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## Studying the impact of intracardiac neurons on cardiac electrophysiology and arrhythmogenesis in an ex-vivo Langendorff system

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**TITLE:**

Impact of Intracardiac Neurons on Cardiac Electrophysiology and Arrhythmogenesis in an *Ex Vivo* Langendorff System

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**KEYWORDS:**

Autonomic nervous system, ventricular tachycardia, autonomic ganglia, intracardiac nervous system, ablation, sudden cardiac death

**SUMMARY:**

Here, we present a protocol for the modulation of the intracardiac autonomic nervous system and the assessment of its influence on basic electrophysiology, arrhythmogenesis, and cAMP dynamics using an *ex vivo* Langendorff setup.

**ABSTRACT:**

Since its invention in the late 19<sup>th</sup> century, the Langendorff *ex vivo* heart perfusion system continues to be a relevant tool for studying a broad spectrum of physiological, biochemical, morphological, and pharmacological parameters in centrally denervated hearts. Here, we

describe a setup for the modulation of the intracardiac autonomic nervous system and the assessment of its influence on basic electrophysiology, arrhythmogenesis, and cyclic adenosine monophosphate (cAMP) dynamics. The intracardiac autonomic nervous system is modulated by the mechanical dissection of atrial fat pads—in which murine ganglia are located mainly—or by the usage of global as well as targeted pharmacological interventions. An octapolar electrophysiological catheter is introduced into the right atrium and the right ventricle, and epicardial-placed multi-electrode arrays (MEA) for high-resolution mapping are used to determine cardiac electrophysiology and arrhythmogenesis. Förster resonance energy transfer (FRET) imaging is performed for the real-time monitoring of cAMP levels in different cardiac regions. Neuromorphology is studied by means of antibody-based staining of whole hearts using neuronal markers to guide the identification and modulation of specific targets of the intracardiac autonomic nervous system in the performed studies. The *ex vivo* Langendorff setup allows for a high number of reproducible experiments in a short time. Nevertheless, the partly open nature of the setup (*e.g.*, during MEA measurements) makes constant temperature control difficult and should be kept to a minimum. This described method makes it possible to analyze and modulate the intracardiac autonomic nervous system in decentralized hearts.

## INTRODUCTION:

The Langendorff *ex vivo* heart perfusion system continues to be a relevant tool for performing a broad spectrum of physiological, biochemical, morphological, and pharmacological studies in centrally denervated hearts<sup>1-5</sup> since its invention in the late 19<sup>th</sup> century<sup>6</sup>. To date, this system is still widely used for various topics (*e.g.*, ischemia reperfusion) or to study cardiac pharmacological effects<sup>7,8</sup>, and is a basic tool in cardiovascular research. The longevity of this method results from several advantages (*e.g.*, measurements are performed without the influence of the central nervous system or other organs, systemic circulation, or circulating hormones). If needed, pharmaceuticals can be added in a controlled manner to the perfusion buffer or applied to specific structures directly. Experiments are reproducible, and a relatively high number of experiments can be performed in a short period of time. The (in part) open nature of the setup can make temperature regulation difficult and needs to be taken into account. Although the Langendorff system is also used in larger species<sup>9</sup>, smaller animals are primarily used as the experimental setup is less complex, and a greater biological variability (*e.g.*, transgenic mouse models) can be used.

In the experimental setup of this protocol, the influence of the intracardiac autonomic nervous system on basic electrophysiological parameters, ventricular arrhythmogenesis, epicardial conduction, and cyclic adenosine monophosphate (cAMP) dynamics is evaluated. A large number of intracardiac ganglia, which are mainly located in the atrial fat pads and are now well known to control cardiac electrophysiology independent from central neural control, are either left intact or manually removed with careful mechanical dissection. A pharmacological modulation of the autonomic nervous system is performed either globally by adding pharmaceuticals to the perfusion buffer or locally by targeted modulation of the atrial ganglia. After the experiments, the hearts are well suited for an immunohistological assessment as all blood cells have been removed due to the continuous perfusion, which can increase the quality of staining.

The overall goal of the described techniques is to offer novel perspectives for detailed studies regarding the impact of the autonomic nervous system on cardiac electrophysiology and arrhythmogenesis in the mouse heart. A reason to use this technique is that it is possible to study and alter the autonomic nervous system without the impact of the central nervous system. One major advantage is the easy employment of pharmacological experiments, in which potential pro- or antiarrhythmic properties of old and new agents can be tested. In addition, transgenic and knockout mouse models of various cardiac diseases are available to investigate the mechanisms underlying arrhythmias, heart failure, or metabolic diseases. This approach has enhanced our understanding of how the autonomic nervous system on the atrial level can impact ventricular cardiac electrophysiology and the induction of arrhythmias.

## **PROTOCOL:**

All procedures involving animals were approved by the local authorities of the State of Hamburg, the University of Hamburg Animal Care and Use Committees.

### **1. Preparation of the Langendorff Apparatus**

Note: A commercially available Langendorff perfusion system is used.

1.1. Prepare a modified Krebs-Henseleit solution (119 mM of sodium chloride, 25 mM of sodium bicarbonate, 4.6 mM of potassium chloride, 1.2 mM of potassium phosphate monobasic, 1.1 mM of magnesium sulfate, 2.5 mM of calcium chloride, 8.3 mM of glucose, and 2 mM of sodium pyruvate). Add a mixture of 95% oxygen/5% carbon dioxide to the perfusion solution to prevent calcium precipitation. Filter the buffer with a pore size of 0.22  $\mu\text{m}$ .

1.2. Add a pharmacological agent to the buffer to investigate its effect on cardiac electrophysiology and arrhythmogenesis as needed.

1.3. Start the water bath and place the perfusion solution including a mixture of 95% oxygen/5% carbon dioxide in it. Adjust the temperature of the water bath, so that the perfusion solution temperature directly before the cannula is  $\sim 37^\circ\text{C}$ .

1.4. Start the roller pump and fill the apparatus with the perfusion solution as soon as the correct temperature is reached.

1.5. Adjust the pump rate before attaching the heart, so that no air bubbles are left in the cannula when mounting it to the apparatus.

### **2. Hard- and Software Preparation**

2.1. Connect a digital data acquisition system and its corresponding software to the Langendorff apparatus for a continuous recording of the perfusion pressure, flow rate, and heart rate.

2.1.1. Set the targeted perfusion pressure to 80 mmHg.

2.1.2. Start recording.

2.2. Use an electrophysiology catheter (**Table of Materials**) with platinum electrodes, an electrode surface of 0.5 x 0.5 mm, and an electrode spacing of 0.5 mm for the data recording and stimulation with a designated digital stimulus generator.

2.2.1. Place the catheter close to the area where the heart will be positioned after the attachment to the apparatus.

2.2.2. Prepare the stimulation by selecting 2 electrodes from the catheter and use a cycle length of 100 ms.

### 3. Preparation of the Heart

3.1. Add the cold (~2 - 4 °C) perfusion buffer (10 - 20 mL and 40 - 50 mL, so that the whole dish is covered) to the 2 Petri dishes (diameter of 6 cm and 10 cm) and the dish with the cannula and place them on ice directly next and under the microscope. Prepare a double knot around the cannula.

3.2. Rapidly excise the heart after cervical dislocation by opening the thorax using Mayo scissors and narrow pattern forceps. Then grip the aorta and vena cava above the diaphragm using the London forceps and excise the heart-lung block by cutting all vessels and connective tissue close to the spine with the Strabismus scissors.

3.3. Transfer the heart-lung block into the first dish (6 cm diameter) filled with the ice-cold buffer and carefully remove the lungs without damaging the heart by using Strabismus scissors and London forceps. Then place the heart under the microscope and carefully remove the thymus, the esophagus and trachea by using Spring scissors and Dumont SS forceps.

3.4. Use the Spring scissors to cut a hole of 1.5 - 2 mm in the upper part of the right atrium for the insertion of the catheter. Cut a hole in the pulmonary artery. Then cut the aorta directly under the supraaortic branches and remove the tissue from the aorta, so that the knot can be attached easily.

3.5. Keep the atrial fat pads, including the major atrially located ganglionated plexi, around the left atrium intact or completely remove them by careful dissection.

3.6. Transfer the heart to the dish with the cannula and place it under the microscope. Pull the aorta over the cannula with the Dumont SS forceps and tie the prepared knot tightly around the aorta. Make sure that the cannula is not placed too deep in the aorta so that the aortic valve and the coronary vessels are left free.

3.7. Attach the cannula quickly to the Langendorff apparatus. Make sure that there are no

bubbles left in the cannula.

3.8. Switch the perfusion pressure to 80 mmHg, allowing a constant pressure perfusion.

3.9. Insert the catheter carefully into the right atrium and right ventricle without touching or damaging the heart and attach the catheter to the cannula with tape.

3.10. Start the stimulation with the prepared cycle length of 100 ms for an initial 20 min equilibration period.

3.11. Close the chamber to allow a stable temperature.

#### **4. Electrophysiological Parameters and Arrhythmogenesis**

4.1. Apply a programmed stimulation via the distal or proximal electrodes of the catheter at twice the atrial or ventricular pacing threshold to evaluate the electrophysiological parameters as described in the following steps.

4.1.1. Determine the sinus node recovery time as the maximum return cycle length after 10 s of fixed-rate pacing at an S1S1 cycle length of 120 ms, 100 ms, and 80 ms.

4.1.2. Determine the Wenckebach point as the longest S1S1 cycle length (8 stimuli; S1S1: 100 ms; 2 ms stepwise reduction) with a loss of 1:1 AV nodal conduction. Determine the atrioventricular nodal refractory periods as the longest S1S2 (12 stimuli; S1S1: 120 ms, 110 ms, and 100ms; one short coupled extrastimulus with a 2 ms stepwise S1S2 reduction) with a loss of AV nodal conduction.

4.1.3. Determine the atrial and ventricular refractory periods as the longest S1S2 (12 stimuli; S1S1: 120 ms, 110 ms, and 100ms; one short coupled extrastimulus with a 2 ms stepwise S1S2 reduction) with an absent atrial or ventricular response<sup>10-11</sup>.

4.2. Perform a programmed extrastimulation (S1S1: 120 ms, 100 ms, and 80 ms, followed by up to 3 extra beats; 60 - 20 ms with a 2 ms stepwise reduction) or burst pacing protocols (5 s at S1S1: 50 - 10 ms with a 10 ms stepwise reduction) in line with the Lambeth Conventions to evaluate the ventricular arrhythmogenesis<sup>10-12</sup>.

#### **5. Epicardial Conduction Measurements**

Note: Record unipolar epicardial electrograms by using a 128-channel, computer-assisted recording system with a sampling rate of 25 kHz for high-resolution mapping. Use a 32 multi-electrode array (MEA; inter-electrode distance: 300  $\mu$ m; 1.8 x 1.8 mm). Note that the data were bandpass filtered (50 Hz) and digitized with 12 bit and a signal range of 20 mV.

5.1. Place the MEA in the designated area of the heart and add the grounding to another part of

the heart<sup>13-15</sup>.

5.2. Place an epicardial stimulation catheter close to the MEA and start a constant stimulation.

5.3. Start recording after the confirmation of good contact of the electrodes by checking the signal quality and amplitude.

5.4. Use an offline analysis for the determination of wave propagation velocity and dispersion in the conduction direction.

## 6. Förster Resonance Energy Transfer (FRET)-based Cyclic Adenosine Monophosphate (cAMP) Imaging

Note: For FRET-based measurements, harvest hearts from CAG-Epac1-camps transgenic mice<sup>16</sup>.

6.1. Use a self-built imaging system around a stereomicroscope<sup>15,17</sup>.

6.2. Place the stereomicroscope in front of the heart and adjust it for acuity.

6.3. Excite the cAMP sensor with a light source [*e.g.*, use a single-wavelength light emitting diode (440 nm)]. Split the emission light into donor and acceptor channels using a beam-splitter (for a cyan fluorescent protein/yellow fluorescent protein pair, use a 565dcxr dichroic mirror and D480/30 and D535/40 emission filters).

6.4. Ensure a stable temperature by putting a plastic wrap around the chamber.

6.5. Take images by using a scientific complementary metal-oxide-semiconductor (sCMOS) camera. Coordinate the light source and camera image capturing with an open source imaging software such as Micro-Manager.

6.6. To start the image acquisition, push the **Multi-D Acq.** button and set up a time-lapse, which acquires an image every 10 s, with the appropriate exposure time, which depends on the strength of the fluorescent signal (around 100 ms).

6.7. Use the previously described and available **FRET online** and **FRET online 2** plugins<sup>17</sup> to split the image into two channels, select the regions of interest, and monitor the ratio tracing.

6.8. During the acquisition, perfuse the heart with the modified Krebs-Henseleit solution containing different substances, depending on the nature of the experiment.

6.9. At the end of the experiment, switch off the acquisition by pushing the **Stop** button and save the stack of images.

6.10. Analyze the FRET data offline using a dedicated analysis software that can split images into

two identical sections for the donor and acceptor channels and can perform FRET analysis in multiple regions-of-interest.

Note: A dedicated plug-in (**FRET offline**) is needed, which is provided in Sprenger *et al.*<sup>17</sup>.

6.10.1. Start the software. Open the analysis by going to the **Plugins** menu, then click on **MicroManager**, and then on **Open Micro-Manager File**.

6.10.2. Run the **FRET offline** plugin in order to split the time-lapse into two identical images for the CFP and YFP channels.

6.10.3. Use the **Freehand Selections** tool to mark the region of interest in the YFP image. Press the **Add** button to add the selection to the **Multi Measure** window.

6.10.4. Choose the region of interest in the **Multi Measure** window and press **Multi** to obtain a table with mean grey values for each frame and region. Copy and paste all the data into an Excel sheet.

6.10.5 Click on the **CFP Image Stack**. Perform the same actions as in step 6.10.4 for the CFP stack and paste the mean grey values into the same Excel sheet.

6.11. Correct the raw data offline for the bleed-through factor of the donor into the acceptor channel<sup>17</sup>.

6.11.1. Use the following formula, where B is the bleed-through factor:

Ratio = (YFP - B x CFP) / CFP

6.11.2. Determine the bleed-through factor B by imaging a heart expressing CFP only and measure the percentage of the donor fluorescence in the YFP channel ( $B = \text{YFP} / \text{CFP}$ ).

## 7. Neuromorphology

Note: Analyze the intracardiac autonomic nervous system by using whole-mount immunostainings of intact murine hearts. Note that the majority of intracardiac ganglia are localized in the epicardial adipose tissue close to the pulmonary veins.

7.1. Use different stainings for the depiction of neurofilament (general neuronal structures; chicken anti-NF-H, 1:3,000), tyrosine hydroxylase (sympathetic neuronal structures; rabbit  $\alpha$  TH, 1:1,000), and choline acetyltransferase (parasympathetic neuronal structures; goat  $\alpha$  ChAT, 1:50).

7.2. After the perfusion in the Langendorff apparatus, fix the mouse hearts in 10 mL of formalin for 24 h and store them in phosphate-buffered saline (PBS) at 4 °C.



7.3. Bleach the hearts in Dent's bleach (4:1:1 methanol: hydrogen peroxide solution 30% (w/w) in H<sub>2</sub>O: dimethyl sulfoxide (DMSO)) for 1 week at 4 °C and rehydrate them subsequently to PBS in a series of descending methanol in PBS (100%, 75%, 50%, 25%; 1 h each)<sup>18</sup>.

7.4. Perform the following incubations in a 24-well-plate format with a gentle agitation at 4 °C:

7.4.1. Permeabilize the hearts in 1% Triton-X-100/PBS (PBS-T) for 3x 1 h at room temperature before blocking them overnight in a blocking buffer [5% bovine serum albumin (BSA) / PBS-T + 0.2% sodium azide].

7.4.2. Dilute the antibodies as follows: goat  $\alpha$  ChAT (1:50), rabbit  $\alpha$  TH (1:1,000), chicken  $\alpha$  neurofilament (1:3,000); secondary antibodies for fluorescent labelling (1:500; or according to the manufacturer's instructions); biotinylated secondary antibodies for chromogenic labelling (1:200; or according to the manufacturer's instructions).

7.5. Incubate the specimens in primary antibodies diluted in a blocking buffer for 1 week at 4 °C.

7.6. Wash the hearts for 3 x 15 min in PBS-T before the secondary antibody incubation in a blocking buffer for 4 days.

7.7. Wash the hearts for 3 x 15 min in PBS-T and store them in a mounting medium for fluorescent staining for 3 h at room temperature or use an avidin-biotin complex detection kit according to the manufacturer's instructions.

7.8. Pre-incubate the hearts for 1 h in a commercial buffer for 3,3'-diaminobenzidine (DAB), before developing them under a visual control in a DAB-containing buffer according to the manufacturer's instructions.

7.9. Store the specimens in double distilled water.

7.10. For paraffin sections, dehydrate and embed the hearts in paraffin.

7.10.1. Cut 4- $\mu$ m thick sections and deparaffinize them according to the laboratory's routine procedures. Whether and how antigen retrieval needs to be performed needs to be established for every individual setup since it depends on the antibody combination.

7.10.2. Permeabilize the sections for 10 min in 0.2% Triton-X-100/Tris-buffered saline (TBS), followed by 3 x 5 min washes in TBS.

7.10.3. Block them with 3% BSA/TBS for 1 h at room temperature.

7.10.4. Incubate them overnight at 4 °C [primary antibodies: goat  $\alpha$  ChAT (1:50), rabbit  $\alpha$  TH (1:500), chicken  $\alpha$  neurofilament (1:1,000)] or 2 h at room temperature (fluorescence-labelled secondary antibodies, 1:500) in 1% BSA/TBS with 3 x 5 min washes TBS in between.

7.10.5. Add 1 µg/mL of bisbenzimidazole H33342 trihydrochloride to the secondary antibody solution or use a different nuclear staining method.

7.10.6. Mount the slides in a mounting medium for fluorescent staining.

#### **REPRESENTATIVE RESULTS:**

**Figure 1** shows an image of the Langendorff setup including 2 multi-electrode arrays (MEAs). Before the experiment, the intracardiac catheter is positioned close to the cannula to facilitate a quick and easy insertion in the right atrium/right ventricle and to ensure a short time period until the equilibration can start. The lower part of the chamber can be heightened (see the arrows in **Figure 1**) so that the chamber is fully closed and guarantees a stable temperature.

**Figure 2** depicts a representative whole-mount staining using an antibody targeting neurofilament, which is a marker for the neuronal cytoskeleton. Neuronal fibers traverse from the atria to the apex on the posterior (**Figure 2A**) or anterior site (**Figure 2B**). Exemplary neurofilament positive fibers are marked by arrowheads. In **Figure 2C** a hematoxylin and eosin (H&E) staining of one heart section is presented. In the exemplary enlargement (**Figure 2D**) an immunohistochemical staining of one single atrial ganglion demonstrates the predominantly parasympathetic cells (red, ChAT-positive) compared to less numerous sympathetic cells (green, TH-positive).

**Figure 3** shows the murine heart connected to the cannula of the Langendorff apparatus with an inserted octapolar catheter in the right atrium and right ventricle and an epicardial multi-electrode array (MEA) placed on the anterior left ventricle (**Figure 3A**). Ventricular arrhythmia susceptibility testing via the electrodes in the RV is presented in **Figure 3B**. The induction of a ventricular tachycardia in hearts occurred more frequently after partial atrial denervation. In the enlarged MEA (**Figure 3C**) the schematic layout of the electrodes is presented. It is important to ensure a stable epicardial contact of all electrodes. In **Figure 3D** the offline-analyzed epicardial conduction recorded by an MEA is depicted.

**Figure 4** shows FRET measurements in a whole heart being retrogradely perfused in the Langendorff apparatus. Different areas of the heart can be analyzed as needed (**Figure 4A**). A global as well as local topical application of pharmaceuticals is easily possible in this setup (**Figure 4B**).

#### **FIGURE AND TABLE LEGENDS:**

**Figure 1: Langendorff setup including multi-electrode arrays (MEAs).** The octapolar stimulation and recording catheter is placed close to the area in which the heart will be attached. The lower part of the chamber will be moved upwards (white arrows) after the heart has been attached to the apparatus so that a stable temperature is ensured.

**Figure 2: Cardiac whole mount staining depicting parts of the autonomic nervous system. A-B.** These panels show how the staining with neurofilament visualizes fibers (arrowheads) of the

autonomic nervous system at the posterior (**Figure 2A**) and anterior (**Figure 2B**) site running from the atria to the apex (scale bars: 1 mm). **C**. This is a depiction of a cardiac H&E-stained paraffin section (scale bar: 1 mm). **D**. An exemplary enlargement of one immunohistochemically stained atrial ganglion demonstrates the predominantly parasympathetic cells (red, ChAT-positive) compared to less numerous sympathetic cells (green, TH-positive; scale bar: 75  $\mu$ m).

**Figure 3: Intra- and epicardial measurements using the Langendorff setup.** **A**. This panel shows an example of a murine heart within the Langendorff system. The intracardiac octapolar catheter, which is inserted in the right atrium and ventricle, and one epicardial multi-electrode array (MEA) are depicted. **B**. Arrhythmia susceptibility testing using burst stimulation without (control) or with the induction of a self-terminating ventricular tachycardia [after partial atrial denervation (PAD)] are depicted. **C**. The epicardial MEA is depicted with an enlargement of the schematic electrode layout. **D**. Wave propagation velocity was analyzed using a custom-made software. The distance between the isochrones is 2 m/s.

**Figure 4: FRET measurements in a Langendorff setup.** **A**. The two different cAMP biosensor fluorescence channels [yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP)] during FRET measurements in a retrograde perfused heart are depicted. If needed, different parts of the heart (*e.g.*, atria and ventricle) can be analyzed (scale bar: 1 mm). **B**. This panel shows a representative FRET experiment, which measures cAMP levels during a pharmacological stimulation in the atrium and left ventricle. First, the heart was systemically perfused with the adenylyl cyclase activator NKH477, a forskolin analogon, to increase cAMP levels. Then nicotine was topically applied and targeted at the atrial ganglia, which acutely reduced cAMP levels.

## DISCUSSION:

In this manuscript, the well-known Langendorff *ex vivo* heart perfusion system is presented as a tool to study the impact of intracardiac neurons on cardiac electrophysiology and arrhythmogenesis by using different mapping and stimulation techniques including endocardial and epicardial approaches.

Several parts of the protocol are crucial for the setup. First, it is important to use a preparation technique in which the atrial fat pads stay intact or are removed quickly without injuring the myocardium. Second, a properly sized opening has to be cut in the right atrium for an easy insertion of the octapolar catheter into the right atrium and right ventricle. The catheter should slip easily into the right ventricle without generating any pressure. During the attachment of the catheter to the cannula, the catheter should not dip deeper into the ventricle, to avoid cardiac injury. Third, temperature control is a crucial part of all Langendorff setups<sup>1,2,5</sup>. The thermal chamber is closed during the arrhythmia testing, ensuring a stable temperature. But for MEA or FRET recordings, the chamber needs to be at least partly open to allow measurements. Either recording time should be kept to a minimum, or other techniques to reduce temperature loss, like putting a plastic wrap around the chamber during longer measurements, should be performed. Fourth, MEAs should be placed at the same anatomical locations in all experiments. Good surface contact, which is confirmed by large amplitudes in the real-time analysis, can be achieved by using two MEAs on opposite sites so that a counterbalance is produced. Fifth, FRET

measurements are influenced by movement. To reduce spontaneous movement, the heart is paced at a stable frequency by the intracardiac catheter. For additional stabilization, a tube with a slight vacuum can stabilize the apex.

One advantage of the Langendorff system is that the hearts can be used subsequently for immunohistological assessments of the cardiac nervous system. The continuous perfusion removes most red blood cells which have a high level of autofluorescence<sup>19</sup>, improving the quality of staining. After formalin fixation, the hearts can be stored in a temperature controlled (4 °C) environment in phosphate-buffered saline for up to a year without noticeable changes in staining quality.

The most important feature of this setup is that all measurements are performed in a centrally denervated heart. The predominantly parasympathetic atrial intracardiac ganglia are the last relay station within the heart<sup>20</sup> as the sympathetic ganglion stellatum is located intrathoracically and is therefore removed during preparation. Although the intracardiac neurons get no central input, it has been shown that they are still active in a physiological way as the photoactivation of cardiac sympathetic nerves increases the heart rate and cardiac contractile force<sup>21</sup>. In line with these findings supporting the functional importance of intracardiac neurons in the centrally denervated heart, we recently demonstrated their impact on ventricular function and arrhythmogenesis<sup>15</sup>.

One advantage of this centrally denervated setup is that it allows the researcher to study the communication between different intracardiac regional neural networks (*e.g.*, the interaction between atrium and ventricle)<sup>15</sup>. These differences might be relevant for patients after heart transplantation in whom treatment with the selective sinus node modulator ivabradine improves survival, compared to treatment with the beta-blocker metoprolol succinate<sup>22</sup>. In a future step, direct electrical stimulation of parasympathetic (vagus nerve) or sympathetic structures (Ggl. stellatum<sup>23</sup>) will help to improve our knowledge of the interaction between the extra- and intracardiac autonomic nervous system.

It is important to keep in mind that parasympathetic and sympathetic fibers are mostly co-localized so that current therapies like catheter ablation of atrial or ventricular arrhythmias will inevitably modify both structures. In the here described setup, local pharmaceutical modification of targeted structures (*e.g.*, specific stimulation of parasympathetic ganglia) can be studied. Besides targeted modifications, global perfusion with different pharmaceuticals (*e.g.*, beta-blockers) is easily possible, so that potential proarrhythmic or antiarrhythmic properties of various agents can be studied. Using this setup, interventions and different techniques can be tested during the stimulation or inhibition of different parts of the intracardiac autonomic nervous system, revealing information of the impact of specific parts of the autonomic nervous system on cardiac function and arrhythmogenesis. Further, the murine setup allows studying the cardiac autonomic nervous system in states of disease like myocardial infarction, heart failure or diabetes.

In conclusion, the simple and well-known Langendorff *ex vivo* heart perfusion system provides a

flexible basis to modify and study the impact of intracardiac neurons on cardiac electrophysiology and arrhythmogenesis.

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#### **DISCLOSURES:**

The authors have nothing to disclose.

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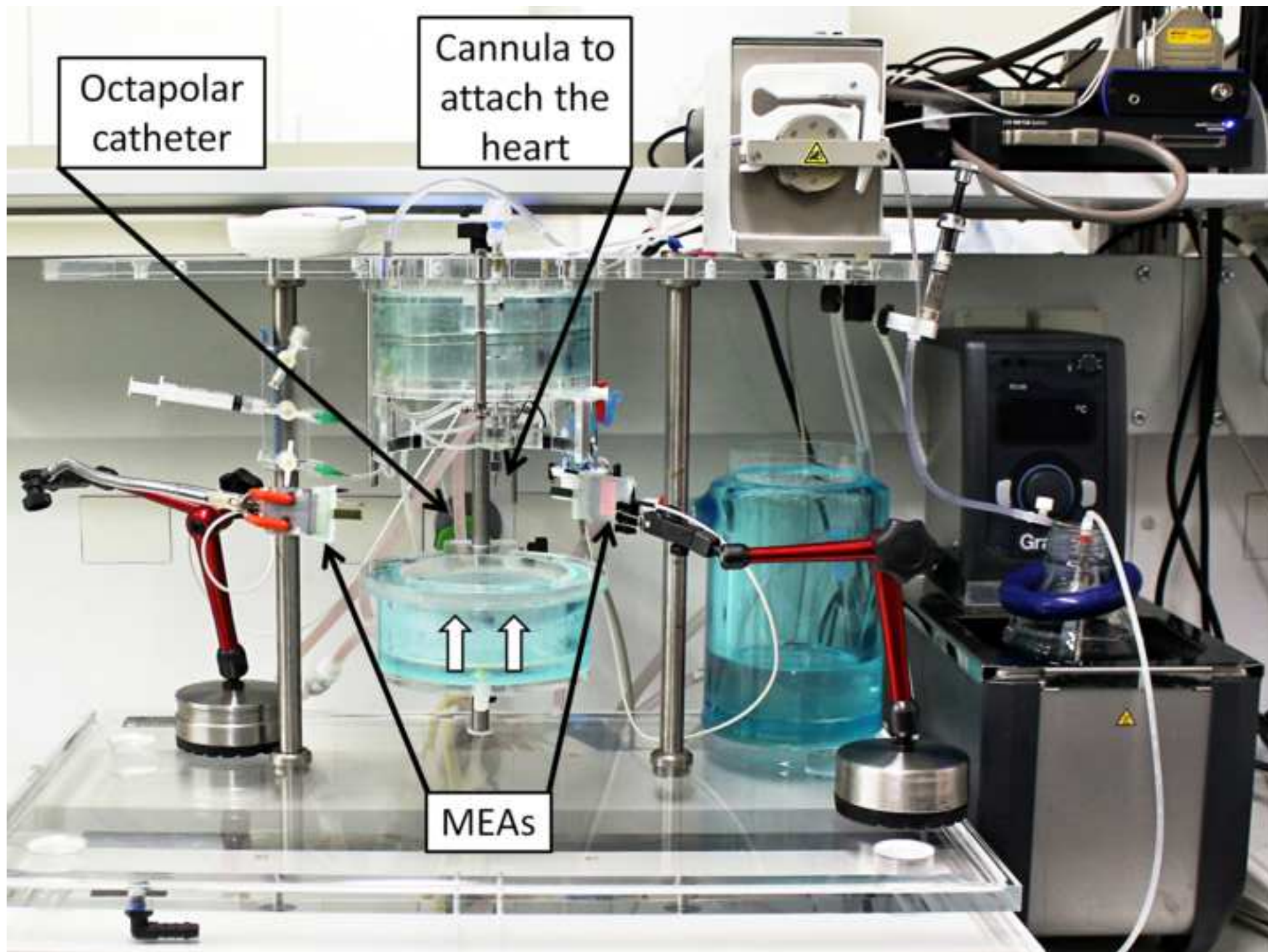
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Figure 1





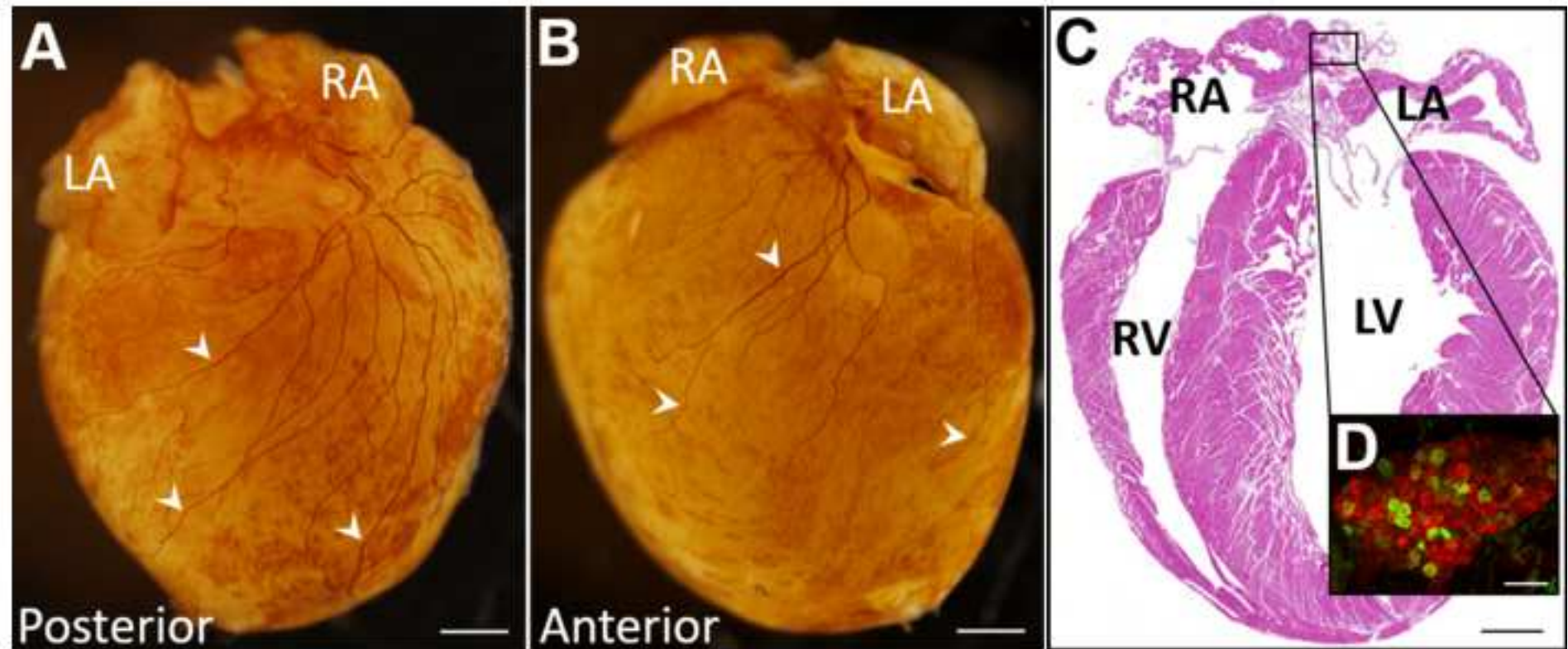
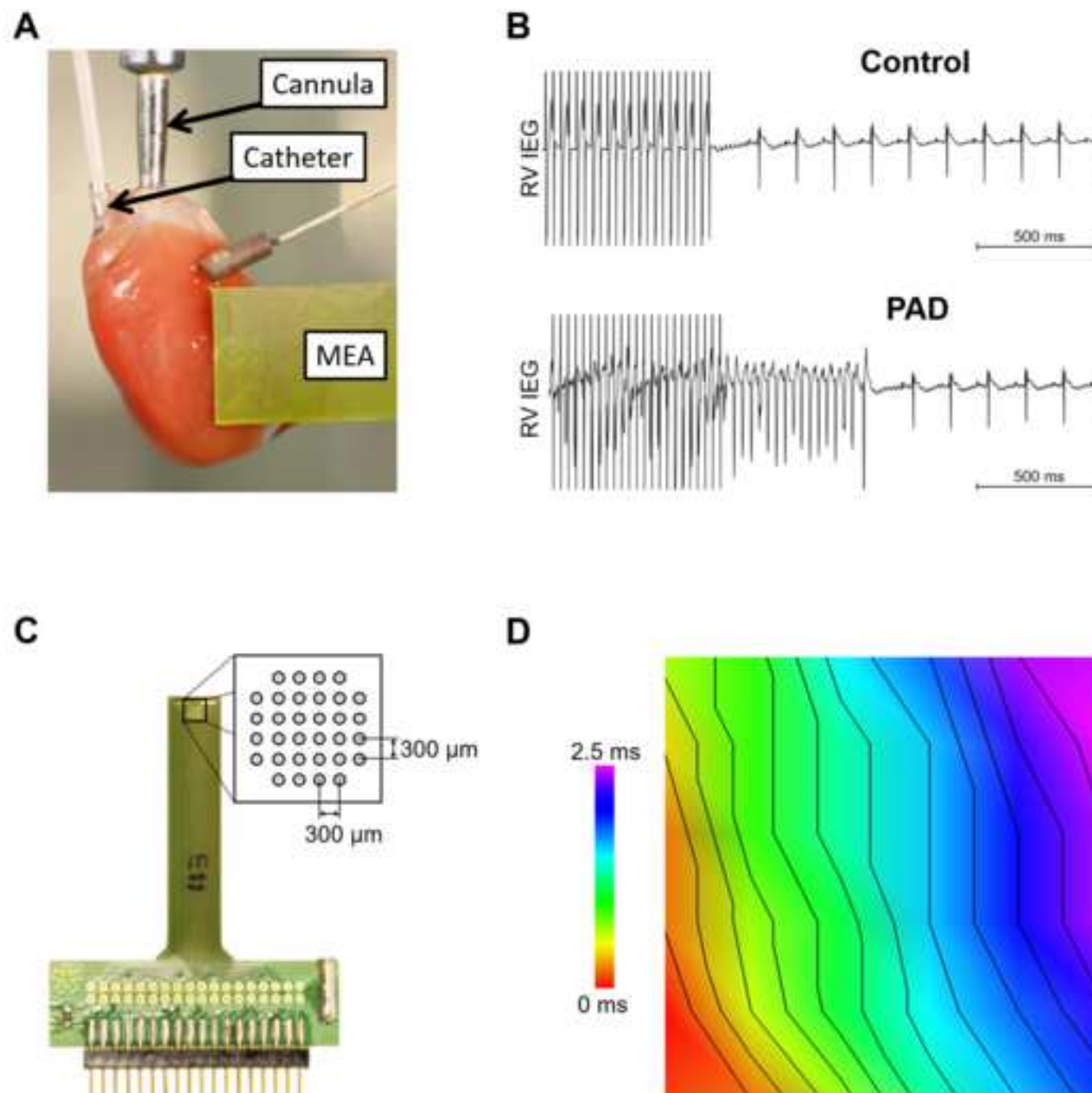
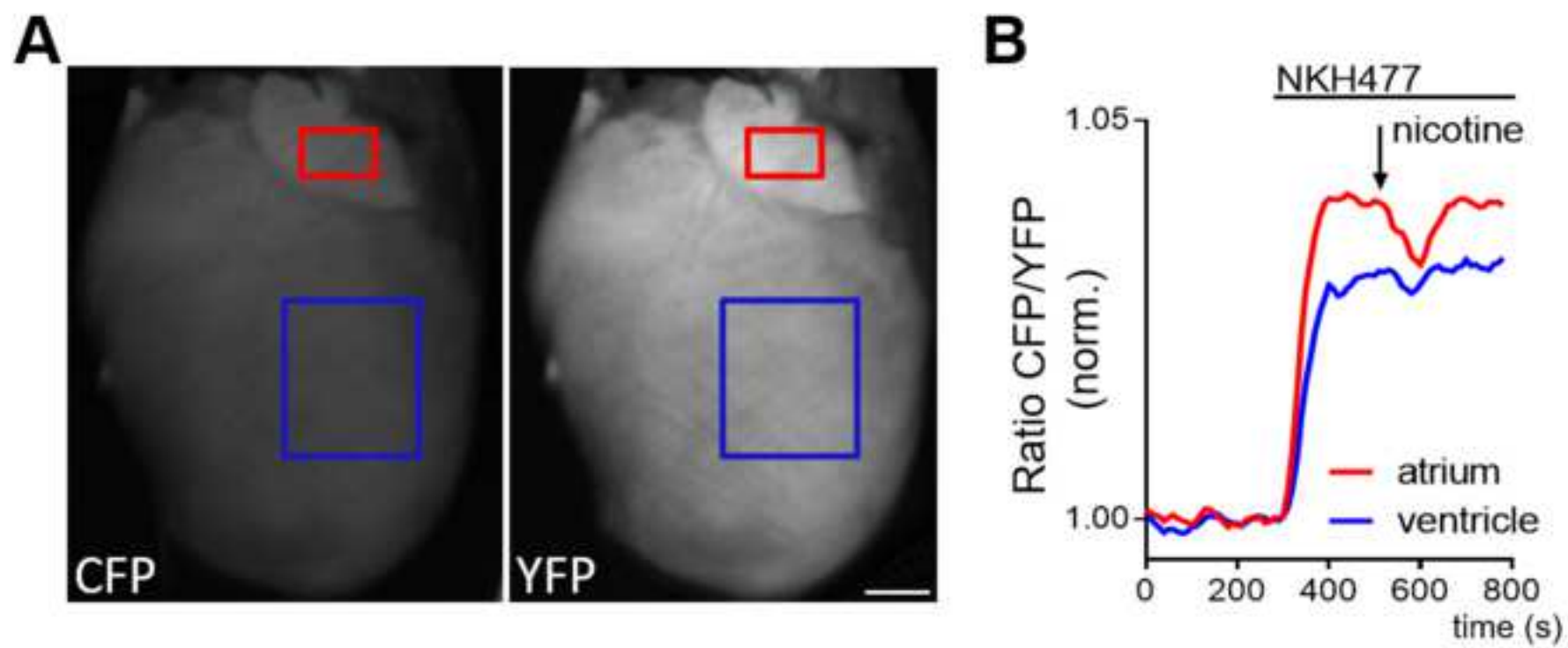


Figure 3





**Name of Material/ Equipment**

Sodium chloride  
Sodium hydrogencarbonate  
Potassium chloride  
Potassium phosphate monobasic  
Magnesium sulfate heptahydrate  
Calcium chloride dihydrate  
Glucose  
Sodium pyruvate bioXtra  
Carbogen (95% O<sub>2</sub> / 5% CO<sub>2</sub>)  
Sterile filter steritop-GP 0.22  
Atropine sulfate  
Hexamethonium chloride  
Nicotine free base 98-100%  
Formalin solution neutral buffered 10%  
Tris(hydroxymethyl)aminomethane  
Methanol  
Hydrogen peroxide solution 30% (w/w) in H<sub>2</sub>O  
Dimethyl sulfoxide  
Phosphate-buffered saline tablets  
Triton-x-100  
Albumin bovine fraction V  
Chicken anti neurofilament  
Rabbit anti tyrosine hydroxylase  
Goat anti choline acetyltransferase  
Donkey  $\alpha$  rabbit IgG Alexa 488  
Donkey  $\alpha$  goat IgG Alexa 568  
Donkey  $\alpha$  chicken IgY Alexa 647  
Biotin-conjugated donkey  $\alpha$  rabbit igG  
Biotin-conjugated donkey  $\alpha$  goat igG  
Biotin-conjugated goat  $\alpha$  chicken igY  
Vectashield mounting medium  
Vectastain ABC kit  
Steady DAB/Plus  
HistoClear  
BisBenzimide H33342 trihydrochloride (Hoechst)  
Vectashield HardSet mounting medium

Perfusion system  
Data acquisition system and corresponding software for catheter and physiological parameter  
Octapolar catheter

Stimulus generator  
Stimulation software  
Data acquisition system and corresponding software for epicardial electrograms  
Multi-electrode array  
Multi-electrode array recording software

Spring scissors  
Strabismus Scissors  
Mayo Scissors  
Dumont SS Forceps  
London Forceps  
Narrow Pattern Forceps  
Plastic Wrap

Stereomicroscope  
LED  
DualView  
DualView filter set  
optiMOS scientific CMOS camera  
Imaging software  
Analysis Software

## Company

Sigma-Aldrich

Sigma-Aldrich

Sigma-Aldrich

Sigma-Aldrich

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SOL-Group, TMG Technische und Medizinische Gas GmbH, Krefeld, Gersthofen, Germany

EMD Millipore

Sigma-Aldrich

Sigma-Aldrich

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Merck, KGA, Darmstadt, Germany

Merck, KGA, Darmstadt, Germany

Gibco / Invitrogen

Sigma-Aldrich

Biomol, Hamburg, Germany

EMD Millipore

EMD Millipore

EMD Millipore

Thermo Fisher Scientific

Thermo Fisher Scientific

Merck, KGA, Darmstadt, Germany

R&D Systems

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R&D Systems

Vector laboratories, Burlingame, CA, USA

Vector laboratories, Burlingame, CA, USA

Abcam plc, Cambridge, UK

DiaTec, Bamberg, Germany

Sigma-Aldrich, St. Louis, MO, USA

Vector laboratories, Burlingame, CA, USA

HUGO SACHS ELEKTRONIK - HARVARD APPARATUS GmbH, March-Hugstetten, Ger

Powerlab 8/30 & Labchart, ADInstruments, Dunedin, New Zealand

CIB'ER Mouse, NuMed Inc., Hopkinton, NY, USA

STG4002, Multi Channel Systems, Reutlingen, Germany  
Multi Channel Systems, Reutlingen, Germany  
ME128-FAI-MPA-System, Multi Channel Systems, Reutlingen, Germany  
MEA, EcoFlexMEA36, Multi Channel Systems, Reutlingen, Germany  
Multi Channel Systems, Reutlingen, Germany

Fine Science Tools GmbH, Heidelberg, Germany  
Fine Science Tools GmbH, Heidelberg, Germany  
Fine Science Tools GmbH, Heidelberg, Germany  
Fine Science Tools GmbH, Heidelberg, Germany  
Fine Science Tools GmbH, Heidelberg, Germany  
Fine Science Tools GmbH, Heidelberg, Germany  
Parafilm M, Bemis NA, based in Neenah, WI, United States

Leica M165FC; Leica Microsystems GmbH, Wetzlar, Germany  
CoolLED, Andover, UK  
Photometrics, Tucson, AZ, USA  
Photometrics, Tucson, AZ, USA  
Qimaging, Surrey, BC, Canada  
Micro-Manager; Vale Lab, University of California San Francisco, CA, USA  
Image J software; Public Domain, NIH, USA

<b>Catalog Number</b>	<b>Comments/Description</b>
S3014	Modified Krebs-Henleit solution
401676	Modified Krebs-Henleit solution
P5405	Modified Krebs-Henleit solution
P5655	Modified Krebs-Henleit solution
M1880	Modified Krebs-Henleit solution
C7902	Modified Krebs-Henleit solution
G5767	Modified Krebs-Henleit solution
P8574	Modified Krebs-Henleit solution
	Modified Krebs-Henleit solution
SCGPT05RE	Modified Krebs-Henleit solution
A0257	Neuromodulation
H2138	Neuromodulation
N3876	Neuromodulation
HT501128	Whole mount staining
252859	Whole mount staining
34860	Whole mount staining
H1009	Whole mount staining
D8418	Whole mount staining
18912-014	Whole mount staining
T8787	Whole mount staining
11924.03	Whole mount staining
AB5539	Whole mount staining
AB152	Whole mount staining
AP144P	Whole mount staining
A21206	Whole mount staining
A11057	Whole mount staining
AP194SA6	Whole mount staining
AP182B	Whole mount staining
AP192P	Whole mount staining
BAD010	Whole mount staining
H-1000	Immunohistochemistry
PK-4000	Immunohistochemistry
ab103723	Whole mount staining
HS2002	Immunohistochemistry
B2261	Immunohistochemistry
VEC-H-1400	Immunohistochemistry
73-4343	Langendorff apparatus
PL3508 PowerLab 8/35	Langendorff setup
custom	Langendorff setup



STG4002-160 $\mu$ A	Stimulation setup
MC_Stimulus II	Stimulation setup
USB-ME128-System	MEA setup
EcoFlexMEA36	MEA setup
MC_Rack	MEA setup
15003-08	Heart Preparation
14575-09	Heart Preparation
14110-15	Heart Preparation
11203-25	Heart Preparation
11080-02	Heart Preparation
11003-13	Heart Preparation
	Consumable Materials
	FRET
<i>p</i> E-100	FRET
DV2-SYS	FRET
05-EM	FRET
01-OPTIMOS-R-M-16-C	FRET
	FRET
	FRET



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Author(s): C. JÜNGEN, K. SCHERSCHEL, N. I. BORK, P. KUKLIK, N. KLATT, H. KNIEP, C. EICKHOLT, S. WILLEMS, V. O. MKOLAEV, C. MEYER

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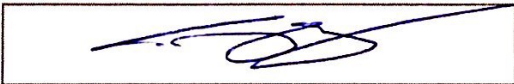
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Notes are not highlighted.

- 6. Please define all abbreviations before use, e.g., DMSO, BSA, PBS-T**

All abbreviations have been defined before first usage.

- 7. Protocol step 3.2, 4.2: Please split this step into three or more steps so that each step contain only 2-3 actions.**

These protocol steps have been split.

- 8. 2.2.2.: Please specify the electrodes. What type of electrode is used? What's the surface area?**

The electrodes have been specified under 2.2.

Platinum electrodes with a surface area of 0.55 x 0.55 mm each were used.

- 9. 4.1: Please describe the programmed stimulation in detail.**

The programmed stimulation has been described in the next steps. This was clarified by adjusting the enumeration.

- 10.4.2: Please ensure that all text is written in imperative tense.**

All text is now written in imperative tense.

- 11.6.6, 6.7: For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.**

A step-wise description of the software usage has been added.