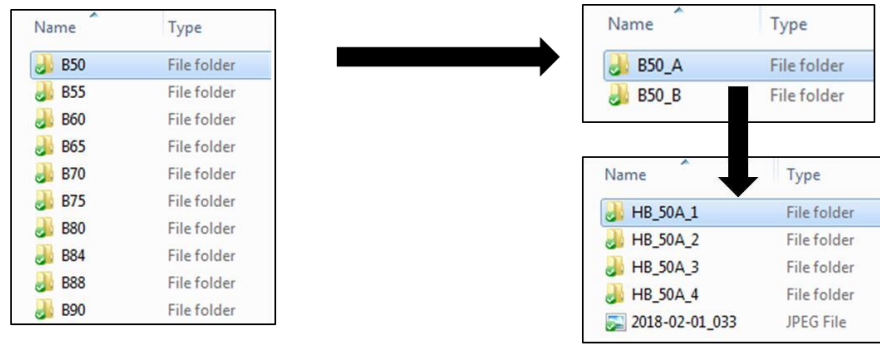**Supplemental Information**

A Comprehensive Integrated Anatomical

and Molecular Atlas of Rat

Intrinsic Cardiac Nervous System

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Figure S1: Microscopic images are available for all the collected samples, related to Figure 1. In the dataset on the SPARC Data Portal, within the "Samples" folder, there is a document called "Sample_Collection_Information" (Bottom Panel) and another folder titled "Supplementary Sample Acquisition Images". The top level of this folder is shown on the top panel (left), where the folder names correspond to the slide number of the sample in question (top panel, left and bottom panel, column A). Within that folder is a folder for each slide region (top panel, top right, and bottom panel, column B). Within each folder for a slide region is a folder for each sample containing the images taken for the acquisition of that sample (top panel, bottom right, and bottom panel, column C). For each sample, the corresponding folder contains images showing the area of interest, the neurons marked before they are collected, the neuron after it was collected on the collection cap, the remaining tissue after the neurons had been picked, as well as images showing the tissue before and after an additional UV cut which aided in the mapping of samples later on (bottom panel columns F-K). All images are labeled with the date they were collected followed by a number that is listed in the spreadsheet to identify the images.

TRANSPARENT METHODS

All animal work was approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Figure 1 illustrates our two approaches as two graphical workflows. We established two multi-component pipelines to map neurons mapped from histological heart tissue sections. One approach (Figure 1A) optimizes precision of 3D heart shape and tissue section alignment for establishing a 3D reference framework. The second (Figure 2B) trades resolution of cardiac structure for, in addition to mapping neuron positions, the acquisition of projections/connectomic data and/or acquisition of neuron samples for molecular profiling.

Figure 1A shows the anatomy-focused pipeline that acquires sections and images by using Knife Edge Scanning Microscopy system (KESM, 3Scan) which maintains precision and eliminates most artifacts greatly improving anatomical rigor and reproducibility. The present results show data from one representative heart from a Fischer (RRID:RGD_1547866; Charles River, USA) 250 g male. Such data supports mapping into a scaffold that provides a reference framework, or atlas, suitable for aligning data from other types of experiments into the 3D structure of the ICN. All 3D model figures in the present work use data from this pipeline, including Figures 2-7.

Figure 1B shows the molecular-focused pipeline, using cryostat sectioning, used here for two 300 g Sprague-Dawley (RRID: RGL_1566457; Envigo, USA) male (Heart A) and female (Heart B) rat hearts. These two hearts were exhaustively cryosectioned producing sections useful not just for comprehensive mapping but also for Laser Capture Microdissection (LCM) of both tracing-labeled and non-connected neurons for transcriptional single neuron profiling. For consistency, the transcriptional profiles of anatomically localized neurons were drawn from one heart, Heart B. Images of these sections, including cell positions, can be stacked to create a whole heart volume with data that can be brought into the 3D reference systems created by the first approach. All transcriptional data and 3D position of these neurons are from Heart B, including Figures 8-13.

Knife Edge Scanning Microscopy

Images taken by KESM have great advantages for precision of gross morphology while resting on cell-level histology, ideal for developing 3D frameworks to hold additional data types from other approaches. Using Tissue Mapper software (MBF Bioscience) to map the position of each neuron as illustrated in Figure 2 we develop a comprehensive mapping of the precise extent and distribution of the ICN in the 3D framework of the heart, as described below:

Sample Preparation: In brief, a normal male rat heart was obtained fresh and subsequently immersed in 4% paraformaldehyde prior to whole-mount diffusion staining with cresyl violet stain (0.05g cresyl echt violet in 50ml dH2O + 150µl glacial acetic acid, for 7 days) to enable visualization of the intrinsic cardiac neurons and ganglia.The tissue sample was subsequently paraffin embedded and KESM digitized.

Image Acquisition: The FFPE block was KESM digitized at slice thickness of 5 µm per z-slice. The FFPE block was mounted to a nano-precision XYZ robotic stage. The robotic stage moves samples along the XY axis towards a diamond knife ultramicrotome, which is coupled to a fiber optic cable. Hence, the cutting blade also serves as a source of

illumination. There is custom-built objective, with a 5 mm field of view, and a tube lens that equates to a magnification of approximately 10x trained on the bevel of the diamond knife. The end of the tube lens is coupled to a CMOS TDI line scan color sensor with a 16K pixel resolution RGB output and a 5 µm x 5 µm pixel size.

The 5mm blade moves across the surface of the FFPE block, slicing and scanning simultaneously, capturing one continuous line (or strip) of image data at a time, to generate an image tile comprised of 10,000 pixels, with each pixel representing 0.5 µm. After the strip has been fully sectioned and imaged, the stage is moved to position the adjacent region of the heart in line for sectioning and imaging. This process is repeated until the entire heart has been sectioned.

Image Processing: Post-processing of the data utilizes the precise spatial alignment provided by the KESM technique to generate 2D image planes, which were subjected to denoising and artefact reduction. Individual image planes were assembled together into a 3D volume, enabling quantification of morphological details over large anatomical distances. To achieve this, individual KESM image tiles acquired from each XY location at each Z position were automatically aligned and stitched into 2D image planes, cropped to remove excess image data that did not contain the heart, and then assembled into a 3D image volume with 35:1 JPEG2000 compression using Biolucida Converter. These image volumes were annotated using Tissue Mapper as described below.

Software Development and Neuron Mapping
A custom suite of computational mapping programs have been developed for mapping neurons (or any cell of interest) in organs including the heart: TissueMaker and Tissue Mapper. These were, and continue to be, evolved from the tools MBF Bioscience has developed for brain mapping, such as Neurolucida and BrainMaker.

The rat heart sectioned at TJU as in Figure 1B was run through the TissueMaker and Tissue Mapper pipeline. By contrast, as in Figure 1A the KESM image data from 3Scan did not require the alignment step in TissueMaker because it was already spatially aligned (see imaging description above).

Using Tissue Mapper software, precise locations of each cardiac neuron were mapped in all sections in which neurons are present. In addition to marking the cell location, numerous regions selected from the comprehensive ontology first generated for the Cardiac Physiome Project (Hunter and Smith, 2016) were mapped. Of the thousands of ontological features that were available, less than 40 were selected for these initial representations in order to simplify the images and to test the pipeline more efficiently. On each section, researchers traced key features (e.g. aorta, pulmonary vessels, atrial borders) and identified neurons based upon a combination of Nissl staining and morphology. The TissueMaker and Tissue Mapper software are then able to generate 3D wireframes of the hearts with neurons positioned in context. Attributes like color and shape can be customized in these reconstructions and quantitative spatial data can be obtained. By mapping anatomical fiducial information alongside the neuron locations,

the extent and location of the neurons within the larger context of the entire heart could be viewed as a 3D representation, as seen in the Figures for the present work.

Cryosectioning and Embedding

In our initial efforts with Heart A (male) and Heart B (female) we learned that, unlike the brain, the distortions of the heart are far less homogenous and symmetric, which can interfere with data visualization and comparison between specimens. Thus we developed the method described below to keep the chambers inflated. We also discovered that the embedding media needs color added to permit image segmentation. Optimal Cutting Temperature media (OCT, TissueTek; VWR 25608-930) is added to an embedding mold, to cover the bottom of the mold and kept on dry ice. Three concentrations of OCT diluted in 1x PBS are prepared 25% 50%, and 100%. The 100% OCT preparation should include a few drops of green food grade dye to permit optimal image segmentation of any subsequent blockface images. The animal is sacrificed using rapid decapitation after 60 seconds of exposure to 5% isoflurane. The heart is immediately excised and submerged in room temperature 1x PBS for 30 seconds or until the majority of blood is pumped out of the chambers. The still beating heart is transferred to 25% OCT, and lightly agitated for 30-60 seconds. This is then transferred to a 50% OCT solution, and lightly agitated for 30-45 seconds. The heart is still beating at this point. The large chambers of the heart were injected with colored OCT via the great vessels using a 14-16 gauge blunt needle on a syringe in order to mitigate structural collapse during cryosectioning. Next, the heart is placed in a chilled embedding mold. Room-temperature OCT is added to completely submerge the heart. The mold is then placed in a slurry of dry ice and methanol to promote rapid freezing. Care was taken to avoid allowing methanol to come into contact with the OCT in the block as this will compromise the structural integrity of the OCT once frozen. Note that holding the block near liquid nitrogen, but not submerging it, is also a means of rapid cooling. After the OCT is completely frozen, it is covered with aluminum foil and then in a plastic wrap to prevent accumulation of condensation in the mold. The mold is transferred to a -80°C freezer. Ideally the entire process should happen within 5-10 minutes to mitigate RNA degradation, which will be necessary for future investigations of single cell transcriptomics using laser capture microdissection.

Slide Preparation and Image Processing

Heart A was sectioned from base to apex at 20µm, yielding nearly 800 sections, with corresponding blockface images, and mounted onto 400 slides (two sections per slide). In order to appreciate a finer level of detail and clarify tissue staining, Heart B was sectioned at 10 um yielding nearly 1600 sections mounted on 800 slides with blockface images for each section.

Each slide was stained with 0.1% Cresyl violet and dehydrated using increasing concentrations of ethanol and xylene. Cover slips were added using mounting media and slides were then imaged using a slide scanner equipped with 20x Olympus objective (N.A.= 0.75; Bliss-200, MBF Bioscience, Williston, VT). On average, 2,000 image tiles were automatically acquired and stitched to create a high-resolution whole slide image containing two heart sections per image. Using TissueMaker software, the

section images were then extracted from the whole slide image, cropped to a uniform size, the perimeter automatically segmented, and aligned spatially by identifying the centroid of each section to generate an image stack. The heart image volume was then shared with TJU and UCF for further mapping and segmentation using custom-developed software (Tissue Mapper; MBF Bioscience). The software application includes annotation tools for automatically or manually drawing regions, placing markers to indicate cell positions and other discrete points, and the ability to import comprehensive lists of regions as a text or comma-delimited file or via direct integration with the SciCrunch database.

Laser Capture Microdissection of Single Neurons
In order to isolate neurons while maintaining their anatomical origin in three dimensional space, it was necessary to use laser capture microdissection (Arcturus, ThermoFisher). Neurons were visualized using a rapid cresyl violet stain that highlighted the histological appearance of neurons and maintained RNA quality. Single neurons were collected on Capsure HS caps and the cells lysed right on the cap within 15 minutes after laser capture using lysis buffer from the CellsDirect DNA extraction kit (Life Technologies).

Transcriptional assay of laser captured single neurons using multiplex RT-qPCR
RNA-seq performed on laser-captured single cells has only recently been demonstrated and was not available during these experiments (Foley et al. 2019). In order to assay single neurons from laser capture, it was necessary to apply multiplex RT-qPCR to ensure the sensitivity to detect several genes of interest given the small amount of RNA per sample. The Biomark microfluidic system (Fluidigm, San Francisco, CA) was used for all gene expression assays. After reverse transcription and whole transcriptome amplification (Qiagen, Hilden, Germany), the samples were processed through the Biomark system following manufacturer suggested protocols. Quality control of RT-qPCR results included filtering through melt-curve analysis along with automatic C_t thresholding to determine the limit of detection.