

All-Optical Closed-Loop Control of Human Cardiomyocyte Networks Exploiting Holographic Optogenetics

Corresponding Author: Mr Robert Wendland

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript presents an elegant and technically sophisticated demonstration of spatiotemporal closed-loop control of cardiac wavefront dynamics in human iPSC-derived cardiomyocyte monolayers. By integrating label-free speckle-based imaging with high-speed holographic optogenetic stimulation, the authors establish a minimally invasive, all-optical feedback system capable of detecting and correcting aberrant propagation directions in real time. The primary conceptual contribution lies in the use of sparsely sampled regions of interest (ROIs) to estimate wavefront type, direction, and velocity from phase delays. The authors convincingly demonstrate that planar and rotating waves can be discriminated using a small number of spatially distributed measurement points, and they provide both experimental validation and error analysis to justify the chosen phase threshold. This reduced-data framework is particularly compelling in the context of implantable or energy-constrained systems, where data throughput and processing cost represent major limitations.

Several aspects could be further clarified or expanded.

- a) While the study demonstrates sparse ROI-based wavefront estimation and closed-loop control, it lacks systematic evaluation of key hyperparameters. The impact of ROI number, on estimation accuracy, robustness, and latency is not analyzed, leaving the “minimal sensing” claim insufficiently supported. Additionally, the choice of stimulation delay ($T/2$) and angular threshold (45°) appears empirical, without sensitivity analysis or justification of operating margins. A parametric study examining how these parameters affect correction success, stability, and energy efficiency would significantly strengthen the methodological rigor and generalizability of the proposed framework.
- b) The authors should further address the impact of common optical imaging artifacts, particularly imaging noise and motion-related disturbances, on the reliability of the proposed method. Speckle-based approaches are inherently sensitive to global motion, mechanical drift, and vibration, which may introduce false temporal differences unrelated to cardiac contraction. It would be important to clarify how the system distinguishes true contraction-induced speckle fluctuations from external motion artifacts, and whether additional motion compensation, spatial registration, or hardware stabilization was employed. A quantitative robustness analysis under controlled motion perturbations would strengthen confidence in the method's applicability beyond ideal in vitro conditions.
- c) The manuscript would also benefit from a clearer quantitative comparison between the proposed closed-loop strategy and an appropriate open-loop baseline. Although the authors demonstrate high correction success under feedback control, it remains unclear how much improvement is specifically attributable to the closed-loop architecture. For example, would periodic or predefined patterned stimulation (open-loop) achieve comparable directional correction rates? Including controlled experiments comparing correction accuracy, number of stimuli required, recovery time, and robustness to spontaneous variability would more convincingly demonstrate the added value of real-time feedback and substantiate the claim that closed-loop control provides superior spatiotemporal regulation.

Overall, this study represents a significant step toward spatially resolved optogenetic rhythm control. By showing that minimal sensing can suffice for effective closed-loop modulation of wavefront orientation, the authors provide both a conceptual and technological framework that may inform future optical pacemaker or cardioversion strategies. The work will be of interest to researchers in cardiac electrophysiology, optogenetics, and closed-loop control of excitable biological systems.

Reviewer #2

(Remarks to the Author)

I have read the manuscript with interest and would like to raise several points for the authors to clarify. I would appreciate the authors' responses to the following questions.

1. You picked 25° as your threshold for distinguishing planar from rotating waves, based on observing 13 independent cardiomyocyte networks where the maximum measured phase delay stayed below 19.7°. I don't think thirteen is a large sample. What happens if you encounter a culture with unusually slow conduction velocity or a shorter contraction period than anything in your current dataset — couldn't a perfectly planar wave then exceed 25° and get misclassified as rotating? Did you consider making the threshold adaptive on a per-culture basis rather than fixing it as a universal constant?
2. Four ROIs is elegant for simple cases, but what about other fragmented wavefronts, like multiple wavelets, or figure-of-eight reentry — patterns that actually matter in clinical arrhythmia? With only 4 sensors, what does the system output when the plane wave assumption breaks down entirely?
3. Both of the two failures you report occurred at the very end of their respective experiments, where the control loop was terminated before enough corrective stimuli could be applied. So those are not really failures of the control strategy itself — but I think are artifacts of experiment duration. If you had let the loop run longer, would those contractions have been corrected too? I think you should be more careful about how you present this number, because as it stands it conflates the effectiveness of the control scheme with practical constraints in your experiment design. Could you clarify whether there were any genuine cases where the stimulation simply could not redirect the wavefront, independent of timing cutoffs?
4. Your speckle-based approach tracks contraction, which is a mechanical readout downstream of the electrical activation. There is necessarily an electromechanical delay between the action potential wavefront and what you actually measure. May I know how large is this delay, and if it is spatially uniform across the culture? If it varies from region to region, your propagation angle measurements could carry a systematic bias I think. Your manuscript did not show any direct comparison with voltage- or calcium-sensitive dye recordings from the same preparations. In my opinion, such a comparison would go a long way toward validating the fidelity of your approach.
5. You motivate the paper with tachycardia at 100–250 bpm, but your camera runs at 60 Hz. At 250 bpm that gives you roughly 14 frames per cardiac cycle right? But your own Eq. 2 shows the phase delay error scales inversely with sampling rate. So the question is have you estimated the measurement uncertainty at tachycardia rates, and does it stay comfortably below your 25° threshold?
6. In the manuscript, you noted that both uncorrected contractions were preceded by sequences requiring multiple consecutive corrective stimuli. I think this is a pattern that worth exploring. I would like to know does repeated optogenetic pulsing alter local excitability or refractoriness in the tissue? And could there be like opsin desensitization or ionic loading effects that make each subsequent correction progressively harder? If so, that would be a meaningful limitation for any sustained or long-duration application of this approach, and it would be helpful to see some discussion or data on this point.

Reviewer #3

(Remarks to the Author)

In the submitted manuscript entitled 'All-Optical Closed-Loop Control of Networks Exploiting Holographic Optogenetics', Wendland et al. describe the development of a novel experimental setup to assess the possibility of optical steering of propagating electrical waves, with the vision to provide optimised protocols for optogenetic termination of tachyarrhythmia. The proposed system relies on the use of 2D monolayers of hiPSC-derived cardiomyocytes expressing the red-light-gated cation channel Chrimson, allowing them to precisely trigger membrane depolarisation in the illuminated region. There are two main outcomes/achievements from the study: (1) Propagating electrical waves can be estimated from cell deformations, i.e. contractions, and Wendland et al. showed how sparse spatial sampling is sufficient to deduce the deviation of the direction of conduction from 'normal' conduction. (2) A line ('strip') of light applied during the 'diastolic' phase and perpendicular to the propagating wavefront is sufficient to restore normal conduction patterns. The provided technology, especially the implementation of sparse sampling, is compelling, providing a step towards closed-loop control of cardiac activity. However, the feasibility of the proposed approach in 3D tissue, as well as the underlying mechanisms of wavefront steering, remain to be still elucidated.

Please find my suggestions for improving the manuscript below:

- Introduction, but also in the discussion: Please carefully distinguish between electrical activation/deactivation and contraction/relaxation throughout the study, especially in the introduction. While these two processes are physiologically coupled via excitation-contraction coupling, they may not be coupled in the case of tachyarrhythmia, and such coupling might be absent in fibrillating tissue. Please revise the entire introduction accordingly, and explain in which cases deformation can be used as a surrogate for electrical activation (of course, with a temporal delay). Please also revise terminology, for example 'contraction rhythm' is not a commonly used term in the field.
- The distinction between focal tachycardia and reentry tachycardia is unclear given the scope of the study. In the introduction, you may want to distinguish between atrial and ventricular arrhythmia, given that contraction of the heart is mainly driven by the ventricular walls. I would advise to first explain the different electrical activation patterns, before discussing the resulting alterations in (ventricular) contraction-relaxation behaviour. Please also adjust passages in the results part/discussion, e.g. lines 234-36 and lines 338-340. In case of a not sinus-node driven atrial tachyarrhythmia, ventricular activation maps might still appear normal, as ventricular activation would be AV-node driven. Depending on the atrial rhythm, there might be a partial or even full dissociation between atrial and ventricular activation.
- Throughout the study, the authors claim to have implemented the first all-optical, closed-loop strategy for cardiac arrhythmia termination. However, closed-loop optogenetic approaches have been suggested more than ten years ago (e.g. see <https://pmc.ncbi.nlm.nih.gov/articles/PMC4775736/>) and have been successfully applied to cardiac tissue or cells (e.g. see <https://pmc.ncbi.nlm.nih.gov/articles/PMC10753386/>; and very recently: <https://pubmed.ncbi.nlm.nih.gov/41684280/>; <https://www.science.org/doi/10.1126/sciadv.abq7469>)
- Please also refer to the pioneering work by Jan Christoph, demonstrating the feasibility of using mechanical activity to infer electrical activity, e.g. <https://pubmed.ncbi.nlm.nih.gov/33380038/>

- The electrical and mechanical connectivity of hiPSC-cardiomyocytes strongly depend on the density of the cells in the culture. Please provide the number of cells seeded by area, and please provide a representative image of the cells within the measurement chamber.
- You choose a phase delay of 25° to distinguish a planar wave from a rotating wave. How translatable is this distinction to cardiac tissue with physiologically occurring conduction pathways and their variations? Why did you use 45° as threshold for applying light later on?
- If I understand correctly, the light stimulus is delivered in-between two contractions (e.g. at T/2). Please discuss limitations of this approach, especially in the case of fast rhythms when full diastolic repolarisation/relaxation might not be reached.
- In addition, you apply a light strip perpendicular to the desired wavefront propagation to reset the direction of the wavefront. In Figure 3, you state two different light intensities and two different durations of light applied. How did you determine the optimal parameters? Why did they differ between individual experiments? Which wavelength was used? What were the x-y dimensions of the applied light strip, and did it always extend to the boundaries of the field of view/chamber? To further understand the methodology, it would be beneficial to include a time-resolved schematic with a propagating wavefront and the corresponding illumination pattern(s), both depicting a case with single illumination and one where multiple attempts were needed.
- Please explain the underlying mechanism by which the wavefront was re-directed. Did you test different light intensities (e.g. sub-threshold and above-threshold regimes)?
- In Figure 2, the provided conduction velocities are significantly lower than in normal myocardium, and the provided rates are unphysiologically low. Have you tested the presented cardioversion approach at frequencies as present in tachyarrhythmia e.g. at 2 Hz or faster?
- Please discuss challenges of translating the presented cardioversion approach into 3D tissue and myocardium. In this regard you mention the use of 3D organoids. How representative are currently available organoid models in respect to anisotropic conduction velocity as present in normal myocardium? Why not use tissue slices instead?
- Please make sure to label/explain all variables used in the Figures in the Figure legends. In Figure 2f-h please label the x-axes of the graphs provided.
- Please correct spelling mistakes/typos.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have made a substantial effort to address the reviewers' concerns. In particular, the added simulation-based analysis of ROI number, ROI geometry, noise sensitivity, wavefront curvature, and achievable cycle time strengthens the methodological basis of the sparse-sensing framework. However, one concern remains insufficiently resolved. The added analyses do not fully address the need for an appropriate open-loop baseline. The rebuttal explains why open-loop stimulation is expected to be less robust, but this is not equivalent to a controlled comparison using the same directional-correction endpoint. The manuscript should either include a matched open-loop comparison, even if limited, or substantially soften claims that the closed-loop strategy provides superior spatiotemporal regulation. In summary, the revision improves the manuscript, I would recommend a minor revision before acceptance.

Reviewer #2

(Remarks to the Author)

Thank you for the thorough revision. Most concerns have been satisfactorily addressed.

Two minor textual additions I think would help the final version: (1) an explicit acknowledgement that the spatial uniformity of the electromechanical delay was not measured, and that spatially heterogeneous delay could bias propagation angle estimates; and (2) inclusion of the quantitative phase delay error at tachycardia rates (~52% at 250 bpm) discussed in the rebuttal but absent from the manuscript.

I do not anticipate a further round of review.

Reviewer #3

(Remarks to the Author)

The authors have carefully revised the manuscript, addressing all points raised during the initial review.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have carefully revised the manuscript, addressing all points raised during the initial review.

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Response to the Reviewers

Nature Communications Engineering submission,
Manuscript ID: COMMS-ENG-26-0039,
Type: research article

Title: All-Optical Closed-Loop Control of Human Cardiomyocyte Networks Exploiting Holographic Optogenetics

Authors: Robert Wendland, Felix Schmieder, Muhammad Atif Sikandar, Frithjof Paul Knüppel, Wolfram-Hubertus Zimmermann, Olaf Bergmann, Lars Büttner, Jürgen Czarske

black: Comments of the Reviewers

blue: the authors' reply to these comments

Please note that the referenced text passages refer to the revised manuscript (**with markup**).

Reviewer 1:

1. While the study demonstrates sparse ROI-based wavefront estimation and closed-loop control, it lacks systematic evaluation of key hyperparameters. The impact of ROI number, on estimation accuracy, robustness, and latency is not analyzed, leaving the “minimal sensing” claim insufficiently supported. Additionally, the choice of stimulation delay ($T/2$) and angular threshold (45°) appears empirical, without sensitivity analysis or justification of operating margins. A parametric study examining how these parameters affect correction success, stability, and energy efficiency would significantly strengthen the methodological rigor and generalizability of the proposed framework.

To strengthen the methodological evaluation of the proposed sparse sensing approach, we added a simulation-based study investigating the impact of key hyperparameters of this framework. Specifically, we evaluated the influence of the number/ geometry of ROIs on angle and velocity measurement accuracy, robustness against noise and the impact of deviations from ideal planar wave propagation. We further added achievable cycle times/ latencies for different ROI numbers. These results are presented in section 2.3 and figure 3 of the revised manuscript.

Regarding the choice of stimulation delay and the angular threshold, we agree that a systematic optimization and sensitivity analysis of these parameters would be highly valuable. In the present proof-of-concept study, these parameters were selected empirically to demonstrate the feasibility of the closed-loop framework. We therefore clarified the rationale underlying the selection of these and mentioned their systematic evaluation and optimization for future work in the discussion (see lines 519-521, 525-527).

However, we analyzed the relationship between stimulation parameters (intensity and fluence) and the necessity of multiple corrective stimuli and added it to the manuscript (see section 2.4 and figure 5).

2. The authors should further address the impact of common optical imaging artifacts, particularly imaging noise and motion-related disturbances, on the reliability of the proposed method. Specklebased approaches are inherently sensitive to global motion, mechanical drift, and vibration, which may introduce false temporal differences unrelated to cardiac contraction. It would be important to clarify how the system distinguishes true contraction-induced speckle fluctuations from external motion artifacts, and whether additional motion compensation, spatial registration, or hardware stabilization was employed. A quantitative robustness analysis under controlled motion

perturbations would strengthen confidence in the method's applicability beyond ideal *in vitro* conditions.

Since speckle-based approaches are inherently sensitive to externally induced motion, the experimental setup was mounted on a damped optical table and potential vibration sources such as fan-cooled power supplies were removed from the table to minimize these artifacts. In addition, remaining high-frequency disturbances and slow drift components were suppressed by temporal filtering within the evaluation algorithms. These hardware- and software-based stabilization strategies are now described (with references to respective supplementary information) in the revised Methods section 4.5.

Further, we agree that controlled experimental perturbation studies would provide additional insight into the robustness of the method. However, such experiments were considered beyond the scope of the study, as the proposed speckle-based imaging approach is intended for *in vitro* applications, where environmental disturbances can generally be minimized effectively. Nevertheless, as mentioned in the response to comment 1, we incorporated a simulation-based analysis of noise sensitivity. We integrated a simulation-based study on the influence of noise as mentioned in the response to 1.). The simulated noise was modeled as random phase perturbations in the sample plane, representing high-frequency externally induced motion artifacts (a full methodological description of the simulation can be found in Supplementary Note 7).

3. The manuscript would also benefit from a clearer quantitative comparison between the proposed closed-loop strategy and an appropriate open-loop baseline. Although the authors demonstrate high correction success under feedback control, it remains unclear how much improvement is specifically attributable to the closed-loop architecture. For example, would periodic or predefined patterned stimulation (open-loop) achieve comparable directional correction rates? Including controlled experiments comparing correction accuracy, number of stimuli required, recovery time, and robustness to spontaneous variability would more convincingly demonstrate the added value of real-time feedback and substantiate the claim that closed-loop control provides superior spatiotemporal regulation.

A comparison to an open-loop strategy is essential to highlight the benefit of feedback control. In an open-loop configuration, successful correction/ pacing depends on prior knowledge of contraction parameters, in particular the period of spontaneous contraction and time relative to the refractory state of the cell network. This claim is supported by the data presented in Supplementary Fig. S3, which shows that successful pacing and capture strongly depends on the ratio between stimulation and spontaneous contraction frequency. The proposed closed-loop approach does not require a priori knowledge of these temporal dynamics and can adapt stimulation-timing-based feedback about contractions in the ROIs, thereby ensuring stimulation outside refractory periods. Moreover, open-loop strategies inherently lack the ability to adapt to spatially varying propagation characteristics, such as changes in wavefront direction.

We have clarified this comparison in the revised manuscript and further emphasized the role of feedback control (see lines 502-509).

Reviewer 2:

1. You picked 25° as your threshold for distinguishing planar from rotating waves, based on observing 13 independent cardiomyocyte networks where the maximum measured phase delay stayed below 19.7°. I don't think thirteen is a large sample. What happens if you encounter a culture with unusually slow conduction velocity or a shorter contraction period than anything in your current dataset —couldn't a perfectly planar wave then exceed 25° and get misclassified as rotating? Did you

consider making the threshold adaptive on a per-culture basis rather than fixing it as a universal constant?

We fully agree, that the chosen threshold should not be understood as a universal constant. This value depends on parameters like wavefront velocity, contraction period and ROI geometry. This has been additionally clarified in the discussion of the revised manuscript (see lines 480-486). In particular, we emphasized that the 25° threshold was selected based on experimentally observed cardiomyocyte networks and is therefore appropriate for the investigated conditions, rather than intended as a general fixed value.

Importantly, this threshold is already partially adaptive, as phase delays which are compared to the threshold values are calculated relative to the last observed culture-specific contraction period (see Eq. 1).

Furthermore, as noted by the reviewer, in extreme cases with slow velocity v_c or a short contraction period T (compared to the last observed one), a perfectly planar wave could in principle exceed this threshold. Within our ROI configuration (assuming an ROI spacing of 7 mm), such cases would correspond to $v_c \cdot T \leq 100$ mm and were not observed within our dataset. This consideration is included in the Discussion of the revised manuscript (see lines 486-490).

2. Four ROIs is elegant for simple cases, but what about other fragmented wavefronts, like multiple wavelets, or figure-of-eight reentry — patterns that actually matter in clinical arrhythmia? With only 4 sensors, what does the system output when the plane wave assumption breaks down entirely?

We agree that more complex contraction patterns represent clinically highly relevant scenarios that are not fully captured by the present 4-ROI implementation. The current approach was designed for characterization of predominantly planar and rotating patterns. In principle, the sparse sensing approach can be expanded to larger ROI arrays (e.g., 3×3 or 4×4 configurations), enabling estimation of propagation angle and velocity within spatial subregions and thereby facilitating reconstruction of more fragmented or spatially heterogeneous wave dynamics. We included this potential modification in the Discussion (see lines 495-499).

When the plane wave assumption breaks down, the 4 ROI configuration remains capable of identifying abnormalities through inconsistencies, such as spatially varying contraction periods or in general elevated contraction frequencies. Spiral wave propagation can be distinguished based on the phase delays as described in section 2.2. In such cases, alternative stimulation strategies could be triggered, for example global reset stimuli. This is now also mentioned in the Discussion of the revised manuscript (see lines 490-495).

In addition, to better characterize the limitations of the plane-wave assumption, we performed a simulation-based analysis investigating the influence of wavefront curvature and apex displacement on the uncertainty of propagation angle and velocity estimation (section 2.3 and figure 3 in the revised manuscript).

3. Both of the two failures you report occurred at the very end of their respective experiments, where the control loop was terminated before enough corrective stimuli could be applied. So those are not really failures of the control strategy itself — but I think are artifacts of experiment duration. If you had let the loop run longer, would those contractions have been corrected too? I think you should be more careful about how you present this number, because as it stands it conflates the effectiveness of the control scheme with practical constraints in your experiment design. Could you clarify whether there were any genuine cases where the stimulation simply could not redirect the wavefront, independent of timing cutoffs?

We thank the reviewer for this helpful remark, to which we totally agree. The two instances of non-correction occurred at the very end of their respective experiments, where the control loop had already been terminated and no further corrective stimuli could be applied. To avoid conflating these

timing-related limitations with the performance of the control strategies, we have clarified in the Discussion that the two cases do not reflect a failure of the control algorithm itself (see lines 509-511) and added “at least” in the abstract where we presented the number of correction success.

4. Your speckle-based approach tracks contraction, which is a mechanical readout downstream of the electrical activation. There is necessarily an electromechanical delay between the action potential wavefront and what you actually measure. May I know how large is this delay, and if it is spatially uniform across the culture? If it varies from region to region, your propagation angle measurements could carry a systematic bias I think. Your manuscript did not show any direct comparison with voltage- or calcium-sensitive dye recordings from the same preparations. In my opinion, such a comparison would go a long way toward validating the fidelity of your approach.

We agree that our speckle-based approach measures contraction-associated mechanical deformation downstream of electrical activation, rather than membrane voltage or calcium directly. We have clarified in Section 2.1 that this readout can serve as a surrogate for electrical activation when excitation–contraction coupling is preserved (see lines 108-111). Previous studies support this concept. Christoph et al. (doi: 10.1063/5.0023751) and Liu et al. (doi: 10.1021/acsphotonics.2c01644) demonstrated that activation dynamics can be inferred from mechanical motion under controlled conditions. In particular, Liu et al. showed comparable wavefront velocities and similar spatial patterns when using electrical and mechanical readouts in cardiac monolayers. We have included these references as suggested by Reviewer 3. Based on these references, the electromechanical delay is on the order of <300 ms, but its magnitude and spatial uniformity were not measured. We now include the possibility of differences between electrical excitation and mechanical activity based on spatial heterogeneity (see lines 437-439). In addition, we included lines 434-441 in the Discussion addressing that in case of fibrillating tissue, severe conduction heterogeneity or impaired calcium handling, the excitation-contraction coupling may be no longer valid.

5. You motivate the paper with tachycardia at 100–250 bpm, but your camera runs at 60 Hz. At 250 bpm that gives you roughly 14 frames per cardiac cycle right? But your own Eq. 2 shows the phase delay error scales inversely with sampling rate. So the question is have you estimated the measurement uncertainty at tachycardia rates, and does it stay comfortably below your 25° threshold?

We agree that at tachycardia rates, the phase-delay error becomes a practical limitation, when the camera caps the measurement frequency at its framerate of 60 fps. For example, using exemplary values of $v_c = 80$ mm/s, $R_{p,q} \cdot \cos(\vartheta - \alpha_{p,q}) = 7$ mm and $1/T = 250$ bpm, Eq. 2 yields a relative error of approx. 52 %. We now mention the framerate-related limitation in the Discussion (see lines 566-571). We further clarified that, in our implementation, the computation can run at up to 3.86 kHz. Thus, if the camera is replaced by a sensor with higher sampling rate, the relative error of phase delay measurement would decrease to approx. 0.8 %. This indicates that the limitation arises primarily from the imaging frame rate rather than from the computation algorithm itself.

Regarding the 25° threshold, we clarified that this value was suitable for our observations but should not be interpreted as a universal constant (see lines 480-490). For example, for a ROI spacing of 7 mm, a plane wave satisfying $v_c \cdot T \leq 100$ mm could be misclassified. Therefore, particularly when investigating different samples or using a different ROI geometry, this threshold needs to be adapted accordingly.

6. In the manuscript, you noted that both uncorrected contractions were preceded by sequences requiring multiple consecutive corrective stimuli. I think this is a pattern that worth exploring. I would like to know does repeated optogenetic pulsing alter local excitability or refractoriness in the tissue? And could there be like opsin desensitization or ionic loading effects that make each subsequent correction progressively harder? If so, that would be a meaningful limitation for any sustained or longduration application of this approach, and it would be helpful to see some discussion or data on this point.

The occurrence of uncorrected contractions after sequences requiring multiple consecutive corrective stimuli is an important pattern and may indicate a relevant limitation for sustained or long-duration applications. We have therefore expanded the Discussion to address the possibility that repetitive optogenetic stimulation could alter local excitability or refractoriness of the cardiomyocytes, for example due to incomplete recovery of ion channels, ionic accumulation, or the finite recovery kinetics of the employed opsin (see lines 515-519). Such effects could, in principle, reduce the efficacy of subsequent corrective stimuli and make repeated corrections progressively more difficult. In addition, we investigated whether different stimulation intensities/fluences affected the need for multiple corrective stimuli. The corresponding results have been added to Section 2.4.

Reviewer 3:

1. Introduction, but also in the discussion: Please carefully distinguish between electrical activation/deactivation and contraction/relaxation throughout the study, especially in the introduction. While these two processes are physiologically coupled via excitation-contraction coupling, they may not be coupled in the case of tachyarrhythmia, and such coupling might be absent in fibrillating tissue. Please revise the entire introduction accordingly, and explain in which cases deformation can be used as a surrogate for electrical activation (of course, with a temporal delay). Please also revise terminology, for example ‘contraction rhythm’ is not a commonly used term in the field.

We thank the reviewer for this important comment. We agree that electrical activation/deactivation and mechanical contraction/relaxation should be clearly distinguished throughout the manuscript. Although these processes are linked through excitation–contraction coupling, where electrical activation precedes and initiates mechanical contraction, this relationship may not always be preserved, particularly in tachyarrhythmic or fibrillating tissue. We have revised the Introduction and Section 2.1 to state more explicitly that our approach uses mechanical motion as surrogate for electrical activation, with an associated temporal delay (lines 82-84 and 108-111). In the Discussion, we now additionally address the limitation that, for example in fibrillating tissue, the coupling between electrical activation and mechanical deformation may not be guaranteed, which limits the applicability of deformation-based inference of electrical activation (see lines 434-441). We also revised the terminology throughout the manuscript to consistently distinguish between electrical activity and mechanical motion/ contraction, and replaced non-standard wording such as “contraction rhythm”.

2. The distinction between focal tachycardia and reentry tachycardia is unclear given the scope of the study. In the introduction, you may want to distinguish between atrial and ventricular arrhythmia, given that contraction of the heart is mainly driven by the ventricular walls. I would advise to first explain the different electrical activation patterns, before discussing the resulting alterations in (ventricular) contraction-relaxation behaviour. Please also adjust passages in the results part/discussion, e.g. lines 234-36 and lines 338-340. In case of a not sinus-node driven atrial tachyarrhythmia, ventricular activation maps might still appear normal, as ventricular activation would be AV-node driven. Depending on the atrial rhythm, there might be a partial or even full dissociation between atrial and ventricular activation.

We revised the Introduction to more clearly distinguish between focal and reentrant activation patterns, as well as between atrial and ventricular arrhythmias. In particular, we now first describe the underlying electrical activation patterns before discussing their possible consequences for contraction. We also clarified that our study focuses on local activation dynamics in simplified *in vitro* monolayers, while whole-heart atrial and ventricular activation can be partially or fully decoupled due to AV-node

conduction (see lines 51-59). Furthermore, we now explicitly state that mechanical deformation is used as a surrogate for electrical activation based on excitation–contraction coupling, while the associated temporal delay and the limitations of this surrogate relationship are integrated in Section 2.1 (see lines 108-111) and in the Discussion (see lines 434-441). We also adjusted the mentioned passages in the Results part and the Discussion (see lines 347-350 and 539-542).

3. Throughout the study, the authors claim to have implemented the first all-optical, closed-loop strategy for cardiac arrhythmia termination. However, closed-loop optogenetic approaches have been suggested more than ten years ago (e.g. see <https://pmc.ncbi.nlm.nih.gov/articles/PMC4775736/>) and have been successfully applied to cardiac tissue or cells (e.g. see <https://pmc.ncbi.nlm.nih.gov/articles/PMC10753386/>; and very recently: <https://pubmed.ncbi.nlm.nih.gov/41684280/>; <https://www.science.org/doi/10.1126/sciadv.abq7469>)

We agree that closed-loop optogenetic approaches have previously been proposed and applied in cardiac research, including real-time all-optical stimulation strategies for controlling cardiac rhythm and closed-loop all-optical cardiac electrophysiology approaches. We have therefore revised the manuscript to avoid overstating the novelty of our work. Specifically, we now mention the concept of all-optical closed-loop strategies in the Introduction and have integrated the relevant literature suggested by the reviewer (see lines 71-73). In addition, we added the recent all-optical closed-loop approach for spiral wave termination by Deng *et al.* in the Discussion (see lines 546-548). To avoid any ambiguity, we have also removed the phrase “for the first time” throughout the manuscript. The focus of our work is the implementation of closed-loop control not only in the temporal domain, i.e., stimulus timing, but also in the spatial domain, i.e., feedback-based adaptation of the illumination pattern according to the detected wavefront orientation. This spatial feedback component enables the system to respond to ongoing changes in propagation direction and thereby extends beyond closed-loop strategies based solely on temporal triggering.

4. Please also refer to the pioneering work by Jan Christoph, demonstrating the feasibility of using mechanical activity to infer electrical activity, e.g. <https://pubmed.ncbi.nlm.nih.gov/33380038/>

Thanks for this hint. We have now included the reference by Christoph and Lebert when describing the surrogate approach in section 2.1 (see line 110), namely the use of mechanical activity to infer electrical activity.

5. The electrical and mechanical connectivity of hiPSC-cardiomyocytes strongly depend on the density of the cells in the culture. Please provide the number of cells seeded by area, and please provide a representative image of the cells within the measurement chamber.

Since the electrical and mechanical connectivity of hiPSC-CMs depends on cell density, we have added the seeding density to the Methods section (see lines 613-614). Specifically, 2-3 million cells were seeded per well (area: 9.6 cm²).

We have included a representative image of the cells within the measurement chamber in Fig S1 of the Supplementary Information and mentioned the name of the utilized incubator in the Methods section 4.4.

6. You choose a phase delay of 25° to distinguish a planar wave from a rotating wave. How translatable is this distinction to cardiac tissue with physiologically occurring conduction pathways and their variations? Why did you use 45° as threshold for applying light later on?

The 25° phase-delay threshold should not be interpreted as a universal constant. As mentioned in the respective Results section (see lines 223-227) and now additionally clarified in the Discussion (see lines 480-490), this threshold is suitable for the local observations and ROI geometry used in the present

implementation, but it depends on parameters such as ROI spacing, wavefront velocity, and contraction period. Therefore, when applying the approach to different samples, different geometries, or tissue with physiologically occurring conduction pathways and greater heterogeneity, the threshold would need to be adapted accordingly.

In more complex propagation patterns, the current sparse sampling strategy based on a 2×2 ROI arrangement may need to be extended, for example to a 3×3 or 4×4 grid. Such an extension would allow a more detailed reconstruction of the local propagation direction and could also support rotor identification by comparing propagation angles across multiple local regions. We now mention this as a possible direction for future studies (see lines 495-499).

We would like to clarify the distinction between the two thresholds, as both are expressed in degrees but refer to different quantities. The 25° threshold refers to a phase-delay threshold used to distinguish between planar and rotating contraction wavefronts. In contrast, the 45° threshold used later for light application is an angular threshold related to the spatial propagation direction of the wavefront. This threshold was chosen because the goal of the closed-loop intervention is to control spatial wavefront properties — specifically, the propagation direction — in a proof-of-concept framework.

7. If I understand correctly, the light stimulus is delivered in-between two contractions (e.g. at $T/2$). Please discuss limitations of this approach, especially in the case of fast rhythms when full diastolic repolarisation/relaxation might not be reached.

Applying the corrective stimulus at $T/2$ after the last observed contraction assumes sufficient recovery between successive contractions. We have now added a discussion of this limitation (see lines 519-523). In particular, at high beating frequencies, full diastolic repolarization and mechanical relaxation may not be reached before the next stimulus, which could reduce local excitability and thereby affect both the efficacy and the optimal timing of the corrective stimulation. We further note in the Discussion that such high-frequency regimes could be detected by the individual sensors in our closed-loop framework (see lines 523-525). This would allow future implementations to adapt the stimulation timing or strategy accordingly, for example to better handle cases in which incomplete recovery between contractions limits the effectiveness of stimulation.

8. In addition, you apply a light strip perpendicular to the desired wavefront propagation to reset the direction of the wavefront. In Figure 3, you state two different light intensities and two different durations of light applied. How did you determine the optimal parameters? Why did they differ between individual experiments? Which wavelength was used? What were the x-y dimensions of the applied light strip, and did it always extend to the boundaries of the field of view/chamber? To further understand the methodology, it would be beneficial to include a time-resolved schematic with a propagating wavefront and the corresponding illumination pattern(s), both depicting a case with single illumination and one where multiple attempts were needed.

The stimulation was applied in the above-threshold regime. The corresponding strength-duration curve is provided in Supplementary Note 2. Different stimulation durations and intensities were used across the closed-loop experiments. To assess whether these stimulation parameters influenced the number of required corrective stimuli, we have now added an evaluation of this relationship in section 2.4.

The wavelength used for optogenetic stimulation throughout the manuscript was 589 nm, corresponding to the action spectrum peak of Chrimson. This was already stated in section 2.1 (see lines 96-97) and now added to the Methods section (see lines 624 and 625).

Further, we have added a paragraph in section 2.4 describing the geometry of the applied light stripe (see lines 363-366). The light stripe had an area of 3.65 mm² and always extended to the boundaries of the FOV.

Following the reviewer's suggestion, we added a time-resolved schematic illustrating the relationship between the propagating wavefront and the corresponding illumination pattern. This schematic is now

included in Figure 4 and depicts a case in which a single corrective stimulus was sufficient and a case in which two corrective stimuli were required.

9. Please explain the underlying mechanism by which the wavefront was re-directed. Did you test different light intensities (e.g. sub-threshold and above-threshold regimes)?

We have clarified in section 2.4 that wavefront redirection was achieved using above-threshold optogenetic stimulation, initiating a new contraction wavefront (see lines 360-362).

The stimulation parameters were chosen in the above-threshold regime to ensure reliable activation, as supported by the strength-duration curve provided in Supplementary Note 2. We tested different light intensities within this above-threshold regime and, as mentioned in our response to the previous comment, we now included in section 2.4 an evaluation of the impact of stimulation intensity/ fluence on the need for multiple corrective stimuli.

10. In Figure 2, the provided conduction velocities are significantly lower than in normal myocardium, and the provided rates are unphysiologically low. Have you tested the presented cardioversion approach at frequencies as present in tachyarrhythmia e.g. at 2 Hz or faster?

We thank the reviewer for raising this point. No, the presented cardioversion approach was not tested at frequencies of 2 Hz or higher in the current study. The experiments were conducted at the spontaneous beating rates of the hiPSC-CM monolayers, which were lower than typical tachyarrhythmic rates, and the conduction velocities were also lower than those observed in normal myocardium. We now acknowledge this as a limitation of the present proof-of-concept study and clarify that future experiments should assess the performance of the approach under faster pacing conditions that better reflect tachyarrhythmia-relevant frequencies (see lines 527-530). However, we consider the challenges associated with higher frequencies to be primarily technical rather than conceptual, since the approach could in principle be extended to faster regimes using components with sufficiently high temporal resolution, including faster sensors.

11. Please discuss challenges of translating the presented cardioversion approach into 3D tissue and myocardium. In this regard you mention the use of 3D organoids. How representative are currently available organoid models in respect to anisotropic conduction velocity as present in normal myocardium? Why not use tissue slices instead?

We thank the reviewer for this important comment. We agree that translation of the presented approach from monolayers to 3D tissue and ultimately myocardium poses additional challenges, particularly because anisotropic conduction, three-dimensional tissue architecture, and physiologically occurring conduction pathways need to be considered. To address this point, we revised the Discussion and no longer explicitly refer to organoids as the primary next model system (see lines 548-560). Instead, we now discuss 3D cardiac constructs more generally and specifically mention tissue-engineered heart muscle and cardiac slice cultures as intermediate models for future validation. In particular, cardiac slice cultures are advantageous in this context because they preserve the native three-dimensional cellular and fiber architecture and are therefore expected to better maintain anisotropic conduction behavior than current organoid models. We further clarify in the revised Discussion that clinical translation will require homogeneous expression of an optogenetic tool and additional adaptation of the sparse-sampling-based wavefront characterization approach to account for anisotropic conduction in 3D tissue.

12. Please make sure to label/explain all variables used in the Figures in the Figure legends. In Figure 2fh please label the x-axes of the graphs provided

We thank the reviewer for pointing this out. We have revised the figure legends to ensure that all variables used in the figures are defined and explained. In addition, we have labeled the x-axes in Figure 2f,h as requested.

13. Please correct spelling mistakes/typos

We have carefully proofread the revised manuscript and corrected any spelling mistakes or typographical errors identified during revision.

The authors thank the reviewers for their valuable comments.

On behalf of all authors,
Robert Wendland

Response to the Reviewers

Nature Communications Engineering submission,
Manuscript ID: COMMS-ENG-26-0039,
Type: research article

Title: All-Optical Closed-Loop Control of Human Cardiomyocyte Networks Exploiting Holographic Optogenetics

Authors: Robert Wendland, Felix Schmieder, Muhammad Atif Sikandar, Frithjof Paul Knüppel, Wolfram-Hubertus Zimmermann, Olaf Bergmann, Lars Büttner, Jürgen Czarske

black: Comments of the Reviewers

[blue: the authors' reply to these comments](#)

Please note that the referenced text passages refer to the revised manuscript (**with markup**).

Reviewer 1:

The authors have made a substantial effort to address the reviewers' concerns. In particular, the added simulation-based analysis of ROI number, ROI geometry, noise sensitivity, wavefront curvature, and achievable cycle time strengthens the methodological basis of the sparse-sensing framework. However, one concern remains insufficiently resolved. The added analyses do not fully address the need for an appropriate open-loop baseline. The rebuttal explains why open-loop stimulation is expected to be less robust, but this is not equivalent to a controlled comparison using the same directional-correction endpoint. The manuscript should either include a matched open-loop comparison, even if limited, or substantially soften claims that the closed-loop strategy provides superior spatiotemporal regulation. In summary, the revision improves the manuscript, I would recommend a minor revision before acceptance.

[We agree that the added open-loop pacing analysis does not constitute a matched open-loop comparison using the same directional-correction endpoint. To address this concern, we have revised the corresponding Discussion paragraph and substantially softened the claims regarding the benefit of closed-loop control. Specifically, we no longer state that the closed-loop strategy provides superior spatiotemporal regulation compared with an open-loop approach. Instead, we now describe the rationale for using feedback-guided stimulation in the present proof-of-concept implementation \(see lines 504-511\).](#)

Reviewer 2:

Thank you for the thorough revision. Most concerns have been satisfactorily addressed.

Two minor textual additions I think would help the final version: (1) an explicit acknowledgement that the spatial uniformity of the electromechanical delay was not measured, and that spatially heterogeneous delay could bias propagation angle estimates; and (2) inclusion of the quantitative phase delay error at tachycardia rates (~52% at 250 bpm) discussed in the rebuttal but absent from the manuscript.

I do not anticipate a further round of review.

We thank the reviewer for the positive assessment of the revised manuscript and for pointing out these two remaining textual clarifications. We have now incorporated both points into the Discussion. First, we now explicitly acknowledge that the magnitude and spatial uniformity of the electromechanical delay were not quantitatively assessed in the present study. We further clarify that spatial heterogeneities in this delay could bias mechanical-motion-derived estimates of electrical propagation, including the propagation angle (see lines 430 and 436). Second, we have added the quantitative phase-delay error at tachycardia-relevant rates to the Discussion. Specifically, we now state that the relative phase-delay error decreases with increasing sampling frequency but increases at higher contraction frequencies and may therefore become substantial at tachycardia-relevant rates. To make this limitation quantitative, we included the previously discussed illustrative estimate of approximately 52% at 250 bpm for the current 60 Hz camera-based implementation (see lines 568-575).

Reviewer 3:

The authors have carefully revised the manuscript, addressing all points raised during the initial review.

We thank the reviewer for the positive assessment of our revised manuscript and are pleased that the revisions have addressed the points raised during the initial review.

The authors thank the reviewers for their valuable comments.

On behalf of all authors,
Robert Wendland

Supplementary Information

“All-Optical Closed-Loop Control of Human Cardiomyocyte Networks Exploiting Holographic Optogenetics”

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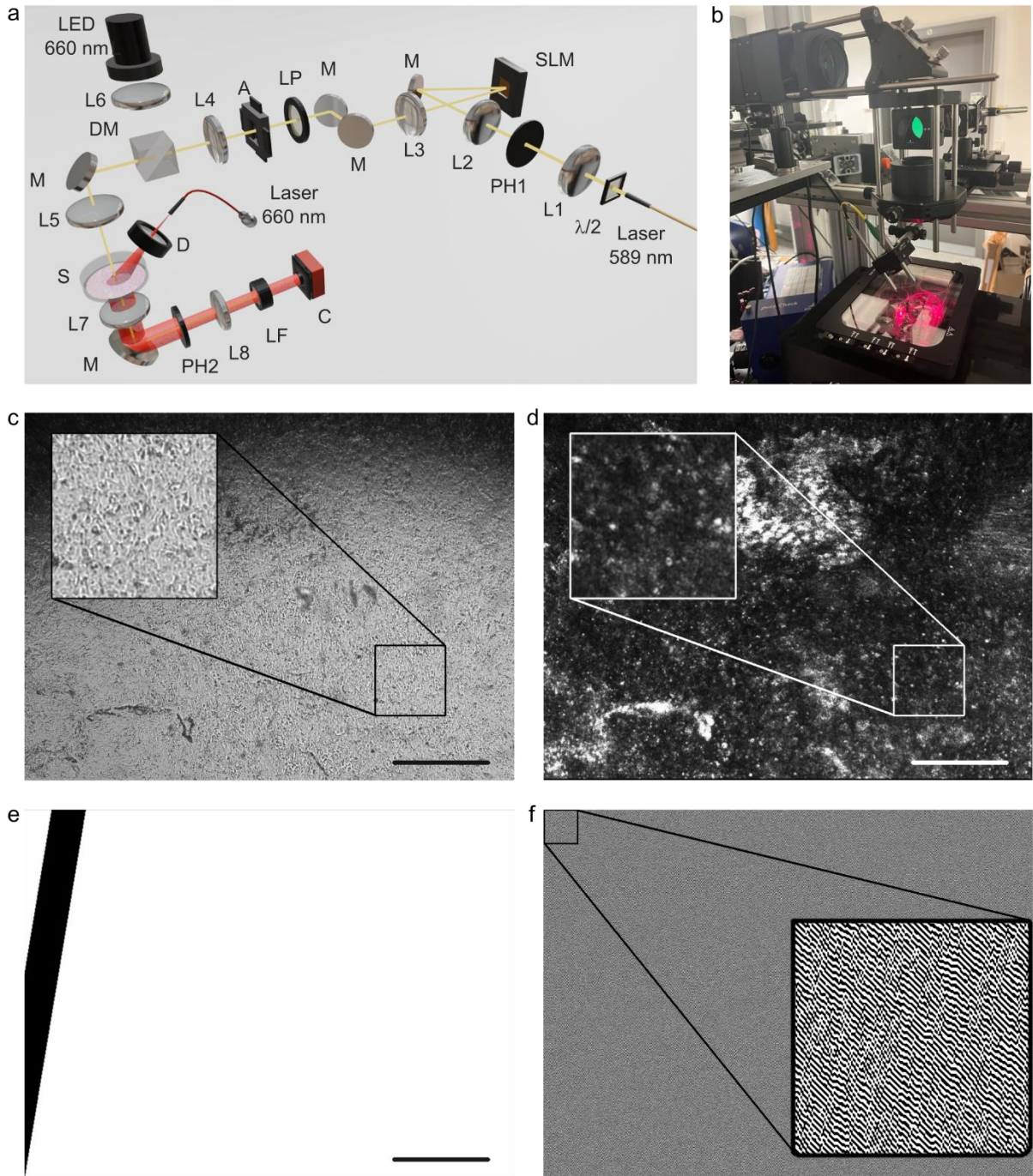
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Supplementary note 1: Optical Setup for Label-Free Imaging and Holographic Stimulation

The complete optical setup is visualized schematically in supplementary figure S1a. The stimulation path is highlighted in yellow, while the imaging path is visualized in red. To realize holographic stimulation of the f-Chrimson¹ modified human cardiomyocytes, a fiber-coupled (Thorlabs P5-405BPM-FC-2; Thorlabs PAF2A-18A) laser (MPB Communications 2RU-VFL-Series, $\lambda=589$ nm; 200 mW) is used. The collimated beam is expanded by the lenses L1 (Thorlabs AC254-030-A) and L2 (Thorlabs AC508-150-A) to illuminate a fast binary ferroelectric SLM (Forth Dimension Displays QXGA-3DM M175). The half wave plate $\lambda/2$ (Thorlabs WPH10M-588) is used to ensure the SLM is illuminated in its preferred polarization angle. Pinhole PH1 (Thorlabs VRC4CPT) acts as spatial frequency filter located in the Fourier plane between lenses L1 and L2. The SLM displays binary phase only Fourier computer-generated holograms (CGHs), generated as described in the Methods section of this paper. The holograms' first diffraction order is selected as the stimulation source of the sample using a rectangular aperture A (homebuilt, 0-3 cm $\times$ 0-4 cm). The binary SLM allows polarization shifts of approx. 60°. The linear polarizer LP (Thorlabs LPVISE200-A) is used to project the polarization of the light coming from the SLM onto its polarization axes, yielding a binary 180° phase modulation. The lens L3 (Thorlabs ACT508-250-A) performs the transformation of the holograms from the Fourier domain. The resulting stimulation pattern is magnified and projected onto the sample S by the lenses L4 (lens combination of Thorlabs AC508-080-A and Thorlabs LC1611-A) and L5 (Thorlabs AC508-150-A). To acquire widefield images of the cardiomyocytes, a 660 nm LED (Thorlabs M660L4) together with lens L6 (Thorlabs AC254-030-A) is integrated into the setup. The beam is aligned with the stimulation beam on the optical axis by a dichroitic mirror DM (Thorlabs DMSP605R). To realize the tracking of contraction wavefronts label-free, a 660 nm laser source (Quantum MPC6000 IGNIS 660) is coupled into a fiber (Thorlabs S1FCA, Thorlabs FT030-Y), whose output is obliquely mounted above the sample (see Fig. S1b). In front of the fiber output a diffuser D (Thorlabs DG10-600) is mounted to realize homogenous illumination of the sample in the FOV. The oblique laser illumination results in speckle patterns, changing with the contractions of the sample. These speckle patterns are projected onto the camera C (IDS uEye UI-3040SE-M-GL) by the lenses L7 (Thorlabs AC508-150-A) and L8 (Thorlabs AC508-075-A). The pinhole PH2 (Thorlabs ID25) allows for spatial filtering in the Fourier plane. To stop stimulation light from reaching the camera, a laser line filter LF (2x Thorlabs NF594-23) is mounted in front of the

camera. All mirrors are Thorlabs PF10-03-F01 or Thorlabs PF20-03-F01. The PC used for
executing closed loop control and data analysis uses an Intel Core i5-9500 CPU at 3.00 GHz as
well as 16 GB of RAM at 2667 MHz.



Supplementary Figure S1 Setup and exemplary images for data acquisition and conduction of experiment. (a) Schematic of the complete setup. The schematic was created using assets from the Optical Components Pack v1 by Ryo Mizuta Graphics. (b) Representative image of the samples in the incubator during an

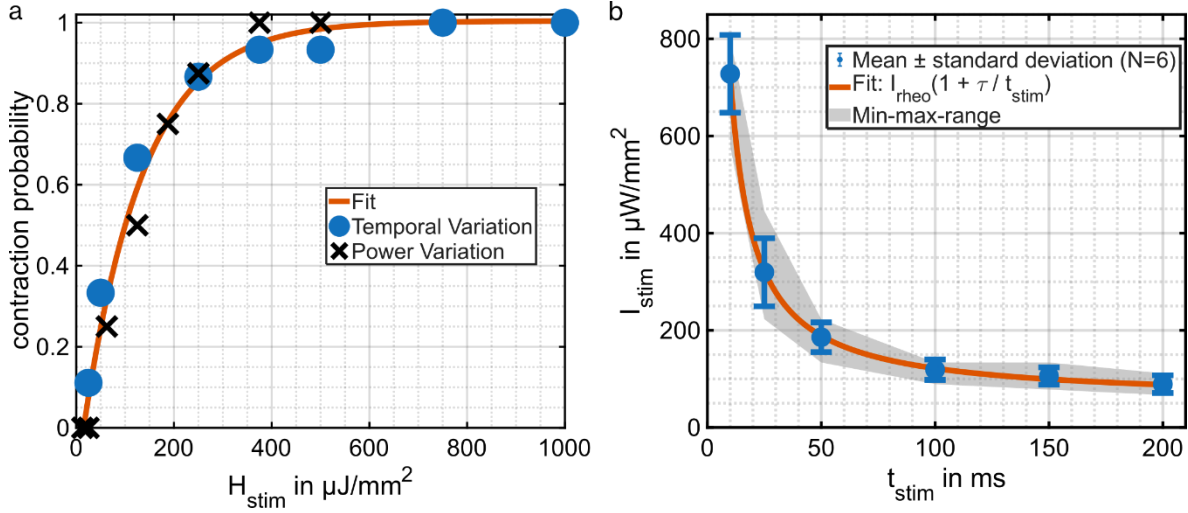
experiment. (c) Widefield image of LED-illuminated cardiomyocyte monolayer. (d) Speckle pattern resulting from oblique laser illumination at the same center wavelength. (e) desired stimulation pattern, the angled black bar denotes the illuminated area. (f) Computer-generated binary phase hologram to create the pattern shown in (d). All insets magnified from the small framed areas. Scale bars: 2 mm.

The all-optical tracking of contraction wavefronts relies on contraction-induced local changes of the intensity of speckles created by 660 nm laser light passing the investigated cell networks, an approach similar to temporal difference speckle methods². As shown in supplementary figure S1c, widefield illumination with 660 nm provides little contrast for contractions, especially for increasing FOVs, since intensity changes get mapped to ever less pixels. Supplementary figure S1d shows an example of the speckle contrast achieved with our setup for the same location on the same sample as in supplementary figure S1c, resulting in better dynamic range of intensity changes on the camera. Experiment-specific stimulation patterns are generated as images denoting the desired locations for stimulation marked in black as in supplementary figure S1e. For the calculation of the wavefront-shaping holograms, these images are converted to complex matrices by adding a random phase to each pixel. Then, images are transformed according to Eq. 3, taking into account the physical properties of our setup, and then Fourier-transformed and binarized. This process results in a computer-generated hologram as in panel f in supplementary figure S1, where the inset shows a magnified portion of the hologram. Scale bars for all marked images represent a distance of 2 mm.

Supplementary note 2: Characterization of Optical Stimulation Efficiency

We first characterized the stimulation efficiency of cardiomyocytes by exposing a round culture patch of $A_{\text{stim}} = 0.56 \text{ mm}^2$ for $t_{\text{stim}} = 100 \text{ ms}$ to increasing levels of intensity I_{stim} from 0 to 5 mW/mm^2 , measured at the sample plane, which resulted in guaranteed contractions starting from 3.75 mW/mm^2 . Similarly, we tested the dependency of the contraction on the duration of the stimulus t_{stim} by exposing 0.56 mm^2 culture patches to increasing durations of 3.75 mW/mm^2 light stimuli ranging from 10 ms to 200 ms, resulting in guaranteed contractions starting from 150 ms. Both experiments can be summarized using the radiant exposure $H_{\text{stim}} = I_{\text{stim}} \cdot t_{\text{stim}} / A_{\text{stim}}$ required to elicit contractions, as can be seen in supplementary figure S2a. Both data agree well with an inverse exponential fit, suggesting that in the investigated regime the energy supplied to the cells is the crucial parameter. According to the fit, a 95 % probability to stimulate contraction can be achieved by illuminating cell cultures with a radiant exposure of approx. $380 \text{ } \mu\text{J/mm}^2$.

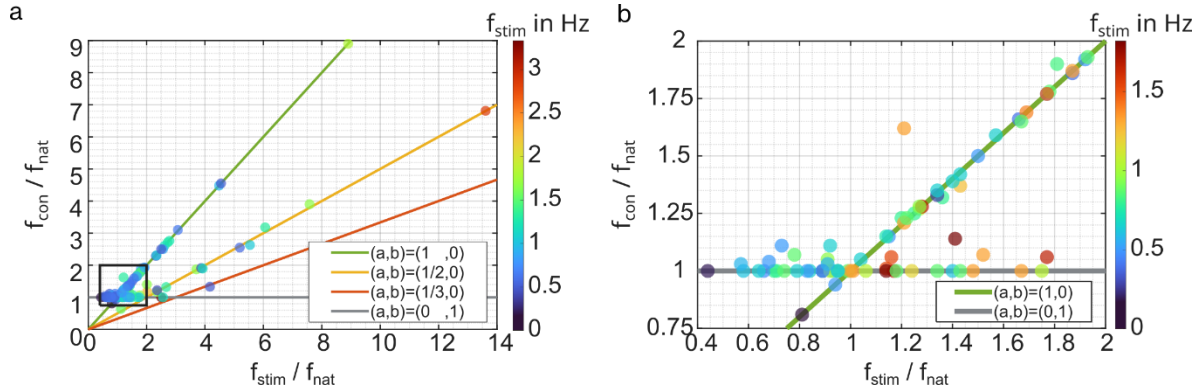
Further, we determined rheobase and chronaxie of our cell cultures^{3,4}. Rheobase is defined as the “minimal [impulse strength] of infinite duration leading to excitation”. Chronaxie is the excitation pulse duration resulting in an excitation for a pulse strength of twice the rheobase (both from Ref. 3). We determined these parameters by increasing the stimulation intensity for each stimulation time (stimulation frequency $f_{\text{stim}} = 1 \text{ Hz}$) until we elicited a response in $N=6$ samples. Here, an illumination area of 3.14 mm^2 was used. The results of these experiments can be seen in supplementary figure S2b as mean $\pm$ standard deviation indicated in blue. The minimum and maximum stimulation intensity for each stimulation time is indicated by the gray-shaded area. Fitting a Weiss-Lapicque-curve $I_{\text{stim}} = I_{\text{rheo}}(1 + \tau/t_{\text{stim}})$ to the data^{3,4}, we can extract the rheobase $I_{\text{rheo}} = 54.5 \text{ } \mu\text{W/mm}^2$ and chronaxie $\tau = 123.3 \text{ ms}$.



Supplementary Figure S2 Characterization of contraction response as a function of optical stimulation strength. (a) Probability of eliciting cell contraction as a function of radiant exposure used for stimulation, summarizing two independent experiments altering the stimulation power and duration, respectively, for a constant size of the illuminated area of 0.56 mm^2 . Dots show constant power data with the time being varied; crosses show constant time data with power being varied. Red line shows an inverse exponential fit to which all data agree well (mean squared error $MSE = 2.5 \cdot 10^{-3}$). (b) Stimulation intensity I_{stim} over stimulation time t_{stim} for the determination of rheobase I_{rheo} and chronaxie τ of Chrimson-expressing human cardiomyocyte monolayers. Blue dots and error bars indicate mean and standard deviation measured over $N=6$ samples. Minimum and maximum stimulation intensities required for each stimulation time are indicated by gray shading. Orange curve represents the fit of the Weiss-Lapicque-curve $I_{stim} = I_{rheo}(1 + \tau/t_{stim})$. All experiments were conducted using an illumination area of 3.14 mm^2 .

We further investigated the effect of varying stimulation frequencies on the ability to elicit cell contractions sequences (see supplementary figure S3). Here, stimulation frequencies f_{stim} were selected as multiples of the spontaneously occurring contraction frequency f_{nat} of the respective cell culture. Across $N=141$ experiments, we observed spontaneous contraction frequencies ranging from 0.16 Hz to 2 Hz with a median of 0.85 Hz . Supplementary figure 3 shows the elicited contraction frequency f_{con} over the stimulation frequency, both normalized to the spontaneous frequency. All cultures following the linear function $f_{con}/f_{nat} = a \cdot f_{stim}/f_{nat} + b$ with $a = 1$ and $b = 0$ show a 1:1 coupling of reaction to stimulation, whereas data on the function with $a = 0$ and $b = 1$ do not react to stimulation at all. As can be seen, stimulation below the spontaneously occurring contraction frequency is largely ineffective. However, for very low

spontaneous frequencies, some cultures react to every second ($a = 1/2$) or third ($a = 1/3$) stimulus, see supplementary figure S3a.



Supplementary Figure S3 Characterization of contraction response as a function of stimulation frequency.

(a) Observed contraction frequency f_{con} over stimulation frequency f_{stim} , both normalized to the spontaneously occurring contraction frequency f_{nat} . Color codes the stimulation frequency in Hz. (b) Magnified section of (a), visualized by the black box in (a). Several separate regimes can be observed, divided into four regimes following linear functions $f_{\text{con}}/f_{\text{nat}} = a \cdot f_{\text{stim}}/f_{\text{nat}} + b$: Cultures following every stimulation ($a = 1; b = 0$), every second ($a = 1/2; b = 0$) or every third ($a = 1/3; b = 0$) stimulus and cultures not following stimulations at all ($a = 0; b = 1$). For all experiments an $H_{\text{stim}} = 195 \mu\text{J}/\text{mm}^2$ was used. Note that this low radiant exposure level may be the explanation why not all datapoints lay on the straight lines with positive slopes for $f_{\text{stim}}/f_{\text{nat}} > 1$.

Chrimson and its variants have, to the day of the writing of this manuscript, a more widespread application as a tool for the manipulation of neural populations with a recent increase in use in cardiology, in some instances combined with other tools for simultaneous stimulation and inhibition^{1,5-12}. Stimulus response observation methods vary between direct observation of action potentials^{1,6,9,10} and indirect macroscopic responses like muscle contractions^{7,8}, which, in addition to the varying Chrimson variants with different kinetics, complicates a direct comparison with the results of this study. Compared to directly stimulated neurons ($0.75 \mu\text{J}/\text{mm}^2$ to $100 \mu\text{J}/\text{mm}^2$), f-Chrimson-activated cardiomyocytes in our study require higher energies to elicit 1:1 pacing. In comparison to indirectly activated muscle tissue, our samples required either comparable radiant exposure, around $300 \mu\text{J}/\text{mm}^2$ (Ref. 8), or less, as values up to $500 \text{ mJ}/\text{mm}^2$ have been reported for indirect activation⁷. Studies also investigating the direct optical stimulation of f-Chrimson found much lower requirements with $0.1 \mu\text{J}/\text{mm}^2$ using atrial-engineered human myocardium¹¹. Other studies using the opsin Channelrhodopsin-2 (ChR2) found that stimulating larger areas of samples

results in comparably lower required stimulation intensities¹³, a fact which is reflected by our results shown in supplementary figure S2. This suggests that in future experiments, larger areas could be illuminated to lower response thresholds, if necessary, i.e. to limit possible effects of phototoxicity. Regarding the chronaxie of our monolayers, the values we find are approximately two to three times as high as reported for Chrimson as expressed in the construct BiPoles in human cardiomyocyte monolayers¹⁴. Compared to Ref. 11, the rheobase value we find is half as high (54 $\mu\text{W}/\text{mm}^2$ vs. 118 $\mu\text{W}/\text{mm}^2$) at much higher chronaxie (123 ms vs. 5 ms), which is likely due to differences in the number of stimulated cells¹⁵.

Since our setup for the optical detection of contraction waves uses 660 nm light, there is an overlap with the action spectrum of Chrimson at roughly 20 % of the maximum (extrapolated from Ref. 1 and Ref. 9) which might lead to subthreshold excitation of our samples. As a consequence, stimulation thresholds reported here might be too low and refractory periods of the cells are possibly higher than without illumination¹⁶, possibly leading to lower achievable maximum stimulation frequencies.

164 **Supplementary note 3: Monitoring of the Contraction Activity and Beating Frequency**
 165 **Measurement**

166 The contraction activity of the cell cultures was measured by extracting a one-dimensional time
 167 signal from videos of contraction-induced temporal speckle fluctuations. The procedure is
 168 summarized in supplementary algorithm A1. It relies on evaluating the difference between each
 169 video frame F and a slowly varying background B , which is computed as the temporal mean within
 170 a window of size $(2 \cdot L_{BG} + 1)$ to account for general intensity changes. We set $2 \cdot L_{BG} + 1 =$
 171 101, corresponding to 1683 ms in our configuration. The absolute difference to the background
 172 AD is summed up over all pixels to obtain values of the time trace I_{raw} . A Tukey apodization
 173 window TW (75 % taper width) is applied to I_{raw} , yielding the windowed signal I_{win} . The signal
 174 is then transformed into the frequency domain, denoted by a hat ($\hat{I}_{win}$), where a second-order
 175 Butterworth bandpass filter BP is applied to remove low- and high- frequency artefacts unrelated
 176 to cardiomyocyte contractions. The filter was parametrized with a lower cutoff frequency of 0.2
 177 Hz and an upper cutoff frequency of 3 Hz.

Supplementary algorithm A1: Contraction activity extraction from speckle patterns

Input: video recording of speckle pattern F

Output: normalized time signal I corresponding to the contraction activity of the monolayers

1 **FOR** each video frame $F(m, n, i)$

2 update background: $B(n_x, n_y, i) = \frac{1}{2 \cdot L_{BG} + 1} \sum_{i-L_{BG}}^{i+L_{BG}} F(n_x, n_y, i)$

3 calculate absolute difference: $AD(n_x, n_y, i) = |F(n_x, n_y, i) - B(n_x, n_y, i)|$

4 sum up all pixels: $I_{raw}(i) = \sum_{\forall(n_x, n_y)} AD(n_x, n_y, i)$

5 **ENDFOR**

6 apply Tukey window: $I_{win}(i) = I_{raw}(i) \cdot TW(i)$

7 transform into frequency domain: $\hat{I}_{win}(f) = \mathcal{F}\{I_{win}(i)\}$

8 apply bandpass filter: $\hat{I}_{filtered}(f) = \hat{I}_{win}(f) \cdot BP(f)$

9 inverse transformation into time domain: $I_{\text{filtered}}(i) = \mathcal{F}^{-1}\{\hat{I}_{\text{filtered}}(f)\}$

10 normalize signal: $I(i) = \text{norm}_{\forall i}[I_{\text{filtered}}(i)]$

Algorithm 1: Contraction activity extraction from speckle patterns. i denotes a discrete time stamp, f a frequency component, and n_x and n_y denote the horizontal and vertical pixel indices within a frame.

178

179 This procedure enables the extraction of the contraction activity as can be seen exemplarily in Fig.
180 1d. Since the shape of the contraction peaks may vary, we calculated the beating frequency not
181 from the maxima but based on the temporal distance between the maxima of the temporal
182 derivative of the time signal I , which is plotted in Fig. 1e.

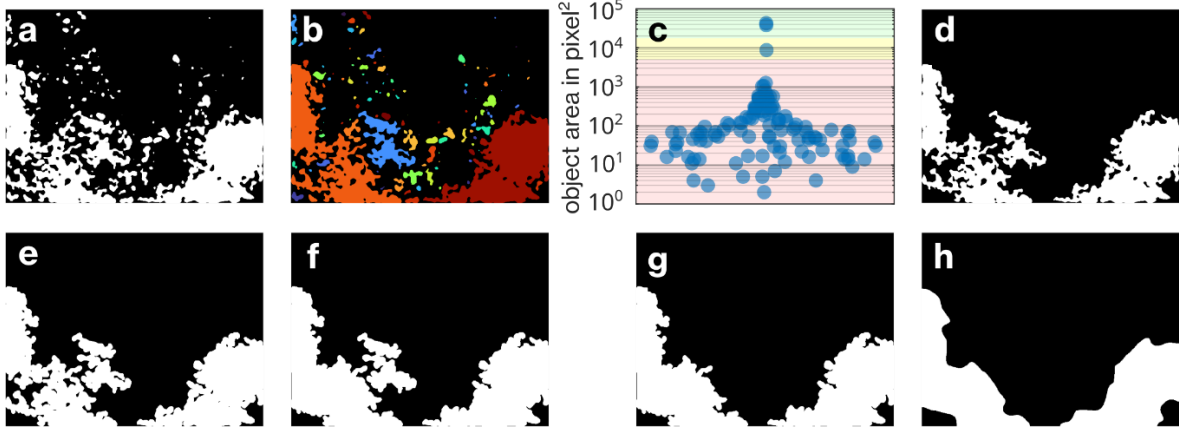
183

5	eliminate negative values:	$D_{\text{box}}^+(i) = \max_{\forall i}[D_{\text{box}}(i), 0]$
6	assign to spatial position	$F_{\text{der}}(n_x, n_y, i)$
7	ENDFOR	
8	FOR each derivative frame $F_{\text{der}}(m, n, i)$	
9	spatial smoothing:	$F_{\text{smo}}(n_x, n_y, i) = G(n_x, n_y) * F_{\text{der}}(n_x, n_y, i)$
10	ENDFOR	
11	normalization:	$F_{\text{res}}(n_x, n_y, i) = \text{norm}_{i-L_{\text{norm}} \leq i' \leq i}[F_{\text{smo}}(n_x, n_y, i')]$
Supplementary algorithm A2: Contraction wavefront visualization based on contraction induced intensity changes. i denotes a discrete time stamp, and n_x and n_y denote the horizontal and vertical pixel indices within a frame.		

200

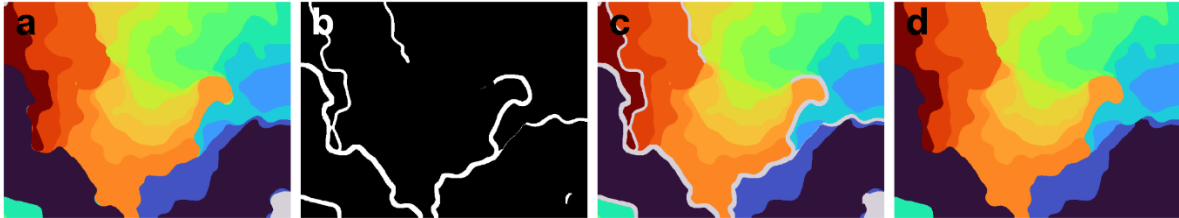
201 To reduce the computational effort of the following steps, only frame stacks from the processed
202 video F_{res} containing contraction events were analyzed. The mechanical activity was determined
203 using supplementary algorithm A1, and the start and end points of each contraction were identified
204 from the extrema of its temporal derivatives. For each contraction stack, an activity map was
205 generated as outlined in supplementary algorithm A4. First, the frames were binarized
206 (supplementary figure S4a). If the total excited area in one frame did not exceed a predefined
207 threshold (described below), the content of this frame was merged with the next one. Connected
208 pixel regions (“objects”) were then identified, their areas calculated, and sorted in descending order
209 (supplementary figure S4b). To limit computation, only the 20 largest objects were retained. From
210 these, only upper outliers exceeding a minimum area threshold defined as $A_{\text{obj}} \geq A_{\text{mean}} +$
211 $\frac{1}{3}[A_{\text{max}} - A_{\text{mean}}]$ were considered as part of the wavefront, where A_{mean} is the mean area and
212 A_{max} the maximum area within this subset (supplementary figure S4c,d). When the total excited
213 area exceeded a threshold, morphological dilation (disk-shaped structuring element, radius = 4 px)
214 was applied to connect wavefront segments (supplementary figure S4e). Holes within the
215 wavefront were filled (supplementary figure S4f), and the inverse image was also filled to remove

residual artefacts not connected to the wavefront (supplementary figure S4g). The result was smoothed using a Gaussian kernel (FWHM = 40 px). If regions of the FOV had not previously



Supplementary Figure S4 Image processing steps for wavefront shape extraction. (a) Processed frame F_{res} after binarization. (b) Identified objects in the binarized frame labeled in different colors. (c) Distribution of the areas of the objects from (b); retained large-area objects are highlighted in green; for demonstration purposes, additional retained objects are highlighted in yellow; discarded objects are highlighted in red. (d) Remaining objects in frame after area-thresholding. (e) Frame after dilation. (f) Frame after filling operation. (g) Frame after removal of the filled inverse. (h) Smoothed wavefront shape.

been active, the corresponding time stamp was written to the respective location in the isochrone map (supplementary figure S4h). This process was repeated for each frame in the contraction stack, yielding a temporary map (supplementary figure S5a).



Supplementary Figure S5 Image processing steps for isochrone map generation. (a) Superposed isochrone map extracted from a frame stack corresponding to a single contraction peak. (b) Regions with high local standard deviation are marked in white. (c) Isochrone map after removal of the regions identified in (b). (d) Final isochrone map.

In this map, pixels with a local standard deviation greater than $5 / f_{\text{cam}}$ were removed, where f_{cam} denotes the camera frame rate (supplementary figure S5b,c). The local standard deviation was calculated within an 11×11 px window. This threshold reflects the assumption that wavefronts propagate continuously, with each sampling step ($1 / f_{\text{cam}}$) yielding a follow-up image

239 of the wavefront located alongside the previous one. Remaining artefacts smaller than a minimum
 240 area threshold were removed. Pixels with missing values were finally filled by extending the
 241 borders of neighboring wavefronts into the corresponding regions (supplementary figure S5d),
 242 yielding the final activity map of the contraction event.

Supplementary algorithm A3: Creation of activity maps

Input: videos with visualized wavefronts F_{res} and time signal with mechanical activity signal I

Output: activity maps depicting contraction timing within the FOV for each contraction

```

1  calculate temporal derivatives of  $I$ 
2  find extrema in derivatives
3  derive frame stacks within a contraction event
4  FOR each frame stack
5      FOR each frame within stack
6          binarize video frames  $F_{\text{res}}$ 
7          (optional: cumulate with last frame)
8          identify connected pixels (objects)
9          FOR each object
10             calculate area
11          ENDFOR
12          sort objects by area
13          consider 20 largest objects
14          search for upper outliers and remove other objects
15          IF total excited area < minimal excited area
16             cumulate with next frame
17          ELSE
```

```

18         dilate objects
19         filling operation
20         remove artefacts, not connected to the wavefront
21         spatial smoothing
22         insert wavefront in temporary map with corresponding time stamp
23     ENDIF
24 ENDFOR
25     localize pixels with high local standard deviation in temporary map and replace
        them with NaNs
26     FOR each wavefront in temporary map
27         calculate area of the wavefront
28         IF area < minimal structure size
29             remove wavefront and replace with NaNs
30         ENDIF
31     ENDFOR
32     interpolate areas of NaNs
33     save final map of contraction activity
34 ENDFOR

```

Supplementary algorithm A3: Creation of activity maps. Note that in case of a rotating contraction frame stacks are derived from a local mechanical activity.

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Supplementary note 5: Real-Time Contraction Rhythm Extraction from Sparsely Sampled Mechanical Activity

To reduce computation time and enable real-time operation, time signals are extracted only from a limited set of predefined regions of interest (ROIs) within the field of view. Recorded videos are hardware-binned by a factor of two to further decrease processing load. The general principle of signal extraction follows the fundamental procedure described in Algorithm 1 and is summarized here in supplementary algorithm A4. For each ROI p of the currently recorded camera frame (i), the corresponding image segment F_p is extracted from the speckle pattern. This segment is then used to update the background estimate B_p for every ROI. Unlike in the offline approach, only preceding frames are available in the real-time setting. Therefore, a cumulative mean is employed for background estimation. Once B_p is estimated, the current value of the time signal $I_{p,\text{raw}}$ is computed as the pixelwise sum of absolute differences with respect to the background. Due to the sensitivity of the laser-speckle-based label-free imaging approach to externally induced motion—particularly external vibrations—the extracted signals are smoothed, yielding $I_{p,\text{smo}}$. This is achieved through an online convolution using a boxcar kernel BC of length L_{conv} , which introduces a temporal delay between contraction activity and its detection of $(L_{\text{conv}} - 1)/2$ frames (assuming L_{conv} is odd). A kernel length of 19 frames was selected, resulting in a delay of 150 ms. Following smoothing, the resulting signal I_p is obtained by normalizing $I_{p,\text{smo}}$ to the range $[0, 1]$ relative to the preceding $L_{\text{norm}} = 180$ frames. The binary activity state of each ROI is then determined by thresholding the normalized signal at $I_{\text{th}} = 1/2$. The timestamps of positive threshold crossings t_p are extracted and further used to analyze the propagation of the contraction wavefront.

Supplementary algorithm A4: Real-time capable signal extraction from ROIs

Input: current frame F , captured by the camera, activity threshold I_{th}

Output: time signals I_p corresponding to the mechanical activity and contraction times t_p for each ROI

1 **FOR** each ROI p

2	extract ROI image segment from F	$F_p(n_x, n_y, i)$
3	update background:	$B_p(n_x, n_y, i) = \frac{1}{i} \left[\frac{B_p(n_x, n_y, i-1)}{i-1} + F_p(n_x, n_y, i) \right]$
4	extract time signal:	$I_{p,\text{raw}}(i) = \sum_{\forall(n_x, n_y)} F_p(n_x, n_y, i) - B_p(n_x, n_y, i) $
5	smooth signal:	$I_{p,\text{smo}}(i) = I_{p,\text{raw}}(i) * BC(L_{\text{conv}})$
6	normalize signal:	$I_p(i) = \text{norm}_{i-L_{\text{norm}} \leq i' \leq i} [I_{p,\text{smo}}(i')]$
7	ENDFOR	
8	threshold signals with I_{th}	t_p
Supplementary algorithm A4: Real-time capable signal extraction from ROIs. i denotes a discrete time stamp, and n_x and n_y denote the horizontal and vertical pixel indices within a frame.		

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Supplementary note 6: Quantitative Real-Time Analysis of Planar Wavefronts Based on Sparsely Sampled Mechanical Activity

Supplementary algorithm 4 enables the acquisition of time stamps of contraction at distinct spatial locations. By modeling the contraction propagation as a plane wave, this enables the determination of characteristics, like propagation angle and wavefront velocity. If the wavefront passes through two ROIs p and q , located at $\vec{r}_p$ and $\vec{r}_q$, at times t_p and t_q with the velocity v_c the temporal lag $\Delta t_{p,q} = [t_p - t_q]$ fulfills:

$$\vec{k} \cdot [\vec{r}_p - \vec{r}_q] = v_c \cdot |\vec{k}| \cdot \Delta t_{p,q}, \quad (\text{S1})$$

where $\vec{k}$ denotes the wave vector. Using multiple ROIs, a linear system of equations,

$$\underline{A} \cdot \vec{b} = \vec{c}, \quad \text{with:} \quad (\text{S2a})$$

$$\underline{A} = \begin{bmatrix} \vec{r}_1 - \vec{r}_2 \\ \vdots \\ \vec{r}_p - \vec{r}_q \\ \vdots \end{bmatrix}, \quad \vec{b} = \frac{\vec{k}}{|\vec{k}|} \cdot \frac{1}{v_c} \quad \text{and} \quad \vec{c} = \begin{bmatrix} \Delta t_{1,2} \\ \vdots \\ \Delta t_{p,q} \\ \vdots \end{bmatrix}, \quad (\text{S2b})$$

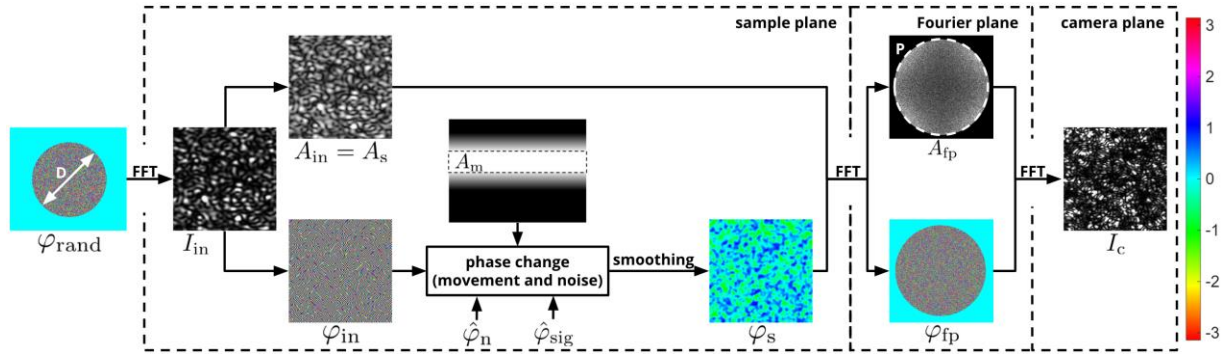
is created, where $\vec{b}$ is estimated by a minimum-norm least squares solution, yielding the propagation direction $\tan(\vartheta) = b_y/b_x$. The velocity of the wavefront v_c is then obtained from:

$$v_c = \frac{s_{p,q}}{\Delta t_{p,q}}, \quad \text{with:} \quad s_{p,q} = (\vec{r}_p - \vec{r}_q) \cdot \begin{pmatrix} \cos(\vartheta) \\ \sin(\vartheta) \end{pmatrix}, \quad (\text{S3})$$

where $s_{p,q}$ is the projection of the inter-ROI distance onto the propagation direction.

Supplementary note 7: Simulation of the Optical Setup for Label-Free Imaging

To systematically validate and assess limitations of the ROI-based sparse sensing approach (described in detail in section "Wavefront Characteristics from Sparsely Sampled Mechanical Activity", supplementary note 5 and 6), we simulated the optical setup described in supplementary note 1. The general idea is to simulate motion-induced intensity changes in the recorded speckle patterns based on predefined regions representing areas of cellular contraction. Subsequently, time traces—forming the basis of the ROI-based measurement approach—will be extracted, and the measured parameters will be compared with the simulation input. The simulation environment for motion-induced changes in speckle patterns is illustrated schematically in Fig. S6.



Supplementary Figure S6 Random phase values, φ_{rand} , are assigned within a circular region of diameter D . A Fourier transform is then used to generate a static speckle pattern in the sample plane, described by the input amplitude A_{in} and phase φ_{in} . To simulate motion, the phase φ_{in} is locally modulated within a defined modulation area A_{m} , with a maximum motion-induced phase change of $\hat{\varphi}_{\text{sig}}$. Noise is introduced by adding random phase variations across the full field, with a maximum noise-induced phase change of $\hat{\varphi}_{\text{n}}$. The resulting complex field in the sample plane, $A_{\text{s}} \cdot \exp(j \cdot \varphi_{\text{s}})$, including both motion-induced and noise-related phase variations, is propagated to the Fourier plane. In the Fourier plane, a circular pupil function P is applied, yielding the complex field $A_{\text{fp}} \cdot \exp(j \cdot \varphi_{\text{fp}})$. This field is subsequently propagated to the camera plane, where the simulated camera intensity distribution I_{c} is obtained.

First, a static speckle pattern is created with the approach described in Ref. 17: A circle with a diameter $D = 500$ px is filled with uniformly distributed random phase values φ_{rand} . The speckle pattern, corresponding to the intensity in the sample plane I_{in} is then calculated as:

$$I_{\text{in}} = \underline{E}_{\text{in}} \cdot \underline{E}_{\text{in}}^* \quad \text{with:} \quad \underline{E}_{\text{in}} = \mathcal{F}\{e^{j\varphi_{\text{rand}}}\}. \quad (\text{S4})$$

Here, $\underline{E}_{\text{in}} = A_{\text{in}} \cdot e^{j\varphi_{\text{in}}}$ denotes the initial simulated complex electrical field in the sample plane. The whole simulated plane has the dimensions $(759 \text{ px}) \times (1012 \text{ px})$, where the FOV covers $(543 \text{ px}) \times (724 \text{ px})$. To simulate motion within a defined area, a mask A_{m} defining the area of

sample motion is used. The binary mask is smoothed using a gaussian kernel. The phase values of φ_{in} where the smoothed mask has values greater than zero are varied and the difference to the initial phase value is scaled with the respective value of the smoothed mask. Further, all values of φ_{in} are additionally varied to model different noise levels (see Eq. S6). The electrical field in the sample plane $\underline{E}_s = A_s \cdot e^{j\varphi_s}$ consists of:

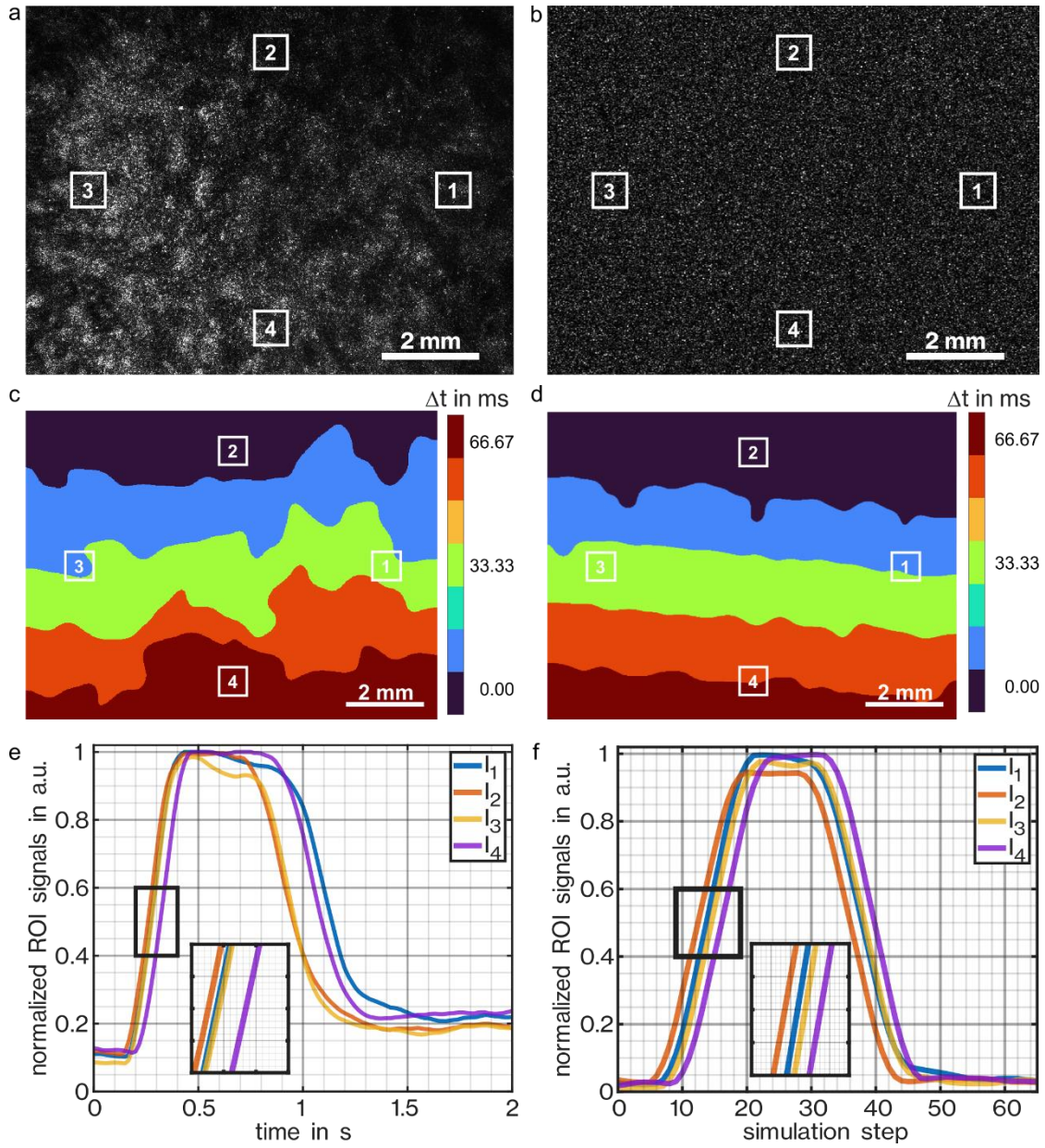
$$A_s = A_{\text{in}} , \quad (\text{S5})$$

$$\varphi_s = \varphi_{\text{in}} + [\hat{\varphi}_{\text{sig}} \cdot 2\pi \cdot U(0,1)]_{\text{motion}} + \hat{\varphi}_{\text{n}} \cdot 2\pi \cdot U(0,1) . \quad (\text{S6})$$

The symbol $U(0,1)$ denotes uniformly distributed random values between 0 and 1, and $\hat{\varphi}_{\text{sig}}$ and $\hat{\varphi}_{\text{n}}$ indicate the maximum phase changes due to the movement (signal) and noise respectively. To eliminate numerical artefacts of later Fourier transforms, the phase is smoothed using a Gaussian kernel with a standard deviation of 1 px. The field $\underline{E}_s$ is then propagated to the Fourier plane of the 4f-system, where the point spread function of the optical system is taken into account as multiplication with a circular pupil function P . Finally, the intensity distribution on the camera I_c is calculated via the Fourier transform as:

$$I_c = \underline{E}_c \cdot \underline{E}_c^* \quad \text{with:} \quad \underline{E}_c = \mathcal{F}\{P \cdot \mathcal{F}\{\underline{E}_s\}\} . \quad (\text{S7})$$

To validate the simulation environment, spontaneous contractions in a cardiomyocyte monolayer are evaluated using both label-free and ROI-based sparse sensing approaches, with the aim of sufficiently reproducing the experimental results through simulation of the optical setup. In Fig. S7a,b is the speckle pattern recorded during the experiment (left) compared with the simulated speckle pattern (right). The main observable differences are the intensity gradient and certain structural features in the experimental image that are not included in the simulation. However, as these are sample-specific and as long as the ROIs are sufficiently illuminated, they do not affect algorithm performance, since time traces corresponding to the motion of the cardiomyocytes can still be extracted. The activation map, shown in Fig. S7c corresponds to a spontaneous contraction of the cardiomyocyte monolayer.



Supplementary Figure S7 Comparison of an experimentally recorded spontaneous contraction with a simulated plane wave propagation. (a) Speckle pattern, acquired with the optical system. (b) Simulated speckle pattern. (c) Activity map derived from experimental measurement. (d) Activity map obtained from a simulated contraction using experimentally measured parameters. White boxes indicate the positions of ROIs (e) ROI signals I_1 to I_4 recorded during the experiment. (f) ROI signals obtained from the simulated setup.

Wavefront parameters were measured based on time traces extracted from the ROIs indicated as white boxes in the activity map. The propagation angle ϑ_{exp} was measured as 265° and the wavefront velocity $v_{c,\text{exp}}$ as 91 mm/s from the temporal delay of the rising edges of the four ROI

signals, shown in Fig. S7e. To mimic the dynamics of the plane wave, A_m was set to a stripe moving through the simulated FOV with speed $v_{c,exp}$ and orientation ϑ_{exp} . Then, the simulated speckle movement was processed using the same algorithm as for the experimental data. The resulting activity map is shown in Fig. S7d. As expected, the movement steps of the visualized wavefront are similar to the experiment, visualized in Fig. S7c. Only slight differences, originating from the assumption of an ideal plane wavefront can be seen, when comparing Fig. S7c with Fig. S7d. Also, time signals from the same ROIs were extracted from the simulation (Fig. S7f). Based on these time traces, the propagation angle and wavefront velocity were measured, yielding an orientation of the wavefront $\vartheta_{sim} = 263^\circ$ and a velocity of $v_{c,sim}$ of 90 mm/s, showing only a little difference compared to the experiment. When comparing the simulated time traces with Fig. S7e, the experimental traces show slight differences in peak shape. In particular, time traces I_1 and I_3 exhibit a small decrease during the plateau phase whereas the simulated traces remain close to their maximum. The shape of these experimental time traces is related to the prolonged contraction of the cardiomyocytes. During this time, the monolayers remain tense and the difference relative to the ROI-specific moving background approximation may decrease. This can lead to the local minima or slight decreases of the time traces in Fig. S7e after the first maxima. This does not pose a problem for evaluating the performance of the ROI-based measurement approach with the simulation, since the measurement only relies on the rising edges of the recorded time signals which corresponds to the propagation of the contraction wavefronts (not relaxation). As can be seen from the comparison of the rising edges of the time traces, the inputs for the measurement, namely the activation order of the ROIs and the respective temporal differences, are well matched between simulation and experiment. Therefore, the simulation environment proves to be suited to investigate limitations of the proposed ROI-based techniques, as well as for general investigations that are described in section “*Wavefront Characteristics from Sparsely Sampled Mechanical Activity*”.

Further, we estimated the practically relevant amplitude ratios $\hat{\phi}_n/\hat{\phi}_{sig}$. For this purpose, we analyzed no-contraction time windows in the experimentally recorded contraction-derived ROI signals, assuming that the remaining signal fluctuations during these intervals predominantly represent noise. We then compared the corresponding standard deviations with those obtained from simulations performed with defined noise amplitude ratios, as described above. For the

368 simulated signals, we found an approximately linear relationship between the imposed noise-to-
369 signal amplitude ratio and the resulting standard deviation σ_I of the time traces ($R^2 = 0.97$):

$$\hat{\varphi}_n / \hat{\varphi}_{\text{sig}}(\sigma_I) = 8.681 \cdot \sigma_I - 0.016. \quad (\text{S7})$$

370 Using this regression model, we estimated the experimental noise-to-signal amplitude ratios from
371 the standard deviations σ_I measured during no-contraction time windows in the experiments
372 presented in Fig. 2. One of the 13 experiments was excluded from this analysis because the
373 available no-contraction interval was very short, which would have resulted in a high uncertainty
374 of the estimated σ_I . Therefore, this analysis was performed with $N = 12$ experiments. The
375 resulting experimental noise-to-signal amplitude ratio was $\hat{\varphi}_n / \hat{\varphi}_{\text{sig}} = 0.23 \pm 0.11$
376 (mean $\pm$ standard deviation).

Supplementary note 8: Phase Delay Measurement Error

The maximum relative error in the phase delay measurement between two points p and q , resulting from uncertainties in the detection of the respective activation times, can be derived from Eq. 1 using standard error propagation:

$$\left| \frac{\Delta(\hat{\phi}_{p,q})}{\Delta\phi_{p,q}} \right| = \frac{1}{|\Delta\phi_{p,q}|} \left[\left| \frac{\partial(\Delta\phi_{p,q})}{\partial t_p} \cdot \Delta\hat{t}_p \right| + \left| \frac{\partial(\Delta\phi_{p,q})}{\partial t_q} \cdot \Delta\hat{t}_q \right| + \left| \frac{\partial(\Delta\phi_{p,q})}{\partial T} \cdot \Delta\hat{T} \right| \right], \quad (\text{S9})$$

where hats (^) denote maximum possible errors. The maximum uncertainty in activation time detection is the same for both points ($|\Delta\hat{t}_p| = |\Delta\hat{t}_q| = |\hat{t}|$). The period T is calculated as the time difference between two consecutive contractions at a single location and therefore doubly impacted by the error in activation time detection ($|\hat{T}| = 2 \cdot |\hat{t}|$), which is determined by the frame rate of the camera ($|\hat{t}| = 1/f_{\text{cam}}$). Applying this and expressing $|\Delta t_{p,q}|$ in terms of the projected distance between p and q in the direction of wave propagation and the wavefront velocity v_c , supplementary equation (S4) leads to the final formulation of the maximum relative error:

$$\left| \frac{\Delta(\Delta\hat{\phi}_{p,q})}{\Delta\phi_{p,q}} \right| = \frac{2}{f_{\text{cam}}} \cdot \left| \frac{v_c}{R_{p,q} \cdot \cos(\vartheta - \alpha_{p,q})} + \frac{1}{T} \right|, \quad (\text{S10})$$

where $R_{p,q}$ is the Euclidian distance between p and q , ϑ is the direction of wave propagation and $\alpha_{p,q}$ the angle of the vector connecting p and q . In supplementary equation (S5), the error of phase delay measurement is influenced, on the one hand, by the parameters v_c and T , which arise from the biophysical properties of the cardiomyocytes. On the other hand, controllable factors such as the chosen alignment of the ROIs and, above all, the sampling rate also affect the error. The sampling rate is of particular importance, as the measurement error is inversely proportional to it. Therefore, f_{cam} offers a powerful means to reduce uncertainty. Assuming our hardware and ROI alignment as shown in Fig. 2b, we set $R_{1,2} = 4.7$ mm, $\alpha_{1,2} = 127^\circ$ and $f_{\text{cam}} = 60$ Hz. For illustration we further use exemplarily values of v_c and T and $|\Delta\phi_{1,2}|$ obtained from one experiment in Fig. 2g,h, yielding $v_c = 70$ mm/s, $T = 3.57$ s and $|\Delta\phi_{1,2}| = 6.7^\circ$. The corresponding propagation angle was $\vartheta = -39^\circ$. Inserting all this in supplementary equation (S4), we get $|\Delta(\Delta\hat{\phi}_{1,2})| = 3.36^\circ$. Although the relative error may appear large in comparison to $|\Delta\phi_{1,2}| = 6.7^\circ$, it is important to note that the primary objective is to distinguish planar and rotating waves, where phase delays typically exceed single-digit values. Another noteworthy aspect is that the maximum relative error approaches infinity when the planar wave propagates

403 perpendicular to the interconnection of ROIs p and q . In this case, a phase delay may not be
404 detected if $|\Delta t_{p,q}| < 1/f_{\text{cam}}$. However, this does not pose a practical limitation for wavefront
405 classification: the phase delay $|\Delta \varphi_{p,q}|$ is then effectively treated as zero, leading to the correct
406 identification of a planar wavefront within our framework.

407

Supplementary note 9: Validation of Phase Delay Measurements

The phase delay $\Delta\varphi_{p,q}$ between two points p and q can be extracted from the temporal delay $\Delta t_{p,q}$ of a wavefront, passing these points ($\Delta\varphi_{p,q} = 360^\circ \cdot \Delta t_{p,q}/T$) relative to the contraction period T . This phase delay depends on several parameters. In the following, we describe the influence of both biological properties of cardiomyocytes and factors related to the ROI alignment. One influential parameter is the contraction period T , the inverse of the contraction frequency f_{con} . Since $\Delta\varphi_{p,q}$ is calculated relative to T , a longer contraction period results in a smaller phase delay, and *vice versa*. In cardiac monolayers, the contraction period is strongly affected by environmental factors such as temperature. Importantly, although T may vary, it is bounded from below by the effective refractory period (ERP) of the cardiomyocytes. Beyond the contraction frequency, the propagation velocity v_c of the contraction wavefronts also influences the measured phase delay. Variations in v_c may arise from sample inhomogeneities. Assuming that T and the ROI alignment remain constant, an increase in v_c reduces the measured phase delay between two points. The phase delay further depends on the interplay between biological properties and ROI alignment. Specifically, the relative angle between the straight line connecting ROIs p and q and the propagation angle $\vartheta - \alpha_{p,q}$ has impact on $\Delta\varphi_{p,q}$. In addition, the spatial separation $R_{p,q}$, which is directly determined by the ROI alignment, also affects the phase delay: greater spacing yields larger delays if all other parameters remain constant. These dependencies are summarized in the following table.

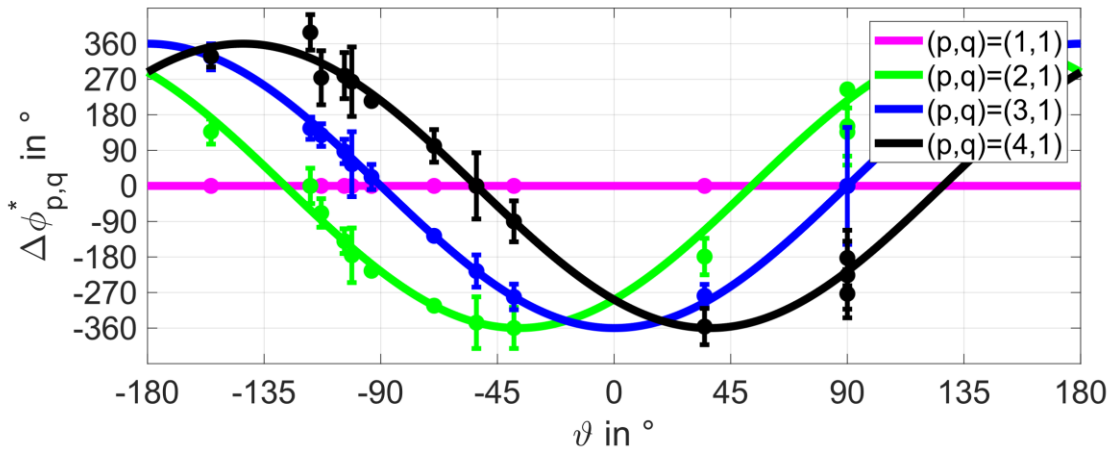
Parameter	Impact on phase delay
period T	longer contraction period leads to a decrease of the phase delay, since: $\Delta\varphi_{p,q} \sim \frac{1}{T}.$
propagation velocity v_c	higher propagation velocity leads to a decrease of the phase delay, since: $\Delta\varphi_{p,q} \sim \Delta t_{p,q} \sim 1/v_c _{s_{p,q}=\text{const.}}$
angle difference $\vartheta - \alpha_{p,q}$	change of the propagation direction affects the effective (projected) distance $s_{p,q}$ between the observation points and therefore: $\Delta\varphi_{p,q} \sim \Delta t_{p,q} \sim s_{p,q} _{v_c=\text{const.}} \sim \cos(\vartheta - \alpha_{p,q}).$
spatial distance $R_{p,q}$	increased distance between the observation points leads to an increase of the phase delay, since:

$$\Delta\varphi_{p,q} \sim \Delta t_{p,q} \sim s_{p,q} \Big|_{v_c=\text{const.}} \sim R_{p,q}.$$

Although many parameters vary and influence the measured phase delay, a phase delay threshold for distinguishing planar and rotational waves remains reasonable, since the variation of these parameters is limited, especially of the period T by the ERP. However, to validate the phase delay measurements between sparsely sampled signals across different experiments, a scaling of the measured phase delay with respect to the varying parameters is necessary. This scaled phase $\Delta\varphi_{p,q}^*$ can be derived as:

$$\Delta\varphi_{p,q}^* = \frac{T \cdot v_c}{R_{p,q}} \cdot \Delta\varphi_{p,q} = 360^\circ \cdot \cos(\vartheta - \alpha_{p,q}), \quad (\text{S11})$$

and is corrected with respect to the contraction wave parameters (T, v_c) as well as the geometrical arrangement of the ROI $(R_{p,q})$, enabling the validation of measured contraction wavefront characteristics. The angle between the ROI p and q (Fig. 2a) is denoted by $\alpha_{p,q}$. We then plotted the scaled phase measurements against the measured propagation angle and compared it with the theoretical expectation (right side of Eq. S11). The result is shown in supplementary figure S8. Each group of four vertically aligned data points belongs to one experiment, where propagation angle, velocity, period, and phase delay for each ROI with respect to ROI 1 were measured. The close agreement between the data and the theoretical model (mean average error MAE = 18.16°, equivalent to 5 % of the scaled phase amplitude) confirms the validity of our measurement approach and the resulting data.



Supplementary Figure S8 Scaled phase (see Eq. 11) for ROI pairs (p, q) plotted as median $\pm$ standard deviation over the measured propagation angle, based on multiple contractions of one cell culture; error bars

447 denote standard deviation. Solid lines indicate theoretical model of scaled phase:
448 $\Delta\varphi_{p,q}^* = 360^\circ \cdot \cos(\vartheta - \alpha_{p,q})$.
449

Supplementary note 10: Maximum Distance of a Detectable Rotor Core Based on Phase Delay Measurements

To quantify the detection limit of a rotational wave, we used a simple model, an uncurved wavefront rotating around $(x_c|y_c)$. Due to the symmetry of the ROI placement (diamond shape, as described in the main section), it is sufficient to observe only one quadrant of the coordinate system. The maximum phase delay,

$$\Delta\varphi_{p,q}^{\max} = \max_{p,q \in \mathcal{M}_{\text{ROI}}} |\Delta\varphi_{p,q}|, \text{ with: } \mathcal{M}_{\text{ROI}} = \{1, 2, 3, 4\}, \quad (\text{S12})$$

measured between two ROIs p and q as a function of the rotor core position $(x_c|y_c)$, normalized by the maximum distance $d_{\max}$ between any ROI and the central reference point $(x_0|y_0)$ is visualized in Fig. 2i.

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Data collection Matlab version 2023b, Matlab version 2025a, Microsoft Excel version 2016, uEye Cockpit version 4.96.0650

Data analysis Matlab and toolboxes: Curve Fitting Toolbox, Image Acquisition Toolbox, Image Processing Toolbox
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Replication All reported results were derived from experiments performed on at least six independent cell cultures.

Randomization Randomization for different experimental groups was not applicable as experiments were performed on uniform biological material, i.e. genetically modified cardiac cell cultures.

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Novel plant genotypes	n/a
Authentication	n/a