

A Wearable Noninvasive Sonogenetic Pacemaker

Corresponding Author: Professor Qifa Zhou

Version 0:

Decision Letter:

Dear Professor Zhou,

Thank you again for submitting to *Nature Biomedical Engineering* your manuscript, "A Noninvasive Sonogenetic Pacemaker". The manuscript has been seen by 3 experts, whose reports you will find at the end of this message.

You will see that the reviewers appreciate aspects of the work. However, they articulate concerns about the degree of support for some of the claims and about the advance that the work represents over relevant published studies, and provide useful suggestions for improvement. We hope that with substantial further effort you can address the criticisms, increase the strength of the evidence, and convince the reviewers of the merits of the study. In particular, we would expect that a revised version of the manuscript provides:

- * Additional investigation on the long term effects of overexpression of mechanosensitive channels in the heart, as per the many comments of all the reviewers.
- * Extensive biosafety and methodological reports as well as discussion on safety regulation.
- * Clarification of the employed viral vector, with rationale to explain its choice.
- * Validation of device integration in the in vivo sonogenetic experiments.

When you are ready to resubmit your manuscript, please [upload](#) the revised files, a point-by-point rebuttal to the comments from all reviewers, the [reporting summary](https://www.nature.com/authors/policies/ReportingSummary.pdf), and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

- * Please submit your revised manuscript in a word doc or LaTeX format.
- * Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).
- * If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).
- * If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).
- * Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.
- * The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).
- * Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We expect that you will be able to resubmit the manuscript within **20 weeks** of receiving this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical*

Engineering.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Valeria

Dr Valeria Caprettini

Senior Editor, Nature Biomedical Engineering

Reviewer #1 (Report for the authors (Required)):

The manuscript by Gong et al. reports the development of a noninvasive ultrasound pacemaker (UNP) system based on the sonogenetic activation of cardiomyocytes. The authors employed intravenous delivery of adeno-associated viral vectors (AAVs) to express the sonosensitive mechanosensitive ion channel variant MscL G22S in cardiomyocytes. They used focused ultrasound to noninvasively stimulate these cells noninvasively, achieving spatiotemporally controlled cardiac pacing. The system's performance was evaluated in vitro using transfected human cardiomyocytes and in vivo in rodent models. Furthermore, the authors demonstrate the ability of the UNP system to restore sinus rhythm in an arrhythmia rat model. They also present the design of a wearable ultrasound device for potential human application, and demonstrate ultrasound's ability to penetrate deep tissue using an ex vivo porcine heart model. The reviewer appreciates the significance of advancing noninvasive cardiac pacing technologies and recognizes the innovation in extending the application of sonogenetics beyond previous studies focused primarily on neurons and T cells. While the manuscript presents an important advance in the field of sonogenetics, it falls short of representing a sufficiently transformative advance in cardiac pacing to meet the standards of Nature BME, due to the following major criticisms.

Major technical criticisms

1. The authors present a wearable multi-channel ultrasound system capable of real-time imaging and steerable stimulation, which is positioned as a key innovation. However, the wearable system, designed for human applications, was not fully integrated into the in vivo rodent studies. The unique features of the wearable device, such as the cloud-based AI model, hydrogel interface, and image-guided targeting, were not utilized for the sonogenetic experiments in rats. Instead, device validation was limited to ultrasound penetration in an ex vivo porcine heart. This lack of integration creates a disconnection between the engineering innovation and the biological proof-of-concept.
2. The study demonstrates sonogenetic pacing in anesthetized rats. However, noninvasive, cell-type-specific cardiac pacing using optogenetics in freely moving mice has been previously reported. Thus, while the application of sonogenetics to cardiac pacing is novel, the degree of technological breakthrough in achieving noninvasive, precise cardiac control by the work reported in this manuscript is considered moderate in the context of existing literature.
3. Fig. 3d shows significant enrichment of transcriptional regulatory pathways in cardiomyocytes expressing MscL G22S, with multiple GO terms displaying high statistical significance. These transcriptional alterations raise concerns about the biological impact of overexpressing foreign mechanosensitive ion channels in the heart, and the long-term consequences for cardiac function are not addressed.
4. The demonstration of cardiac rhythm restoration in a rat arrhythmia model is the highlight of this manuscript; however, the data presented in Fig. 4 raises several issues. First, the baseline heart rate in the model (180 bpm) differs markedly from the baseline reported elsewhere in the manuscript (~240 bpm), raising questions about the consistency of the model. Second, there is no control group consisting of animals expressing MscL-G22S without ultrasound stimulation. Given the mechanosensitivity of MscL, it remains unclear whether mechanical forces from normal heart contractions could activate the channel independently of ultrasound. This is critical for interpreting therapeutic potential and safety. Third, the number of animals used in the study is not provided, making it difficult to evaluate the scientific rigor of the data.
5. While the authors claim that "comprehensive biosafety assessment" supports clinical translation, the biosafety data provided is limited. The requirement for genetic modification via viral transduction presents a major translational hurdle that is not adequately discussed. Furthermore, clinical pacemakers are intended for continuous or frequent long-term stimulation (hours per day, for years), whereas the safety evaluation presented is limited to acute, single-session stimulation. Chronic safety assessment would be essential for substantiating translational relevance.

Minor technical criticisms

1. There is an inconsistency regarding the viral vector used. The Methods section mentions AAV2-cTnT, while the Supplementary Materials reference AAV9 and AAV2/9 in Extended Fig. 4. Given that AAV9 is known for its cardiac tropism and AAV2 is not, clarification of the serotype used is critical. Additionally, while Fig. 3b shows expression of MscL G22S, quantitative analysis of cell-type specificity is not provided, which is essential for validating the precision of the sonogenetic approach.
2. The manuscript states that the sonogenetic system achieves <1 mm spatial resolution using a 3 MHz single-element focused transducer. However, detailed characterization of the ultrasound focal zone size is not provided. Furthermore, spatial resolution was assessed under open-chest conditions; transmission through the chest wall would likely degrade

spatial precision, and this effect is not characterized.

3. The study transitions from mice to rats for different experiments without a rationale. Similarly, the ultrasound stimulation setup changes from the single-element system to the NUP device without a clear explanation, complicating the interpretation of the results.

Reviewer #2 (Report for the authors (Required)):

This manuscript presents an exceptionally innovative and technically outstanding platform—the Noninvasive Ultrasound Pacemaker (NUP)—poised to redefine the landscape of cardiac rhythm management. By seamlessly combining sonogenetic sensitization of cardiomyocytes via the MscL G22S channel with a compact, AI-driven, wearable ultrasound array, the authors achieve a remarkable milestone: truly noninvasive, chamber-specific cardiac pacing. This is a first in the field and represents a bold leap toward implant-free, patient-friendly cardiac therapies.

The study is underpinned by a rich tapestry of methodologically sound experiments, including rigorous in vitro calcium imaging, multi-species (rodent and porcine) validations, and comprehensive safety assessments, all orchestrated within an advanced closed-loop system guided by real-time imaging. The work stands out not only in concept but also in execution, embodying the highest standards in biomedical engineering and translational research.

I strongly recommend acceptance pending revisions. The following comments aim to further elevate this already exceptional manuscript:

Major Comments

1. Quantitative Characterization of MscL G22S Channel Sensitivity

To strengthen the mechanistic insight, please include quantitative activation data for the MscL G22S channel. Specifically, pressure–response curves (e.g., EC_{50} , Hill coefficient) obtained via patch-clamp electrophysiology or quantitative calcium-flux assays would greatly enhance our understanding of its mechanical sensitivity. In addition, the inclusion of a plasmid control group lacking the MscL G22S insert is essential to verify specificity.

2. Safety Thresholds and Regulatory Relevance

Given the authors' claim of preclinical validation, it is important to contextualize the chosen acoustic pressure (~2 MPa) against FDA diagnostic ultrasound limits (e.g., $MI \leq 1.9$, $ISPTA \leq 720 \text{ mW/cm}^2$). Please include a quantitative discussion of safety margins, particularly after accounting for chest-wall attenuation in porcine models, and reference the FDA's "Marketing Clearance of Diagnostic Ultrasound Systems and Transducers" guidance.

3. Mechanosensitive Channel Comparisons and Biophysical Mechanisms

In Supplementary Text 2, I recommend a direct experimental or literature-based comparison of MscL G22S with other mechanosensitive channels relevant at cardiac ultrasound frequencies (e.g., Piezo1, TREK-1, TRPA1). This will reinforce MscL G22S as the optimal choice. Additionally, given that MscL is central to the biological mechanism of the NUP, please provide a deeper biophysical discussion on how its function is modulated in cardiac membranes. Comparative insights between cardiomyocytes and other cell types (e.g., neurons) in terms of membrane tension coupling, lipid raft distribution, and cytoskeletal interactions would be highly valuable.

4. Ex Vivo Porcine Pacing Validation

To confirm true myocardial depolarization, please supplement the current ex vivo validation with ECG recordings from porcine hearts under NUP stimulation. This would offer compelling functional evidence of pacing efficacy.

5. Long-Term Calcium Handling Stability

To ensure safety under repeated stimulation, it is recommended to perform immunostaining and quantitative analyses of key calcium-handling proteins (e.g., RyR2, SERCA2a, PLB) to assess any long-term effects on intracellular Ca^{2+} cycling.

Minor Comments

1. Define Abbreviations Clearly

Please define all acronyms (e.g., PRF, ST, MEA, FWHM, MI, ISPTA) at first mention in both the main text and supplementary materials to improve readability for interdisciplinary audiences.

2. Standardize and Clarify Data Presentation

Several bar graphs lack consistency in sample size indication (e.g., Fig. 2e, Extended Data Fig. 4b). Ensure that sample sizes (n), error bars ($\pm SD$ or 95% CI), statistical tests, and p-values or significance levels are provided in all figure panels. Unify error bar styles across figures for visual consistency.

3. Confirm qPCR Analysis Methodology

Please confirm that the $2^{-\Delta\Delta CT}$ method was used for RT-qPCR analysis. GAPDH does not need to appear in the final figure (e.g., Fig. S10) but should be clearly stated as the internal control in the text and calculations.

4. Minor Typographic Corrections

Fix typographical errors—for example, “strqess” should be corrected to “stress” in Fig. S10.

This work is a tour de force in noninvasive biomedical device engineering. The integration of synthetic biology, acoustics,

and intelligent control exemplifies next-generation, systems-level thinking in translational medicine. With the suggested enhancements, this manuscript is poised to become a landmark contribution to the field of bioelectronic medicine and cardiac therapeutics.

Reviewer #3 (Report for the authors (Required)):

This study presents an innovative and integrated approach to noninvasive cardiac pacing by combining mechanosensitive ion channels (MscL G22S) with an AI-regulated, cloud-connected ultrasound system. By harnessing the ability of MscL G22S to modulate Ca^{2+} signaling in cardiomyocytes upon ultrasound stimulation—a key trigger for cardiac contraction—the authors demonstrate spatially and temporally precise cardiac activation. Cardiac-specific expression of the channel, achieved via AAV vectors, was rigorously evaluated in animal models, confirming both safety and responsiveness to ultrasound. The AI-driven ultrasound system autonomously manages stimulation site selection, cardiac function monitoring, and delivery of optimized pacing stimuli. Its ability to target specific cardiac regions, regardless of depth relative to the body surface, offers unprecedented flexibility in pacing site selection without the need for surgically implanted leads.

I commend the authors for translating bold concepts into a cohesive, clinically relevant framework. The potential to select pacing sites without invasive procedures is particularly compelling. Moreover, integrating ultrasound—a cornerstone of cardiology practice—with sonogenetics and real-time cloud-based data analysis elevates both the diagnostic and therapeutic potential of this platform.

This work provides important insights into noninvasive pacemaker technology and employs sophisticated analytical approaches; however, several key areas warrant further clarification and refinement.

Major Comments

1. Chronic Ca^{2+} Overload Risk:

The pacemaking system relies on Ca^{2+} influx induced by the MscL G22S channel. While the authors demonstrate minimal effects on the expression of genes related to harmful responses such as inflammation or apoptosis, the long-term implications remain uncertain. Clinical scenarios such as complete atrioventricular (AV) block or severe sick sinus syndrome require continuous pacing, raising concerns about potential cardiac remodeling or fibrosis due to chronic Ca^{2+} overload. This issue should be addressed using mid- to long-term pacing models. Additionally, in such models, it would be valuable to assess potential changes in the expression of other ion channel genes.

2. Variability in Channel Expression:

In a clinical setting, MscL G22S expression levels are likely to vary both within an individual heart and between patients. Is there any available data on the distribution or variability of transgene expression following AAV-mediated delivery? Furthermore, the relationship between ion channel density in targeted cardiomyocytes and their responsiveness to ultrasound stimulation should be explored.

3. Choice of AAV Serotype and Off-Target Risks:

The study employs AAV2-cTNT-MscL G22S for selective cardiac expression in mice, but the rationale for using the non-cardiotropic AAV2 serotype remains unclear. For potential clinical translation, tissue-specific targeting is critical. Future strategies could include incorporating cardiac-specific enhancers or using alternative promoters to enhance cardiac specificity. Additionally, if the channel is expressed in non-cardiac tissues such as skeletal muscle or neurons, there may be a risk of abnormal contractions upon ultrasound stimulation. Do the authors have additional data on off-target expression levels or activation thresholds in non-cardiac tissues?

4. Unintentional Activation Scenarios:

Please provide an example scenario in which the ultrasound-triggered pacing system might be unintentionally activated, analogous to how conventional electrical pacemakers must be safeguarded against electromagnetic interference.

5. Dyssynchrony and Multi-site Pacing:

This technology's ability to selectively pace specific cardiac regions is highly promising. However, even slight deviations in pacing site can induce ventricular dyssynchrony, potentially worsening cardiac function or precipitating heart failure. Are there plans to evaluate the risk of dyssynchrony in future studies? Additionally, please discuss the feasibility of multi-site pacing (e.g., simultaneous or staged atrioventricular [A-V] and left-right ventricular [LV-RV] pacing) from the standpoints of technological complexity and power consumption.

6. Tolerability of External Ultrasound Module:

While the minimally invasive nature of this system is commendable, the external ultrasound module raises concerns regarding skin tolerability, potential skin damage, and long-term maintenance. Detailed information on these aspects would be useful. Furthermore, patient movement could significantly alter heart positioning, potentially moving it outside the ultrasound monitoring zone. How does the system detect such displacement, and what safeguards or recovery mechanisms are in place to maintain capture if the heart moves out of the effective imaging field?

7. Temperature Effects with Prolonged Use:

Although the authors report temperature data for up to 1200 stimulations, is there additional data on temperature variations during prolonged, continuous use over days or weeks?

Minor Comments

1. Typographical Errors:

There are typographical issues in several figures. For example, in Extended Data Fig. 6d, one X-axis label is incorrectly written as "3830" instead of "3837." Also, in Extended Data Fig. 7j, "Atrial Fibrillation" should be spelled correctly.

2. Figure Panel Order:

The order of figure panel labels is inconsistent. For example, Fig. 2b and Fig. 5b appear after panels labeled with later letters.

3. Battery Life and Feasibility:

Given the inherent limitations in battery life, how do the authors envision the clinical feasibility and sustainability of this system?

Version 1:

Decision Letter:

Dear Dr. Zhou,

Thank you for submitting your revised manuscript "A Noninvasive Sonogenetic Pacemaker" (NBME-25-1039A). Having consulted with the original reviewers (whose comment you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in Nature Biomedical Engineering, pending minor revisions to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements. In order to simplify these checks, please **provide the manuscript in a word file by reply of this email (we need to have the figures included, only the captions at the end of the document)**. Please do not upload the final materials and make any revisions until you receive this additional information from us.

TRANSPARENT PEER REVIEW

Nature Biomedical Engineering offers a transparent peer review option for new original research manuscripts submitted from 17th February 2021. We encourage increased transparency in peer review by publishing the reviewer comments, author rebuttal letters and editorial decision letters if the authors agree. Such peer review material is made available as a supplementary peer review file. **Please state in the cover letter 'I wish to participate in transparent peer review' if you want to opt in, or 'I do not wish to participate in transparent peer review' if you don't.** Failure to state your preference will result in delays in accepting your manuscript for publication.

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Sincerely,

Valeria

Dr Valeria Caprettini

Senior Editor, <http://www.nature.com/nbme> >Nature Biomedical Engineering

Reviewer #1 (Report for the authors (Required)):

My comments have been carefully addressed. I appreciate the authors' efforts in adding 16 new figures, four supplementary tables, and two supplementary texts to address my concerns. I have no further comments.

Reviewer #2 (Report for the authors (Required)):

The article can be accepted as is. The authors did a great job addressing the reviewers' comments.

Reviewer #3 (Report for the authors (Required)):

The authors have addressed my previous comments and have significantly improved the manuscript. I have no further comments.

Version 2:

Decision Letter:

Dear Qifa,

I am happy to inform you that your manuscript, "A Wearable Noninvasive Sonogenetic Pacemaker", has now been accepted for publication in *Nature Biomedical Engineering*.

Over the next few weeks, the figures will be checked for production quality, the text edited to ensure that it conforms to house style, and the manuscript typeset.

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Thank you for having submitted this work to *Nature Biomedical Engineering*.

Best wishes,

Valeria

Dr Valeria Caprettini

Senior Editor, [Nature Biomedical Engineering](http://www.nature.com/nbme)

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Response to Referees' Comments (manuscript nBME-25-1039)

We sincerely thank the editors and reviewers for their insightful and constructive feedback on our manuscript, which has been invaluable in guiding our revisions. In response to the primary concerns, we have carefully revised the manuscript to address every point. Specifically, we incorporated a new investigation into the potential long-term effects of overexpressing mechanosensitive channels in the heart, a key point raised by all reviewers. Furthermore, we expanded the manuscript to include extensive biosafety and methodological reports, along with a detailed discussion of the safety and regulatory landscape pertinent to this technology. We also provided a clearer explanation of the employed viral vector, with a thorough rationale for its choice in this application. We are confident that these additions have substantially strengthened our findings, solidifying the novelty and importance of our work on a non-invasive sonogenetic pacemaker.

A detailed point-by-point response is provided below, and all changes have been highlighted in the revised manuscript for your convenience. Thank you once again for your expertise and for your significant contribution to improving our work.

Reviewer #1:

The manuscript by Gong et al. reports the development of a noninvasive ultrasound pacemaker (UNP) system based on the sonogenetic activation of cardiomyocytes. The authors employed intravenous delivery of adeno-associated viral vectors (AAVs) to express the sonosensitive mechanosensitive ion channel variant MscL G22S in cardiomyocytes. They used focused ultrasound to noninvasively stimulate these cells noninvasively, achieving spatiotemporally controlled cardiac pacing. The system's performance was evaluated in vitro using transfected human cardiomyocytes and in vivo in rodent models. Furthermore, the authors demonstrate the ability of the UNP system to restore sinus rhythm in an arrhythmia rat model. They also present the design of a wearable ultrasound device for potential human application, and demonstrate ultrasound's ability to penetrate deep tissue using an ex vivo porcine heart model. The reviewer appreciates the significance of advancing noninvasive cardiac pacing technologies and recognizes the innovation in extending the application of sonogenetics beyond previous studies focused primarily on neurons and T cells. While the manuscript presents an important advance in the field of sonogenetics, it falls short of representing a sufficiently transformative advance in cardiac pacing to meet the standards of Nature BME, due to the following major criticisms.

We are very grateful to the reviewer for the detailed feedback and for recognizing our NUP system as a key innovation. This acknowledgment is greatly appreciated, and we appreciate the opportunity to further clarify our system's advanced capabilities, experimental design choices, and the overall significance of our findings. To address all reviewers' comments, we added **16 new figures, 4 supplementary tables, and 2 supplementary texts**. Responses to each specific comment are provided below. Newly added content in the manuscript is highlighted in [blue](#).

Comment 1. The authors present a wearable multi-channel ultrasound system capable of real-time imaging and steerable stimulation, which is positioned as a key innovation. **However, the wearable system, designed for human applications, was not fully integrated into the in vivo rodent studies.** The unique features of the wearable device, such as the cloud-based AI model, hydrogel interface, and image-guided targeting, were not utilized for the sonogenetic experiments in rats. Instead, device validation was limited to ultrasound penetration in an ex vivo porcine heart. This lack of integration creates a disconnection between engineering innovation and the biological proof-of-concept.

Response:

We thank the reviewer for this important comment on the integration of the NUP system in our in vivo studies. We also appreciate the opportunity to emphasize that the NUP system, in its core function as an integrated imaging and therapeutic device, was indeed fully utilized for biological validation in our in vivo rodent studies. The **fundamental features** of the NUP system, including stimulation and imaging, were validated in these models. Specifically, we used the NUP to successfully demonstrate noninvasive overdrive pacing, increasing

the heart rate from a baseline of 240 bpm to 540 bpm (**Fig. 3e**). We also demonstrated different chamber pacing (**Fig. 3h-i**) and rescue of chemically induced arrhythmias (**Fig. 4**), confirming the biological efficacy of the sonogenetic approach. The system's imaging capabilities were also used on these animals to acquire the B-mode and M-mode images that guided the arrhythmia recovery experiments (**Fig. 4b, f, j**). Using these images, the operator could "point-and-click" on specific cardiac locations on the screen to trigger stimulation. These critical experiments validated our primary biological hypothesis: that the NUP can deliver precise, image-guided sonogenetic stimulation to achieve effective cardiac control in a living animal.

We agree with the reviewer that several **human-centric features** were not applied in rodent experiments. This was a deliberate choice based on relevance and feasibility. Developing a robust AI model to detect various cardiac disease states requires a massive clinically relevant dataset, typically thousands of videos. It was neither practical or necessary to create such a resource for a rodent model, since the device is not intended for that use. Instead, we focused on the translational path by training our models on a large existing database of human cardiac ultrasound videos, supplemented with hundreds of videos collected using the NUP system. This approach ensures that the AI pipeline is clinically relevant.

Similarly, the bioadhesive hydrogel was specifically engineered for long-term daily wear in humans to avoid the inconvenience of reapplying standard ultrasound gel. This long-term adhesion was not essential for acute rodent experiments, in which animals were anesthetized and immobilized in a stereotaxic frame. For reasons of cost-effectiveness and convenience during this preclinical validation phase, standard ultrasound gel was used. We confirmed that the acoustic coupling and stimulation results were equivalent between the two mediums, ensuring this choice did not impact our scientific conclusions. As the advantages of this hydrogel for wearable applications have been previously reported (Wang, et al. *Science*[1]. Liu, et al. *Science Advance*[2]), we further emphasized this component in the animal validation section.

Therefore, we adopted a rigorous, two-pronged validation strategy. We used the rodent model to validate the core biological principle and therapeutic efficacy of NUP-driven sonogenetics, demonstrating noninvasive, image-guided pacing and arrhythmia rescue in living animals. Simultaneously, we validated the human-centric engineering innovations using the most appropriate models: demonstrating sufficient energy penetration in ex vivo porcine hearts and validating the AI diagnostic pipeline on human clinical imaging data. We believe this approach, which prioritizes the development of a complete, clinically relevant platform over a simplified, animal-specific device, is a strength of our study.

References:

1. Wang, C., et al., *Bioadhesive ultrasound for long-term continuous imaging of diverse organs*. *Science*, 2022. **377**(6605): p. 517-523.
2. Liu, H.-C., et al., *Wearable bioadhesive ultrasound shear wave elastography*. *Science Advances*, 2024. **10**(6): p. eadk8426.

Comment 2. The study demonstrates sonogenetic pacing in anesthetized rats. However, noninvasive, cell-type-specific cardiac pacing using optogenetics in freely moving mice has been previously reported. Thus, while the application of sonogenetics to cardiac pacing is novel, the degree of technological breakthrough in achieving noninvasive, precise cardiac control by the work reported in this manuscript is considered moderate in the context of existing literature.

Response:

We thank the reviewer for this insightful comment. We acknowledge that optogenetic approaches have previously demonstrated cell-type-specific pacing in freely moving mice. However, our work establishes **one fundamental discovery: sonogenetics can be harnessed for precise, noninvasive cardiac pacing**. By sensitizing cardiomyocytes with the mechanosensitive ion channel MscL G22S and activating them with externally delivered ultrasound, we demonstrate for the first time that deep-penetrating acoustic energy can achieve robust, rhythm-restoring cardiac control.

Compared with optogenetics, our approach has a distinct translational advantage. Light scattering and attenuation in tissue severely limit the feasibility of external cardiac pacing in humans, requiring invasive optical fibers. In contrast, ultrasound provides excellent tissue penetration (attenuation <15 dB at clinically relevant depths), making it uniquely suited for noninvasive therapy at the human scale. Thus, while optogenetics has been highly valuable in basic research, our sonogenetic platform offers a tangible path toward clinical translation. A direct comparison of traditional pacemakers, optogenetics, and our sonogenetic NUP is provided in **Table R1**, contrasts our sonogenetic NUP with commercial lead [1-3] and leadless [4] devices, self-powered (triboelectric [5] or piezoelectric [6]), other wirelessly powered systems (electromagnetic induction [7, 8] or light [9]), and optogenetic systems, which highlights the unique combination of noninvasiveness, imaging guidance, multi-chamber control, and cellular precision achieved by our system.

To realize this breakthrough, we developed **two key technological innovations**:

1. The First Wearable Ultrasound Imaging-Neuromodulation System

Our NUP platform integrates a 64-channel wearable ultrasound array that enables real-time B-mode imaging (~7 fps, sub-millimeter resolution) together with electronically steerable, high-energy stimulation (1–3.8 MPa, PRF up to 9 Hz) in a compact, battery-powered device operating up to 8 hours. Unlike previous wearable patches that focused on imaging only and often relied on bulky systems such as Verasonics[10] or Iconeus[11], our system combines diagnostic and therapeutic functions in a fully integrated, low-cost, and accessible format. Prior efforts have demonstrated impressive wearable imaging technologies—from continuous cardiac imaging (Hu et al., *Nature*[12]) and bioadhesive shear wave elastography (Liu, et al. *Science Advance*[13]) to portable hemodynamic monitoring (Lin et al., *Nature Biotechnology*[14]) and skin-conformable wrist electronics (Wang et al., *Nature Electronics*[15])—yet all remained limited by reliance on external hardware, single-channel operation, or the absence of stimulation. In contrast, the NUP supports 64 fully parallel channels with low latency, high frame rate, and robust stimulation capability, making it not only a diagnostic tool but also the first compact and wearable therapeutic ultrasound neuromodulation platform.

2. AI-enabled Imaging Guidance and Closed-Loop Control

The system incorporates AI-driven segmentation and feedback control, allowing imaging-guided pacing, chamber-specific targeting, and multi-site stimulation, and disattachment detection. This feature provides a new level of precision and safety, ensuring robust rhythm restoration and continuous monitoring under realistic usage scenarios.

Taken together, these innovations establish a foundation for a new class of wearable sonogenetic devices that merge diagnostics and therapy, offering transformative potential for cardiac care and beyond. We thank the reviewer for this insightful question, which has led to a more enriched and balanced discussion of our work.

REDACTED

References:

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 12. Hu, H., et al., *A wearable cardiac ultrasound imager*. Nature, 2023. **613**(7945): p. 667-675.
 13. Liu, H.-C., et al., *Wearable bioadhesive ultrasound shear wave elastography*. Science Advances, 2024. **10**(6): p. eadk8426.
 14. Lin, M., et al., *A fully integrated wearable ultrasound system to monitor deep tissues in moving subjects*. Nature Biotechnology, 2023.
 15. Gao, X., et al., *A wearable echomyography system based on a single transducer*. Nature Electronics, 2024. **7**(11): p. 1035-1046.

Comment 3. Fig. 3d shows significant enrichment of transcriptional regulatory pathways in cardiomyocytes expressing MscL G22S, with multiple GO terms displaying high statistical significance. These transcriptional alterations raise concerns about the biological impact of overexpressing foreign mechanosensitive ion channels in the heart, and the long-term consequences for cardiac function are not addressed.

Response:

3.1 Explain the Observed Transcriptional Changes

We thank the reviewer for this important comment. Regarding the biosafety of transcriptional GO alterations, although some GO terms (e.g., “transcription by RNA Pol II” and “chromosome organization”) were enriched, they were driven by modest fold changes and small gene sets, consistent with the sustained expression of an AAV transgene. Crucially, cell-cycle and proliferation signatures were not enriched (GSEA $q \geq 0.1$; no change in Mki67, Pcna, or Top2a), and immune or apoptosis panels remained unchanged (**Supplementary. Fig. 22, 23 and 27**).

The GO terms identified in **Fig. 3d**, such as “transcription by RNA polymerase II” (GO 6, 7), “cell cycle process” (GO 4, 5, 8, 9, 10), and “chromosome organization” (GO 1, 3), are intrinsically linked to the expression of a new protein from a virally delivered vector. A point-by-point explanation is provided below:

- **Transcription by RNA Polymerase II (GO 6, 7):** This is the most direct and expected finding. Our AAV vector places the MscL G22S gene under the control of the cTnT promoter, which recruits RNA Polymerase II to transcribe the gene into mRNA. The high-level expression of this new gene naturally leads to a statistically significant enrichment of the pathway responsible for its expression. This reflects normal cellular function rather than dysregulation.
- **Cell Cycle and Chromosome Organization (GO 1, 2, 3, 4, 5, 8-10):** The slight enrichment in these pathways does not indicate that cardiomyocytes—which are terminally differentiated—are re-entering the cell cycle or proliferating. Instead, these findings represent minor fluctuations in the complex machinery that regulates genetic material. The introduction and high-level expression of a foreign gene can create a “transcriptional load,” leading to minor compensatory adjustments in pathways related to chromosome maintenance and transcriptional regulation. This represents a subtle cellular response, not a fundamental change in the cell’s state or a sign of genomic instability.

Crucially, although the transcriptional machinery was active, we performed extensive analyses to confirm that this did not trigger any adverse pathways. Our data demonstrates that the expression of MscL G22S is remarkably safe:

- **No Evidence of Cardiac Malfunction or Disease:** Differential gene expression analysis, visualized in the volcano plots in **Extended Data Fig. 10f-g**, showed that apart from the intended MscL G22S and mCherry genes, there were no significant changes in genes associated with cardiac malfunction, heart disease, or apoptosis.
- **No Inflammatory or Immune Response:** No upregulation of key inflammatory markers was observed (**Extended Data Fig. 10e**). Immunofluorescence staining of cardiac tissue showed no increase in inflammatory markers such as TNF- α or the fibroblast/fibrosis marker vimentin/ α -SMA. This was further supported by RNA-seq data, which showed no significant differential expression of genes related to major immune or inflammatory responses.
- **Global Transcriptional Stability:** The overall gene expression profiles of ST and WT hearts (**Extended Data Fig. 10c, d**) were highly similar. Heatmaps showed consistent expression patterns, and correlation coefficient plots revealed an extremely high correlation ($R \geq 0.96$) between WT and ST samples, indicating no significant global transcriptional disruption.

- **Normal Tissue Morphology:** Histological analysis with H&E staining of all major organs (**Supplementary Fig. 21**), including the heart, revealed no significant morphological changes or signs of cellular damage in the ST group compared to controls.

In conclusion, the GO term enrichment reflects the cell's active and normal transcriptional machinery at work to express the MscL G22S transgene.

3.2 Long-term Consequences Valuation for Heart Function

We thank the reviewer for raising this important concern regarding the long-term consequences of sonogenetic therapy on cardiac function. To comprehensively address this critical point, we conducted a multi-faceted, long-term evaluation encompassing **in vivo functional, genetic, and proteomic assessments**.

3.2.1 Assessment of Long-Term Cardiac Function in vivo

We assessed whether chronic MscL G22S expression had adverse effects on cardiac function at the whole-organism level, particularly under conditions of elevated physiological demand. A critical safety benchmark is the ability of the heart to perform normally during physical exercise, a commonly used approach for characterizing the safety of cardiac therapy.

To this end, we conducted a treadmill endurance test, a standard method for assessing cardiovascular performance (**Fig. R1a-c**). WT and ST rats at 2, 6, and 8 months post-transduction were subjected to a continuous 45-minute run. During exercise, we noninvasively monitored key physiological parameters in real time—including heart rate, blood oxygen saturation (SpO₂), respiratory rate, and the P–P interval—using a collar-based sensor system.

This functional stress test revealed no discernible differences between ST groups and WT controls at any time point, supporting long-term functional safety:

- **Heart Rate and P-P Interval:** The continuous monitoring plots (**Fig. R1d, g**) and the quantitative analysis broken down into 9-minute intervals (**Fig. R1h, k**) showed that heart rate and corresponding P–P intervals in all ST groups were statistically indistinguishable from those in the WT group across the 45-minute exercise period. This indicates that the chronotropic response to exercise was not impaired.
- **Oxygen Saturation:** All animals, regardless of treatment or age, maintained stable and high blood oxygen saturation levels (typically >95%) throughout the endurance test (**Fig. R1e, i**). This indicates that cardiovascular and respiratory function in ST animals was sufficient to meet the metabolic demands of strenuous exercise, with no evidence of compromised cardiac output.
- **Respiratory Rate:** Respiratory rate patterns were comparable across groups, reflecting a normal physiological response to exertion with no significant differences (**Fig. R1f, j**).

In conclusion, the comparable performance of sonogenetically treated rats and wild-type controls under sustained cardiovascular workload indicates that chronic MscL G22S expression does not compromise the heart's intrinsic ability to adapt to physiological stress.

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Fig. R1 (add as 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-month 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, $p > 0.05$; $n=5$ 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. No statistically significant differences (ns, $p > 0.05$) were observed between any of the groups in any of the measured parameters.

3.2.2 Spatial Transcriptomic Analysis at Long-Term 6 Months Post-Transduction

Next, we present results from spatial transcriptomic sequencing of ST mouse hearts at 6 months post-transduction, alongside age-matched WT controls.

Spatial transcriptomics provides an unbiased, high-resolution approach to assess gene expression changes at single-cell resolution within the native architecture of the heart. To minimize technical variability, tissue sections from both a WT and an ST mouse were co-processed on the same slide (sample placement shown in [Fig. R2a](#)). The analysis was performed using Stomics Stereo-seq technology. The workflow included sample preparation, staining, and imaging of tissue sections, followed by tissue permeabilization for mRNA capture and cDNA synthesis. Libraries were prepared using the Stereo-seq Library Preparation Kit (V1.3) and sequenced on a DNBseq-T7 platform (2×75 bp paired-end reads) to a depth of up to 3 billion reads.

First, we confirmed the identity of each tissue section by mapping expression of the MscL G22S transgene. As shown in [Fig. R2b](#), a robust MscL G22S signal was detected throughout the right-sided heart sample, confirming it as the ST sample, whereas the left-sided sample showed an almost complete absence of expression and was identified as the WT control. We then performed cell-type annotation, which successfully delineated the primary cardiac structures, including distinct populations of atrial cardiomyocytes (aCMs), ventricular cardiomyocytes (vCMs), terminal cardiomyocytes, and Purkinje cells ([Fig. R2c](#)). The overall spatial organization and cellular composition of the hearts were qualitatively indistinguishable between ST and WT samples, indicating that long-term expression of the sonogenetic construct does not induce gross structural remodeling at the transcriptomic level.

The comprehensive nature of spatial transcriptomics allowed us to extend the analysis to key ion channel families to determine whether exogenous MscL G22S altered the expression of endogenous channels. As shown in the [Fig. R2d-h](#), we observed no significant differences in the expression patterns of major calcium, potassium, sodium, or endogenous mechanosensitive channels, or other related channel families, between ST and WT hearts. These results indicate that long-term overexpression of the MscL G22S channel does not significantly alter the transcriptional profile of essential components for Ca^{2+} , K^{+} , Cl^{-} , and Na^{+} cycling, demonstrating that the cardiac ion channel transcriptome remains stable.

To investigate the immunological safety of long-term transgene expression, we profiled the distribution and abundance of major immune cell populations, including T cells, B cells, natural killer (NK) cells, macrophages, and neutrophils. Spatial mapping showed that all identified immune cells were sparsely and evenly distributed throughout the cardiac tissue in both samples ([Fig. R3](#)). Crucially, we observed no evidence of focal immune cell infiltration or inflammatory clustering in the ST heart.

In conclusion, these spatial transcriptomic results provide strong evidence for the long-term molecular safety and biocompatibility of our sonogenetic approach. The data demonstrate that sustained expression of the MscL G22S transgene for 6 months does not alter the fundamental cellular architecture of the heart or elicit a significant immune response.

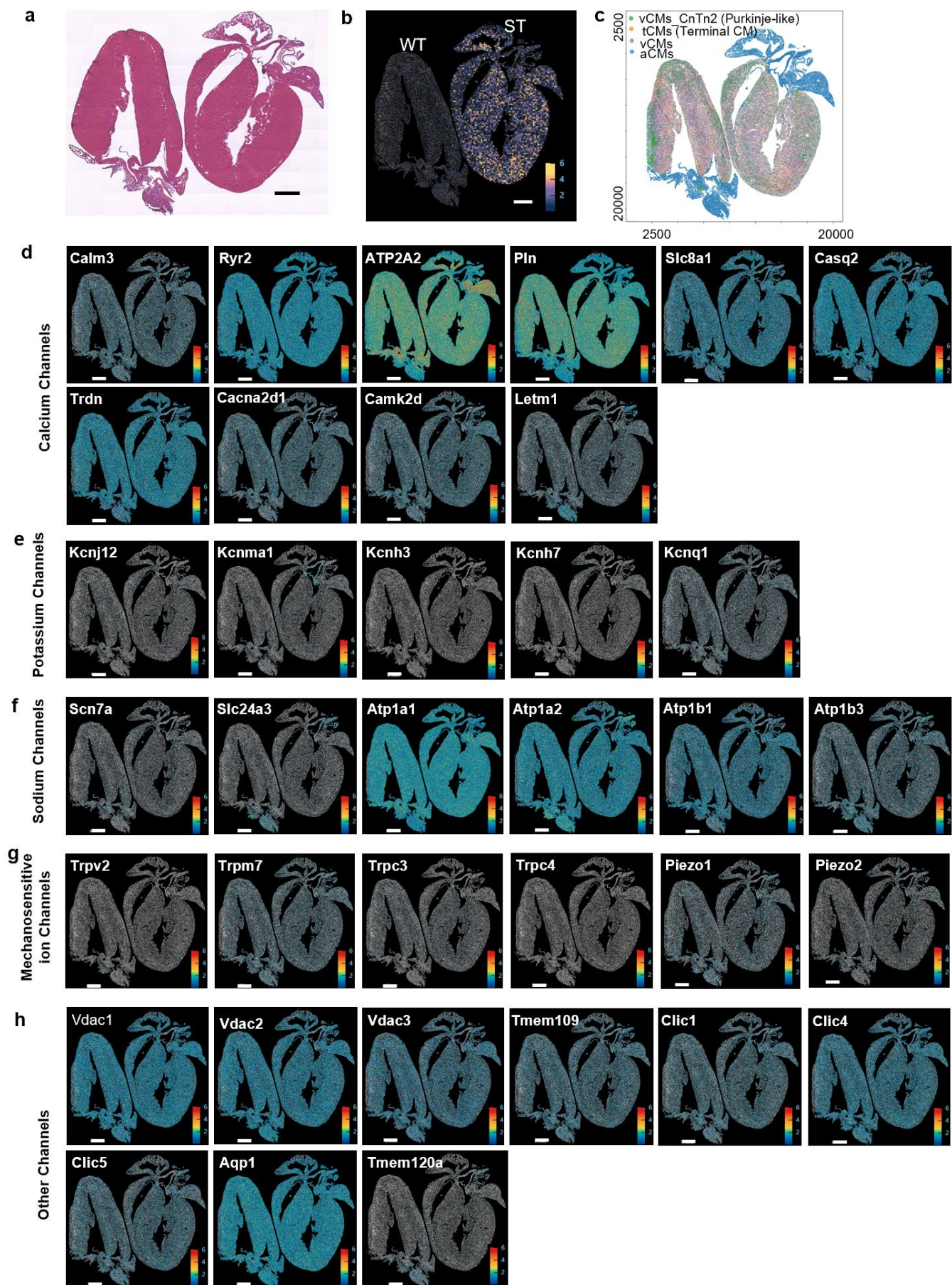


Fig. R2 (add as Supplementary Fig. 26) | Comprehensive spatial transcriptomic analysis of key ion channel genes in WT and ST hearts. **a**, H&E staining of adjacent wild-type (WT, left) and sonogenetically transduced (ST, right) cardiac tissue sections. **b**, Spatial plot confirming transgene expression 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.

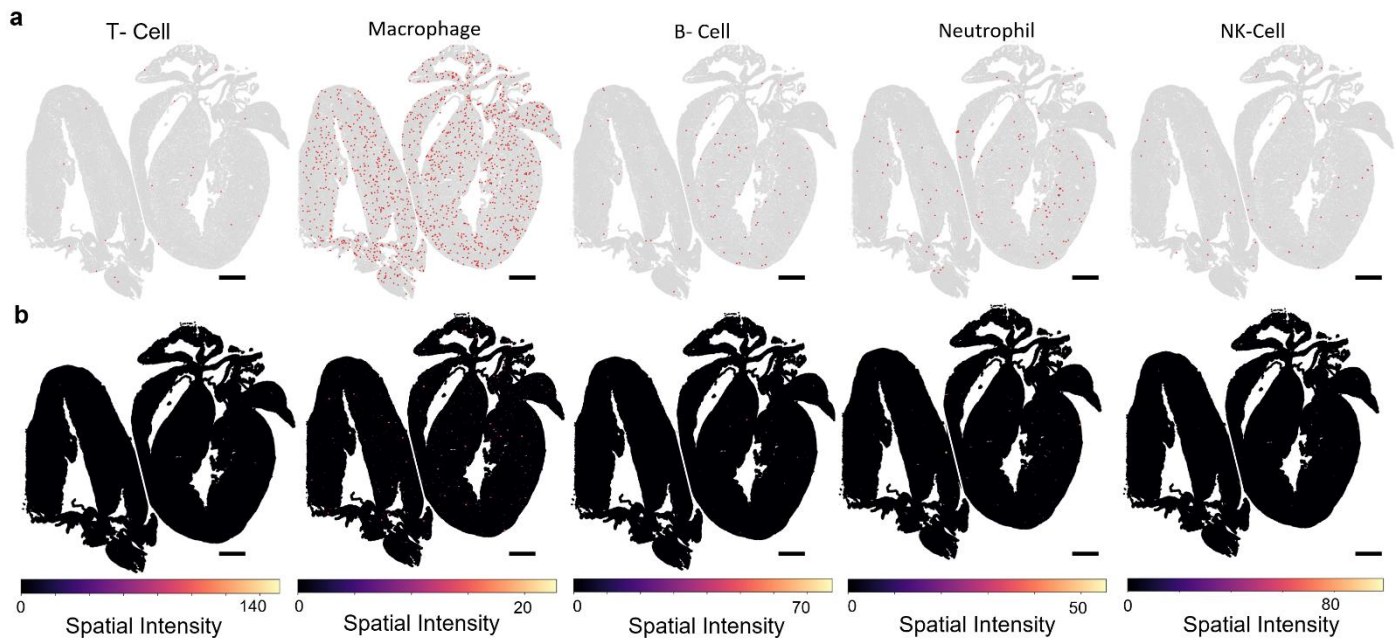


Fig. R3 (add as Supplementary Fig. 27) | Spatial transcriptomic analysis of inflammatory cells in wild-type and sonogenetically transduced cardiac tissues. **a**, Spatial plots indicating the locations of individual cells for five major immune cell types: T-Cells, Macrophages, B-Cells, Neutrophils, and 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.

3.2.3 Proteomic Analysis of Long-Term Transduction Stability

We also performed a comprehensive proteomic analysis, which provides a more direct assessment of cellular phenotype compared with transcriptomics. Whereas RNA levels indicate genetic intent, protein levels represent the functional molecules that execute cellular processes. A total of nine rat heart tissue samples ($n = 3$ per group: ST 6-month, ST 8-month, and age-matched WT controls) were processed for protein extraction, followed by enzymatic digestion with Trypsin/Lys-C. The resulting peptides were analyzed by LC-MS/MS, and the raw data were processed using Thermo Proteome Discoverer 2.5 against the *Rattus norvegicus* protein database.

First, we assessed the overall stability of the cardiac proteome. The correlation plots (**Fig. R4a-c**) compared the average protein expression levels between ST and WT hearts at 2, 6, and 8 months. Across all time points, the correlation coefficient was exceptionally high ($R \geq 0.99$), indicating that the global protein landscape was virtually identical between the two groups. This remarkable consistency provides strong evidence that long-term expression of the MscL G22S transgene does not cause widespread off-target disruption of the cardiac proteome.

To provide a detailed safety assessment, we analyzed proteins that were differentially expressed at the 6- and 8-month time points. The volcano plots (**Fig. R4d, e**) showed that nearly all cardiac proteins remained unchanged, indicating a highly specific and targeted effect of the transgene. The few proteins that showed statistically significant changes did not indicate a pathological response; rather, they reflected a logical and benign adaptation of the cellular machinery to the presence of a new functional ion channel.

- **Analysis of Upregulated Proteins:** The upregulated proteins were primarily involved in cellular structure, membrane trafficking, and metabolism—functions directly related to producing and managing a new membrane protein.

Structural and Cytoskeletal Components: We observed a modest increase in proteins such as Col1a1 (Collagen Type I Alpha 1), Myh9 (Non-muscle Myosin IIA), and Myo18a (Myosin XVIIIa). Although Col1a1 upregulation can be a marker for fibrosis, this was an isolated finding. There was no corresponding upregulation of a broader fibrotic protein network, and, importantly, H&E staining of the cardiac tissue showed no evidence of fibrosis or adverse

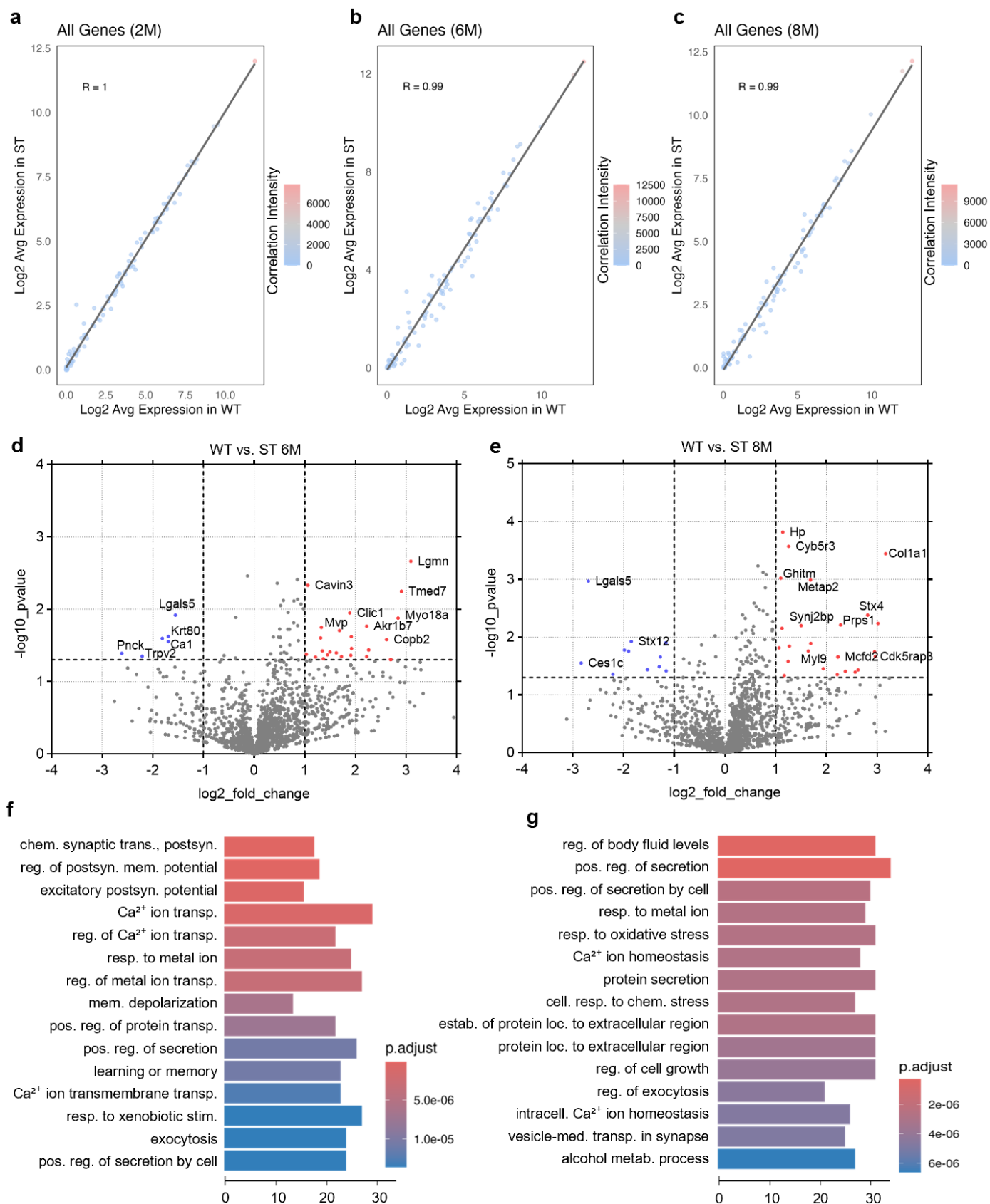


Fig. R4 (add as Supplementary Fig. 28) | Proteomic analysis of chronic MscL G22S expression in cardiomyocytes. a-c, Correlation plots of the average protein expression levels (Log2) between sonogenetically treated (ST) and wild-type (WT) mouse heart samples at 2 months (a), 6 months (b), and 8 months (c) post-transduction, showing high consistency ($R \geq 0.99$). Each dot represents a single protein, and the color gradient indicates the correlation intensity. **d-e,** Volcano plots of differentially expressed proteins in ST and WT mice hearts at 6 months (d) and 8 months (e). **f-g,** Bar plots showing the top enriched biological process pathways identified from the differentially expressed proteins at 6 months (f) and 8 months (g). The x-axis represents the statistical significance of the enrichment ($-\log_{10}$ adjusted p-value). $n=3$ for each condition.

tissue remodeling. The changes in myosin motors likely represent an adaptive cytoskeletal response to support the trafficking and membrane integration of the new MscL G22S protein.

- **Vesicle Trafficking and Membrane Organization:** A notable group of upregulated proteins—including Cavin3, Cltc (Clathrin), Copb2 (Coatomer), and the syntaxins Stx4 and Stx12—were core components of the cellular machinery for vesicle formation, transport, and membrane fusion. Their upregulation represents a direct and logical consequence of the increased demand on the cell to synthesize, process, fold, and transport the MscL G22S channel from the endoplasmic reticulum to its final destination at the cell surface. This reflects a normal and productive cellular process rather than a stress response.
- **Metabolic and Processing Enzymes:** Proteins such as Lgmn (Legumain, a lysosomal protease) and Cyb5r3 (a reductase involved in fatty acid metabolism) showed minor upregulation, likely reflecting increased protein turnover and modest metabolic adjustments required to support the sustained expression of the transgene.
- **Analysis of Downregulated Proteins:** The few consistently downregulated proteins were not associated with core cardiac health pathways, and their minor changes did not indicate adverse cellular events.
 - The most consistently downregulated protein, Lgals5 (Galectin-5), is not typically expressed at high levels in the heart, and its role in cardiac pathology is not well-defined. Therefore, its downregulation is of unknown significance and is not linked to any known adverse outcome.
 - Other downregulated proteins such as Krt8/18 (Keratins)—intermediate filaments not prominent in cardiomyocytes—or proteins involved in specific signaling pathways, including Pnck and Synj2bp, represented minor and isolated regulatory shifts. Importantly, these changes did not converge on any known pathways of apoptosis, inflammation, or cardiac failure.

A detailed examination of all significantly altered proteins revealed a consistent pattern: the changes were minor, typically related to the expected task of producing a new protein, and did not trigger any coordinated pathological programs.

Next, we analyzed the biological pathways associated with differentially expressed proteins (**Fig. R4f, g**). The results indicated that these cellular changes represented a logical and benign adaptation to the presence of a new functional ion channel, rather than a sign of stress or pathology.

- **Expected Functional Pathways:** Many of the most significantly enriched terms were directly related to ion channel function, including “regulation of Ca²⁺ ion transport,” “Ca²⁺ ion homeostasis,” “membrane depolarization,” and “excitatory postsynaptic potential.” The enrichment of these pathways provides compelling evidence that the MscL G22S channel was functionally integrated and active within the cardiomyocyte membrane, influencing ion flux as intended.
- **Protein Handling Pathways:** Expected terms included “exocytosis,” “protein secretion,” and “vesicle-mediated transport.” The cell actively produced, transported, and inserted the new MscL G22S protein into the cell membrane, and these pathways reflected normal cellular activity.
- **Absence of Pathological Pathways:** Importantly, the list of enriched pathways is notable for what was absent. There was no significant enrichment of pathways related to “inflammatory response,” “apoptotic process,” “cell death,” “response to injury,” or “fibrosis.”

Collectively, these data provide proteomic evidence that long-term MscL G22S expression does not induce compensatory up- or downregulation in the heart’s native ion channel landscape. The preserved integrity of these proteins—which govern cardiac excitability, signaling, and contraction—supports the biological safety and specificity of the sonogenetic approach for chronic therapeutic applications.

In response to **Comment 3**, we added new figures to the revised Supplementary Materials. We also included a new paragraph in the main text (page 7, lines 20–37) to present the results of our long-term safety assessments.

“We further validated the long-term impact and safety of chronic MscL G22S expression. First, bulk RNA-seq analysis was performed on cardiac tissues collected at 6- and 8-month post-transduction to examine potential effects of the exogenous MscL G22S gene on endogenous ion channel expression. The results showed no significant alterations in the transcriptional landscape of key ion channel families (**Supplementary Fig. 24**). Next, immunofluorescence staining was conducted to evaluate representative calcium-handling proteins, including RyR2, SERCA2a, and PLB, in order to assess potential long-term effects on intracellular Ca^{2+} cycling (**Supplementary Fig. 25**). We additionally employed spatial transcriptomics to map the expression and distribution of ion channel genes with spatial resolution, confirming that the qualitative and quantitative expression patterns of calcium, potassium, and sodium channels were comparable between ST and WT hearts (**Supplementary Fig. 26**). Moreover, immune cell profiling revealed no evidence of focal inflammatory infiltration or clustering in ST cardiac tissue (**Supplementary Fig. 27**). Finally, at the protein level, we performed global proteomic analysis at 2-, 6-, and 8-month post-transduction. These datasets confirmed long-term safety, showing strong correlations between the proteomic profiles of ST and WT hearts (Pearson’s $R \geq 0.99$), with no significant perturbations in the expression of major ion channel families (**Supplementary Fig. 28 and 29**). Taken together, these multi-level assessments demonstrate that chronic sonogenetic transduction preserves transcriptional integrity, maintains proteomic stability, and does not elicit inflammatory or immune responses. Collectively, these findings establish the long-term safety and translational feasibility of sonogenetics for cardiac applications.”

Comment 4. The demonstration of cardiac rhythm restoration in a rat arrhythmia model is the highlight of this manuscript; however, the data presented in Fig. 4 raises several issues. First, the baseline heart rate in the model (180 bpm) differs markedly from the baseline reported elsewhere in the manuscript (~240 bpm), raising questions about the consistency of the model. Second, there is no control group consisting of animals expressing MscL-G22S without ultrasound stimulation. Given the mechanosensitivity of MscL, it remains unclear whether mechanical forces from normal heart contractions could activate the channel independently of ultrasound. This is critical for interpreting therapeutic potential and safety. Third, the **number of animals** used in the study is not provided, making it difficult to evaluate the scientific rigor of the data.

Reply to Comment 4:

4.1 Explain of baseline heart rate

We thank the reviewer for this important question. The baseline heart rate of healthy, anesthetized rats exhibits a normal physiological range, typically between 180 and 300 bpm, as reported by others[1, 2]. The different baseline of heart rates observed between the experiments in **Fig.3** (~240 bpm) and **Fig. 4** (~180 bpm) are both falling within this healthy range.

It is important to note the distinct objectives of these two experiments. The experiment shown in **Fig. 3** was designed to demonstrate **overdrive pacing** in healthy animals. In this case, we showed that the NUP increased the heart rate from a baseline of ~240 bpm to as high as 540 bpm, confirming its ability to control and override the native sinus rhythm.

The experiment shown in **Fig. 4** was designed to demonstrate **therapeutic rescue** from a pathological state. For this arrhythmia model, a separate cohort of animals was used, which consistently exhibited a pre-induction baseline of ~180 bpm. Following chemical induction of arrhythmia, the heart rate dropped into severe bradycardia (~60 bpm). The NUP was then used to restore the heart rate to the original baseline of ~180 bpm.

Therefore, the different baselines of ~180 bpm and ~240 bpm reflect normal physiological variation between distinct animal cohorts used for different experimental purposes. Both values are within the healthy range for this species, and this variation does not introduce bias into our data or conclusions.

4.2 Add new control group

Following the reviewer's suggestion, we added a new control group of ST rats that were subjected to the same CaCl₂-induced arrhythmia protocol but, critically, did not receive any therapeutic ultrasound stimulation.

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Fig. R5 (add as Supplementary Fig. 18) | ECG recordings and analysis of chemically-induced arrhythmia in ST rats without ultrasound pacing. This figure shows control experiments for a ST rat (a-c) and a previous WT rat (d-f) after a chemically induced arrhythmia, with no ultrasound pacing applied. The ECG traces in panels **b** and **e** 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. Values are normalized by Z-scores (mean=0, SD=1). n=5 for each condition (d-f are graphs from Fig. 4, placing here for a reference).

This experiment was designed to directly test whether the mechanosensitive MscL G22S channel could be activated by the intrinsic mechanical forces of the arrhythmic heart, potentially leading to spontaneous recovery.

As shown by the new data (Fig. R5a-c), the baseline heart rate in this group was ~180 bpm, consistent with the previous experiment group (Fig. R5e) in the arrhythmia study and addressing concerns regarding model consistency. Following CaCl₂ injection, the animals developed severe bradycardia and erratic ECG waveforms, consistent with the disease model onset in the treatment groups. In the absence of ultrasound pacing, the ST animals did not spontaneously recover, and arrhythmia with severe bradycardia persisted throughout the post-modeling observation period.

These results indicate that the endogenous mechanical forces of the heart were insufficient to activate the MscL G22S channel to a level that could elicit a therapeutic effect. They further demonstrate that the restoration of sinus rhythm in the treatment group was attributable to focused ultrasound application, rather than a spontaneous event facilitated by the channel alone.

While these in vivo rat data are informative, we also addressed the potential for endogenous activation in the human context. Although direct human experiments are not currently feasible, our in vitro studies on human cardiomyocytes provide supporting evidence. Under baseline, unstimulated conditions, the spontaneous activity of ST cells was indistinguishable from that of WT cells (Fig. 2d), indicating that the MscL G22S channel was not activated by intrinsic contractile forces.

Furthermore, there are some huge mismatches between the pressure and frequency generated by the heart and the activation threshold of the MscL G22S ion channel.

- **Pressure Mismatch:** A normal human heart generates peak systolic pressures of ~0.016 MPa (120 mmHg). In contrast, the acoustic pressure required to activate the MscL G22S channel exceeds 0.5 MPa (based on our in vitro human cardiomyocyte study). This ~50-fold difference makes spontaneous activation by the heartbeat physically implausible.
- **Frequency Mismatch:** The MscL G22S channel responds optimally to high-frequency mechanical waves (2.2-3 MHz[3, 4]). In contrast, the frequency of a human heartbeat (~1–2 Hz) is several orders of magnitude lower, providing an additional layer of biophysical safety since the channel is insensitive to the slow pressure changes of a heartbeat.

In summary, three independent lines of evidence—a dedicated in vivo control experiment, baseline analyses of human cardiomyocytes, and quantitative biophysical assessments—confirmed that the MscL G22S channel is not activated by the heart's endogenous mechanical forces. Therapeutic rhythm restoration relied exclusively on focused ultrasound application, supporting both the efficacy of our approach and its safety with respect to off-target activation.

We added detailed information to the Supplementary Materials (page 21) and included a new description of this control group in the main text (page 6, line 1).

“To confirm these observations were not due to spontaneous recovery, we performed control experiments on WT (Fig. 4i-l) and ST (Supplementary Fig. 18) rats with induced arrhythmia that received no ultrasound pacing; in these animals, cardiac arrhythmias persisted for the entire monitoring period without spontaneous resolution. These findings confirm the effectiveness of sonogenetics-enabled NUP cardiac pacing under arrhythmic conditions, highlighting its clinical applicability and broader therapeutic potential of NUP.”

4.4 More description of the number of animals

We have added the number of animals (n) used in each experiment to the main text and Supplementary Materials to ensure scientific rigor.

For the in vivo validation in **Fig. 3**, the viral transduction and off-target analyses used n=6 mice per dose. The rat RNA sequencing was performed on n=3 WT and n=3 ST rats. The noninvasive pacing validations (**e-p**) were conducted with n=5 rats.

For the arrhythmia treatment in **Fig. 4**, each condition was validated with n=5 rats. We have now added the statement "(n=5 for each condition)" to the main text (Page 22, Line 16).

For the in vivo characterization of the tissue distribution of sonogenetics shown in **Extended Data Fig. 4**, we used n=3 rats. We have now added the statement "n=5 for each organ" to the main text (Page 29, Line 7).

For the open-chest direct stimulation shown in **Extended Data Fig. 5**, we used n=3 rats. We have now added the statement "n=3 for each condition" to the main text (Page 30, Line 7).

For the stimulation of WT rats in **Extended Data Fig. 6**, we used n=10 rats to confirm the results. We have now added the statement "n=10" to the main text (Page 31, Line 15).

For the test of stimulation at a lower frequency in **Supplementary Fig. 18**, n=5 ST rats were used. We have now added the statement "(n=5)" to the supplementary materials (Page 21, Line 9).

For the spatial transcriptomics analysis, one WT and one ST mouse were analyzed, a number limited by the physical constraints of the sequencing chip.

For the proteomics analysis, we used n=3 rats for each group: 2-month WT and ST; 6-month WT and ST; and 8-month ST.

For the temperature monitoring experiments in **Supplementary Fig. 30 and 31**, n=3 rats were used for each condition. We have now added the statement "(n=3 for each condition)" to the supplementary materials (Page 37, Line 12 and Page 38, Line 11).

For the cardiac function assessment in **Supplementary Fig. 32**, we used n=5 animals per group. We have now added the statement "(n=5 per group)" to the supplementary materials (Page 35, Line 10).

References:

1. Rao, P., et al., *Near-infrared light driven tissue-penetrating cardiac optogenetics via upconversion nanoparticles in vivo*. Biomed Opt Express, 2020. **11**(3): p. 1401-1416.
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4. Cadoni, S., et al., *Ectopic expression of a mechanosensitive channel confers spatiotemporal resolution to ultrasound stimulations of neurons for visual restoration*. Nature Nanotechnology, 2023.

Comment 5. While the authors claim that "comprehensive biosafety assessment" supports clinical translation, the biosafety data provided is limited. The requirement for genetic modification via viral transduction presents a major translational hurdle that is not adequately discussed. Furthermore, clinical pacemakers are intended for continuous or frequent long-term stimulation (hours per day, for years), whereas the safety evaluation presented is limited to acute, single-session stimulation. Chronic safety assessment would be essential for substantiating translational relevance.

Response:

5.1 Comprehensive Biosafety Assessment

We thank the reviewer for raising this important concern regarding biosafety. In the revised manuscript, we substantially expanded the biosafety analysis with several new long-term studies to address this point directly. The new data include:

- (1) A longitudinal assessment of cardiac function (2, 6, and 8 months post-transduction) under high physiological demand using a treadmill stress test (**comment 3.2, Fig. R1**).
- (2) A long-term (6 months post-transduction) spatial transcriptomic analysis assessing endogenous ion channel expression (see response **comment 3.2, Fig. R2**) and immune cell distribution (see response **comment 3.2, Fig. R3**) following introduction of the MscL G22S transgene.
- (3) A longitudinal (2-, 6-, and 8-months post-transduction) proteomic analysis to evaluate the stability of the cardiac ion channel landscape and the global proteome. This included correlation plots (**comment 3.2, Fig. R4a-c**) demonstrating high concordance ($R \geq 0.99$) between ST and WT groups, as well as volcano plots (**comment 3.2, Fig. R4d-e**) with GO analysis (**comment 3.2, Fig. R4f-g**) to assess the functional impact of any differentially expressed proteins.

When combined with the biosafety data already presented in the original manuscript—including bulk RNA sequencing (**Extended Data Fig.10, Supplementary Fig. 23**), in vivo thermal monitoring during stimulation (**Supplementary Fig. 30 and 31**), H&E histology (**Supplementary Fig. 21**), and immunofluorescence for inflammatory markers such as TNF- α and GFAP (**Supplementary Fig. 22**)—this extensive new dataset provides a robust and comprehensive biosafety evaluation.

5.2 Discussion of AAV-Mediated Gene Delivery translational hurdles

We thank the reviewer for raising this critical point regarding the translational hurdles of viral vector-based gene therapy. To address this and provide greater context for our methodological choices, we added a new detailed section to the manuscript: **Supplementary Text 3**. This supplementary text provides a comprehensive overview covering:

- The current landscape of gene therapy methods for cardiac applications.
- The rationale for selecting the AAV2/9 serotype, emphasizing its superior specificity and efficiency for cardiac transduction.
- A broader discussion of the current challenges, progress, and future directions in clinical gene therapy, including issues of immunogenicity and manufacturing.

We believe this supplementary text thoroughly addresses the reviewer's concerns.

5.3 Assessment of Chronic Stimulation Safety

To directly address the need for chronic safety data, we performed additional long-term stimulation experiments. In a new group of ST rats, we applied intermittent sonogenetic pacing (80 minutes per day) for two weeks. At the end of this chronic pacing regimen, we assessed cardiac health using ECG and histological analyses.

As shown in **Fig. R6**, the ECG trace from the final day of stimulation demonstrated that sonogenetic pacing was robust and effective, with no evidence of induced arrhythmia or conduction abnormalities. Following each stimulation session, the heart rate returned to a stable sinus rhythm.

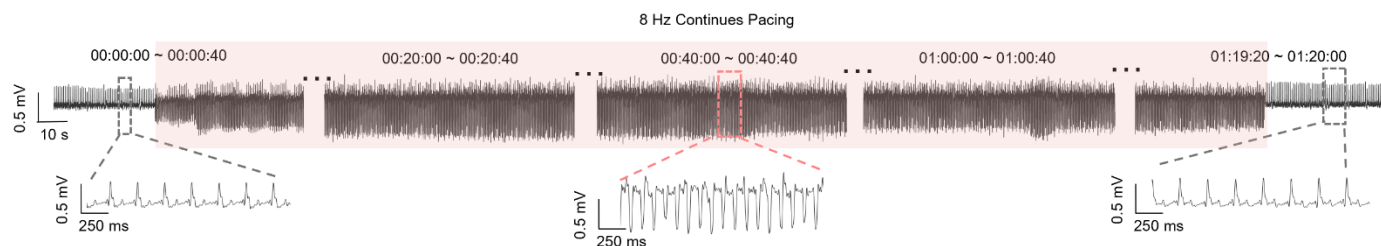


Fig. R6 | Long-term sustained cardiac pacing at 8 Hz. A representative electrocardiogram (ECG) trace from a rat undergoing continuous 8 Hz cardiac pacing for 1 hour and 20 minutes. The pink shaded area indicates the full duration of ultrasound stimulation. Magnified insets show the successful cardiac capture at the onset of stimulation (left), the stable paced rhythm at the 40-minute mark (middle), and the immediate return to sinus rhythm upon cessation of stimulation (right).

Histological analysis of hearts harvested after the two-week chronic pacing protocol revealed no evidence of significant tissue changes or cardiomyocyte damage (see attached **Fig.R7**).

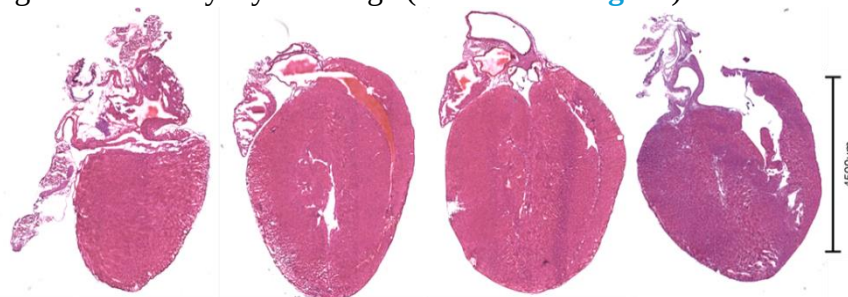


Fig. R7 | Histological analysis of cardiac tissue sections. Representative images of four mice heart cross-sections stained with Hematoxylin and Eosin (H&E). The staining illustrates the overall tissue morphology and cellular architecture. The scale bar represents 4500 μm . (n=4).

This new dataset provides direct evidence that long-term, repeated application of sonogenetic pacing is well tolerated and does not cause cumulative cardiac damage or electrophysiological instability. These findings significantly strengthens the case for the safety and translational potential of our NUP system for chronic cardiac rhythm management.

In addition to showing that the rats' heart rates and overall physiological status remained stable during prolonged stimulation sessions, we also examined the effects of tissue heating caused by long-duration ultrasound application. While our previous acute safety tests, comprising over 1200 stimulation pulses at various intensities, established an initial safety margin, we recognized that this did not constitute a true long-term evaluation. To rigorously assess thermal safety under chronic-use conditions, we designed an experiment

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Fig. R8 (add as Supplementary Fig. 31) | Long-duration in vivo thermal monitoring under continuous sonogenetic pacing. Top: schematic of the experimental setup for in vivo temperature monitoring. Fine-gauge thermocouple probes (0.23 mm) were positioned to measure temperature directly within the heart (intracardiac) and on the skin surface of an anesthetized rat, while the NUP system delivered continuous ultrasound stimulation. A syringe pump provided continuous intravenous infusion to maintain hydration, and a heating pad was used to maintain a stable baseline physiological temperature. **Middle:** A sustained 13-hour session of sonogenetic pacing was applied (indicated by the shaded region). The different traces represent the thermal effect of continuous stimulation at three different acoustic pressures: **2.0 MPa (black)**, **1.5 MPa (red)**, and **1.1 MPa (light red)**. **Bottom:** Insets show magnified views of the temperature profiles at the onset of stimulation (left, 0.6-1.4 hours) and after its cessation (right, 12.8-13.2 hours) for all three pressure levels. n=3 for each condition.

in which sonogenetic pacing was applied continuously for over 13 hours while direct temperature measurements were performed simultaneously (**Fig. R8**).

This extended *in vivo* thermal safety assessment was conducted on anesthetized rats placed on a heating pad to maintain a stable core body temperature. To prevent dehydration and ensure physiological stability during prolonged anesthesia, a continuous intravenous infusion of saline was delivered via the tail vein. The NUP system was configured to deliver uninterrupted sonogenetic pacing for over 13 consecutive hours. To precisely measure any thermal effects, fine-gauge thermocouple probes (0.23 mm) were used to monitor temperature directly within the heart (intracardiac).

The results from this experiment demonstrate the thermal safety of our platform. Upon initiation of the 13-hour stimulation, we observed a small initial temperature rise of approximately 1°C in the heart, which then remained stable for the entire duration of the session. There was no evidence of cumulative or runaway tissue heating. This observation was further confirmed with FDA-recommended thermal index (TI) calculations, a standard metric used to evaluate the clinical safety of ultrasound devices. The calculated TIS values for our stimulation ranged from ~0.4 to 1.2, depending on the focal area and acoustic pressure, corresponding to a maximal temperature rise of no more than 1.2 °C. Crucially, once ultrasound stimulation was terminated, intracardiac temperature promptly returned to its pre-stimulation baseline. This experiment provides direct and compelling evidence that the NUP system can be operated for prolonged periods without inducing significant or hazardous thermal effects, thereby strongly supporting its safety profile for chronic therapeutic applications.

As detailed in our response to **Comment 3**, our investigation demonstrates safety from the molecular level—where transcriptomic and proteomic analyses revealed no pathological changes over eight months—to the whole-organism level, where sonogenetically treated animals exhibited normal cardiopulmonary function identical to controls during high-intensity exercise. Furthermore, as outlined here in our response to **Comment 5**, we provide a thorough discussion of the clinical translation pathway for our AAV vector strategy and present new data from chronic stimulation protocols. These experiments confirm that more than 13 hours of pacing does not induce adverse cardiac remodeling, functional decline, or significant thermal effects.

We are confident that we have thoroughly addressed the reviewer's concerns and have substantially strengthened the case for the translational potential of this technology.

This supplementary figure has been added to the Supplementary Materials on page 34 and has been referenced in the main text on page 7, line 45, where we describe the results of long-term stimulation-induced heating.

“Under 1.5 MPa ultrasound stimulation, the temperature of heart increased by 0.05°C after 360 stimulations, 0.25°C after 720 stimulations, 0.33°C after 1,200 stimulations and 0.7°C after 12 hours of continuous stimulation (**Supplementary Fig. 30, 31**). ”

Minor technical criticisms

1. There is an inconsistency regarding the viral vector used. The Methods section mentions AAV2-cTnT, while the Supplementary Materials reference AAV9 and AAV2/9 in Extended Fig. 4. Given that AAV9 is known for its cardiac tropism and AAV2 is not, clarification of the serotype used is critical. Additionally, while Fig. 3b shows expression of MscL G22S, quantitative analysis of cell-type specificity is not provided, which is essential for validating the precision of the sonogenetic approach.

Response:

M1.1 Clarification of the serotype

We apologize for the confusion. All viral vectors used in this study were AAV2/9-cTnT, and we have corrected the description in the relevant experimental details.

The AAV2/9 pseudotype [1, 2] uses the AAV9 capsid, which provides strong cardiac tropism and efficient systemic transduction of cardiomyocytes, and the AAV2 genome backbone, which enables stable recombinant vector construction and high-titer packaging. This combination has become standard in cardiac research because it ensures both robust myocardial expression and reproducible vector production for translational studies.

We have summarized the comparison in detail in [Table 2](#), and this discussion was included in new [Supplementary Text 3.1](#).

Table 2 (add as Supplementary Data 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	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)

M1.2 Quantitative analysis of cell type specificity.

To quantitatively assess the cell-type specificity of the AAV2/9-cTnT delivery system, we used spatial transcriptomic analysis (see Methods and **Response 3.2**). Using established molecular markers, we identified four major cardiomyocyte subtypes: ventricular cardiomyocytes (vCMs), atrial cardiomyocytes (aCMs), Purkinje-like cells and terminal cardiomyocytes (tCMs). Several non-myocyte populations, including macrophages, sinoatrial node (SAN)-like cells, T cells, and B cells, were also annotated. ([Fig. R9 d-g](#))

The spatial distribution and expression intensity of MscL G22S-positive cells were visualized across the tissue section. Quantitative analysis confirmed that the transgene was expressed with high specificity in ventricular cardiomyocytes, around 60% MscL G22S positive vCMs (2545 cells with counts), 100% MscL G22S positive tCMs (822 cells with counts). The transduction efficiency in Purkinje-like cells and atrial cardiomyocytes was moderate, at approximately 35.8% for aCMs (967 cells with counts) and 26.4% for Purkinje-like cells (710 cells with counts). Critically, expression in non-myocyte populations, including SAN-like pacemaker cells, T cells, B cells, and macrophages, was negligible (less than 2%) ([Fig. R9 k](#)). These data collectively demonstrate the high cardiomyocyte specificity of this delivery strategy.

We have added this figure to the Supplementary Materials on page 17 as Supplementary Fig. 14, and in the main text (page 4, line 31) we have included a new description.

[“We further corroborated these findings using spatial transcriptomics, which confirmed the highly specific expression of the MscL G22S transgene within cardiomyocyte populations \(Supplementary Fig. 14\).”](#)

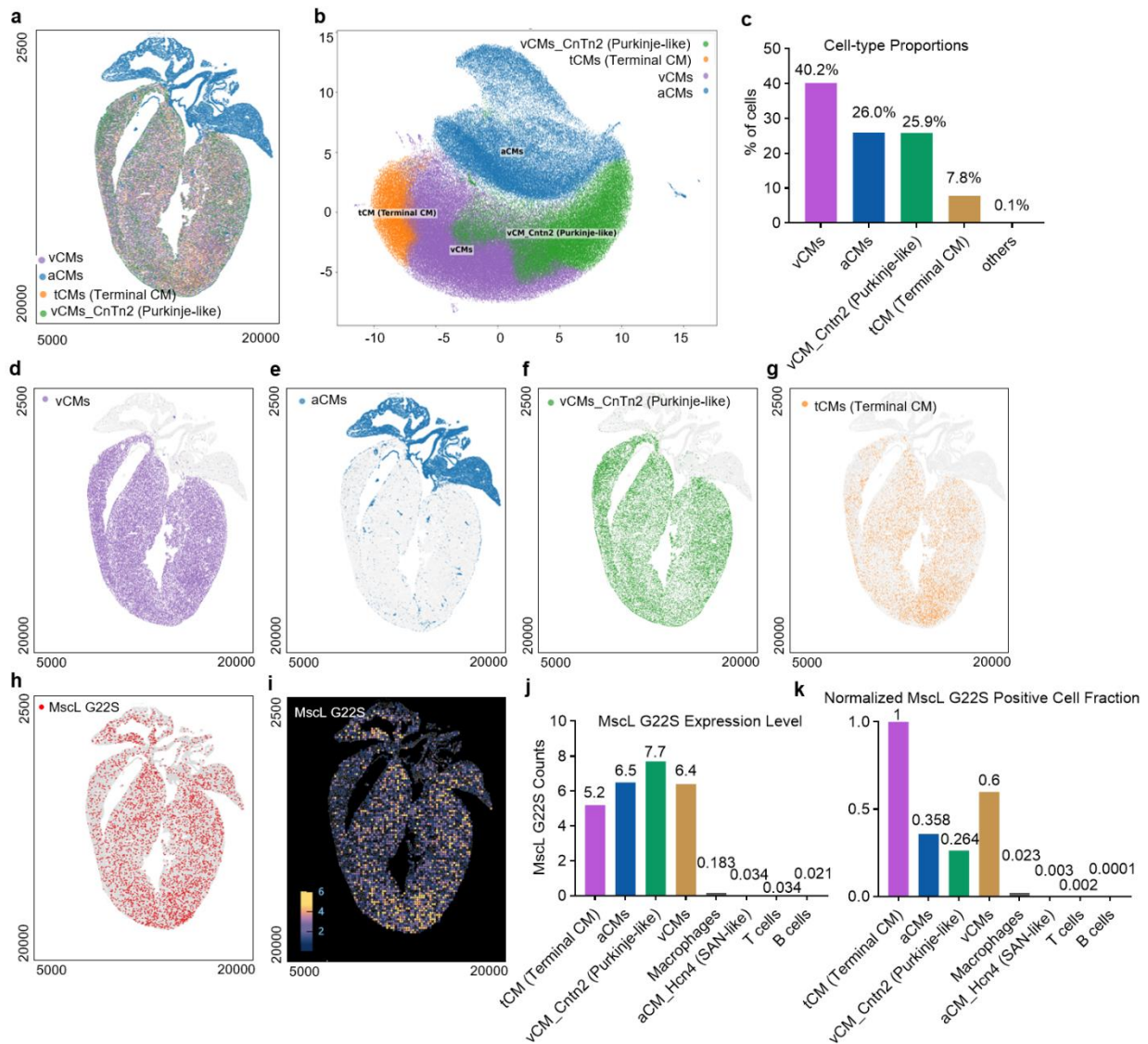


Fig. R9 (add as Supplementary Fig. 14) | Spatial transcriptomic analysis of cell populations and MscL G22S expression in the mice transduced heart. **a**, Spatial plot of annotated cell types overlaid on the cardiac tissue section. **b**, UMAP plot identifying distinct cell type clusters. **c**, Bar 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.

References:

1. Katz, M.G., et al., *Use of Adeno-Associated Virus Vector for Cardiac Gene Delivery in Large-Animal Surgical Models of Heart Failure*. Hum Gene Ther Clin Dev, 2017. **28**(3): p. 157-164.
2. Pankajakshan, D., et al., *Successful transfection of genes using AAV-2/9 vector in swine coronary and peripheral arteries*. J Surg Res, 2012. **175**(1): p. 169-75.

2. The manuscript states that the sonogenetic system achieves <1 mm spatial resolution using a 3 MHz single-element focused transducer. However, detailed characterization of the ultrasound focal zone size is not provided. Furthermore, spatial resolution was assessed under open-chest conditions; transmission through the chest wall would likely degrade spatial precision, and this effect is not characterized.

Response:

We thank the reviewer for this valuable comment and agree that explicit focal-zone characterization is important. We wish to clarify that the simulations presented in the main text (**Fig. 5f**) were performed under realistic trans-thoracic conditions, not in an open-chest model. To provide the requested detailed characterization, we have included a new supplementary figure illustrating our simulation methodology and results.

Our simulations were conducted using a heterogeneous finite-element model of the human thorax created from an open-source 3D anatomical dataset. This model incorporated the specific acoustic impedance, density, and attenuation parameters for all relevant tissues, including heart, blood, heterogeneous rib and lung layers, to accurately predict beam propagation (see **Fig. R10a-c**).

In an ideal, attenuation-free medium, the -3 dB focal width of our 3-MHz phased array at the target is approximately 0.8–1.0 mm laterally, with a depth of focus of 5–7 mm.

For most target sites, such as the right ventricle (RV), left bundle branch area (LBBAP), and His bundle (HBP), the apical four-chamber view provides a clear acoustic window. This allows the ultrasound beam to be steered through an intercostal path that avoids the lungs and ribs. Under these realistic, unobstructed conditions, the model shows that although the peak pressure is reduced by 8–12 dB due to tissue attenuation, the lateral resolution is preserved at <1 mm. The simulated beam profiles confirm that the acoustic field shape is not significantly distorted upon reaching the ventricular wall (see **Fig. R10d, f**).

For more challenging targets where the beam path may be partially obstructed by lung tissue, such as at the cardiac resynchronization therapy (CRT) site, the simulations show a modest degradation in focus. In these scenarios, the lateral width broadens to approximately 1.5–1.8 mm. This finding highlights the importance of integrated imaging, which enables the operator to select the optimal unobstructed acoustic window in real time to achieve the highest possible resolution for each target.

In summary, these simulations provide the requested focal-zone characterization and confirm that sub-millimeter resolution is achievable under realistic, image-guided trans-thoracic conditions when an unobstructed acoustic path is selected.

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Fig. R10 | Simulation of trans-thoracic focused ultrasound for cardiac pacing. (a-c) Schematics showing the placement of the NUP system on a human subject and the anatomical cross-section of the thorax used for the finite-element simulation model. (d-e) Representative simulations of focused ultrasound beams for left bundle branch area pacing (LBBAP) (d) and cardiac resynchronization therapy (CRT) (e) stimulation, shown as both acoustic pressure and normalized sound level. (f) A schematic of four clinically relevant cardiac pacing sites (left) and the corresponding simulation results demonstrating the system's ability to electronically steer the acoustic focus to each target (right).

3. The study transitions from mice to rats for different experiments without a rationale. Similarly, the ultrasound stimulation setup changes from the single-element system to the NUP device without a clear explanation, complicating the interpretation of the results.

Response:

We thank the reviewer for their careful attention to our methodology and for the opportunity to clarify these important details. The selection of animal models and ultrasound systems was determined by the specific requirements of each experimental stage. We have now added this rationale to the methods section of the manuscript to ensure clarity for the reader.

3.1. Rationale for the Use of Mouse and Rat Models

Mice for Initial Validation and Screening: We used mice for initial validation, including optimization of viral transduction efficiency and determination of effective viral concentrations. Their smaller size makes them a more efficient model for these preliminary screening purposes.

Rats as the Primary Model for In Vivo Functional Studies: The majority of our in vivo functional assessments, including the noninvasive pacing (Fig. 3e-p), arrhythmia rescue (Fig. 4), bulk RNA-sequencing, and multi-chamber stimulation experiments, were conducted in rats. The larger size of the rat heart and thoracic cavity facilitates the use of the NUP system, allowing clear ultrasound imaging and precise image-guided targeting of specific cardiac chambers.

To this end, while we presented a representative immunofluorescence image from a mouse in **Fig. 3a** to demonstrate successful MscL G22S expression. The same validation was performed in rats, and the results are shown in **Extended Data Fig. 4d**. To avoid confusion, we clarify that all functional data (such as ECG and capture rate) in the main figures were acquired from rats. These complementary datasets demonstrate the robustness of our approach across species.

3.2. Rationale for the Use of Single-Element and NUP Ultrasound Systems

The transition between the single-element transducer and the NUP system was determined by the physical constraints of each experimental setup.

Single-Element Transducer for In Vitro and Open-Chest Stimulation:

For experiments on cultured human cardiomyocytes (**Fig. 2**), we used a 3-MHz ring-shaped single-element transducer. This choice was necessary because the culture dishes were too small to accommodate the larger 64-channel phased-array transducer. Similarly, in our initial open-chest rat experiments, we also used a small single-element 3-MHz probe. In this setting, the heart was exposed and directly interfaced with MEA electrodes. A compact transducer enabled stimulation while minimizing the risk of electrode displacement during ultrasound application. In contrast, use of a larger phased-array transducer would have obstructed the surgical field and impaired direct visualization of electrode movement.

NUP System for In Vivo and Ex Vivo Studies:

The fully integrated NUP system was used for all noninvasive in vivo stimulation experiments in mice and rats, as well as for ex vivo porcine heart attenuation studies. This underscores its role as the core platform for translational and animal research.

Importantly, we ensured consistency between the two setups. Both the single-element and phased-array transducers were operated at the same 3-MHz center frequency, and all acoustic pressure outputs were precisely measured and calibrated with a hydrophone. The stimulation parameters applied in vitro were well within the operational capabilities of the NUP system, ensuring methodological consistency across conditions.

Reviewer #2:

This manuscript presents an exceptionally innovative and technically outstanding platform—the Noninvasive Ultrasound Pacemaker (NUP)—poised to redefine the landscape of cardiac rhythm management. By seamlessly combining sonogenetic sensitization of cardiomyocytes via the MscL G22S channel with a compact, AI-driven, wearable ultrasound array, the authors achieve a remarkable milestone: truly noninvasive, chamber-specific cardiac pacing. This is a first in the field and represents a bold leap toward implant-free, patient-friendly cardiac therapies.

The study is underpinned by a rich tapestry of methodologically sound experiments, including rigorous in vitro calcium imaging, multi-species (rodent and porcine) validations, and comprehensive safety assessments, all orchestrated within an advanced closed-loop system guided by real-time imaging. The work stands out not only in concept but also in execution, embodying the highest standards in biomedical engineering and translational research.

I strongly recommend acceptance pending revisions. The following comments aim to further elevate this already exceptional manuscript.

We sincerely thank the reviewer for the highly positive evaluation of our manuscript and for recognizing the innovative potential of our work. We also greatly appreciate the reviewer's insightful comments and valuable suggestions, which have helped us further enhance the quality and impact of our study. We believe these revisions strengthen the manuscript's contribution to the field of biomedical engineering. In response to the reviewer's feedback, we have added **16** new figures and **4** new tables to the manuscript. All textual revisions have been marked in blue for clarity. We thank the reviewer again for their time and constructive feedback.

Major Comments 1. Quantitative Characterization of MscL G22S Channel Sensitivity

To strengthen the mechanistic insight, please include quantitative activation data for the MscL G22S channel. Specifically, pressure–response curves (e.g., EC_{50} , Hill coefficient) obtained via patch-clamp electrophysiology or quantitative calcium-flux assays would greatly enhance our understanding of its mechanical sensitivity. In addition, the inclusion of a plasmid control group lacking the MscL G22S insert is essential to verify specificity.

Response to Comments 1:

We thank the reviewer for this important suggestion. Characterizing the pressure–response relationship of the MscL G22S channel is indeed crucial for elucidating the underlying mechanism and will serve as a valuable guide for future applications of sonogenetics.

Due to technical challenges, patch-clamp electrophysiology and quantitative calcium-flux assays were not feasible in this study. Because cardiomyocytes are contractile cells, both their intrinsic beating and the applied ultrasound would generate strong motion artifacts and electrical noise, making it difficult to maintain stable recordings. We also did not use quantitative calcium-flux assays, as they lack the single-cell resolution and direct spatiotemporal information provided by calcium imaging.

To answer the reviewer's question, as an alternative, as an alternative, we employed quantitative calcium imaging, a well-established method that offers several advantages for addressing this specific question. This approach enables simultaneous monitoring of large cell populations, providing statistically robust data on response rates and synchrony. Furthermore, this technique provides sufficient temporal resolution to precisely determine the timing of intracellular Ca^{2+} rise and the resulting paced contraction. Changes in intracellular Ca^{2+} concentration are the immediate downstream consequence of MscL G22S channel activation and serve as the trigger for cardiomyocyte contraction, making them a highly relevant physiological readout for pacing efficacy. We therefore performed additional experiments using calcium imaging to investigate this relationship.

We performed a dose-response experiment using five different acoustic pressures: 0.21, 0.40, 0.59, 0.73, 0.94, and 1.126 MPa. We also included the suggested plasmid control group to confirm the specificity of

sonogenetic activation, as the reviewer suggested. For this control, we used a lentivirus expressing only mCherry without the MscL G22S channel (LV-Ef1a-mCherry-WPRE). The construction of this control plasmid is detailed in **Fig. R1** of the revised manuscript. This control vector shares the same backbone and drives mCherry expression in cardiomyocytes, serving as an effective transduction marker while lacking the mechanosensitive channel.

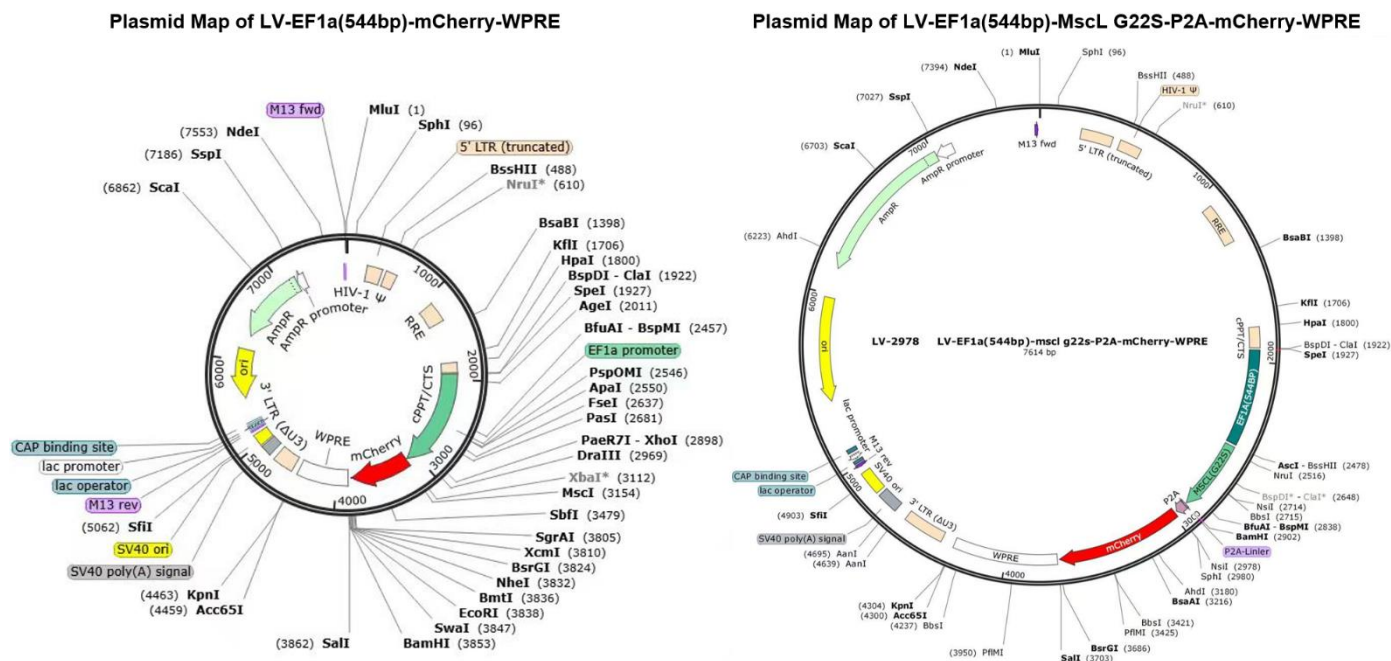


Fig. R1 (add as Supplementary Fig. 7) | Schematic diagram of the viral plasmid construction. This figure displays plasmid maps for the experimental and control lentiviral vectors used in this study. (Right) Utilizes P2A to connect MscL G22S, GCaMp8m and mCherry for non-fusion expression, which allow 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 can be used to indicate intensity of GCaMp8m protein expression. (Left) The plasmid LV-EF1a-mCherry-WPRE served as the negative control.

Our experimental results are as follows:

For cardiomyocytes transduced with MscL G22S, we selected culture dishes that exhibited minimal spontaneous activity at baseline. At lower pressures of 0.21 MPa and 0.40 MPa, we observed only sporadic calcium transients that were not synchronized with our 1 Hz stimulation frequency. A clear activation threshold was reached at 0.59 MPa, where MscL G22S-expressing cells exhibited a significant and reliable increase in calcium transient frequency (**Fig. R2e**). This reliable pacing effect persisted at 0.73 MPa (**Fig. R2f**). However, when the pressure was further increased to 0.94 MPa, pacing was still observed, but signs of cell detachment emerged. Specifically, cells detached from the culture surface, resulting in an unstable imaging plane and an upward drift in the $\Delta F/F$ baseline (**Fig. R2g**).

In contrast, the control group showed no discernible increase in activation frequency across the entire pressure range. Even at the higher pressures of 0.94 and 1.126 MPa—which caused significant cell detachment and baseline artifacts from mechanical vibration—the control cells failed to produce increased rhythmic responses.

In summary, our new data clearly define the optimal in vitro operating window. For cultured cardiomyocytes, robust and reliable activation without observable detachment or damage occurs at pressures of approximately 0.59–0.79 MPa. Activation appears to follow a threshold-like phenomenon. Pressures at or above 0.94 MPa induced detrimental effects, primarily cell detachment, due to the relatively weak adhesion of the cell monolayer to the culture surface. The in vitro results demonstrated the pressure-dependent and channel-specific mechanism of activation.

However, while we showed the channel dependent activation of cardiomyocytes, we also noticed a discrepancy between the optimal in vitro pressures and the higher pressures used in our in vivo experiments. This difference is expected and can be attributed to two main factors. The first factor is acoustic attenuation: the pressures reported in our in vivo experiments (1.2–2.0 MPa) represent values generated at the transducer

surface, before the ultrasound propagates through tissue. Intervening tissues such as skin, muscle, and rib cause attenuation of acoustic energy. Therefore, a much higher initial pressure is required to ensure that the pressure delivered to target cardiomyocytes in the heart falls within the effective activation range (e.g., ~0.6–0.8 MPa). This range is consistent with our direct open-chest rat heart stimulation experiments, where an initial pressure of 0.699 MPa was sufficient to induce pacing (**Extended Data Fig. 5**). The second factor is the difference in structural and mechanical environments between *in vitro* and *in vivo* conditions. *In vivo*, cells are embedded within a robust three-dimensional extracellular matrix, which provides strong structural support and intercellular connections. This renders them far more resilient to mechanical forces than a 2D monolayer of cells sparsely adhered to a plastic surface. The *in vitro* model is therefore more susceptible to mechanical disruption such as detachment, exhibiting a lower damage threshold. The precise pressure for *in vivo* applications must be empirically optimized to account for the acoustic attenuation properties of tissues in each animal model.

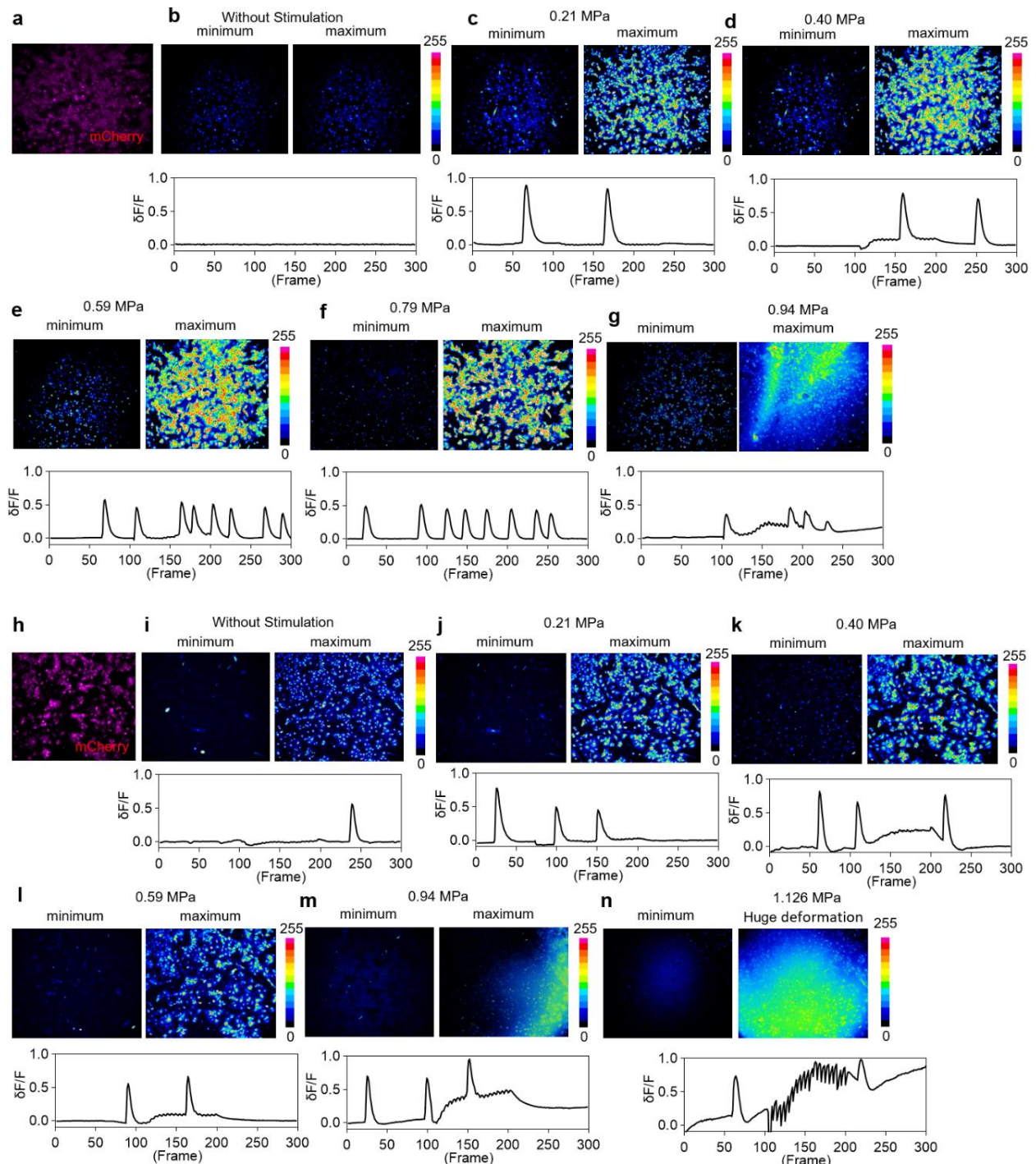


Fig. R2 (add as Supplementary Fig. 10) | Calcium imaging of MscL G22S-mediated sonogenetic activation in cardiomyocytes at varying acoustic pressures. Each condition includes minimum and maximum fluorescence intensity images and a corresponding $\Delta F/F$ trace. **a** is a representative mCherry fluorescence image. **b** shows the baseline recording without ultrasound stimulation. **c**

through 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**. All figures were captured with 20× objective lens. n=3 for ST and controlled group.

We have updated the description of this dataset in the main text (page 4, lines 9–16) by adding the following passage:

“We further quantitatively characterized the response curve of the MscL G22S channel in human cardiomyocytes exposed to varying ultrasound pressures. Calcium imaging was performed at six stimulation levels (0.21, 0.40, 0.59, 0.73, 0.94, and 1.126 MPa), revealing a dose-response relationship with an optimal stimulation range of 0.5-0.8 MPa (**Supplementary Fig. 10a-g**). To confirm the specificity of this effect, cardiomyocytes were transduced with a control lentivirus (LV-Ef1a-P2A-mCherry-WPRE, **Supplementary Fig. 7**). Similar to WT cells, these control cardiomyocytes exhibited no synchronized pacing signals (**Supplementary Fig. 10h-n**), thereby confirming that the observed sonogenetic pacing was specifically mediated by MscL G22S rather than artifacts from the viral vector or mCherry expression.”

2. Safety Thresholds and Regulatory Relevance

Given the authors' claim of preclinical validation, it is important to contextualize the chosen acoustic pressure (~2 MPa) against FDA diagnostic ultrasound limits (e.g., $MI \leq 1.9$, $ISPTA \leq 720 \text{ mW/cm}^2$). Please include a quantitative discussion of safety margins, particularly after accounting for chest-wall attenuation in porcine models, and reference the FDA's "Marketing Clearance of Diagnostic Ultrasound Systems and Transducers".

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 [1]; Nelson, 2009 [2]; AIUM, 2004 [3]; [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 **~2.03 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.

Table 1 | The actual measurements of the NUP system output acoustic power, combined with the pre-amplified 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

Furthermore, we assessed thermal safety during continuous ultrasound stimulation for over 12 hours (**Fig. R3**). This extended in vivo safety assessment was conducted on anesthetized rats placed on a heating pad to maintain a stable core body temperature. A continuous intravenous infusion of saline was administered to ensure physiological stability throughout the prolonged procedure. The NUP system delivered uninterrupted pacing for 12 consecutive hours while fine-gauge (0.23 mm) thermocouple probes monitored the intracardiac temperature. The results show that for all three acoustic pressures tested (1.1, 1.5, and 2.0 MPa), the maximum temperature increase was less than 1.2 °C. This observation is consistent with and further confirmed by FDA-required thermal index (TI) calculations, one of the key indices used to evaluate the clinical safety of ultrasound devices. The calculated TI of our stimulation is $TIS \approx 0.4\text{--}1.2$, depending on focal area and acoustic pressure, indicating that the temperature increase does not exceed 1.2 °C. Crucially, once ultrasound stimulation was terminated, the intracardiac temperature promptly returned to its baseline level. This experiment provides direct and compelling evidence that our NUP system can be operated for prolonged periods without inducing significant or hazardous thermal effects, strongly supporting its safety profile for chronic therapeutic applications.

The complete response to this issue has been integrated into **Supplementary Text 7: Safety Thresholds and Regulatory Relevance** in the Supplementary Materials page 50.

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Fig. R3 (add as Supplementary Fig. 31) | Long-duration in vivo thermal monitoring under continuous pacing. Top: schematic of the experimental setup for in vivo temperature monitoring. Fine-gauge thermocouple probes (0.23 mm) were positioned to measure temperature directly within the heart (intracardiac) and on the skin surface of an anesthetized rat, while the NUP system delivered continuous ultrasound stimulation. A syringe pump provided continuous intravenous infusion to maintain hydration, and a heating pad was used to maintain a stable baseline physiological temperature. **Middle:** A sustained 13-hour session of pacing was applied (indicated by the shaded region). The different traces represent the thermal effect of continuous stimulation at three different acoustic pressures: **2.0 MPa (black)**, **1.5 MPa (red)**, and **1.1 MPa (light red)**. **Bottom:** Insets show magnified views of the temperature profiles at the onset of stimulation (left, 0.6-1.4 hours) and after its cessation (right, 12.8-13.2 hours) for all three pressure levels. $n=3$ for each condition.

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3. Mechanosensitive Channel Comparisons and Biophysical Mechanisms

In Supplementary Text 2, I recommend a direct experimental or literature-based comparison of MscL G22S with other mechanosensitive channels relevant at cardiac ultrasound frequencies (e.g., Piezo1, TREK-1, TRPA1). This will reinforce MscL G22S as the optimal choice. Additionally, given that MscL is central to the biological mechanism of the NUP, please provide a deeper biophysical discussion on how its function is modulated in cardiac membranes. Comparative insights between cardiomyocytes and other cell types (e.g., neurons) in terms of membrane tension coupling, lipid raft distribution, and cytoskeletal interactions would be highly valuable.

We thank the reviewer for this insightful suggestion. In response, we have included a comprehensive literature-based comparison of mechanosensitive ion channels relevant to ultrasound pacing at cardiac frequencies, together with an expanded biophysical analysis of MscL-G22S in cardiomyocytes.

3.1 Literature-Based Comparison of Mechanosensitive Channels Relevant to Cardiac Ultrasound Frequencies

Several endogenous mechanosensitive ion channels, including Piezo1, TREK-1, TRPA1, and TRPV1, are expressed in cardiac tissues and have been shown to respond to mechanical or ultrasound stimuli. We added a summarized table to introduce them ([Table 2](#)). However, their intrinsic electrophysiological properties and activation thresholds make them suboptimal for high-frequency ultrasound cardiac pacing (2–4 MHz).

Piezo1, a non-selective cation channel, is expressed in cardiomyocytes and responds to both mechanical stretch and ultrasound stimulation. However, it exhibits rapid desensitization within tens of milliseconds, which limits its suitability for repetitive cardiac pacing. Moreover, ultrasound activation of Piezo1 typically requires either low frequencies (<1 MHz) or very high-frequency, high-intensity conditions, such as 43-MHz in vitro paradigms[1, 2]. These frequency–intensity mismatches, together with its rapid inactivation, preclude its practical use for sustained cardiac pacing.

TREK-1 and related K2P family channels (e.g., TREK-2, TRAAK) are activated by mechanical stress and ultrasound typically below 1 MHz. However, these channels mediate K⁺ efflux that hyperpolarizes the membrane and thereby opposes the depolarization required for action potential initiation in cardiomyocytes[3, 4]. Although they contribute to cardiac repolarization and arrhythmogenesis, their electrophysiological role is fundamentally incompatible with pacing through depolarizing mechanisms.

TRPA1 channels, although mechanosensitive, respond to ultrasound only at non-cardiac frequencies such as 430 kHz or 6.9 MHz. Their expression in cardiomyocytes and potential contribution to pacing remain uncertain. Studies validating TRPA1 for ultrasound neuromodulation in astrocytes highlight its utility for central nervous system (CNS) applications but not for cardiac use[5, 6].

TRPV1, a thermosensitive channel, is primarily activated by high-intensity ultrasound through thermal mechanisms. Its activation latency is slow (often >500 ms), making it unsuitable for temporally precise, rapid cardiac pacing[7].

In contrast, the engineered bacterial mechanosensitive channel MscL-G22S exhibits properties uniquely suited for cardiac sonogenetics. It operates efficiently within the 2–3 MHz range—the optimal frequency window for both imaging and therapeutic cardiac ultrasound. It demonstrates rapid activation latencies of 5–10 ms and does not undergo inactivation, enabling consistent and repeatable pacing. The G22S mutation destabilizes inter-subunit hydrophobic interactions in the closed-state conformation, thereby lowering the tension threshold required for gating[8]. MscL-G22S forms a large-conductance, non-selective cation pore that facilitates robust Na⁺ and Ca²⁺ influx to initiate action potentials. Furthermore, its exogenous origin permits selective expression in cardiomyocytes using tissue-specific promoters (e.g., cTnT or mTnT) delivered via AAV2/9 vectors, thereby minimizing off-target effects and ensuring cell-specific activation[9, 10].

Table 2 (add as Supplementary Table 2) | Comparative Analysis of Mechanosensitive Ion Channels for Ultrasound Cardiac Pacing Suitability

Channel	Activation Threshold	Activation Frequency	Ion Selectivity/Conductance Type	Membrane Potential Effect	Activation Latency (US)	Inactivation Time (US)	Cardiomyocyte Expression & Endogenous Role	Suitable for 2-4 MHz Pacing	Notes/Key References
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. [10, 11]
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 [12].	No	Hyperpolarizing effect is unsuitable for pacing [13].
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 [3].
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 [3].
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[14].	Unclear	It may be suitable for pacing, its optimal frequency is less appropriate for this application than that of MscL G22S [1, 2].
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 [2].
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[5, 15].	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[16].	Unclear	Slower kinetics and less defined US response for pacing compared to MscL-G22S [17].
TRPP2 (PKD2)	Not well-defined for US	Unknown	Ca ²⁺ /Na ⁺ influx; primarily ER Ca ²⁺ 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 [18].	No	Primarily an intracellular channel, complex regulation, not a primary plasma membrane pacer for US [2].
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[19].	No (as primary)	Acts as an amplifier of Ca ²⁺ signals; not a primary US sensor itself for pacing [2].
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 [20].	No (for precise pacing)	Primarily thermal activation by US, slow latency, not suitable for precise, rapid pacing [7].
Prestin (N7T/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 [21].

This table has been added to **Supplementary Table 3. Comparative Analysis of Mechanosensitive Ion Channels for Ultrasound Cardiac Pacing Suitability** in Supplementary materials page 56.

3.2 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[22]. 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[23]. 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[24, 25].

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[19].

3.3 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[26, 27]. 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[28].

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.

This discussion has been added to **Supplementary Text 4**, in Supplementary Materials Page 47 line 15 to 43.

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4. Ex Vivo Porcine Pacing Validation

To confirm true myocardial depolarization, please supplement the current ex vivo validation with ECG recordings from porcine hearts under NUP stimulation. This would offer compelling functional evidence of pacing efficacy.

Response:

We thank the reviewer for this excellent suggestion. We agree that supplementing our ex vivo validation with ECG recordings would provide compelling functional evidence of genuine myocardial depolarization and pacing efficacy.

However, performing an ex vivo ECG recording on a porcine heart presents significant technical and logistical challenges that are beyond the scope of our current capabilities. This experiment requires a highly specialized large-animal working heart apparatus (such as the Radnoti or Langendorff perfusion system, see attached fig from AD Instruments, Australia), which is a complex setup that includes water-jacketed reservoirs, bubble traps, a thermostat, circulation and peristaltic pumps, compliance chambers, and stainless-steel aortic and atrial cannulas, among other critical components. The proper operation of this system necessitates a dedicated laboratory facility and personnel with extensive, specialized training in large animal heart harvesting, surgical cannulation, and management of ex vivo perfusion to maintain cardiac viability.

Our preliminary assessment indicated that the financial investment for the perfusion system alone would be considerable, with vendor quotations (e.g., AD Instruments) exceeding tens of thousands of dollars and requiring a long lead time. This figure does not account for the additional time and resources required for personnel training, protocol development and refinement, and the complex upstream procedures, which include in vivo AAV transduction in live pigs followed by open-chest surgery to explant the heart.

Given these substantial constraints in cost, infrastructure, and time, we were unable to incorporate this experiment into the current study. Instead, we are actively preparing for the next phase of this research, which will focus on in vivo validation in a large-animal model. We are in the process of submitting a grant application to the NIH to secure the necessary funding for these comprehensive studies. We believe that advancing directly to an in vivo model, although challenging, represents a more physiologically relevant and strategically aligned step for validating our NUP technology and achieving our long-term translational objectives.

We hope this explanation clarifies the practical limitations we currently face. We are grateful for the reviewer's thoughtful suggestion and trust that our strategic decision to prioritize in vivo validation as the next major milestone will be appreciated.



Fig. R4 | Langendorff perfusion system. This figure from AD Instruments' official webpage, showing the components of the perfusion system, includes water-jacketed reservoirs, bubble traps, a thermostat, circulation, peristaltic pumps, compliance chambers, stainless-steel aortic and atrial cannulas.

5. Long-Term Calcium Handling Stability

To ensure safety under repeated stimulation, it is recommended to perform immunostaining and quantitative analyses of key calcium-handling proteins (e.g., RyR2, SERCA2a, PLB) to assess any long-term effects on intracellular Ca^{2+} cycling.

Response:

We sincerely appreciate the reviewer's insightful suggestion, which prompted us to investigate the biosafety of our sonogenetic system in greater depth and detail. To this end, we performed both immunofluorescence staining and proteomic analyses of rat hearts 6 and 8 months after AAV-mediated MscL-G22S transduction, compared with age-matched WT controls.

5.1 Immunofluorescence staining

We performed whole-mount immunostaining of three key calcium-handling proteins recommended by the reviewer—RyR2, SERCA2a, and PLB—to assess whether long-term MscL-G22S expression alters the endogenous cardiac calcium signaling machinery.

- RyR2: Proteintech™, Cat# 19765-1-AP (Rabbit anti-rat)
- SERCA2a (ATP2A2): Proteintech™, Cat# 27311-1-AP (Rabbit anti-rat)
- PLB: Invitrogen™, Cat# PA5-26004 (Rabbit anti-rat)

Representative whole-heart images are shown in [Fig. R5](#). For quantitative comparison, we analyzed identical 2 mm × 2 mm regions from each heart section, measuring both the mean fluorescence intensity and the positive area fraction of each target protein. The statistical results (right panels) showed no significant differences (ns, $p > 0.05$, t-test) between the transduced and control groups across all three markers. These findings suggest that long-term expression of MscL-G22S does not alter the native expression or spatial distribution of key calcium-handling proteins.

This figure has been added to the Supplementary Materials (page 28). In the main text (page 7, line 24), we have included the following description:

“Next, immunofluorescence staining was performed to evaluate representative calcium-handling proteins, including RyR2, SERCA2a, and PLB, to assess potential long-term effects on intracellular Ca^{2+} cycling ([Supplementary Fig. 25](#)).”

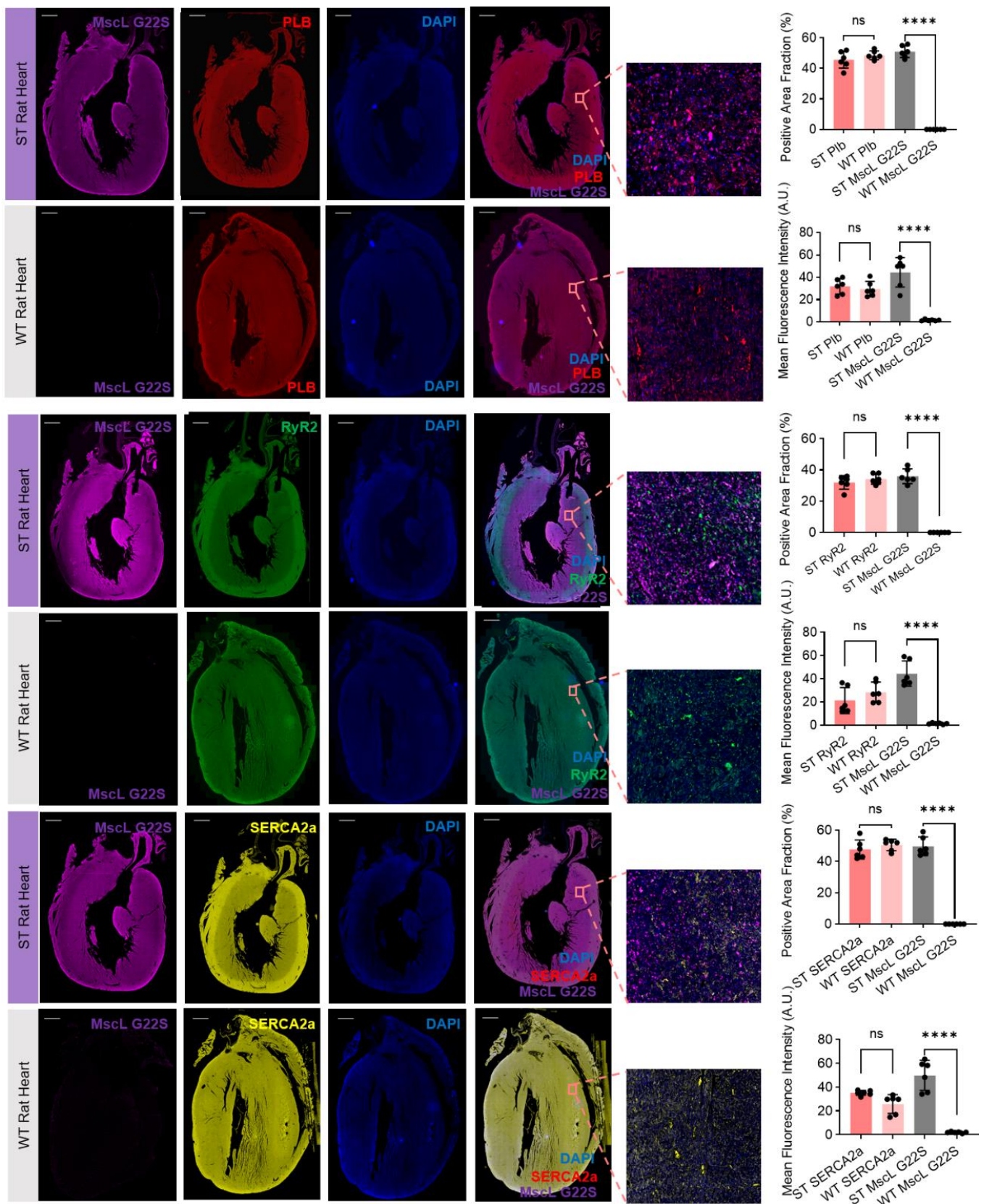


Fig. R5 (add as Supplementary Fig. 25) | Immunofluorescence analysis reveals upregulation of RyR2 and SERCA2a in hearts with chronic MscL G22S expression. Representative immunofluorescence images and corresponding quantitative analysis of key calcium-handling proteins—phospholamban (PLB), ryanodine receptor 2 (RyR2), and sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a)—in heart sections from ST and WT rats. For each protein, images show the expression of the MscL G22S transgene (magenta, present only in ST hearts), the target protein (PLB in red, RyR2 in green, SERCA2a in yellow), and cell nuclei (DAPI, blue). Merged images and magnified insets are provided to visualize protein expression and tissue architecture. Scale bar = 1 mm for whole heart images, 50 μ m for insets. Bar charts on the right quantify the Positive Area Fraction (%) and Mean Fluorescence Intensity (A.U.) for each target protein, comparing ST and WT groups (n=6 per group). (ns = not significant, ****p < 0.0001; one-way ANOVA).

5.2 Spatial Transcriptomic Analysis

We also performed spatial transcriptomics to evaluate the long-term effects of MscL G22S expression on the cardiac ion channel transcriptome. This technique provides an unbiased, high-resolution approach to assess single-cell-level gene expression within the native structural context of the heart. We analyzed cardiac tissue from ST mice 6 months post-transduction, using age-matched WT mice as controls. To minimize technical variability, tissue sections from both a WT and an ST mouse were co-processed on the same slide (sample placement shown in [Fig. R6a](#)). The analysis was conducted using Stomics' Stereo-seq technology. The workflow included sample preparation, staining and imaging of the tissue section, tissue permeabilization for mRNA capture and cDNA synthesis, followed by library preparation with the Stereo-seq Library Preparation Kit (V1.3) and sequencing on a DNBseq-T7 platform (2 × 75 bp paired-end reads) to a depth of up to 3 billion reads.

First, we confirmed the identity of each tissue section by mapping the expression of the MscL G22S transgene. As shown in [Fig. R6b](#), a robust signal for MscL G22S was detected throughout the right-sided heart sample, confirming it as the ST sample, whereas the left-sided sample showed an almost complete absence of expression, confirming it as the WT control. We then performed cell type annotation, which successfully delineated the primary cardiac structures, including distinct populations of atrial cardiomyocytes (aCMs), ventricular cardiomyocytes (vCMs), terminal cardiomyocytes, and Purkinje cells ([Fig. R6c](#)).

In response to the reviewer's suggestion, our analysis focused on key genes involved in intracellular Ca²⁺ cycling. The spatial expression maps for genes encoding the ryanodine receptor 2 (RyR2), phospholamban (Pln), and other critical calcium-handling proteins ([Fig. R6d](#)) showed that their expression levels and distribution patterns are qualitatively and quantitatively similar between the ST and WT cardiac tissues.

Furthermore, the comprehensive nature of spatial transcriptomics allowed us to extend our analysis to other key families of ion channels. As shown in the [Fig. R6e-h](#), we observed no significant differences in the expression patterns of major potassium channels, sodium channels, endogenous mechanosensitive channels, or other related channel families between the ST and WT hearts.

These results indicate that long-term overexpression of the MscL G22S channel does not significantly alter the transcriptional profile of essential components of Ca²⁺ cycling, and that the broader cardiac ion channel transcriptome remains stable.

This figure has been added to the Supplementary Materials (page 29). In the main text (page 7, line 26), we have also added the following description:

“We additionally employed spatial transcriptomics to map the expression and distribution of ion channel genes with spatial resolution, confirming that the qualitative and quantitative expression patterns of calcium, potassium, and sodium channels were comparable between ST and WT hearts ([Supplementary Fig. 26](#)).”

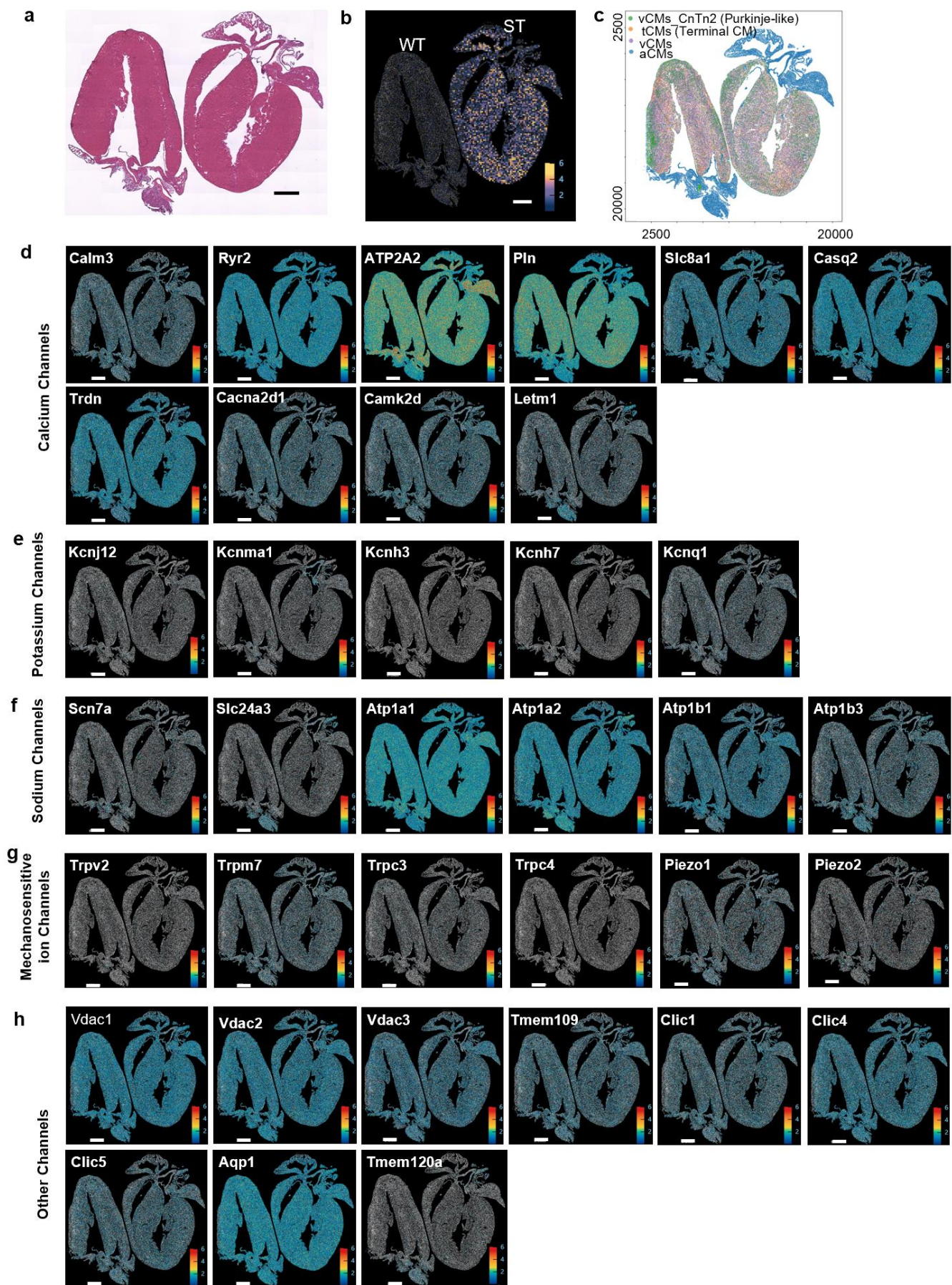


Fig. R6 (add as Supplementary Fig. 26) | Comprehensive spatial transcriptomic analysis of key ion channel genes in wild-type and sonogenetically transduced hearts. **a**, H&E staining of adjacent wild-type (WT, left) and sonogenetically transduced (ST, right) cardiac tissue sections. **b**, Spatial plot 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.

5.3 Proteomic Analysis

In addition to the key calcium-handling proteins RyR2, SERCA2a, and phospholamban (PLB) mentioned by the reviewer, we further extracted and analyzed several other representative calcium signaling-related proteins identified from the proteomics data. These include Calm3 (Calmodulin), Calml3, Slc8a1 (NCX1), Casq2, TRDN, Cacna2d1, Camk2d, and Letm1. We summarized the normalized relative abundance (%) and corresponding T-test statistics for these proteins in **Table 3** and visualized the expression patterns using a heatmap (see attached **Fig. R7**).

Table 3 | Proteomic analysis of calcium ion channels in long-term ST hearts

	Gene Symbol	6 months ST.# PSMs normalized relative abundance (%)	8 months WT.# PSMs normalized relative abundance (%)	WT.# PSMs normalized relative abundance (%)	T-test Ratio (ST6/W T)	T-test Ratio (ST8/W T)	T-test Ratio (ST8/S T6)
	MscL G22S [1, 2]	0.0459/0.0254/0.0354	0.0100/0.0208/0.0187	0.0000/0.0000/0.0000	0.0038	0.0075	0.0485
Calcium Channels	Calm3 [3]	0.1327/0.1711/0.1545	0.1839/0.1530/0.1550	0.1228/0.2183/0.1558	0.6916	0.9584	0.4955
	Ryr2 [4]	0.0372/0.0454/0.0489	0.0451/0.0480/0.0554	0.0346/0.0346/0.0503	0.5648	0.1883	0.2878
	SERCA2 [5]	0.2181/0.2367/0.2463	0.2483/0.2541/0.2572	0.2315/0.2732/0.2615	0.2197	0.8710	0.0881
	PLB [6]	0.2074/0.2898/0.2330	0.4217/0.2466/0.2962	0.2179/0.2752/0.2901	0.6185	0.3458	0.2461
	Slc8a1 [7]	0.0046/0.0083/0.0087	0.0042/0.0088/0.0066	0.0053/0.0080/0.0095	0.8251	0.5690	0.7289
	Casq2 [8]	0.1676/0.1291/0.1614	0.1858/0.1552/0.1429	0.1435/0.1354/0.1405	0.3498	0.1724	0.6463
	TRDN [9]	0.0026/0.0016/0.0070	0.0099/0.0041/0.0018	0.0038/0.0094/0.0118	0.1893	0.4209	0.6258
	Cacna2d1 [10]	0.0016/0.0042/0.0011	0/0/0.0011	0.0008/0.0023/0.0042	0.9227	0.1235	0.1365
	Camk2d [11]	0.0152/0.0131/0.0159	0.0103/0.0214/0.0193	0.0164/0.0098/0.0174	0.9408	0.5853	0.5539
	Letm1 [12]	0.0280/0.0345/0.0426	0.04081/0.0386/0.0365	0.0283/0.0282/0.0314	0.2591	0.0047	0.4646

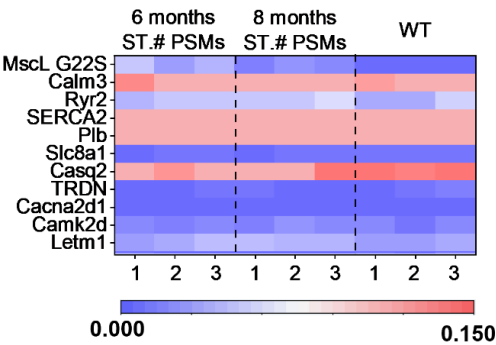


Fig. R7 | Proteomic heatmap analysis of key calcium-handling proteins in long-term sonogenetically treated hearts. Heatmap displaying the normalized relative abundance of key proteins involved in cardiac calcium handling, based on label-free quantitative proteomics. Rows represent individual proteins, including the MscL G22S transgene and major endogenous calcium-handling proteins. Columns represent individual biological replicates (n=3 per group) from ST rat 6 and 8 months post-transduction, and age-matched WT controls. The color intensity corresponds to the normalized relative abundance of each protein, as indicated by the scale bar (blue = low abundance, red = high abundance). The heatmap confirms the specific and robust expression of MscL G22S only in the ST samples. The expression levels of most endogenous calcium-handling proteins, such as Calmodulin-3 (**Calm3**), Phospholamban (**PLB**), and the Na⁺/Ca²⁺ exchanger (**Slc8a1**), remain stable across all groups.

Our results showed that, apart from the genetically introduced sonogenetic actuator MscL G22S, which exhibited statistically significant differences in expression between groups (t-test, p < 0.01), the expression levels of endogenous calcium-handling proteins remained stable. Specifically, when comparing ST samples at both 6 and 8 months post-transduction with their respective WT controls, no significant alterations were observed in the abundance of these native calcium regulators.

These findings indicate that long-term expression of MscL-G22S and subsequent sonogenetic stimulation do not appreciably disturb the physiological expression of calcium cycling proteins in the heart. The minimal proteomic perturbation, combined with the preserved expression of critical calcium-handling channels, underscores the biosafety of our sonogenetic approach for chronic cardiac applications.

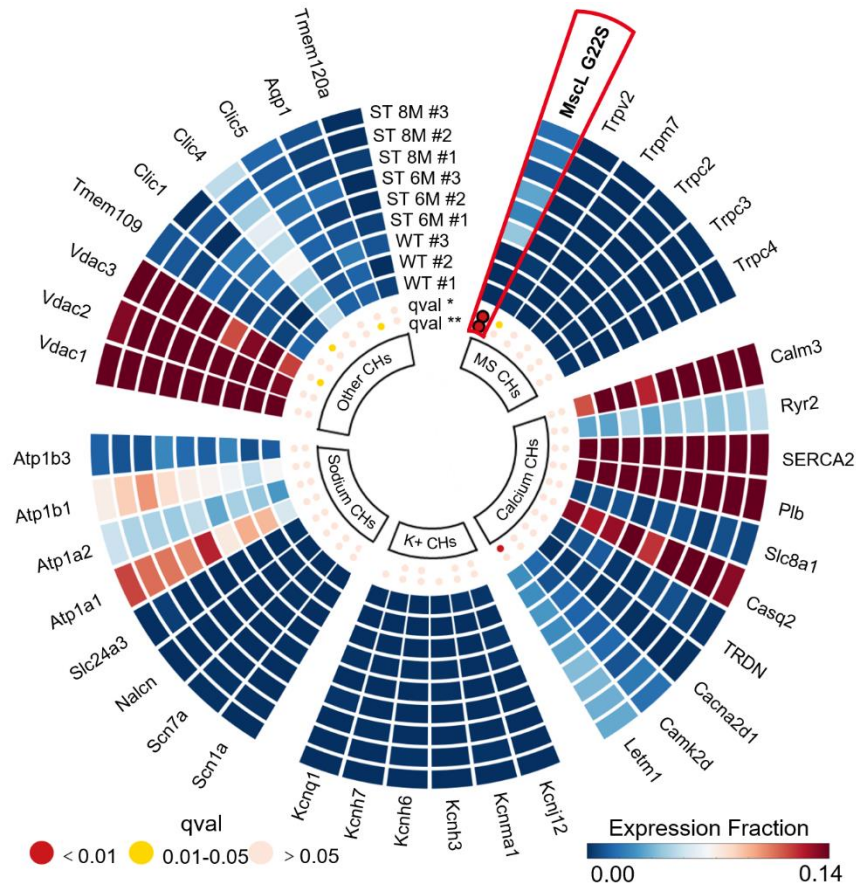


Fig. R8 (add as Supplementary Fig. 29) | Proteomic profiling of the cardiac ion channel landscape following sonogenetic transduction. Proteomic analysis of major ion channel families in heart tissue from WT, ST 6M, and ST 8M rat. The plot is organized by ion channel class: Mechanosensitive (MS), Calcium (Ca^{2+}), Potassium (K^{+}), Sodium (Na^{+}), and other channels. Each concentric ring corresponds to a specific protein as labeled, and each radial segment represents an individual biological replicate ($n=3$ for each group). The color intensity of each cell indicates the normalized expression fraction of the protein within that sample, with red representing higher expression and blue representing lower expression. The central ring of dots displays the statistical significance (q-value) for the comparison between ST and WT groups, where gray indicates no significant difference ($q > 0.05$).

To comprehensively evaluate long-term biosafety in cardiac tissues, we extended our proteomic analysis to include all major classes of ion channel proteins identified in the LC-MS/MS dataset. In addition to calcium channels (e.g., RyR2, SERCA2a, PLB, Calm3, etc.), we systematically profiled potassium channels (Kcnj12, Kcnma1, Kcnh3, Kcnh6, Kcnh7, Kcnq1), sodium channels (Scn1a, Scn7a, Nalcn, Slc24a3, Atp1a1, Atp1a2, Atp1b1, Atp1b3), mechanosensitive ion channels (Trpv2, Trpm7, Trpc2, Trpc3, Trpc4), and other ion transport-related proteins (e.g., Vdac1-3, Tmem109, Clic1/4/5, Aqp1, Tmem120a). Normalized relative abundances of these proteins across three groups (ST 6-month, ST 8-month, and WT controls) were quantified and summarized in **Table 4**, with statistical comparisons (T-test ratios and q-values) calculated for each condition. Their expression patterns are visualized in **Fig. R8**.

Overall, most of these ion channel proteins exhibited highly consistent expression levels across all groups. While a few channels (e.g., Nalcn, Kcnh3, or Tmem120a) showed very low or undetectable expression in WT samples but detectable levels in occasional ST replicates, their absolute abundance remained minimal, and the differences did not reach statistical significance ($q > 0.05$). The only protein that consistently showed significant differences ($p < 0.01$) was the sonogenetically introduced MscL-G22S, confirming its specific expression and the effectiveness of AAV delivery.

These findings collectively suggest that chronic expression of MscL-G22S does not appreciably alter the endogenous ion channel landscape in the heart. From a proteomic standpoint, the integrity of critical electrophysiological signaling pathways is preserved. This molecular evidence is further supported by our behavioral and functional assessments of long-term sonogenetic stimulation in rats, where no abnormalities in cardiac function or physiology were observed. Thus, our results provide compelling evidence for the safety and specificity of the sonogenetic system in long-term cardiac applications.

Table 4 (add as Supplementary Table 4) | Proteomic analysis of all ion channels in long-term ST hearts

	Gene Symbol	6 months ST.# PSMs normalized relative abundance (%)	8 months WT.# PSMs normalized relative abundance (%)	WT.# PSMs normalized relative abundance (%)	T-test Ratio (ST6/W T)	T-test Ratio (ST8/W T)	T-test Ratio (ST8/S T6)
	MscL G22S [1, 2, 13]	0.0459/0.0254/0.0354	0.0100/0.0208/0.0187	0.0000/0.0000/0.0000	0.0038	0.0075	0.0485
Calcium Channels	Calm3 [3]	0.1327/0.1711/0.1545	0.1839/0.1530/0.1550	0.1228/0.2183/0.1558	0.6916	0.9584	0.4955
	Ryr2 [4]	0.0372/0.0454/0.0489	0.0451/0.0480/0.0554	0.0346/0.0346/0.0503	0.5648	0.1883	0.2878
	SERCA2 [5]	0.2181/0.2367/0.2463	0.2483/0.2541/0.2572	0.2315/0.2732/0.2615	0.2197	0.8710	0.0881
	PLB [6]	0.2074/0.2898/0.2330	0.4217/0.2466/0.2962	0.2179/0.2752/0.2901	0.6185	0.3458	0.2461
	Slc8a1 [7]	0.0046/0.0083/0.0087	0.0042/0.0088/0.0066	0.0053/0.0080/0.0095	0.8251	0.5690	0.7289
	Casq2 [8]	0.1676/0.1291/0.1614	0.1858/0.1552/0.1429	0.1435/0.1354/0.1405	0.3498	0.1724	0.6463
	TRDN [9]	0.0026/0.0016/0.0070	0.0099/0.0041/0.0018	0.0038/0.0094/0.0118	0.1893	0.4209	0.6258
	Cacna2d1 [10]	0.0016/0.0042/0.0011	0/0/0.0011	0.0008/0.0023/0.0042	0.9227	0.1235	0.1365
	Camk2d [11]	0.0152/0.0131/0.0159	0.0103/0.0214/0.0193	0.0164/0.0098/0.0174	0.9408	0.5853	0.5539
	Letm1 [12]	0.0280/0.0345/0.0426	0.04081/0.0386/0.0365	0.0283/0.0282/0.0314	0.2591	0.0047	0.4646
Potassium Channels	Kcnj12 [14]	0.0021/0/0	0/0/0	0.0020/0/0.0027	0.4575	0.1241	0.3739
	Kcnma1 [15]	0/0/0	0/0.0012/0	0.0022/0/0	0.3740	0.7098	0.3739
	Kcnh3 [16]	0.0008/0.0010/0	0/0/0	0/0/0	0.1224	N/A	0.1224
	Kcnh6 [17]	0.0009/0/0	0/0.0015/0	0/0/0	0.3739	0.3739	0.7705
	Kcnh7 [18]	0.0008/0/0	0.0011/0/0	0/0/0	0.3739	0.3739	0.7878
	Kcnq1 [19]	0.0013/0/0.0018	0/0/0	0/0/0	0.1248	N/A	0.1248
Sodium Channels	Scn1a [20]	0.0004/0.0011/0	0/0.0007/0	0/0/0	0.1872	0.3739	0.5091
	Scn7a [21]	0.0005/0/0	0/0.0017/0.00076405	0/0.0008/0.0007	0.3645	0.5780	0.2862
	Nalc1 [22]	0/0/0	0/0/0	0.0005/0.0007/0	0.1314	0.1314	N/A
	Slc24a3 [23]	0/0/0	0/0.0046/0	0.0014/0/0	0.3739	0.5432	0.3739
	Atp1a1 [24]	0.0826/0.1326/0.1114	0.1152/0.1170/0.1255	0.0622/0.0992/0.1021	0.3389	0.0766	0.5222
	Atp1a2 [25]	0.0361/0.0557/0.0475	0.0511/0.0517/0.0591	0.0316/0.0459/0.0501	0.64897	0.13733	0.29461
	Atp1b1 [26]	0.0769/0.0801/0.0877	0.1082/0.0937/0.0802	0.0746/0.0556/0.0725	0.1092	0.0581	0.2239
	Atp1b3 [27]	0.0129/0.0166/0.0261	0.0098/0.0102/0.0138	0.0125/0.0093/0.0250	0.6601	0.4314	0.1535
Mechanosensitive Ion Channels	Trpv2 [28]	0/0.0015/0	0/0.0019/0	0.0023/0.0017/0.0031	0.0449	0.0786	0.8920
	Trpm7 [29]	0.0005/0.0006/0	0.0007/0/0	0/0/0	0.1224	0.3739	0.7112
	Trpc2 [30]	0/0.0020/0	0/0.0012/0.0011	0.0007/0/0.0010	0.9159	0.7137	0.8916
	Trpc3 [31]	0.0010/0.00135/0	0/0/0.0014	0.0010/0.0014/0	0.9448	0.6328	0.6651
	Trpc4 [30]	0/0/0	0.0014/0.0015/0	0.0018/0/0	0.3739	0.6624	0.1163
Other Channels	Vdac1 [32]	0.6131/0.3810/0.4882	0.4408/0.4834/0.4762	0.7085/0.4460/0.4348	0.7656	0.5244	0.7100
	Vdac2 [33]	0.1493/0.1533/0.1931	0.1626/0.1787/0.1436	0.1448/0.1500/0.1889	0.8487	0.9813	0.8459
	Vdac3 [34]	0.1239/0.1802/0.1799	0.1937/0.1813/0.2041	0.1263/0.1609/0.1436	0.4503	0.0145	0.1855
	Tmem109 [35]	0.0074/0.0095/0.0150	0.0056/0.0117/0.0106	0.0144/0.0054/0.0096	0.8103	0.8968	0.6742
	Clic1 [36]	0.0187/0.0192/0.0201	0/0.0118/0	0.0109/0/0.0048	0.0112	0.8118	0.0178
	Clic4 [37]	0.0746/0.0550/0.0671	0.0488/0.0169/0.0558	0.0551/0.0463/0.0505	0.0757	0.4539	0.1318
	Clic5 [38]	0.0143/0.0139/0.0193	0.0164/0.0114/0.0153	0.0139/0.0104/0.0092	0.1056	0.1991	0.5606
	Aqp1 [39]	0.0100/0.0043/0.0180	0.0051/0.0053/0.0095	0.0130/0.0145/0.0216	0.3090	0.0325	0.3834
	Tmem120a [40]	0.0052/0/0	0.0040/0/0	0.0051/0/0.0102	0.3842	0.3092	0.8593

Fig. R8 (add as Supplementary Fig. 29) has been added to the Supplementary Materials (page 32). In the main text (page 7, lines 31–37), we have also included the following description:

“These datasets confirmed long-term safety, showing strong correlations between the proteomic profiles of ST and WT hearts (Pearson’s $R \geq 0.99$), with no significant perturbations in the expression of major ion channel families (**Supplementary Fig. 28 and 29**). Taken together, these multi-level assessments demonstrate that chronic sonogenetic transduction preserves transcriptional integrity, maintains proteomic stability, and does not elicit inflammatory or immune responses. Collectively, these findings establish the long-term safety and translational feasibility of sonogenetics for cardiac applications.”

In addition, **Table 3** (add as **Supplementary Table 4**), presenting proteomic analysis of all ion channels in long-term ST hearts, has been added to the Supplementary Materials.

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Define Abbreviations Clearly

1. Please define all acronyms (e.g., PRF, ST, MEA, FWHM, MI, ISPTA) at first mention in both the main text and supplementary materials to improve readability for interdisciplinary audiences.

Response:

We thank the reviewer for this helpful suggestion. To improve readability, we have defined all acronyms at their first occurrence in both the main text and the Supplementary Materials. The detailed revisions are as follows:

PRF: Defined as pulse repetition frequency (PRF) at its first occurrence in the main text (page 3, line 37) and in the Supplementary Materials (page 20, line 1). Subsequent mentions use only “PRF.”

ST: Defined as sonogenetics-transfected (ST) at its first occurrence in the main text (page 3, line 44) and in the Supplementary Materials (page 18, line 3). Subsequent mentions use only “ST.”

MEA: Defined as microelectrode arrays (MEA) at its first occurrence in the main text (page 4, line 41) and in the Supplementary Materials (page 19, line 2). Subsequent mentions use only “MEA.”

MI: Defined as Mechanical Index (MI) at its first occurrence in the main text (page 7, line 40) and in the Supplementary Materials (page 50, line 8). Subsequent mentions use only “MI.”

ISPTA: Defined as spatial peak temporal average intensity ($I_{\text{spta.3}}$) at its first occurrence in the main text (page 7, line 39) and in the Supplementary Materials (page 50, line 9). Subsequent mentions use only “ $I_{\text{spta.3}}$ ”

2. Standardize and Clarify Data Presentation

Several bar graphs lack consistency in sample size indication (e.g., Fig. 2e, Extended Data Fig. 4b). Ensure that sample sizes (n), error bars ($\pm$ SD or 95% CI), statistical tests, and p-values or significance levels are provided in all figure panels. Unify error bar styles across figures for visual consistency.

Response:

Thank you for your reminder and suggestions. We have carefully reviewed all the figures and confirmed that each of them includes the sample sizes (n), error bars ($\pm$ SD), the statistical test method, and the significance levels.

3. Confirm qPCR Analysis Methodology

Please confirm that the $2^{-\Delta\Delta CT}$ method was used for RT-qPCR analysis. GAPDH does not need to appear in the final figure (e.g., Fig. S10) but should be clearly stated as the internal control in the text and calculations.

Response:

Thank you for pointing out this issue. We confirm that the $2^{-\Delta\Delta CT}$ method was indeed used. GAPDH has been removed from the figure, and the following description has been added to the Methods section of the Supplementary Materials (page 36, line 16) to ensure that your suggestion has been incorporated into the manuscript:

“GAPDH was used as the internal control, and gene expression fold changes were calculated using the $2^{-\Delta\Delta CT}$ method, normalized to GAPDH expression levels.”

4. Minor Typographic Corrections

Fix typographical errors—for example, “strqess” should be corrected to “stress” in Fig. S10.

Response:

Thanks for pointing out this error, we have corrected the “strqess” to “stress” in supplementary materials page 14 line 8.

Reviewer #3

I commend the authors for translating bold concepts into a cohesive, clinically relevant framework. The potential to select pacing sites without invasive procedures is particularly compelling. Moreover, integrating ultrasound—a cornerstone of cardiology practice—with sonogenetics and real-time cloud-based data analysis elevates both the diagnostic and therapeutic potential of this platform.

This work provides important insights into noninvasive pacemaker technology and employs sophisticated analytical approaches; however, several key areas warrant further clarification and refinement.

We thank the reviewer for finding our approach to be a "cohesive, clinically relevant framework" and recognizing the potential of integrating ultrasound with sonogenetics and cloud-based analysis. We also appreciate the insightful and constructive feedback, which has allowed us to substantially enhance the manuscript. To address the reviewers' comments, we have added **16 new figures and 4 supplementary Tables**. We believe these revisions have greatly improved the paper and will contribute to the ongoing development of non-invasive pacemaker technology. Our detailed, point-by-point responses are provided below, and all major changes in the revised manuscript have been marked in [blue](#).

Comment 1. Chronic Ca^{2+} Overload Risk:

The pacemaking system relies on Ca^{2+} influx induced by the MscL G22S channel. While the authors demonstrate minimal effects on the expression of genes related to harmful responses such as inflammation or apoptosis, the long-term implications remain uncertain. Clinical scenarios such as complete atrioventricular (AV) block or severe sick sinus syndrome require continuous pacing, raising concerns about potential cardiac remodeling or fibrosis due to chronic Ca^{2+} overload. This issue should be addressed using mid- to long-term pacing models. Additionally, in such models, it would be valuable to assess potential changes in the expression of other ion channel genes.

Response:

1.1 Evaluation of the Long-term Effects of Sonogenetic Transduction on the Heart

We thank the reviewer for raising the important concern regarding the long-term consequences of sonogenetic transduction. To comprehensively address this, we have conducted a multi-faceted long-term evaluation, encompassing transcriptomic, proteomic, and in vivo functional assessments.

1.1.1 Spatial Transcriptomic Analysis at 6 Months Post-Transduction

First, we present the results of our spatial transcriptomic sequencing analysis performed on the hearts of ST mice at 6 months post-transduction, alongside age-matched WT control mice.

Spatial transcriptomics provides an unbiased, high-resolution methodology to assess single-cell level gene expression changes within the native architectural context of the heart. To minimize technical variability, tissue sections from both a WT and an ST mouse were co-processed on the same. The analysis was performed using Stomics' Stereo-seq technology. The workflow included sample preparation, staining and imaging of the tissue section, followed by tissue permeabilization for mRNA capture and cDNA synthesis. Libraries were prepared using the Stereo-seq Library Preparation Kit (V1.3) and sequenced on a DNBseq-T7 platform (2x75 bp paired-end reads) to a depth of up to 3 billion reads.

To investigate the immunological safety of long-term transgene expression, we profiled the distribution and abundance of major immune cell populations, including T cells, B cells, NK cells, macrophages, and neutrophils. Spatial mapping demonstrated that all identified immune cells were sparsely and evenly distributed throughout the cardiac tissue in both samples ([Fig. R1](#), left side sample is WT and right-side sample is ST). Crucially, we observed no evidence of focal immune cell infiltration or clustering inflammatory in the ST heart.

In conclusion, these spatial transcriptomic results provide strong evidence for the long-term safety and biocompatibility of our sonogenetic approach at the molecular level. The data demonstrate that sustained

expression of the MscL G22S transgene for 6 months does not alter the fundamental cellular architecture of the heart or elicit a significant, widespread immune response.

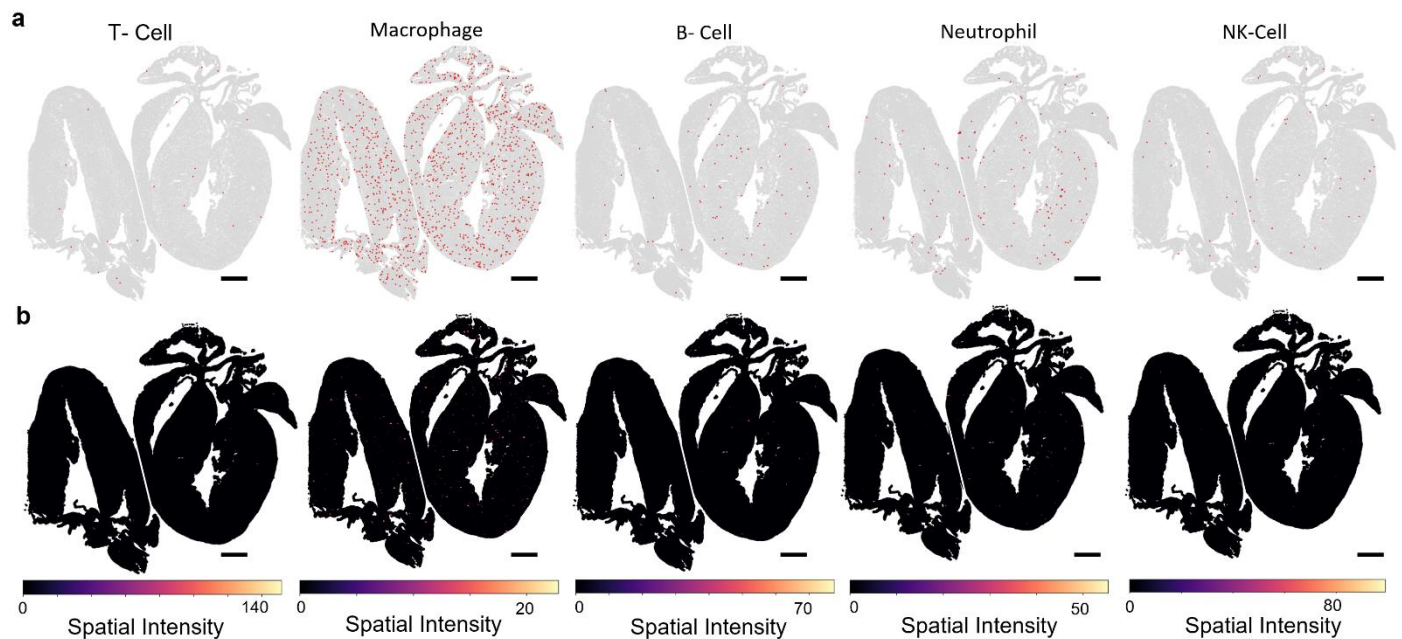


Fig. R1 (add as Supplementary Fig 27) | Spatial transcriptomic analysis of inflammatory cells in wild-type and sonogenetically transduced cardiac tissues. a, Spatial plots indicating the locations of individual cells for five major immune cell types: T-Cells, Macrophages, B-Cells, Neutrophils, and Natural 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.

3.2.2 Proteomic Analysis of Long-Term Transduction Stability

We also performed a comprehensive proteomic analysis, which provides a more direct assessment of cellular phenotype compared with transcriptomics. Whereas RNA levels indicate genetic intent, protein levels represent the functional molecules that execute cellular processes. A total of nine rat heart tissue samples ($n = 3$ per group: ST 6-month, ST 8-month, and age-matched WT controls) were processed for protein extraction, followed by enzymatic digestion with Trypsin/Lys-C. The resulting peptides were analyzed by LC-MS/MS, and the raw data were processed using Thermo Proteome Discoverer 2.5 against the *Rattus norvegicus* protein database.

First, we assessed the overall stability of the cardiac proteome. The correlation plots (**Fig. R2a-c**) compared the average protein expression levels between ST and WT hearts at 2, 6, and 8 months. Across all time points, the correlation coefficient was exceptionally high ($R \geq 0.99$), indicating that the global protein landscape was virtually identical between the two groups. This remarkable consistency provides strong evidence that long-term expression of the MscL G22S transgene does not cause widespread off-target disruption of the cardiac proteome.

To provide a detailed safety assessment, we analyzed proteins that were differentially expressed at the 6- and 8-month time points. The volcano plots (**Fig. R2d, e**) showed that nearly all cardiac proteins remained unchanged, indicating a highly specific and targeted effect of the transgene. The few proteins that showed statistically significant changes did not indicate a pathological response; rather, they reflected a logical and benign adaptation of the cellular machinery to the presence of a new functional ion channel.

- **Analysis of Upregulated Proteins:** The upregulated proteins were primarily involved in cellular structure, membrane trafficking, and metabolism—functions directly related to producing and managing a new membrane protein.

Structural and Cytoskeletal Components: We observed a modest increase in proteins such as Col1a1 (Collagen Type I Alpha 1), Myh9 (Non-muscle Myosin IIA), and Myo18a (Myosin XVIIIa). Although Col1a1 upregulation can be a marker for fibrosis, this was an isolated

finding. There was no corresponding upregulation of a broader fibrotic protein network, and, importantly, H&E staining of the cardiac tissue showed no evidence of fibrosis or adverse tissue remodeling. The changes in myosin motors likely represent an adaptive cytoskeletal response to support the trafficking and membrane integration of the new MscL G22S protein.

- **Vesicle Trafficking and Membrane Organization:** A notable group of upregulated proteins—including Cavin3, Cltc (Clathrin), Copb2 (Coatomer), and the syntaxins Stx4 and Stx12—were core components of the cellular machinery for vesicle formation, transport, and membrane fusion. Their upregulation represents a direct and logical consequence of the increased demand on the cell to synthesize, process, fold, and transport the MscL G22S channel from the endoplasmic reticulum to its final destination at the cell surface. This reflects a normal and productive cellular process rather than a stress response.
- **Metabolic and Processing Enzymes:** Proteins such as Lgmn (Legumain, a lysosomal protease) and Cyb5r3 (a reductase involved in fatty acid metabolism) showed minor upregulation, likely reflecting increased protein turnover and modest metabolic adjustments required to support the sustained expression of the transgene.
- **Analysis of Downregulated Proteins:** The few consistently downregulated proteins were not associated with core cardiac health pathways, and their minor changes did not indicate adverse cellular events.
 - The most consistently downregulated protein, Lgals5 (Galectin-5), is not typically expressed at high levels in the heart, and its role in cardiac pathology is not well-defined. Therefore, its downregulation is of unknown significance and is not linked to any known adverse outcome.
 - Other downregulated proteins such as Krt8/18 (Keratins)—intermediate filaments not prominent in cardiomyocytes—or proteins involved in specific signaling pathways, including Pnck and Synj2bp, represented minor and isolated regulatory shifts. Importantly, these changes did not converge on any known pathways of apoptosis, inflammation, or cardiac failure.

A detailed examination of all significantly altered proteins revealed a consistent pattern: the changes were minor, typically related to the expected task of producing a new protein, and did not trigger any coordinated pathological programs.

Next, we analyzed the biological pathways associated with differentially expressed proteins ([Fig. R2f, g](#)). The results indicated that these cellular changes represented a logical and benign adaptation to the presence of a new functional ion channel, rather than a sign of stress or pathology.

- **Expected Functional Pathways:** Many of the most significantly enriched terms were directly related to ion channel function, including “regulation of Ca²⁺ ion transport,” “Ca²⁺ ion homeostasis,” “membrane depolarization,” and “excitatory postsynaptic potential.” The enrichment of these pathways provides compelling evidence that the MscL G22S channel was functionally integrated and active within the cardiomyocyte membrane, influencing ion flux as intended.
- **Protein Handling Pathways:** Expected terms included “exocytosis,” “protein secretion,” and “vesicle-mediated transport.” The cell actively produced, transported, and inserted the new MscL G22S protein into the cell membrane, and these pathways reflected normal cellular activity.
- **Absence of Pathological Pathways:** Importantly, the list of enriched pathways is notable for what was absent. There was no significant enrichment of pathways related to “inflammatory response,” “apoptotic process,” “cell death,” “response to injury,” or “fibrosis.”

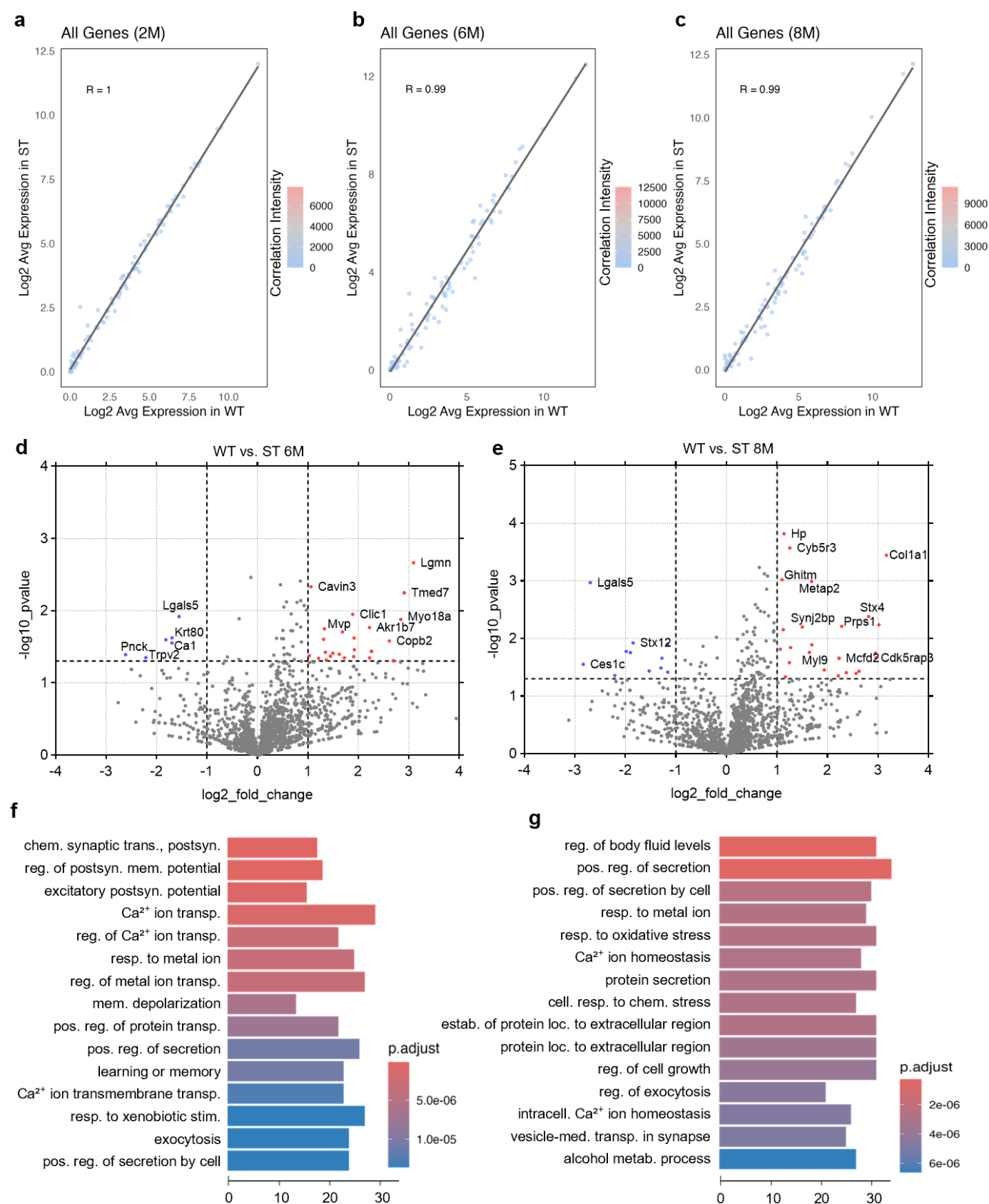


Fig. R2 (add as Supplementary Fig. 28) | Proteomic analysis confirms long-term biological safety of MscL G22S expression in cardiomyocytes. **a-c**, Correlation plots of the average protein expression levels (Log2) between ST and WT rat heart samples at 2 months (a), 6 months (b), and 8 months (c) post-transduction, showing high consistency ($R \geq 0.99$). Each dot represents a single protein, and the color gradient indicates the correlation intensity. **d-e**, Volcano plots of differentially expressed proteins in ST and WT mice hearts at 6 months (d) and 8 months (e). **f-g**, Bar plots showing the top enriched biological process pathways identified from the differentially expressed proteins at 6 months (f) and 8 months (g). The x-axis represents the statistical significance of the enrichment ($-\log_{10}$ adjusted p-value). $n=3$ for each condition.

3.2.3 Assessment of Long-Term Cardiac Function

We next assessed whether chronic MscL G22S expression had adverse effects on cardiac function at the whole-organism level, particularly under conditions of elevated physiological demand. A critical safety benchmark is the ability of the heart to perform normally during physical exercise.

To this end, we conducted a treadmill endurance test, a standard method for assessing cardiovascular performance (**Fig. R3**). WT and ST rats at 2, 6, and 8 months post-transduction were subjected to a continuous 45-minute run. During exercise, we noninvasively monitored key physiological parameters in real time—including heart rate, blood oxygen saturation (SpO₂), respiratory rate, and the P–P interval—using a collar-based sensor system.

This functional stress test revealed no discernible differences between ST groups and WT controls at any time point, supporting long-term functional safety:

- **Heart Rate and P-P Interval:** The continuous monitoring plots (**Fig. R3d, g**) and the quantitative analysis broken down into 9-minute intervals (**Fig. R3h, k**) showed that heart rate and corresponding P–P intervals in all ST groups were statistically indistinguishable from those in the WT group across the 45-minute exercise period. This indicates that the chronotropic response to exercise was not impaired.
- **Oxygen Saturation:** All animals, regardless of treatment or age, maintained stable and high blood oxygen saturation levels (typically >95%) throughout the endurance test (**Fig. R3e, i**). This indicates that cardiovascular and respiratory function in ST animals was sufficient to meet the metabolic demands of strenuous exercise, with no evidence of compromised cardiac output.
- **Respiratory Rate:** Respiratory rate patterns were comparable across groups, reflecting a normal physiological response to exertion with no significant differences (**Fig. R3f, j**).

In conclusion, the comparable performance of sonogenetically treated rats and wild-type controls under sustained cardiovascular workload indicates that chronic MscL G22S expression does not compromise the heart's intrinsic ability to adapt to physiological stress.

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Fig. R3 (add as 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, $p > 0.05$; $n = 5$ 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.

1.2 Exploration of Potential Cardiac Remodeling and Fibrosis Induced by Continuous US Pacing

To directly address the need for chronic safety data, we performed additional long-term stimulation experiments. In a new group of ST rats, we applied intermittent sonogenetic pacing (80 minutes per day) for two weeks. At the end of this chronic pacing regimen, we assessed cardiac health using ECG and histological analyses.

As shown in the new figure, the ECG trace from the final day of stimulation demonstrated that sonogenetic pacing was robust and effective, with no evidence of induced arrhythmia or conduction abnormalities. Following each stimulation session, the heart rate returned to a stable sinus rhythm.

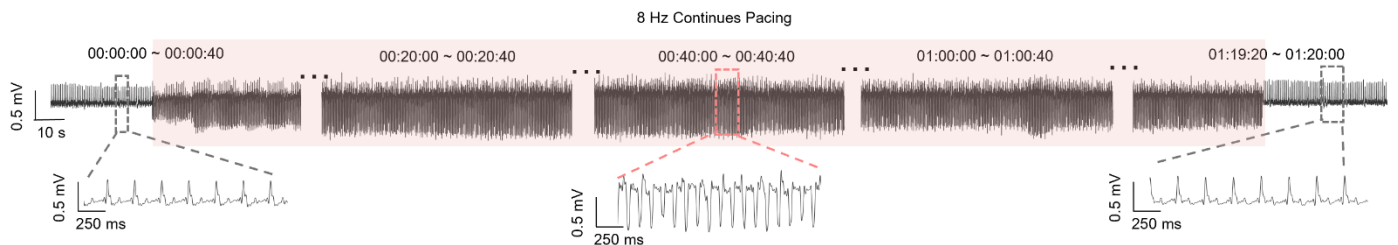


Fig. R4 | Long-term sustained cardiac pacing at 8 Hz. A representative electrocardiogram (ECG) trace from a rat undergoing continuous 8 Hz cardiac pacing for 1 hour and 20 minutes. The pink shaded area indicates the full duration of ultrasound stimulation. Magnified insets show the successful cardiac capture at the onset of stimulation (left), the stable paced rhythm at the 40-minute mark (middle), and the immediate return to sinus rhythm upon cessation of stimulation (right).

Histological analysis of hearts harvested after the two-week chronic pacing protocol revealed no evidence of significant tissue changes or cardiomyocyte damage (see attached [Fig. R5](#)).

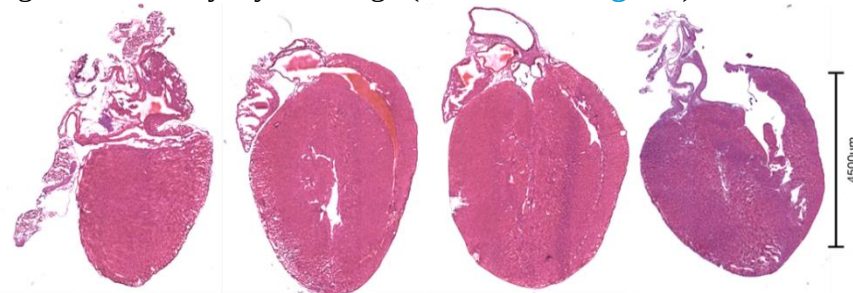


Fig. R5 | Histological analysis of cardiac tissue sections. Representative images of four mice heart cross-sections stained with Hematoxylin and Eosin (H&E). The staining illustrates the overall tissue morphology and cellular architecture. The scale bar represents 4500 μm . (n=4).

This new data provides direct evidence that long-term, repeated application of sonogenetic pacing is well-tolerated and does not cause cumulative cardiac damage or electrophysiological instability. These findings support the hypothesis that a non-contact stimulation method like ultrasound may avoid common triggers for fibrosis, such as the chronic mechanical irritation and foreign body response associated with implanted electrodes and leads[1, 2]. Our noninvasive approach avoids this direct physical interaction, potentially reducing the risk of such adverse remodeling. While these results are promising, we agree that dedicated long-term continuous pacing studies are the definitive next step and a key focus of our future work. This evidence significantly strengthens the case for the safety and translational potential of our NUP system for chronic cardiac rhythm management.

1.3 Evaluation of Ion Channel Protein Expression in Long-Term Sonogenetics Models

In response to the reviewer's valuable suggestion regarding the potential changes in the expression of other ion channel genes, we performed a comprehensive evaluation using both quantitative proteomic analysis and transcriptomic validation to assess the long-term molecular safety of the sonogenetics model.

1.3.1 Bulk RNA Sequencing Analysis of Heart Related Ion Channel Genes

To assess the long-term impact of MscL G22S expression on the heart's essential electrophysiological machinery, we performed bulk RNA sequencing to create a comprehensive transcriptomic profile of ion

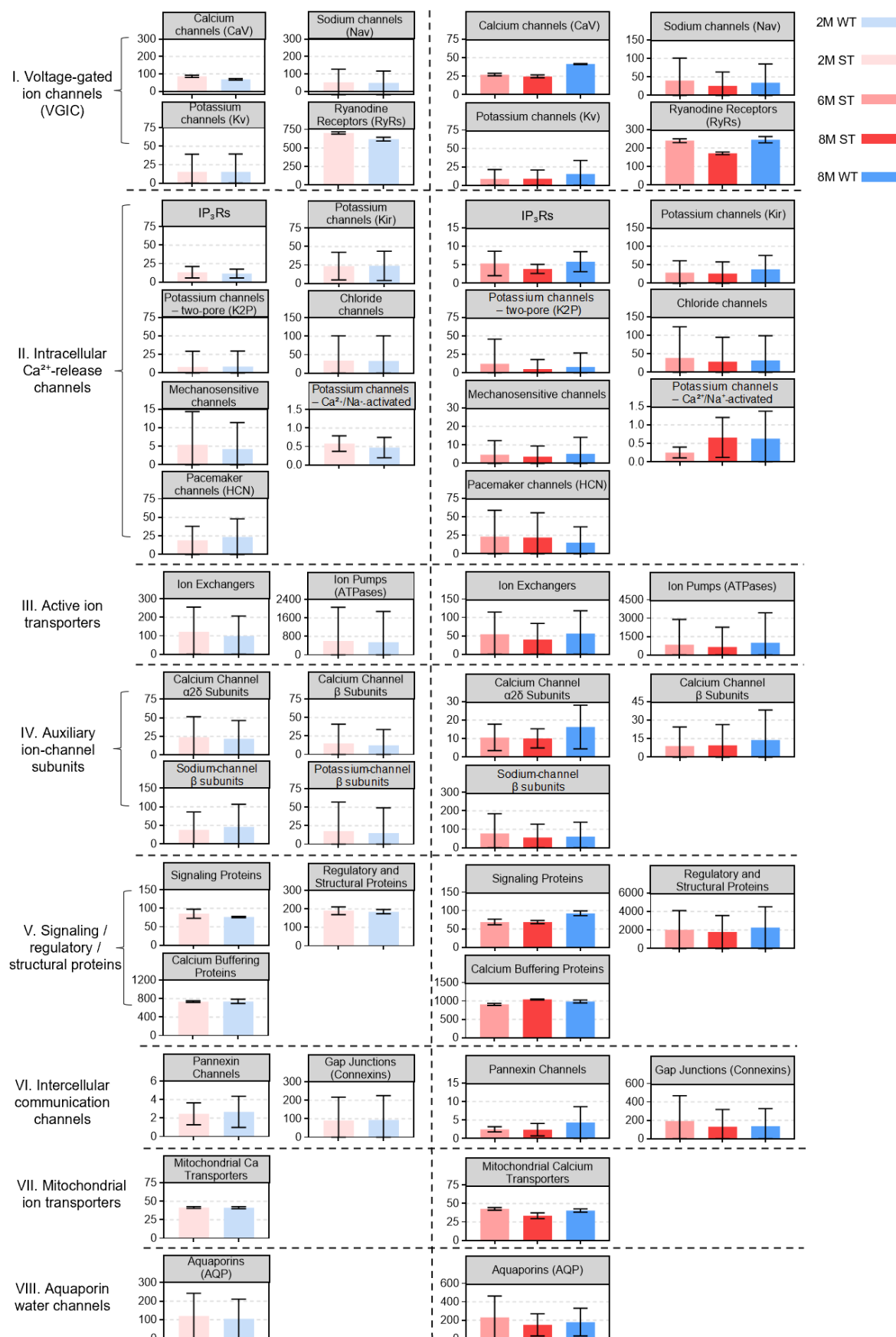


Fig. R6 (add as Supplementary Fig. 24) | Transcriptional profiling of cardiac ion channels and related genes confirms long-term safety of MscL G22S expression. Bulk RNA sequencing quantitative analysis of gene expression levels for a comprehensive panel of ion channels and related proteins in heart tissue from WT and ST rat. The bar charts are organized into eight functional categories (I-VIII), encompassing voltage-gated ion channels, intracellular Ca²⁺ release channels, active ion transporters, auxiliary subunits, signaling proteins, intercellular communication channels, mitochondrial transporters, and aquaporins. The left column of charts compares expression levels at 2 months post-transduction (2M WT vs. 2M ST). The right column compares expression at 8 months (8M WT vs. 8M ST), and the 6-month ST group (6M ST) to show longitudinal expression in treated animals. Each bar represents the mean relative expression level for a specific gene family, with error bars indicating the standard deviation. n=3 for each condition.

channel genes. Expanding on our initial analysis, we profiled ST rat at 6 and 8 months post-transduction, using age-matched WT rat as controls (n=3 biological replicates per group at each time point). The sequencing and analysis were conducted using an established methodology consistent with our previous analysis (sample sequencing by Azenta, MA, USA, to a depth of 40M paired-end reads). The results of this extensive longitudinal analysis revealed a remarkable degree of transcriptional stability across the cardiac ion channel landscape.

As shown in [Fig. R6](#), we quantified the expression of all major ion channel families and their associated proteins. Across nearly all categories—including voltage-gated channels (NaV, CaV, Kv) responsible for action potential generation, intracellular Ca²⁺ release channels (RyRs) essential for excitation–contraction coupling, and connexins that mediate intercellular electrical communication—expression levels showed no statistically significant differences between the ST and WT groups, even at 8 months post-transduction. This transcriptional stability indicates that chronic expression of MscL G22S does not induce compensatory remodeling or dysregulation of the heart’s endogenous electrophysiological machinery, thereby supporting the long-term transcript-level safety of the sonogenetic approach.

1.3.2 Spatial Transcriptomic Analysis of Cardiac Ion Channel Genes

We performed spatial transcriptomic analysis to evaluate the long-term effects of MscL G22S expression on cardiac ion channels, including potassium, sodium, endogenous mechanosensitive channels, and other related families. Spatial transcriptomics offers an unbiased, high-resolution approach to assess single-cell gene expression within the intact architectural context of the heart. Cardiac tissue from ST mice at 6 months post-transduction was analyzed alongside age-matched WT controls. To minimize technical variability, tissue sections from both WT and ST hearts were co-processed on the same slide (sample placement shown in [Fig. R7a](#)).

We first confirmed the identity of each tissue section by mapping MscL G22S expression. As shown in [Fig. R7b](#), a robust signal was detected across the right-sided heart sample, confirming it as the ST group, whereas the left-sided sample showed minimal expression, consistent with WT controls. We then performed cell-type annotation, which delineated the primary cardiac populations, including atrial cardiomyocytes (aCMs), ventricular cardiomyocytes (vCMs), terminal cardiomyocytes, and Purkinje cells ([Fig. R7c](#)).

Furthermore, the comprehensive resolution of spatial transcriptomics allowed us to examine the major ion channel families in detail. As shown in [Fig. R7d-h](#), there were no significant differences in the expression patterns of calcium, potassium, sodium, endogenous mechanosensitive, or other channel families between ST and WT hearts.

These findings indicate that long-term overexpression of MscL G22S does not significantly alter the transcriptional profile of essential Ca²⁺-handling components and that the broader cardiac ion channel transcriptome remains stable.

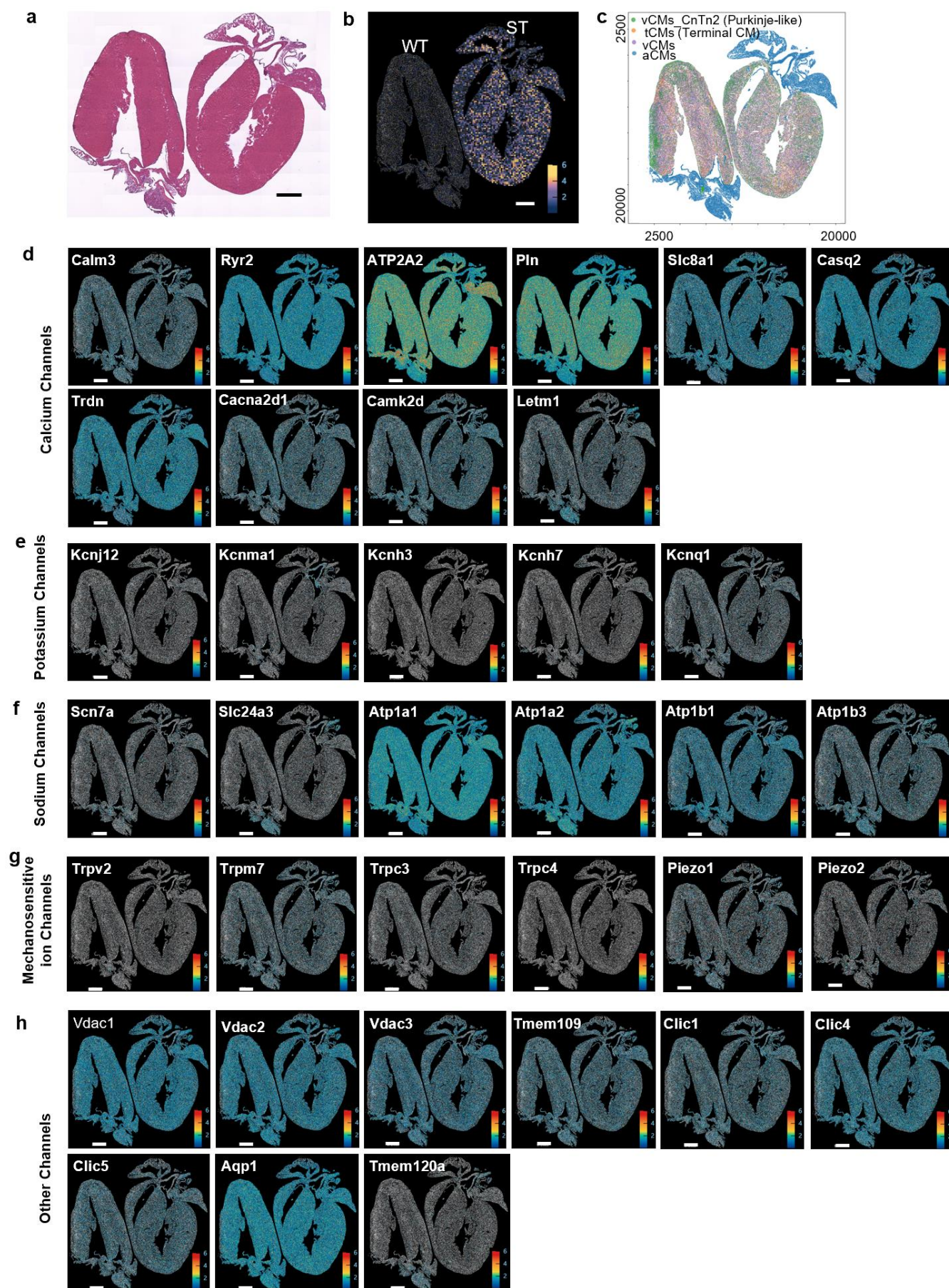


Fig. R7 (add as Supplementary Fig. 26) | Comprehensive spatial transcriptomic analysis of key ion channel genes in wild-type and sonogenetically transduced hearts. **a**, H&E staining of adjacent wild-type (WT, left) and sonogenetically transduced (ST, right) cardiac tissue sections. **b**, Spatial plot 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.

1.3.3 LC-MS/MS Proteomic Profiling across Ion Channel Families

We also conducted high-resolution LC-MS/MS proteomic analysis on heart tissues collected from three experimental groups: ST rats at 6 months, ST rats at 8 months, and age-matched WT controls (n = 3 per group). To ensure a thorough assessment, we expanded our analysis beyond calcium-handling proteins to include all major ion channel classes detected in the dataset:

- Calcium channels: RyR2, SERCA2a, PLB, Calm3, TRDN, Casq2, Slc8a1, etc.
- Potassium channels: Kcnj12, Kcnma1, Kcnh3, Kcnh6, Kcnh7, Kcnq1
- Sodium channels: Scn1a, Scn7a, Nalcn, Slc24a3, Atp1a1, Atp1a2, Atp1b1, Atp1b3
- Mechanosensitive ion channels: Trpv2, Trpm7, Trpc2, Trpc3, Trpc4
- Other ion-related proteins: Vdac1–3, Tmem109, Clic1/4/5, Aqp1, Tmem120a

Normalized relative abundances were calculated and summarized in [Table 1](#), with corresponding T-test statistics and q-values across comparisons (ST6 vs WT, ST8 vs WT, ST8 vs ST6). Protein expression heatmaps are shown in [Fig. R8](#).

Importantly, most of ion channels exhibited consistent expression profiles across all groups, with no statistically significant differences ($q > 0.05$). Although a few proteins (e.g., Nalcn, Kcnh3, Tmem120a) were weakly detected in select ST samples but absent in WT, these observations were rare, of very low abundance, and not statistically significant. As expected from AAV-mediated transgene delivery, the only protein showing significant differential expression was MscL G22S ($p < 0.01$).

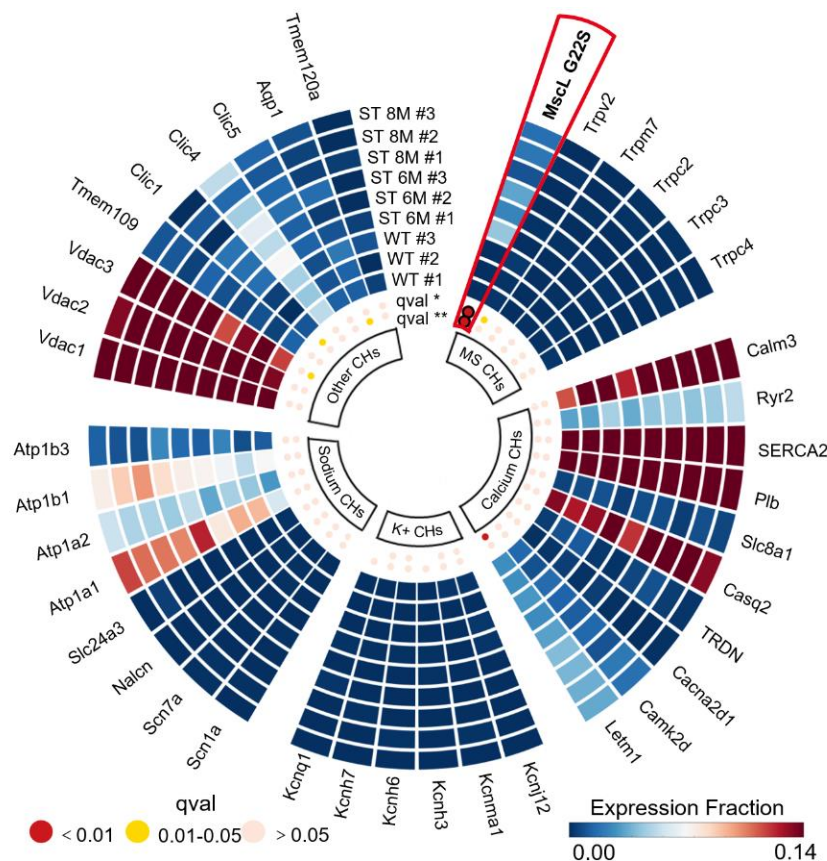


Fig. R8 (add as Supplementary Fig. 29) | Proteomic Profiling of the Cardiac Ion Channel Landscape Following Sonogenetic Transduction. Proteomic analysis of major ion channel families in heart tissue from WT, ST 6M, and ST 8M rat. The plot is organized by ion channel class: Mechanosensitive (MS), Calcium (Ca^{2+}), Potassium (K^{+}), Sodium (Na^{+}), and other channels. Each concentric ring corresponds to a specific protein as labeled, and each radial segment represents an individual biological replicate (n=3 for each ST and WT condition). The color intensity of each cell indicates the normalized expression fraction of the protein within that sample, with red representing higher expression and blue representing lower expression. The central ring of dots displays the statistical significance (q-value) for the comparison between ST and WT groups, where gray indicates no significant difference ($q > 0.05$).

Table 1 (add as Supplementary Table 4) | Proteomic analysis of all ion channels in long-term ST hearts.

	Gene Symbol	6 months ST.# PSMs normalized relative abundance (%)	8 months WT.# PSMs normalized relative abundance (%)	WT.# PSMs normalized relative abundance (%)	T-test Ratio (ST6/W T)	T-test Ratio (ST8/W T)	T-test Ratio (ST8/S T6)
	MscL G22S [3, 4]	0.0459/0.0254/0.0354	0.0100/0.0208/0.0187	0.0000/0.0000/0.0000	0.0038	0.0075	0.0485
Calcium Channels	Calm3 [5]	0.1327/0.1711/0.1545	0.1839/0.1530/0.1550	0.1228/0.2183/0.1558	0.6916	0.9584	0.4955
	Ryr2 [6]	0.0372/0.0454/0.0489	0.0451/0.0480/0.0554	0.0346/0.0346/0.0503	0.5648	0.1883	0.2878
	SERCA2 [7]	0.2181/0.2367/0.2463	0.2483/0.2541/0.2572	0.2315/0.2732/0.2615	0.2197	0.8710	0.0881
	PLB [8]	0.2074/0.2898/0.2330	0.4217/0.2466/0.2962	0.2179/0.2752/0.2901	0.6185	0.3458	0.2461
	Slc8a1 [9]	0.0046/0.0083/0.0087	0.0042/0.0088/0.0066	0.0053/0.0080/0.0095	0.8251	0.5690	0.7289
	Casq2 [10]	0.1676/0.1291/0.1614	0.1858/0.1552/0.1429	0.1435/0.1354/0.1405	0.3498	0.1724	0.6463
	TRDN [11]	0.0026/0.0016/0.0070	0.0099/0.0041/0.0018	0.0038/0.0094/0.0118	0.1893	0.4209	0.6258
	Cacna2d1 [12]	0.0016/0.0042/0.0011	0/0/0.0011	0.0008/0.0023/0.0042	0.9227	0.1235	0.1365
	Camk2d [13]	0.0152/0.0131/0.0159	0.0103/0.0214/0.0193	0.0164/0.0098/0.0174	0.9408	0.5853	0.5539
	Letm1 [14]	0.0280/0.0345/0.0426	0.04081/0.0386/0.0365	0.0283/0.0282/0.0314	0.2591	0.0047	0.4646
Potassium Channels	Kcnj12 [15]	0.0021/0/0	0/0/0	0.0020/0/0.0027	0.4575	0.1241	0.3739
	Kcnma1 [16]	0/0/0	0/0.0012/0	0.0022/0/0	0.3740	0.7098	0.3739
	Kcnh3 [17]	0.0008/0.0010/0	0/0/0	0/0/0	0.1224	N/A	0.1224
	Kcnh6 [18]	0.0009/0/0	0/0.0015/0	0/0/0	0.3739	0.3739	0.7705
	Kcnh7 [19]	0.0008/0/0	0.0011/0/0	0/0/0	0.3739	0.3739	0.7878
	Kcnq1 [20]	0.0013/0/0.0018	0/0/0	0/0/0	0.1248	N/A	0.1248
Sodium Channels	Scn1a [21]	0.0004/0.0011/0	0/0.0007/0	0/0/0	0.1872	0.3739	0.5091
	Scn7a [22]	0.0005/0/0	0/0.0017/0.00076405	0/0.0008/0.0007	0.3645	0.5780	0.2862
	Nalcn [23]	0/0/0	0/0/0	0.0005/0.0007/0	0.1314	0.1314	N/A
	Slc24a3 [24]	0/0/0	0/0.0046/0	0.0014/0/0	0.3739	0.5432	0.3739
	Atp1a1 [25]	0.0826/0.1326/0.1114	0.1152/0.1170/0.1255	0.0622/0.0992/0.1021	0.3389	0.0766	0.5222
	Atp1a2 [26]	0.0361/0.0557/0.0475	0.0511/0.0517/0.0591	0.0316/0.0459/0.0501	0.64897	0.13733	0.29461
	Atp1b1 [27]	0.0769/0.0801/0.0877	0.1082/0.0937/0.0802	0.0746/0.0556/0.0725	0.1092	0.0581	0.2239
	Atp1b3 [28]	0.0129/0.0166/0.0261	0.0098/0.0102/0.0138	0.0125/0.0093/0.0250	0.6601	0.4314	0.1535
Mechanosensitive Ion Channels	Trpv2 [29]	0/0.0015/0	0/0.0019/0	0.0023/0.0017/0.0031	0.0449	0.0786	0.8920
	Trpm7 [30]	0.0005/0.0006/0	0.0007/0/0	0/0/0	0.1224	0.3739	0.7112
	Trpc2 [31]	0/0.0020/0	0/0.0012/0.0011	0.0007/0/0.0010	0.9159	0.7137	0.8916
	Trpc3 [32]	0.0010/0.00135/0	0/0/0.0014	0.0010/0.0014/0	0.9448	0.6328	0.6651
	Trpc4 [31]	0/0/0	0.0014/0.0015/0	0.0018/0/0	0.3739	0.6624	0.1163
Other Channels	Vdac1 [33]	0.6131/0.3810/0.4882	0.4408/0.4834/0.4762	0.7085/0.4460/0.4348	0.7656	0.5244	0.7100
	Vdac2 [34]	0.1493/0.1533/0.1931	0.1626/0.1787/0.1436	0.1448/0.1500/0.1889	0.8487	0.9813	0.8459
	Vdac3 [35]	0.1239/0.1802/0.1799	0.1937/0.1813/0.2041	0.1263/0.1609/0.1436	0.4503	0.0145	0.1855
	Tmem109 [36]	0.0074/0.0095/0.0150	0.0056/0.0117/0.0106	0.0144/0.0054/0.0096	0.8103	0.8968	0.6742
	Clic1 [37]	0.0187/0.0192/0.0201	0/0.0118/0	0.0109/0/0.0048	0.0112	0.8118	0.0178
	Clic4 [38]	0.0746/0.0550/0.0671	0.0488/0.0169/0.0558	0.0551/0.0463/0.0505	0.0757	0.4539	0.1318
	Clic5 [39]	0.0143/0.0139/0.0193	0.0164/0.0114/0.0153	0.0139/0.0104/0.0092	0.1056	0.1991	0.5606
	Aqp1 [40]	0.0100/0.0043/0.0180	0.0051/0.0053/0.0095	0.0130/0.0145/0.0216	0.3090	0.0325	0.3834
	Tmem120a [41]	0.0052/0/0	0.0040/0/0	0.0051/0/0.0102	0.3842	0.3092	0.8593

In response to Comment 1, we have added new figures as well as table, to the Supplementary Materials. In addition, we have included a new paragraph in the main text (page 7, lines 20–37) to present the results of our long-term safety assessments.

“We further validated the long-term impact and safety of chronic MscL G22S expression. First, bulk RNA-seq analysis was performed on cardiac tissues collected at 6- and 8-months post-transduction to examine potential effects of the exogenous MscL G22S gene on endogenous ion channel expression. The results showed no significant alterations in the transcriptional landscape of key ion channel families (**Supplementary Fig. 24**). Next, immunofluorescence staining was conducted to evaluate representative calcium-handling

proteins, including RyR2, SERCA2a, and PLB, in order to assess potential long-term effects on intracellular Ca^{2+} cycling (**Supplementary Fig. 25**). We additionally employed spatial transcriptomics to map the expression and distribution of ion channel genes with spatial resolution, confirming that the qualitative and quantitative expression patterns of calcium, potassium, and sodium channels were comparable between ST and WT hearts (**Supplementary Fig. 26**). Moreover, immune cell profiling revealed no evidence of focal inflammatory infiltration or clustering in ST cardiac tissue (**Supplementary Fig. 27**). Finally, at the protein level, we performed global proteomic analysis at 2-, 6-, and 8-months post-transduction. These datasets confirmed long-term safety, showing strong correlations between the proteomic profiles of ST and WT hearts (Pearson's $R \geq 0.99$), with no significant perturbations in the expression of major ion channel families (**Supplementary Fig. 28 and 29**). Taken together, these multi-level assessments demonstrate that chronic sonogenetic transduction preserves transcriptional integrity, maintains proteomic stability, and does not elicit inflammatory or immune responses. Collectively, these findings establish the long-term safety and translational feasibility of sonogenetics for cardiac applications.”

References:

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Comment 2. Variability in Channel Expression:

In a clinical setting, MscL G22S expression levels are likely to vary both within an individual heart and between patients. Is there any available data on the distribution or variability of transgene expression following AAV-mediated delivery? Furthermore, the relationship between ion channel density in targeted cardiomyocytes and their responsiveness to ultrasound stimulation should be explored.

Response:

We thank the reviewer for this important question regarding the variability of transgene expression and its functional consequences. To address this, we first present new data on inter-animal variability in our vector's transduction efficiency, followed by a discussion of the relationship between expression levels and responsiveness to ultrasound.

2.1 Multi-Mouse Transfection Efficiency Test

To validate the stability and efficiency of our AAV9-cTnT vector, an additional group of six mice was administered a single 100 μ L dose of the vector at a titer of 1.0×10^{13} vg/mL via tail vein injection. After a two-month period to allow stable transgene expression, the mice were perfused, and their hearts were harvested and prepared for cryosectioning. Sections were subsequently stained with DAPI to visualize nuclei and analyzed by fluorescence imaging to quantify transduction efficiency.

Analysis of heart tissue sections revealed highly consistent and robust transduction across all animals (see attached [Fig. R9](#)). The transduction efficiency averaged **95.0%**, with a mean absolute deviation of only **2.0%** and a standard deviation of **2.37%**, indicating exceptional reliability and low inter-animal variability of the vector.

This high level of efficiency is consistent with previous reports for AAV2/9, which is known for strong cardiotropism and potent transduction of cardiomyocytes in mice [1-3]. Efficiencies exceeding 90% are considered successful outcomes for preclinical validation in rodent models. Furthermore, this result is highly encouraging for translation to larger animal models, where the therapeutic threshold for cardiac gene therapy may be substantially lower. For instance, studies in porcine models of heart disease have suggested that transduction of approximately 30-40% of cardiomyocytes can be sufficient to achieve a significant therapeutic effect [4-8].

High inter-subject variability is a common challenge in gene therapy, often necessitating the use of higher vector doses to ensure a therapeutic effect in all patients. This can increase the risk of dose-dependent toxicity, such as adverse immune responses. By establishing a vector that achieves robust and stable expression at a moderate dose, we mitigate this risk and increase the predictability of therapeutic outcomes. These results provide a strong foundation for future investigations into optimizing delivery routes and dose-finding for clinical applications.

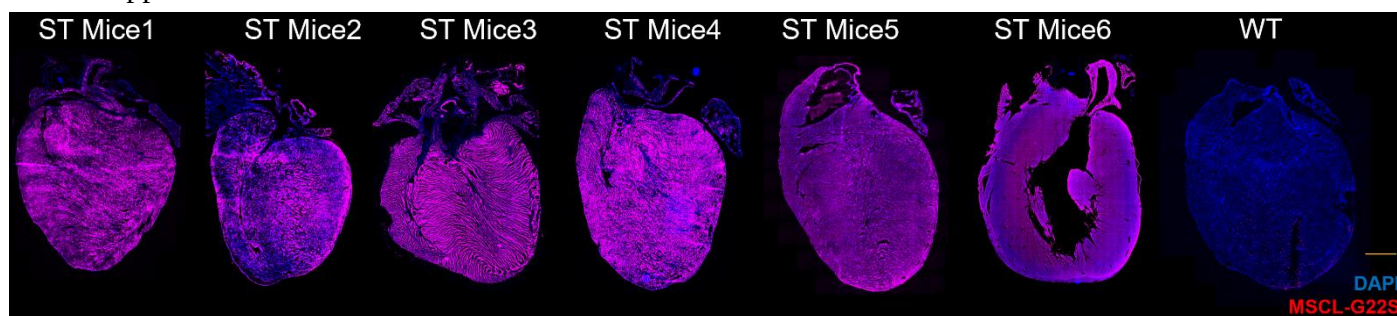


Fig. R9 | Consistent in vivo transduction efficiency of MscL G22S AAV in individual mice | The bar chart displays the percentage of MscL G22S-positive cardiomyocytes in the hearts of six individual mice (1-6) and the group average. Transduction efficiency was quantified from 20 $\times$ magnification immunofluorescence images of heart sections. Scale bar represents 1mm.

2.2 Multi Density of Titers Expression Evaluation:

To determine how expression density governs sonogenetic responsiveness, we conducted an in-vivo dose response study of AAV2/9-cTnT-MscL G22S-mCherry in mouse hearts using five titers (0.20, 0.40, 0.75,

1.00, and 1.50×10^{12} vg per mouse; see **Fig. R10**). The figure panels are accompanied by bar plots showing the MscL G22S expression area and mean fluorescence intensity across doses, demonstrating a dose-dependent increase that plateaus at higher titers.

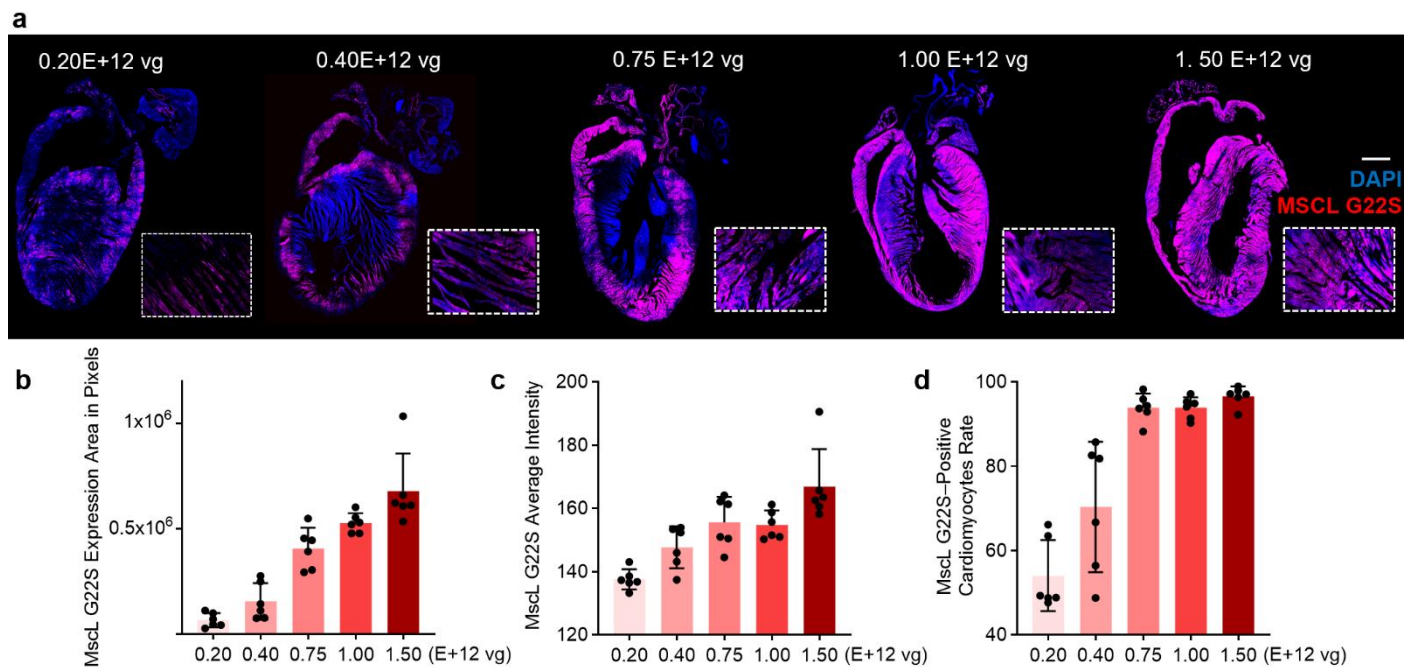


Fig. R10 (add as Supplementary Fig. 13) | Dose-dependent transduction efficiency of MscL G22S viral vector in the mouse heart | a, Representative whole-heart sections with higher-magnification insets (DAPI nuclei, blue; MscL-G22S/mCherry, magenta) following systemic delivery at 0.20, 0.40, 0.75, 1.00, and 1.50×10^{12} vg per mouse. **b-d**, Quantification across $n = 6$ fields of view per group (images acquired at 20 $\times$). **b**, MscL G22S positive area (pixels $\times 10^6$); **c**, mean fluorescence intensity of the MscL G22S signal; **d**, percentage of MscL G22S positive cardiomyocytes. Bars show mean $\pm$ SEM; dots represent individual fields of view. Scale bar represents 1mm. $n = 6$ for each dose.

For cell-level quantification, total nuclei were identified by DAPI. A cell was scored mCherry-positive when MscL G22S signal (magenta) was detected within the nuclear ROI or its immediately adjacent perinuclear region. The transduction ratio for each field was therefore calculated as: (mCherry-positive cells / total DAPI-positive cells) $\times$ 100%. This procedure allowed us to quantify how viral dose affects the fraction of transduced cardiomyocytes. Quantitative histology revealed a monotonic, near-linear rise in cardiomyocyte transduction at lower doses, with clear signs of saturation beyond $\sim 1.0 \times 10^{12}$ vg. Across cohorts, the fraction of MscL G22S positive cardiomyocytes increased from $\sim 48\%$ at the lowest dose to $>90\%$ at $\geq 1.0 \times 10^{12}$ vg, with only a marginal additional gain at 1.50×10^{12} vg (**Fig. R10d**). Mean fluorescence intensity followed the same trend (**Fig. R10c**). These data identify approximately 0.75×10^{12} vg as a practical inflection point, whereas 1.0×10^{12} vg consistently achieves $>90\%$ cardiomyocyte transduction, which expected to provide robust ultrasound responsiveness while avoiding unnecessary vector load.

Prior optogenetic cardiac pacing studies—also based on AAV-mediated delivery of ion channels to cardiomyocytes, provide quantitative benchmarks linking expression density to functional capture. In adult mice, systemic AAV-ChR2 enabled in-vivo ventricular optical capture in 74% of hearts when on average $\sim 58\%$ of cardiomyocytes were opsin-positive, and the functional threshold for reliable capture in the illuminated region was estimated at $\sim 35\text{--}40\%$ opsin-expressing myocytes[9]. In adult rats, systemic AAV9 at 0.5×10^{12} vp produced robust, predominantly cardiomyocyte-specific expression within 4 weeks, supporting the feasibility of whole-heart modification at clinically tractable doses[10]. In neonatal mice, AAV9 can achieve $96 \pm 2\%$ cardiomyocyte transduction at 3.15×10^{10} vg/mouse (cTnT promoter), underscoring strong cardiac tropism and the age-dependence of transduction efficiency[1]. More broadly, typical systemic cardiotropic doses in mice reported in recent gene-delivery studies are on the order of $3 \times 10^{11} \sim 5 \times 10^{12}$ vg/mouse[11].

In our study, the working titers are higher than those commonly used in the optogenetic literature (mice: 1×10^{12} vg; rats: 3×10^{12} vg), and they yielded very strong expression; with appropriate ultrasound parameters

(**Fig. 3g**), we achieved high pacing success. Consistent with the dose–response, reducing the dose to $\sim 0.75 \times 10^{12}$ vg did not markedly diminish the transduction fraction, whereas $\sim 0.20\text{--}0.25 \times 10^{12}$ vg lowered the transduction ratio to below $\sim 50\%$. We therefore recommend using $\geq 0.75 \times 10^{12}$ vg to maximize the likelihood of successful pacing. Increasing the dose beyond 1.0×10^{12} vg appears to yield minimal additional benefit in transduction efficiency.

In response to **Comment 2**, we have added **Fig. R10** (add as **Supplementary Fig. 13**) to the Supplementary Materials (page 16) and cited this figure in the main text (page 4, line 27). In addition, it has also been referenced in the Methods section of the main text (page 9, line 31).

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Comment 3. Choice of AAV Serotype and Off-Target Risks:

The study employs AAV2-cTNT-MscL G22S for selective cardiac expression in mice, but the rationale for using the non-cardiotropic AAV2 serotype remains unclear. For potential clinical translation, tissue-specific targeting is critical. Future strategies could include incorporating cardiac-specific enhancers or using alternative promoters to enhance cardiac specificity.

Additionally, if the channel is expressed in non-cardiac tissues such as skeletal muscle or neurons, there may be a risk of abnormal contractions upon ultrasound stimulation. Do the authors have additional data on off-target expression levels or activation thresholds in non-cardiac tissues?

Response:

3.1 Specific Rationale for the AAV2/9 Serotype and the cTnT Promoter

3.1.1 The selection of a pseudotyped AAV2/9 vector

We thank the reviewer for raising this question. First, we would like to clarify that all vectors used in this study were AAV2/9, and we have corrected all related descriptions in both the Methods and the main manuscript. The AAV2/9 pseudotype [1, 2] uses the AAV9 capsid, which provides strong cardiac tropism and efficient systemic transduction of cardiomyocytes, and the AAV2 genome backbone, which enables stable recombinant vector construction and high-titer packaging. This combination has become standard in cardiac research because it ensures both robust myocardial expression and reproducible vector production for translational studies.

We have summarized the comparison in detail in Table 2.

Table 2 (add as 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	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)

3.1.2 The selection of a cTnT Promoter

The cTnT protein is a fundamental and exclusive component of the cardiac sarcomere's thin filament, making its endogenous promoter one of the most specific regulators of cardiomyocyte gene expression known[14-16]. Our choice was supported by a robust body of literature[9, 17-21] showing that even minimal promoter fragments can drive potent and highly specific transgene expression at both the whole-organ and single-cell levels.

Previous studies have shown that the cTnT promoter ensures transgene expression is overwhelmingly confined to the heart at the whole-organ level[9, 22, 23]. This exceptional specificity is not a passive trait but an active, mechanistic property rooted in transcriptional regulation. This mechanism stands in stark contrast to ubiquitous viral promoters, such as the widely used cytomegalovirus (CMV) promoter[9, 24]. In contrast, the activity of the cTnT promoter is governed by a “lock-and-key” system[18, 25]. Its DNA sequence contains specific cis-regulatory binding sites for a unique combination of cardiomyocyte-restricted transcription factors, primarily GATA4, Nkx2.5, and members of the MEF2 family[26]. These master regulatory proteins are abundant in cardiomyocytes but are absent or present only at negligible levels in other tissues such as the liver, skeletal muscle, and neurons[21, 27]. Consequently, the cTnT promoter remains transcriptionally silent in non-cardiac tissues, providing a powerful first layer of containment. Because of this reliability, the cTnT promoter has become a workhorse tool used extensively in cardiac gene therapy and validated in numerous

preclinical studies, spanning conditions from heart failure and post-infarction remodeling to genetic disorders such as catecholaminergic polymorphic ventricular tachycardia (CPVT), in models ranging from rodents to large animals such as pigs[9, 21, 28-30].

This specificity is further refined at the cellular level within the heart, which is a critical feature for both safety and efficacy. The heart is a heterogeneous organ, with cardiomyocytes constituting only a fraction of the total cell population[31-34]. The same "lock-and-key" transcriptional mechanism that confers organ-level specificity also ensures that within the heart, the cTnT promoter is active exclusively in cardiomyocytes. Other abundant cell types, such as cardiac fibroblasts, endothelial cells, and smooth muscle cells, do not express the necessary combination of GATA4/Nkx2.5/MEF2 transcription factors and thus cannot activate the promoter[17, 35, 36]. This cellular fidelity is indispensable for ensuring both safety and therapeutic efficacy. Expression of a therapeutic gene, such as an ion channel or a calcium-handling protein, in non-target cells like fibroblasts would be ineffective and could dangerously alter their signaling, proliferation, or contribution to fibrosis[37, 38]. Likewise, misexpression in vascular cells could compromise vascular tone and integrity. By restricting expression to the intended cell type, the cTnT promoter ensures that the therapeutic dose is concentrated where it is needed, thereby maximizing efficacy and preventing unintended pathology in neighboring cells[39-41].

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

We have integrated all these related discussions into the new [Supplementary Text 3](#).

Although the combination of the cTnT promoter and the AAV2/9 vector already provides strong cardiac specificity at the transcriptional level, we acknowledge the reviewer's point that further refinements could enhance translational safety. Future strategies, such as incorporating cardiac-specific enhancers or employing alternative promoters, may further improve targeting precision and reduce the risk of off-target expression. This represents an important direction that we plan to pursue in future work, building upon the current platform.

3.2 Evaluation of Off-Target Expression in Non-Cardiac Tissues

To address the potential risks of off-target expression and activation in non-cardiac tissues, we performed a new, multi-scale analysis of transgene biodistribution, which has been included in the revised manuscript.

First, our biodistribution data demonstrate minimal off-target expression. Ex vivo fluorescence imaging was performed on all major organs from ST rats 3 months post-transduction. As shown in the new [Fig. R11](#) (top panel), the mCherry reporter signal was overwhelmingly localized to the heart. Other major organs—including the liver, spleen, stomach, kidney, and lung—exhibited negligible fluorescence, confirming minimal systemic leakage and strong cardiac specificity. Furthermore, to specifically investigate skeletal muscle and gastrointestinal tissues, we conducted fluorescence microscopy on sections from the abdominal wall, back, chest, and leg muscles, as well as the intestine and stomach. As shown in representative images and quantitative analyses ([Fig. R11](#), bottom), mCherry expression in these non-cardiac tissues was minimal and statistically indistinguishable from the background levels of wild-type (WT) controls (n = 4 per group). Together, these findings demonstrate that our dual-targeting approach—combining the intrinsic cardiotropism

of the AAV2/9 vector with the stringent regulation of the cardiomyocyte-specific cTnT promoter—confers exceptionally high cardiac specificity. This strongly supports the overall safety profile of our system and indicates a very low likelihood of unintended ultrasound-induced activation in non-cardiac tissues.

Second, even if trace off-target expression were present, ultrasound stimulation itself provides an additional layer of safety control, since channel activation requires surpassing defined acoustic thresholds that are not reached under normal physiological conditions.

Third, future strategies could further enhance cardiac specificity, for example through the incorporation of additional cardiac-specific enhancers or alternative promoters. These refinements would further strengthen the therapeutic safety profile of the system.

In summary, these results collectively demonstrate that our system achieves highly specific cardiac expression with negligible off-target risks, and is further safeguarded by ultrasound activation thresholds and potential future refinements.

This figure has been added on page 18 of the Supplementary Materials and has been cited in the main text on page 4, line 34.

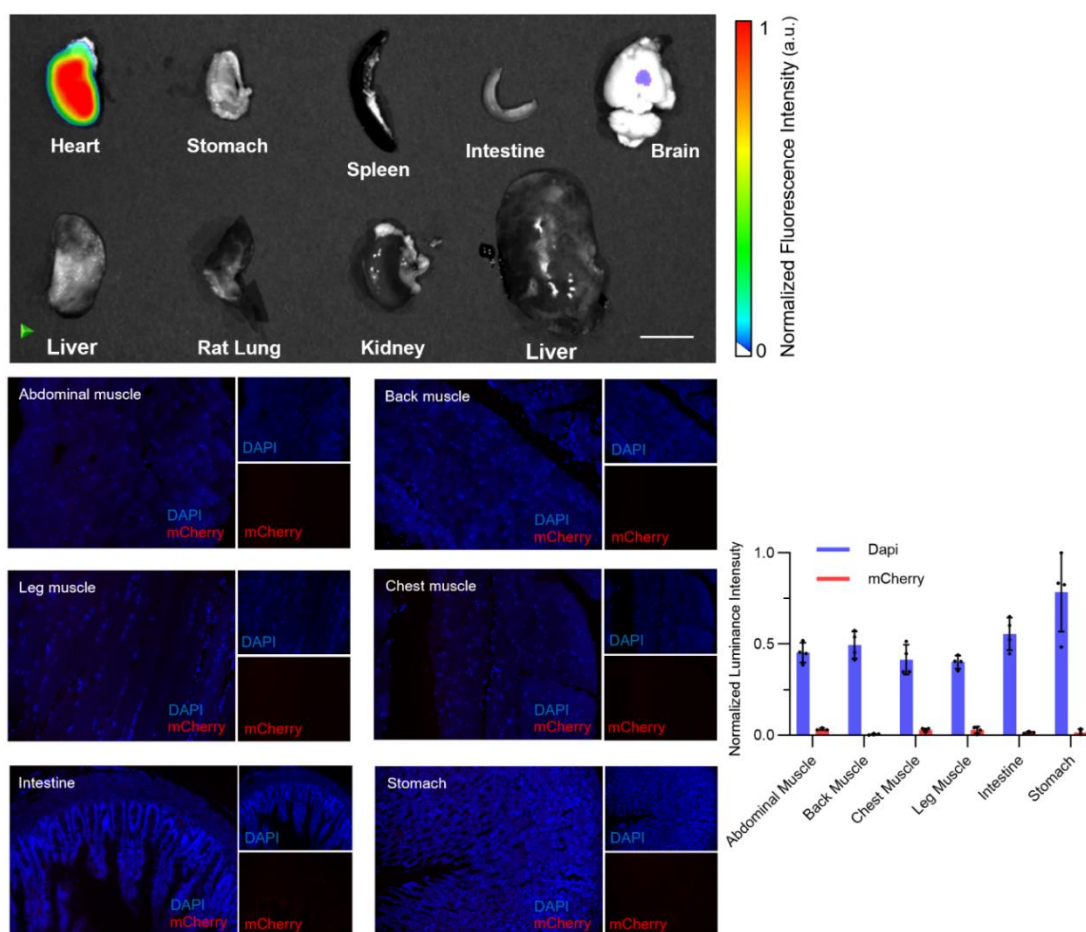


Fig. R11 (add as Supplementary Fig. 15) | Systemic and tissue-level biodistribution analysis of mCherry. **Top Panel**, representative *ex vivo* mCherry fluorescence imaging of major organs harvested from a ST rat 3 months post-transduction using IVIS. Scale bar, 10 mm. **Bottom Left Panels**, representative fluorescence microscopy images of various non-cardiac tissues, including abdominal, back, chest, and leg muscle, as well as intestine and stomach. For each tissue, insets show DAPI staining for cell nuclei (blue) and mCherry fluorescence for transgene expression (red). **Bottom Right Panel**, quantitative analysis of normalized luminance intensity for DAPI and mCherry signals across the different tissue types (n=4 per tissue).

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Comment 4. Unintentional Activation Scenarios:

Please provide an example scenario in which the ultrasound-triggered pacing system might be unintentionally activated, analogous to how conventional electrical pacemakers must be safeguarded against electromagnetic interference.

Response:

We thank the reviewer for this important question regarding the safety and specificity of our pacemaker. First, we would like to emphasize that the proposed sonogenetic pacemaker operates by using ultrasound to activate the MscL G22S channel for heart rate control, which is largely unaffected by external environmental factors and is highly unlikely to be triggered unintentionally. In contrast, electromagnetic waves can travel long distances in air and penetrate human tissues, making conventional electronic pacemakers susceptible to electromagnetic interference (EMI). However, ultrasound waves propagate poorly in air and do not readily transmit from air into biological tissues. Therefore, it is virtually impossible for an external ultrasound source to unintentionally trigger sonogenetic activation. The only conceivable scenario would be during a clinical ultrasound scan or therapeutic procedure that directly transmits ultrasound waves into the patient's body. However, such medical procedures are specialized and tightly controlled. Furthermore, effective activation requires a highly specific set of ultrasound parameters to be met simultaneously. This multi-layered specificity provides a robust safeguard against unintentional activation from external sources.

For an external acoustic source to induce unintentional activation, it would need to replicate the following highly specific parameters:

- **Specific Frequency Requirement:** Our system operates at 3 MHz, which aligns with reports in the literature identifying the optimal sensitivity range of the MscL G22S channel as approximately 2.2–3 MHz. Common environmental sounds occur in the low kHz range and therefore lack the frequency necessary for activation.
- **High Acoustic Pressure Threshold:** The sonogenetically modified cardiomyocytes require a focused acoustic pressure of approximately 0.5 MPa to trigger a stable response. This therapeutic pressure level is substantially higher than any physiological pressures generated during strenuous physical activity. This is supported by our in vivo and in vitro experiments, in which rats subjected to continuous treadmill running exhibited stable cardiac function without any evidence of unintended activation from physical exertion.
- **Specific Pulse Duration:** Effective cardiac capture requires a controlled pulse duration. Our data indicates that a robust response is typically achieved with pulse durations exceeding 30 ms. In contrast, typical ultrasound imaging pulses are on the order of 1 microsecond, far below the threshold required to activate sonogenetic ion channels.

Given these stringent requirements, accidental activation under real-world conditions—such as vigorous running, jumping, or even chest impacts—is highly improbable. The most plausible, though still highly remote, scenario for unintentional activation would stem not from an external source but from an internal system malfunction. Examples include faults in the stimulation hardware leading to uncontrolled output, or failures in the diagnostic algorithm resulting in pacing when not clinically indicated or at inappropriate frequencies.

This possibility highlights the critical importance of the system's internal safety mechanisms. We addressed this by implementing a multi-step verification protocol that must be completed before any stimulation is delivered. As detailed in our updated workflow ([Fig. 5a](#) and [Extended Data Fig. 8](#)) according to your **Comments 6**, the system first performs a critical left ventricular (LV) integrity check to confirm that the probe is correctly positioned and has not been displaced. Only after confirming correct placement does the system proceed to verify that the heart's condition meets the predefined criteria for pacing. Stimulation is delivered only after all steps in this verification sequence have been successfully completed. This rigorous, software-

controlled logic ensures safe and appropriate system operation, providing a robust safeguard against malfunction-induced activation.

Comment 5. Dyssynchrony and Multi-site Pacing:

This technology's ability to selectively pace specific cardiac regions is highly promising. However, even slight deviations in pacing site can induce ventricular dyssynchrony, potentially worsening cardiac function or precipitating heart failure. Are there plans to evaluate the risk of dyssynchrony in future studies? Additionally, please discuss the feasibility of multi-site pacing (e.g., simultaneous or staged atrioventricular [A-V] and left-right ventricular [LV-RV] pacing) from the standpoints of technological complexity and power consumption.

Response:

5.1 Plans to evaluate the risk of dyssynchrony in future studies

We thank the reviewer for raising this critical and clinically relevant point. We agree that the management of dyssynchrony is paramount for the clinical success of any advanced pacing technology. Our NUP system was specifically engineered as an integrated diagnostic and therapeutic platform to address this very challenge. Rather than being merely a risk to avoid, the detection and correction of dyssynchrony constitute a key application for which our platform is uniquely suited.

In the present study, we demonstrated this flexibility in both simulations and rodent experiments by alternating stimulation between the LV and RV (**Fig. 3h-i**), thereby supporting the feasibility of dynamic multi-site pacing. Furthermore, our integrated B-mode imaging and M-mode analysis are sufficiently sensitive to detect dyssynchronous activation. This demonstrates that our platform possesses the requisite spatial precision and diagnostic sensitivity to form the foundation of a comprehensive synchrony management system.

The AI-centric, closed-loop architecture of our system provides a direct pathway to not only prevent dyssynchrony but to actively diagnose and treat it in real time. Our immediate **future work** is focused on leveraging these inherent capabilities:

- **AI-Powered Dyssynchrony Detection:** We will leverage the NUP's real-time, four-chamber imaging to train machine-learning models for rapid, automated detection of asynchronous wall motion and inter-chamber timing abnormalities. This advances the system's diagnostic function beyond basic metrics toward sophisticated, real-time assessment of cardiac mechanics.
- **Closed-Loop Resynchronization:** Once dyssynchrony is detected, the AI will calculate the optimal timing and location for compensatory stimulation. Leveraging the NUP's ability to electronically steer the ultrasound beam to single or multiple sites, the system can autonomously deliver corrective pacing protocols to restore synchrony.
- **Immediate Therapeutic Verification:** A key advantage of our platform is the capacity to instantly verify the mechanical effects of corrective pacing via integrated ultrasound imaging, establishing a genuine "detect-treat-verify" theranostic loop. Our current 3–7 fps feedback rate already supports this capability, and planned hardware upgrades will further enhance responsiveness.
- **Technological Feasibility and Power Consumption:** Multi-site pacing is technologically straightforward for our phased-array system, as it only requires adjusting electronic phase delays, which adds minimal power overhead. Our current power budget (0.187 J per pulse group) and 8-hour battery life with a commercial power bank comfortably support dual-site pacing protocols, confirming the feasibility of this advanced therapeutic approach (**Comment 5.3.**).

In summary, the NUP system is designed to function as more than a pacemaker; it is a comprehensive, noninvasive platform for managing cardiac synchrony. Its unique capacity to image, diagnose with AI, and deliver corrective multi-site pacing within a closed loop positions it as a promising tool for personalized Cardiac Resynchronization Therapy (CRT)

5.2 The feasibility of multi-site pacing

To address the reviewer's request on multi-site pacing, we constructed a heterogeneous finite-element model of the human thorax in COMSOL using an open-source 3D anatomical dataset. This model incorporated the specific acoustic impedance, density, and attenuation parameters for all relevant tissues, including heart, blood, heterogeneous rib and lung layers, to accurately predict beam propagation generated by the 64-element, 3-MHz phased array coupled on the chest surface.

The simulations show that both simultaneous dual-focus and single-focus modes form compact, well-localized focus at the intended myocardial targets (**Fig. R12**). As expected from aperture sharing, the peak pressure and acoustic intensity at each focus in dual-site mode were modestly reduced relative to single-site delivery, yet focal integrity and spatial selectivity were preserved. This attenuation can be compensated by increasing in drive voltage. This adjustment preserves focal quality, minimizes crosstalk, and maintains the average duty factor close to that of single-site operation, thereby limiting incremental power and thermal load. Taken together, these results support the technical feasibility of dual-site pacing (A–V or LV–RV) with the same wearable phased array.

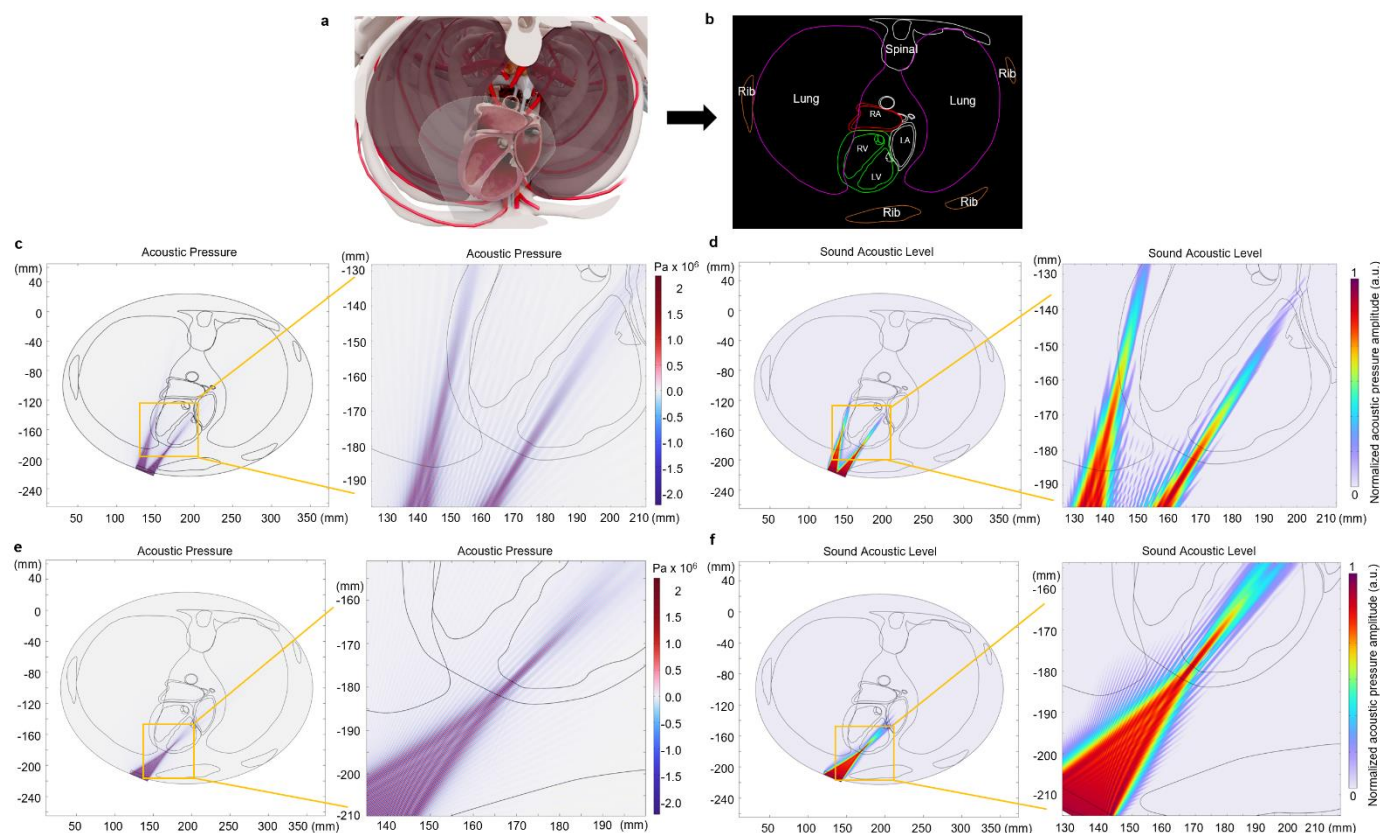


Fig. R12 (add as Supplementary Fig. 20) | Ultrasound multi-site pacing simulation in a human-thorax model. (a, b) A 3D thoracic rendering (a) and the corresponding axial segmentation (b) used for the finite-element simulations. **(c, d)** Simulation of a dual-site steering solution that places two foci 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).

This figure has been added to the Supplementary Materials on page 23 and has been described in the main text on page 6, lines 36–38.

“Simulations of the acoustic field within a human thoracic model confirmed that sufficient energy remained after tissue attenuation to enable stimulation at clinically relevant pacemaker sites, including single-site (**Fig. 5f**) and simultaneous multi-site pacing (**Supplementary Fig. 20**).”

5.3 The power consumption of multi-site pacing

To quantify the energy overhead of dual-site pacing, we modeled the driver load under the same acoustic requirement per focus (3 MHz center frequency, 3,000 cycles per pulse ≈ 1 ms, PRF = 1 Hz per site; 64-

channel array). The total hourly energy is the sum of a base system term (electronics, telemetry, control; approximately constant with the number of foci) and a **pulse term** (energy delivered by the array during acoustic bursts):

$$E_{\text{hour}} = E_{\text{base}} + E_{\text{pulse}}$$

Single Focus Stimulation Mode Pulse Output Power Consumption

Time	Pulse power Consumption(mWh)	Base Power Consumption(mWh)	Total(mWh)	Required Capacity for 3.7V battery (mAh)	Duration if use 3.7V 10000mAh battery	Duration if use 3.7V 20000mAh battery
1 H	187.2	3630	3817.2	1031.675676		
2 H	374.4	7260	7634.4	2063.351351		
4 H	748.8	14520	15268.8	4126.702703	9.69 H	19.38 H
8 H	1497.6	29040	30537.6	8253.405405		
12 H	2246.4	43560	45806.4	12380.10811		

With the above parameters we obtain:

- **Single focus:** $E_{\text{base}}=3,630$ mWh; $E_{\text{pulse}}=187.2$ mWh; $E_{\text{hour}}=3,817.2$ mWh
- **Dual focus (simultaneous):** due to aperture sharing/apodization, the E_{pulse} increases by **~50%** relative to single-focus for equal peak pressure at each focus ($187.2 \rightarrow 280.8$ mWh), while E_{base} is unchanged; thus $E_{\text{hour}}=3,910.8$ mWh.

Expressed as a total system overhead, dual-site pacing raises hourly energy by only 2.5%, because the base term dominates the budget. Consequently, with a 3.7 V, 10,000 mAh pack (≈ 37 Wh), the runtime decreases only modestly from 9.69 h (single-site) to 9.46 h (dual-site). For a 20,000 mAh pack, the runtime decreases from 19.38 h to 18.92 h.

From an acoustic standpoint, if a 30 Vpp excitation yields ~ 2 MPa at a single focus, achieving the same peak pressure at two simultaneous foci requires $\sim 50\%$ more array pulse energy. However, once base power is included, this corresponds to only $\sim 2\text{--}3\%$ additional total system energy. Interleaving the two targets in PRF (alternating pulses to LV and RV) maintains an average duty factor comparable to single-focus operation, preserves focal quality, and does not materially alter the energy budget relative to simultaneous delivery at the same per-site PRF. Dual-site pacing is energetically feasible on the wearable platform: meeting the same per-focus pressure costs $\sim 50\%$ more pulse energy but adds only $\sim 2.5\%$ to total hourly energy, resulting in a negligible reduction in battery life while enabling LV–RV (or A–V) multi-site stimulation.

Double Focus Same Time Stimulation Mode Pulse Output Power Consumption (Single Focus Mode Pulse Power Consumption*1.5)

Time	Pulse power Consumption(mWh)	Base Power Consumption(mWh)	Total(mWh)	Required Capacity for 3.7V battery (mAh)	Duration if use 3.7V 10000mAh	Duration if use 3.7V 20000mAh
1 H	280.8	3630	3910.8	1056.972973		
2 H	561.6	7260	7821.6	2113.945946		
4 H	1123.2	14520	15643.2	4227.891892	9.46 H	18.92 H
8 H	2246.4	29040	31286.4	8455.783784		
12 H	3369.6	43560	46929.6	12683.67568		

Rapid-switch (PRF-doubled) mode.

After completing stimulation at one site, there is another condition is that the array immediately switches to the second site within the same cardiac cycle while keeping the per-site acoustic requirement unchanged (3 MHz carrier, 3,000 cycles ≈ 1 ms, same Vpp to reach the target pressure). In this case, the array PRF doubles (1 Hz $\rightarrow$ 2 Hz). Because the energy per acoustic burst is fixed by the drive voltage and pulse width, the hourly pulse energy scales linearly with PRF, whereas the continuous baseline electronics power remains constant. Formally:

Let E_{base} be the hourly baseline energy and $E_{pulse,1Hz}$ be the hourly pulse energy at PRF = 1 Hz (single-focus). Then $E_{hour} (PRF) = E_{base} + E_{pulse,1Hz} \times PRF/1 \text{ Hz}$.

Required battery capacity for t hours at 3.7 V:

$$\text{mAh}_{\text{req}}(t) = t \cdot E_{hour} / 3.7.$$

Runtime for a battery of capacity C (mAh):

$$T = C \times 3.7 / E_{hour}.$$

Numbers used (assumptions: 64-ch array, 3 MHz, 3 000 cycles $\approx$ 1 ms, same peak pressure per site):

Single-focus (PRF = 1 Hz): $E_{base} = 3,630 \text{ mWh}$; $E_{pulse,1Hz} = 187.2 \text{ mWh}$; $E_{hour} = 3,817.2 \text{ mWh}$.

Rapid-switch (PRF = 2 Hz): $E_{hour} = 3,630 + 2 \times 187.2 = 4,004.4 \text{ mWh}$.

Rapid-switch dual-site pacing (PRF doubled to 2 Hz) increases the pulse energy twofold but adds only $\sim 4.9\%$ to the total system energy because the base term dominates. The battery-life penalty is modest ($\approx 9.69 \text{ h} \rightarrow 9.24 \text{ h}$ for 10 000 mAh; $19.38 \text{ h} \rightarrow 18.48 \text{ h}$ for 20 000 mAh), supporting the practicality of this operating mode.

Double Focus Switch Stimulation Mode Pulse Output Power Consumption (Single Focus Basic Power Consumption*1.2)

Time	Pulse power Consumption(mWh)	Base Power Consumption(mWh)	Total(mWh)	Required Capacity for 3.7V battery (mAh)	Duration if use 3.7V 10000mAh	Duration if use 3.7V 20000mAh
1 H	187.2	4356	4543.2	1227.891892		
2 H	374.4	8712	9086.4	2455.783784		
4 H	748.8	17424	18172.8	4911.567568	8.14 H	16.28 H
8 H	1497.6	34848	36345.6	9823.135135		
12 H	2246.4	52272	54518.4	14734.7027		

6. Tolerability of External Ultrasound Module:

While the minimally invasive nature of this system is commendable, the external ultrasound module raises concerns regarding skin tolerability, potential skin damage, and long-term maintenance. Detailed information on these aspects would be useful. Furthermore, patient movement could significantly alter heart positioning, potentially moving it outside the ultrasound monitoring zone. How does the system detect such displacement, and what safeguards or recovery mechanisms are in place to maintain capture if the heart moves out of the effective imaging field?

Response:

6.1 Skin tolerability, skin damage, Long-term Maintenance:

We thank the reviewer for this important question. To evaluate the long-term skin tolerability and practical robustness of the external ultrasound module, we employed a bioadhesive ultrasound (BAUS) couplant developed in Dr. Zhao's laboratory, as previously reported in *Science* (Wang et al., 2022; DOI: 10.1126/science.abo2542). This couplant was specifically engineered to maintain intimate and stable skin contact over extended periods while avoiding discomfort or skin damage. We systematically evaluated its performance by applying it to multiple anatomical regions—including the neck, chest, arm, and waist—and monitored skin responses for up to 48 hours (see attached [Fig. R13](#) from that paper). Throughout the duration of application, no allergic reactions, blistering, or discoloration were observed. The central contact region, where the couplant adhered directly to the skin, remained visually indistinguishable from baseline skin appearance. Minor erythema was occasionally observed at the adhesive ring edge—particularly in high-mobility areas such as the arm and waist, but it was transient and non-inflammatory in nature. Upon removal, no visible residue, peeling, or irritation was noted, indicating excellent biocompatibility of the BAUS couplant.

To benchmark the skin compatibility of BAUS, we previously conducted comparative dermatological assessments using patch tests across multiple commercial couplants, including liquid and solid hydrogels, silicone films, polyurethane layers, vinyl tapes, and conventional ultrasound gels. Dermatological outcomes were classified using standard criteria, where “+?” denoted a doubtful positive reaction and “—” indicated no observable response. Among all tested materials, the BAUS couplant demonstrated the highest proportion of negative skin reactions and the lowest incidence of doubtful positives ([Fig. R13d](#)), indicating a superior safety profile. Subjective skin comfort was further assessed using the Visual Analogue Scale (VAS; 0–10, where lower scores indicate higher comfort), with the BAUS couplant again ranking highest in user-reported comfort ([Fig. R13e](#)). These findings affirm the BAUS couplant's suitability for prolonged and repeatable use in wearable applications.

Regarding mechanical reliability under real-world conditions, we recognize that patient movement especially involving torso flexion or rotation may transiently alter the heart's spatial location relative to the fixed ultrasound array. To mitigate potential signal loss from such displacement, our system employs a wide-beam acoustic configuration that provides a large effective field of view, thereby enhancing robustness to anatomical shifts. In addition, real-time monitoring algorithms detect the absence of physiological motion signatures (e.g., periodic myocardial contraction), which triggers an automated reacquisition procedure. This procedure involves rapid lateral scanning to locate the heart and restore the imaging window without user intervention. In ongoing work, we are integrating inertial measurement unit (IMU) feedback to dynamically estimate patient posture, thereby enabling further improvements in motion-adaptive imaging. Collectively, these features ensure that the system maintains continuous and stable cardiac capture even under variable ambulatory conditions, without compromising user safety or comfort.

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Fig. R13 | Stable adhesion of the BAUS couplant on the skin over 48 hours. **a.** Chemical structure of the bioadhesive couplant. **b.** Schematic illustration of stable adhesion of the bioadhesive couplant on various locations on the body (arm, neck, chest, waist). **c.** Photos of stable adhesion of the bioadhesive couplant on various locations on the body (arm, neck, chest, waist) over 48 hours (relative humidity, 30-50%; temperature, 24°C). Scale bar, 10 mm. **d-e.** Comparison of 48-hour wearing comfort of the BAUS couplant, typical liquid (Aquasonic Clear, Parker Labs) and solid (Aquaflex, Parker Labs) hydrogel couplants, and elastomer couplants including silicone (Ecoflex, Smooth-On), acrylic tape (VHB, 3M), polyurethane (Sigma-Aldrich), and Aqualene (Olympus) on the market. **d.** wearing comfort tests. Evaluations for different couplants according to the patch test criteria of the International Contact Dermatitis Research Group (ICDRG). '+' and '-' stand for doubtful positive reaction and negative reaction, respectively. **e.** Participants reported their feelings of discomfort while the couplants were attached to the skin. The feelings were then evaluated by the visual analogue scale (VAS), ranging from 0 (most comfortable) –10 (most uncomfortable). Error bar values represent the mean $\pm$ SD (n = 15 independent samples). Statistical significance and P values are determined by one-way ANOVA followed by Tukey's multiple comparison test; ***P $\leq$ 0.001.

6.2 System detects such displacement caused by patient movement.

To address the critical issue of potential cardiac displacement arising from patient movement, we have implemented a significant upgrade to our system.

Building upon our established DeepLabV3 machine learning model for left ventricle (LV) segmentation, we have integrated a real-time centroid detection algorithm. Following precise segmentation of the LV, the algorithm automatically calculates the geometric centroid of this region and tracks its coordinates in real-time. To account for anatomical variations in heart size and position among individuals, a personalized threshold for the valid centroid coordinate range can be established for each user prior to use. This calibration process is straightforward: under the system's guidance, the user positions the probe at the leftmost and rightmost limits where the LV wall remains fully visualized. The system then automatically records these boundary coordinates to define a personalized and safe operational window. The corresponding code has been updated and integrated into our publicly available model files.

Should the probe become displaced during daily use, the system provides immediate visual feedback via the companion application to guide the user in correcting its position (as shown in [Fig. R14](#)):

- **Centered (a):** When the probe is correctly positioned, the system confirms its central alignment, and all functions remain operational.
- **Left-Shifted (c):** If the probe shifts excessively to the left, causing a portion of the LV wall to move outside the imaging field of view, the centroid's coordinates will exceed the predefined threshold, and the application will display a "Left-Shifted" alert.

- **Right-Shifted (d):** Similarly, a rightward displacement that compromises the complete visualization of the LV will trigger a "Right-Shifted" alert, prompting user adjustment.
- **Connection Lost (b):** In the event of complete probe detachment, the system will notify the user of a lost connection.

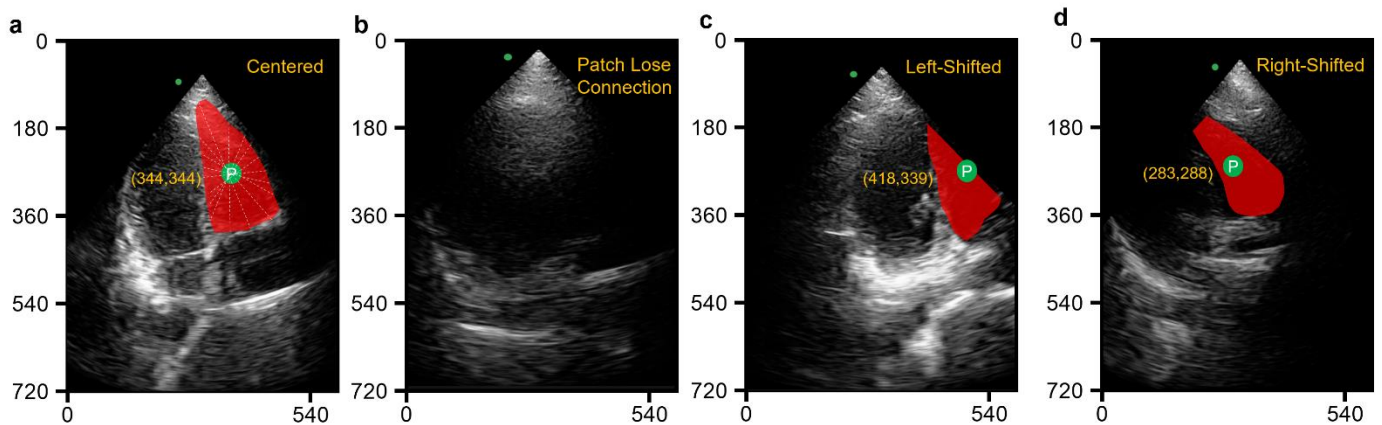


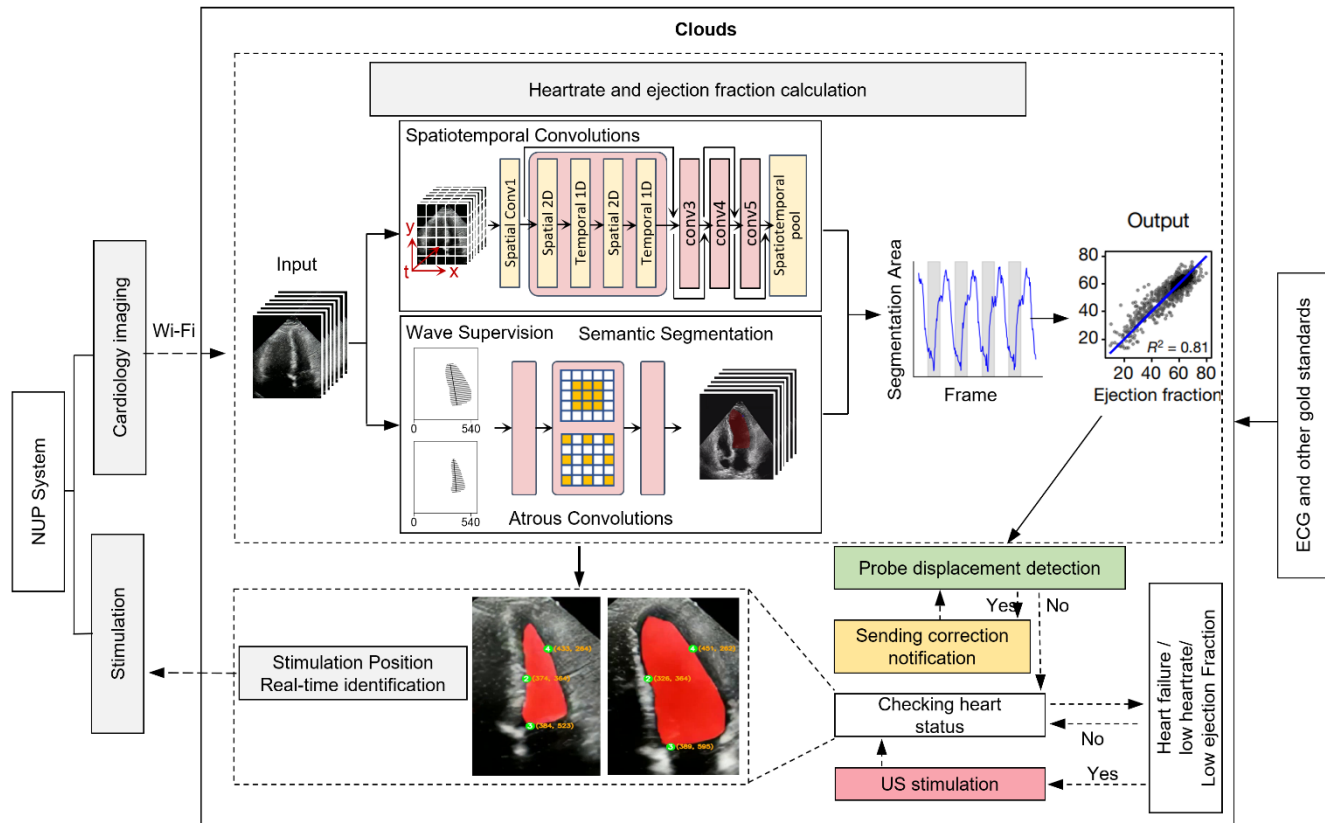
Fig. R14 (add as Supplementary Fig. 19) | Real-time AI-guided feedback for probe displacement. Representative ultrasound images showing the automated feedback system for maintaining correct probe alignment. **a**, Centered probe with full visualization of the left ventricle (red overlay). **b**, Connection 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.

Crucially, we have designed a core safety interlock to address this issue: should the system detect that the centroid has moved beyond the preset threshold, all stimulation functions are immediately and automatically suspended. This prevents off-target stimulation resulting from positional drift. Importantly, the imaging function remains active, providing the user with real-time visual feedback to guide manual repositioning of the patch. Stimulation can only be resumed once the patch is correctly repositioned and the centroid returns to within the safe operational window.

This figure has been added to the Supplementary Materials and has been described in the main text on page 6, lines 23–26.

“As a critical safety feature, we also incorporated an automated probe displacement detection function. It uses the AI model to continuously monitor the position of the left ventricle; if a significant shift is detected, stimulation is automatically paused, and the user is prompted with directional guidance for correction (Supplementary Fig. 19).”

We have fully integrated this safety check into the system's primary operational workflow, as depicted in the flowchart in **Fig. 5a** and **Extended Data Fig. 8** (see updated figure below) The decision-making logic for initiating cardiac stimulation now mandatorily includes a check for LV displacement and ensures the centroid's coordinates are within the established threshold, in addition to assessing physiological metrics such as heart rate and ejection fraction. A stimulation command is executed only when all of these conditions are met. This series of enhancements directly addresses the reviewer's concerns and ensures the long-term safety and reliability of our system.



Extended Data Fig. 8 | 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.

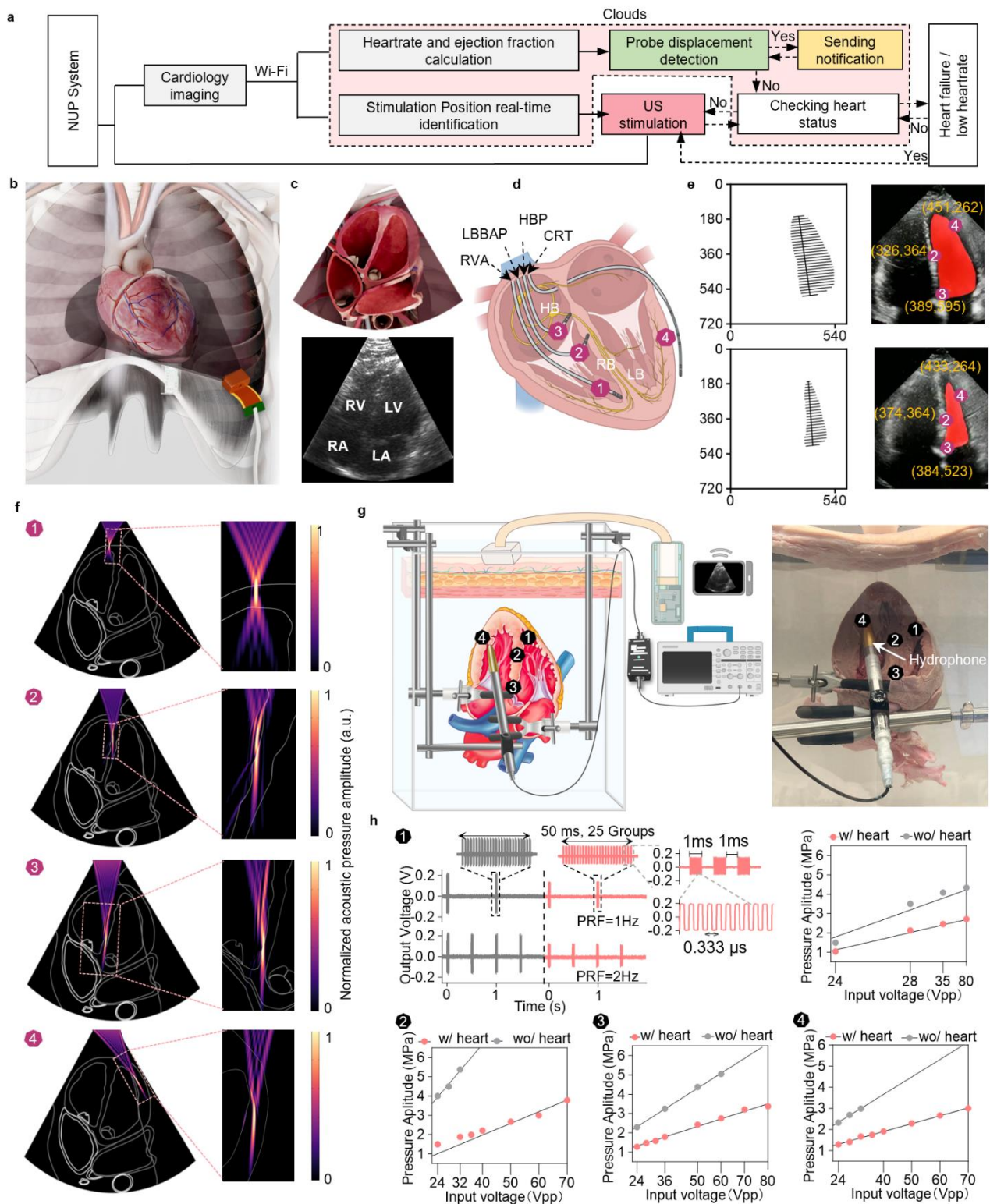


Fig. 5 | Feasibility investigation of NUP in clinical applications. **a**, Diagram of the wearable system operation, showing real-time imaging of the heart, left chamber volume calculations, heart rate monitoring, and the decision-making process for initiating ultrasound stimulation based on heart status. This process includes a critical initial safety check for probe displacement to ensure accurate targeting before the system performs stimulation. **b**, Schematic of the wearable ultrasound stimulation system positioned on a human subject's chest, showing device placement and size for effective heart stimulation. **c**, Illustration of the phased array stimulation performing an apical four-chamber view and ultrasound B-mode imaging, identifying the RA, LA, RV and LV. **d**, Schematic of traditional pacemaker cardiac stimulation sites, including His-bundle pacing (HBP), right ventricular apical pacing (RVA), left bundle branch area pacing (LBBAP), and cardiac resynchronization therapy (CRT). **e**, Real-time tracking of the left ventricular cross-sectional area with an automated algorithm visualizing cardiac cycle dynamics and computational analysis of LV volume changes, including output of three stimulation point positions. **f**, Simulated ultrasound energy transmission in the heart, showing the distribution of acoustic pressure amplitude at different stimulation sites to demonstrate targeted stimulation efficacy. **g**, Diagram of the hydrophone system simulating ultrasonic energy transmission through an ex vivo pig heart, illustrating energy distribution, probe positioning, and ultrasound-mediated cardiac stimulation fidelity. **h**, Analysis of ultrasound output voltage and acoustic pressure amplitude at various cardiac sites under different operational conditions, including pulse repetition frequency (PRF) and input voltage, comparing results with and without heart tissue.

7. Temperature Effects with Prolonged Use:

Although the authors report temperature data for up to 1200 stimulations, is there additional data on temperature variations during prolonged, continuous use over days or weeks?

Response:

We thank the reviewer for this suggestion. We have performed new experiments to assess the effects of prolonged stimulation, in which continuous stimulation was applied for over 13 hours with simultaneous direct temperature measurements (**Fig. R15**)

This extended *in vivo* thermal safety assessment was conducted on anesthetized rats placed on a heating pad to maintain a stable core body temperature. To counteract dehydration and ensure physiological stability throughout the prolonged anesthesia, a continuous intravenous infusion of saline was administered via the tail vein. The NUP system was configured to deliver uninterrupted pacing for over 12 consecutive hours. To precisely measure any thermal effects, we used fine-gauge (0.23 mm) thermocouple probes to monitor the temperature directly within the heart (intracardiac).

REDACTED

Fig. R15 (add as Supplementary Fig. 31) | Long-duration *in vivo* thermal monitoring under continuous pacing. Top: schematic of the experimental setup for *in vivo* temperature monitoring. Fine-gauge thermocouple probes (0.23 mm) were positioned to measure temperature directly within the heart (intracardiac) and on the skin surface of an anesthetized rat, while the NUP system delivered continuous ultrasound stimulation. A syringe pump provided continuous intravenous infusion to maintain hydration, and a heating pad was used to maintain a stable baseline physiological temperature. **Middle:** A sustained 12-hour session of pacing was applied (indicated by the shaded region). The different traces represent the thermal effect of continuous stimulation at three different acoustic pressures: **2.0 MPa (black)**, **1.5 MPa (red)**, and **1.1 MPa (light red)**. **Bottom:** Insets show magnified views of the temperature profiles at the onset of stimulation (left, 0.6-1.4 hours) and after its cessation (right, 12.8-13.2 hours) for all three pressure levels. $n=3$ for each condition.

The results from this experiment demonstrate the exceptional thermal safety of our platform. Upon initiation of the 13-hour stimulation, we observed a small initial temperature rise of approximately 1°C in the heart, which then remained remarkably stable for the entire duration of the session. There was no evidence of cumulative or runaway tissue heating. This observation is consistent and further confirmed with FDA-required thermal index (TI) calculation, one of the significant index to evaluate the clinical safety of ultrasound devices. The calculated TI of our stimulation is $TIS = \sim 0.4$ to 1.2 depending on the focal area and acoustic pressure, meaning the temperature increase will be no more 1.2 °C. Crucially, as soon as the ultrasound stimulation was terminated, the intracardiac temperature promptly returned to its pre-stimulation baseline level. This experiment provides direct and compelling evidence that our NUP system can be operated for prolonged periods without inducing significant or hazardous thermal effects, strongly supporting its safety profile for chronic therapeutic applications.

This supplementary figure has been added to the Supplementary Materials on page 34, and it has been referenced in the main text on page 7, line 45, where we describe the results of long-term stimulation-induced heating.

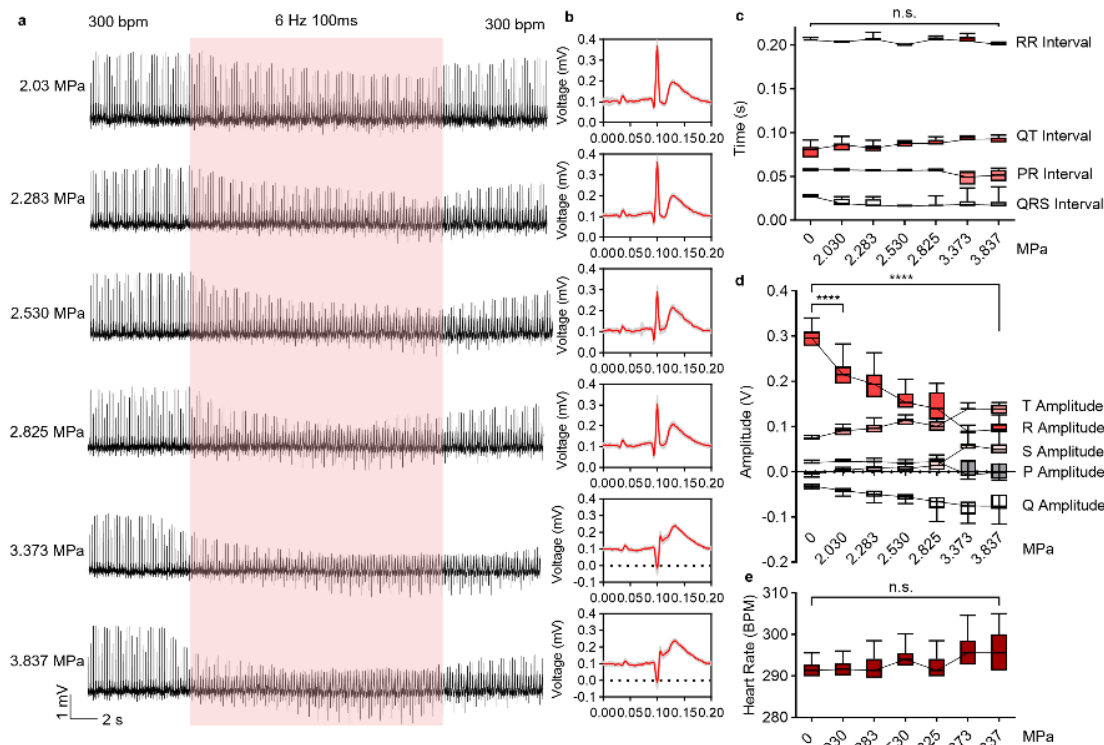
“and 0.7°C after 12 hours stimulations (**Supplementary Fig. 30, 31**).”

Comments

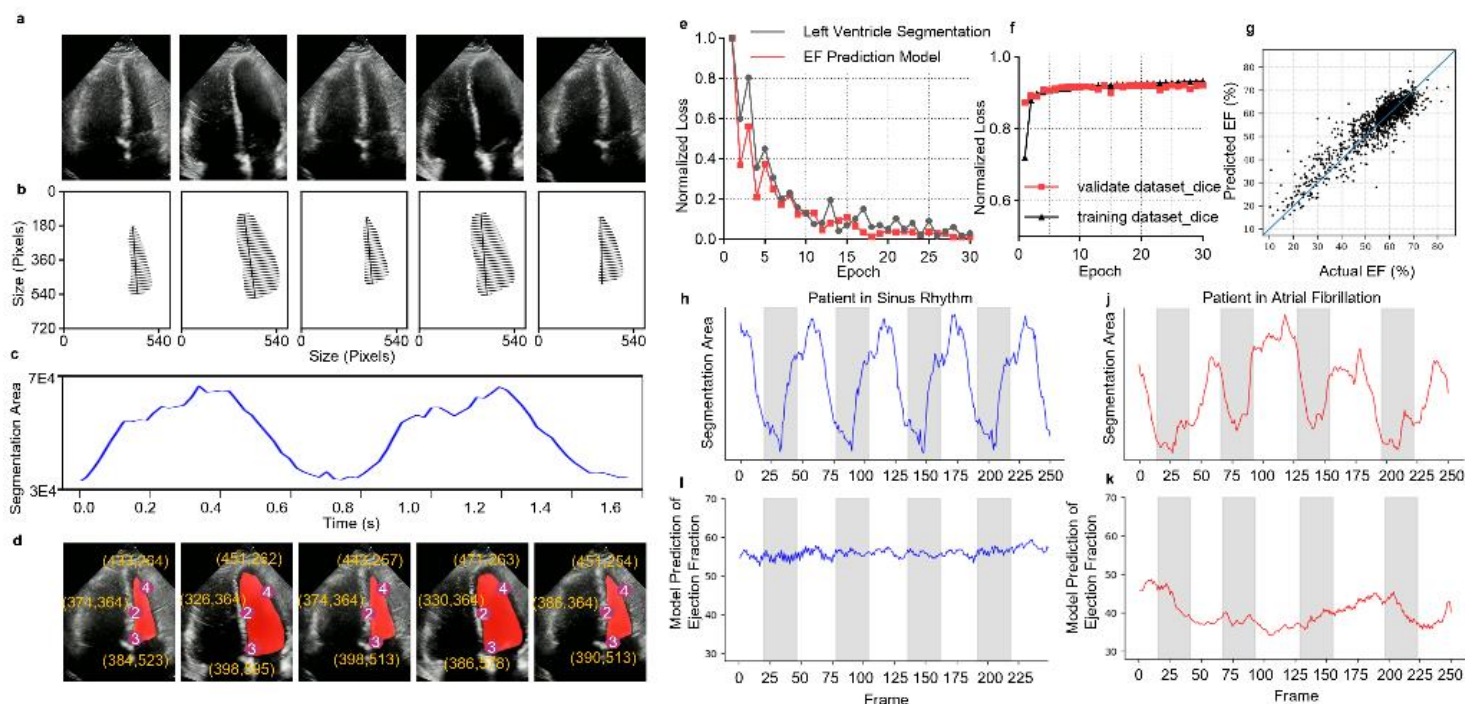
1. Typographical Errors:

There are typographical issues in several figures. For example, in Extended Data Fig. 6d, one X-axis label is incorrectly written as “3830” instead of “3837.” Also, in Extended Data Fig. 7j, “Atrial Fibrillation” should be spelled correctly.

Thanks for pointing out these issues, for **Extended Data Fig. 6d**, we have corrected the x-axis label from “3.830” to “3.837” MPa.



For **Extended Data Fig. 7j**, we have corrected the figure title as suggested, and the revised figure is attached here. We sincerely appreciate the reviewer’s constructive comments.



2. Figure Panel Order:

The order of figure panel labels is inconsistent. For example, Fig. 2b and Fig. 5b appear after panels labeled with later letters.

For Fig. 2b:

Thank you for pointing out this error. We found that **Fig. 2b** was mislabeled, and we have corrected it. To improve the overall consistency and flow of the figure sequence, we have also made additional revisions. The updated version is provided below.

REDACTED

Fig. 2 | Real-time spatiotemporal analysis of ultrasound-stimulated calcium dynamics in human cardiomyocytes. **a**, Schematic of the experimental setup for ultrasound stimulation of in vitro cultured cardiomyocytes (CMs), combined with real-time fluorescence imaging. The inset shows representative CMs expressing MscL G22S, mCherry, with data collected from $n = 8$ independent culture groups. Scale bar, 100 μm . **b**, Schematic depiction of the differentiation and transfection process for human cardiomyocytes expressing MscL G22S. **c-d**, Representative $\Delta F/F$ traces and heatmaps showing calcium dynamics in WT (**c**) and ST (**d**) CMs under ultrasound stimulation. **e**, Time-lapse fluorescence images showing calcium influx in WT and ST CMs during ultrasound stimulation, right panels display maps of the maximal $\Delta F/F$ changes. **f**, Quantification of normalized excited area and excitation rate ($n = 25$ cells per group). Scale bars, 100 μm . **** indicate statistical significance ($p < 0.0001$).

For Fig. 5b:

We thank the reviewer for the guidance on Fig. 5. In the original version, **b** represented the implantation sites of conventional cardiac pacemakers, followed directly by our NUP implantation site, which created a somewhat confusing sequence. In the revised figure, we have moved the original panel **b** to the new position as panel **d**, so that the figure sequence is more logical: new panel **b** now introduces the NUP implantation position along with the corresponding B-mode imaging plane; panel **c** presents the imaging results; panel **d** transitions to the conventional pacemaker implantation sites; panel **e** demonstrates that these conventional pacing sites can be tracked in real time and stimulated by the NUP system; and panel **f** illustrates the simulated acoustic field distribution and attenuation across the four pacing sites.

Due to space limitations and to avoid redundancy, we have included the revised **Fig. 5** and its updated legend in our response to **Comment 6.2** as well as in the main manuscript.

3. Battery Life and Feasibility:

Given the inherent limitations in battery life, how do the authors envision the clinical feasibility and sustainability of this system?

We thank the reviewer for raising this important point regarding battery life and clinical feasibility. Power consumption and sustainability are indeed critical considerations in the design of wearable biomedical devices, particularly those intended for long-term or continuous clinical use.

To address this, we carefully evaluated and optimized the energy efficiency of the NUP system. Based on our measurements (**Extended Data Fig. 3**), under a representative stimulation condition of 60 pulses per minute, 50% duty cycle, and a typical pulse duration of 50 ms, the system exhibits a total energy consumption of approximately 30.5 Wh over 8 hours. This corresponds to an average power consumption of ~3.81 W. When powered by a standard 3.7V 10,000 mAh lithium-ion battery, the system can operate continuously for 8 hours, which aligns with daily use durations such as hospital shifts, outpatient monitoring, or therapeutic sessions.

Table in **Comment 5** further illustrates how total power consumption scales with output voltage, ranging from 30.01 Wh at 20 Vpp to 33.00 Wh at 80 Vpp. At the most commonly used setting (50 Vpp), the system consumes 31.51 Wh, requiring approximately 8,500 mAh capacity, which is comfortably within the capabilities of our selected battery.

To further enhance practicality and reliability, we implemented several power management strategies:

1. **Energy buffering:** We incorporated high-capacitance components (16,000 μ F), which stabilize high-voltage pulses and mitigate transient loads without significantly increasing continuous current draw.
2. **Duty-cycled or adaptive operation:** Many use scenarios, such as arrhythmia correction, do not require continuous stimulation. By enabling event-triggered or algorithm-controlled pacing, the average power usage can be **reduced by 30–70%**, significantly extending battery life under real-world usage.
3. **Compact and wearable design:** The full device (including battery, driver circuits, and transducer array) weighs 116 g, with a volume of 13 cm $\times$ 3.5 cm $\times$ 2.2 cm, making it suitable for mobile and ambulatory use without compromising patient comfort.
4. **Modular upgradeability:** The power system is modular, supporting potential integration of higher-capacity batteries, external DC power sources, or inductive charging systems, allowing further extension of operational time for continuous 24/7 monitoring if needed.

Finally, during preliminary human-factors testing, the system maintained stable output performance throughout the 8-hour window without voltage droop or heating, and the hydrogel interface allowed consistent skin coupling throughout prolonged wear.

In summary, the current battery-powered design supports sustained, real-world operation without interruption, and our measurements demonstrate a clear alignment between power supply and functional use. We believe the NUP system represents a clinically viable and sustainable solution for wearable, noninvasive cardiac rhythm modulation. Future iterations can further extend runtime using adaptive control strategies, optimized waveform delivery, and next-generation power sources.