Remote focusing for 2-photon microscopy to follow action potential propagation transmurally in acute rabbit cardiac slices

Giedrė Astrauskaitė*[a], Sharika Mohanan[a], Lewis Williamson[a], Erin Boland[b], Eline Huethorst[b], Ryo Kinegawa[a], Godfrey Smith[b], Caroline Müllenbroich[a]

[a]School of Physics and Astronomy, University of Glasgow, Glasgow, United Kingdom, G12 8QQ;
[b]Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, United Kingdom, G12 8QQ
*g.astrauskaite.1@research.gla.ac.uk

ABSTRACT

In the heart, action potentials travel through the tissue to orchestrate muscle contraction and make the organ an efficient pump. Scar tissues caused by myocardial infarction impairs electrical conduction. In the rabbit heart, action potentials propagate from the endocardium to the epicardium with a conduction velocity of 30 cm/s. Therefore, a rapid vertical scan is necessary to observe this transmural cardiac conduction at cellular resolution. Here we present an implementation of a versatile remote focusing module, compatible with retrofitting to commercial two-photon microscopes and capable of 0.3 kHz rate axial scanning over the range of 100 µm in cardiac tissue without disturbing the sample or the sample objective. We discuss the necessary optimization to compensate for pulse broadening, power losses and optical aberrations. We demonstrate fast imaging of cardiac cell structure in functionally viable rabbit ventricular slice model. We will apply this system to resolve cardiac electrical signal propagation transmurally in healthy and infarcted hearts.

Keywords: 2-photon microscopy, cardiac electrophysiology, remote focusing, fluovolt, voltage sensitive dye, fast axial refocusing

1 INTRODUCTION

In the heart, natural pacemaker cells generate electrical wavefronts or action potentials (AP) which are transmitted through the organ in an orchestrated fashion. This electrical conduction governs muscle contraction making the heart an efficient pump. However, natural electrical pathways can be obstructed by scar tissue after myocardial infarction (MI). To study how post-MI structural remodeling leads to arrythmias, new methods to probe electrical conduction in healthy and diseased cardiac preparations are essential.

Optical mapping [1] with voltage sensitive dyes (VSDs) is widely applied to investigate cardiac electrophysiology *ex-vivo*, revealing electrical wavefront propagation in large-scale intact tissue. Nevertheless, this method does not allow to investigate cardiac cell layer conduction in depth below the epicardium. On the contrary, cellular resolution at deep tissue layers and optical sectioning can be achieved with two-photon fluorescence microscopy (2PM), where scattering robust infrared wavelengths are utilized [2]. In the heart, conduction velocity differs depending on direction. Along the long axis of rabbit cardiomyocytes it is around 75 cm/s and 2PM was used to resolve AP characteristics as deep as 500 µm in longitudinal planes in the ventricles of intact Langendorff-perfused hearts [3-5]. However, the slower (30 cm/s) transmural cardiac conduction from endocardium to the epicardium has not yet been investigated. This is because the longitudinal planes lie along the

galvanometric mirror scan axis, while conventional microscopes are not capable of rapid vertical scanning needed to resolve AP propagation transmurally. Traditional axial refocusing methods are slow or cause mechanical disturbance of the sample.

Several techniques have been investigated for fast axial refocusing. Electrically tunable lenses offer cost effective solutions that are easily implemented into existing hardware, however, they are not suited for high NA systems [6-7]. Adaptive optics such as spatial light modulators (SLMs) and deformable membrane mirrors (DMMs) can be used for axial refocusing [6, 8, 9], though active elements like SLMs and DMMs are costly, can introduce significant power losses and require complex calibration.

In remote focusing (RF) [10], an additional objective is introduced, creating a 3D optical copy of the sample. If a small mirror is placed in the focus of this remote objective, axial refocusing is enabled by repositioning the mirror. For rapid refocusing, voice coil actuators can be utilized, as they can execute small amplitude wavefronts approaching several hundred of Hertz range. Additionally, the spherical aberration introduced by imaging an image plane away from the objective's native focal plane is cleverly compensated by an equal but opposite amount of aberration in the second remote objective, yielding a self-correcting refocusing system. RF therefore affords the necessary spatial and temporal resolution necessary to follow the propagation of electrical wavefronts. It is possible to maintain cellular spatial resolution over a large dynamic range and the technique is compatible with 2PM [10].

Few RF-2PM microscopes have been reported [8, 11-13], with applications mainly in neural tissue [8, 11]. For instance, calcium transient responses were measured from neurons in a RF system capable of axial scan rate of 2.7 kHz [11]. However, light scatters significantly more in brain tissue than in cardiac preparations [14]. Slow (7 Hz) RF was used also with 3PM [15] as deep as 600 µm deep in mouse brain. In [12], sarcomere length of cardiomyocytes was measured at 2.7 kHz rate scanning with RF; while it was done in an intact, Langendorff-perfused heart, only cardiac structure, not electrical function was investigated. Electrophysiology of isolated cardiomyocytes and small cardiac preparations were probed with a bi-modal single-photon light sheet microscope (LSM) system [16-18]. While RF increased the throughput of the system, the geometry of oblique LSM does not accommodate larger preparations.

We report an implementation of a remote focusing module retrofitted to a commercial 2-photon microscope and optimised for investigating cardiac electrophysiology in terms of resolution, pulse duration and power retention. The system is capable of 0.3 kHz axial scanning over a range of 100 µm in cardiac tissue without disturbing the sample. Using acute rabbit ventricular slice model, we present preliminary data to validate the use of remote focusing to probe AP propagation transmurally. We will use this system to quantitatively discern how post-MI electrically inert cells disturb cardiac conduction.

2 METHODS

2.1 Experimental set up

The experimental setup is shown in Figure 1. The power output of a pulsed Ti:Sa laser source (Coherent, Vision 2, 80MHz, 800nm) is attenuated with a Pockels cell (Conoptics, 50-80 LA) and is directed to a custom built single-prism pulse compressor. Efficient power coupling into the folded-geometry RF module is ensured using waveplates. Horizontally polarized beam is transmitted by the polarizing beam splitter. The quarter wave plate in RF module is positioned with its fast axis at 45 degrees relative to the incident polarization to obtain a circularly polarized state. The beam undergoes expansion to overfill the back aperture of the remote objective (Nikon Plan Fluor, 40X, NA 0.75, air). At its focal plane, the light-weight remote mirror (Thorlabs, UM05-0A, low-GDD mirror, 720 nm - 900 nm, 0° AOI) is positioned on a voice coil actuator (Equipment Solutions LFA-2010 Linear actuator). Upon reflection, the circular state handedness is flipped. The quarter wave plate retrieves vertically polarised light, which the polarising beam splitter reroutes to a commercial 2-photon microscope (Scientifica, HyperScope). Here the sample objective (Olympus XLUMPLFLN, 20X, NA 1, 2mm WD, water dipping) is used to probe the preparation in a custom designed sample chamber filled with Tyrode's solution. The solution is kept at 21 degrees Celsius and is enriched with medical grade oxygen before the experiment for 20 minutes to maintain tissue viability.

The two objectives meet the requirement that the remote objective must have an angular acceptance greater or equal to that of the sample objective, or $NA_{RF} \geq NA_{sample}/n_{sample}$ [10]. Additionally, high NA objectives are designed to obey the sine condition, where at a single nominal focal plane imaging is without distortion. This includes off-axis points within the plane. In RF microscopes, Herschel condition must be simultaneously met for distortion free imaging of objects positioned along the optical axis. This can only be achieved, if the magnification between the remote copy and the sample is $n_{sample}/n_{RF} = 1.33$ [10]. As deviations from this magnification matching lead to considerable deterioration of refocusing range [19], the lenses L_3, L_4, L_5 and L_6 with focal lengths of 125, 200, 175 and 100 mm were carefully chosen with consideration also of the lenses inside the microscope itself. The waveform generated for the microscope's fast galvanometric mirror is re-scaled and used to drive the voice coil actuator in RF imaging.

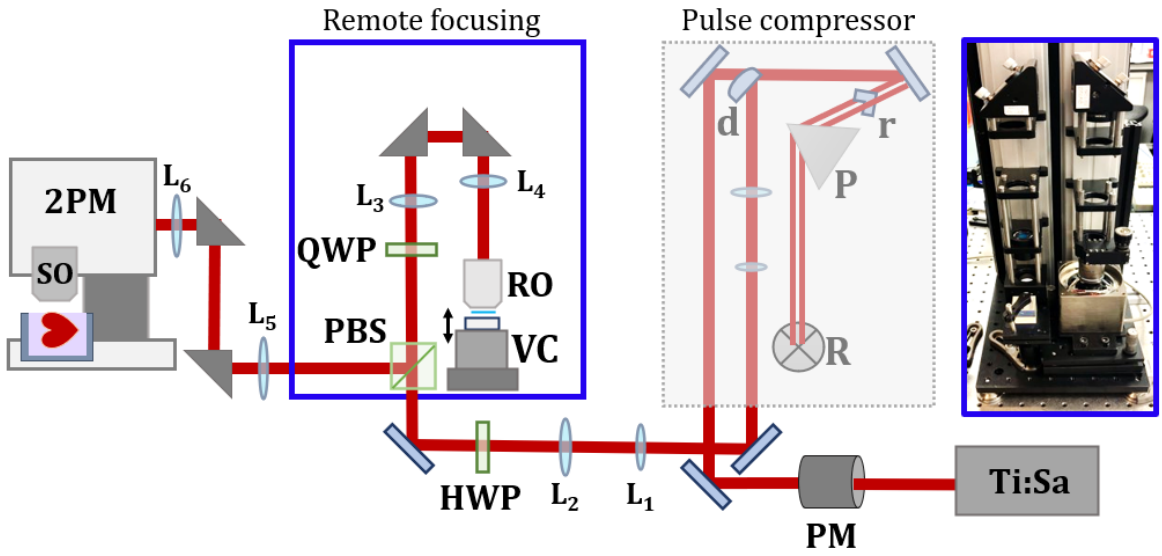

Figure 1. Experimental set up with an ultrafast laser as excitation source for a commercial 2-photon microscope with custom-built remote focusing and pulse compression units. QWP, HWP: quarter- and half-wave plates. PBS: polarizing beam splitter. VC: voice coil actuator. PM: power modulation. RO: remote objective. SO: sample objective. d, r: d-shaped and roof mirrors, respectively. P: prism, R: retroreflector. The arrow indicates the direction of remote mirror actuation.

2.2 Point spread function measurement

Microscope resolution was evaluated by imaging sub-resolution 0.196nm diameter Dragon Green fluorescent beads (Bangs laboratories, FCDG003). The beads were suspended in distilled water and to prevent formation of clumps; the Eppendorf was submerged in an ultrasonic bath (Grantt instruments) for 10 minutes. A small drop of water-bead solution was then placed on a microscope coverslip which was then allowed to evaporate for the beads to adhere to the coverslip. A droplet of water was then added carefully for the water dipping objective. Some beads were detached from the coverslip however most remained stuck for imaging. This method was more reliable than suspending the beads in 1% agarose as the focal spot of the high NA 2PM melted the agar and caused the beads to drift.

Z-stacks were obtained at different positions of the remote mirror. The coverslip with beads was placed on a vertical translation stage to bring it back to focus. Z-stacks were resliced and then z-projected in ImageJ. A line profile was used to select intensity values axially and laterally over the visible beads. MATLAB was used to fit a Gaussian function and retrieve the axial and lateral FWHMs. The uncertainty was estimated as the standard deviation of the FWHM values of the beads visible in each resliced z-projection for a given RF depth.

2.3 Preparation of acute rabbit ventricular slices

Intact hearts were labelled with Fluovolt (Thermo Fisher Scientific, F10488) via Langendorff-perfusion at a concentration of 1:1000 with Tyrode's perfusate (12-15µL for a rabbit heart). Viable ventricular slices were prepared closely following the protocol in [20], with some steps shown in Figure 2. Immediately after removing the heart from Langendorff-perfusion the organ was submerged in ice cold slicing solution with BDM (2,3-Butanedione monoxime) to ensure electromechanical uncoupling and reduction of metabolic needs. Left ventricle was excised and sections approximately 2 x 2 cm in size were glued on agarose – coated vibratome (Leica VT1200S) stage. The preparation was glued epicardium down to ensure the flatness of the tissue. Hystoacryl based glue was used. The tissue was sliced using stainless steel blades to 350 µm thick sections at 0.03 mm/s speed. The slices were then kept in icy slicing solution. 1 x 1 cm area of aligned fibers was then selected and cut under the dissection microscope. Small 3D printed plastic hooks were glued on the tissue edges for convenient mounting to a Sylgard coated sample holder.

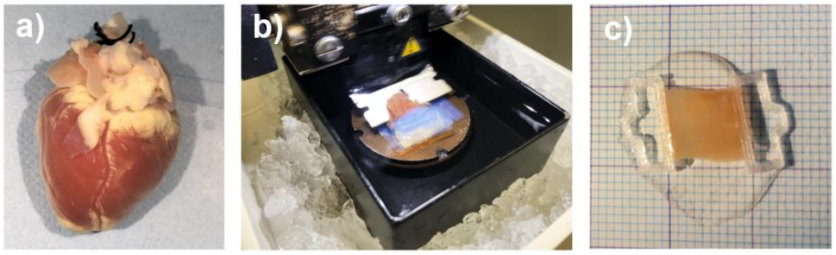

Figure 2. Preparation of acute ventricular rabbit slices. A) Fluovolt stained intact heart, b) slicing ventricular sections using a vibratome (Leica VT1200S) and c) ventricular slice prepared for mounting.

3 RESULTS

Characterisation of the system resolution is shown in Figure 3. As the remote mirror is moved from its nominal position, the resolution is deteriorated. Nevertheless, it is evident, that the system maintains cellular resolution throughout the range of 200 µm refocus, as cardiac cells are approximately 200 µm long and 20 µm thick.

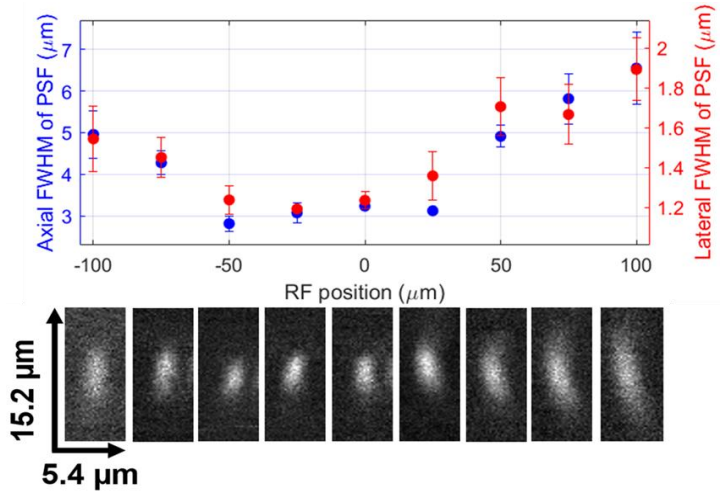

Figure 3. RF-2PM system resolution across the dynamic range of refocusing.

In Figure 4, cell structure of the ventricular preparation was imaged with the RF module and compared with the conventional microscope scan (800 nm excitation wavelength and 22 mW average power at sample). Both scans show qualitative agreement of features and the Fluovolt-stained boundaries between the cardiomyocytes are clearly resolved in RF scan. The reslice of the conventional xy-z stack (Figure 4a) took 14 minutes to obtain, while the 4 RF zy frames summed in b) image were acquired in 8.4 s (4.09 ms/vertical line in individual frame).

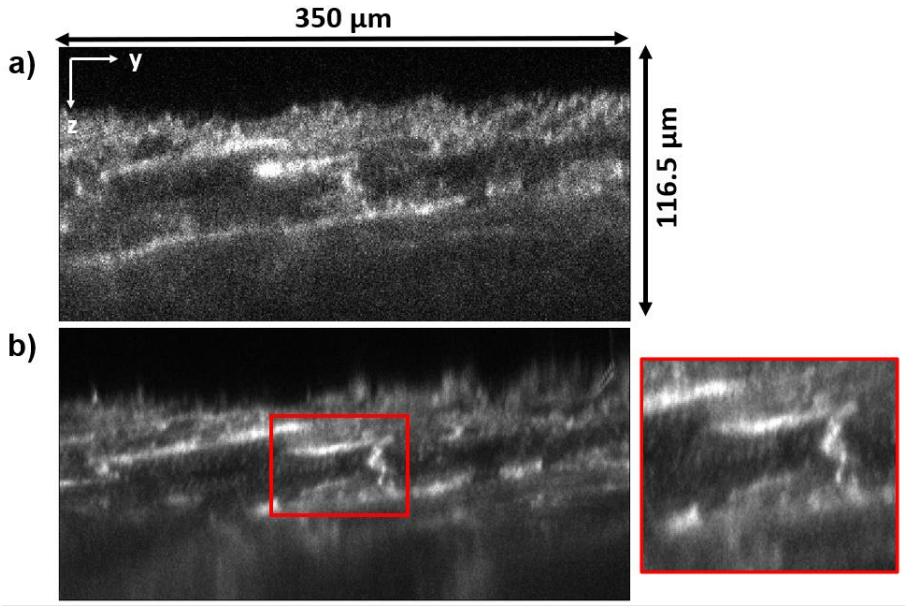

Figure 4. Fluovolt stained ventricular slice imaged by a) conventionally translating the sample objective and b) remote refocusing z-y frame scanned at 122 Hz axially. Qualitatively the structures are comparable and cell demarcations (highlighted red) are clearly visible.

4 DISCUSSION

Firstly, RF allows visualizing axial cardiac structures directly and in significantly shorter timescales compared to traditional scanning. Recording several frames per plane in seconds allows improving the signal to noise ratio. While a hint at sarcomere striations appears in the scan, with a more suitable staining protocol, the contrast of structures can be improved.

Importantly, as an individual axial line can be acquired in milliseconds (refocusing frequency up to 300 Hz), our 2PM-RF system will be applied to investigate how electrical wavefronts propagate transmurally. Acute cardiac slices will be electrically stimulated with a Digitimer (MK-2DSA) voltage stimulator and Grass platinum electrodes placed parallel to the long fibre orientation of the slices. It should be noted that it is not necessary to resolve the structure at a given depth to observe Fluovolt spike with potential difference and we anticipate that our module will be able to trace the electrical wavefront.

5 CONCLUSION

We report a custom built, compact and portable RF system compatible with retrofitting to commercial two-photon microscopes. Below 5 µm axial resolution maintained over the range of 100 µm refocus and distortions are negligible compared to the size

of cardiomyocytes. The system achieves up to 300 Hz axial. We optimise the system for power efficiency and dispersion for cardiac imaging. We validate the 2P-RF microscope by resolving cardiac cell structures in a fast axial scan in viable rabbit ventricular slice model. This system will be applied to resolve cardiac electrical signal propagation transmurally in healthy and infarcted hearts and discern at cellular resolution how post-MI fibrotic scar alters electrical conduction.

REFERENCES

[1] O'Shea, C., Kabir, S. N., Holmes, A. P., Lei, M., Fabritz, L., Rajpoot, K., & Pavlovic, D. (2020). Cardiac optical mapping – state-of-the-art and future challenges. The International Journal of Biochemistry & Cell Biology, 126, 105804-105804. https://doi.org/10.1016/j.biocel.2020.105804

[2] Zipfel, W., Williams, R. & Webb, W. Nonlinear magic: multiphoton microscopy in the biosciences. Nat Biotechnol 21, 1369–1377 (2003). https://doi.org/10.1038/nbt899

[3] Ghouri, I. A., Kelly, A., Burton, F. L., Smith, G. L., & Kemi, O. J. (2015). 2-photon excitation fluorescence microscopy enables deeper high-resolution imaging of voltage and Ca2+ in intact mice, rat, and rabbit hearts. Journal of Biophotonics, 8(1-2), 112-123. https://doi.org/10.1002/jbio.201300109

[4] Salerno, S., Garten, K., Smith, G. L., Stølen, T., & Kelly, A. (2020). Two-photon excitation of FluoVolt allows improved interrogation of transmural electrophysiological function in the intact mouse heart. Progress in Biophysics and Molecular Biology, 154, 11-20. https://doi.org/10.1016/j.pbiomolbio.2019.08.007

[5] Ghouri, I. A., Kelly, A., Salerno, S., Garten, K., Stølen, T., Kemi, O., & Smith, G. L. (2018). Characterization of electrical activity in post-myocardial infarction scar tissue in rat hearts using multiphoton microscopy. Frontiers in Physiology, 9, 1454-1454. https://doi.org/10.3389/fphys.2018.01454

[6] SeungYeon, K., Martí, D., & Arnold, C. B. (2020). Variable optical elements for fast focus control. Nature Photonics, 14(9), 533-542. https://doi.org/10.1038/s41566-020-0684-z

[7] Voigt, F.F., Kirschenbaum, D., Platonova, E. et al. The mesoSPIM initiative: open-source light-sheet microscopes for imaging cleared tissue. Nat Methods 16, 1105–1108 (2019). https://doi.org/10.1038/s41592-019-0554-0

[8] Žurauskas, M., Barnstedt, O., Frade-Rodriguez, M., Waddell, S., & Booth, M. J. (2017). Rapid adaptive remote focusing microscope for sensing of volumetric neural activity. Biomedical Optics Express, 8(10), 4369-4379. https://doi.org/10.1364/BOE.8.004369

[9] William J. Shain, Nicholas A. Vickers, Bennett B. Goldberg, Thomas Bifano, and Jerome Mertz, "Extended depth-of-field microscopy with a high-speed deformable mirror," Opt. Lett. 42, 995-998 (2017)

[10] Botcherby, E. J., Juškaitis, R., Booth, M. J., & Wilson, T. (2008). An optical technique for remote focusing in microscopy. Optics Communications, 281(4), 880-887. https://doi:10.1016/j.optcom.2007.10.007

[11] Botcherby, E. J., Smith, C. W., Kohl, M. M., Debarre, D., Booth, M. J., Juskaitis, R., . . . Wilson, T. (2012;2011;). Aberration-free three-dimensional multiphoton imaging of neuronal activity at kHz rates. Proceedings of the National Academy of Sciences – PNAS, 109(8), 2919-2924. https://doi:10.1073/pnas.1111662109

[12] Botcherby, E. J., Corbett, A., Burton, R. A. B., Smith, C. W., Bollensdorff, C., Booth, M. J., Kohl, P., Wilson, T., & Bub, G. (2013). Fast measurement of sarcomere length and cell orientation in langendorff-perfused hearts using remote focusing microscopy. Circulation Research, 113(7), 863-870. https://doi.org/10.1161/CIRCRESAHA.113.301704

[13] Sofroniew, N. J., Flickinger, D., King, J., & Svoboda, K. (2016). A large field of view two-photon mesoscope with subcellular resolution for in vivo imaging. Elife, 5 https://doi.org/10.7554/eLife.14472

[14] Jacques, S. L. (2013). Optical properties of biological tissues: A review. Physics in Medicine & Biology, 58(11), R37-R61. https://doi.org/10.1088/0031-9155/58/11/R37

[15] Takasaki, K. T., Tsyboulski, D., & Waters, J. (2019). Dual-plane 3-photon microscopy with remote focusing. Biomedical Optics Express, 10(11), 5585-5599. https://doi.org/10.1364/BOE.10.005585

[16] Sparks, H., Dvinskikh, L., Firth, J. M., Francis, A. J., Harding, S. E., Paterson, C., MacLeod, K. T., & Dunsby, C. (2020). Development a flexible light-sheet fluorescence microscope for high-speed 3D imaging of calcium dynamics and 3D imaging of cellular microstructure. Journal of Biophotonics, 13(6), e201960239-n/a. https://doi.org/10.1002/jbio.201960239

[17] Dvinskikh L, Sparks H, MacLeod KT, Dunsby C. High-speed 2D light-sheet fluorescence microscopy enables quantification of spatially varying calcium dynamics in ventricular cardiomyocytes. Front Physiol. 2023 Feb 14;14:1079727. doi: 10.3389/fphys.2023.1079727. PMID: 36866170; PMCID: PMC9971815.

[18] Dvinskikh, L., Sparks, H., Brito, L., MacLeod, K. T., Harding, S. E., & Dunsby, C. (2023). Remote-refocusing light-sheet fluorescence microscopy enables 3D imaging of electromechanical coupling of hiPSC-derived and adult cardiomyocytes in co-culture. Scientific Reports, 13(1), 3342-3342. https://doi.org/10.1038/s41598-023-29419-w

[19] Sharika Mohanan, Alexander D. Corbett, Sensitivity of remote focusing microscopes to magnification mismatch. (2020). Journal of Microscopy (Oxford), https://doi.org/10.1111/jmi.12991

[20] Watson, S., Scigliano, M., Bardi, I. et al. Preparation of viable adult ventricular myocardial slices from large and small mammals. Nat Protoc 12, 2623–2639 (2017). https://doi.org/10.1038/nprot.2017.139