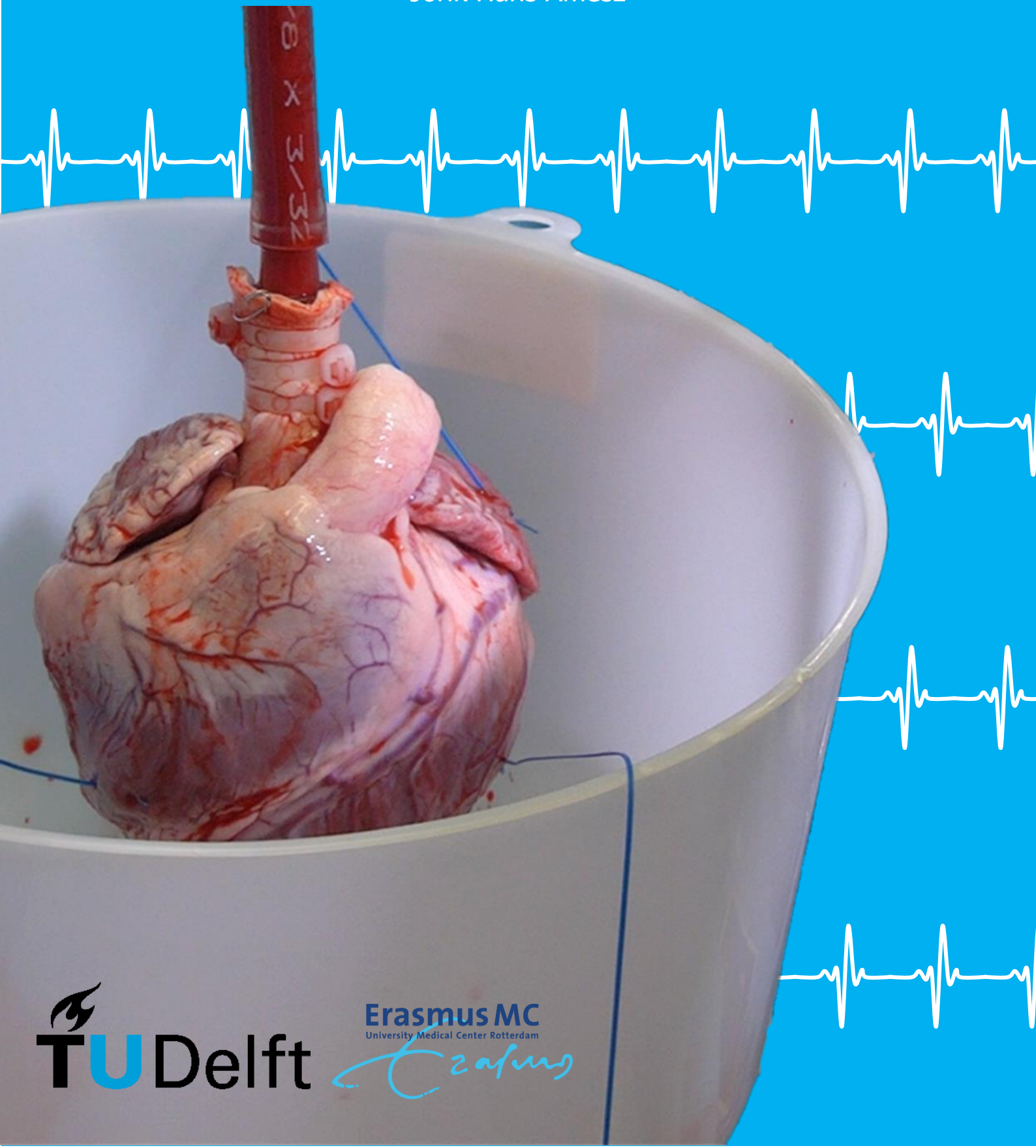
Cardiac mapping on *ex vivo* perfused porcine slaughterhouse hearts

Jorik Hans Amesz



TU Delft
Erasmus MC
University Medical Center Rotterdam

Cardiac mapping on *ex vivo* perfused porcine slaughterhouse hearts

A Langendorff perfusion protocol for epicardial mapping on isolated beating hearts

Jorik Hans Amesz

A thesis presented for the degree of Master of Science in Biomedical Engineering

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Thesis Committee

Prof. Dr. Ir. R. Dekker
Prof. Dr. J. Dankelman
Prof. Dr. N.M.S. de Groot
Dr. Y.J.H.J. Taverne
Ir. P. Knops

Delft University of Technology
The Netherlands

Summary

This thesis report is written for the master degree of Biomedical Engineering from the Delft University of Technology. The presented research was conducted in the Erasmus Medical Center at the departments of Translational Electrophysiology and Cardiothoracic Surgery.

Cardiac arrhythmias are associated with severe complications such as stroke, heart failure and even sudden cardiac death. To unravel the underlying electropathology of those rhythm disorders, epicardial mapping techniques in patients undergoing open-heart surgery were developed. However, not all mapping locations and conduction inquiries can be tested in the operation room. Therefore, the project goal was to develop a research platform in which this mapping technique could be applied on an isolated beating heart.

An *ex vivo* heart perfusion setup was designed to facilitate this platform and a protocol was written to resuscitate hearts on this system. The basic setup consisted of a cardiotomy reservoir, roller pump and oxygenator and heat exchanger. Blood and hearts were collected from pigs at a local abattoir and cardioplegia was given to minimize the warm ischemic time and protect the hearts during transport. Hearts were revived with Langendorff perfusion, retrogadely into the aorta, which resupplied the coronary arteries with an oxygenated blood mixture. This blood perfusate was circulated and its blood gas, electrolyte and metabolite values were closely monitored. Once the hearts started contracting, mapping protocols were performed on the cardiac surface with a high-definition 128-electrode array. Those mapping signals were analyzed with custom-made software of the Erasmus MC, from which electro(patho)physiological activation maps were constructed.

This is the first study to perform epicardial mapping on large animal slaughterhouse hearts. The presented platform is ideal for a variety of electrophysiological studies, because interventions can be performed that would be hazardous to perform *in vivo*. For example, mapping can be performed on endocardial locations, lesions can be created in conductive tissue and stimulation can be given to relatively unknown anatomies. This research will provide more information about arrhythmogenic structures and can eventually lead to better diagnosis and treatment of patients with cardiac arrhythmias.

The first part of this report contains a methods paper about ‘Cardiac mapping on Langendorff perfused porcine slaughterhouse hearts’. The second part contains appendices with background information, a flow chart, a detailed protocol and a logbook in which the protocol’s design process is represented. Especially, the flowchart in Appendix B comprises a good summary of the full procedure, which is also visualized with an online supplementary video.

Acknowledgements

This thesis is the final assessment for the completion of the master degree of biomedical engineering. During this project, I gained a lot of knowledge and practical skills on organ perfusion and electrophysiology and I was able to build a working heart model. But obviously I could not have done this on my own and therefore I want to express my deep gratitude to the people that helped me along the way.

I want to thank Dr. Yannick Taverne for the opportunity to carry out this project and the fine collaboration. I would like to thank Prof. Dr. Ir. Ronald Dekker for his external supervision, although we could not meet very often, I always felt that I could easily reach out to you. And I would like to thank Prof. Dr. Natasja de Groot for the warm welcome in the research group.

A big thank you to Paul Knops for his daily guidance and problem solving during the project. And I want to thank all other colleagues from the department of ‘’translationele elektrofysiologie’’, in particular Rohit, Matthijs and Nawin for their help with the trips to the abattoir, the experiments and the cardiac signal analysis.

Then I was helped by many others during this project so I also want to thank the departments of ‘cardiothorocale chirurgie’, ‘klinische perfusie’, ‘medische instrumentatie techiek’, ‘experimentele cardiologie’ and ‘experimentele intensive care’ for their advice and for the use of their materials.

The conducted research would not have been possible without the collaboration of the local butcher. I am very thankful to Ferry and his colleagues for their contribution to excise the hearts as fast as possible and their willingness to improve the protocol of my experiments.

Nomenclature

Abbreviations

AF	Atrial fibrillation
AV	Atrioventricular
CIT	Cold ischemic time
DCD	Donation after cardiac death
EVHP	*Ex vivo* heart perfusion
KH	Krebs-Henseleit
LAD	Left anterior descending (artery)
LCA	Left coronary artery
LV	Left ventricle
RCA	Right coronary artery
RV	Right ventricle
SA	Sinoatrial (node)
WIT	Warm ischemic time

Contents

I Methods paper

Cardiac mapping on *ex vivo* perfused porcine slaughterhouse hearts

Jorik H. Amesz

Abstract

Cardiac mapping techniques, in which an electrode-array is placed directly on the surface of the heart, were developed to gain a better insight in the underlying electropathology of cardiac arrhythmias. This procedure is performed in patients undergoing cardiac surgery, but here not all relevant cardiac structures and conduction inquiries can be tested due to possible complications. Hence, to fulfill this gap, an *ex vivo* Langendorff perfused heart model was designed in this study. Porcine hearts were collected at an abattoir and resuscitated on this setup, after which cardiac mapping was performed. The presented method is the first to perform epicardial mapping on an isolated beating porcine slaughterhouse heart and can be used for a large variety of electrophysiology studies.

Keywords

Ex vivo heart perfusion;
Langendorff perfusion;
cardiac mapping;
electrophysiology

Introduction

Cardiac arrhythmias are the cardiovascular epidemic of the 21st century and are associated with severe complications such as stroke and heart failure (Morillo et al., 2017). The number of patients with arrhythmias is rapidly increasing due to ageing, obesity, diabetes and hypertension (Aronow & Banach, 2009). It is estimated that the number of adults with atrial fibrillation (AF), the most common type of arrhythmia, will more than double to 549 600 patients in the Netherlands in 2060 (Krijthe et al., 2013). Despite the increasing prevalence, knowledge on the underlying pathology of rhythm disorders remains limited.

For this reason, cardiac mapping approaches were developed, which are defined as a method by which potentials recorded directly from multiple sites of the heart's surface are spatially depicted as a function of time in an integrated manner (de Groot & Allessie, 2001). In the Erasmus MC a custom electrode-array was developed for high-resolution epicardial mapping of the cardiac surface during open-chest surgery (van der Does et al., 2016). This technique led to new insights about the identification and localization of electropathology associated with atrial fibrillation. However, not all relevant structures, e.g. the interatrial septum, can be accessed during surgery and some conduction inquiries are difficult to test in a patient due to ethical or even possible hazardous issues. Therefore, an alternative manner using an *ex vivo* heart perfusion (EVHP) setup in Langendorff mode was designed in order to create a new platform for electrophysiological testing, and to unravel electropathologies of cardiac arrhythmias. In this isolated beating heart model, cardiac mapping can be performed on all desirable anatomical locations and even lesions can be created in conductive tissues.

Ex vivo heart perfusion was pioneered by Oskar Langendorff in 1895, who used a blood-based circulatory system to revive hearts of small mammals (Langendorff, 1895) as presented in Appendix A. Briefly, by pumping warm blood retrogadely into the aorta, the coronary arteries got reperfused, which supplied the cardiomyocytes with nutrients and oxygen and subsequently resuscitated the arrested heart. Currently, Langendorff's method is standard practice in many cardiology labs, but the use of large hearts remains challenging. The reason to use large animal hearts is due to the inherent anatomical similarities with human hearts which is absent in rodent or other small animals (Crick

et al., 1998). Therefore porcine hearts were used for this study and these organs were collected at an abattoir, because slaughterhouse materials are cheap and contribute to the minimization of animal experiments (Görge et al., 1994).

This is the first study that combines an EVHP setup for large animal hearts with cardiac mapping using a high definition 128-electrode array. The paper describes a methodology for Langendorff perfusion of porcine slaughterhouse hearts and different mapping modalities for future experimental testing of conduction abnormalities. This technique will provide further information about arrhythmogenic structures and may lead to the development of novel diagnostic tools and treatment modalities for cardiac arrhythmias.

Methods & Preliminary Results

A summary of this method is presented in a flowchart in Appendix B, along with a detailed protocol of all steps in Appendix C. The procedure is also visualized with online video content.

Langendorff system

The EVHP setup operated in Langendorff mode with a cardiotomy reservoir (Medtronic EL402), roller pump (Stockert Shiley) and a combined oxygenator and heat exchanger (Maquet quadrox-i adult).

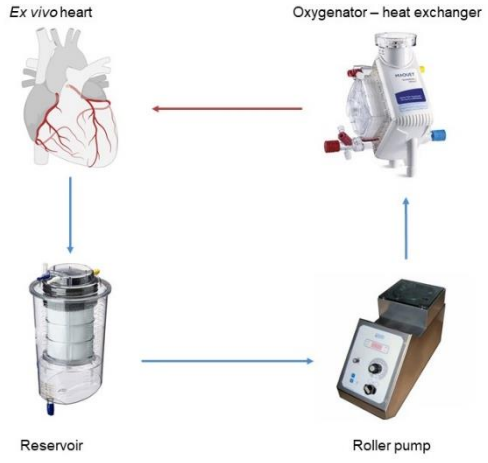


Figure 1. Schematic representation of the EVHP setup.

Blood flowed from the reservoir to the oxygenator, where it got heated and oxygenated, into the heart (Figure 1). The blood was returned to the reservoir by means of gravitational venous drainage into a funnel (Figure 2).

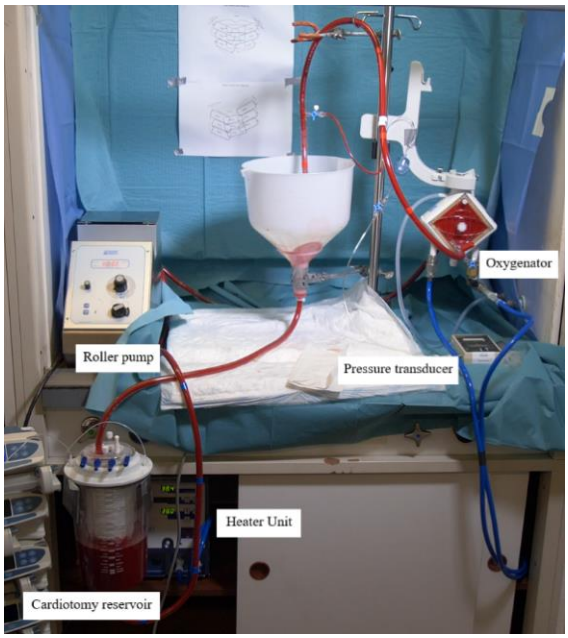


Figure 2. Picture of parts assembled in the EVHP setup.

System preparation

The Langendorff system was primed with one liter of adjusted Krebs-Henseleit (KH) solution (Table 1). Krebs-Henseleit is the most widely used perfusion buffer because it contains ions that mimic the plasma content as emphasized by Mouren et al. (2010). Heparin was added to form a coating along the lining of the tubing and mannitol to increase colloid-osmotic pressure and decrease edema formation in the heart (Miles et al., 2017).

Table 1. Composition of adjusted Krebs-Henseleit solution (Krebs & Henseleit, 1932).

Chemical	Quantity	
Sodium chloride	689.6	mg/L
Potassium chloride	35	mg/L
Magnesium sulfate heptahydrate	29.6	mg/L
Monopotassium phosphate	16.3	mg/L
Calcium chloride	27.7	mg/L
Sodium bicarbonate	2100	mg/L
D(+)-Glucose monohydrate	1650	mg/L
Heparin	2500	units/L
Mannitol	16	mmol/L

After priming, two liters of autologous blood were inserted into the system and mixed with the Krebs-Henseleit solution to lower the blood's viscosity, resulting in a hematocrit of 25 – 30%. This perfusate mixture was gradually heated to 38 °C with the oxygenator connected to a water bath (Maquet HU 35), because of the porcine body temperature of approximately 38.5 °C (Hannon et al., 1989). The blood was filtered with a 20 µm filter integrated in the cardiotomy reservoir to remove large impurities, e.g. clots and cell aggregates, subsequent to exsanguination.

Blood & heart collection

The hearts and blood used in this study were obtained from 4 slaughterhouse pigs, weighing approximately 90 kilograms at an age of 6 to 7 months. All animals were examined by a veterinarian, and the hearts were isolated without disturbing the usual slaughtering procedure of the local butcher. The animals were killed by shooting a pin through the skull after which a cut was immediately made in the neck for exsanguination. Blood was collected in storage containers, and 5000 U/L of heparin was rapidly added to prevent blood clotting (Howell & Holt, 1918). After exsanguination and blood collection, the animals were placed in a steam bath for hair removal. Next, the pig was quickly opened and the heart was removed according to the in-house protocol for human heart explantation. After heart excision, the heart was directly immersed in a cold (4 °C) 0.9% saline solution for topological cooling and to prevent thrombi formation because of air contact (Skrzypiec-Spring et al., 2007). The hearts were first inspected on quality and were excluded in case of stiffened ventricles, big lesions or epicardial bleedings. The heart was then positioned in supine orientation, the ascending aorta clamped before the first bifurcation and a needle was inserted between the clamp and the aortic valve to flush two liters of heparinized (2500 U/L) St. Thomas cardioplegic solution through the coronary arteries under gravitational pressure. This resulted in complete electromechanical arrest of the heart and the period between this arrest and animal shooting was noted as the warm ischemic time (Figure 3 & Table 3). The heart was then inserted in a transportation bag filled with cold saline and placed in an icy slurry after which it was transported to the Erasmus MC together with the heparinized blood.

Heart preparation

In the lab, a 3/8 inch ribbed cannula was fixed in the aorta, a few millimeters above the aortic valve, with multiple tie wraps, allowing the heart to freely hang above the funnel without external compression. The heart was weighed before assembly on the perfusion system (Table 3). Once the temperature of the perfusion system reached the set point value, the heart was flushed with 0.5 liters of warm physiological saline (38 °C) to wash out cardioplegia and metabolic waste products and to remove large blood clots from the heart chambers.

Heart connection on setup

After warm flushing, the heart was connected to the setup for reperfusion. The aortic cannula was filled with perfusate dripping from the EVHP system before attachment to ensure an air-free connection. The period between cardioplegic flush and reperfusion was accomplished within 109 ± 8 minutes (Table 3) and was noted as cold ischemic time (Figure 3).

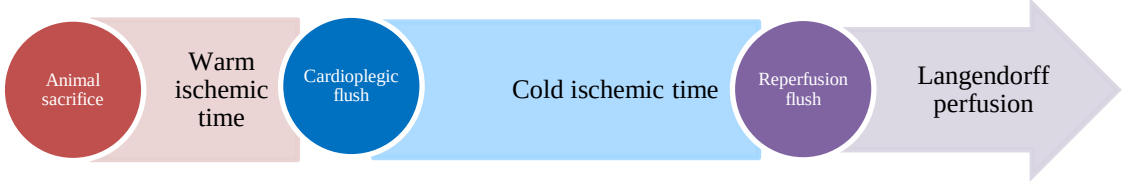


Figure 3. Definitions of warm ischemic time (WIT) and cold ischemic time (CIT).

Langendorff perfusion

Hearts were perfused in Langendorff mode with retrogade perfusion into the aorta. This resulted in active coronary perfusion and blood exiting the coronary sinus passively flowed without resistance from the opened vena cava into the funnel below. Retrograde aortic perfusion, resulted in spontaneous recontractions due to coronary and myocardial reperfusion. The blood-KH perfusate was circulated with a constant flow rate based on the heart mass of 1.0 – 1.5 mL/g/min (Sutherland & Hearse, 2000). This flow rate resulted in a pressure build up in the coronary arteries of approximately 80 – 100 mmHg. With constant flow, the pressure is mainly dependent on the resistance of the coronary circulation according to Ohms law and even more on the vessel diameter according to Hagen-Poiseuille's law (Appendix A). Therefore, the pressure differences are mainly caused by changes in coronary diameter due to either vasodilation or vasoconstriction. Pressure was monitored in the aortic root with a pressure isolator line (Medtronic 5/B) connected to a pressure transducer (Fluke Biomedical DPM1B). It is important that the balloon of this line is positioned at the same height as the heart for an accurate pressure measurement of coronary perfusion (Figure 4).

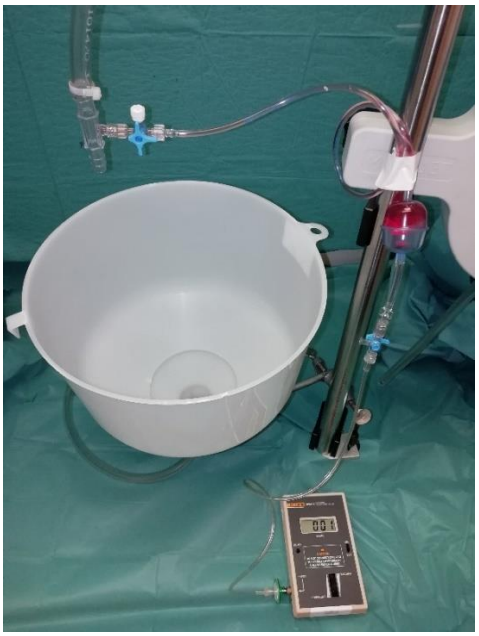


Figure 4. Pressure measurement in the aortic root. A pressure isolator line is connected to a side-branch of the arterial tubing. A membrane in this balloon separates the two sides of the line, of which one is filled with perfusate while the other contains air. Pressure builds up in the coronaries, leading to a deformation of the membrane which compresses the air column on the other side. This compression is registered by the pressure transducer.

The aortic pressure was not allowed to exceed 140 mmHg, as coronary vessel diameter autoregulation fails above this limit. It is also the maximum pressure allowed during cardiopulmonary bypass in the Erasmus MC. The flow rate on the roller pump was decreased if the pressure exceeded this cut-off value.

Defibrillation

A defibrillator (Philips Heartstart XL M4735A) was used to overcome malignant arrhythmias, e.g. ventricular tachycardia or fibrillation. Metal paddles were placed directly on the heart for a series of shocks of 10 – 30 J until cardioversion.

Blood gas monitoring

Oxygenation was provided via the oxygenator connected to a standard air source (21% oxygen, 78% nitrogen, RT) of the fume hood. The air supply was measured with an air flow sensor (Ohio Medical Flowmeter). A baseline blood sample was taken five minutes before heart connection to the setup to measure blood gas (pH, pO_2, pCO_2, cHb, sO_2), electrolyte ($cHCO_3^-$, cK^+, cNa^+, cCa^{2+}) and metabolite (cGluc and cLac) values on a blood gas analysis system (Siemens RAPIDPoint 500). Five minutes after connection and subsequently every 30 minutes these analyses were repeated for monitoring of the perfusate composition. The perfusate values were compared to *in vivo* physiological reference values and were maintained between the borders of a self-defined supranormal range (Table 2).

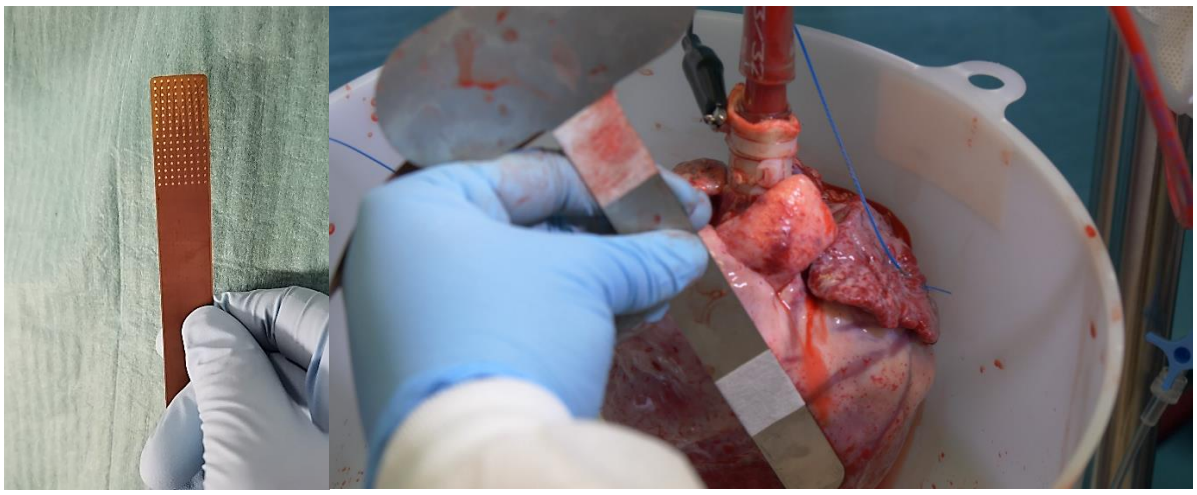
Table 2. Physiological range and supranormal range of blood gas, electrolytes and metabolite values for Langendorff experiment.

Parameter	Physiological range	Supranormal range
pH	7.35 – 7.45	7.35 – 7.70
pCO_2	35 – 45 mmHg	10 – 45 mmHg
pO_2	100 – 300 mmHg	100 – 300 mmHg
$cHCO_3^-$	21 – 27 mmol/L	12 – 27 mmo/L
cHb	11.5 – 17.4 g/dL	8.0 – 17.4 g/dL
sO_2	94 – 100 %	94 – 100 %
cNa^+	135 – 145 mmol/L	130 – 145 mmol/L
cK^+	3.5 – 5.1 mmol/L	3.5 – 9.5 mmol/L
cCa^{2+}	1.1 – 1.3 mmol/L	1.1 – 1.3 mmol/L
cGluc	60 - 140 mg/dL	60 – 140 mg/dL
cLac	0 – 2.0 mmol/L	0 – 9.0 mmol/L

Calcium concentrations were supplemented with calcium gluconate to facilitate normal contractility of the heart. A glucose-insulin mixture was infused (20 U/h) to lower extracellular potassium concentrations because of insulin-driven potassium influx and glucose was added to provide the heart with nutrients and prevent hypoglycemia. Dobutamine was infused (8 µg/min) to increase the contractility of the heart. One gram of magnesium sulfate was added to the blood to prevent arrhythmias (White et al., 2015).

Mapping protocol

When a heart was successfully resuscitated, cardiac unipolar signals were collected with a mapping system (EPMed Workmate) connected to a 128-electrode array (Figure 5) following a method previously described by Yaksh et al. (2015). In short, bipolar pacemaker leads were placed on the high right atrial sinus node region and basal ventricular regions of the heart. These wires were connected to an external pacemaker (Medtronic 5375) for stimulation if required, but they also served as reference electrodes when connected to the EPsystem. A steel wire was fixed in distal aortic tissue serving as indifferent grounding electrode (Figure 6). The total surface of the *ex vivo* heart was mapped by systematically placing the electrode on predefined cardiac locations (Appendix A).



Figures 5 & 6. Left: 128-electrode array. Right: Electrode array on Langendorff heart with crocodile clip attached to steel wire as indifferent electrode and blue pacemaker wires as reference electrodes.

Each electrode in the 128-array records an electrical signal. An example of recorded cardiac signals during intrinsic rhythm is shown in Figure 7.

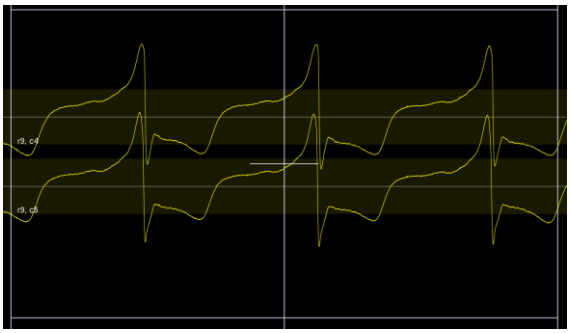


Figure 7. Ventricular signals from two electrodes in the 128-electrode array, recorded during intrinsic rhythm at the anterior left ventricular surface.

An example of signals recorded during stimulation with the external pacemaker is shown in Figure 8. The sharp downward spike prior to the cardiac potential complex is defined as the 'pacing spike', the following cardiac deflection is a result of signal capture. Capture occurs when the pacing stimulus leads to depolarization and consequently contraction of the tissue.

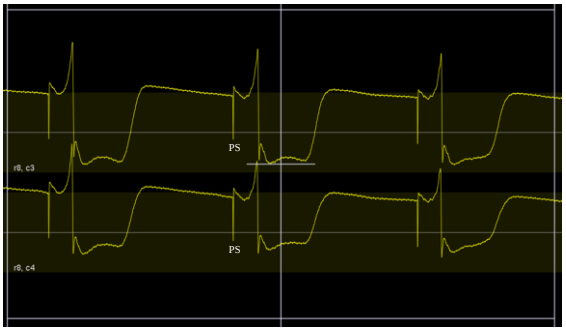


Figure 8. Ventricular signals from two electrodes in the 128-electrode array, recorded during pacemaker stimulated rhythm at the anterior left ventricular surface. Capture occurs after the pacemaker spikes (PS).

The signals from the previous Figures were recorded at the anterior left ventricle surface (Figure 9).

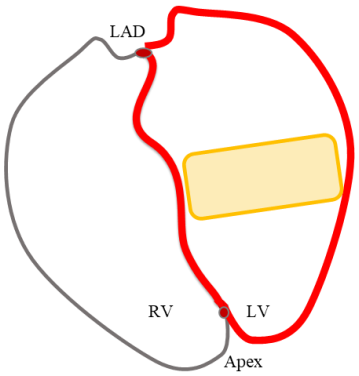


Figure 9. Anterior view of the heart with right and left ventricle (RV & LV) that shows the location of the electrode array during recording of the potentials from Figures 7, 8, 10, 11 & 12.

Local activation times of these recorded unipolar cardiac signals were marked, from which color-coded activation maps were reconstructed by custom-made software which has previously been described in more detail by Allessie et al. (2010). Briefly, the ventricular complexes of all recordings are automatically detected and marked at the steepest negative deflection of each cardiac complex by the software. The electrode with the earliest marking is set as 0-reference for the activation times of the other electrodes. The Figures below (10 & 11) illustrate the construction of activation maps during intrinsic rhythm at the anterior left ventricular surface from two different Langendorff procedures. These activation patterns are compared with the activation map of an anesthetized pig from previous *in vivo* experiments (Figure 12) at the same location to compare electrophysiological properties between the *ex vivo* and *in vivo* experiments. Figure 10 demonstrates a slow propagating wave that took approximately 100 milliseconds to run through the array, while in the sedated pig of Figure 12 it only took 25 milliseconds to cross the same distance. Figure 11 shows a faster wave that propagated in approximately 35 milliseconds, but contained lines of block. A block line is drawn when a delay of more than 12 milliseconds is present between neighboring electrodes.

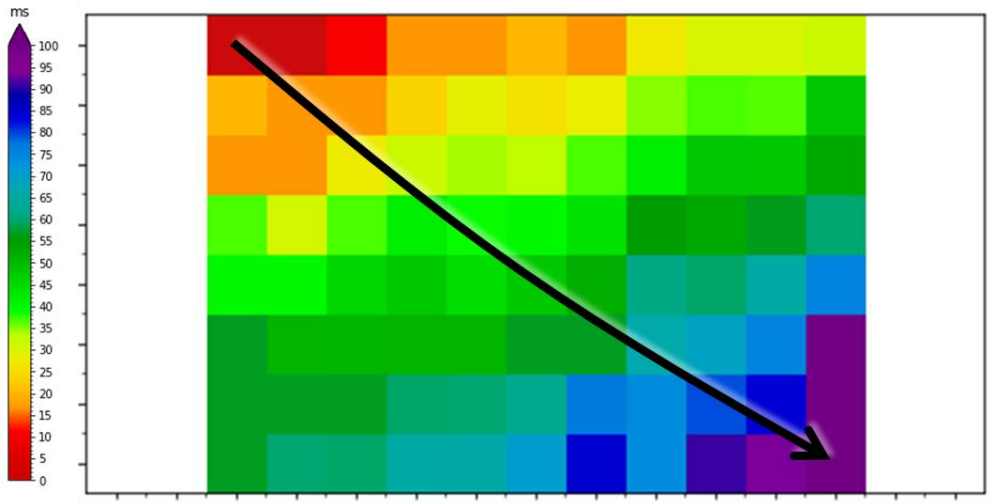


Figure 10. Activation map of intrinsic rhythm on the left ventricle of an isolated heart with slow activation pattern of approximately 100 milliseconds. Timescale: 0 – 100 milliseconds. Columns were deleted because of noisy signals.

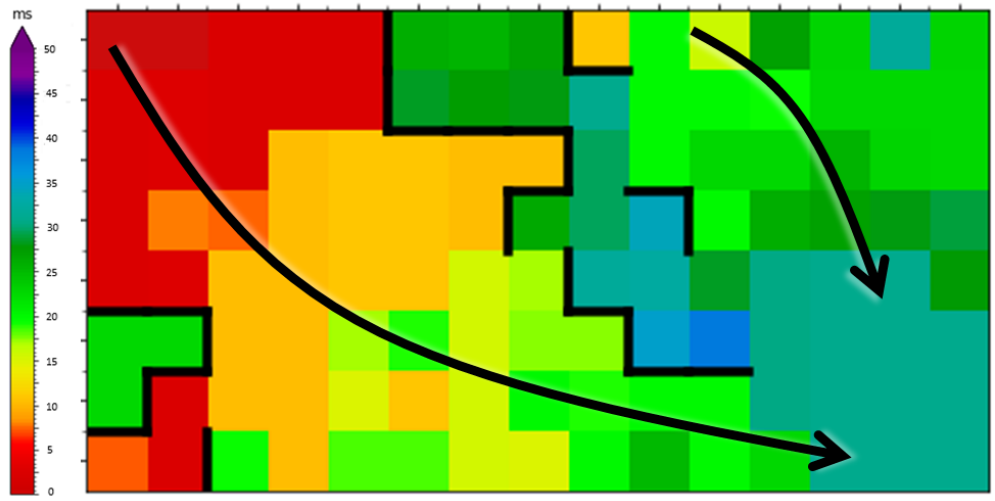


Figure 11. Activation map of intrinsic rhythm on the left ventricle of an isolated heart with faster activation pattern, but lines of block. Timescale: 0 – 50 milliseconds.

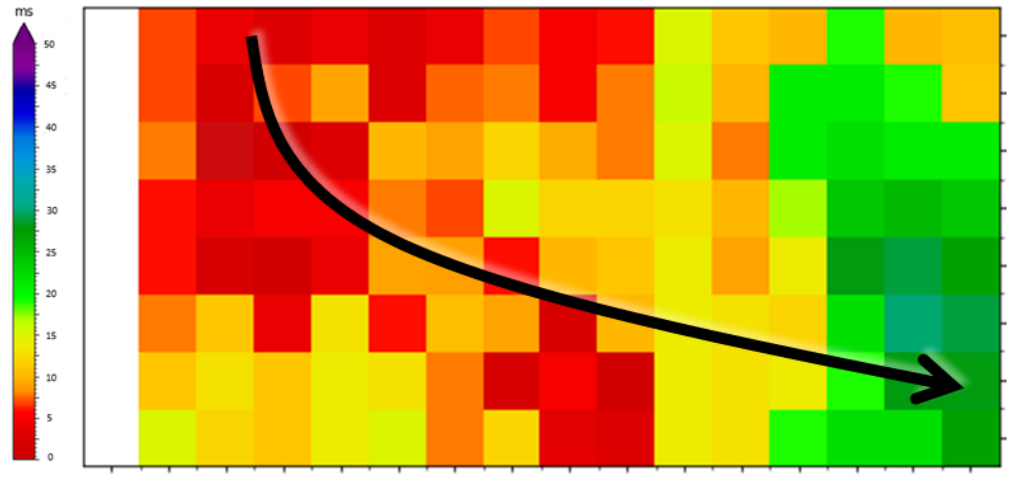


Figure 12. Activation map of intrinsic rhythm on the left ventricle of an anesthetized pig with fast activation pattern. Timescale: 0 – 50 milliseconds.

Post perfusion analysis

Heart survival and perfusion times were dependent on the type of experiment, but all hearts were beating for at least 2 hours with a maximum of 5 hours. Different types of mapping experiments can be found in the logbook in Appendix D. After the experiments, the hearts were arrested by administering an overdose of potassium chloride. The hearts were removed from the setup and were weighed to calculate the difference in mass before and after perfusion (Table 3).

Table 3. Details of ischemic times and heart mass of n=4 experiments.

	Mean ± SD
Warm ischemic time (min)	9.8 ± 1.5
Cold ischemic time (min)	109 ± 8
Mass before perfusion (g)	474 ± 4
Mass after perfusion (g)	640 ± 23
Mass gain (g)	166 ± 26

This mass gain is an indication for the amount of edema formation (Kappler et al., 2019).

Discussion

The present study is the first to perform epicardial mapping on an isolated beating porcine abattoir heart. With this protocol, large hearts can be resuscitated in Langendorff mode and cardiac conduction potentials can be recorded with a high-definition electrode array. This perfusion model is an ideal platform for a large variety of electrophysiology studies.

Ventricles spontaneously started recontracting after connection to the setup and cardiac mapping was performed once this occurred. Figure 7 shows that ventricles beat with a normal intrinsic rhythm and Figure 8 shows an example of cardiac signals in which electrical signal capture was present, indicating that the tissue was vital. This phenomenon is known as cardiac excitation-contraction coupling (Bers, 2002) and means that mechanical contractions were observed for each consecutive beat in the electrical signals. After signal analysis, two features were typical for the activation pattern of Langendorff perfused hearts. Firstly, slow activation patterns (Figure 10) were observed in comparison with *in vivo* activation patterns (Figure 12) and secondly, activation patterns contained lines of block (Figure 11). These differences are probably caused by local regions of ischemia in the cardiac tissue. So, cardiac signals have been demonstrated in vital Langendorff perfused hearts, but due to tissue damage the signals propagate slower or are affected by lines of block.

Current epicardial mapping procedures can only be performed on diseased patients during open-chest cardiac surgery. However, with this model, it is possible to perform epicardial mapping on porcine hearts without heart disease. Admittedly, the porcine heart has some minor anatomical differences to the human heart, but it is generally accepted in literature that it is similar to that of man (Crick et al., 1998) and thus this porcine model is appropriate for various cardiac mapping inquiries. To start, mapping procedures can be performed on locations that would be potentially hazardous in patients, e.g. the interatrial septum. Likewise, stimulation can be given to structures, e.g. His-bundle to study their role in cardiac conductance. And finally, lesions can be created with e.g. ablation, to precisely study their effect on conduction. In summary, this Langendorff perfusion mapping technique uses *ex vivo* experiments for translation to relevant *in vivo* findings and serves as an experimental window into cardiac electrophysiology.

Protocol optimization

The described protocol was developed over the course of seven experiments, and after each experiment adjustments were made for optimization of the procedure. These optimizations resulted in resuscitated hearts in the last four experiments. A view on the design process of this protocol can be found in the logbook in Appendix D and a summary of the most important actions can be found in the Table below.

Table 4. Summary of the most important optimization steps that lead to the final protocol.

n=7	Action	Underlying problem	Outcome
N2	Integration of water bath heater unit.	Difficult to regulate perfusion temperature.	Constant perfusion temperature of 38 °C.
	Priming of system and tubing with heparinized Krebs-Henseleit solution.	High blood viscosity.	Better flow in system.
N4	Heart immediately immersed in cold saline after excision.	Long WIT	Topological cooling of heart and less thrombi formation because of air contact.
	Cardioplegia administration via an infuse needle instead of aortic cannula.	Long WIT	Shortened WIT and easier to perform cardioplegic flush.
	Warm saline flush to wash out cardioplegia.	High potassium values in perfusate after reperfusion.	Initial physiological potassium values.
N5	Installation of syringe pumps for constant infusion of glucose-insulin.	Increasing potassium values during perfusion.	Delay in the buildup of extracellular potassium ions.
N7	Integration of air flow sensor at oxygenator.	High pH and low carbon dioxide pressure.	Decrease in pH values and better control of air supply in L/min.

The quality of the experiment is highly dependent on two important factors, namely the minimization of the warm ischemic time (WIT) and the status of the perfusate with regard to blood gas, electrolyte and metabolite values.

The WIT was reduced to 9.8 ± 1.5 minutes by performing the cardioplegic flush with an infuse needle and aortic clamping instead of aortic cannulation at the abattoir (Table 4). This falls within the time limit of 30 minutes for human donation after cardiac death (DCD) transplantation (Niederberger et al., 2019). But remarkably, in our experience, hearts with a WIT longer than 15 minutes were already unfit for this procedure, because the ventricles already stiffened significantly. Rapid stiffening could be explained by the uncontrolled way of death and the employment of a high temperature steam bath for hair removal in slaughterhouse pigs. The use of slaughterhouse materials could therefore be seen as suboptimal compared to hearts excised from pigs under anesthesia, as in such a controlled setting, cardioplegia can be administered directly in the thorax to arrest the heart and because slaughterhouse animals are more prone to external stress factors. On the other hand, using abattoir hearts minimizes animal instrumentation and costs, because offal is used (Görge et al., 1994).Yet, macroscopic inspection was carried out before cardioplegic infusion to determine the chance of heart revival. If the quality of a heart seemed insufficient, the heart was rejected and the heart of a next animal was inspected. This quality control resulted in consistent cardiac resuscitation after its implementation in the protocol. Hence, a meticulous inspection and the reduction of warm ischemic time are essential for a successful Langendorff experiment. The cold ischemic time, however less important because of the cardioplegic protection in this phase, was also kept well below the guideline limit of 4 hours for DCD transplantation (Niederberger et al., 2019).

With regard to the electrolyte balance, it was difficult to maintain potassium values within the physiological range of 3.5 to 5.1 mM, but in our experience this did not affect the contractility as long as the extracellular ion buildup could be reduced to a supranormal maximum of 9.5 mM (Table 2). Physiologically, potassium concentrations are high intracellular, but during EVHP these ions end up extracellular because of cell necrosis due to acute ischemia, leading to hyperkalemia. Severe hyperkalemia can lead to

heart block, asystole, ventricular tachycardia and ventricular fibrillation (Weiss, Qu & Shivkumar, 2017). Accordingly, this explains why the ventricular contractions worsened over time. High potassium concentrations were counteracted by infusing insulin (Table 4), which promotes the potassium uptake by the heart (Kones & Phillips, 1975).

Calcium is considered the most important ion involved in the process of excitation-contraction coupling (Bers, 2002) therefore calcium levels should be monitored with special care. In this study, calcium was maintained within the physiological range by the addition of calcium gluconate once the concentration dropped below 1.1 mM.

The pH of the blood-KH mixture was very alkaline in the first experiments with a pH of around 8, which is far from porcine physiological levels of 7.48 ± 0.03 (Hannon et al., 1989). In comparison, the pH of the full blood was measured at 7.46 in a blood sample taken at the abattoir. This increase in pH during EVHP was caused by an elevated gas flow exchange, resulting in a low carbon dioxide pressure, also known as respiratory alkalosis. Respiratory alkalosis has many cardiac effects, such as tachycardia and other arrhythmias and thus should also be prevented (Foster et al., 2001). The pH was eventually reduced with the integration of an air flow sensor (Table 4) to a value of approximately 7.6. However, the air supply could not be further reduced with the current air flow sensor, thus the author recommends the use of an air source with higher carbon dioxide concentrations, such as 5% CO_2 as demonstrated by Prall et al. (2015), to increase the carbon dioxide pressure and lower the pH. A 100% oxygen source was tested in one experiment (Appendix D), but these hyperoxic conditions with oxygen pressures of more than 500 mmHg, have the potential to lead to the oxygen toxicity syndrome (Bostek, 1989) and is therefore not recommended.

Heart mass was increased after the experiments as a result of edema formation in the heart muscle. Edema is defined as a swelling due to the expansion of interstitial fluid volume (Lent-Schochet & Jialal, 2020). In the Langendorff experiment, fluid extravasation is probably caused by two factors. Firstly, due to a decreased plasma oncotic pressure, because full blood is diluted with KH. The author does not recommend to use a full-blood perfusate, which has a higher viscosity because flow obstruction was observed in one of the first experiments with sole blood (Table 4).Secondly due to an incorrect balance of sodium and potassium concentrations leading to abnormal Starling forces (Navas & Martinez-Maldonado, 1993). Oncotic pressure was increased with the addition of mannitol in the KH and the electrolyte balance was regulated with close blood gas monitoring, nevertheless heart weight was significantly increased. Remarkably, the mass gain was approximately equal while the perfusion time differed between experiments. Probably, the edema formation resulted in contraction loss because of the stiffening of cardiac structures leading to cardiac death. So to summarize, the oncotic pressure and the electrolyte balance are considered as important factors for a prolonged Langendorff experiment of multiple hours.

Conclusion

This is the first study to perform cardiac mapping on *ex vivo* perfused porcine slaughterhouse hearts. The presented method was optimized over time and can now be used to study a wide variety of cardiac conduction inquiries in an experimental setting.

Future perspectives

Eventually, this Langendorff model can be developed into a working mode model, in which the left heart is loaded resulting in an antegrade aortic flow, first described by Neely et al. (1967). In Langendorff mode, electrophysiology is studied without preload and afterload in the heart chambers, but the effect of myocardial stretch on cardiac conductance can be studied with a pump-supported working mode (Xin et al., 2017) as previously done by Franz et al. (1992) in rabbit

hearts. This circulation model will integrate the role of mechano-electric feedback, defined as the transduction of a mechanical stimulus into an electrical signal (Lab, 1996). Such models are already optimized and available in the industry, e.g. LifeTec Group's PhysioHeart (de Hart, 2011). However these models are expensive and not readily accessible, thus necessitating the construct of our cost effective alternative using in-house available parts of the heart-lung machine.

Limitations

This study contained a small sample size of 4 resuscitated hearts with a total of 7 experiments. Due to the Covid-19 pandemic and problems with the blood gas analyzer system, it was impossible to perform more experiments. However, in the short time period of my master thesis project, I showed resuscitation of porcine slaughterhouse hearts and epicardial mapping on those hearts. The protocol was developed by the author and serves as guide for EVHP experiments in the Erasmus MC.

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II Appendices

A Background information

Heart anatomy

The heart's main function is pumping blood through the body to supply all organs and tissues with oxygen and nutrients. The heart is made of four chambers that are naturally divided between a right side and a left side by septum. The upper chambers are called the atria and the lower chambers are known as the ventricles. The right ventricle pushes blood into the pulmonary artery towards the lungs in which the blood gets oxygenated for return to the left heart. The left ventricle exits its blood in the aorta which brings the oxygenated blood to the body after which it returns deoxygenated in the right atrium. The atria and ventricles and ventricles and arteries are separated by valves (Figure 1).

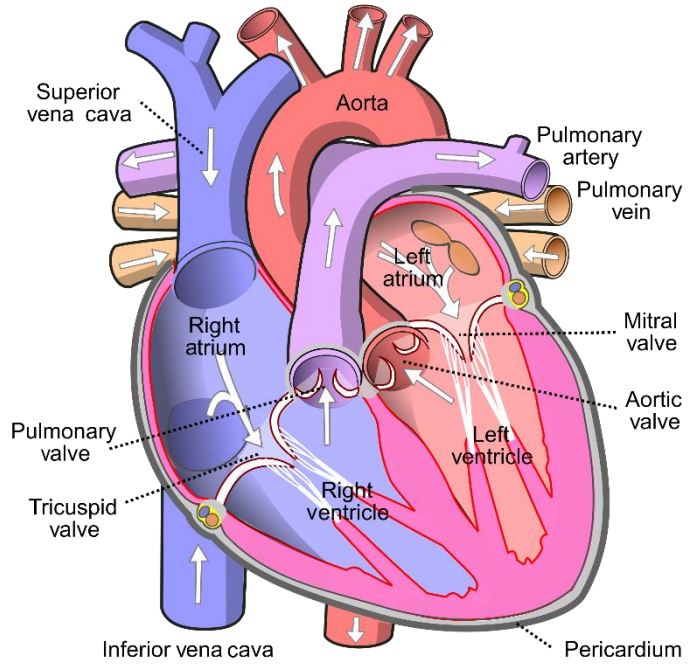


Figure 1. Heart anatomy and physiology (Wapcaplet).

The period in which the ventricles contract and thus pump blood away from the heart is called systole and the period in which the muscle relaxes and refills with blood is called diastole. If the pressure in the aorta becomes higher than in the ventricle, which is at the start of diastole, the aortic valve closes. The closure of this valve prevents backflow of blood into the heart, but in the meantime provides the coronaries, which both rise from the aorta, with oxygenated blood (Vander et al., 2008).

The coronary arteries are the blood vessels that transport oxygenated blood to the heart muscle. A decreased flow in the coronary arteries results in a reduction of oxygen and nutrients delivery to the myocardium, which can lead to a heart attack. The coronary arteries are of major importance and reduced function can have a serious impact on health.

The left and right coronary arteries (LCA & RCA) both rise from the aorta after which they have several bifurcations that wrap around the entire heart (Figure 2). The LCA supplies the left side of the heart and

branches into the LAD and the left circumflex. The right coronary artery feeds the right side of the heart and branches into the right marginal arteries (Vander et al., 2008).

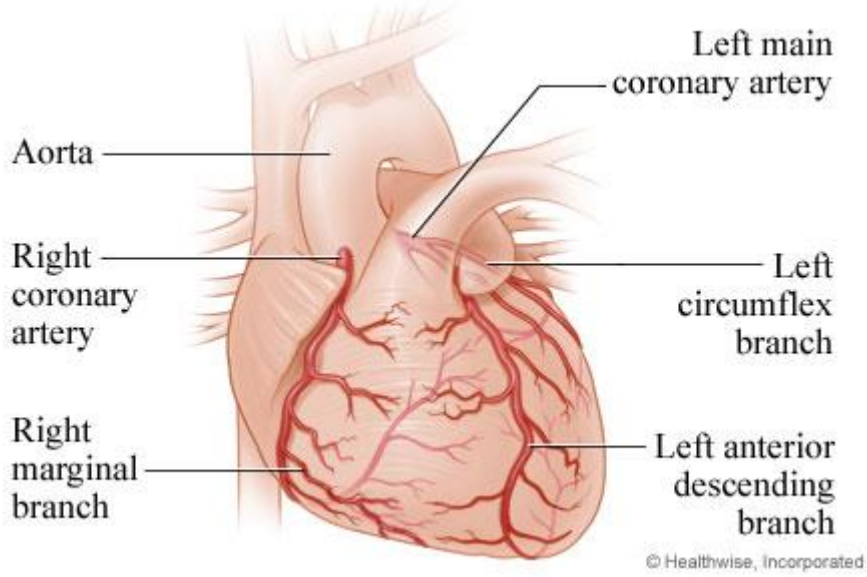
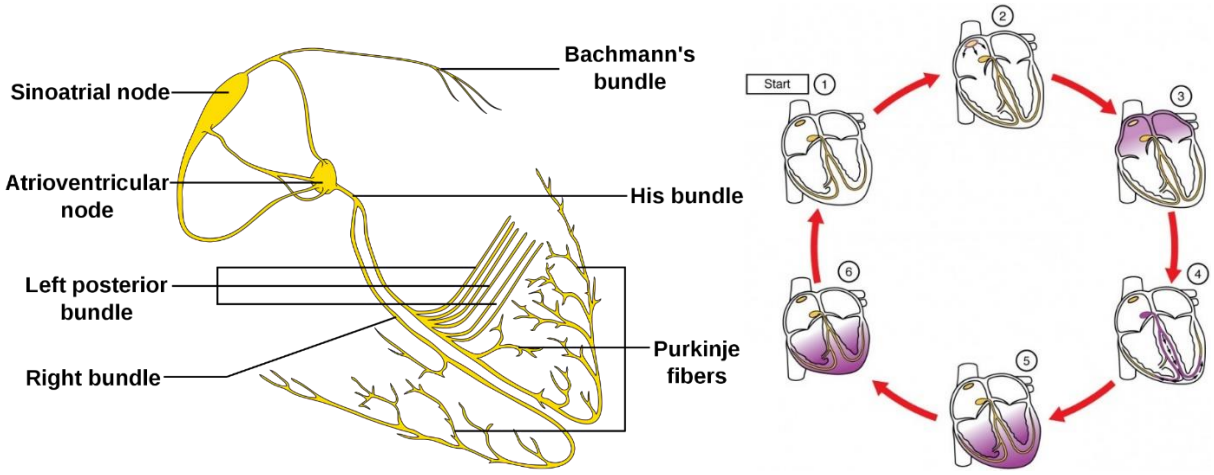


Figure 2. Coronary anatomy (Healthwise, Incorporated).

Heart electrophysiology

The pump function of the heart is regulated by its cardiac conduction system. Electrical signals travel along the heart muscle and subsequently activate contraction in different parts of the heart which leads to the typical cardiac pumping cycle. The electrical system is made up of three main parts (Figure 3), being: the sinoatrial (SA) node, the atrioventricular (AV) node and the His-Purkinje system.



Figures 3 & 4. Left: Main parts of the cardiac conduction system (Wikimedia). Right: Cardiac conduction cycle (OpenStax).

Figure 4 summarizes the sequence of repetitive action potentials for each individual heartbeat. The sequence starts in the SA node (1) which creates an excitation signal (2) that spreads across the atria and causes atrial contraction (3). Here, an interesting structure is Bachmann's bundle which is an important pathway in the interatrial conduction system. When the signal reaches the AV node, it is delayed to make sure the atria and ventricles do not contract simultaneously. The signal then travels further through the bundle of His and the Purkinje fibres (4) which causes ventricular contraction (5 & 6).

An action potential that enters a contractile cell causes calcium-channels to open, so calcium ions can move into the cell, which lowers the electrochemical gradient of that cell. Those calcium ions are essential for heart muscle contraction and play a major role in the process of excitation-contraction coupling. Repolarization is again caused by the outflow of calcium ions with the help of Ca^{2+}-ATPase, but also via the Na^{+}-Ca^{2+}-exchanger. This influx of sodium ions is counterbalanced by Na^{+}-K^{+}-ATPase, so the right concentration of sodium and potassium ions is also crucial for normal heart function (Silverthorn, 2010) and should be monitored during EVHP experiments. Now, it is also understandable why cardioplegia, with an overdose of potassium ions, is given to protect the heart during donor transport. This extracellular overdose disturbs the electrochemical gradient of the cells, because potassium is normally high intracellular, and thus pauses the metabolism of the cardiomyocytes.

Cardiac arrhythmias

Cardiac arrhythmias are a group of conditions in which the heartbeat is abnormal. Bradycardia and tachycardia are conditions in which the normal resting heart rate of roughly 60 to 100 beats per minute is either decreased or increased. Fibrillation is the medical term for an irregular heartbeat (Vander et al., 2008).

In the research group of Professor de Groot et al., special attention is given to atrial fibrillation. Atrial fibrillation is the most common type of arrhythmia and is characterized by beat-to-beat changes in the pattern of activation in the atria. It may cause stroke and heart failure and is even associated with death. The exact pathophysiology of atrial fibrillation is not yet discovered, but it is known that the regular impulses produced by the sinus node are overwhelmed by rapid electrical discharges in other parts of the atria and parts of the pulmonary veins (Figure 6).

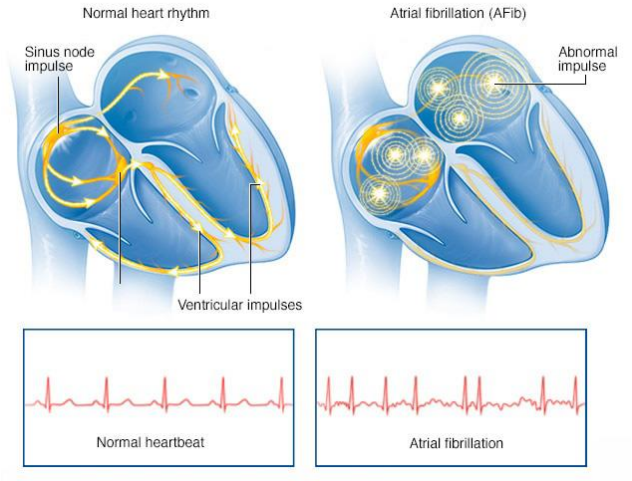


Figure 6. Comparison of electrical discharge in normal heart rhythm and atrial fibrillation (MAYO 2016).

Cardiac mapping

The translational electrophysiology group of de Groot et al. tries to understand the underlying mechanisms of atrial fibrillation, to eventually improve its prevention and treatment outcomes. The group developed a method for epicardial mapping during open-heart surgery with a 192-electrode array (Figure 7).

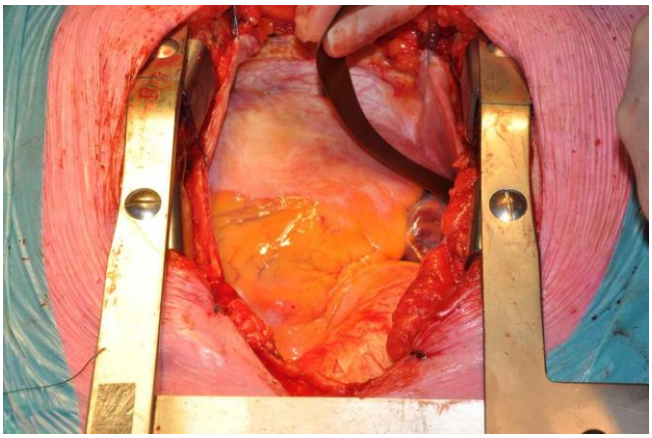


Figure 7. Electrode-array on heart during cardiac surgery.

Mapping is performed before going on extracorporeal circulation with a custom-made electrode array. Signals are recorded at different cardiac locations for 5-10 seconds (Figure 8).

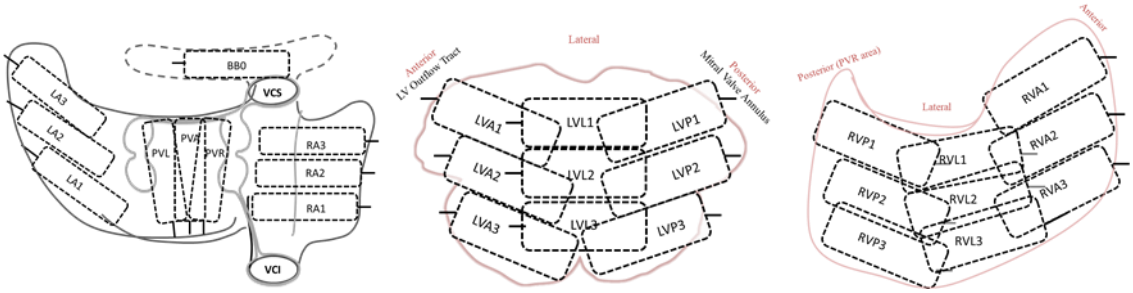
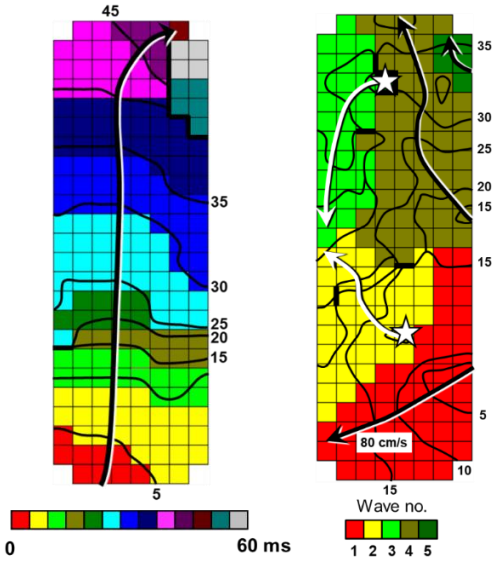


Figure 8. Different mapping locations on left and right atrium (LA & RA), left ventricle (LV) and right ventricle (RV).

From this data, local activation times are marked, from which colour-coded activation and wave maps are reconstructed (van der Does et al., 2016). In Figure 9, examples of an activation map during normal sinus rhythm and atrial fibrillation are shown. In sinus rhythm the wave smoothly travels along the electrode, but with AF this pattern is disturbed.



Figures 9. Left: activation map during normal sinus rhythm with one single wave. Right: activation map during atrial fibrillation with a total of 5 waves (van der Does et al., 2016).

By collecting mapping data from cardiac patients, de Groot et al. hope to discover the underlying mechanisms of arrhythmias. Furthermore, they also want to study certain locations and conduction inquiries in an experimental setting and therefore an *ex vivo* heart model, in which the heart is isolated outside the organism and beats on a machine setup, is a great alternative to study these locations.

Ex Vivo Heart Perfusion & Langendorff

In 1895, Oskar Langendorff invented the Langendorff apparatus, which was the first *ex vivo* heart perfusion (EVHP) system and a major breakthrough in cardiovascular research. The Langendorff apparatus is a setup to perfuse an isolated heart in order to revive it. It formed the basis for understanding that the heart derives its oxygen and nutrient supply via the coronary circulation (Langendorff, 1895).

The basic principle of the Langendorff heart is inserting a cannula into the ascending aorta which perfuses the heart via the coronary arteries. This perfusion is in retrograde direction and perfuses the coronary arteries on the premises that the aortic valve closes (Figure 10), contrary to the *in vivo* setting where blood flows in an antegrade fashion, from the left ventricle into the aorta. This Langendorff perfusion leads to the resuscitation of a stopped heart by delivering oxygen and nutrients to the heart muscle cells (Dhein, 2005).

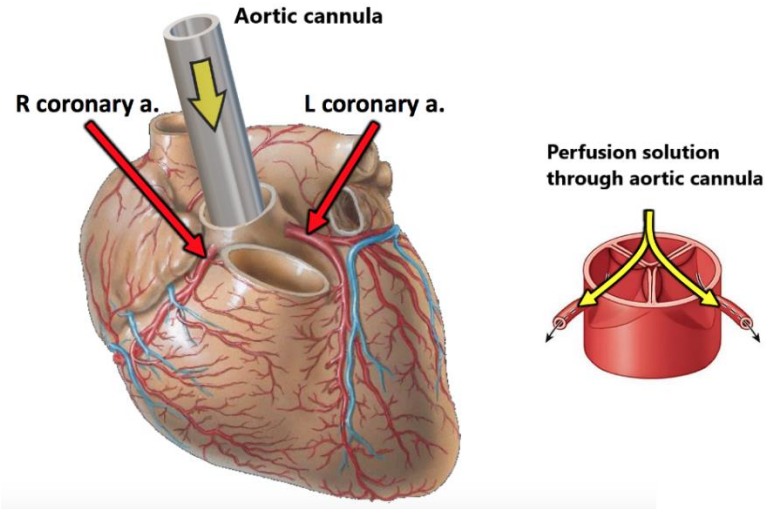


Figure 10. Retrograde perfusion through aortic cannula closes the aortic valve which makes the perfusion solution flow into the coronary arteries, supplying the myocardium with oxygen and nutrients (Image adjusted by Jorik Amesz from F. Netter).

A standard Langendorff setup consists of a blood reservoir, an oxygenator, a heat exchanger and a pump. A pump is needed to move the medium through the system. The perfusion solution is stored in a reservoir and needs to be heated to the appropriate temperature in the heat exchanger. The oxygenator supplies the perfusate with the appropriate gas mixture of oxygen and carbon dioxide. As previously mentioned, in Langendorff mode (Figure 11), the perfusate is pumped in retrograde way into the aorta, which perfuses the coronary arteries. Coronary circulation ends in the right atrium through the coronary sinus in which the venous blood is ejected via the right ventricle into the pulmonary artery and led back to the reservoir.

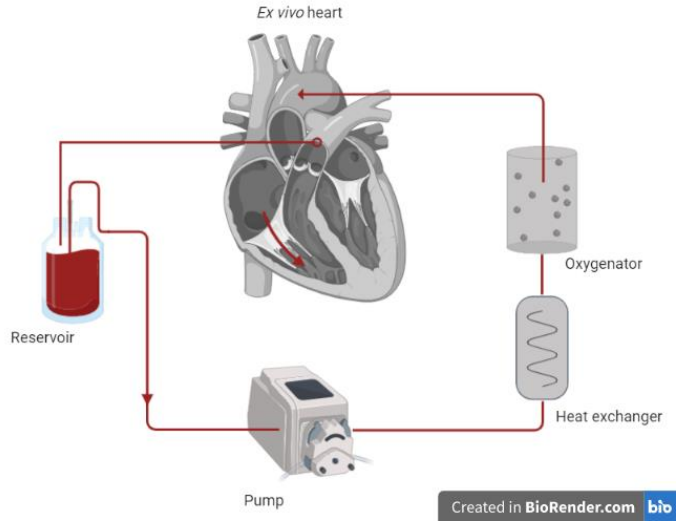


Figure 11. Basic Langendorff setup (created in Biorender by Jorik Amesz).

Once the heart is resuscitated in Langendorff mode, some EVHP systems switch to a working mode in which the left atrium is filled, leading to filling of the left ventricle and isovolumetric contraction, thereby ejecting blood into the aorta.

Parameters

The myocardium needs oxygen and nutrients to survive, but for EVHP the heart needs to be transferred from the donor to the EVHP setup, this results in a period with a lack of oxygen which is called hypoxia, and can lead to ischemia. Cardioplegia is given to prevent ischemia by arresting the cardiomyocytes, with an overdose of potassium ions, and thus protect the heart. Lactate is a major product of anaerobic metabolism and appears in the blood during hypoxic conditions which lowers the pH (Dhein, 2005).The pH of the blood is dependent on the balance between the concentration acid and base, according to the Henderson-Hasselbalch equation (Equation 1).

$$pH = pKa + \log(\frac{[Base]}{[Acid]}) \qquad \text{(Eq. 1)}$$

The bicarbonate buffer system in the blood (Equation 2) ensures this balance maintains 7.40 ± 0.05. Ventilation also plays a role because this determines the amount of carbon dioxide that is exchanged in the lungs. An increased or decreased carbon dioxide pressure can lead to either respiratory acidosis or alkalosis (Vander et al., 2008). In EVHP experiments, the pH can be influenced by supplementing with sodium bicarbonate or altering the gas flow.

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+ \qquad \text{(Eq. 2)}$$

Different types of carriers can be used for EVHP, which can be classified in three basic categories, being a crystalloid perfusate, a blood perfusate and an erythrocyte perfusate. A crystalloid perfusate is a transparent solution that mimics the ionic composition of the blood, e.g. Krebs-Henseleit. Often a sugar, like glucose needs to be added to those crystalloid solutions. Whole blood is the most physiologic identical perfusate but needs to be drained from a donor animal. Erythrocytes of donors can be added to crystalline perfusates to provide the solution with the oxygen-carrying protein haemoglobin. Albumin or another protein is added to account for the low oncotic pressure of crystalline carriers (White, 30 July 2019).

When reperfusion is started on the setup, tissue damage occurs because the restoration of circulation results in inflammation and oxidative stress, which is called ischemia-reperfusion injury. This injury should be minimized to protect the myocardium and to result in a successful transplantation from donor to the EVHP setup (Osaki et al., 2006).

The flow in the coronaries can be explained with Ohm's law (Equation 3), which gives the correlation between resistance (R), pressure (P) and flow (Q).

$$Q = \frac{\Delta P}{R} \qquad \text{(Eq. 3)}$$

The flow is dependent of the resistance, which is highly dependent of the vessel diameter (r) according to Poiseuille's law (Equation 4).

$$Q = \frac{\pi \Delta P r^4}{8 \eta L} \qquad \text{(Eq. 4)}$$

A small change in vessel diameter has a huge impact on blood flow and thus on pressure via Ohm's law (Figure 12). The right perfusion pressure of 60 – 140 mmHg is important to prevent damage to the coronaries and myocardium (Nocke, Meyer & Lessmann, 2014).

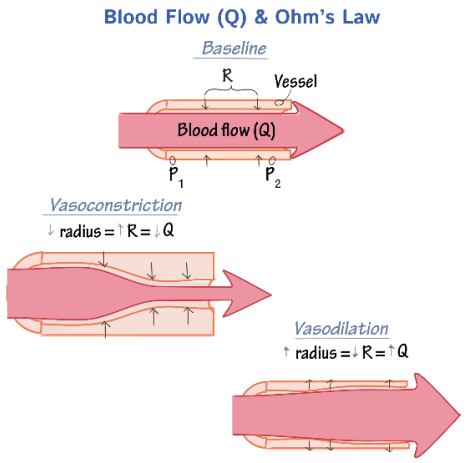


Figure 12. Relationship between blood flow and Ohm's law, flow is highly dependent on the vessel diameter (Draw It to Know It, Creations, LLC).

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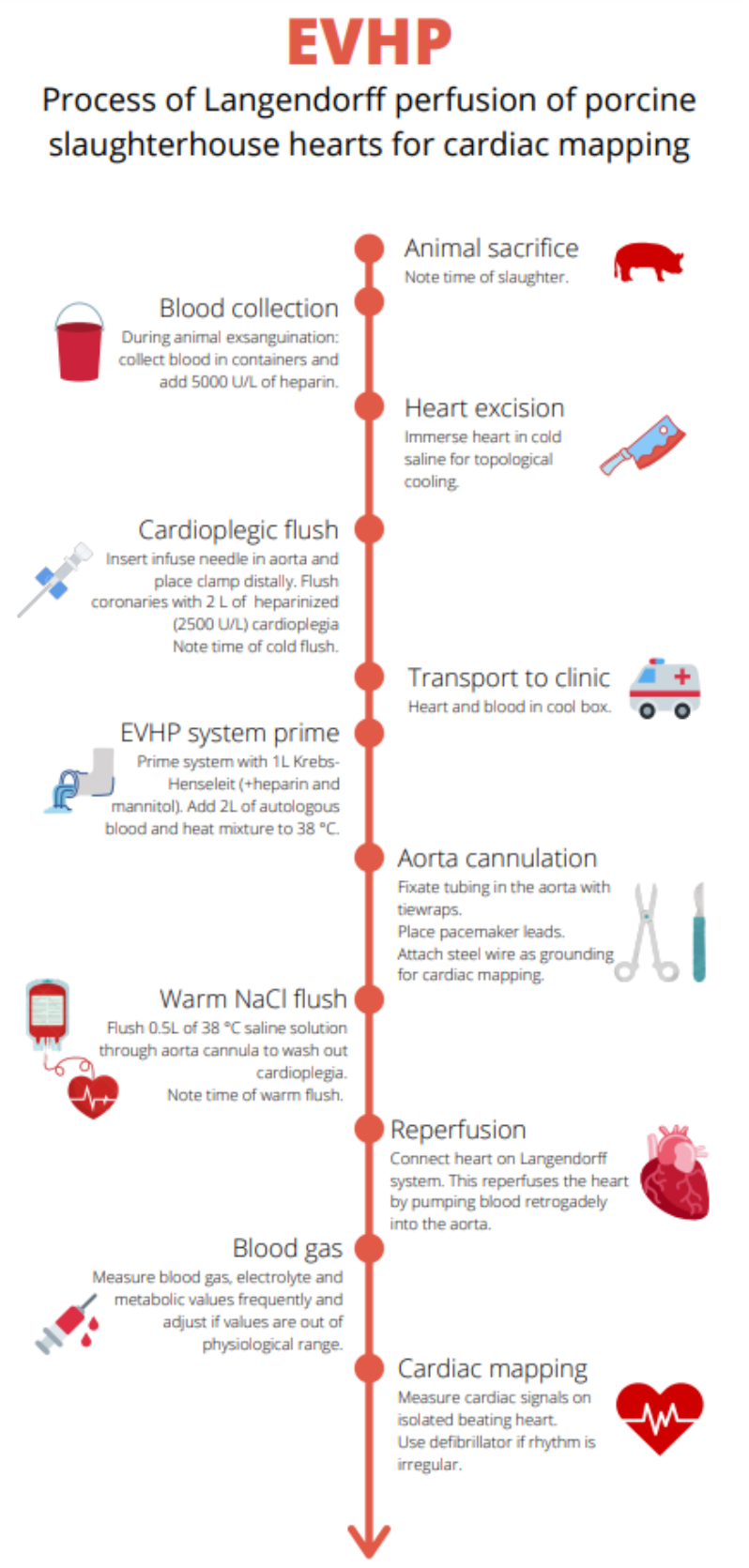
B Flowchart


EVHP
Process of Langendorff perfusion of porcine slaughterhouse hearts for cardiac mapping
Animal sacrifice
Note time of slaughter.
Blood collection
During animal exsanguination: collect blood in containers and add 5000 U/L of heparin.
Heart excision
Immerse heart in cold saline for topological cooling.
Cardioplegic flush
Insert infuse needle in aorta and place clamp distally. Flush coronaries with 2 L of heparinized (2500 U/L) cardioplegia
Note time of cold flush.
Transport to clinic
Heart and blood in cool box.
EVHP system prime
Prime system with 1L Krebs-Henseleit (+heparin and mannitol). Add 2L of autologous blood and heat mixture to 38 °C.
Aorta cannulation
Fixate tubing in the aorta with tiewraps.
Place pacemaker leads.
Attach steel wire as grounding for cardiac mapping.
Warm NaCl flush
Flush 0.5L of 38 °C saline solution through aorta cannula to wash out cardioplegia.
Note time of warm flush.
Reperfusion
Connect heart on Langendorff system. This reperfuses the heart by pumping blood retrogadely into the aorta.
Blood gas
Measure blood gas, electrolyte and metabolic values frequently and adjust if values are out of physiological range.
Cardiac mapping
Measure cardiac signals on isolated beating heart.
Use defibrillator if rhythm is irregular.

Protocol for Langendorff perfusion of porcine slaughterhouse hearts

Jorik H. Amesz

Departments of Translational Electrophysiology & Cardiothoracic Surgery, Erasmus Medical Center

Summary

This protocol describes in detail the steps identified to resuscitate porcine hearts obtained from a slaughterhouse for cardiac mapping purposes. The protocol is developed for employees of the above stated departments as guidance for Langendorff experiments. A sheet with time tables is attached to this document in which values of the procedure can be noted.

Preparations before experiment

Building the Langendorff apparatus

1. Hang cardiotomy reservoir, oxygenator and funnel in their holders.
2. Connect one end of 3/8 inch tubing to the outflow at the bottom of the reservoir.
3. Insert this tubing in the roller pump and check the flow direction on the roller pump.
4. Connect the other end of this tubing to the blood inflow of the oxygenator.
5. Connect another 3/8 inch tube to the blood outflow of the oxygenator.
6. Hang the other end of this tube in the the funnel with the support of a tripod hook.
7. Insert a 3/8 x 3/8 inch connector with luer-lock side-branch in the end of this tube.
8. Connect a three-way valve to this side-branch and connect a pressure isolator balloon to another side of this valve.
9. Connect a last 3/8 inch tube to the outflow of the funnel.
10. Connect the other end of this tube to the inflow at the top of the cardiotomy reservoir.
11. Connect the blue tubing of the heater unit to the water inflow and outflow of the oxygenator. And fill the heater unit with water.
12. Connect the air flow sensor to the standard air source of the hood with a 3/8 inch tube and tie wrap. Make sure the air source is turned off to avoid excessive pressure buildup in the sensor.
13. Connect a flexible tube to the gas inflow of the oxygenator and connect the other end to the air flow sensor.

Day of experiment

Blood and heart collection at abattoir

1. Note the time of animal slaughter.
2. Collect at least 2 L of blood in small (≈1 L) buckets during animal exsanguination.
3. Add 5000 U/L of heparin to this blood.
4. Note the time of heart excision.
5. Immerse the heart immediately in cold physiological saline.
6. Inspect the heart visually and manually on quality. Check if no epicardial bleedings or big lesions are present, especially in the coronaries. Does the heart feel flexible, not stiffened? If the quality seems insufficient, discard this heart and try another animal by restarting at Step 1 of this section.
7. Place a clamp on the ascending aorta proximal to the first bifurcation as distal as possible. To do so, identify anatomy and dissect the aortapulmonary window if needed. Place clamp when branches can be identified and it is certain that the clamp will obstruct all flow in the aorta.

8. Insert an infuse needle between the aortic valve and this aortic clamp, close to the clamp to prevent valve damage.
9. Connect an infuse to this needle and flush 2 L of cold St .Thomas cardioplegia (+2500 U heparin) through the coronary arteries with gravitational force. Before the flush, make sure the infuse system is de-aired to prevent air entering the coronaries.
10. Note the time of cold cardioplegia flush.
11. Check if the cardioplegia exits from the coronary sinus into the right atrium and check if there is pressure on the aortic root to make sure cardioplegia flows through the coronary arteries.
12. After flush, place the heart in a transportation bag filled with cold saline.
13. Transport the blood and heart in cool boxes with ice back to the Erasmus MC.

Preparing the Langendorff apparatus

1. Turn on the roller pump and prime the EVHP system with 1 L of Krebs-Henseleit solution (+2500 U heparin & 16 mM mannitol). This solution should be prepared prior to the experiment.
2. De-air the whole system including the pressure isolator line. Connect the pressure transducer to this isolator line.
3. Add 2 L of autologous blood to the system.
    a. If not enough volume was collected from one animal, blood from another animal can be added. Or the priming can be done with less Krebs-Henseleit, as long as the dilution blood:KH remains 2:1 and the total volume in the system is sufficient.
4. Turn on the heater unit and heat the blood-KH mixture to 38 degrees Celsius.
5. Take a baseline sample of the blood-KH mixture for blood gas, electrolyte and metabolite values.

Preparing the heart for cardiac mapping and for attachment to the Langendorff apparatus

1. Insert a 3/8 inch tube and connector in the aorta, a few millimeters above the valve and fixate this cannula with multiple tie wraps. A 3/8 inch connector with tubing can be used as cannula because of its ribbed surface.
2. Stitch a steel wire in distal aortic tissue between the tie wraps as indifferent electrode and wrap it around the aorta for a bigger contact surface.
3. Stitch bipolar pacemaker wires in atrial and ventricular tissue (with no fat or incisions) as reference or for stimulation.
4. Measure the heart mass.
5. Attach an infuse system on the aortic cannula and flush at least 0.5 L of warm 0,9% NaCl in the aorta to wash out cardioplegia. Avoid air entering the circulation by making an air-free connection between the cannula and infuse.
6. Note the time of this warm flush.

Attaching the heart to the Langendorff apparatus

1. Hold the heart in the funnel underneath the tube from which the perfusate drips.
2. Fill the aortic cannula with this perfusate.
3. Make an air-free connection between the tubing and cannula to attach the heart on the system, making sure no air enters the coronary circulation.
4. Note the time of heart connection on the setup.

5. Recirculate the perfusate with a flow rate on the roller pump based on heart mass of 1 – 1.5 mL/g/min.
6. Monitor the pressure on the pressure transducer with a normal pressure around 100 mmHg, make sure the pressure does not exceed 140 mmHg.
7. Turn on the gas flow by opening the flow on the sensor and turning on the air flow of the hood. Try to reduce the gas flow to less than 1 L/min to prevent respiratory alkalosis.
8. Take another blood sample for blood gas analysis.

Defibrillation

1. Hearts (ventricles) should start contracting spontaneously after reperfusion on the Langendorff system.
2. In case of ventricular fibrillation, perform electrical cardioversion (10 – 30 J) with metal defibrillation paddles placed directly on the heart.

Addition and infusion

1. Add 1 gram of magnesium sulfate to the blood-KH mixture to prevent arrhythmias.
2. Start a glucose-insulin infuse with 50 mL G-50% with 20 Units insulin per hour. Place the syringe in a syringe pump with a infuse line directly on the reservoir. This syringe should be prepared before the experiment.
3. Start a dobutamine infuse with 8 µg dobutamin per minute.

Blood gas analysis and correction

1. Take a blood sample from the arterial tubing, just after the oxygenator, 5 minutes before heart-setup connection, 5 minutes after connection and subsequently every 30 minutes. Note the times of these samples.
2. Insert the blood samples on a blood gas analysis system.
3. Note the blood gas, electrolyte and metabolite values on the time table sheet and compare these with the reference values.
4. Correction
    a. Low calcium: add calcium gluconate to the system.
    b. High potassium: add extra insulin
    c. Low pH, low oxygen pressure (respiratory acidosis): increase gas flow
    d. Low pH, normal oxygen pressure (metabolic acidosis): add sodium bicarbonate
    e. High pH (respiratory alkalosis): decrease gas flow
    f. Low glucose: add extra glucose

Cardiac mapping

With good contractions and a stable heart rhythm, mapping can be started.

1. Attach crocodile clip from mapping system to steel wire for grounding.
2. Attach pacemaker leads to an external pacemaker for stimulation.
3. Place the electrode-array on the heart on previously defined positions and perform the epicardial mapping procedure. Detailed mapping protocols have been written by the department of translational electrophysiology.

Attachment to Appendix C: Time table

Datum:

Timing	Slacht	
	Hart ondergedompeld op ijs	
	Toedienen cardioplegie	
	Start auto	
	Aankomst EMC Ee-19	
	Flush	
	Connectie Langendorff	
	Stop experiment	

Hart massa	Voor perfusie	g
	Na perfusie	g

Mix	Bloed	L
	Krebs-Hens	L
		L

			- 5'	5'	30'	60'	90'	120'	150'	180'
Waarden		Range	:	:	:	:	:	:	:	:
Arterieel bloedgas	pH	7.35 – 7.45								
	pCO_2	35 – 45 mmHg								
	pO_2	100 – 300 mmHg								
	$cHCO_3^-$	21 – 27 mmol/L								
	Hct	20 – 30 %								
	ctHb	11,5 – 17,4 g/dL								
	sO_2	94 – 100 %								
	cNa^+	135 – 145 mmol/L								
	cK^+	3,5 – 5,1 mmol/L								
	cCa^{2+}	1,1 – 1,3 mmol/L								
	cGluc	60 - 140 mg/dL								
	cLac	0 – 2.0 mmol/L								

		X1	X2	X3
Pacing	Start tijd			
	Stop tijd			
	BPM			
	Output (mA)			

		X1	X2
Defi	Tijd		
	Joule		
	Aantal		

D Series of experiments

Logbook

This section contains a logbook of the seven different experiments. The first three experiments failed but were very informative and contributed to the success of the last four experiments (Table 1). Each experiment led to the optimization of the Langendorff protocol and this learning curve will become clear in this logbook. Cardiac mapping on *ex vivo* hearts was a new project in the Erasmus MC and no one had experience with these experiments, yet the project started from zero and ended with a beating heart model. This process comprised the design of the optimal setup and the optimization of the different clinical parameters, e.g. ischemia times and blood gas status.

Table 1. Number and date of seven different experiments. Resuscitation of porcine slaughterhouse hearts was shown in the last four experiments.

Number of experiment	Date	Heart resuscitation
N1	07-07-2020	✗
N2	21-07-2020	✗
N3	27-07-2020	✗
N4	31-08-2020	✓
N5	14-09-2020	✓
N6	28-09-2020	✓
N7	19-10-2020	✓

Initial preparations

In the previous weeks, materials were collected and assembled on the Langendorff setup. A storage room was cleaned out and converted into a lab facility for experimental research. Test runs were done with water on the setup to make sure no leaks were present in the system. The cannulation procedure was practiced in the dissecting room.

N1	7 July 2020
Blood and Heart Collection	Two hearts and a few liters of blood were collected at an industrial pork slaughterhouse. The hearts were still warm and flexible upon reception, indicating a short WIT. This was the first time that cannulation and cardioplegic flush had to be performed at the slaughterhouse, which was a bit unhandy despite the training period. Nevertheless, aortic cannulation was performed after which cardioplegia was pumped through the cannula with a pressure bag.
Reperfusion	The Langendorff system was filled with 2 liters of non-autologous blood. The blood perfusate was heated in the oxygenator with a continuously running warm water tap. The temperature of this warm water tap was regulated with a temperature sensor in the water tubing, but temperature fluctuated and a lot of water had to be used. The first heart that was connected to the system, seemed to show some minimal atrial fibrillations. But unfortunately the heart snapped loose before the electrode could be placed on the atria. The heart slipped from the cannula because one tie wrap was not enough to support the heart's weight. The snap resulted in a lot of blood loss (Figure 1).  *Figure 1. A lot of blood loss due to snapping of heart from the aortic tubing.* A second heart was connected, but no contractions were here observed. The cold cardioplegic flush was probably not performed properly at the abattoir due to problems with the cannulation.
Blood gas	One blood gas measurement was done for the second heart, with increased potassium and lactate levels and decreased carbon dioxide levels.
Discussion	The outflow of the funnel was very small which resulted in fluid accumulation in the funnel and a decreased venous return to the reservoir. This led to obstruction of the tubing. This first experiment resulted in a lot of cleaning of the lab facility, but was very useful to identify steps that could be taken to improve the protocol.

N2	**21 July 2020**
Adjustments in comparison with the previous experiment	- The hood was covered with surgical cloths (Figure 2).  *Figure 2. Setup covered with surgical cloths.* - The aortic cannula was fixated with multiple tiewraps to fully support its weight. - A water bath was connected to the oxygenator instead of a running water tap to heat the mixture in the oxygenator. This allowed for a better temperature regulation and decreased the amount of used water, because the water was recirculated. - The system was primed with 1L of adjusted Krebs-Henseleit solution (+2500 U/L heparin and 16 mmol/L mannitol). Heparin could form a coating along the lining of the tubing to reduce blood clotting because of contact with foreign substances. Two liters of non-autologous blood were later added and mixed with this priming solution, which resulted in a lower viscosity and better flow. - A 100% oxygen bottle was connected to the oxygenator instead of standard air (21% oxygen) of the fume hood. - The outflow diameter of the funnel was increased.
Blood and Heart Collection	Two hearts were collected from the same industrial abattoir, but the hearts were already stiffened upon reception. The butcher declared that the slaughtering process was changed and that we could only receive hearts approximately 20 minutes after the animal's sacrifice. Nevertheless cardioplegic flush was quickly done and the hearts were transported back to the Erasmus MC.
Reperfusion	Despite the rapid cardioplegic flush, both hearts would not revive, probably because of a long WIT.
Blood gas	The oxygen bottle was disconnected after the first blood sample analysis, because the pO_2 was measured at 523 mmHg, which is far above the physiological range.
Discussion	The experiment failed, but a lot of improvements were made in comparison with the first try. Aortic cannulation was rapidly performed.

N3	**27 July 2020**
Adjustments in comparison with the previous experiment	- Another abattoir was chosen in which the cardioplegic flush could be performed directly next to the slaughtering process. Collaboration was easier with this small local slaughterhouse facility. - Hearts were first inspected on quality before continuation of the cardioplegic procedure. - No connection with 100% oxygen bottle but with standard air source.
Blood and Heart Collection	Hearts were excised by the butcher with the other organs, e.g. lungs and liver still attached to the aorta. The WIT was approximately 15 minutes in those hearts. It was difficult for the butcher to collect multiple liters of blood.
Reperfusion	Perfusion was therefore performed with 0.5 L of KH solution and 1 L of autologous blood, so the ratio blood:KH remained 2:1.
Blood gas	The blood gas analysis machine did not work, because of a rinsing error, so no information could be obtained about the physiological status of the heart.
Discussion	The failure of the experiment is probably due to a disturbed ion balance.

N4	31 August 2020
Adjustments in comparison with the previous experiment	- Extensive literature study for optimizations in the protocol. - Only one heart was collected instead of multiple hearts. - Heart was immediately immersed in cold physiological saline for topological cooling. - Heart was flushed with 2 L of cardioplegia at the abattoir, instead of 1 L at the abattoir, and another liter the next hour. - Heart was placed in a transportation bag with cold saline in a cool box with icy slurry for transport. - Aortic cannulation was not performed at the abattoir, but was calmly performed in the clinic. - Cardioplegia was administered through a needle and the aorta was cross-clamped distally. - The infuse system contained a bubble chamber to prevent air flowing through the coronaries. - Blood was collected in smaller buckets of 1 L to better evaluate its volume and to make collection easier during exsanguination. - A timetable sheet with reference ranges for electrolyte, blood gas and metabolite values was used. WIT & CIT were also registered on this sheet. - Heart was flushed with warm saline solution prior to connection on the setup. - Medication with dobutamin, insulin, glucose and calcium gluconate was added.
Blood and Heart Collection	The one heart was still fibrillating when it was immersed in cold NaCl, 9 minutes after the animal shooting. The WIT was less than 10 minutes, because cardioplegia could be rapidly administered via a needle. This needle should be inserted with caution to prevent perforation of the backside of the aortic wall. Two liters of blood were easily collected and heparinized in collaboration with the butcher, which resulted in fresh blood for perfusion.
Reperfusion	Upon arrival at the Erasmus MC, the heart was cannulated and prepared for Langendorff perfusion. The perfusion system was primed with heparinized Krebs-Henseleit after which two liters of blood were added and this mixture was heated to 38 degrees Celsius. Before connection, the heart was flushed with 500 mL of warm (T = 38°C) saline solution to wash out the overdose of potassium ions from the cardioplegia solution. After this flush, the heart was connected to the Langendorff system, which resulted in a cold ischemic time of 105 minutes. Immediately after connection, the heart started fibrillating and started showing signs of contractions (Figure 3).

Figure 3. First successful experiment in which a heart was resuscitated, which brought a lot of joy in the lab.

Blood gas

A blood gas sample was taken prior to heart connection and no adjustments were made after analysis of the concentrations, because these were in physiological ranges. Another blood gas sample was taken after connection, which showed elevated potassium concentrations. It was tried to lower the K^+-concentrations by adding one ampule of calcium-gluconate and by infusing a glucose-insulin mixture. Also, every 10 minutes, a shot dobutamine was given, which significantly improved the contractility of the heart.

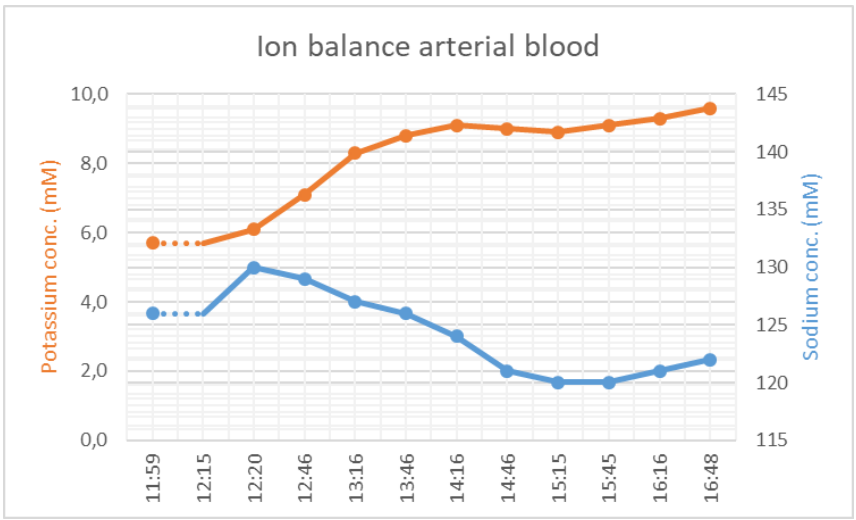
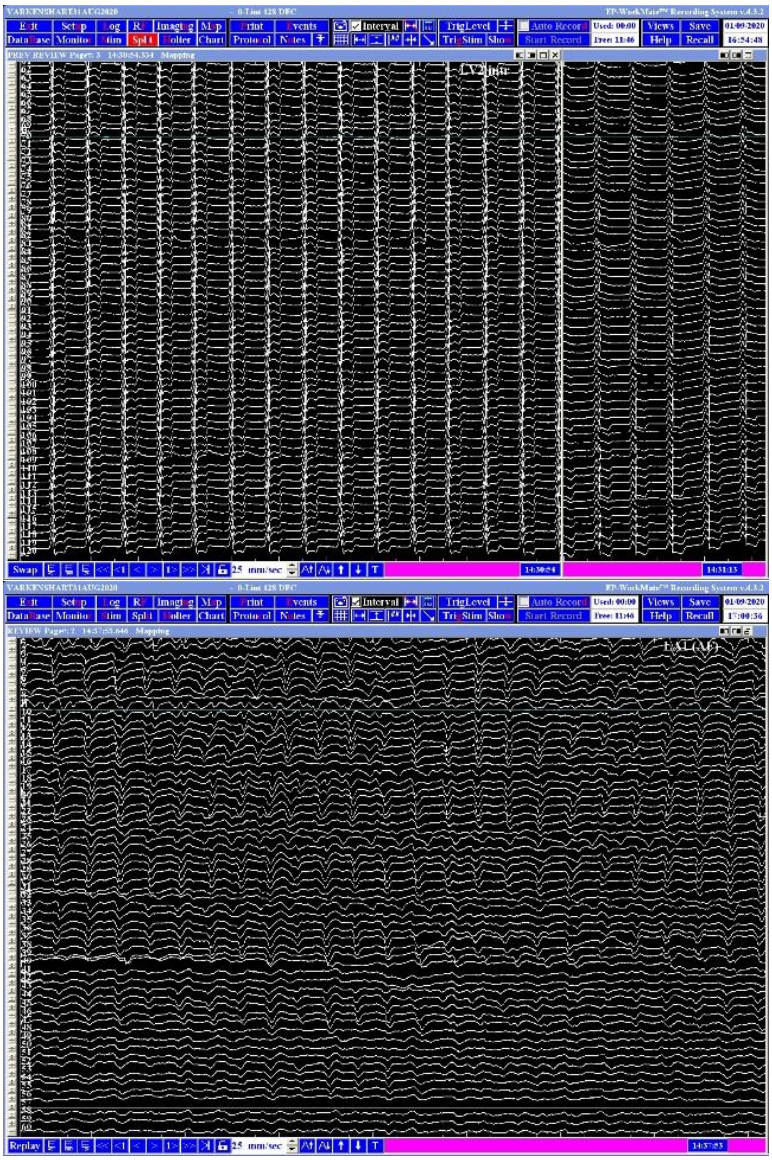
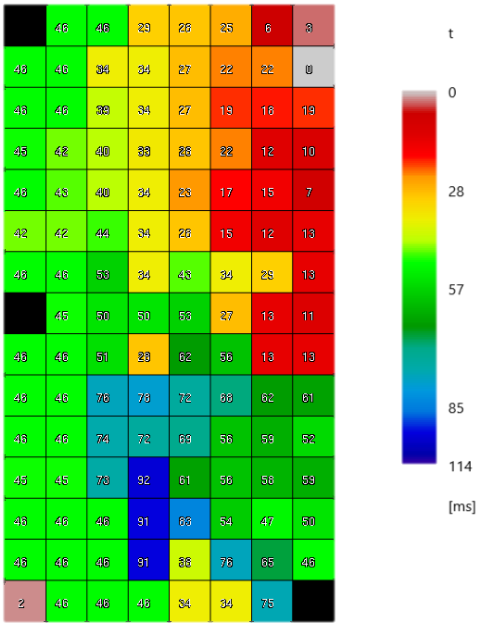


Figure 4. Overview of the potassium (orange) and sodium (blue) concentrations during the 5-hour experiment. Potassium levels are increasing, while the sodium concentrations are slightly low. The first points were measured before heart connection on the setup.

No success was accomplished in lowering the potassium concentration, instead it kept increasing during the experiment (Figure 4).

Mapping	A pacemaker wire was fixated and a steel wire was connected as reference for the mapping system. Cardiac mapping data signals were recorded (Figure 5). The ventricles beat in a constant rhythm, while the atria were only fibrillating. In the beginning, stimulation with the pacemaker was needed, but eventually the ventricles could beat normally without external stimulation. A few times, a shot magnesium sulfate was given to prevent arrhythmias. Three times, the defibrillator was used to reset the heart in normal rhythm.  *Figure 5. Raw signals with 128-electrode array of intrinsic rhythm. Above: Left ventricular signals. Below: Left atrial signals.* The heart beat for 5 hours, after which the experiment was stopped. Initial heart weight before connection was 470 grams and increased to 672 grams after disconnection from the Langendorff setup. This was the first successful experiment in which a porcine heart was resuscitated in Langendorff mode and epicardial mapping signals were registered. This data was analyzed for a first idea of the signal quality (Figure 6).

	 *Figure 6. Activation map of a location on the left ventricle, a global activation pattern can be seen.*
Discussion	Despite the success of this experiment, calcium and glucose concentrations could not be monitored with the current blood gas analyzer and medication strategies had to be improved.

N5	**14 September 2020**
Adjustments in comparison with the previous experiment	- Infuse needle was used for cardioplegic flush instead of normal needle to prevent unwanted perforations. - Syringe pumps were installed for a constant infusion of dobutamin and insulin in the reservoir. - Another blood gas analyzer was used to also measure calcium and glucose concentrations.
Blood and Heart Collection	One heart was collected and cardioplegic flush was easier with the new needle, resulting in a short WIT of 8 minutes. Blood collection was difficult so only 0.75 L of autologous blood could be brought back to the EMC.
Reperfusion	This blood volume was diluted with 1 L of KH, but this resulted in a low hematocrit percentage. The heart started beating after reperfusion, which made this the second successful experiment. Especially the left ventricle made powerful contractions, but the other chambers showed less contractions.
Blood gas	It was possible to measure the calcium and glucose concentrations of the perfusate. Syringe pumps were started with a dobutamin and glucosine-insulin infuse to better control the electrolyte concentration of the perfusate (Figure 7). With the infusion of insulin, it was easier to minimize the build-up of extracellular potassium ions, although the heart was still dying. The lactate concentration was increasing rapidly during the experiment (Figure 8), probably because of the low amount of erythrocytes, resulting in ischemia. Glucose concentration was too high at the end of the experiment.  *Figure 7. Syringe pumps for infusion of dobutamin and glucose-insulin directly in the reservoir.*

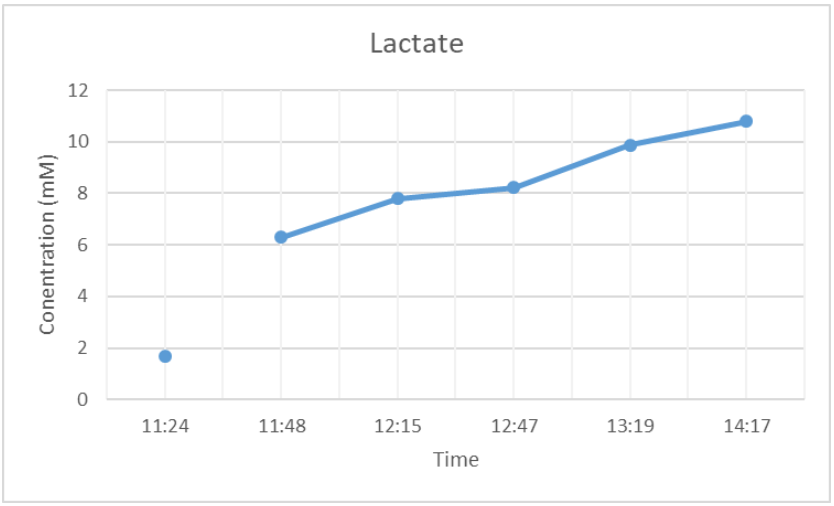


Figure 8. Increase of lactate in the experiment. The first point was measured before heart connection on the setup.

pH values were initially high in the blood-KH perfusate with values above 8.0, but this lowered to a value of 7.45 after stopping the air inflow. Restarting the air supply increased the pH back to 7.78, indicating a respiratory alkalosis.

Mapping

One location was chosen on the left ventricle for a mapping and pacing protocol. From four sides of this location mapping was performed both in sinus rhythm and during paced rhythm. Then a linear lesion was made with an ablation catheter and the procedure was repeated (Figure 9). Unfortunately after the creation of the lesion, the heart started fibrillating and no relevant signals could be recorded.

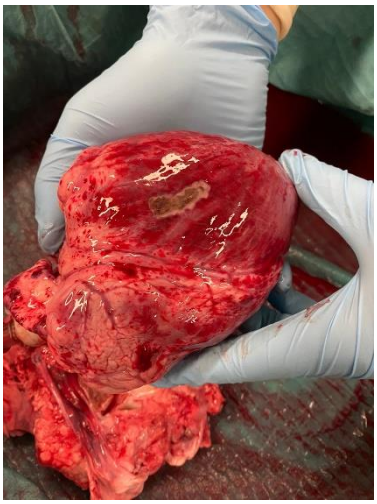
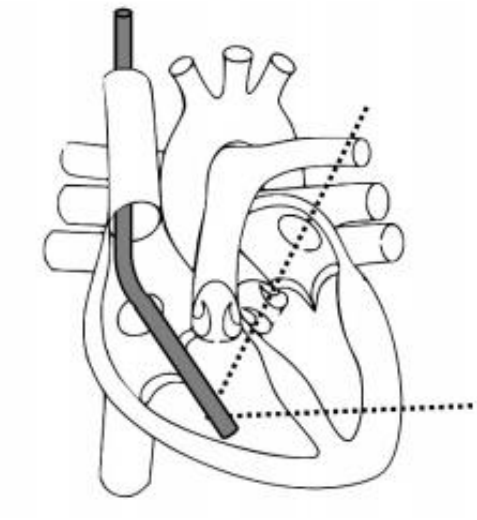
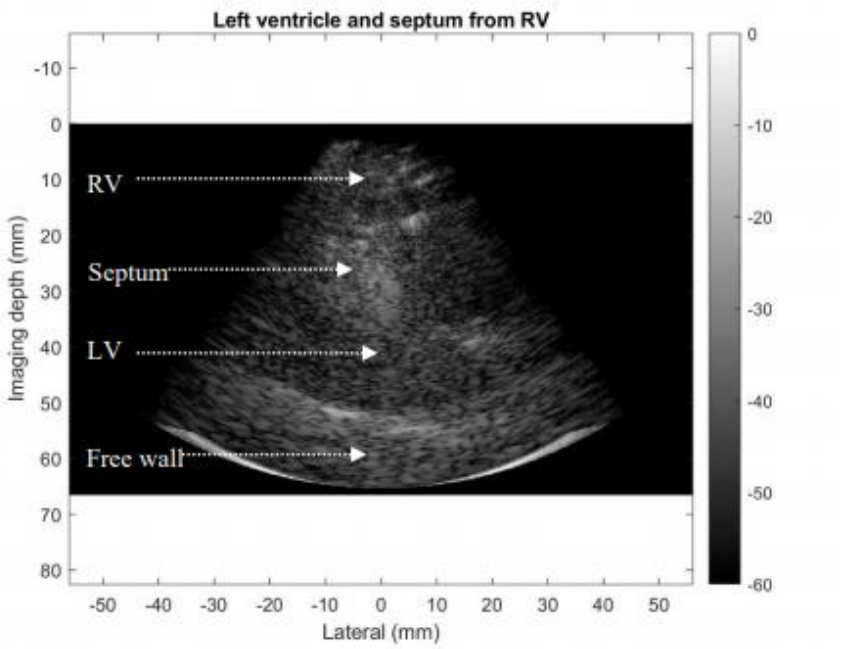


Figure 9. Lesion created on left ventricle. Picture taken after the experiment.

An ultrasound probe was tested on the beating heart (Figure 10) and it was possible to record initial high frame rate data for an experimental echocardiography study (Figure 11). The aim of this study is to track

	electromechanical wave patterns and compare this with the activation patterns from the cardiac mapping studies.  *Figure 10. Position of the catheter in the right ventricle*  *Figure 11. A sample of an image acquired with the high frame rate method where the catheter was in the right ventricle. Anatomic markings are guessed.* The heart mass increased from 480 to 624 grams.
Discussion	Another reservoir and oxygenator were used, which were difficult to completely de-air with the priming solution.

N6	28 September 2020
Adjustments in comparison with the previous experiment	-
Blood and Heart Collection	The first heart was rejected at the slaughterhouse because the ventricles felt stiff. Another heart was brought back to the lab. No autologous blood was used, because of this first rejection and later difficulties with blood collection.
Reperfusion	Heart started contracting directly after reperfusion.
Blood gas	Problems occurred with the blood gas analyzer so it was difficult to analyze the status of the blood perfusate. When these problems were solved the pH was again very alkaline, around 8 and potassium levels increased rapidly. This was probably the reason why the contractions were not strong and the heart stopped beating after two hours.
Discussion	Problems with the roller pump occurred, the flow rate was not constant and could rapidly change without altering the settings on the pump. Professional videos were recorded of the procedure to visualize the procedure with an explanatory movie.

N7	**19 October 2020**
Adjustments in comparison with the previous experiment	- An air flow sensor (Ohio Medical Corporation) was integrated for a more precise control of the air supply (Figure 12). Reason for this is the high pH in previous experiments, probably due to a low carbon dioxide pressure. With this sensor the air flow can be reduced to increase the carbon dioxide pressure.  *Figure 12. An air flow sensor for a better regulation of the gas flow in the oxygenator.*
Blood and Heart Collection	Blood was easily collected from one pig (aprox. 1.5 L) and the heart from the next pig was chosen for cardioplegic flush, which was easily performed, resulting in a warm ischemic time of 12 minutes. The butcher declared, because the temperature is dropping outside, the pigs get a thicker fur, which prolongs the time needed up to heart excision and explains the longer warm ischemic time.
Reperfusion	The heart immediately started contracting after connection on the setup. Very strong contractions on the left ventricle and waves could be seen on the atria.
Blood gas	Blood gas could be monitored. Calcium levels could be maintained within the physiological ranges, resulting in strong contractions. pH was around 7.6 – 7.7, which is still high, but already better compared to the previous experiments. Carbon dioxide pressure was around 7 – 17 mmHg, which is low, but also better than before. This is the result of a reduced gas flow with the air flow sensor flow of approximately 1 L/min, preventing a huge respiratory alkalosis.
Mapping	Mapping was performed on the total surface of the heart. Pacing protocol: Electrode on one location on the left ventricle. Pacing around the electrode at four locations (above, underneath, right, left). Creation of a lesion on the chosen ventricular location with an ablation catheter. And again pacing around the electrode to examine the effect of the lesion.

Discussion	To further decrease the pH, a gas bottle with higher carbon dioxide percentage could be considered, like the one below with 95% standard air and 5% carbon dioxide. Unfortunately, this gas source could not be tested because the butcher had problems with his equipment and could not sacrifice pigs for a few weeks.  *Figure 13. Bottle with 95% standard air and 5% carbon dioxide gas.*