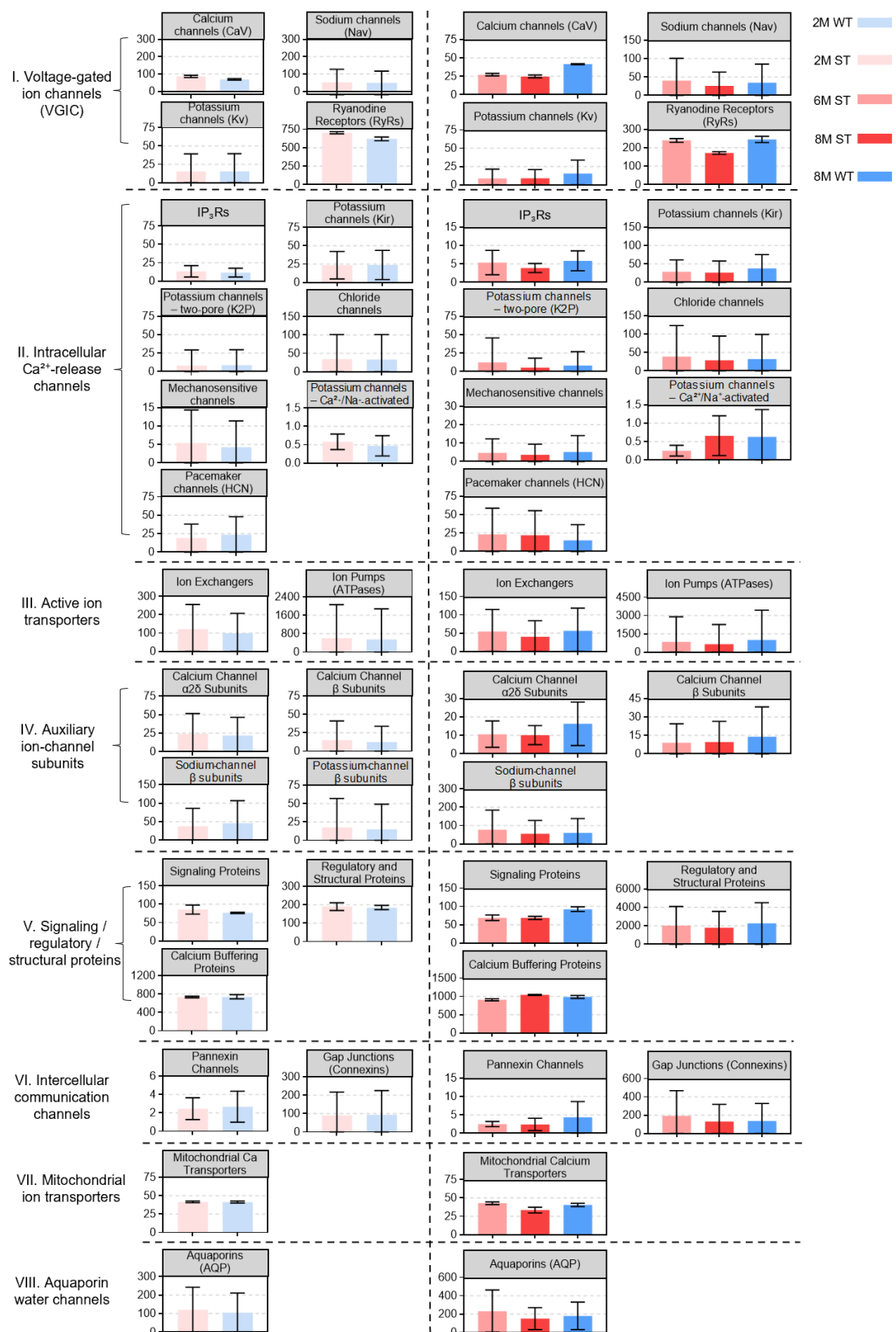
6



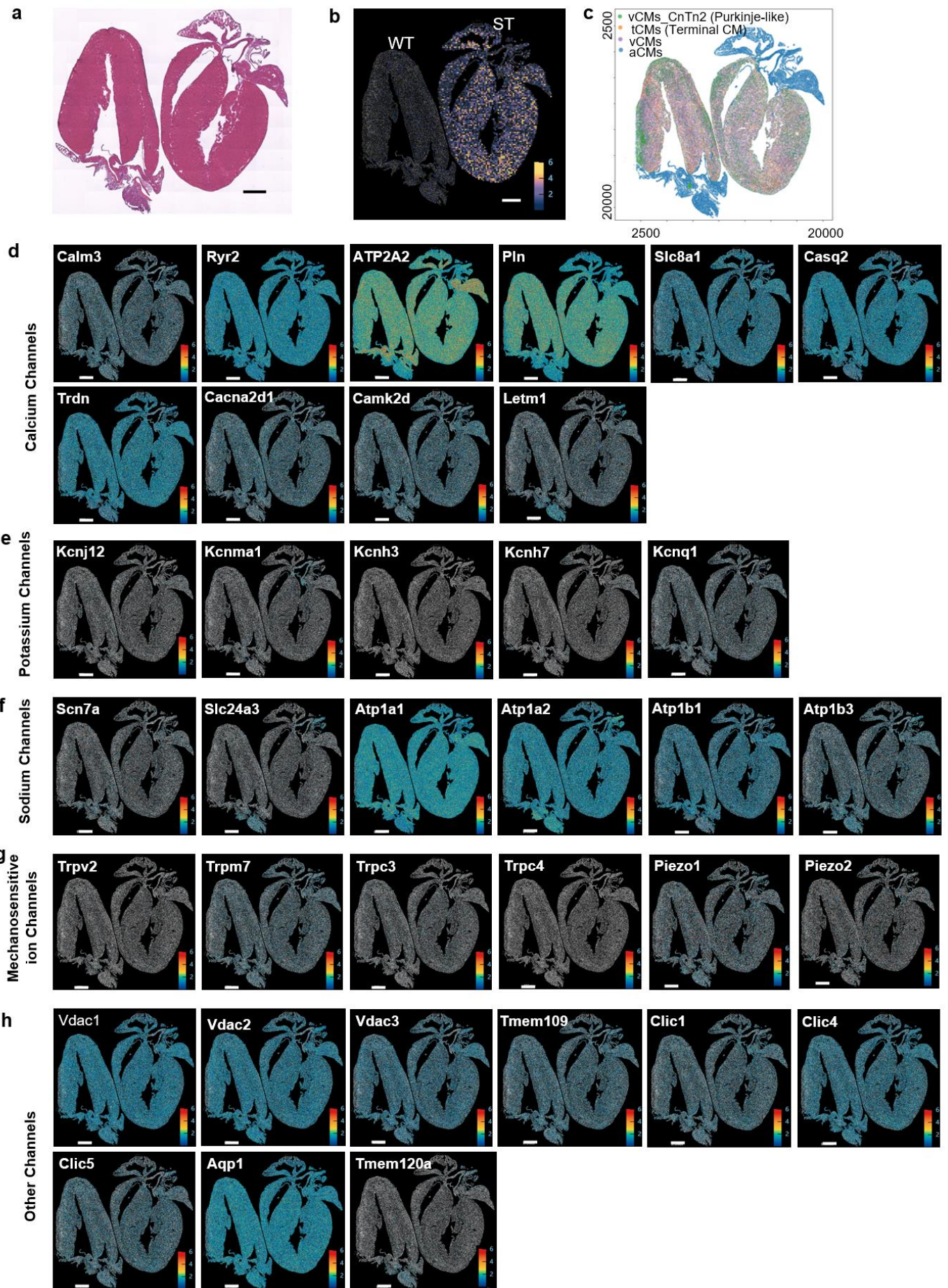
Supplementary Fig. 26 | Genetic safety assessment of sonogenetic transfection. **a**, Heatmap of 50 selected genes across four sample groups (WT-ventricle, WT-atrium, ST-ventricle, and ST-atrium). Rows are Z-score normalized; red indicates higher and blue indicates lower relative expression. Transgene-derived transcripts (mCherry and MscL-G22S) are highlighted. Each group includes ≥ 3 biological replicates. **b**, Heatmap of the top 200 most highly expressed genes with hierarchical clustering, with the accompanying histogram showing the distribution of row Z-scores across samples. **c,d**, Correlation (coefficient) plots comparing WT and ST samples for ventricle (**c**, Pearson's $R = 0.96$) and atrium (**d**, Pearson's $R = 0.99$). Each point represents one gene; dashed lines indicate linear regression fits, and color indicates correlation intensity. **e**, Bubble plot summarizing expression patterns of gene sets associated with immune response and cardiac function across groups. **f,g**, Volcano plots of differential gene expression between ST and WT. Each point represents one gene; the y-axis shows $-\log_{10}$ adjusted P value. Genes related to cardiac disease/immune pathways are highlighted, and transgene-derived transcripts (mCherry and MscL-G22S) are labeled.



1 **Supplementary Fig. 27 | Cluster expression profile bubble plots of cardiac gene expression.** Expression
2 profile plot using bubble heatmap to indicate the expression level and bubble size indicating the relative
3 abundance across groups. **a**, Cluster expression profile plot showing the average log2 expression levels, and
4 the proportion of expressed genes related to various cardiac conditions, including heart block, heart failure
5 (systolic, diastolic, and congestive), and myocardial infarction, across WT atrium, WT ventricle, ST atrium,
6 and ST ventricle groups. Circle sizes represent the percentage of genes expressed in each condition, and color
7 indicates the average expression levels. **b**, Cluster expression profile plot depicting genes associated with
8 immune dysregulation, apoptosis, cardiomyocyte necrosis, and T-cell proliferation. The visualization
9 highlights comparable gene expression patterns between WT and ST groups, with no notable differences in
10 immune or pathological responses. These findings support that sonogenetics transfection does not induce
11 immune activation or negatively impact cardiac function, maintaining a consistent transcriptional profile
12 across experimental conditions.

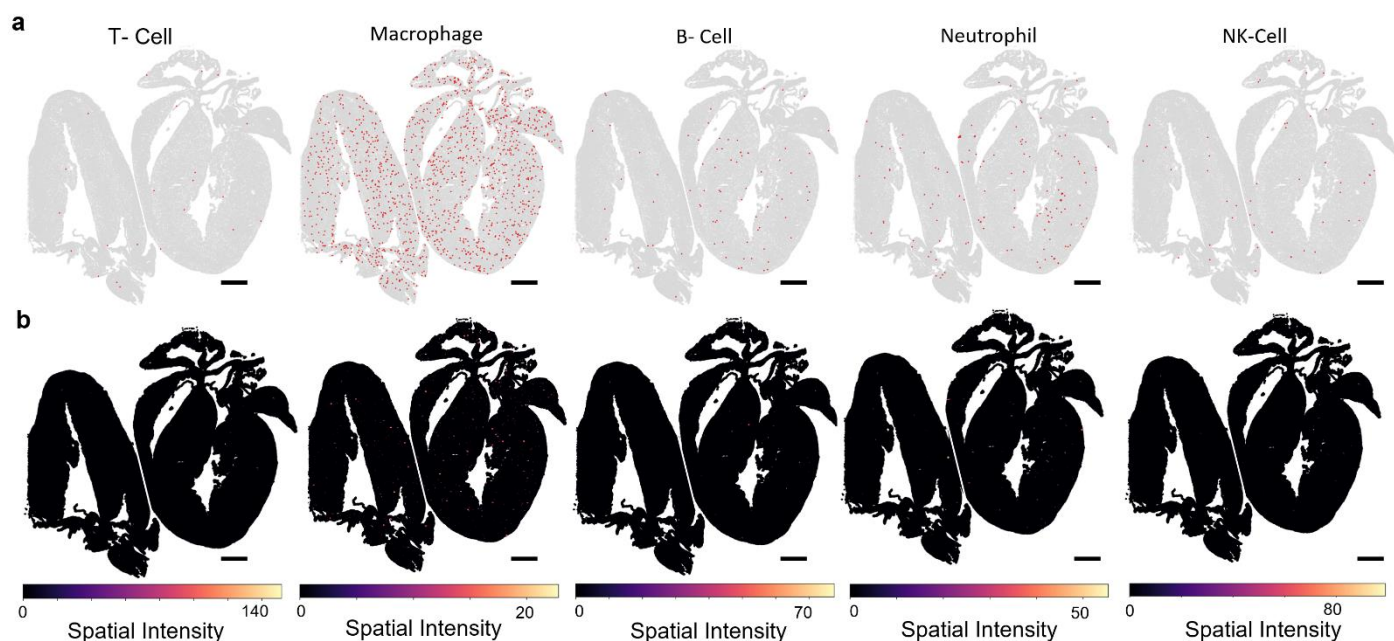


1 **Supplementary Fig. 28 | Long-term analysis of cardiac ion channels by bulk RNA sequencing.**
2 Quantitative analysis of gene expression levels for a comprehensive panel of ion channels and related proteins
3 in heart tissue from WT and ST rat. The bar charts are organized into eight functional categories (I-VIII),
4 encompassing voltage-gated ion channels, intracellular Ca^{2+} release channels, active ion transporters, auxiliary
5 subunits, signaling proteins, intercellular communication channels, mitochondrial transporters, and
6 aquaporins. The left column of charts compares expression levels at 2 months post-transduction (2M WT vs.
7 2M ST). The right column compares expression at 8 months (8M WT vs. 8M ST), and the 6-month ST group
8 (6M ST) to show longitudinal expression in treated animals. Each bar represents the mean relative expression
9 level for a specific gene family, with error bars indicating the standard deviation. n=3 for each condition.

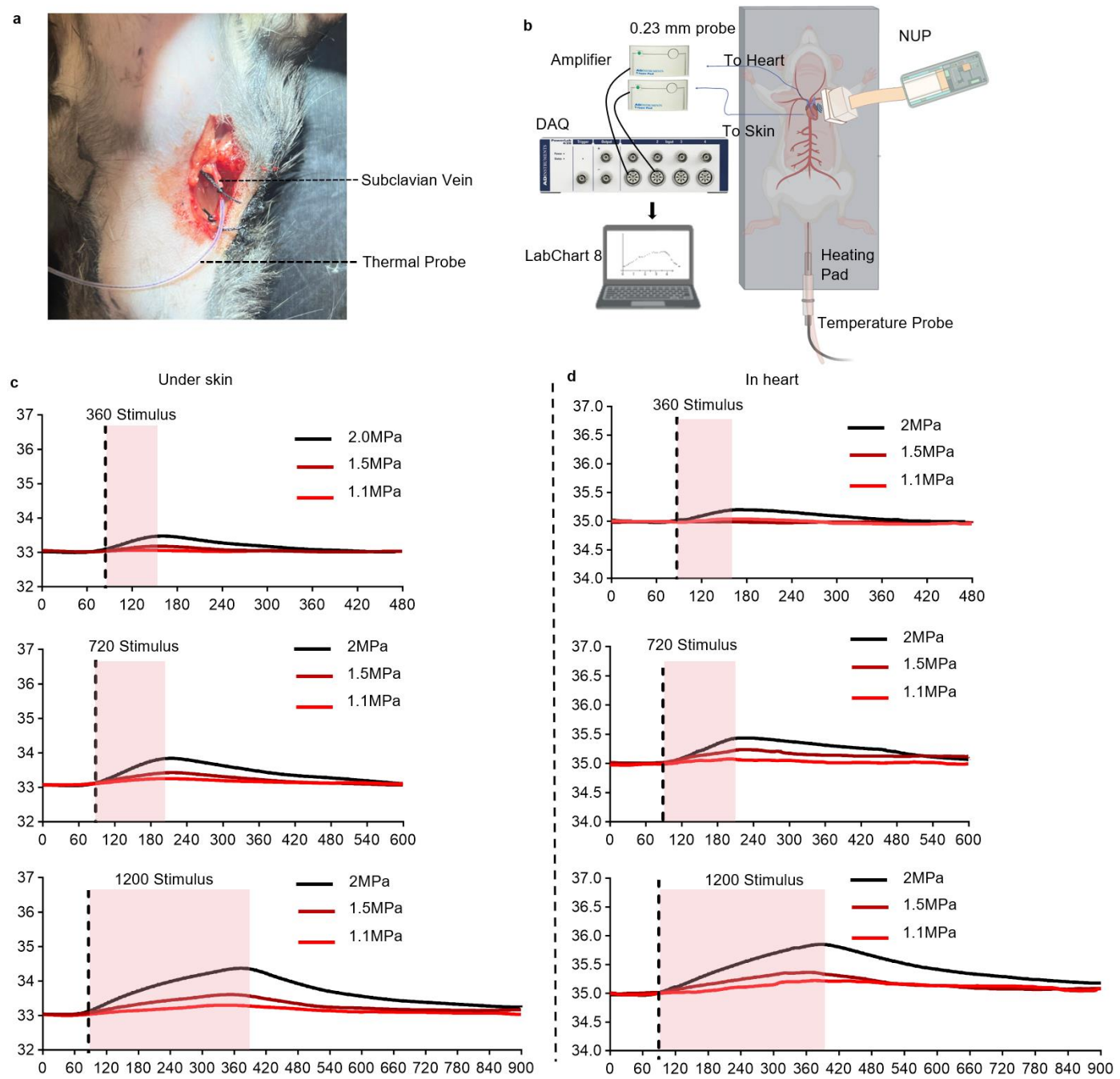


1 **Supplementary Fig. 29 | Comprehensive spatial transcriptomic analysis of key ion channel genes in WT**
2 **and ST hearts. a**, H&E staining of adjacent wild-type (WT, left) and sonogenetically transduced (ST, right)
3 cardiac tissue sections. **b**, Spatial plot confirming transgene expression is primarily localized to the ST section.

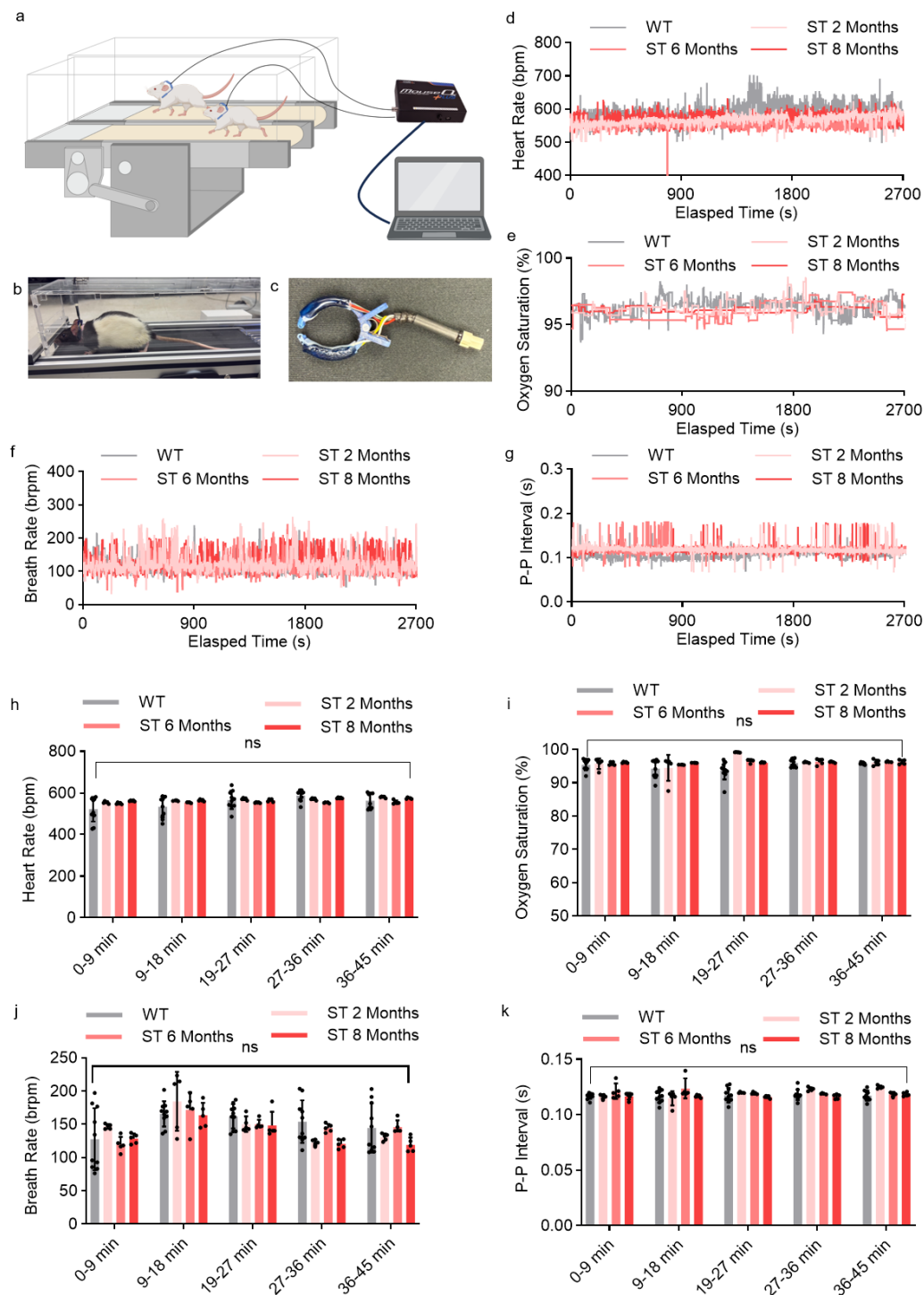
c, Spatial distribution of annotated cardiomyocyte subtypes across both sections. **d-h**, Spatial expression intensity maps are displayed for genes associated with distinct ion channel families across both WT and ST sections: **d**, Calcium channels and handling proteins; **e**, Potassium channels; **f**, Sodium channels and transporters; **g**, Endogenous mechanosensitive ion channels; and **h**, Other related channels. The color scales indicate normalized expression counts. The scale bar represents 1 mm.



Supplementary Fig. 30 | Spatial transcriptomic analysis of inflammatory cells in wild-type and sonogenetically transduced cardiac tissues. a, Spatial plots indicating the locations of individual cells for five major immune cell types: T-Cells, Macrophages, B-Cells, Neutrophils, and Natural Killer (NK) Cells. **b**, Spatial intensity plots corresponding to the immune cell types. The color scale represents the expression intensity of the gene signature for each cell type across both tissue sections. Scale bar is 1 mm.



Supplementary Fig. 31 | Temperature monitoring in ST rat heart under ultrasound stimulation. **a**, Representative image showing the surgical placement of the thermal probe through the subclavian vein into the rat heart, demonstrating the precise positioning required for accurate temperature measurement. **b**, Schematic of the experimental setup used for ultrasound stimulation, depicting the placement of the temperature probe, the heating pad, amplifier, RF power amplifier, function generator, and data acquisition system (DAQ). LabChart 8 software was used to monitor temperature changes during the experiment. **c**, Temperature profiles recorded under the chest skin at different ultrasound pressures (1.1 MPa, 1.5 MPa, 2.0 MPa) during 360, 720, and 1200 ultrasound stimuli. The shaded pink area represents the duration of ultrasound exposure, and temperature changes are shown over time for each stimulus condition. **d**, Temperature profiles recorded in the heart under different ultrasound pressures (1.1 MPa, 1.5 MPa, 2.0 MPa) during 360, 720, and 1200 ultrasound stimuli. The shaded pink area indicates the period of ultrasound exposure, highlighting the temperature response within the cardiac tissue. n=3 for each condition. Parts of this figure (**b**) was created with BioRender.com.



Supplementary Fig. 32 | Cardiopulmonary monitoring of WT and ST rats during treadmill exercise. **a**, Schematic of the experimental setup utilizing the Mousepox Plus® system for real-time monitoring of physiological parameters in rats during treadmill exercise. **b**, Illustration of a rat running on the treadmill while being monitored by the MouseOx Plus® system, showing the placement and connection of the monitoring device. **c**, Close-up view of the MouseOx Plus® collar sensor used for pulse oximetry measurements, designed to provide non-invasive, continuous monitoring of oxygen levels and other physiological metrics. **d-g**, Representative real-time physiological tracings over a 45-minute (2700 s) exercise period from individual rats in the WT and ST groups at 2, 6, and 8 months post-transduction. Parameters monitored include Heart Rate (**d**), Oxygen Saturation (**e**), Breath Rate (**f**), and P-P Interval (**g**). **h-k**, Quantitative analysis of physiological parameters, averaged over 9-minute intervals, for the entire cohort of animals (statistics by two-way ANOVA with Sidak's multiple comparison test; ns, $p > 0.05$; $n = 5$ rat per group). Bar charts compare Heart Rate (**h**), Oxygen Saturation (**i**), Breath Rate (**j**), and P-P Interval (**k**) between WT and ST groups at different time points. Parts of this figure (**a**) was created with BioRender.com.

1 **Supplementary Methods**

2 **RT-qPCR analysis of cardiomyocyte gene expression post-ultrasound stimulation**

3 One day after ultrasound stimulation, total RNA was extracted from each group using the Direct-zol™ RNA
4 Miniprep Kit (R2053, Zymo Research, USA) according to the manufacturer's instructions. RNA was reverse-
5 transcribed into cDNA using the ProtoScript® II First Strand cDNA Synthesis Kit (E6560S, New England
6 Biolabs, USA). Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) on
7 a CFX Opus 96 Real-Time PCR System (Bio-Rad) with Hard-Shell 96-Well PCR Plates (HSP9601, Bio-Rad).
8 Each 10 µL reaction contained 1 µL cDNA, 0.25 µL each primer (10 µM), 5 µL SYBR Green Supermix (2×),
9 and nuclease-free water to volume. GAPDH was used as the internal control, and gene expression fold changes
10 were calculated using the $2^{-\Delta\Delta CT}$ method, normalized to GAPDH expression levels.

11 Custom DNA oligonucleotide primers were purchased from Integrated DNA Technologies (IDT; 25 nmol
12 scale; standard desalting). Primers targeted GAPDH, FOS (RefSeq: NM_005252.4), TNNT2 (RefSeq:
13 XM_011509943.3), NRF1 (NM_001293163.2), GATA4 (NM_001308094.2), GJA1 (NM_000165.5), ACTC1
14 (NM_005159.5), MYH7 (NM_001407004.1), FABP3 (NM_004102.5), and TNF (NM_000594.4). Full primer
15 sequences are provided in **Supplementary Data**. Primer sequences are available for reuse and can also be
16 obtained from the corresponding author upon reasonable request.

17 **Fabrication and characterization of flexible MEA**

18 The preparation of the flexible MEA sensor began with sputtering a sacrificial layer (Ti/Cu; 10nm/200 nm in
19 thickness) on a silicon wafer. A 3 µm bottom layer of polyimide (PI-2611, HD Microsystems, Inc.) was spin-
20 coated (4000 rpm for 60 s), followed by a multistep curing process (room temperature for 15 minutes, 110°C
21 for 15 minutes, 150°C for 15 minutes, 180°C for 15 minutes, and 260°C for 1 hour) in a vacuum oven. The
22 thin film electrodes (Cr/Au; 20 nm/200 nm in thickness) were fabricated via a lift-off process using a negative
23 photoresist. The top polyimide encapsulation was fabricated following the same procedure as the bottom layer.
24 Lithographic patterning of a Cu hard mask (100 nm in thickness) on the PI/metal/PI trilayer film followed by
25 oxygen plasma etching (March RIE) of the polyimide defined the outline of MEA and exposed the metal
26 electrodes and the contact pads. Undercutting the Cu sacrificial layer in a Cu etchant released the MEA from
27 the wafer. Electrical connection between the flexible MEA's contact pads and a 34-position flat flex cable
28 (FFC, Molex) was achieved using an anisotropic conductive film.

29 Electrochemical deposition of platinum black (PtB) was performed using a potentiostat/impedance analyzer
30 (PalmSens4), with a Pt electrode (BASMW1032; BASi) as the counter electrode and an Ag/AgCl electrode
31 (BASMF2052; BASi) as the reference electrode. The MEA was immersed in an electrolyte solution consisting
32 of 0.6 g of chloroplatinic acid (PtCl₆H₂; Sigma-Aldrich), 5 mg of lead acetate [Pb(C₂H₃O₂)₂; Sigma-Aldrich],
33 and 20 mL of deionized water. Chronoamperometry was used at -0.1 V for 20 seconds to complete the
34 deposition process. Impedance measurements were conducted after PtB deposition using the
35 potentiostat/impedance analyzer (PalmSens4) in phosphate-buffered saline (PBS, pH 7.4), with scanning
36 frequencies ranging from 5 Hz to 10 kHz.

37 **ECG signal analysis**

38 The ECG signals were analyzed using LabChart 8 software (ADInstruments, Colorado Springs, CO, USA).
39 The alignment of ECG traces was set based on the QRS maximum value to ensure consistency in evaluating

1 waveform morphology across different experimental conditions. The corrected QT interval (QTc) was
2 computed using Bazett's formula: $QTc = QT / \sqrt{RR}$, which adjusts for heart rate variability.

3 The software's "Baseline Removal" function was utilized to correct for low-frequency drift and noise,
4 enhancing the clarity of individual waveform components. After the ECG signal acquisition and initial analysis,
5 the extracted parameters—including the amplitudes of the P, Q, R, S, and T waves, ST segment height, T peak-
6 to-T end interval, JT interval, QT interval, QRS duration, PR interval, RR interval, QTc, P wave duration, and
7 heart rate—were exported to OriginPro software (OriginLab Corporation, Northampton, MA, USA) for
8 further processing. Before generating the heatmap, each data point was normalized using the Z-score N (0,1)
9 method, defined as $Z = (X - \mu) / \sigma$.

10 For heatmap visualization, the normalized data was divided into three sections—average, minimum, and
11 maximum—according to the ECG analysis data. These segments were color-coded using a red-green-blue
12 gradient scheme. The heatmaps were generated using the built-in matrix plot function in OriginPro, with the
13 color scaling adjusted to emphasize clinically significant variations. Statistical analysis, including paired t-
14 tests and one-way ANOVA, was confirmed using OriginPro to compare ECG parameters across different
15 conditions.

16 **H&E staining**

17 Following the experiments, animals underwent cardiac perfusion. Hearts and other tissues were isolated, fixed
18 in 4% paraformaldehyde at 4°C for 3 days, and embedded in paraffin for sectioning. After the deparaffinization
19 process, sections were stained with filtered Harris hematoxylin (10 s), rinsed with tap water until clear, and
20 counterstained with eosin (30 s), followed by additional tap water rinses. Dehydration involved two 5-min
21 cycles in absolute ethanol, followed by two 5-min cycles in xylene for clearing. Finally, sections were mounted
22 with a resin-based medium.

23 **RNA-sequencing and data analysis**

24 After euthanizing the rats, bulk RNA was extracted and purified from different rat groups using the Direct-
25 zol™ RNA Miniprep Kit (R2053, Zymo Research, USA), following the manufacturer's protocol. The RNA
26 was stored at -80°C for short-term preservation. In total, 14 rat tissue samples were analyzed, including 3 atrial
27 samples from wild type (WT) rats, 3 ventricular samples from WT rats, 4 atrial samples from sonogenetics-
28 transfected (ST) rats, and 4 ventricular samples from ST rats. The RNA samples were then sent to Azenta
29 (Chelmsford, MA, USA) for comprehensive analysis, with a sequencing depth of 40M PE reads (20M in each
30 direction). Since MscL G22S and mCherry genes are not present in the rat genome, we added the sequences
31 of these two genes to the reference library. The specific sequence of MscL G22S is as follows:

32 ATGGCGAGCATTATTAAAGAATTCGCGAATTTGCAGATGCACGGGAACGTGGTGGATTGCGCGG
33 GTGGGTGTCATTATCGGTGCGGCATTGGGAAAGATTGTCTCTTCACTGGTTGCCGATATCATCATG
34 CCTCCTCTGGGCTTATTAATTGGCGGGATCGATTGTAAACAGTTTGCTGTCACGCTACGCAGAT
35 GCAGAGGGGGATATCCCTGCTGTTGTGATGCATTACGGTGTCTTCATTCAAACGTCTTTGATTTC
36 TGATTGTGGCCCTTTGCCATCTTTATGGCGATTAAGCTAATCAACAACTGAATCGGAAAAAAGA
37 AAGAACCAGCAGCCGCACCTGCACCAACTAAAGAAGAAGTATTACAGAAATTCGTGATTGGCTG
38 AAAGAGCAGAATAACCGCTCT

39 MscL (G22S) qPCR primers:

1 Forward Primer: 5' cgcggaacgtggtggattg 3'

2 Reverse Primer: 5' cccgccaattaataagcccagagg 3'

3 Product Length: from 40 to 156, length: 117

4 For data analysis, we used Gene Ontology (GO) analysis, heatmap plotting, coefficient plots comparing ST
5 and WT samples, volcano plots, cluster plots. The reference genes were obtained from publicly available
6 databases (<https://www.ncbi.nlm.nih.gov/gene> and <https://useast.ensembl.org/index.html>).

7 GO enrichment analysis. The GO enrichment analysis was conducted to identify significantly enriched
8 biological processes, molecular functions, and cellular components among the genes differentially expressed
9 between WT and ST conditions. The enrichment analysis was specifically targeted to pathways and processes
10 most prominently affected by the treatments. The GO terms were filtered based on the p-value threshold (<
11 0.05), and the top pathways were visualized using chord diagrams and histograms. For the chord diagrams,
12 we visualized the relationships between GO terms and the genes contributing to them, highlighting the
13 interconnectedness between biological functions. GO pathway bar plots were also generated to provide a
14 quantitative representation of the enrichment levels of each GO term, where the $-\log_{10}(\text{p-value})$ of each term
15 was plotted to indicate the significance of the enrichment. The color of bars reflected the enrichment p-values,
16 with warmer colors indicating stronger significance.

17 Heatmap generation. Heatmaps visualized gene expression patterns and hierarchical clustering grouped genes
18 by similarity across conditions (WT atrium/ventricle and ST atrium/ventricle). Genes with similar expression
19 patterns in WT and ST groups, regardless of tissue type, were filtered based on a tolerance value, thereby
20 allowing us to focus on genes with consistent behavior across treatments.

21 Coefficient plot between ST and WT samples. These scatter plots depicted the $\log_2$ fold change in gene
22 expression between the two conditions, allowing us to identify trends and relationships between gene
23 expression levels. The correlation coefficient (R value) was calculated and displayed on each plot to provide
24 a quantitative measure of the correlation. We used linear regression to fit a trendline through the data points,
25 and the strength of the correlation was visually represented using color gradients applied to individual data
26 points, where stronger correlations were represented with darker shades. The trendline was drawn in a light
27 gray, dashed format to emphasize the general trend while minimizing visual distraction from the individual
28 data points.

29 Volcano plot for differential gene expression. Volcano plots were used to depict the relationship between the
30 statistical significance ($-\log_{10}$ adjusted p-value) and the magnitude of expression changes ($\log_2$ fold change)
31 for each gene. The x-axis represented the $\log_2$ fold change, while the y-axis represented the $-\log_{10}$ adjusted
32 p-value. For notable genes such as mCherry and MscL-G22S, which were explicitly highlighted in green to
33 indicate their importance. Threshold lines representing significant p-values and fold changes were also plotted
34 as dashed lines to delineate the criteria for differential expression.

35 Cluster analysis and expression profile plot. Cluster analysis was performed to identify groups of genes with
36 similar expression profiles under different conditions. For the expression profile plot, we utilized cluster data
37 to group genes and plotted their expression levels across various experimental groups, including WT atrium,
38 WT ventricle, ST atrium, and ST ventricle. These bubble plots visualized the average $\log_2$ expression levels
39 of genes in each cluster, as well as the proportion of genes expressed in each condition. Circle sizes represented

the percentage of expressed genes, while color indicated average expression levels.

Simulation of ultrasound acoustic field energy in human heart

The human heart model was redrawn based on an existing publicly available model (<https://ai.cgmodel.com/>). Simulation based on Finite Element Analysis was performed by COMSOL (COMSOL Multiphysics 6.2, Burlington, USA). Defined parameters included the rib, muscle, atrium, ventricle, water, vessel wall, and blood density and speed of sound. The simulation used a 3 MHz phased array with a pitch of 0.254 mm, consisting of 64 elements. The array's lateral dimension is 13 mm with a lateral focus at 80 mm, with each element measuring 0.254 mm $\times$ 13 mm. The kerf width was set to 0.01 mm. The focal point was chosen based on the placement locations of commercial pacemakers (**Fig. 5d**). The plotted data represents magnitude of acoustic pressure.

We used Field II for beamforming simulations of the focused acoustic field generated by the phased array. We simulated the acoustic field focusing for a 64-channel phased array at distances of 30 mm, 50 mm, 80 mm, and 100 mm, and we also simulated the field shape at a focus of 100 mm with steering angles of 10°, 20°, and 30°.

PCB design for an integrated ultrasound imaging and stimulation system

The system consists of two main components: a data acquisition board and a power board. The data acquisition board is a highly integrated circuit board that combines analog signals, digital signals, and power modules, with dimensions of 105 mm $\times$ 32.5mm. Given the system's stringent requirements for noise control and image quality, the layout and routing design were carefully planned. In terms of layout, the signal flow is optimized by arranging the FPGA, AFE, and TX modules sequentially along the signal path to reduce signal delay and transmission loss, ensuring that the signal paths are kept as short as possible. To minimize interference, high-frequency and low-frequency signals, as well as analog and digital signals, are partitioned into distinct regions. The board utilizes a 14-layer stack-up, comprising 6 ground layers, 2 power layers, and 6 signal layers, maintaining the integrity of the ground and power layers to reduce power noise and the resistance of return paths. In routing, differential signals (such as LVDS between the AFE and FPGA, USB, and clock signals) are designed to be matched in length and spacing to minimize signal skew and reflection. Sensitive and high-speed signals (like clock signals) are shielded to mitigate EMI. To meet high-speed signal transmission requirements, the board material selected is Taconic's RF-35™, which offers excellent high-frequency properties including a low dielectric constant, low dielectric loss factor, and low thermal expansion coefficient. Impedance is designed to meet the requirements based on the material and signal characteristics.

The power board is responsible for providing high and low voltages to the system, playing a crucial role in noise control and thermal management. The high-voltage module provides 80Vpp for transmission, while the low-voltage module supplies 1.0V, 2.5V, 3.3V, and 5V to modules like the FPGA, AFE, TX, Wi-Fi, and USB. In the circuit layout, the high-voltage area is physically isolated from the low-voltage control circuits to prevent noise interference and signal crosstalk. The current path from input to output is kept as short as possible to minimize parasitic resistance and inductance, reducing losses and heat buildup. The power board, measuring 50mm $\times$ 18mm, uses a 4-layer stack-up and is made from FR-4, a material with good heat resistance and mechanical strength. In terms of routing, high-current paths (such as power outputs and ground) are widened, with a line width of 60 mil and a copper thickness of 1 oz. Grounding follows a star ground design to separate the high-current ground points from the signal ground points. Ferrite beads or common-

mode inductors are added at the input and output to effectively reduce high-frequency noise. The entire system design takes into account signal integrity, noise control, and thermal management to ensure stable performance and enhanced image quality.

1 **Supplementary Text**

2 **Supplementary Text 1: Introduction to traditional pacemakers**

3 Pacemakers emerged in response to the growing understanding of cardiac arrhythmias, particularly heart block,
4 a condition characterized by the failure of the heart's natural conduction system to propagate electrical
5 impulses from the sinoatrial (SA) node to the ventricles, leading to life-threatening bradycardia or asystole[10].
6 The myocardium, the muscular layer of the heart, contracts in response to electrical stimulation. In a healthy
7 heart, the SA node initiates an electrical impulse that travels through the conduction pathways, coordinating a
8 rhythmic and efficient contraction cycle essential for blood circulation[11]. Damage to these pathways, as seen
9 in heart block or post-surgical conditions, disrupts this rhythm, resulting in insufficient cardiac output and
10 symptoms such as blackouts, syncope, or cardiac arrest[12].

11 Early pacemakers were external, bulky devices powered by large batteries and delivered electrical pulses
12 transcutaneous or via direct myocardial electrodes. These rudimentary external systems, though revolutionary,
13 were highly restrictive for patients due to limited mobility and infection risks[13]. A significant milestone in
14 pacemaker evolution came with the development of implantable devices. In 1958, Åke Senning and Rune
15 Elmqvist successfully implanted the first fully enclosed pacemaker in a human patient, marking the transition
16 from external to internal systems[14]. By the 1960s, physicians were routinely implanting transvenous
17 pacemakers, wherein leads were inserted through veins into the right ventricle and connected to pulse
18 generators placed subcutaneously[15]. These systems became the standard for decades, evolving to include
19 features such as dual-chamber pacing, rate adaptation, and algorithms for managing complex arrhythmias.
20 However, transvenous pacemakers still carry the risk of lead failure, infection, and complications related to
21 lead implantation, such as thrombosis or cardiac perforation.

22 Advancements in rate-responsive pacemakers and microprocessor-driven systems addressed some of the
23 shortcomings of earlier devices by incorporating sensors and algorithms that adjusted pacing rates based on
24 patient activity [16]. These devices featured the ability to detect, analyze, and store data on cardiac events
25 using sophisticated algorithms. By continuously monitoring the heart's activity, microprocessor-driven
26 pacemakers could adjust their parameters automatically to meet the patient's changing needs[17]. However,
27 these systems were not without challenges. The reliance on movement-based sensors as a surrogate for
28 physiological demand sometimes resulted in inappropriate rate adjustments, particularly in scenarios where
29 physical activity did not correlate with metabolic needs[18, 19]. Moreover, the complexity of microprocessor-
30 driven pacemakers introduced concerns about device longevity and the potential for software or hardware
31 failures.

32 A fundamental limitation of all traditional pacemakers is their inability to achieve cell-type-specific
33 stimulation or seamlessly integrate with the body's natural electrochemical processes. These devices rely on
34 indiscriminate electrical stimulation, which, while effective for generating contractions, lacks the precision
35 needed to mimic the intricate signaling patterns of the heart's conduction system[20]. This gap has driven
36 ongoing efforts to explore alternative pacing technologies, such as ultrasound-based systems or sonogenetics,
37 which promise to offer non-invasive, cell-specific, and dynamically adaptive solutions for cardiac rhythm
38 management.

39

1 **Supplementary Text 2: Why MscL G22S? Selection of sonogenetics candidates.**

2 The primary hypothesis regarding the effects of ultrasound on neurons suggests that pressure waves can
3 deform the cell membrane, indirectly causing conformational changes in channel structures or directly
4 affecting channel components to create ion gradients across the membrane [21, 22]. Recent studies have shown
5 that ultrasound induces neuronal depolarization by activating mechanosensitive ion channels when cell
6 membranes are deflected[23]. Several ion channel types have been identified as preferentially activated by
7 pressure changes, including members of the two-pore-domain potassium (K2P) family (TREK-1, TREK-2,
8 and TRAAK)[24], Piezo family (Piezo1[25, 26] and Piezo2[27]), and TRP family (TRPA1[28], TRPC1[29],
9 TRPP2[29], TRPM4[29], and TRPV1[30]), which have been implicated in ultrasound neuromodulation both
10 in vitro and in vivo. While the dominant channels remain unclear, these findings lay the foundation for
11 developing sonogenetics by leveraging mechanosensitive ion channels as mediators.

12 Ultrasound does not universally modulate all neurons; neurons with low expression of mechanosensitive ion
13 channels require overexpression of these channels to respond to ultrasound stimuli. This has given rise to the
14 concept of "sonogenetics." First introduced by Ibsen et al. (2015), sonogenetics demonstrated that low-
15 frequency ultrasound (2 MHz) in the presence of microbubbles could activate specific neurons overexpressing
16 the mechanosensitive ion channel TRP-4, thereby controlling *Caenorhabditis elegans* behavior [31]. However,
17 the reliance on microbubbles to amplify ultrasound's mechanical effects on TRP-4 has limited its translation
18 to mammalian models.

19 Subsequent proof-of-concept studies explored various candidate molecules to enhance neuronal responses to
20 ultrasound stimuli, including TRPV1, Prestin, gas vesicles (GVs), hsTRPA1, and MscL. Each candidate has
21 distinct advantages and limitations depending on the application.

22 The TRP channels play critical roles in various physiological processes, including pain, touch, and
23 thermosensation. TRPV1, a hallmark thermosensitive channel, has been artificially overexpressed in neurons
24 to mediate targeted stimulation via photothermal[32], magnetothermal, and more recently, ultrasound
25 thermomechanical techniques[30]. When tissues absorb ultrasound, part of the mechanical energy converts to
26 heat, potentially raising local temperatures, particularly at high frequencies and long durations[33]. Studies
27 have shown that 3.2 MHz ultrasound neuromodulation can increase deep brain temperatures by up to 2°C[34].
28 Yang et al. (2021)[30] demonstrated that ultrasound-induced local brain heating in mice could be visualized
29 through MR thermometry and controlled by adjusting ultrasound frequency and pulse length. While this
30 approach provides high cellular specificity and millimeter-level spatial resolution, its temporal resolution (<5
31 seconds) is insufficient for applications like cardiac pacing.

32 Prestin, a hearing-related mechanosensitive protein, has been engineered for cell-specific ultrasound
33 neuromodulation in the mouse brain[35]. Huang et al. (2019)[35] screened prestin's ultrasound sensitivity
34 across non-echolocating and echolocating species, identifying two ultrasound-responsive mutations (N7T and
35 N308S) conserved in echolocating species. They demonstrated that mPrestin (N7T, N308S) overexpression in
36 ventral tegmental area neurons rendered them sensitive to ultrasound, with c-fos expression confirming
37 activation. However, mPrestin activation peaked at 0.5 MHz, which is outside the typical range (2–4 MHz)
38 for cardiac ultrasound, making it less suitable for cardiac applications.

39 Gas vesicles (GVs), nanometer-scale protein structures derived from cyanobacteria, have been used as
40 actuators for targeted deep ultrasound stimulation[36]. GV's possess a hollow protein shell that enhances

1 ultrasound contrast, allowing them to induce mechanical perturbations in surrounding tissues. GV-mediated
2 ultrasound stimulation demonstrated strong neuronal activation in vitro, with responses directly correlated to
3 stimulation frequency, providing excellent temporal resolution. However, challenges in expressing GVs in
4 mammalian cells, due to the involvement of multiple genes, limit their utility.

5 Mammalian TRPA1 channels respond significantly to 6.9 MHz ultrasound stimulation[37]. Interestingly,
6 human homologs are more effective candidates, exhibiting threefold higher ultrasound sensitivity than their
7 murine counterparts. The N-terminal tip domain of TRPA1 appears critical for ultrasound sensitivity.
8 Overexpression of hsTRPA1 in vitro significantly enhanced neuronal responsiveness to ultrasound, with in
9 vivo experiments confirming cortical expression of hsTRPA1 induced localized neuronal activation. However,
10 the 6.9 MHz frequency is outside the typical range for cardiac ultrasound, and the small focus size relative to
11 cardiac volume limits its applicability as a candidate for cardiac pacing.

12 MscL, a mechanosensitive ion channel, emerges as a particularly promising candidate. MscL rapidly converts
13 mechanical stimuli into neuronal responses without relying on membrane potential, endogenous factors, or
14 thermal deposition. Inducing MscL expression has been reported to enhance cellular responsiveness to
15 ultrasound stimulation[38]. Previous studies demonstrated that MscL expression in mice enabled neurons to
16 respond to ultrasound stimulation, inducing neuronal activation in target regions without affecting the resting
17 membrane potential[39].

18 Among MscL variants, MscL-G22S performs exceptionally well at 2.2 MHz[39], a frequency compatible with
19 cardiac ultrasound imaging. This technology is compatible with millisecond-level temporal resolution,
20 enabling applications such as cortical-level visual restoration. Thus, MscL-G22S is well-suited for current
21 applications involving transcription in cardiomyocytes for cardiac pacing.

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1 **Supplementary Text 3: Rationale for AAV-Mediated Gene Delivery and Discussion of Clinical**
2 **Translation**

3 1. The Promise and Challenge of Gene Therapy for Cardiac Disorders

4 The successful application of our sonogenetic pacemaker relies on the efficient and safe delivery of the MscL
5 G22S gene to cardiomyocytes. Gene therapy represents a paradigm-shifting approach for cardiac diseases,
6 offering the potential for durable, long-term therapeutic effects where conventional treatments may fall short.
7 However, the translation of any gene therapy from bench to bedside requires careful consideration of the
8 delivery vector. This section details the rationale for our choice of an Adeno-Associated Virus (AAV) vector,
9 specifically the AAV2/9 serotype, and discusses the current landscape of challenges and progress in clinical
10 gene therapy.

11 2. Rationale for Selecting AAV as the Delivery Vector

12 We selected AAV as our delivery vector due to its well-established and superior profile for in vivo gene therapy
13 applications, particularly when compared to other viral systems like lentiviruses or adenoviruses. The key
14 advantages of AAVs include:

- 15 • **Excellent Safety Profile:** AAVs are non-pathogenic in humans and elicit a significantly lower immune
16 response than other viral vectors. This reduces the risk of inflammatory side effects and vector
17 clearance, allowing for stable, long-term transgene expression.
- 18 • **Transduction of Non-Dividing Cells:** AAVs can efficiently transduce terminally differentiated cells,
19 such as adult cardiomyocytes, which do not divide. This is a critical requirement for any cardiac gene
20 therapy.
- 21 • **Episomal Persistence and Reduced Mutagenesis Risk:** Upon entering the nucleus, the AAV genome
22 typically persists as a stable, non-integrating episome. This provides durable expression of the
23 therapeutic gene without the risk of insertional mutagenesis that is associated with integrating viruses,
24 a major safety advantage.

25 3. Specific Rationale for the AAV2/9 Serotype and the cTnT Promoter

26 3.1 The selection of a pseudotyped AAV2/9 vector

27 The selection of a pseudotyped AAV2/9 vector—which combines the AAV2 genome backbone with the
28 AAV9 capsid—was a strategic decision to leverage the distinct advantages of both serotypes, creating an
29 optimal system for cardiac gene transfer[40, 41]. The AAV2/9 pseudotype uses the AAV9 capsid, which
30 provides strong cardiac tropism and efficient systemic transduction of cardiomyocytes, and the AAV2 genome
31 backbone, which enables stable recombinant vector construction and high-titer packaging. This combination
32 has become standard in cardiac research because it ensures both robust myocardial expression and
33 reproducible vector production for translational studies.

34 We have summarized the comparison in detail in the Table.

Vector Type	Capsid Source	Genome Backbone/ITRs Source	Cardiac Tropism	Systemic Delivery Efficiency	Pre-existing Antibodies (Human)	Required Delivery Method	Transduction Efficiency (Cardiomyocytes)	Packaging/GMP Production Considerations
AAV2 (Native)	AAV2	AAV2	Poor	Inefficient	High prevalence	Invasive/Local	Low	Established/Complex
AAV9 (Native)	AAV9	AAV9	Excellent	High	Relatively lower	Minimally Invasive/Systemic	High	Established/Complex
AAV2/9 (Pseudo-typed)	AAV9	AAV2	Excellent (via AAV9 capsid)	High (via AAV9 capsid)	Associated with AAV9 capsid	Minimally Invasive/Systemic	High	Simplified/Scalable (via AAV2 ITRs)

1 3.2 The selection of a cTnT Promoter

2 The cTnT protein is a fundamental and exclusive component of the cardiac sarcomere's thin filament, making
3 its endogenous promoter one of the most specific regulators of cardiomyocyte gene expression known[42-44].
4 Our choice was supported by a robust body of literature[45-50] showing that even minimal promoter
5 fragments can drive potent and highly specific transgene expression at both the whole-organ and single-cell
6 levels.

7 Previous studies have shown that the cTnT promoter ensures transgene expression is overwhelmingly confined
8 to the heart at the whole-organ level[47, 51, 52]. This exceptional specificity is not a passive trait but an active,
9 mechanistic property rooted in transcriptional regulation. This mechanism stands in stark contrast to
10 ubiquitous viral promoters, such as the widely used cytomegalovirus (CMV) promoter[47, 53]. In contrast,
11 the activity of the cTnT promoter is governed by a “lock-and-key” system[46, 54]. Its DNA sequence contains
12 specific cis-regulatory binding sites for a unique combination of cardiomyocyte-restricted transcription factors,
13 primarily GATA4, Nkx2.5, and members of the MEF2 family[55]. These master regulatory proteins are
14 abundant in cardiomyocytes but are absent or present only at negligible levels in other tissues such as the liver,
15 skeletal muscle, and neurons[50, 56]. Consequently, the cTnT promoter remains transcriptionally silent in
16 non-cardiac tissues, providing a powerful first layer of containment. Because of this reliability, the cTnT
17 promoter has become a workhorse tool used extensively in cardiac gene therapy and validated in numerous
18 preclinical studies, spanning conditions from heart failure and post-infarction remodeling to genetic disorders
19 such as catecholaminergic polymorphic ventricular tachycardia (CPVT), in models ranging from rodents to
20 large animals such as pigs[47, 50, 57-59].

21 This specificity is further refined at the cellular level within the heart, which is a critical feature for both safety
22 and efficacy. The heart is a heterogeneous organ, with cardiomyocytes constituting only a fraction of the total
23 cell population[60-63]. The same "lock-and-key" transcriptional mechanism that confers organ-level
24 specificity also ensures that within the heart, the cTnT promoter is active exclusively in cardiomyocytes. Other
25 abundant cell types, such as cardiac fibroblasts, endothelial cells, and smooth muscle cells, do not express the
26 necessary combination of GATA4/Nkx2.5/MEF2 transcription factors and thus cannot activate the
27 promoter[45, 64, 65]. This cellular fidelity is indispensable for ensuring both safety and therapeutic efficacy.
28 Expression of a therapeutic gene, such as an ion channel or a calcium-handling protein, in non-target cells like
29 fibroblasts would be ineffective and could dangerously alter their signaling, proliferation, or contribution to
30 fibrosis[66, 67]. Likewise, misexpression in vascular cells could compromise vascular tone and integrity. By
31 restricting expression to the intended cell type, the cTnT promoter ensures that the therapeutic dose is
32 concentrated where it is needed, thereby maximizing efficacy and preventing unintended pathology in
33 neighboring cells[68-70].

34 This level of precision has been foundational for nearly all modern cardiac gene therapies aimed at correcting
35 intrinsic defects within cardiomyocytes. While the general cTnT promoter broadly targets the cardiomyocyte
36 population, more specialized promoters are available for targeting specific cardiomyocyte subtypes. For
37 instance, the myosin light chain-2v (MLC-2v) promoter is restricted to ventricular cardiomyocytes, whereas
38 the atrial natriuretic factor (ANF) promoter preferentially targets atrial cells[71, 72]. This higher-order
39 specificity is critical for developing treatments for chamber-specific diseases, such as atrial fibrillation and
40 ventricular arrhythmias. By leveraging this property, our sonogenetics approach enables more selective
41 transgene expression, thereby allowing the targeting of specific cardiac conditions with greater precision.
42 Therefore, the use of the general cTnT promoter represents a robust and well-validated strategy for broadly
43 and safely targeting the entire cardiomyocyte population, which constitutes the primary goal of our current
44 sonogenetic approach.

45 4. Discussion of Broader Translational Hurdles and the Path Forward

1 The clinical translation of any viral vector-based therapy faces significant hurdles, which the field is actively
2 working to overcome.

- 3 • Pre-existing Immunity: A major challenge is the presence of pre-existing neutralizing antibodies
4 (NAbs) against various AAV serotypes in a significant portion of the human population. Patients with
5 high NAb titers may not be eligible for treatment, as the antibodies would neutralize the vector before
6 it reaches its target. Strategies to address this include developing novel AAV capsids that evade
7 common antibodies and the potential use of transient immunosuppression regimens.
- 8 • Manufacturing and Cost: The large-scale manufacturing of clinical-grade AAV vectors is a highly
9 complex and expensive process. Reducing the cost and scaling up production to meet potential clinical
10 demand remain significant logistical challenges for the entire gene therapy field.
- 11 • Regulatory Pathway: As with any novel therapeutic modality, the regulatory pathway for a
12 sonogenetic-based gene therapy would be rigorous, requiring extensive preclinical safety and
13 toxicology studies followed by carefully designed clinical trials.

14 Despite these challenges, the clinical landscape for gene therapy is optimistic. The FDA approval of AAV9-
15 based therapies like Zolgensma for spinal muscular atrophy has paved a regulatory and clinical path forward.
16 The continued progress in vector engineering, manufacturing, and our understanding of the immune response
17 to AAVs provides confidence that these hurdles can be surmounted. Our work, by leveraging a clinically
18 relevant vector system and demonstrating high cardiac specificity, represents a crucial step in aligning the
19 exciting potential of sonogenetics with a feasible strategy for clinical translation.
20

Supplementary Text 4: Basis and Mechanistic Insights into MscL-G22S-Mediated Ultrasound Cardiac Pacing

1. Principles of Sonogenetic Cardiac Pacing:

Spontaneous calcium signaling is a hallmark of pacemaker cells in the sinoatrial (SA) node, where rhythmic calcium cycling, driven by calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR) and coupled with membrane depolarization, regulates natural pace making activity.

Here, we present ultrasound-mediated cardiac pacing via the mechanically sensitive ion channel MscL G22S as a novel method for heart rhythm regulation. Unlike electrical pacing, this approach utilizes mechanically induced calcium influx to initiate CICR, driving synchronized myocardial contractions. Following MscL-G22S transfection into the heart, ultrasound stimulation activates the ion channels, inducing calcium and sodium ion influx and subsequent depolarization. This depolarization triggers action potentials that propagate through gap junctions, ensuring synchronized cardiomyocyte contraction and replicating the effects of traditional pacemakers. Our results confirm that MscL G22S enables robust, externally controlled calcium signaling and precise cardiac pacing.

2. Biophysical Modulation of MscL-G22S in Cardiomyocytes

MscL gating is intrinsically mediated by membrane tension, rendering its function highly sensitive to the mechanical environment of the host cell. Cardiomyocytes provide a structurally and biophysically favorable substrate for this mechanism. The cardiac sarcolemma is mechanically reinforced by the costamere–integrin–dystrophin–glycoprotein complex, which links the contractile apparatus to the extracellular matrix through the Z-disks and sarcolemmal membrane[73]. This architecture efficiently transmits both intrinsic contractile and externally applied forces—such as those induced by ultrasound—into localized membrane deformations and tension gradients capable of gating MscL-G22S.

Furthermore, cardiomyocytes possess an elaborate T-tubule system that increases membrane surface area and enables subcellular compartmentalization of mechanical and electrical signaling. These invaginations are enriched in ion channels and may serve as focal hubs for ultrasound-induced membrane curvature and tension amplification[74]. Lipid raft microdomains, abundant in the cardiac sarcolemma, modulate membrane curvature, fluidity, and thickness, thereby influencing the mechanical properties of the membrane and lowering activation thresholds for mechanosensitive ion channels[75, 76].

Mechanical models indicate that cytoskeletal stiffness and membrane curvature profoundly influence channel-gating thresholds. Compared with other cell types such as neurons, cardiomyocytes exhibit greater stiffness and mechanical anisotropy, resulting in more efficient mechanical coupling and more consistent MscL activation during ultrasound exposure[77].

3.Comparative Biophysical Insights: Cardiomyocytes vs. Neurons

Neurons are structurally more compliant than cardiomyocytes. Their plasma membranes and underlying cytoskeletons are softer and exhibit reduced mechanical reinforcement. Consequently, neurons require higher ultrasound intensities or longer stimulation durations to generate sufficient membrane tension to gate mechanosensitive channels[78, 79]. In contrast, the stiffer cardiomyocyte membrane, denser cytoskeletal meshwork, and the presence of mechanical focal points such as intercalated discs enhance ultrasound energy transduction and provide a more conducive environment for MscL-G22S gating[80].

Moreover, cardiomyocytes display robust mechanical junctions and a three-dimensional sarcomeric organization that facilitate the spatial focusing of mechanical energy. These structural features are largely absent in neuronal tissues, further supporting the superior performance of MscL-G22S in cardiac pacing applications compared with CNS neuromodulation.

Supplementary Text 5: Different types of heart disease and corresponding pacemakers

There are various types of traditional pacemakers. The choice of these pacemaker is carefully determined by the physician based on the patient's specific condition and heart rhythm. For symptomatic bradycardia, characterized by abnormally slow heart rates that lead to symptoms such as fatigue, dizziness, or fainting, a dual-chamber or single-chamber pacemaker is often implanted depend on the situation to maintain a heart rate that ensures adequate blood flow[81]. The right atrium is stimulated if the patient's conduction system can still transmit signals to the ventricles, while the right ventricle is paced if the connection between the atria and ventricles is impaired.

For Mobitz Type II atrioventricular (AV) block, a condition where there is an intermittent failure of electrical signals to travel from the atria to the ventricles, a more sophisticated approach is needed[82]. This type of heart block can cause unpredictable drops in heart rate, leading to episodes of lightheadedness, fatigue, or even loss of consciousness. In such cases, a dual-chamber pacemaker is often used. This device has two leads: one placed in the atrium and another in the ventricle. By coordinating the contractions of the atria and ventricles, the pacemaker ensures that the ventricles contract immediately after the atria, mimicking the heart's natural rhythm [83].

In addition, patients with dyssynchrony between the left and right ventricles, symptoms such as shortness of breath, fluid retention, and exercise intolerance are common[84]. These symptoms occur because the ventricles are not contracting in a synchronized manner, reducing the heart's efficiency[85]. In these cases, a biventricular pacemaker, or cardiac resynchronization therapy (CRT) device, is used[86]. This device has an additional lead placed in the left ventricle via the coronary sinus, allowing it to stimulate both ventricles simultaneously. By resynchronizing the contractions of the left and right ventricles, the pacemaker improves cardiac output, reduces symptoms, and even enhances survival in patients with advanced heart failure[87].

Furthermore, hypertrophic obstructive cardiomyopathy with abnormal thickening of the heart muscle will obstruct blood flow. In such cases, an implantable cardioverter-defibrillator (ICD) is often the best option. This device not only provides pacing support but also continuously monitors the heart's rhythm. If a life-threatening arrhythmia is detected, the ICD delivers an electric shock to restore a normal rhythm, significantly reducing the risk of sudden cardiac death in these high-risk patients[88, 89].

Supplementary Text 6: Safety Thresholds and Regulatory Relevance

The NUP system operates in two distinct modes: diagnostic imaging and therapeutic stimulation, each subject to different FDA acoustic output regulations.

2.1 Imaging Mode

For diagnostic guidance, the system operates in standard B-mode with a peak rarefactional pressure of 2.2 MPa and a duty cycle of 0.1%. The primary goal is to achieve clear anatomical visualization while keeping acoustic exposure As Low as Reasonably Achievable (ALARA). Safety compliance is ensured by adhering to the FDA's stringent limits for cardiac diagnostic applications, which are a **Mechanical Index (MI) ≤ 1.9** and a derated spatial-peak temporal-average intensity ($I_{spta.3}$) $\leq 720 \text{ mW/cm}^2$ (Abbott, 1999 [96]; Nelson, 2009 [97]; AIUM, 2004 [98]; FDA, 2008).

The MI, a unitless metric indicating the likelihood of mechanical bioeffects, is calculated from the derated peak rarefactional pressure (in megapascals, MPa) at the point along the beam axis where the maximum pulse intensity is found, adjusted for tissue attenuation at a rate of 0.3 dB/cm/MHz ($P_{r.3}(Z_{sp})$) divided by the square root of the center frequency (f_c), as the formula listed below:

$$MI = \frac{P_{r.3}(Z_{sp})}{\sqrt{f_c}}$$

The relationship between the key intensity metrics, $I_{sppa.3}$ and $I_{spta.3}$ (Spatial-peak temporal/pulse-average intensities) are defined as:

$$I_{sppa.3} = (\text{hydrophone output})^2 / \text{Sens}(V^2 \text{cm}^2 / W)$$

$$I_{spta.3} = I_{sppa.3} * \text{duty cycle}$$

Where $\text{Sens}(V^2 \text{cm}^2 / W)$ is obtained by quantitative sound field measurement using a calibrated capsule hydrophone. By operating at low acoustic pressure and a minimal duty cycle, our system's resultant $I_{spta.3}$ and MI values remain well within the established diagnostic safety thresholds (**MI = 1.25**, **$I_{spta.3} = 162 \text{ mW/cm}^2$**), confirming the imaging function is fully compliant.

2.2 Stimulation Mode

For its therapeutic function, the NUP system delivers targeted energy to elicit cardiac pacing. The FDA guidance for therapeutic ultrasound limits the derated **spatial-peak pulse-average intensity ($I_{sppa.3}$) to 190 W/cm^2 and the MI to 1.9** (FDA, 2024). In cardiac stimulation mode, the system operates with a lower acoustic pressure of **$\sim 2.03 \text{ MPa}$** , and a corresponding **duty cycle of 2.5%** (25ms per 1s), optimized to achieve efficient pacing while minimizing energy exposure. According to our measurements:

- **MI ≈ 1.153 ,**
- **$I_{sppa.3} \approx 137.353 \text{ W/cm}^2$,**
- **$I_{spta.3} \approx 3.434 \text{ W/cm}^2$,**

—all of which fall **below the FDA therapeutic safety limits**, especially considering that these values are **unattenuated**. When corrected for tissue attenuation (0.3 dB/cm/MHz), actual in situ exposure is even lower, providing a safety margin. Additionally, the use of **minimal effective acoustic energy** aligns with the **ALARA principle** to avoid unnecessary biological effects while preserving efficacy.

1 **Table:** The actual measurements of the NUP system output acoustic power, combined with the pre-amplified
 2 driving conditions, for pressure, I_{spta} , I_{sppa} , and MI do not account for the 0.3 dB attenuation.

Pressure (MPa)	I_{spta} (W/cm ²)	I_{sppa} (W/cm ²)	MI
1.28	1.385	55.416	0.732
1.48	1.828	73.121	0.841
1.62	2.201	88.031	0.920
1.74	2.529	101.144	0.989
2.03	3.434	137.353	1.153
2.20	4.045	161.813	1.250

3 Furthermore, we assessed thermal safety during continuous ultrasound stimulation for over 12 hours
 4 (**Supplementary Fig. 31**). This extended in vivo safety assessment was conducted on anesthetized rats placed
 5 on a heating pad to maintain a stable core body temperature. A continuous intravenous infusion of saline was
 6 administered to ensure physiological stability throughout the prolonged procedure. The NUP system delivered
 7 uninterrupted pacing for 12 consecutive hours while fine-gauge (0.23 mm) thermocouple probes monitored
 8 the intracardiac temperature. The results show that for all three acoustic pressures tested (1.1, 1.5, and 2.0
 9 MPa), the maximum temperature increase was less than 1.2 °C. This observation is consistent with and further
 10 confirmed by FDA-required thermal index (TI) calculations, one of the key indices used to evaluate the clinical
 11 safety of ultrasound devices. The calculated TI of our stimulation is $TIS \approx 0.4\text{--}1.2$, depending on focal area
 12 and acoustic pressure, indicating that the temperature increase does not exceed 1.2 °C. Crucially, once
 13 ultrasound stimulation was terminated, the intracardiac temperature promptly returned to its baseline level.
 14 This experiment provides direct and compelling evidence that our NUP system can be operated for prolonged
 15 periods without inducing significant or hazardous thermal effects, strongly supporting its safety profile for
 16 chronic therapeutic applications.

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1 **Supplementary Movies**

2 **Supplementary Movie 1.** Hydrogel interface ensures stable adhesion.

3 **Supplementary Movie 2.** Ultrasound stimulation of microbeads for localizing the stimulation center.

4 **Supplementary Movie 3.** Ultrasound stimulation of sonogenetically transfected cardiomyocytes.

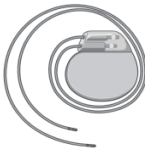
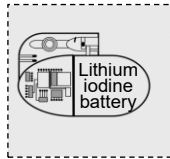
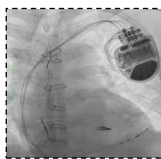
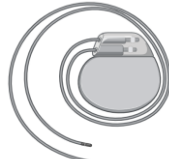
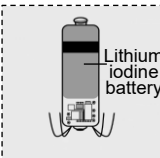
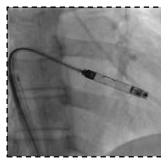
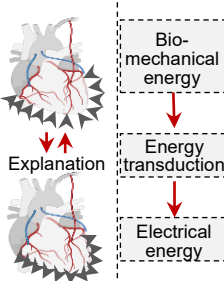
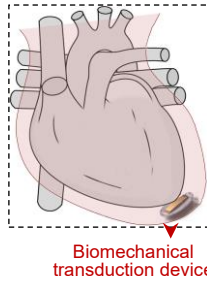
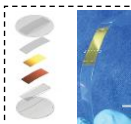
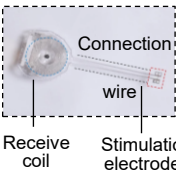
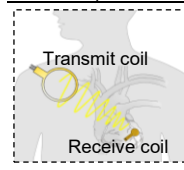
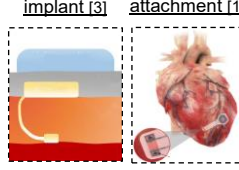
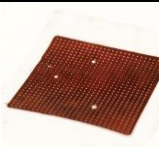
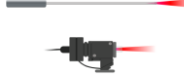
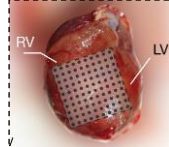
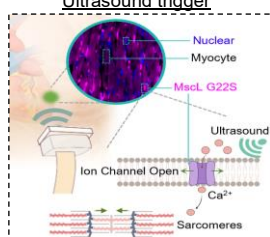
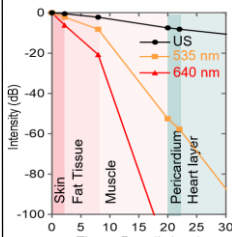
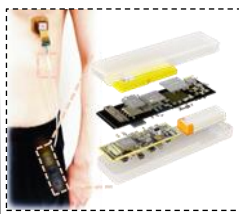
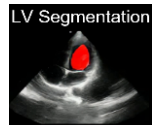
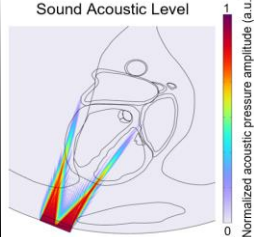
5 **Supplementary Movie 4.** Left ventricle identification and segmentation for area calculation.

6 **Supplementary Movie 5.** Left ventricle identification and output of three stimulation positions.

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1 Supplementary Data Table:

2 Supplementary Table 1. Comparison to conventional pacemakers.

Type	Configuration	Power solution	Implant procedures	Self Heartrate Detection	Real-time imaging guidance	Multi-chamber Pacing capability	Cellular level precision
Commercial pacemaker	<u>Lead Pacemaker</u> 	<u>Lead Pacemaker</u> [4] 	<u>Subcutaneous implant</u> [6] 	✓	✗	<u>Two or Multi Lead Pacemaker</u> 	✗
	<u>Leadless Pacemaker</u> [2] 	<u>Leadless Pacemaker</u> [2] 	<u>Intracardiac implant</u> [10] 	✗	✗	<u>Two or Multi Implant</u> 	✗
Self-powered pacemaker	<u>Triboelectric generator</u> [8] 	<u>Mechanical energy form heartbeat</u> 	<u>Surface attachment</u> [7] 	✗	✗	✓	✗
	<u>Piezoelectric Generator</u> [7] 		<u>Biomechanical transduction device</u> 				
Wirelessly-powered pacemaker	<u>Electromagnetic Induction</u> [1] 	<u>Wirelessly Powered</u> [1] 	<u>Subcutaneous implant</u> [3] <u>Surface attachment</u> [1] 	✗	✗	✗	✗
	<u>Light Source Powered</u> [5] 	<u>Light powered</u> <u>External NIR light controller</u> 	<u>Surface attachment</u> [5] 	✗	✗	<u>Surface attachment</u> [5] 	✗
Sono-genetics pacemaker	<u>Sono-sensitive Ion Channel Overexpress and Ultrasound trigger</u> 	<u>Ultrasound Excellent Tissue Penetration properties</u> 	<u>Wearable Transducer Attach to Chest & Portable System in the Pocket</u> 	<u>LV Segmentation</u> 	<u>Changing the acoustic field emission to target multi regions</u> 	✓	

3

1 **Supplementary Table 2.** Comparative information of AAV vector variants.

Vector Type	Capsid Source	Genome Backbone/ITRs Source	Cardiac Tropism	Systemic Delivery Efficiency	Pre-existing Antibodies (Human)	Required Delivery Method	Transduction Efficiency (Cardiomyocytes)	Packaging/GMP Production Considerations
AAV2 (Native)	AAV2	AAV2	Poor	Inefficient	High prevalence	Invasive/Local	Low	Established/Complex
AAV9 (Native)	AAV9	AAV9	Potent/Natural	High	Relatively lower	Minimally Invasive/Systemic	High	Established/Complex
AAV2/9 (Pseudo-typed)	AAV9	AAV2	Excellent (via AAV9 capsid)	High (via AAV9 capsid)	Associated with AAV9 capsid	Minimally Invasive/Systemic	High	Simplified/Scalable (via AAV2 ITRs)

1 **Supplementary Table 3.** Comparative analysis of mechanosensitive ion channels for ultrasound cardiac
2 pacing suitability

MscL-G22S	0.25-0.45 MPa (0.5 MHz, neurons); ~0.5-10 W/cm ² (intensity varies with setup)	0.5 - 15 MHz; Optimized for 2.2-3.0 MHz for cardiac applications	Large nonselective cation pore (Na ⁺ , Ca ²⁺ , K ⁺)	Depolarizing (+25 to +40 mV)	~5–10 ms	Rapid (~10–20 ms)	Exogenous	Yes	Ideal for pacing due to rapid, sustained depolarization at relevant cardiac US frequencies. [39, 99]
TREK-1	~1.0 MPa	~0.5 MHz	K ⁺ efflux (K2P family)	Hyperpolarizing (-10 to -20 mV)	20–50 ms	Slow (>300 ms)	Endogenous; contributes to resting potential and repolarization [100].	No	Hyperpolarizing effect is unsuitable for pacing [24].
TREK-2	~1.0 MPa	<1 MHz	K ⁺ efflux	Hyperpolarizing (-10 to -30 mV)	~30 ms	Slow (>300 ms)	Endogenous in heart.	No	Similar to TREK-1 [101].
TRAAK	Not well-quantified for US;	<1 MHz	K ⁺ efflux	Hyperpolarizing (~-15 mV)	~30 ms	Moderate-slow (100–300 ms)	Endogenous; less responsive in CMs.	No	Similar to TREK-1 [101].
Piezo1	Highly frequency-dependent; 0.3-0.5 MPa; >50 W/cm ²)	0.2-0.8 MHz / 43 MHz	Nonselective cation (Ca ²⁺ preferred)	Depolarizing (+10 to +20 mV)	10–50 ms	Rapid (~10–50 ms), desensitizes	Endogenous; involved in mechano-electric feedback, stretch sensing, development, and pathology[102].	Unclear	It may be suitable for pacing, its optimal frequency is less appropriate for this application than that of MscL G22S [29, 103].
Piezo2	~0.3 MPa	<1 MHz	Nonselective cation	Depolarizing (+5 to +15 mV)	5–30 ms	Very Rapid (~10–20 ms)	Primarily in sensory neurons; low/no expression in adult CMs.	No	Similar to Piezo2 [29].
TRPA1 (human)	~0.2 MPa (6.9 MHz, Duque et al.); ~0.3 W/cm ²	6.9 MHz / 0.43 MHz	Ca ²⁺ /Na ⁺ influx	Depolarizing (+30 to +40 mV)	5–15 ms	Moderate (~100–200 ms)	Low/debated expression in CMs; validated for US modulation in CNS/astrocytes[104, 105].	No	Activation frequencies generally outside 2-4 MHz cardiac range.
TRPC1	Not precisely defined for US; (4 MPa)	~1 MHz	Ca ²⁺ /Na ⁺ influx	Depolarizing (+10 mV)	>30 ms	Moderate (~100–150 ms est.)	Endogenous; involved in stretch-activated Ca ²⁺ entry, hypertrophy, and fibrosis[106].	Unclear	Slower kinetics and less defined US response for pacing compared to MscL-G22S [107].
TRPP2 (PKD2)	Not well-defined for US	Unknown	Ca ²⁺ /Na ⁺ influx; primarily ER Ca2+ release	Unknown for US	Unknown for US	Unknown for US	Endogenous; interacts with RyR2 in SR, important for Ca ²⁺ homeostasis; mutations cause cardiac defects [108].	No	Primarily an intracellular channel, complex regulation, not a primary plasma membrane pacer for US [29].
TRPM4	~0.3 MPa (indirect, via Ca ²⁺ from primary MS channel)	~1 MHz	Na ⁺ influx only	Depolarizing (+5–10 mV)	10–20 ms	Rapid (~30–50 ms)	Endogenous; Ca ²⁺ activated, contributes to AP shape and arrhythmogenesis[77].	No (as primary)	Acts as an amplifier of Ca2+ signals; not a primary US sensor itself for pacing [29].
TRPV1	≥1.5 MPa / ~50 W/cm ² (thermal effect)	1.7-5 MHz (thermal effect)	Ca ²⁺ /Na ⁺ influx	Depolarizing (+30 mV)	Slow, ≥500 ms to >1s (heat latency)	Slow (>1 s)	Endogenous in cardiac sensory nerves; exogenous expression in CMs possible [109].	No (for precise pacing)	Primarily thermal activation by US, slow latency, not suitable for precise, rapid pacing [30].
Prestin (N71/N308S)	~0.5 MPa	0.5 MHz	Electromotility (not direct ion channel)	Indirect	50–100 ms	Moderate (~100–200 ms)	Unknown	No	Operates at low frequency, indirect mechanism, not suitable for cardiac pacing [35].

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