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Isolation of Cardiomyocytes from Fixed Hearts for Immunocytochemistry and Ploidy Analysis

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RE: revised manuscript submission of JoVE60938

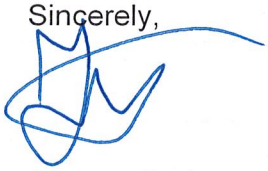
Dear Editors,

Please find attached our revised manuscript JoVE60938 entitled "Isolation of Cardiomyocytes from Fixed Hearts for Immunocytochemistry and Ploidy Analysis".

We have carefully gone through the comments of the reviewers and addressed all of their concerns. We also added more details within the protocol to better explain the precise actions at all steps, as recommended by the editors.

We hope our revised manuscript is now acceptable to move forward in JoVE. Thank you for your time and consideration.

Sincerely,



Jop van Berlo

TITLE:

Isolation of Cardiomyocytes from Fixed Hearts for Immunocytochemistry and Ploidy Analysis

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KEYWORDS:

Heart, cardiomyocyte isolation, ploidy analysis, immunocytochemistry, automated image analysis

SUMMARY:

The goal of this work is to develop a method to reproducibly isolate cardiomyocytes from the adult heart and measure DNA content and nucleation.

ABSTRACT:

The adult mammalian heart is composed of various cell types including cardiomyocytes, endothelial cells and fibroblasts. Since it is difficult to reliably identify nuclei of cardiomyocytes on histological sections, many groups rely on isolating viable cardiomyocytes prior to fixation to perform immunostaining. However, these live cardiomyocyte isolation techniques require optimization to maximize the yield, viability and quality of the samples, with inherent fluctuations from sample to sample despite maximum optimization. Here, we report a reproducible protocol, involving fixation prior to enzymatic digestion of the heart, which leads to maximum yield while preserving the in vivo morphology of individual cardiomyocytes. We further developed an automated analysis platform to determine the number of nuclei and DNA content per nucleus for individual cardiomyocytes. After exposing the chest cavity, the heart was arrested in diastole by perfusion with 60 mM KCl in PBS. Next, the heart was fixed in 4% paraformaldehyde (PFA) solution, and then digested with 60 mg/mL collagenase solution. After digestions, cells were singularized by trituration, and the cardiomyocyte fraction was enriched via differential centrifugation. Isolated cardiomyocytes were stained for Troponin T and α-actinin to assess

purity of the obtained population. Furthermore, we developed an image analysis platform to determine cardiomyocyte nucleation and ploidy status following DAPI staining. Image based ploidy assessments led to consistent and reproducible results. Thus, with this protocol, it is possible to preserve native morphology of individual cardiomyocytes to allow immunocytochemistry and DNA content analysis while achieving maximum yield.

INTRODUCTION:

Heart disease has been the leading cause of death in the majority of western countries for many decades[1,2]. Although many improvements in the treatment of cardiovascular diseases have improved survival, there are currently no treatments that can replace lost cardiomyocytes. Therefore, studies related to cardiomyocyte function, proliferation, apoptosis and hypertrophy have been and continue to be a major focus of the scientific community. Since the adult mammalian heart has a very limited regenerative capacity, with an estimated cardiomyocyte renewal rate of less than 1% per year, it is crucially important to reliably identify cardiomyocyte proliferative events[3,4]. Most strategies that measure proliferative events rely either on staining for incorporated DNA nucleotide analogs to assess previous or current proliferation, or stain for nuclear markers of active proliferation[5]. It is especially important to reliably identify cardiomyocyte proliferative events since the overall number of proliferative cardiomyocytes is so low[3,6]. For example, based on a 1% renewal rate of endogenous cardiomyocytes per year, one can expect to find between 25 and 50 cardiomyocytes to be proliferative at any given time in the adult mouse heart[7,8]. Any inaccuracies in identification of cardiomyocyte nuclei might lead to false positive results. Therefore, it is critical to reliably identify cardiomyocyte nuclei, which has proven difficult and unreliable from histological sections[9]. Identification of cardiomyocytes is much more accurate from single cells than from tissue sections as it might be difficult to distinguish cardiomyocytes from other cell types even when using markers such as α-actinin, although PCM1 might be a reliable marker of cardiomyocyte nuclei in histological sections[10].

Current protocols rely on isolating live cardiomyocytes prior to fixation, which is known to cause death of at least 30% of cardiomyocytes, and might lead to inadvertent selection of specific populations of cardiomyocytes[11]. Furthermore, these protocols are notoriously difficult to optimize to provide reproducible results. Even optimized isolation techniques can typically produce no more than 65% live, rod-shaped cardiomyocytes with varying yields[12].

To overcome these issues, we developed a protocol that allows researchers to isolate fixed cardiomyocytes. Since the samples are fixed prior to isolation, the yield is maximized, and in vivo morphology is well preserved. Moreover, with this protocol it is possible to isolate cardiomyocytes from clinical samples, which are typically fixed immediately after procurement. Furthermore, to identify newly generated cardiomyocytes, it is important to measure the nucleation and ploidy status of individual cardiomyocytes, since only diploid cardiomyocytes are typically assumed to be newly formed. Flow cytometry cannot distinguish multinucleation from polyploidy and is a relatively time and resource-intensive protocol. Manual outlining and measurement of nuclei within images is very low-throughput and prone to human bias. Automated quantification of images of fixed, isolated DAPI-stained cardiomyocytes solves both of these problems. Imaging-based determination of nucleation and ploidy distributions can be

obtained with a minimum of time and reagents using basic equipment.

PROTOCOL:

All animal experiments were performed conform the National Institutes of Health guidelines and approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC).

1. Preparation of the solutions and surgical equipment

1.1. Prior to isolation, sterilize the surgical equipment by using 70% ethanol solution.

1.2. Add 2.24 g of KCl to 500 mL phosphate buffered saline (PBS) solution to obtain a final concentration of 60 mM. Store KCl-PBS solution at room temperature. Use 3 mL of KCl-PBS solution per mouse.

1.3. Dilute 32% paraformaldehyde (PFA) solution with PBS into to obtain final concentration of 4% PFA. Prepare 10 mL of 4% PFA in PBS per mouse. Diluted PFA solution can be stored at 4 °C for 2–3 weeks in a glass container.

NOTE: Prepared 4% PFA solution can be stored at -20 °C for longer periods of time.

1.4. Prepare 1 mL of collagenase solution per mouse by adding 60 mg of collagenase, type 2 per 1 mL of PBS.

2. Perfusion and fixation of the heart

2.1. Anesthetize the animal by using 2–5% isoflurane with an oxygen flow rate of 1 L/min. Confirm the anesthesia by confirming lack of movement and lower rate of breathing.

NOTE: Injecting heparin (100–500 U/kg) before euthanasia can increase the cell quality and yield by preventing blood clots, thereby allowing for more efficient perfusion of the heart with fixative.

2.2. Euthanize the animal according to approved methodologies.

NOTE: We followed the American Veterinary Medical Association guidelines for the euthanasia of animals, and obtained local IACUC approval for euthanasia.

2.3. Place the euthanized animal in supine position, and tape down extended limbs.

2.4. Cut through the chest to expose the heart using blunt-end scissors. Cut descending aorta and inferior caval vein.

2.5. Perfuse the heart by injecting 3 mL of KCl-PBS solution through the left ventricle with a flow rate of 3 mL/min using a peristaltic pump attached to an infusion set with a 23 G butterfly needle (26 G for neonates). Make sure not to pierce through the septum.

NOTE: Alternatively, use a needle attached to a syringe to inject solutions.

2.6. Perfuse the heart by injecting 10 mL of 4% PFA solution for 10 min using a peristaltic pump at a rate of 1 mL/min.

2.7. Remove the whole heart using scissors. After removing the heart, it is possible to isolate a specific region of the heart by incising. Place the heart, or a segment of it in a 1.5 mL microcentrifuge tube containing 1 mL of 4% PFA solution. Incubate the heart on rocker at room temperature with rocking speed between 20–30 rpm for 1 h.

3. Isolation of fixed cardiomyocytes

3.1. Place the heart in a Petri dish containing PBS solution. Squeeze the heart to get rid of any PFA remaining in ventricles, and wash in PBS.

3.2. Put the fixed heart into a new 1.5 mL microcentrifuge tube containing collagenase solution (60 mg/mL). Place the tube on rocker (20–30 rpm) at 37 °C for overnight incubation.

NOTE: Extend the incubation time up to 1 week and replenish the collagenase solution every two days to reduce the possible variation in yield if hearts are anticipated to be fibrotic, which might require longer time of collagenase digestion to digest extracellular collagen.

3.3. Put collagenase solution and the heart into a 35 mm Petri dish. Dissociate the heart into 1 mm pieces by using forceps or scissors.

3.4. Use a transfer pipette to further triturate the dissociated tissue for 2 min. If tissue particles still remain in the dish, use a transfer pipette with narrower opening and continue trituration. Continue until the majority of the tissue is broken down.

NOTE: Over trituration causes individual cardiomyocytes to break. Make sure not to over triturate by checking regularly under a microscope.

3.5. Place a 200–600 µm nylon mesh over opening of 15 mL centrifuge tube.

NOTE: For hypertrophied cardiomyocytes, it is recommended to use 400 µm nylon mesh instead of 200 µm.

3.6. Add 5 mL of PBS to the Petri dish containing dissociated cells and filter the solution through nylon mesh, including tissue particles. Wash the nylon mesh by passing additional 4 mL PBS.

3.7. Centrifuge the filtered solution at 10–100 x *g* for 1 min.

NOTE: 100 x *g* centrifugation will not yield 100% pure cardiomyocyte population, and some non-

cardiomyocyte cells are likely to be included.

3.8. Discard the supernatant unless one wants to stain/evaluate non-cardiomyocytes cardiac cells as well. Resuspend the pellet in 10 mL PBS prior to staining.

4. Staining cardiomyocytes

4.1. Collect the cells by centrifugation at 100 x *g* for 1 min and add 5 mL of permeabilization solution (e.g., 0.5% Triton X-100 in PBS). Incubate for 20 min at room temperature on rocker.

NOTE: For steps 4.1, 4.2 and 4.4 use 15 mL centrifuge tubes as it is easier to remove the supernatant without disturbing the cell pellet compared to 1.5 mL microcentrifuge tubes.

4.2. Collect the cells by centrifugation at 100 x *g* for 1 min, add 5 mL of blocking buffer (e.g., 3% bovine serum albumin [BSA] in PBS) and incubate for 30 min at room temperature on a rocker.

4.3. Collect the cells by centrifugation at 100 x *g* for 1 min and add 1 mL of primary antibody solution (in PBS) with the appropriate dilution ratio. Transfer the solution into 1.5 mL microcentrifuge tube and incubate cardiomyocytes in primary antibody solution under optimized conditions (e.g., 4 °C overnight).

4.4. Transfer cardiomyocytes with primary antibody solution to a 15 mL centrifuge tube and add 9 mL of PBS. Incubate the cardiomyocytes for 10 min at room temperature on a rocker.

4.5. Collect the cells by centrifugation at 100 x *g* for 1 min and add 10 mL of PBS. Incubate the cardiomyocytes for 10 min at room temperature on a rocker. Repeat this step once more.

4.6. Collect the cells by centrifugation at 100 x *g* for 1 min and add the secondary antibody solution containing DAPI. Incubate for 30 min at room temperature on a rocker, followed by repeating step 4.5 twice to wash cardiomyocytes.

4.7. Place the cells either on coverslips or microscope-compatible plates and proceed with imaging.

NOTE: Images included in the manuscript were taken with 10x and 40x objectives. Lasers used were: 405 nm for DAPI, 561 nm for Alpha actinin and 640 nm for Edu.

5. Setup imaging software

NOTE: Follow along with these steps using **Supplementary File 1**-SoftwareScreenshots.pdf.

5.1. Download the Fiji distribution of ImageJ.

5.2. Open Fiji. Click on **Help > Update… > Manage Update Sites**. Check the "IJPB-plugins" and "Biomedgroup" update sites to download the dependencies plugins Ellipse Split and Morpholibj.

5.3. Click **Close**. Fiji should begin downloading the dependencies. Restart Fiji when finished.

5.4. Download Rstudio and open it.

5.5. Copy **install.packages**(**c**(**"ggplot2", "autothresholdr", "dplyr", "purrr", "jsonlite", "shiny"**)) into R console's command line and press the **Enter** key. Type "y" in response to all prompts to install all R dependencies (Screenshot 1 in **Supplementary File 1**).

6. Image quantification

6.1. Open Fiji and drag "AnalyzeNucleation.py" (supplied as a supplementary code file) into Fiji's status bar. This will open a script-editing window. Click **Run** in the lower left corner to begin it (Screenshot 2 in **Supplementary File 1**).

6.2. A dialog box will pop up (**Supplementary File 1**: Screenshot 3), asking for the location of the output data directory. All analysis data, figures, and other data used by this software will be stored in this folder. Another, larger dialog box will pop up, displaying all image analysis settings (**Supplementary File 1**: Screenshot 4).

6.2.1. Select location of directory containing images to be analyzed.

6.2.2. Enter the image filename format using regular expressions. Enter the image filename format, indicating which parts of the filename correspond to row, column, channel, and (optionally) site within braces, using regular expressions. Do not put spaces within the braces. Surround variable parts of the filename format in braces {}. The way files are saved depends on the imaging software, and this step will retrieve relevant information from the image filename.

NOTE: For example, the format string
r" Plate 1-(?P<row>[A-Za-z]+)(?P<column>[0-9]+)-(?P<channel>[A-Za-z]+).tif"
describes a filename that starts with "Plate 1-", which is followed by one or more alphabetical letters indicating the row, which is followed by one or more digits indicating the column, which is followed by "-", which is followed by one or more letters indicating the channel, which is followed by ".tif". The letters inside the angle brackets like "<>" are variable names and are automatically copied into the data when it is collected. One of the variable names must be "<channel>"

6.2.3. Indicate the name of channels in which the nuclear stain is visible and where the cardiomyocytes are visible. These names must be *exactly* as they are in the part matched by the "<channel>" variable in the regular expression filenames.

6.2.4. Indicate how the images should be grouped using comma-separated variable names. All of the images within a given group will be opened and analyzed in one batch. For example, if the images are divided into sets for each well, and there is a well for each unique combination of row and column, then write "row, column" in this field.

NOTE: These grouping variables must be a subset of the variables used in the format string. Do not use "channel" as a grouping variable, this will separate corresponding channel images from each other.

6.2.5. Indicate whether or not images are stitched together in one well image or are separate for each site. In the former case, site should not be indicated in the filename format string.

6.2.6. Choose which thresholding method to use to separate nuclei from the background. All of Fiji's standard thresholding methods are available. Test different thresholding methods to determine which works best for the image set. In this example, choose the Otsu method.

6.2.7. Indicate whether or not the threshold should be recalculated for each site image or if the same threshold should be used for every image in the group. Indicate whether the cardiomyocyte images are brightfield or use a fluorescent marker.

6.2.8. Indicate the cardiomyocyte thresholding method. If brightfield was chosen in the previous step, this thresholding method will be applied to edge-filtered brightfield images. Indicate whether or not the threshold should be recalculated for each site image or if the same threshold should be used for every image in the group.

6.2.9. Indicate the number of rows of site images that cover each well. Indicate the number of columns of site images that cover each well. Indicate the minimum area of nuclei in pixels. Use a generously low minimum size, a higher and more precise threshold will be calculated in the analysis step. Indicate the minimum area of cardiomyocytes.

6.2.10. After choosing the desired settings click **OK**.

6.3. Images resembling those found in **Figure 3** and **Figure 4** will appear on the screen, showing the different stages of the analysis pipeline. Inspect these images to ensure that thresholding and segmentation are occurring properly.

6.4. The selected results folder should now be filled with analysis data (**Supplementary File 1**: Screenshot 5). Files other than analysis data can safely be saved in this folder as long as their names do not begin with "cm_", "nuclei_", or "nucleilink_".

7. Data analysis

NOTE: The csv files that are produced can be analyzed manually. Each analyzed image subset produces a triplet of csv files named "nuclei(metadata).csv", "nucleilink(metadata).csv", and

"cardiomyocytes(metadata),csv", where (metadata) is replaced with a sequence of name-value pairs of the form "_(name)=(value)", where (name) and (value) are sequences of alphanumeric characters derived from strings matched in the regular expression given earlier. (For example, if row and column were indicated in the filenames then strings like "_row=F" and "_column=8" will be present). The unnamed leftmost column of each nuclei and nucleilink file is a nucleus ID number. The "Min" column of the nucleilink file is the id of the cardiomyocyte that contained said nucleus wholly or 0 otherwise. The "Max" column of the nuclei is the ID of the highest-numbered cardiomyocyte that contained said nucleus in part, or 0 otherwise. The "Mean" column of the cardiomyocytes file is the cardiomyocyte id number.

7.1. Open "AnalyzeMultinucleatedServer.R" in Rstudio (provided as supplementary code file).

7.2. At the top of this file is a variable named "folderName". Next to it is a filepath. In here, type the path to the output data folder selected in the last step, without the final slash (**Supplementary File 1**: Screenshot 6).

7.3. In the upper left corner of the script editing window there should be a green arrow labeled **Run App**. Click this arrow. It may take some time for the data to load and for the app to pop up.

7.4. Initially, three gating graphs will be visible, one to indicate the minimum valid nuclear area threshold, one to indicate the minimum valid nuclear mean intensity threshold, and one to indicate the maximum valid minimum feret's diameter for cardiomyocytes. Use the sliders to set these thresholds (**Supplementary File 1**: Screenshot 7).

NOTE: In each of these graphs, a large, broad peak corresponding to valid nuclei or cardiomyocytes should be present, flanked by broad tails representing debris or erroneous segmented cardiomyocytes. Use the thresholds to cut one tail of each of the peaks off.

7.5. Scroll down. Click the button **Apply Selected Thresholds** (bottom of **Supplementary File 1**: Screenshot 7).

7.6. Click the button **Plot Intensity Distribution**. This will render plot of the nuclear intensity distribution of both the entire sample and separate subplots for each grouping variable.

NOTE: For example, if <row> and <column> grouping variables were entered into the regular expression in the Fiji dialog, plots indicating the intensity distribution by row and by column will appear here (**Supplementary File 1**: Screenshot 8). If illumination and staining conditions were constant across the different parts of the sample, these plots should all clearly show two intensity peaks, a dimmer, taller one for the diploid nuclei and a brighter, shorter one for the tetraploid nuclei.

7.7. Intrasample variation will result in this pattern not being visible in the whole-sample plot and there being great variety in the location of the diploid and tetraploid peaks by row, column, or

other grouping variable. In the latter case, scroll down check the checkbox **Normalize Separately by group** to account for this variation (**Supplementary File 1**: Screenshot 9).

7.8. Click the button **Calculate Ploidy** (**Supplementary File 1**: Screenshot 9). Click the button **Plot Estimated Ploidy Distribution**. Graphs will appear in the empty windows to the right. In the normalized whole-sample graph, the two-peak pattern should be visible if it wasn't before.

7.9. Select thresholds to isolate the diploid and tetraploid peaks from both each other and outliers using the sliders (**Supplementary File 1**: Screenshot 9). Scroll down. Click the button **Calculate Ploidy and Nucleation** (**Supplementary File 1**: Screenshot 10).

7.10. Click the button **Plot and Save Into Results Folder**. The plot saved into the selected results folder will also appear in this interactive window (**Supplementary File 1**: Screenshot 10).

REPRESENTATIVE RESULTS:

Cardiomyocytes were isolated according to the protocol described above. Using this method, we typically get uniformly singularized cardiomyocytes that are relatively pure without contaminating non-cardiomyocyte cells (**Figure 1A**). Cardiomyocytes are easily identified under bright field microscopy due to their characteristic size and birefringence. This technique is easy to implement and provides consistent results from different isolations with comparable cardiomyocyte yields and quality (**Figure 1B**). Isolated cardiomyocytes can be stored at 4 °C for several weeks before further use.

Cardiomyocytes that were isolated according to the above protocol can be used for various downstream applications, such as measuring cardiomyocyte size, cardiomyocyte ploidy and immunocytochemistry. As a representative result, we show that cardiomyocytes isolated according to this protocol can be stained using antibodies and fluorochrome-conjugated azides for click chemistry to detect localization of specific proteins or to detect cardiomyocyte DNA replication, respectively. For example, we stained cardiomyocytes with antibodies recognizing α-actinin to show the characteristic z-line staining pattern of sarcomeres (**Figure 2A**). In a separate experiment, we administered the thymidine analog 5-Ethynyl-2'-deoxyuridine (EdU) to mice before isolating fixed cardiomyocytes. After cardiomyocyte isolation, we stained for incorporated EdU using standard protocols[13], and were able to detect cardiomyocytes that had undergone S phase in either mononucleated, binucleated and trinucleated cardiomyocytes (**Figure 2B**).

To further expand the utility of the isolation method, we developed a pipeline that allows quantification of cardiomyocyte ploidy based on integrated DNA staining. To be able to measure ploidy status of cells or nuclei, we needed to segment nuclei and cardiomyocytes. **Figure 3** shows a representation of the strategy we used to identify individual nuclei. First, the original image DNA stained image (**Figure 3A**) is thresholded based on intensity (**Figure 3B**). Here, we used DAPI to stain for DNA, but any other nuclear dye that shows a linear correlation with DNA content would work. The program allows for any of Fiji's intensity thresholding methods to be chosen, but in this example Otsu's method was used. Nuclear masks that are touching the edge of the image or are smaller than the specified minimum pixel area threshold are excluded. Then, ellipses

are fit to the nuclear masks, segmenting individual nuclei. **Figure 3C** shows these ellipses overlaid on the original image. Next, holes are filled in the masks, and the pixels of the image are then partitioned into territories based on which ellipse they are most proximal to (**Figure 3D**). The borders of these territories are then used to draw lines through nuclear clusters, finishing the nuclear segmentation process (**Figure 3E**).

The next step involves detection of cardiomyocytes. For cardiomyocyte images that are obtained based on fluorescently stained cells (**Figure 4A**), the process is very similar to that for nuclei. The image is thresholded based on an intensity value calculated by the selected thresholding method, in this case the triangle method. Identified cardiomyocyte masks that are touching the boundary of the image or are below a certain size are excluded and holes are filled in the masks to provide properly segmented cardiomyocytes (**Figure 4B**). Because cardiomyocytes have a more irregular shape than nuclei, no attempt is made to segment cardiomyocyte clusters. Instead, these clusters are excluded based on their high minimum Feret's diameter during the analysis step. Segmentation from bright field images proceeds slightly differently. First, the original bright field image (**Figure 4C**) is processed with a Sobel edge filter. This filter calculates the absolute value of the gradient of each pixel within the image. Pixels in regions with rapid changes receive high values and pixels in smooth regions of the image receive low values. This edge-filtered image is then thresholded by intensity, using the Triangle method, resulting in masked cardiomyocytes (**Figure 4D**). These highly irregular masks are then smoothed and linked together via morphological closing using a circle with a radius of 2 pixels, which fills in all white regions in the image where the circle cannot fit without overlapping a black region (**Figure 4E**). Finally, holes in the masks are filled, regions touching the border are excluded, and small particles are removed, finishing the cardiomyocyte segmentation process (**Figure 4F**).

Using the outlined segmentation strategy, we can then determine the nucleation status of individual cardiomyocytes. Using this approach, we determined the nucleation status of cardiomyocytes isolated from hearts of outbred CD-1 mice at early postnatal time-points. Hearts of newborn mice (first day of life) showed that the majority of cardiomyocytes at that point are mononucleated (**Figure 5**: neonatal). This high frequency of mononucleated cardiomyocytes is much lower in juvenile mice (2-week old), where mononucleated cardiomyocytes make up about 25% of the total cardiomyocyte population (**Figure 5**: juvenile). Finally, we can measure the ploidy status of individual nuclei within cardiomyocytes, and determine whether they are diploid or tetraploid. These results show higher frequency of tetraploid nuclei in adolescent mice (**Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1: Efficiency of cardiomyocyte isolation after fixation. (**A**) Representative image of isolated cardiomyocytes stained with DAPI to show nuclei. (DAPI (blue), Brightfield (gray)) (**B**) Yield of cardiomyocytes isolated from different mice at 3 months of age. Scale bars = 50 μm.

Figure 2: Immunocytochemistry of isolated cardiomyocytes. (**A**) Representative image of cardiomyocytes stained for α-actinin (α-actinin (red) and DAPI (blue)). (**B**) Cardiomyocytes

stained for incorporated EdU (red) and DAPI (blue). Representative cardiomyocytes that are mononucleated (left), binucleated (middle) and trinucleated (right) and EdU positive are shown. Scale bars = 50 μm.

Figure 3: Strategy for nuclear segmentation. (**A**) Original DAPI channel image. (**B**) Thresholded image (in this example, Otsu's method was used). (**C**) Masks that were identified from the thresholded images overlaid on the original DAPI stained image. (**D**) Voronoi tessellation based on nuclear masks. (**E**) Final segmented nuclei, with split clusters highlighted. Scale bars = 100 μm.

Figure 4: Strategy for cardiomyocyte segmentation. (**A**) Original fluorescent Troponin I stained cardiomyocyte image. (**B**) Triangle-thresholded image, after filling holes and excluding small objects and those touching the border. (**C**) Original bright field cardiomyocyte image (**D**) Edge-filtered and triangle-thresholded cardiomyocyte image (**E**) Edge-filtered image after morphological closing with a radius of two pixels (**F**) Same image after filling holes and excluding small objects and those touching the border. Scale bars = 100 μm.

Figure 5: Classification of cardiomyocytes based on number of nuclei. Neonatal hearts (1 day old) contain more mononucleated cardiomyocytes than juvenile hearts (14 days old).

Figure 6: Distribution of cardiomyocyte DNA content per nucleus. In neonates (left), 13.5% of mononucleated CM nuclei are tetraploid and 11.9% of binucleated CM nuclei are tetraploid. In juveniles (right), 33.9% of mononucleated CM nuclei are tetraploid and 31.2% of binucleated CM nuclei are tetraploid.

DISCUSSION:

Since cardiomyocytes cannot be maintained in culture, it is important to isolate primary cardiomyocytes to be able to study their architecture and function[11]. Hence, cardiomyocyte isolation techniques have been widely used in the cardiac field. If the goal is to determine functional aspects of cardiomyocytes, it is important to isolate viable cardiomyocytes. These live cardiomyocytes can also be used to perform immunostaining on isolated cardiomyocytes. However, optimizing the technique of isolating live cardiomyocytes is technically challenging, and even the best techniques typically only yield 60–65% live rod-shaped cardiomyocytes, and the remaining cardiomyocytes are all balled up and dying or dead[11,12]. Here, we developed a technique that will allow researchers to first fix the heart, and then isolate cardiomyocytes efficiently. This new protocol allows for much higher yields of rod-shaped cardiomyocytes compared to previously published protocols. Furthermore, we developed an imaging analysis platform to categorize cardiomyocytes automatically based on nucleation and ploidy. With these new methodologies, groups can stain cardiomyocytes for different proteins, and study cardiomyocyte ploidy and nucleation status as surrogates for the regenerative potential of the heart.

The protocol described here is relatively straightforward, and can be performed without any advanced equipment. The amount of collagenase and incubation time for digestion might vary depending on the collagenase lot, and the company providing it. We used collagenase type 2,

since this is most widely used to digest the heart for obtaining live cardiomyocytes. Based on our observations, we determined that overnight incubation with 60 mg/mL collagenase type 2 is optimal for almost all mouse hearts regardless of the level of fibrosis. We have never had an issue of overdigestion as intracellular proteins are fixed and not as accessible as extracellular collagen. However, if the heart is not digested properly, more vigorous trituration might be needed, which causes cell fragmentation due to shear stress. Thus, it is crucial to make sure that the heart is digested properly before moving on to trituration. Stiffness of the heart can be tested by squeezing with forceps to assess the degree of digestion. Following incubation with collagenase, hearts should be less stiff and easy to tear apart. Other types of collagenase can also be used. A previous report used a combination of collagenases B and D[14].

Furthermore, we believe that this protocol can be used to assess overall number of cardiomyocytes in the heart[15]. However, if the goal is to obtain and quantify all cardiomyocytes from the heart, it is important to incubate the hearts for extended periods of time in the collagenase solution (e.g., 3–7 days), where the collagenase solution should be replenished once a day. This will minimize inconsistencies in isolation efficiency by eliminating the impact of the degree of trituration on cardiomyocyte yield.

The use of DNA content to measure ploidy is not new, and has been used in flow cytometry for decades. Recently, it was shown that microscopy can similarly be used to estimate DNA content per nucleus[16]. Here, we implemented this strategy to measure ploidy of cardiomyocyte nuclei, as a surrogate for newly formed cardiomyocytes. The dogma in the field of cardiac regeneration is that only mononucleated, diploid cardiomyocytes can undergo cytokinesis and give rise to new cardiomyocytes. Since it is very challenging to measure new cardiomyocyte formation in vivo, isolating cardiomyocytes that have been chased after administration of a DNA nucleotide analog and determining the level of mononucleated, diploid cardiomyocytes has been used as an approximation of the ability of the heart to generate new cardiomyocytes[17]. Here, we provide a macro for ImageJ that allows easy quantification of cardiomyocyte ploidy. At the very minimum, 500 nuclei must be measured to attain an accurate estimate of the location of the G1 peak. If care is taken to ensure that staining and imaging conditions are consistent across every well of the imaged plate, only 500 nuclei across the entire sample need to be imaged, otherwise, there need to be 500 nuclei per image group[18,19]. Limitations of imaging-based measurement of nucleation and ploidy include difficulty to distinguish nuclei from adherent cells from actual cardiomyocyte nuclei, when using two-dimensional images. Such adherent cells might result in overestimation of the quantity of multinucleated cells and decrease the accuracy of measurements of the tetraploid cardiomyocyte nucleus population. One possible strategy to solve this problem would be to use the cardiomyocyte nuclear marker PCM1[6,20]. However, we have had difficulties to obtain reliable PCM1 staining on properly fixed cells or tissues.

Another potential limitation is that some nuclear stain images might have significant background cytoplasmic staining, preventing proper thresholding using Fiji's built in methods without extensive preprocessing. In addition, the irregular contribution of this background fluorescence into ploidy estimates reduces their accuracy. Moreover, if the cells are not left in DNA-staining solution for sufficient time, the fluorescent dye will not bind to saturation within the nuclei and

the assumption of a linear relationship between nuclear integrated intensity and DNA content will no longer be accurate.

It should be noted that the software cannot segment cardiomyocyte clusters and instead removes them from analysis. Therefore, it is critically important to seed cardiomyocytes at a relatively low density (e.g., 1000 cells/cm^2). Further, the software cannot distinguish between two cardiomyocytes lined up end-to-end and long, singular cardiomyocytes. These sorts of clusters might erroneously inflate multinucleation estimates.

Although the described method does not allow for obtaining viable cardiomyocytes and thus cannot be used to measure dynamic cellular processes, if the goal is to perform immunostaining, we believe that the described method is superior to existing protocols with higher yields of cardiomyocytes and better quality in terms of morphology and protein localization. Finally, the described method could be used to isolate cardiomyocytes from clinical samples[14,21]. We believe the described methodology can help different researchers to obtain high-quality cardiomyocytes and measure nucleation and ploidy as surrogates for new cardiomyocyte formation.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

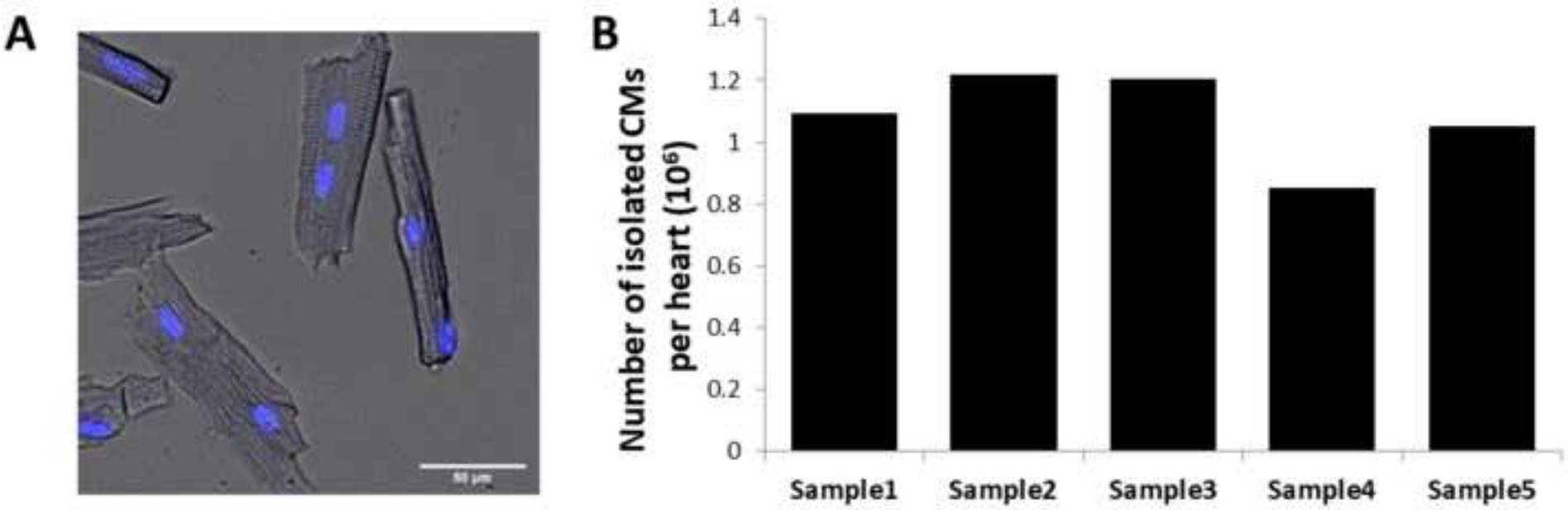

Figure 2

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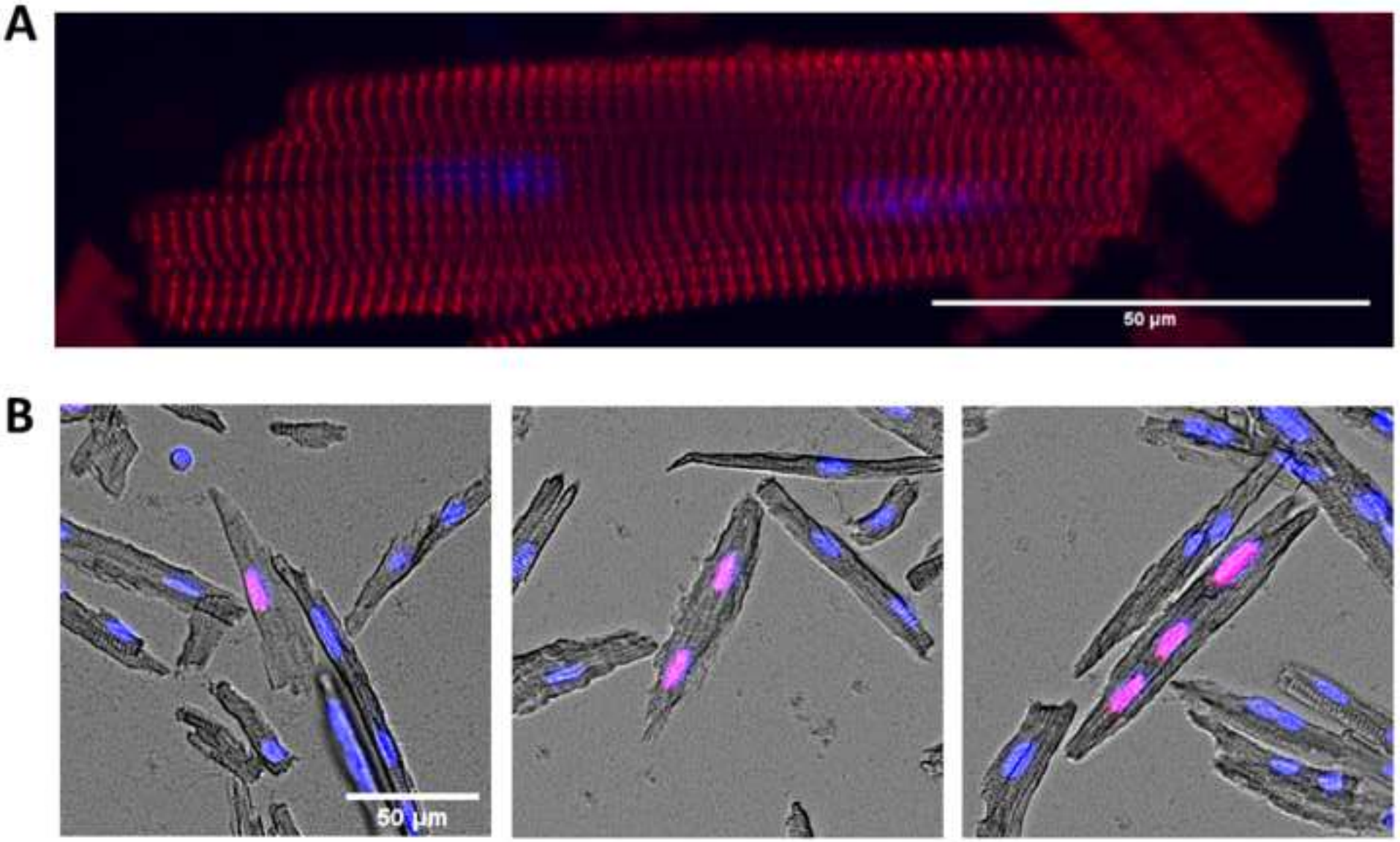

Figure 3

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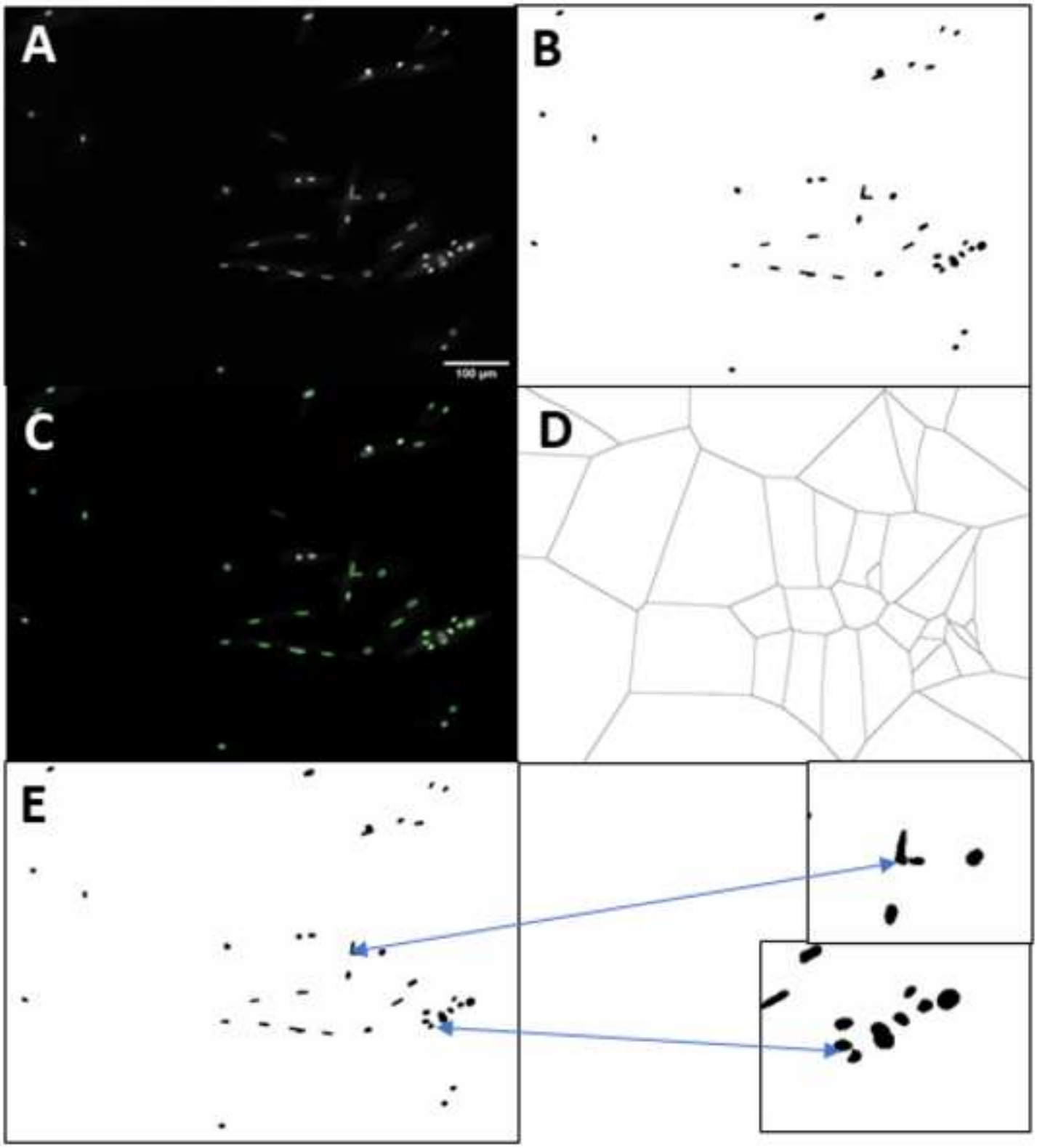

Figure 4

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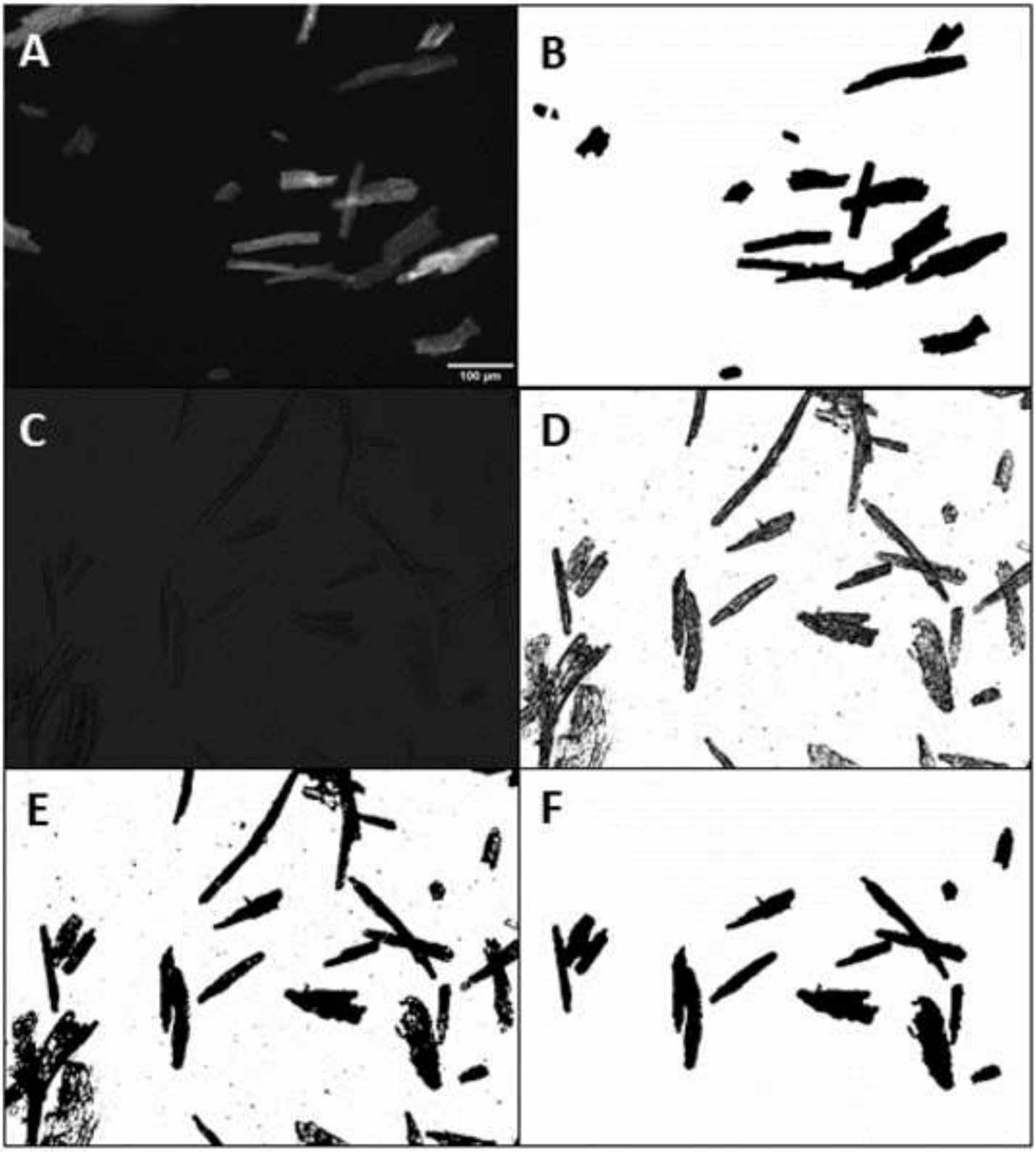


Figure 5

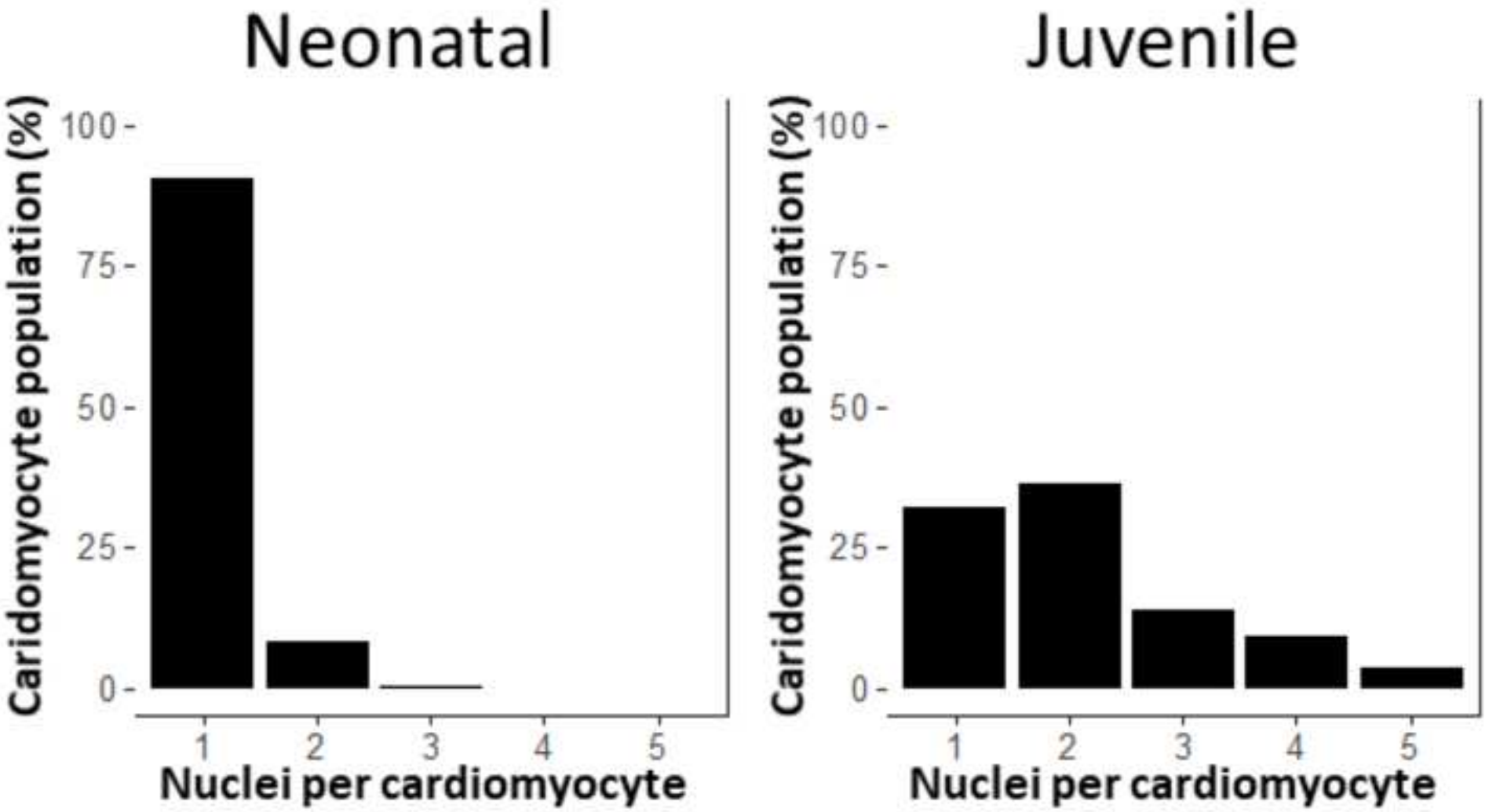
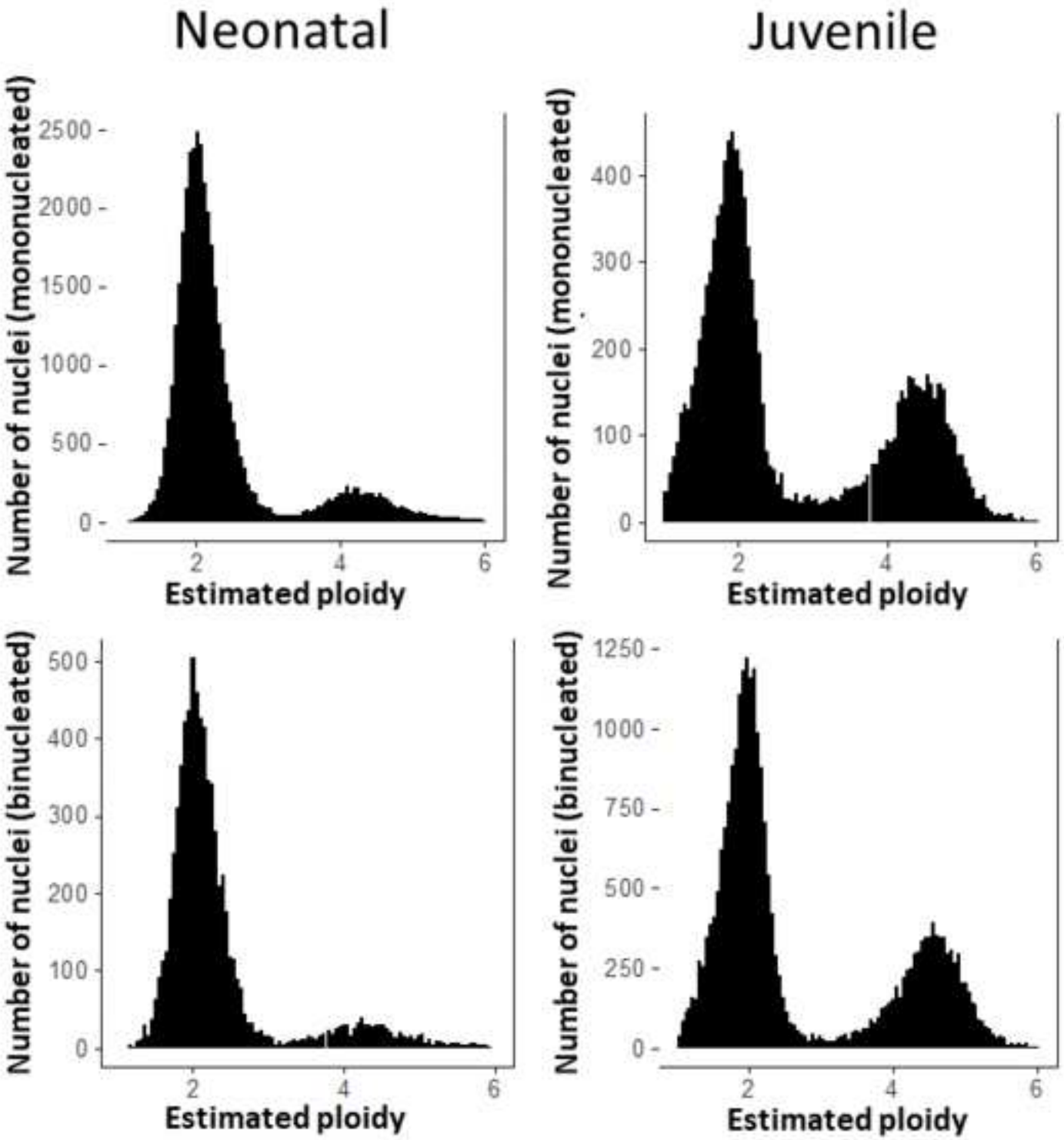


Figure 6



Name of Material/Equipment	Company	Catalog Number
96 wells plate for imaging	Corning	3340
Alpha actinin	Novus Biologicals	NBP1-32462
Blunt scissors	Fine Scissor Tools	14072-10
C57BL/6J	The Jackson Laboratory	664
CD-1 mice	Charles river	22
Collagenase 2	Worthington	LS004177
Copper (II) sulfate pentahydrate	Sigma-Aldrich	203165-10G
Cy5 Picolyl Azide	Click Chemistry Tools	1177-25
Cytation3	BioTek	-
DAPI	Life Technologies	D3571
donkey anti-mouse IgG-Alexa568	Life Technologies	A10037
Forceps	ROBOZ	RS-5137
Hydrochloric acid	Fisher Scientific	A144212
ImageJ	imagej.net/Fiji/Downloads	-
L-ascorbic acid	Sigma-Aldrich	255564-100G
Needle for infusion	TERUMO	SV*23BLK
Nikon A1R HD25	Nikon	-
Nylon mesh 200 micron	Elko filtering	03-200/54
Nylon mesh 400 micron	Elko filtering	06-400/38
Phosphate Buffered Saline (1X)	Corning	21-040-CV
Potassium chloride, Granular	Mallinckrodt	6858
R	r-project.org	-
Tris Base	Fisher Scientific	BP152-5

Comments/Description

We use these plates as they are suitable for imaging, although glass bottom plates would be better for confocal imaging
This antibody is used as a marker of cardiomyocyte sarcomeres
We prefer blunt scissors as the possibility of tearing heart tissue is lower when exposing the heart
Used for imaging, assessing ploidy and nucleation in cardiomyocyte population
Used for imaging, assessing ploidy and nucleation in cardiomyocyte population
For the purpose of this protocol, the batch to batch differences are minimal and don't affect overall yield and quality of the isolation

For edu staining
Azide used for edu staining
Used for automated imaging for DNA analysis
DAPI used for DNA staining. Stocks were dissolved in distilled water.

Secondary antibody used to detect alpha actinin staining within cardiomyocytes
We use these curved, blunt forceps, although straight forceps could also be used
To set pH of Tris-HCl buffer to pH 8.5

Used for analyzing images
For edu staining
We use winged infusion sets throughout the protocol as it is easy to manipulate the position of the needle with these sets during injection
Used to take confocal images of alpha actinin staining
Mesh used for filtering regular cardiomyocytes (not hypertrophied)
Mesh used for filtering hypertrophied adult cardiomyocytes
This can also be prepared in the lab. Although sterility is important in this experiment, we think it is sufficient to prepare PBS and filtering it
Granular potassium chloride was preffered by us as it forms less aggregates when stored in room temperature
Used for data analysis of the measurements obtained from images
Used to buffer EdU staining reaction

We would like to thank the reviewers for critically reading our manuscript and providing their constructive criticisms. We have carefully gone through all comments and addressed them in this revised manuscript. Below is a point-by-point response to the individual remarks.

Reviewer #1:

Manuscript Summary:
This manuscript describes the isolation of individual adult cardiomyocytes by collagenase digestion of perfusion-fixed mouse hearts. This is an extremely interesting approach that may also be useful to isolate cardiomyocytes from fixed human heart samples. The yield is excellent and the structure of the cells, as shown by alpha-actinin staining, is maintained. In addition to this method the authors describe a pipeline for ploidy analysis of isolated cardiomyocytes, which is also a useful technique, since ploidy is known to be affected in cardiomyopathy and is currently understudied due to technical difficulties. The experimental techniques are very well described and should be easy to follow also by less experienced researchers. I just have a few technical comments that should be addressed.

We thank the reviewer for the positive feedback. We have revised the manuscript according to the suggestions of the reviewer as detailed below.

Major Concerns:
None

Minor Concerns:

Line 119: "If the animal is younger than 7 days use decapitation". There are country specific rules on this! In some countries, decapitation is NOT allowed for young animals unless a specific license is obtained. Therefore avoid specifics on this and refer to "local rules".

The reviewer is entirely correct that we specified a method that is approved for our IACUC protocol. We have amended the manuscript and made this description more generic.

Line 128: Mention that a butterfly needle is used and also specify needle size (23 G) here.

The reviewer is correct to point out that we used a butterfly infusion needle to allow for better manipulation of the needle and attached infusion line during infusion. We have revised the manuscript accordingly.

Line 165: I would be a bit worried that a 200 um nylon mesh would trap hypertrophied cardiomyocytes and would therefore go for a larger mesh size?

The reviewer is correct that 200µm mesh is the low end of the spectrum that should be used to remove chunks of myocardium. We have changed the protocol to specify 200 or 400 µm mesh.

List of Materials: also list source for the cell strainers

The source of the mesh is now included.

Reviewer #2:

The JoVE manuscript by Yucel et al describes an improved protocol to isolate adult rodent cardiomyocytes for use in various downstream applications that do not require living cells, such as immunofluorescence and ploidy analysis.

The protocol is clearly written and easy to understand. Several steps in the protocol, and the annotation of the provided scripts could be improved. In addition, the authors could provide sample images with written-out R command line strings to allow readers to easily understand the working principle of their automated image quantification and data analysis.

We thank the reviewer for the positive criticism. We agree with the reviewer that is will be easier to perform the protocol after we implement the changes suggested by the reviewer. We thank the reviewer for the provided improvements of our protocol.

Specific comments (Minor concerns)

- A heparin step in the beginning of the isolation procedure to prevent clotting of blood in the animal may improve yield of cells in the protocol.

The reviewer is absolutely right that this could be important, especially if the time between euthanasia and perfusing the heart is long enough for clotting to initiate. We have included heparin injection prior to euthanasia as an option in our protocol.

- Step 1.2: did the authors mean to prepare 3 liters of KCl-PBS solution per animal?
With a flow rate of 3ml/min as described in step 2.6, it would take more than 16 hours to go through that much KCl solution.

Thanks for pointing out this oversight. We forgot the milli indicator in front of the unit, and have corrected this omission.

- Although the authors make efforts to annotate commands within the R script and the Fiji/ImageJ script, the level/amount of annotations could be improved. This would help to allow users to troubleshoot and customize the scripts more easily.

The reviewer is absolutely right that implementing adaptations and troubleshooting of the R-script is not easily done based on the information that is provided in our protocol. The main issue is that quite a bit of knowledge about R is required to make adjustments to the R script. One of our goals here was to write a script that would not require coding in R, such that it could be easily and broadly implemented. Regardless, to aid in implementing the R script, we now

provide screen shots to show exactly where and how to perform the individual steps in the protocol.

- It would be helpful to include sample images, and exact commands (written-out R command strings) that utilize these specific sample images to help the reader understand the working principle of the automated image processing and data analysis.

We have now included as supplementary materials screen shots of the exact commands and sample images, as requested. We agree that this will aid in understanding the principle of the automated image processing and data analysis.

- Figure 5 and 'Representative Results' text: the authors seem to use 'adult' and 'juvenile' interchangeably. Please correct.

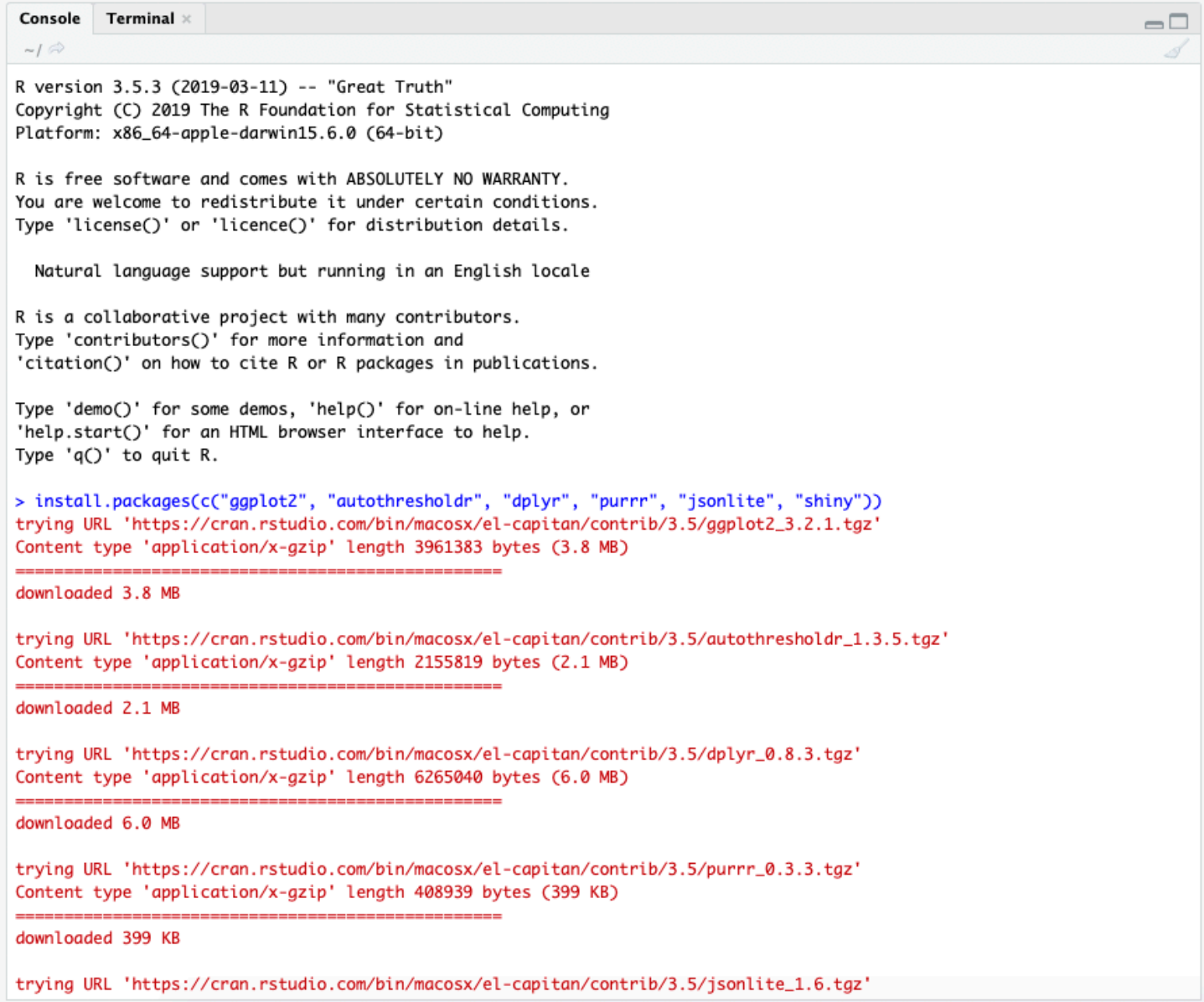
We apologize for the confusion. The reviewer is correct that we use both adult and juvenile mice, as well as neonatal mice. Our goal was to show that this is a broadly applicable protocol that can be used for all ages. However, we failed to define what we called juvenile mice. We have now specified that the juvenile mice are 2 weeks of age at the time of isolation.

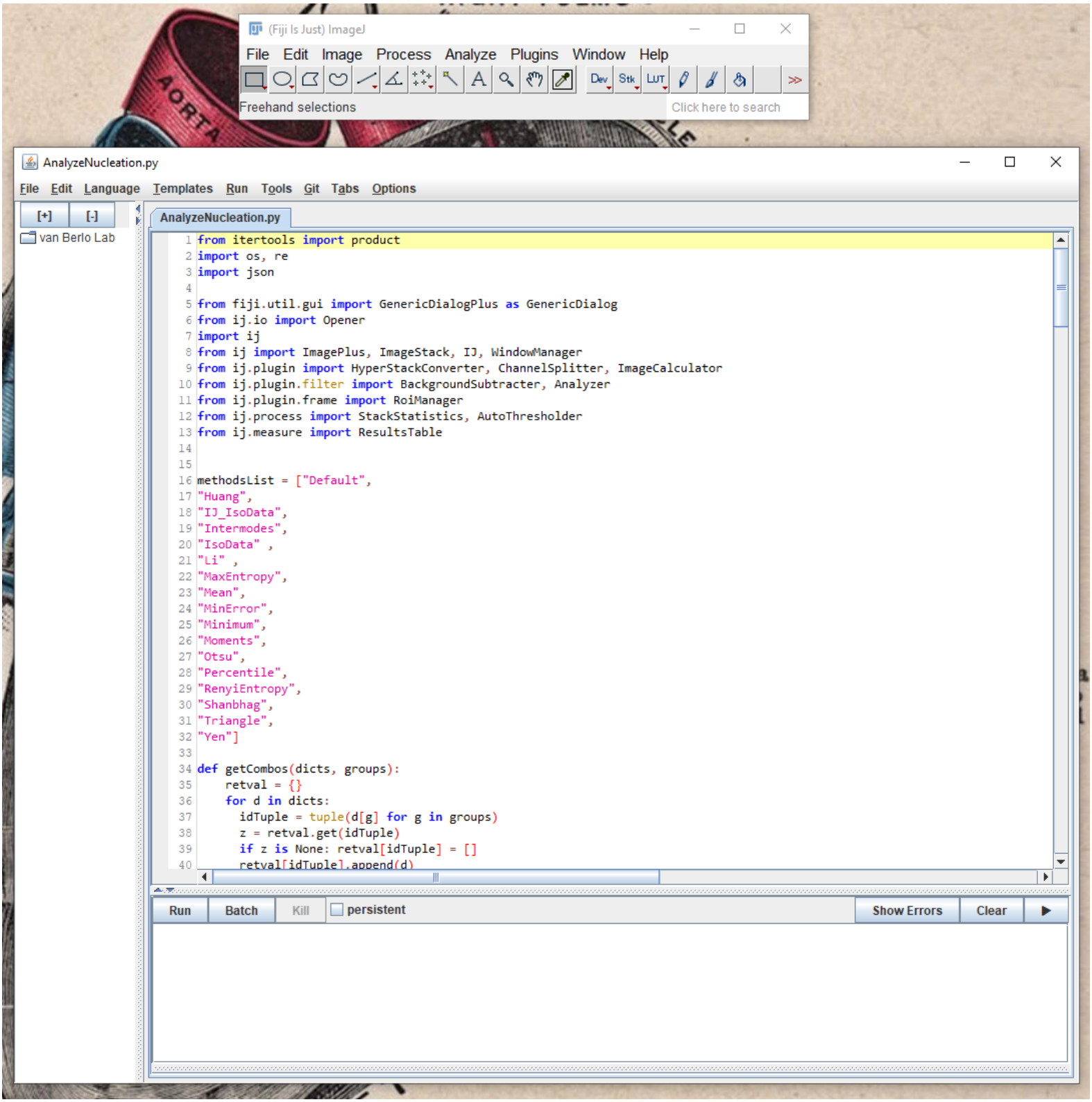
- Discussion: The authors note to 'seed cardiomyocytes at a relatively low density'. Please provide numbers, e.g. cells/cm2.

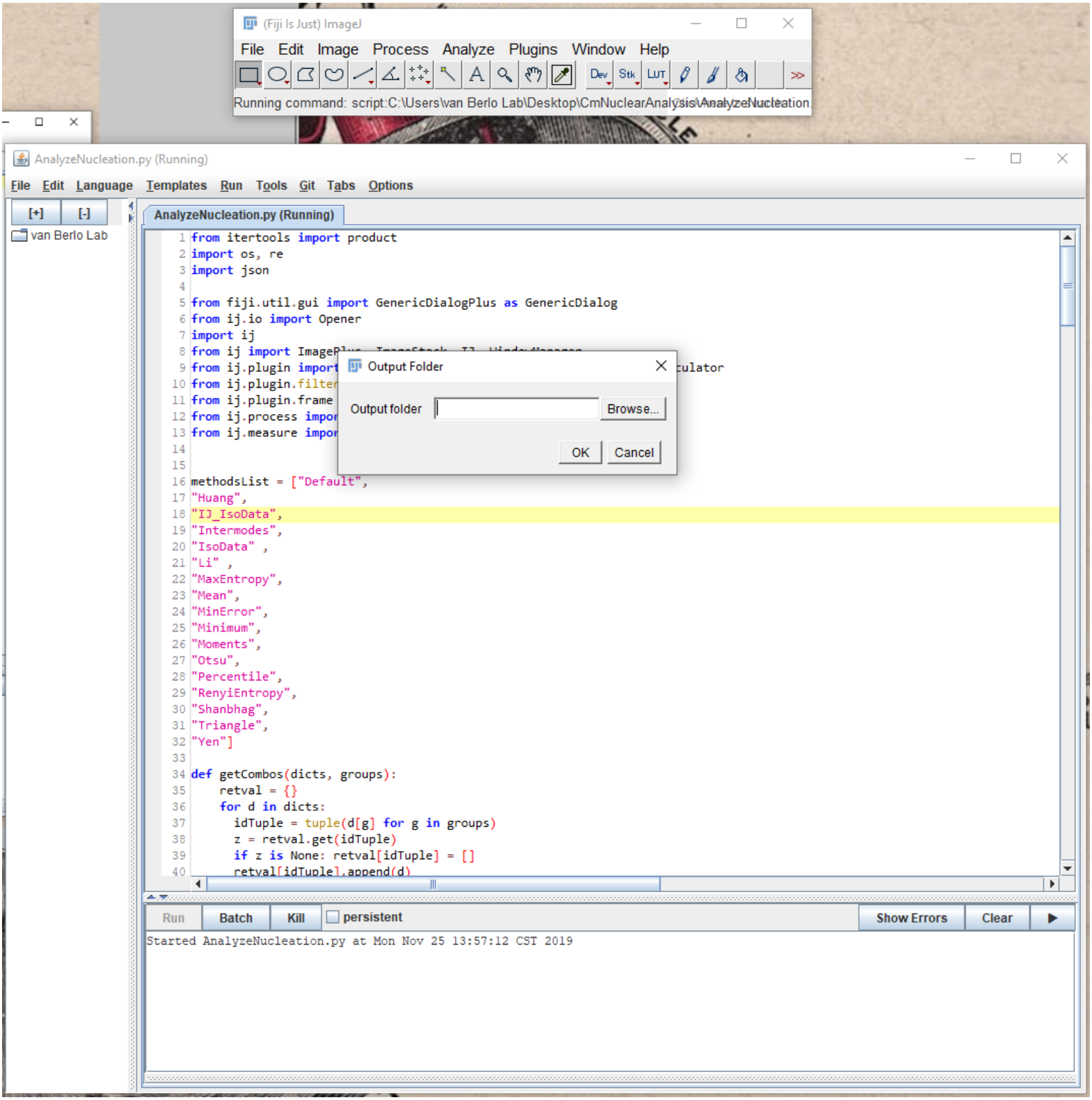
We thank the reviewer for pointing out this problem. We have now specified a recommended seeding density of 1000 cells/cm^2, which in our hands provides optimal separation between cardiomyocytes, while not being too sparse.

Supplemental File 1 (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SoftwareScreenshots final.pdf




(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
Freehand selections
Click here to search
AnalyzeNucleation.py
File Edit Language Templates Run Tools Git Tabs Options
[+] [-]
van Berlo Lab
AnalyzeNucleation.py
1 from itertools import product
2 import os, re
3 import json
4
5 from fiji.util.gui import GenericDialogPlus as GenericDialog
6 from ij.io import Opener
7 import ij
8 from ij import ImagePlus, ImageStack, IJ, WindowManager
9 from ij.plugin import HyperStackConverter, ChannelSplitter, ImageCalculator
10 from ij.plugin.filter import BackgroundSubtracter, Analyzer
11 from ij.plugin.frame import RoiManager
12 from ij.process import StackStatistics, AutoThresholder
13 from ij.measure import ResultsTable
14
15
16 methodsList = ["Default",
17 "Huang",
18 "IJ_IsoData",
19 "Intermodes",
20 "IsoData" ,
21 "Li" ,
22 "MaxEntropy",
23 "Mean",
24 "MinError",
25 "Minimum",
26 "Moments",
27 "Otsu",
28 "Percentile",
29 "RenyiEntropy",
30 "Shanbhag",
31 "Triangle",
32 "Yen"]
33
34 def getCombos(dicts, groups):
35 retval = {}
36 for d in dicts:
37 idTuple = tuple(d[g] for g in groups)
38 z = retval.get(idTuple)
39 if z is None: retval[idTuple] = []
40 retval[idTuple].append(d)
Run Batch Kill persistent Show Errors Clear


(Fiji Is Just) ImageJ
File Edit Image Process Analyze Plugins Window Help
Running command: script:C:\Users\van Berlo Lab\Desktop\CmNuclearAnalysis\AnalyzeNucleation.
AnalyzeNucleation.py (Running)
File Edit Language Templates Run Tools Git Tabs Options
[+] [-]
van Berlo Lab
AnalyzeNucleation.py (Running)
from itertools import product
import os, re
import json
from fiji.util.gui import GenericDialogPlus as GenericDialog
from ij.io import Opener
import ij
Output Folder
Output folder
Browse...
OK
Cancel
methodsList = ["Default",
"Huang",
"IJ_IsoData",
"Intermodes",
"IsoData" ,
"Li" ,
"MaxEntropy",
"Mean",
"MinError",
"Minimum",
"Moments",
"Otsu",
"Percentile",
"RenyiEntropy",
"Shanbhag",
"Triangle",
"Yen"]
def getCombos(dicts, groups):
retval = {}
for d in dicts:
idTuple = tuple(d[g] for g in groups)
z = retval.get(idTuple)
if z is None: retval[idTuple] = []
Run
Batch
Kill
persistent
Show Errors
Clear
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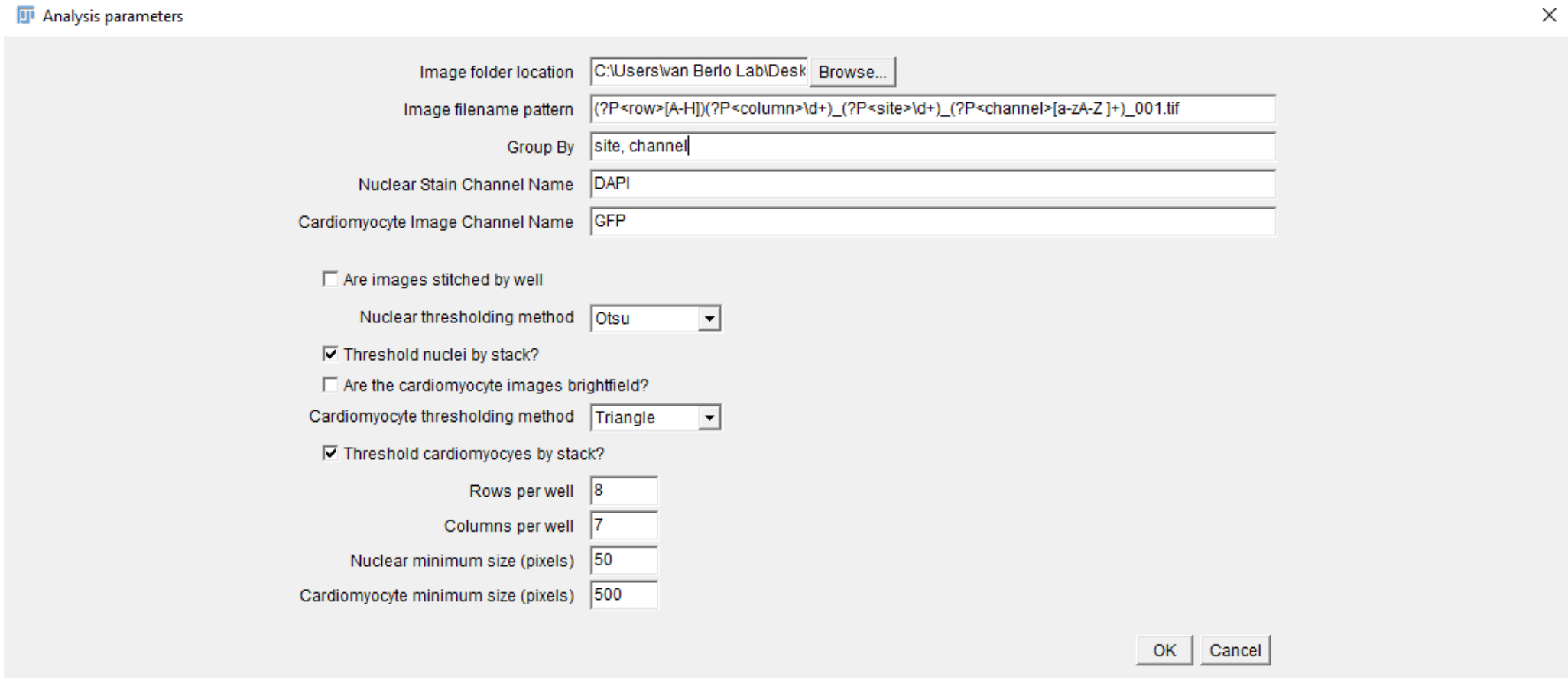
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Group By
site, channel
Nuclear Stain Channel Name
DAPI
Cardiomyocyte Image Channel Name
GFP
Are images stitched by well
Nuclear thresholding method
Otsu
Threshold nuclei by stack?
Are the cardiomyocyte images brightfield?
Cardiomyocyte thresholding method
Triangle
Threshold cardiomyocyes by stack?
Rows per well
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Columns per well
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Nuclear minimum size (pixels)
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Cardiomyocyte minimum size (pixels)
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OK
Cancel

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RStudio

File Edit Code View Plots Session Build Debug Profile Tools Help

Go to file/function | Addins

AnalyzeMultinucleatedServer.R

Run App

```
folderName = "C:/Users/van Berlo Lab/Desktop/Results/BL6 20d 1"
library(shiny)
knitr::opts_chunk$set(echo=FALSE,results='hide',fig.keep='all')
library(jsonlite)
savedParams=F
setwd(folderName)
if (savedParams){
  paramlist = as.list(unlist(read_json("params.json")))
  #This automatically assigns the read analysis parameters to R's global environment.
  list2env(paramlist, environment())
}
library(ggplot2)
th = theme_set(theme_bw() + theme(panel.grid.major = element_blank(),
                                  panel.grid.minor = element_blank(),
                                  text = element_text(size=12),
                                  axis.title = element_text(face="bold")))
library(dplyr)
library(purrr)
library(autothresholdr)
library(rlang)
groupSet = character(0)
find_metadata = function(filename){
  strings = unlist(map(unlist(
    regmatches(filename, gregexpr("_[a-zA-Z1-9]+=[a-zA-Z0-9]+", filename))),
    ~substring(., 2)))
  keys = unlist(map(unlist(
    regmatches(strings, gregexpr("^\\w+=", strings))),
    ~substring(., 1, nchar(.)-1)))
  values = unlist(map(unlist(
    regmatches(strings, gregexpr("=\\w+$", strings))),
    ~substring(., 2)))
  setNames(values, keys)
}

```

19:24 (Top Level) R Script

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Minimum Nuclear Mean Intensity Threshold

10,000 — 63,674.24

3,504 10,004 16,504 29,504 42,504 55,504 63,674.24

Upper X limit of Graph

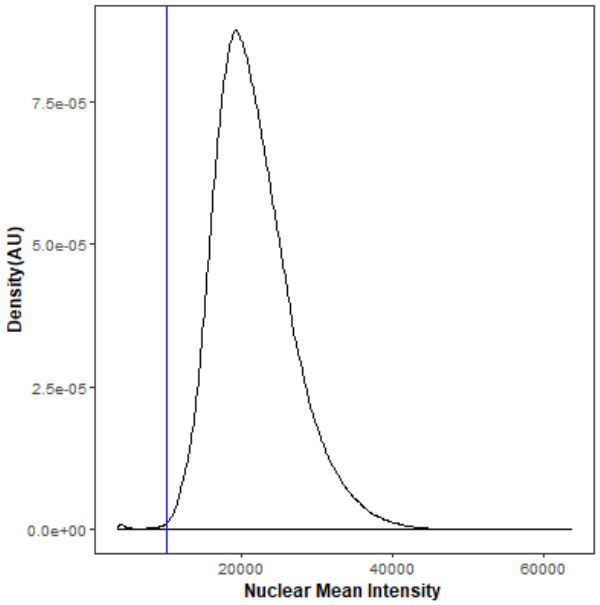
3,504 — 63,674.24

3,504 10,004 16,504 29,504 42,504 55,504 63,674.24

Lower X limit of Graph

3,504 — 63,674.24

3,504 10,004 16,504 29,504 42,504 55,504 63,674.24



Minimum Nuclear Area Threshold

1 — 40 — 768

1 78 155 232 309 385 462 539 616 693 768

Upper X limit of Graph

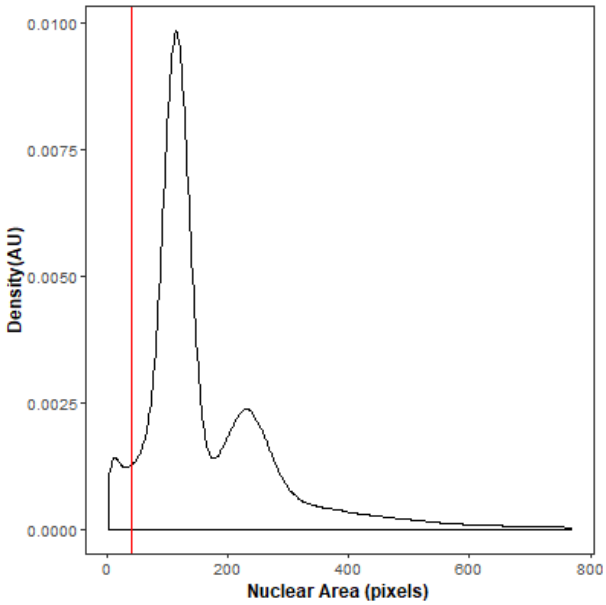
0 — 768

0 77 154 231 308 385 462 539 616 693 768

Lower X limit of Graph

1 — 768

0 77 154 231 308 385 462 539 616 693 768



Maximum Cardiomyocyte Minimum Feret's Diameter Threshold

0 — 100 — 292.197

0 30 60 90 120 150 180 210 240 270 292.197

Upper X limit of Graph

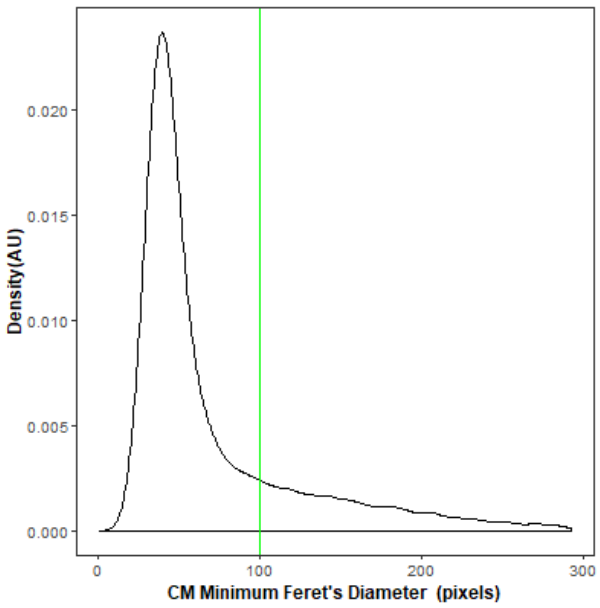
0 — 292.197

0 30 60 90 120 150 180 210 240 270 292.197

Lower X limit of Graph

1 — 292.197

0 30 60 90 120 150 180 210 240 270 292.197



Apply Selected Thresholds?

Plot Intensity Distribution

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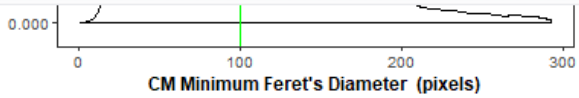
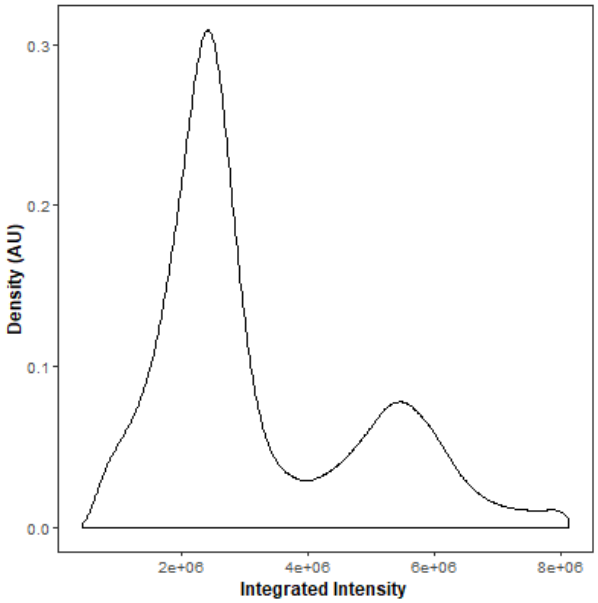
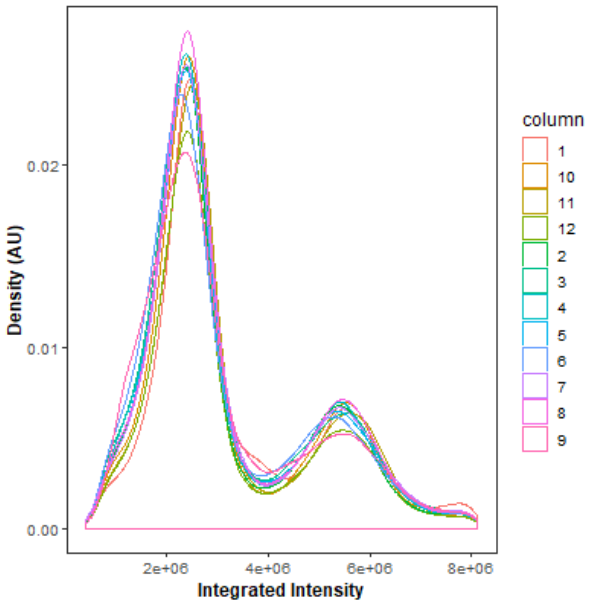
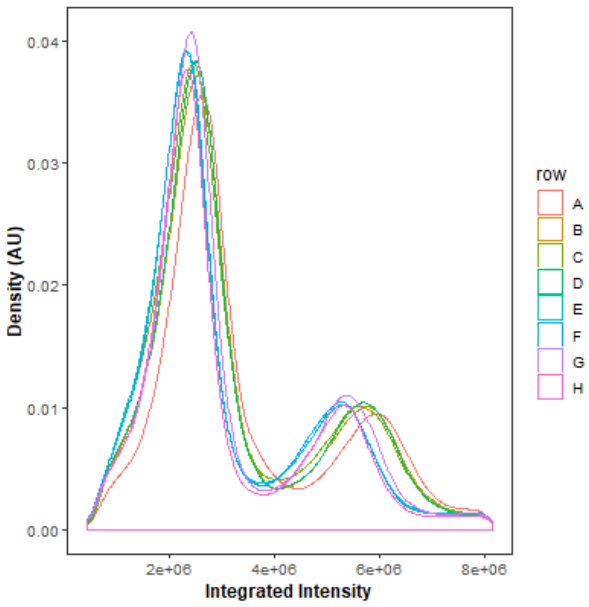


Apply Selected Thresholds?

Plot Intensity Distribution







Normalize Separately by group?

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Normalize Separately by group?

Calculate Ploidy

Plot Estimated Ploidy Distribution

Minimum diploid threshold

0 1 7

0 0.7 1.4 2.1 2.8 3.5 4.2 4.9 5.6 6.3 7

Threshold between diploid and tetraploid

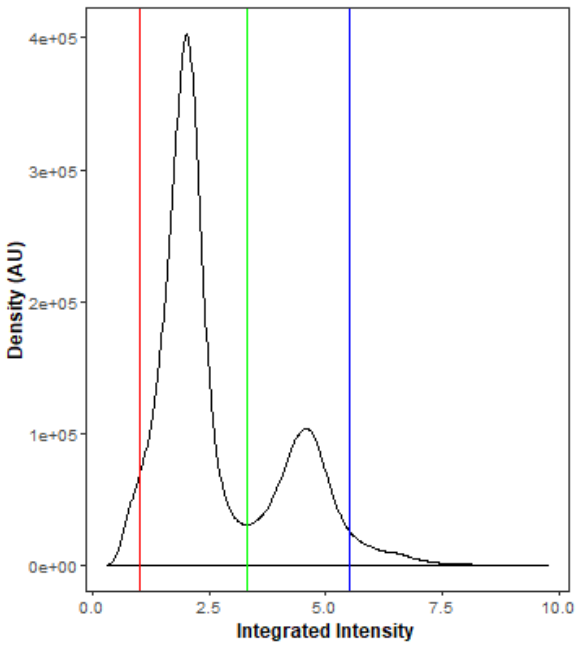
0 3.3 7

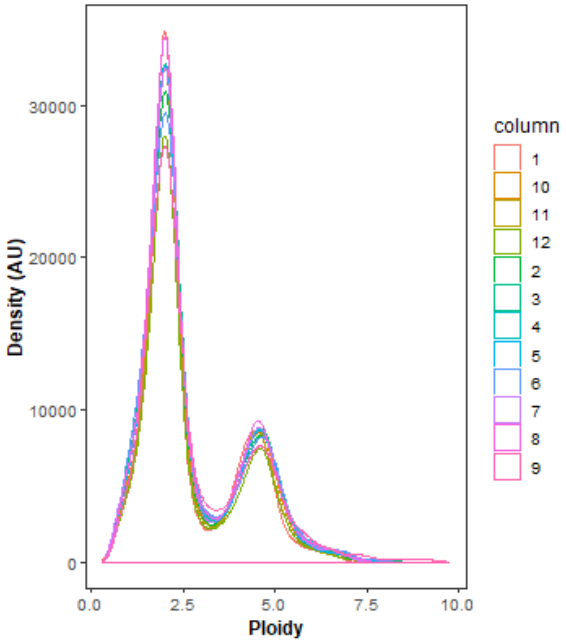
0 0.7 1.4 2.1 2.8 3.5 4.2 4.9 5.6 6.3 7

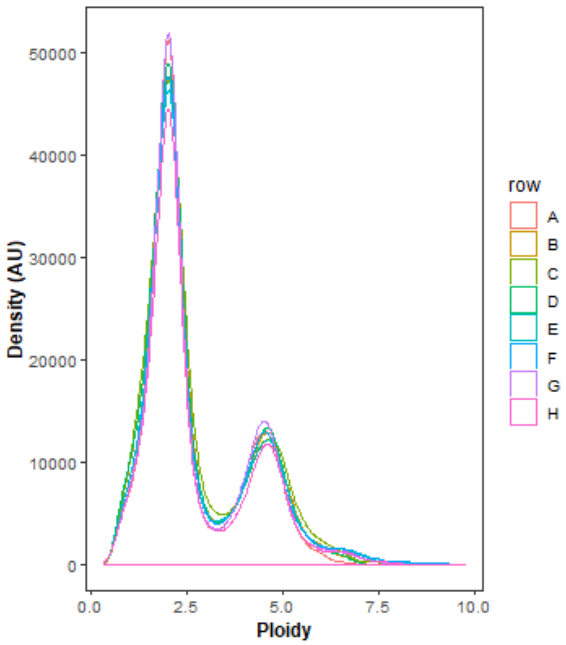
Maximum tetraploid threshold

0 5.5 7

0 0.7 1.4 2.1 2.8 3.5 4.2 4.9 5.6 6.3 7







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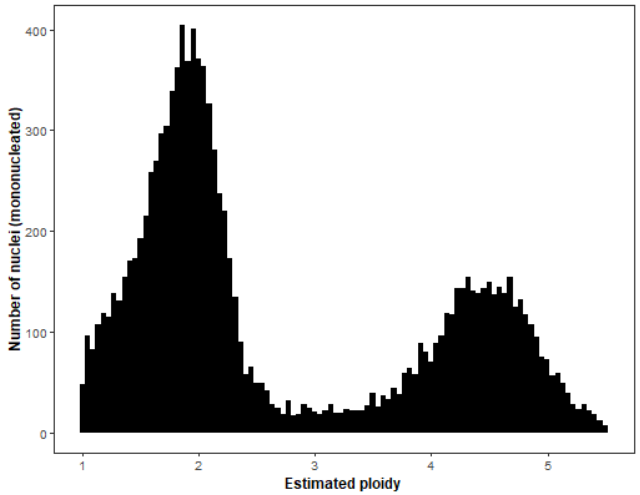
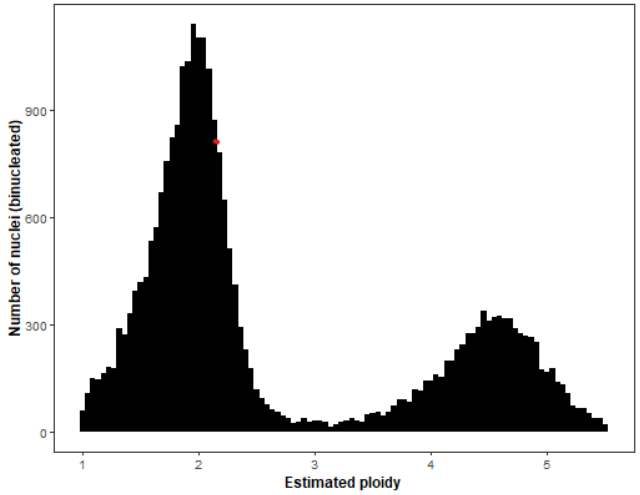
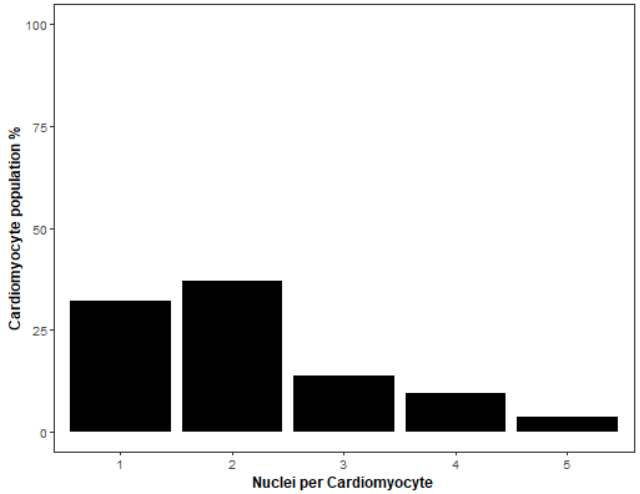
Calculate Cardiomyocyte Ploidy and Nucleation Distributions

Plot and Save Into Output Folder







R script analyze nucleation

Python script analyze nucleation