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Data Article

Experimental data of labeling the heart and cardiac cultures with a retrograde tracer *in vitro* and *in vivo*

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ABSTRACT

Retrograde dyes are often used in basic research to investigate neuronal innervations of an organ. This article describes the experimental data on the application of retrograde dyes on the mouse heart *in vivo* and on the cardiac or neuronal cultures *in vitro*. By providing this information, cardiac or ineninnervations can be evaluated *in vivo*. Therefore, unknown cellular and molecular mechanisms and systemic interactions in the body can be investigated. In particular, we provided practical tips to lower mortality risks following the cardiac surgery and evaluated the staining capacity and fluorescent characteristics of the Di-8-ANEPPQ dye in the cardiac tissue and cell cultures. First, primary cultures of mouse nodose ganglia (NG) neurons and mouse neonatal cardiomyocytes were stained with Di-8-ANEPPQ. The Di-8-ANEPPQ signal from live cultures were visualized using spinning disk confocal microscopy to verify the lipophilic and fluorescent

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labeling capacity of Di-8-ANEPPQ. Next, the excitation and emission data of Di-8-ANEPPQ were collected between 415 nm and 690 nm using power spectrum module of confocal microscopy. This spectrum analysis could be useful for the researchers who plan to use Di-8-ANEPPQ in combination with other fluorescent dyes to eliminate any florescent overlap. In order to label the heart tissue with tracer dyes Di-8-ANEPPQ or DiI *in vivo*, the heart was exposed without damaging lungs or other tissues following anesthetization, then the retrograde dye was applied as a paste for DiI or injected to the apex of the heart for Di-8-ANEPPQ and the operation area was sutured. The surgical procedure required intubation to control the respiratory reflex without the need to perform a tracheotomy and yielded high viability. Following labeling the heart *in vivo*, the heart was dissected, and images of injection area were captured using confocal microscopy. All fluorescent images of Di-8-ANEPPQ labeled cells were analyzed by using the Fiji software. Overall, these data provide applicable data to other investigators to trace the sensory neurons innervating not only the heart but also other organs using Di-8-ANEPPQ. These data support the original research article titled "Evaluation of bilateral cardiac afferent distribution at the spinal and vagal ganglia by retrograde labeling" that was accepted for publication in Brain Research Journal [1].

Specifications Table

Subject	Neuroscience: Sensory Systems
Specific subject area	Application of Di-8-ANEPPQ or other retrograde tracers to the primary neurons or cardiomyocytes *in vitro* and to the murine heart *in vivo*, that could be used to investigate cardiac afferents
Type of data	Image Figure
How data were acquired	Stereomicroscopy, Fluorescent Microscopy Instruments: Fiji software (2.0.0-rc-69/1.52i)
Data format	Raw Analyzed Filtered
Parameters or data collection	Adult BALB/c mice at 8-12 weeks were housed in the animal facility of MEDITAM of Istanbul Medipol University. The animal care was given according to Ethical community approval of HADHEK. The experimental protocol was approved by the Institutional Animal Experimentation Ethical Committee with the approval number 38328770-30. The *in vitro* staining capacity of Di-8-ANEPPQ was explored in primary neonatal mouse cardiomyocyte cultures and primary mouse NG neurons. Di-8-ANEPPQ dye has a lipophilic nature [2,3] and binds to the plasma membrane. The incubation of Di-8-ANEPPQ dye with the cell cultures was kept at RT to allow the staining of only the cell membrane. Furthermore, the images of live Dİ-8-ANEPPQ labeled primary cardiomyocyte cultures were captured following the incubation of 10-15 minutes at 37 °C for the cells to regain their basal activity. To obtain emission and excitation properties of Di-8-ANEPPQ dye, the labeled primary cardiomyocyte culture was excited by not only 488 nm but also 561 nm and 633 nm using the power spectrum module of confocal microscopy. In addition to the application of Di-8-ANEPPQ on the live cultured cells for further

(*continued on next page*)

	labeling evaluations, the mice weighing at least 25 g were chosen to visualize the epiglottis and to insert the intubation tube without tissue damage. First, ketamine (50 mg/kg, Pfizer) and xylazine (5 mg/kg, Bayer) of anesthesia was administrated into the animal, checked vital signs and sedation parameters, another dosage was given if it was necessary. The intubation was verified by checking the synchronization of the animal chest movement. Next, the animal was transferred to an operation area equipped with a heating pad to maintain hemostasis and prevent hypothermic shock. To expose the heart for delivery of the Di-8-ANEPPQ, the third and fourth intercostal muscles were separated using retractors to prevent lung and diagram damage. For the retrograde labeling, 1 µl of 10 mg/ml freshly thawed Di-8-ANEPPQ was used and warmed up to RT before administration. Following the Di-8-ANEPPQ injection into the heart, negative pressure was applied to the lung before suturing the muscle and skin.
Description of data collection	For evaluation of *in vitro* labeling capacity of Di-8-ANEPPQ, primary cultures of NG neurons and cardiomyocytes were stained with 20 µM Di-8-ANEPPQ at RT. After incubation, live images of cultured cells were taken using spinning disk confocal microscopy (Zeiss). The excitation and emission properties of the Di-8-ANEPPQ was obtained using the power spectrum module of confocal microscopy (LSM 780, Zeiss). The images of the *in vivo* heart operation were captured using the stereomicroscope (Discovery 8, Zeiss). The Di-8-ANEPPQ, in 1 µl containing 10 mg/ml, was injected into the apex of the heart. Following 2-3 hrs of the surgery, the heart was dissected out and the injection area was imaged using confocal microscopy (LSM 780, Zeiss). All images were analyzed and prepared using Fiji software.
Data source location	Institution: Istanbul Medipol University City/Town/Region: Istanbul Country: Turkey
Data accessibility	The corresponding data were provided within this article
Related research article	Akgul Caglar T., Durdu Z. B., Turhan M. U., Gunal M. Y., Aydın M. Ş., Ozturk G., Cagavi E., "Evaluation of bilateral cardiac afferent distribution at the spinal and vagal ganglia by retrograde labeling" Brain Research. https://doi.org/10.1016/j.brainres.2020.147201.

Value of the Data

- Our data described a reproducible approach to label the primary cardiac and neural cells *in vitro* and the murine heart *in vivo*. Our data provided important tips for other researchers to enhance labeling efficiency and the recovery after cardiac surgery, in which mortality rates are often high. The evaluation of the fluorescent characteristics of the Di-8-ANEPPQ dye has not been reported elsewhere in such detail that has applicable value.
- The data would be an important reference for the scientists at different disciplines who are interested in tracing neurons *in vivo* not only at the cardiac system but other organs as well. These data would be of great interest to researchers who plan to conduct experiments with Di-8-ANEPPQ or similar dyes for fluorescently staining and tracing cells *in vitro*.
- The data might be used for tracing neurons not only innervating the heart but also other visceral organs using Di-8-ANEPPQ. Researchers who study cardiac physiology, regeneration or nervous system interactions would be expected to benefit and use the provided data.
- The power spectrum data of Di-8-ANEPPQ dye would guide scientist to select the best experimental and imaging settings in combination to other staining methodologies.

1. Data Description

The data in this article described the experimental steps for the application of retrograde dyes on primary cardiac and neuron cultures *in vitro* and the mouse heart *in vivo*. To determine the efficiency of Di-8-ANEPPQ *in vitro;* primary NG neurons and neonatal cardiomyocytes were stained with 20 µM Di-8-ANEPPQ. The live images of cells labeled with Di-8-ANEPPQ were captured using spinning disk confocal microscopy and the green fluorescence signal of Di-8-ANEPPQ

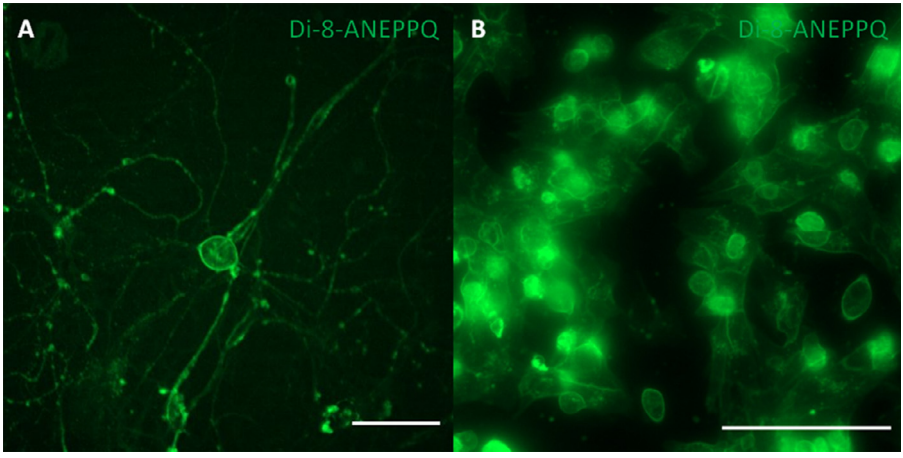

Fig. 1. Imaging data of Di-8-ANEPPQ labeled primary neuronal and cardiac cells *in vitro* The Di-8-ANEPPQ dye labeling efficiency was determined by flourescent imaging of the primary NG neurons **(A)** or primary neonatal cardiomyocytes **(B)** imaged by floresence microscopy. Di-8-ANEPPQ: green, scale bar: 100 µm.

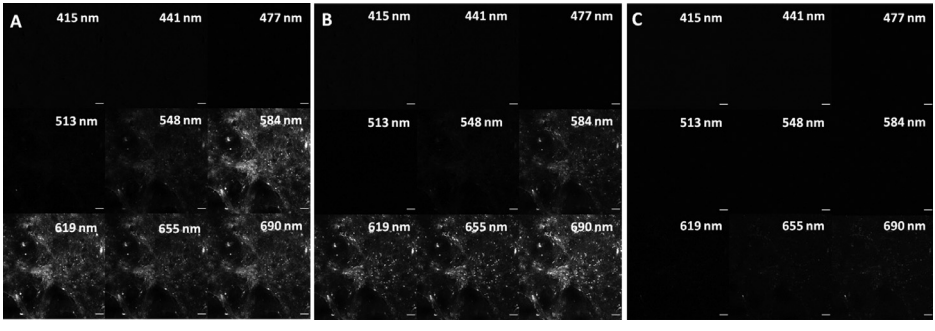

Fig. 2. Emission spectrum analysis of Di-8-ANEPPQ labeled primary cell culture. Di-8-ANEPPQ (gray) labeled cardiac cells were excited **(A)** 488 nm, **(B)** 561 nm, and **(C)** 633 nm. Emission spectrum data between 415 nm and 690 nm were captured and displayed. Scale bars: 100 µm.

was detected at the plasma membrane (Fig. 1). Next, the emission and excitation properties of Di-8-ANEPPQ labeled cardiomyocytes were analyzed by collecting emissions between 415 nm and 690 nm using the power spectrum module of confocal microscopy (Fig. 2). Interestingly, we observed that Di-8-ANEPPQ dye could be excited by both 488 nm (Fig. 2A) and 561 nm (Fig. 2B), but not excited by 633 nm (Fig. 2C) wavelength expanding the previous knowledge [4]. Moreover, the emission spectrum of Di-8-ANEPPQ was found to be at a broad range between 571 and 690 nm (Fig. 2). In addition to the application of Di-8-ANEPPQ on the live primary cultures, 10 mg/ml Di-8-ANEPPQ or DiI paste were applied to the heart apex *in vivo* (Fig. 3). Experimental steps for application of a retrograde dye to the heart *in vivo* were illustrated in Fig. 3. First, the anesthetized mouse was shaved in the operation area, and the skin was aseptically cleaned (Fig. 3A). Then, the mouse was intubated using a cannula and connected to the ventilation apparatus (Fig. 3B). The incisions were made at the left side of the thorax above the xiphoid, and muscles were moved gently to expose the heart (Fig. 3C). The retractors were placed to separate the third and fourth ribs (Fig. 3D). Following stabilization of the vital signs of the mouse and clear visualization of the heart, the Di-8-ANEPPQ dye was injected into the apex (Fig. 3E) or DiI paste was applied onto the heart (Fig. 3F). The application area was observed by the spreading of the retrograde dye in the cardiac tissue (Fig. 3G). The intercostal muscles (Fig. 3H) and

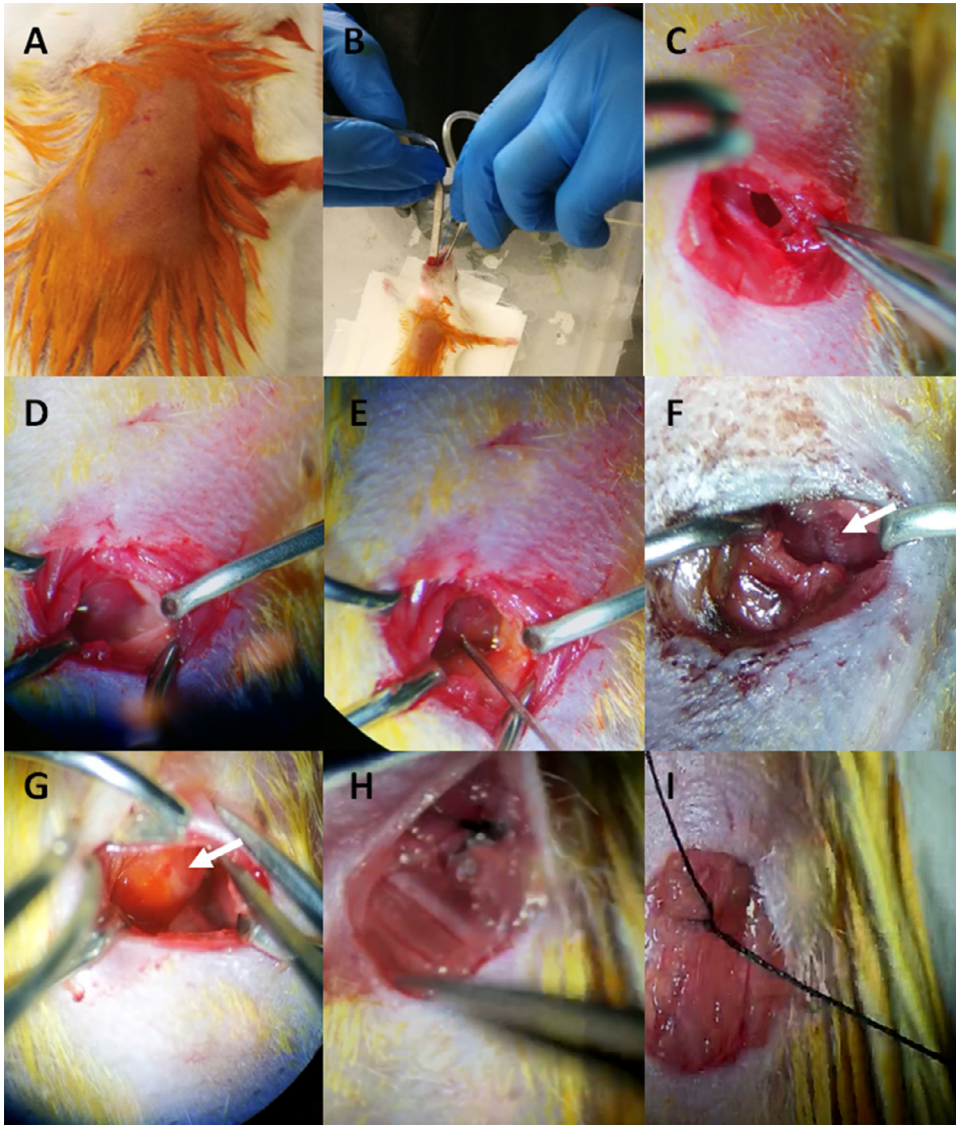

Fig. 3. Experimental steps for application of a retrograde dye to the heart *in vivo*. **(A)** The anesthetized mouse was shaved at the operation area and the skin was aseptically cleaned. **(B)** The mouse was intubated using a cannula and connected to the ventilation apparatus. **(C)** The incisions were made at the left side of the thorax above the xiphoid, and muscles were moved gently to expose the heart. **(D)** The retractors were placed to separate the third and fourth ribs. **(E)** **T**he Di-8-ANEPPQ was injected into apex or **(F)** DiI paste was applied on the heart. Arrow showed the tissue after DiI paste was applied. **(G)** The application area observed by the spread of the retrograde dye in the cardiac tissue. The injection site is shown by the arrow. **(H)** The intercostal muscles and **(I)** the skin were sutured.

the skin were sutured before the mouse was awakened from the anesthesia (Fig. 3I). To verify the proper injection of Di-8-ANEPPQ dye, an unlabeled heart which was dissected for determining the fluorescence background (Fig. 4A), and the heart in which Di-8-ANEPPQ was administrated *in vivo* were imaged by confocal microscopy (Fig. 4B). The green fluorescence signal of Di-8-ANEPPQ dye was detected at the injection site of the heart tissue.

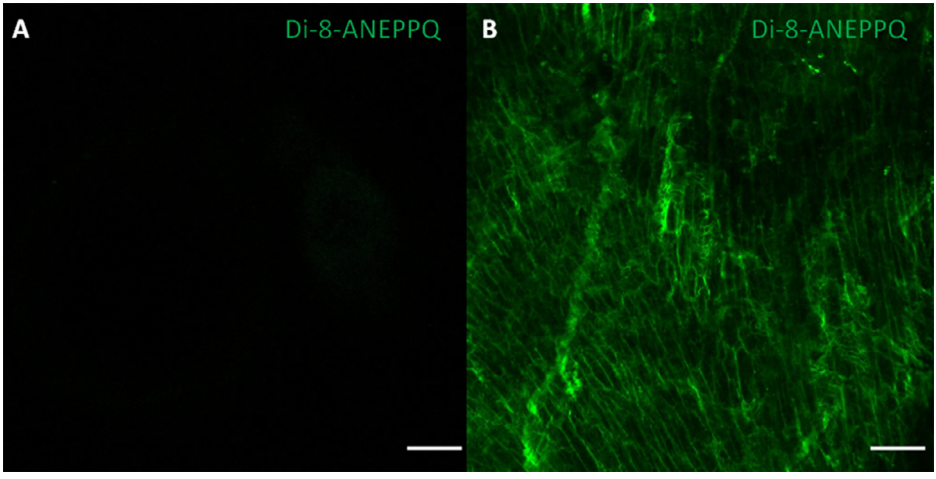

Fig. 4. Imaging data of Di-8-ANEPPQ from *in vivo* labeled cardiac tissue. Fluorescence imaging was performed on unlabeled control heart tissue for determining **(A)** the background or **(B)** following the Di-8-ANEPPQ administration to the murine heart *in vivo* by confocal microcopy. Di-8-ANEPPQ: green. Scale bar: 100 µm.

2. Experimental Design, Materials and Methods

2.1. Preparation of the primary murine cardiomyocyte and neuron cultures for in vitro *labeling*

The primary neonatal cardiomyocytes or adult nodose ganglia (NG) neurons were prepared and cultured as previously described [5,6]. Briefly, the heart of the BALB/c neonatal mice at 1-3 days after birth was dissected out and put in the ice-cold Trypsin solution (T4174, Sigma) for pre-digestion overnight at 4 °C. The next day, the heart tissue was collected and incubated with 450 U/ml collagenase II (17101-015, Gibco) enzyme at a 37 °C water bath for one hour with gentle agitation. The cell suspension was spun at 120 g for 5 min, and the pellet was resuspended with cardiomyocyte medium containing Na-pyruvate (11360-070, Gibco), MEM NEAA (11140-050, Gibco), fetal calf serum (10270-106, Gibco), horse serum (16050-130, Gibco) and newborn calf serum (16010167, Gibco). The neonatal cardiomyocytes were plated on culture dishes coated with 1 mg/ml Fibronectin (F1141, Sigma).

NG neuron cultures were prepared by dissecting the NG from an adult BALB/c mouse at 8-12 weeks as previously described [5]. The mouse was euthanized with cervical dislocation and bilateral NG tissues were dissected out by trimming the ganglia from the neural fibers and extra connective tissue. Next, nerve bodies were enzymatically dissociated using collagenase XI (C7657, Sigma), then trypsin (25300-054, Gibco) at the 37 °C incubator. To dissociate single cells, the NGs were triturated by pipetting and DNAse (D4513, Sigma) was added to the cell suspension to inhibit free DNA fragments. Next, the cell suspension was spun at 120g for 3 min and resuspended using fetal calf serum and trypsin inhibitor (T6522, Sigma). Then, the cell suspension was collected and resuspended with Neural basal medium (10888-022, Gibco) supplemented with B27 (17504-044, Gibco). The collected neurons were plated on the culture dishes coated with 1mg/ml Laminin (L2020, Sigma).

2.2. The labeling of in vitro *primary cultures with Di-8-ANEPPQ*

The cultured cells were stained with Di-8-ANEPPQ to evaluate the staining efficiency of Di-8-ANEPPQ *in vitro* before performing *in vivo* applications. Di-8-ANEPPQ dye was reported to have a lipophilic nature binding to the plasma membrane [2,3]. In the second or third day of the

primary cultures, the neurons or cardiomyocytes were stained with Di-8-ANEPPQ to test the lipophilic staining capacity of Di-8-ANEPPQ for live cells. Di-8-ANEPPQ (61014, Biotium) at a stock solution of 10 mM was prepared to add 697 µl DMSO into the 5 mg dye powder, then vortexed and stored at −20 °C in small aliquots. It is better not to freeze an aliquot once thawed and use fresh aliquots at each time. Di-8-ANEPPQ dye was warmed up to 25 °C for 10-15 min before staining the cells. The NG neuron or cardiomyocyte cultures were incubated with 20 µM Di-8-ANEPPQ in culture media for 20-30 mins at room temperature to allow the staining of only the cell membrane. The dye solution was removed and or the cells to regain their basal activity, cultures were incubated for 10-15 minutes at 37 °C. The images of live Di-8-ANEPPQ labeled primary cultures were captured as detailed below.

2.3. In vivo *labeling of the mouse heart*

For the application of the tracer dyes to the murine heart, adult BALB/c mice at 8-12 weeks of age were used. Before the operation, the mice were weighed, and 25 grams or heavier mice were selected for the operation. This was empirically determined since the visualization of the epiglottis with mice smaller than 25 grams was difficult during intubation, and the mortality rates were higher due to repeated attempts and tissue damage. Before the operation, mice were anesthetized by an intraperitoneal injection of a minimum dose of ketamine (50 mg/kg, Pfizer) and xylazine (5 mg/kg, Bayer) to prevent the possible side effects in difficulty in breathing and sudden death [7,8]. Vital signs and sedation parameters such as pedal reflex and eye movement were checked, and another dose was given if it was necessary. After confirming sedation by monitoring the sedation parameters, the chest of mice was cleaned aseptically with povidone-iodine solution and shaved. The anesthetized mice were positioned into the intubation platform having 60-80 degrees to visualize the epiglottis [9]. After stabilizing the mouth of the mouse, the tongue was lifted by the help of forceps, and a fiber optic cable connected to the light source was placed to the throat to visualize the movement of the epiglottis. When the epiglottis was open, the mouse was endotracheally intubated with an intubation cannula with a Y adapter (1.2 mm od, 27 mm length; 732844, Harvard apparatus), and then the adapter was connected to the ventilation apparatus (Harvard Apparatus Minivent Type 845). In the meantime, stroke volume and stroke rate were adjusted according to the parameters indicated in the manufacturer's manual for the ventilation apparatus [10]. To make sure the air was moving to both lungs, the chest's movement was monitorized. Next, the mouse was transferred to the operation area equipped with a heating pad (WPI) in order to maintain hemostasis and prevent hypothermic shock. In addition, sterile saline drops were applied to the eyes in order to prevent dryness. After the mouse was positioned for the surgery by taping the extremities and the tail, all operational steps were performed under the stereo microscopy (Discovery 8, Zeiss).

To expose the intercostal space, the incisions were made at the left side of the thorax above the xiphoid, and muscles were dissected. The ribcage under the intercostal area was observed and the incision between the third and fourth intercostal muscle was made using fine scissors (FM010R, Aesculap) and forceps (BD329R, Aesculap). To expose the heart for delivery of the Di-8-ANEPPQ dye, the third and fourth intercostal muscles were separated using retractors to prevent any damage to the lung or the diaphragm. The light was spotted on the operational area to visualize the apex. The thin layer of epicardium was removed using fine forceps (FD048R, Aesculap). Next, 1 µl Di-8-ANEPPQ (10 mg/ml) was injected into the apex of the heart to label cardiac afferent retrogradely using 30 G Hamilton injector (5221002, Hamilton). For each dye administration, frozen Di-8-ANEPPQ aliquot was thawed and warmed up at room temperature because storage of Di-8-ANEPPQ at 4 °C could reduce labeling efficieny. Alternatively, another lipophilic retrograde dye DiI Tissue-Labeling Paste (N22880, Thermofisher) was applied on the heart surface using a pipet tip. Following the administration of the retrograde dye to the apex, negative pressure was applied to the lungs before the muscle and skin were sutured. The dye application was verified by the spreading of the Di-8-ANEPPQ over the heart. Next, intercostal muscles and skin were closed using a 6-0 silk suture (Doğsan) and the povidone-iodine solution

was applied at the incision area. The mouse was removed from the intubation apparatus and the chest movement with the rhythmic inhalation was monitorized. Occasionally the breathing was observed to be stopped, then a 25-gauge sterile needle was carefully administered between the operated ribs to remove excess air. While pulling up the plunger to remove air, the needle was constantly checked to be clear from blood to prevent damaging other tissue. Alternatively, a gentle heart message could be performed to restart the breathing. For post-operational care, the 100-200 µl saline was administered subcutaneously. At the end of the operation, the mouse was placed into the recovery cage equipped with a warm bath and was observed until the mouse was awaken from the anesthesia. The food and the water were put into the reachable area of the cage. The cage was placed into post-operative room and the mouse was monitored daily.

2.4. Imaging data collection

Following two or three hours of Di-8-ANEPPQ administration *in vivo*, the mouse was euthanized with cervical dislocation, then the heart was dissected out and put on the ice-cold Roswell Park Memorial Institute (RPMI) 1640 Medium (R0883, Sigma). Live images from the heart tissue was captured using laser scanning confocal microscope (Carl Zeiss, LSM 780) with 488 nm excitation laser, green fluorescence emission detected between 493-630 nm with GaAsP photomultiplier tube, plan-apochromat 10x/0.45 Ph1 objective, 1024×1024 resolution, 1.58 μs pixel dwell time and 20 μm total Z stack divided by five slices. In addition, the primary neuron or neonatal cardiomyocyte cultured cells were stained with 20 µM Di-8-ANEPPQ. Live images from the primary cells were taken under cell observer SD spinning disk time-lapse microscope (Carl Zeiss) with 488 nm excitation laser with 38 HE green flurosecent prot reflector, 500-550 nm filters for emisson detection, C-Apochromat 40x/1.2 water korr objective, 522.4 ms exposure time and 1388×1040 resolution. To detect the excitation and emission spectrum of Di-8-ANEPPQ, cardiomyocyte cells were stained with Di-8-ANEPPQ and imaged using the power spectrum module of LSM 780 confocal microscopy (Zeiss). For excitation of the Di-8-ANEPPQ dye, 488 nm, 561 nm, and 633 nm laser settings were all kept at the pinhole diameter of 90 μm, gain at 800 and the digital gain at 1. Laser power for 633 nm was used at 14%, for 561 nm laser 2% and for the 488nm laser 2%. The emission was captured between 410 nm and 695 nm.

Ethics Statement

The experiments comply with international guidelines and in accordance with HADHEK. Our experimental protocols were approved by the Institutional Animal Experimentation Ethical Committee with the approval number 38328770-30. In this study, two adult BALB/c independent of gender mice and one BALB/c neonatal mouse were used.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have or could be perceived to have influenced the work reported in this article.

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