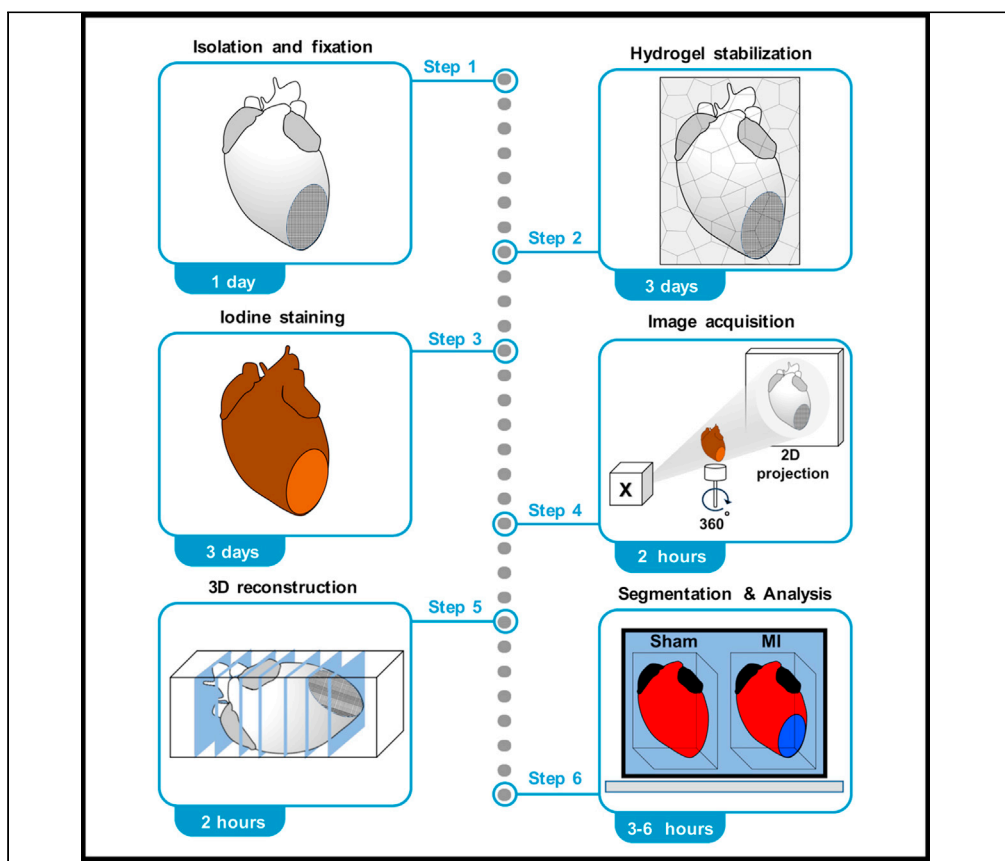**Protocol**

Quantitative 3D analysis and visualization of cardiac fibrosis by microcomputed tomography

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Highlights

A streamlined pipeline for 3D volumetric quantification of scar and myocardium

Focus on sample processing and image acquisition parameters

Description of 3D reconstruction of X-ray microcomputed tomography dataset

Scalable to other cardiac volumetric measurements or other organs

Following myocardial infarction, damaged myocardium is replaced with a fibrotic scar that preserves cardiac structural integrity. Scar area measured from sample 2D images of serial heart sections does not faithfully measure the extent of fibrosis due to structural heterogeneity caused by tissue dynamics. Here, we present an X-ray microcomputed tomography (micro-CT) workflow that generates accurate volumetric quantification of scar and surviving myocardium in infarcted mouse hearts. This workflow could be applied to other fibrotic organs or hearts from other species.

STAR Protocols

Protocol

Quantitative 3D analysis and visualization of cardiac fibrosis by microcomputed tomography

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SUMMARY

Following myocardial infarction, damaged myocardium is replaced with a fibrotic scar that preserves cardiac structural integrity. Scar area measured from sample 2D images of serial heart sections does not faithfully measure the extent of fibrosis due to structural heterogeneity caused by tissue dynamics. Here, we present an X-ray microcomputed tomography (micro-CT) workflow that generates accurate volumetric quantification of scar and surviving myocardium in infarcted mouse hearts. This workflow could be applied to other fibrotic organs or hearts from other species. For complete details on the use and execution of this protocol, please refer to Janbandhu et al. (2021).

BEFORE YOU BEGIN

Solutions are prepared following the recipes in the materials and equipment section. Solutions are prepared in advance and can be stored as indicated. A complete list of materials and equipment required is given in the key resources table.

Note: We have provided a step-by-step manual protocol for assessment of scar volume in infarcted mouse hearts. The availability of mice that have undergone myocardial infarction (MI) surgery is assumed. The presented steps were performed on wild type adult mouse hearts at day-28 post-MI. Details of surgery and isolation of hearts arrested at diastole are described in Janbandhu et al. (2021). Here we provide details on the use of the SkyScan 1272 micro-CT scanner and associated NRecon software (Bruker Corporation); however, micro-CT imaging of isolated hearts could be carried out on other micro-CT systems. Likewise, we describe hydrogel embedding using the X-Clarity Polymerization System; but there are alternatives that do not require this equipment (see Note in "hydrogel stabilization of hearts" section). The image analysis protocol relies on the Amira software package (Thermo Fisher Scientific).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat# 14190250
Lugol's solution	Sigma-Aldrich	Cat# L6146

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
40% Acrylamide	Bio-Rad Laboratories	Cat# 1610140
2% Bis-acrylamide	Bio-Rad Laboratories	Cat# 1610142
VA044	NOVA Chemicals	Cat# 017-19362
Saponin	Sigma-Aldrich	Cat# S4521
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat# P6148
25% Glutaraldehyde	Sigma-Aldrich	Cat# G6257
Experimental models: Organisms/strains		
Mouse: Wild type [C57BL/6J]	The Jackson Laboratory	Stock No: 000664
Software and algorithms		
Skyscan1272 (v 1.1.19)	Bruker Corporation	https://www.bruker.com/products/microtomography/micro-ct-software/3dsuite.html
NRecon (v 1.7.1.0)	Bruker Corporation	
Amira Software (v 2020.2)	Thermo Fisher Scientific	http://www.fei.com/software/amira-3d-for-life-sciences/ RRID:SCR_007353
Other		
X-CLARITY Polymerization System	Logos Biosystems	Cat# C20001
SkyScan 1272	Bruker Corporation	Cat# SkyScan1272
Orthodontic tray wax	Kerr-Dental	Cat# 09246
500 mL Bottle Top Vacuum Filter	Corning	Cat# CLS430513
Kimwipes (lint-free tissue paper)	KIMTECH SCIENCE	Cat# 34120
15 mL Falcon	Corning	Cat# 430791

MATERIALS AND EQUIPMENT

Preparation of 16% paraformaldehyde (PFA) solution		
Reagent	Final concentration	Volume/Mass
PFA	16%	64 g
10× PBS	1×	40 mL
Milli-Q water	n/a	Add to 400 mL
Total	n/a	400 mL

Heat 200 mL of milli-Q water to 60°C–65°C and add 64 g of PFA while stirring. Adjust pH to 7.4 by slowly adding a few drops of 1N NaOH until PFA is well dissolved (clear solution). Add 40 mL of 10× PBS stock solution. Cool the solution to 25°C–30°C and filter using a Corning 500 mL bottle top vacuum filter (0.22 μm). Add milli-Q water to achieve a final volume of 400 mL. Check the pH and if necessary, adjust to 7.4 with 1N NaOH as described above. Aliquot and store at −20°C for 6 months.

Preparation of hydrogel			
Sr no.	Reagent	Final concentration	Volume/Mass
1	16% PFA	4%	125 mL
2	2% Bis-acrylamide	0.05%	12.5 mL
3	40% Acrylamide	4%	50 mL
4	Saponin	0.05%	1.25 g
5	VA044	0.25%	0.25 g
6	Milli-Q water	n/a	Add to 500 mL
	Total	n/a	500 mL

In a 500 mL Schott bottle (on ice), combine reagents 1, 2 and 3. Add Saponin (4) and mix until dissolution. Finally, add the initiator VAO44 (5) and make up to 500 mL with Milli-Q water.

Note: Always use freshly thawed PFA and do not refreeze. Store hydrogel at 4°C for up to 2 weeks.

⚠ CRITICAL: (1) Keep all components on ice at all times. Hydrogel must be prepared on ice and kept cold after adding the initiator (VA044) to prevent acrylamide polymerization. (2) PFA, glutaraldehyde, acrylamide and saponin are toxic and must be handled while wearing appropriate PPE (gloves, goggles, lab coats, etc.) and under a fume hood. Follow the safety data sheet when handling these reagents. Waste must be collected for specific elimination.

STEP-BY-STEP METHOD DETAILS

This micro-CT procedure includes the following steps: sample preparation and mounting, scanner setup and parameter selection, scanning procedure, 3D image reconstruction, and 3D image analysis and visualization.

Isolation and fixation of infarcted mouse hearts

⏱ Timing: 1 day

This section describes isolation and fixation of hearts from mice at 28 days post-MI.

1. Harvest hearts at 28 days post-MI as described in Janbandhu et al. (2021).
2. Fixation: Transfer each heart to a 15 mL falcon tube filled with fixative solution (4% PFA+1% glutaraldehyde in 1× PBS pH 7.4) and place tube on a rocker at 4°C for 1 day.

⚠ CRITICAL: (1) Ensure the heart is immersed in the fixative solution while on the rocker. (2) Use at least 10–15 tissue volumes of fixative solution to achieve adequate fixation.

Hydrogel stabilization of hearts

⏱ Timing: 3 days

Cross-linking of tissue is achieved by PFA fixation followed by binding of hydrogel monomers to biomolecules which supports tissue structure and preserves tissue conformation. Hydrogel stabilization also substantially reduces inter-sample anatomical variation (Wong et al., 2013).

3. Remove the fixative and fill the tube with hydrogel solution and continue rocking at 4°C for 3 days.

⚠ CRITICAL: To allow complete tissue penetration of hydrogel, hearts must be incubated in the hydrogel solution for at least 3 days at 4°C.

4. Initiation of acrylamide polymerization:
   a. Immerse the heart in hydrogel in a 12-well tissue culture plate.
   b. Place the plate in the X-Clarity Polymerization System without the lid and initiate polymerization using the following settings: Pressure: −90 kPa; temperature: 37°C; time: 3 h (Figure 1A).
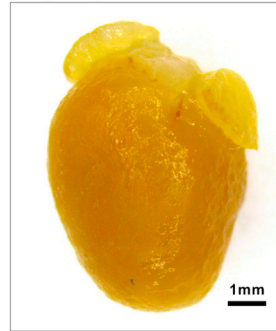
Note: Alternative methods: hydrogel polymerization that does not require the X-Clarity Polymerization system can be found in a handbook published by The University of Newcastle, Australia in association with NSW government: https://openspim.org/documents/3D_Tissue_Clearing_Handbook_3rd_Ed.pdf.

⚠ CRITICAL: Air bubbles can form during hydrogel polymerization under reduced pressure, causing the sample to float and consequently dry out. Allowing the sample to stand under
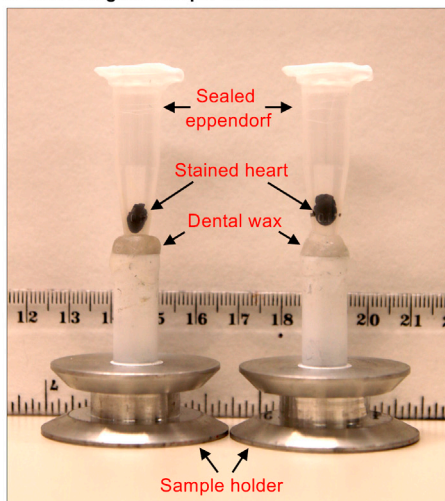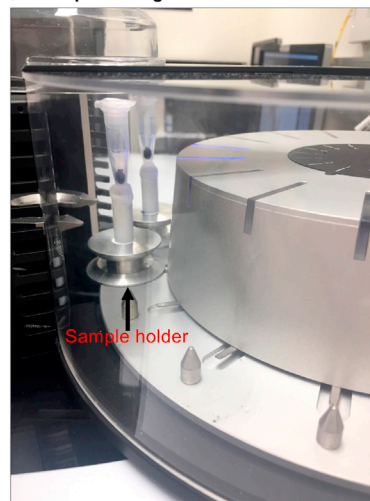
Figure 1. Processing and loading of the sample
(A) X-Clarity Polymerization System used for hydrogel polymerization.
(B) Heart after hydrogel stabilization. Scale bar = 1 mm.
(C) Lugol's-stained hearts mounted on the sample holder.
(D) Sample holders loaded on the sample changer.

> ambient conditions (without rocking) for 1 h prior to polymerization will reduce the likelihood of bubble formation.

5. Separate hearts from the encasing gel and remove any extraneous gel carefully (Figure 1B). Rinse hearts in 1× PBS and keep in PBS for at least 10–15 min to ensure complete removal of hydrogel pieces and PFA traces from the sample (see troubleshooting problem 1).

> ⅠⅠ Pause point: Keep hearts in PBS at 4°C for up to 1 month.

Iodine staining

> ⊙ Timing: 3 days

Myocardium is rich in lipids and glycogen, and scar tissue is mainly composed of collagen-rich ECM. The ability of Lugol's solution (Lugol's iodine) to preferentially bind lipids and glycogen generates an overall staining contrast difference between viable myocardium and scar.

6. Staining of the hearts:
   a. Remove PBS and place hearts in a 15 mL falcon tube containing 15 mL of Lugol's solution
   b. Wrap the tube with aluminum foil to exclude light and place on the rocker at RT for 3 days to ensure penetration of iodine throughout the hydrogel mesh.
   c. Replace Lugol's solution after 2 days (see troubleshooting problem 3).

   △ CRITICAL: (1) Light exposure causes triiodide/iodide (I_3^-/I^-) redox reactions in Lugol's solution which may affect staining quality (Gignac et al., 2016). (2) Incubation times longer than 3 days in Lugol's solution may cause significant tissue shrinkage. Therefore, it is important that the hearts are imaged on the same day when staining is completed.

Micro-CT imaging

⊙ Timing: ~2 h per sample with SkyScan 1272

This section describes the steps for acquiring 3D datasets for each specimen using Bruker/SkyScan 1272 high resolution micro-CT imaging system, fitted with a sample changer capable of holding multiple samples that allows high-throughput batch scanning.

Note: The micro-CT scanners produce large amounts of data; roughly 7–8 gb of data per sample using this protocol. Therefore, sufficient data storage and backup capacity is required.

7. Mounting and loading of the sample:
   a. Remove the heart from the staining solution and place on lint-free tissue paper to remove excess liquid (see troubleshooting problem 2).
   b. Place the heart in 1.5 mL tube in a vertical position.
   c. Gently tap 3–4 times to ensure the heart has settled at the bottom of the tube so that the heart does not move during scanning. We find that the heart is not deformed by the tube wall if mounted in this way. Softer organs or tissues that may be deformed by this mounting technique should be mounted in agarose or with support from plastic gauze.
   d. Seal the tube with parafilm to keep the sample moist during the scanning procedure (see troubleshooting problem 4).
   e. Fix the tube on the sample holder using dental wax (Figure 1C) and load the samples mounted on the specimen holder in the sample changer so that the heart is positioned directly over the axis of rotation, thereby the heart rotates around the central apical-basal axis when the mount is rotated (Figure 1D) (see troubleshooting problem 4).

   Note: (1) Loading of the samples inside the specimen chamber can be controlled by the "Sample changer" control panel of the SkyScan1272 software (Figure 2A-4). (2) Samples should be mounted centrally along the apical-basal axis to avoid orbiting and also to avoid the sample rotating out of the field of view.
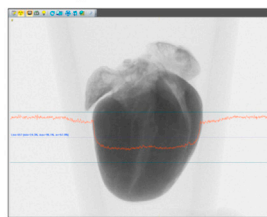
   △ CRITICAL: It is important to load the sample correctly to avoid movement during scanning. If the sample is not properly secured, or if the sample dries out, the resulting movement of the sample will inevitably result in a dataset that cannot be properly reconstructed. Such data will not be suitable for analysis.

8. Image acquisition:
   a. Start SkyScan1272 software. This will open the main screen (Figure 2A) with the main menu (1), toolbar menu (2), display window (3), sample changer control panel (4) and the status bar at the bottom (5).
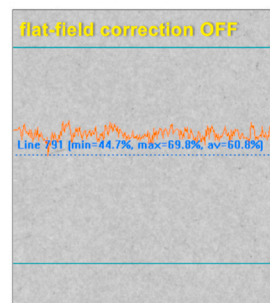   b. Warm-up the X-ray:
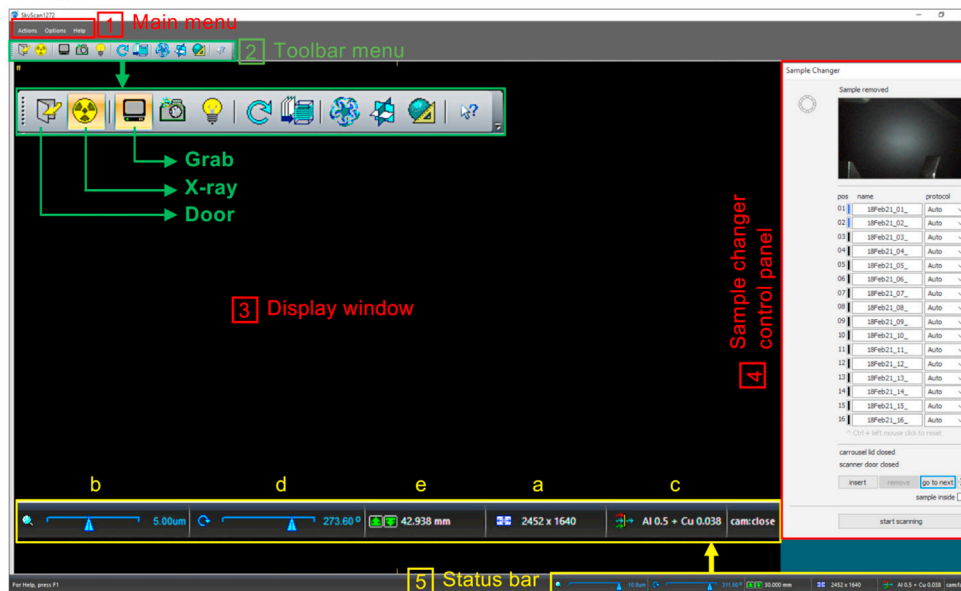
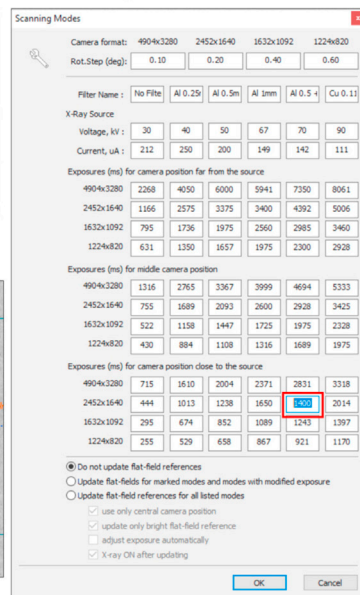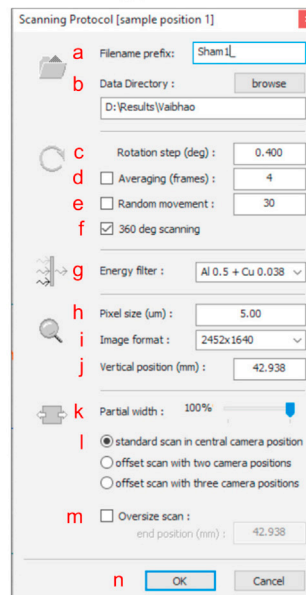**Figure 2. Sample visualization and image acquisition using SkyScan 1272**

(A) Home screen of SkyScan1272 displaying different toolbars and options.

(B) Visualization and positioning the sample in the center of the field of view.

(C) Signal intensity profile of the background.

(D) "Scanning modes" window to set up exposure for updating flat-field reference. Red box indicates the exposure value corresponding to the determined imaging parameters.

(E) "Scanning protocol" window to set up scanning parameters.

    i. Click on the "X-ray" icon in the toolbar menu (Figure 2A-2) to switch on X-ray. Indicator and progress bar then appear on the screen. Warm-up generally takes ∼15–30 min.

    ii. Click on the "X-ray" icon to switch off X-ray.

  c. Insert the sample inside the specimen chamber using the "Sample changer" control panel by:

    i. Click on the "Insert" button in the "Sample changer" control panel (Figure 2A-4). This will insert the first sample inside the specimen chamber.

  d. Once the robotic arm returns back to starting position after inserting the sample in the chamber, click on "Door" icon in the toolbar menu to close the specimen chamber.

Note: Progress of sample loading inside the chamber can be tracked in the Sample Changer window (Figure 2A-4).

⚠ CRITICAL: Do not click on the "Door" icon to close the specimen chamber (Step 8d) until the robotic arm returns to a complete rest at the starting position.

  e. Position the sample in the center of the field of view (Figure 2B):
    i. Click on the "X-ray" and "Grab" icon in the toolbar menu to visualize the X-ray image in the display window continuously.
    ii. Move the sample in a vertical direction by pressing the "Ctrl" button on the keyboard and "left mouse button" simultaneously and dragging the mouse pointer in the desired direction. Alternatively, use arrows in the status bar to position the sample in the center of the field of view (Figure 2A-5e).
     Setting the correct parameters is very critical in order to determine optimal transmission of the X-ray and is achieved by adjusting the filter, resolution, voltage and pixel size. To assess transmission, activate the profile line by right clicking on the sample image in the display window. Transmission values will appear on the screen. The minimum transmission should be between 10 and 50% (30% is ideal).

Note: The following section provides parameters for analyzing infarcted mouse hearts. Settings will need to be adjusted for different micro-CT instruments and sample types.

9. To set up the parameters, in the status bar (Figure 2A-5):
  a. Select resolution: 2452 × 1640 pixels (Figure 2A-5a).
  b. Use slider to achieve 5 μm pixel size (Figure 2A-5b).
  c. Select the filter: 0.5 mm aluminium + Cu 0.038 and note down camera position (e.g., close) (Figure 2A-5c). Here, an X-ray voltage of 73 kV and current of 133 μA is used (see note).
  d. Use the slider to rotate the sample and make sure the sample is not out of field of view at any point of rotation (Figure 2A-5d).
  e. Note down the vertical position of the sample (Figure 2A-5e).

Note: (1) Visualize all samples one-by-one and note down the vertical position for each sample (step 9e). (2) Each energy filter is associated with its own X-ray voltage and current settings. We recommend optimizing voltage/current to achieve optimal transmission.

A flat-field correction procedure is applied to every image to deal with non-linear X-ray attenuation and to increase the signal-to-noise ratio for optimal transmission, which can significantly improve image quality.

10. Once the correct parameters have been determined, a flat-field correction must be performed using the same settings. The flat-field correction ensures an even grey level background that will flatten out any pixel intensity abnormalities that could cause ring artefacts. Perform flat-field correction:
  a. Remove the sample from the specimen chamber by clicking the "Remove" button on the "Sample changer" control panel.
  b. Click on the "Door" icon from the main toolbar menu to close the door.
  c. Click on "X-ray" and "Grab" icon on the toolbar menu, and enter the exact values of all the parameters set for the sample at step 9 in the status bar.

d. Make sure the field of view is completely empty and the background is homogeneous.

e. Double click on "ff" displayed at the upper left corner of the screen to switch off flat-field correction. "flat-field correction OFF" will appear on the screen (Figure 2C).

f. Click the right mouse button in the imaging area to show a signal intensity profile along the line where the mouse pointer is situated (a second right-click will remove the profile from the image) (Figure 2C). Average transmission in air should be around 60%.

g. To adjust the signal intensity value, "Scanning modes" window can be displayed by clicking on "Option" > "Scanning modes" from the main menu (Figure 2D). If the "Scanning modes" option is greyed, this option can be made accessible by pressing Ctrl + Alt + Shift + S on the keyboard.

h. Adjust the exposure time in the correct box (2452 × 1640) in "Exposure (ms) for camera position close to the "source" panel in "Scanning modes" window (Figure 2D). The correct box will be highlighted by default (Figure 2D-red box). Leave the box ticked next to "Do not update flat-field references" and click on "OK" button (see note).

i. Check the signal intensity value, if it is not around 60%, repeat step 10f–g. Once the desired signal intensity value is achieved, open the "Scanning modes" window and tick the box next to "Update flat-fields for marked modes and modes with modified exposure" and click on the "OK" button (Figure 2D). This new flat-field configuration will be applied to all subsequent scans.

j. Double click on "flat-field correction OFF" displayed at the upper left corner of the display window to switch on flat-field correction. "ff" will appear on the screen.

k. Click on the "X-ray" icon in the toolbar menu to turn off X-ray and proceed to acquisition.

Note: Alternatively, adjusting exposure can be done automatically by selecting 'adjust exposure automatically' when updating the flat-field.

⚠ CRITICAL: (1) If any of the acquisition parameters are changed, the flat-field corrections must be performed using the revised setting. (2) If the exposure is changed, then the dark field flat-field correction should also be performed. Dark field flat-field is acquired with X-ray turned off. It contains the distribution of thermal current in every pixel of the image accumulated by the X-ray detector during the selected exposure time. Dark field flat-field corrections can be achieved by- ticking the box next to "Update flat-fields for marked modes and modes with modified exposure" in the "Scanning modes" window (Figure 2D) to make the listed options available; unticking the box next to "Update only bright flat-field reference" and clicking on "OK" button. Re-insert the sample and proceed further.

11. For acquisition of images, in the "Sample changer" control panel (Figure 2A-4), select the "Manual" option under "Protocol". This will open the "Scanning Protocol" dialog that contains a number of scanning parameters (Figure 2E):

a. Enter "Filename prefix" for the sample. This prefix will appear in the file name of each image acquired for the specific sample.

b. Data directory: Click on "browse" button to select a destination folder.

c. Rotation step (deg): 0.4.

d. Averaging (frames): OFF (No tick).

e. Random movement (pix): OFF (No tick).

f. Tick the box next to "360 deg scanning".

g. Energy filter: select filter determined in step 9c.

h. Pixel size (μm): select value determined in step 9b.

i. Image format: select value determined in step 9a.

j. Vertical position: type the value determined for this sample in step 9e.

k. Partial width: 100%. A lower percentage (typically 70%–80%) reduces output file size but can only be used if the sample stays within the new boundary at all angles. Validate this by

setting the partial width and rotating the sample 0/90/180 degrees to make sure it stays within the new boundary.

l. Tick the box next to "standard scan in central camera position".

m. Oversize scan: OFF (No tick).

n. Click on the "OK" button.

⚠ CRITICAL: Vertical position must be set and noted down for each sample (step 9c) and the exact same value must be entered in the acquisition parameters (step 11j) prior to the start of the scan.

Note: (1) If multiple samples are being scanned using a sample changer, "Scanning Protocol" window is called every time and the "Manual" option under "Protocol" needs to be selected in the "Sample changer" control panel for each sample. After entering the values of these parameters for all the samples one-by-one, click on the "Start scanning" button in the "Sample changer" control panel. (2) All acquisition parameters are saved and can be visualized in the log file saved as a text file in the destination folder.

3D data reconstruction

⏱ Timing: ~1–2 h per file with NRecon

The SkyScan NRecon package is used for 3D reconstruction from tomography projection images. Reconstruction parameters: misalignment compensation (post-alignment), ring artifacts reduction, smoothing and beam hardening correction should be adjusted manually to get an optimum image quality.

12. Start NRecon software:
    a. Click on the "Open data" icon in the toolbar menu (Figure 3A-1).
    b. Browse and select 1 file from the selected folder and click "Open" to load the data. This will display the scanned image in the display window (Figure 3A-2) and open the "Reconstruction" window on the right side of the screen (Figure 3A-3).

    Note: If the "Reconstruction" window is not displayed automatically, it can be visualized from the "View" option in the main menu.

13. Establish initial settings by selecting the "Setting" tab in the "Reconstruction" window (Figure 3B).
    a. Tick the box next to "Smoothing" and use an initial value of "1".
    b. Tick the box next to "Misalignment compensation" and use the default value. The software provides an estimate of this value, which is then fine-tuned in step 14a–e.
    c. Tick the box next to "Ring artifacts reduction" and use an initial value of "1".
    d. Tick the box next to "Beam-hardening correction" and set it to an initial value of 10%.
    e. Select the "Start" tab in the "Reconstruction" window. Image of the heart and 3 lines will be displayed in the main window (Figure 3C). Move the outer 2 red lines closer to the heart image to crop as much empty space as possible and keep the middle green line in the center of the heart image.

14. Select "Fine tuning" in the "Start" tab to adjust/improve the above-mentioned settings.
    a. A pop-up window will appear on the main screen (Figure 3D). Select "Post-alignment" in the option menu. Post-Alignment or Misalignment compensation refers to the number of pixels the projected rotation axis is outside the camera midline (Figure 6D).
    b. Set "Number of trials" to "5" and "Parameter step" to "1.0" and click on the "Start" button. Progress bar will appear showing progress of 5 trials of selected parameters i.e., "Post-alignment".
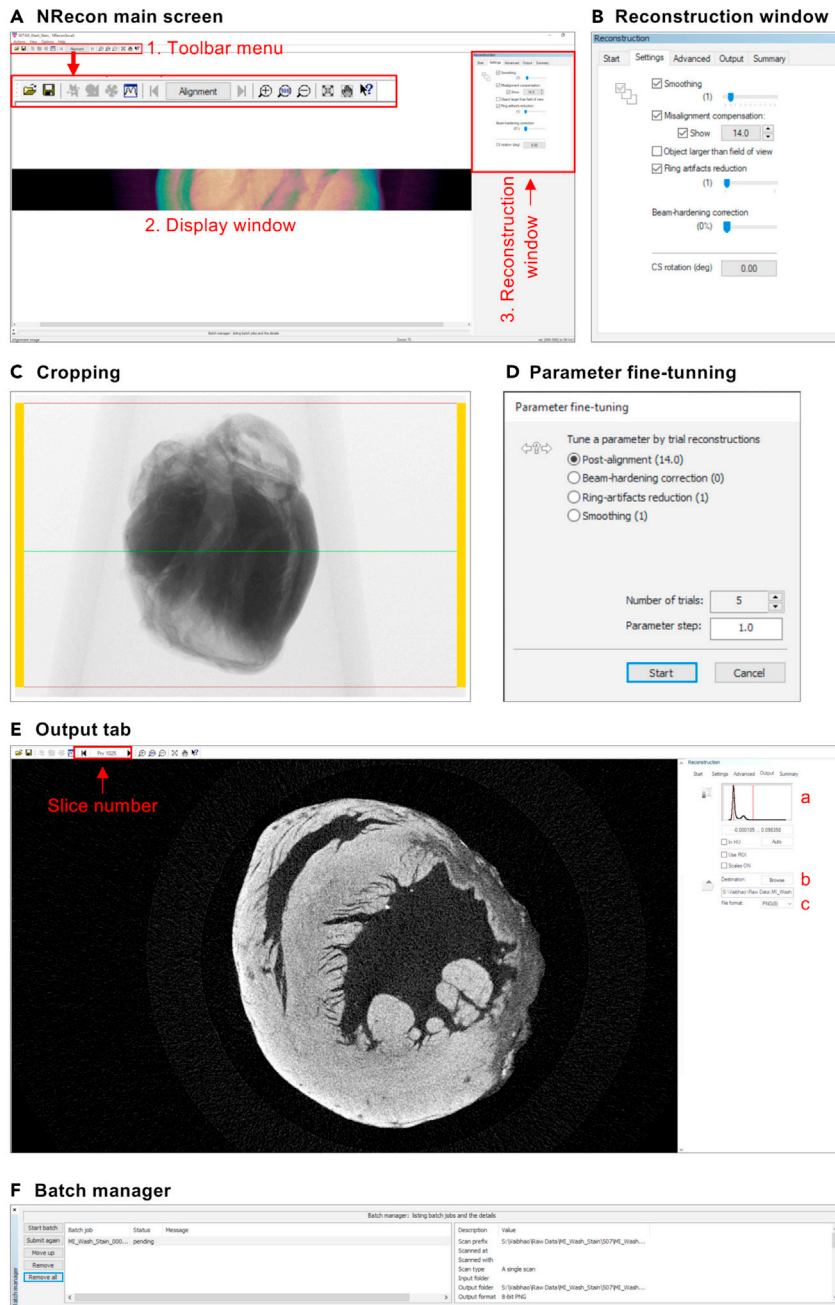
Figure 3. 3D reconstruction using NRecon

(A) Home screen of NRecon displaying different toolbars and options.

(B) Setting tab in the "Reconstruction" window.

(C) Display window showing the selection of the region of interest.

(D) Pop-up window showing options for fine-tuning.

(E) Display screen showing the results of the trials.

(F) Batch manager window showing the list of jobs.

c. This will generate 5 preview reconstructions for the "Post-alignment" parameter with one of the images displayed in the main window resulting from a trial and the "Output" tab will be opened in the "Reconstruction" window (Figure 3E).

    d. To associate the parameters with the results, the slice number (e.g., Prv 1000) is shown in the toolbar menu (Figure 3E, red box). Click on the arrow buttons to the left and right of the slice number to view the different trial images.

    e. Scroll through 5 trial images and select the best image showing the proper alignment (no doubling or blurring in the reconstructed image) (see troubleshooting problem 4). The software will remember the value of the parameter associated with the displayed image (so no need to confirm). Make sure the preferred slice number and corresponding preview image is displayed on the screen (Figure 3E) before moving to the next step as the value is copied to the settings.

    f. Select the "Start" tab in the "Reconstruction" window and click on "Fine tuning". In the pop-up window, select "Ring-artifacts reduction". Ring artefacts can appear due to a heterogenous background image taken by the X-ray camera (for example a dust particle on the camera). During reconstruction, this particle remains in the same place while projection images are taken from the rotating sample giving the appearance of a ring (Figure 6B). Set "Number of trials" to "5" and "Parameter step" to "1.0" and "Start". Repeat steps 14c–14e and select the lowest value that removes ring-artifacts.

    g. Select the "Start" tab in the "Reconstruction" window and click on "Fine tuning". In the pop-up window, select "Smoothing". Smoothing or blurring reduces the image noise. Note that too much smoothing can result in a reduction of image detail (Figures 6E and 6F). Set "Number of trials" to "5" and "Parameter step" to "1.0" and "Start". Repeat steps 14c–14e and choose the image/value that removes the most noise without overly blurring the image (see troubleshooting problem 4).

    h. Select "Start" tab in the "Reconstruction" window and click on "Fine tuning". In the pop-up window, select "Beam hardening correction". Beam hardening refers to the attenuation of low energy X-rays, causing the resulting reconstruction of an object to have reduced internal signal compared to its surface. Set "Number of trials" to "10" and "Parameter step" "10" and "Start". Repeat, steps 14c–14e and select the best image. Beam hardening correction will increase the overall brightness of your scan and improve contrast (Orhan et al., 2019).

Note: We find that the adult mouse heart can be scanned at an acceptable resolution with one field of view. If two or more fields of view are required (oversize scan), misalignment compensation should be carried out separately for all fields of view by moving the green line (step 13e) to each field of view. Once the reconstruction parameters are adjusted manually in a trial-and-error fashion using the preview function, data is now ready for reconstruction.

15. In the "Output" tab in the "Reconstruction" window (Figure 3E):
    a. A histogram of the preview slice will be displayed (Figure 3E-a). Drag the left vertical line in the middle of the first peak and drag the right vertical line just outside the right peak adjusting for voxel intensity.

    b. Select the destination folder after clicking the "Browse" button (Figure 3E-b).

    c. Select the file format: Select PNG file format (Figure 3E-c).

    d. Select the "Start" tab in the "Reconstruction" window, and click on the "Add to batch" button. This will open the "Batch manager" window automatically and the selected data file will appear in the "Batch manager" (Figure 3F).

Note: (1) If the "Batch manager" window is not displayed automatically, it can be visualized from the "View" option in the main menu. (2) Optional: To reduce the size of the dataset, ROI (region of interest) can be selected by ticking the box next to "Use ROI" in the Output tab (Figure 3E) (see troubleshooting problem 5).

    e. Repeat the steps 12a–15d for each sample.

    f. Click on "Start Batch" in the "Batch Manager" to initiate 3D reconstruction.

Note: For each of the data files, the reconstructed data will be saved in the destination folder, selected in step 15b.

Analysis

⏱ Timing: ∼3–6 h per sample with Amira

The Watershed algorithm is a powerful method developed for automated object segmentation or separation based on voxel intensity values. Differential contrast after staining with Lugol's solution allows automated segmentation of scar and myocardium based on voxel grey value thresholds. In this section, step-by-step protocol of initial voxel intensity-based automated segmentation of scar and myocardium by Watershed algorithm and manual curation of minor inaccuracies using Amira software is described.

16. To seed materials for the use of Watershed segmentation tool, start Amira:
    a. Click on "Open Data", select all image files of the reconstructed data and click on "Open" button.
    b. A pop-up window will appear. Enter the correct voxel size in x, y and z (5 μm, set at step 9b) and click on "OK" button. The "Project" view will open along with "Ortho slice" module (Figure 4A).

    ⚠ CRITICAL: Enter the correct voxel size (entered in step 9b) to obtain the correct volumetric measurements generated in downstream analysis.

17. Open the "Segmentation" workroom:
    a. Click on the "Segmentation" tab on the workroom toolbar (Figure 4A-1). This will automatically launch the Segmentation workroom and create a new "Label field" (Figure 4A-2) in the Segmentation editor.
    b. Optional: rename the "Label Field" (e.g., Heart_Segmentation) by clicking on the "Rename" button.

    Note: "Materials" and "Selection" panels in the Segmentation editor (Figure 4A-3 and 4) and 2D viewer (Figure 4A-6) will be displayed on the left and right side of the screen, respectively. Orientation can be changed to cycle through different 2D views (XY, XZ, and YZ) (Figure 4A-6) and 3D view (Figure 4A-7) can be displayed by clicking on icons in the viewer toolbar (Figure 4A-5).

    c. Create 2 new materials by clicking on the "Add" button in the "Materials" panel, rename materials as "Myocardium" and "Scar" by double-clicking and tick the boxes next to "2D" and "3D" options (Figure 4B).
    d. Click on the brush icon in the "Selection" panel (Figure 4A-4) and adjust the brush size with the size slider, as required.
    e. Use the mouse pointer to mark the myocardial area in the 2D (XY) view mode (Figure 4C), select the "Myocardium" material in the "Materials" panel and add the selected voxel by clicking the "+" button in the "Selection" panel. The selected voxels are now assigned to "Myocardium" material and are shown by a colored line in 2D mode and the same color patch in 3D mode (red in Figure 4D).
    f. In a similar fashion, mark the scar area in the same slice and add selected voxels to "Scar" material. The selected voxels are now assigned to "Scar" material and are shown by colored line in 2D mode and same color patch in 3D mode (blue in Figure 4D).
    g. Slide through different slices (2D mode) using a slider (Figure 4A-6), and select voxels throughout the heart and add selected voxels to the respective material as mentioned above (step 17e–f). Visualize all selected voxels in the chosen "Material" in 2D and 3D mode (Figure 4E). We recommend seeding "Materials" in at least 15–20 slices across the entire heart (between apex to base).
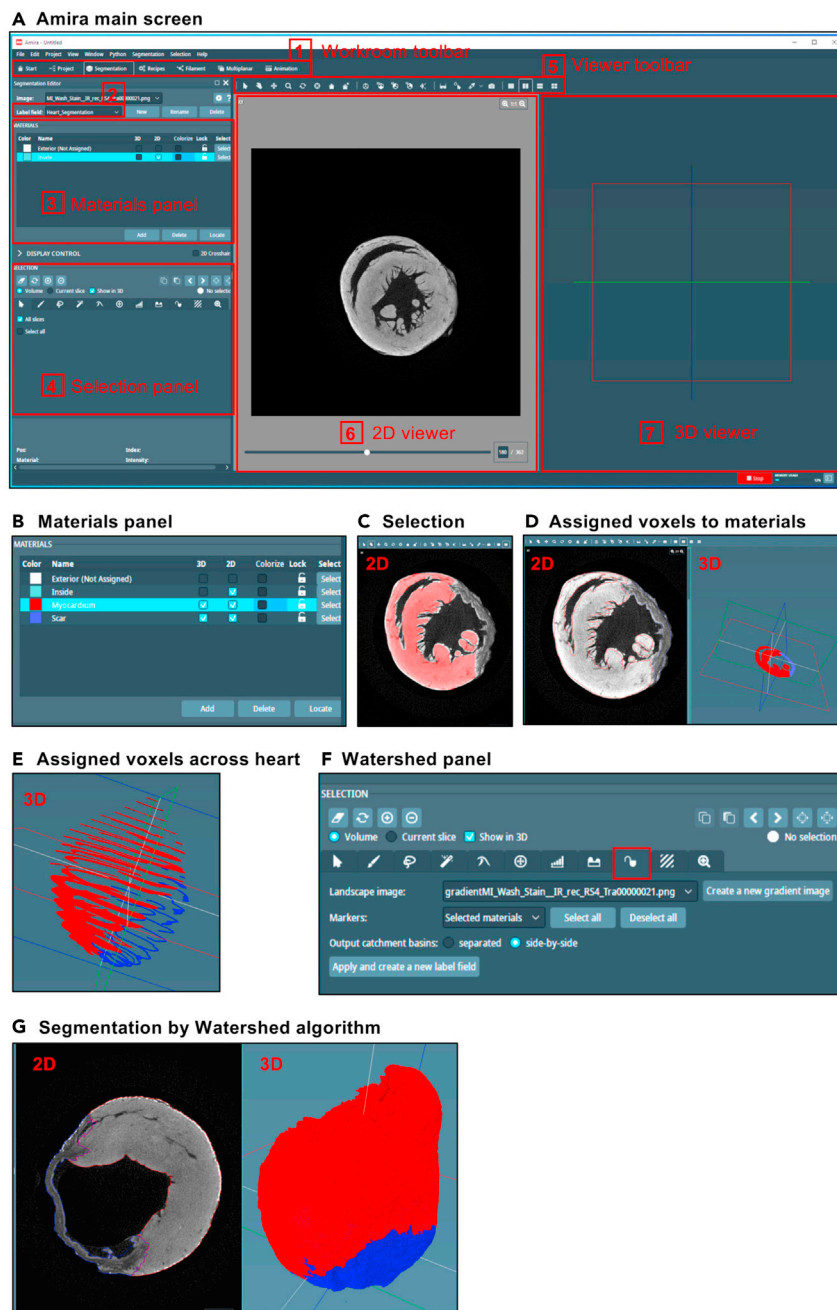
Figure 4. Segmentation and analysis using Amira

(A) Home screen of Amira displaying different toolbars and options.

(B) "Materials" panel.

(C) Selection of voxels in a 2D section.

(D) Assigned voxels in Myocardium (red) and Scar material (blue), shown in 2D and 3D mode.

(E) Assigned voxels in Myocardium and Scar material across the ventricles, shown in 3D mode.

(F) Watershed panel.

(G) Segmentation of Myocardium and Scar by Watershed algorithm, shown in 2D and 3D mode.

> *Note:* Selection of voxels with a brush does not have to be precise but caution should be taken not to select voxels in "unintended" regions while adding in the desired "Material". If the aim is to quantify ventricular volume, do not select and add atria to "Myocardium" material.

18. If satisfied with the selected voxels in the "Myocardium" and "Scar" material, proceed with segmentation using the Watershed tool:
    a. Click on the "Watershed" icon in the "Selection" panel (Figure 4F, red box). This will open the "Watershed" panel.
    b. Click on the "Create new gradient image" button. Progress bar will appear at the bottom.
    c. Click on "Apply and create a new label field" button (Figure 4F). This will create a new label field (Figure 4A-2) with the Watershed results and progress bar will appear at the bottom.
    d. After the Watershed algorithm run is finished, automated segmentation of the scar and myocardium is displayed in 2D/3D mode based on assigned voxels (Figure 4G).

    Note: Slide through all the slices in the 2D/3D view of the segmented heart, generated after the Watershed algorithm. If Watershed works properly and segmentation of scar and myocardium material is mostly satisfactory, minor errors should be corrected by manual curation. However, if seeding of the "Materials" is not adequate for the Watershed algorithm to segment the heart properly, more seeding of materials is required. To achieve this, toggle back to the "Label Field" file (Heart_Segmentation) from drop-down menu in the Segmentation editor tab (Figure 4A-2), and seed the materials with additional voxels in the different 2D views (YZ and XZ) as mentioned in step 8 and 9 and run Watershed algorithm again.

Typically, segmentation by Watershed algorithm is not perfect and minor inaccuracies will occur-e.g., exclusion of specific voxels or inclusion of non-specific voxels in the desired material (scar/myocardium). Such minor imperfections will have to be corrected by manual curation using tools in the Segmentation editor.

19. Examine all the slices in the 2D view:
    a. Identify the non-specific inclusion/exclusion of voxels.
    b. Click the brush icon in the tools in the "Selection" panel, adjust the brush size with the slider and mark the non-specific voxels in the 2D (XY) view mode.
    c. Add or subtract selected voxels from intended material by selecting the material and clicking "+" or "-" button in the "Selection" panel, respectively. The selected voxels are now assigned or omitted from the intended material, and can be tracked and visualized in 2D/3D mode.

    Note: Figure 5A shows an example of inclusion of non-specific voxels in the myocardium material (shown by arrow in the left panel). These voxels were selected by the brush tool (middle panel) and omitted from "Myocardium" material (right panel). Similarly, exclusion of specific voxels can be selected and added in the desired material.

20. Once all mistakes are corrected and the heart is accurately segmented, material statistics can be obtained by selecting "Segmentation" > "Material statistics" in the main menu. A table appears in a dialog box listing the volume of each material (Figure 5B). The data can be imported in most of the commonly used data analysis software such as Excel or GraphPad Prism. The user can also select other parameters of interest for further comparison and statistical analysis.
21. For volume rendering and visualization of a 3D image of the segmented data (Figure 5C):
    a. Click on the "Project" tab in the workroom toolbar menu. Multiple files will be displayed in the "Project view" (1).
    b. Create the "Volume rendering" module by right clicking on the ".labels" file and click on "Display" > "Volume rendering" > "Create". This will generate a 3D impression of the segmented heart highlighting "Myocardium" and "Scar" in selected colors in the 3D view panel (2).
    c. Click on the "Volume rendering setting" module to open the "Properties" panel (3) and adjust the settings to modify the 3D view as required.

A **Manual curation of inaccuracies**
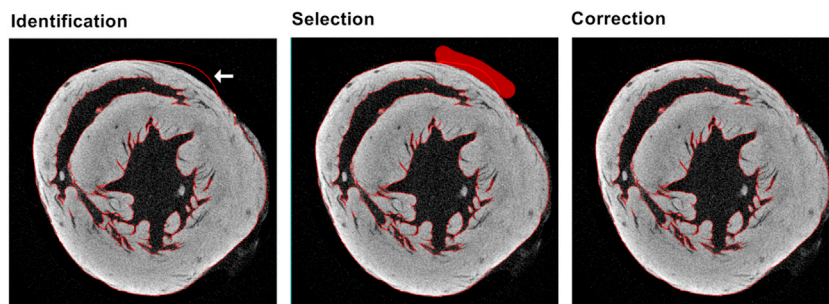
Identification   Selection   Correction

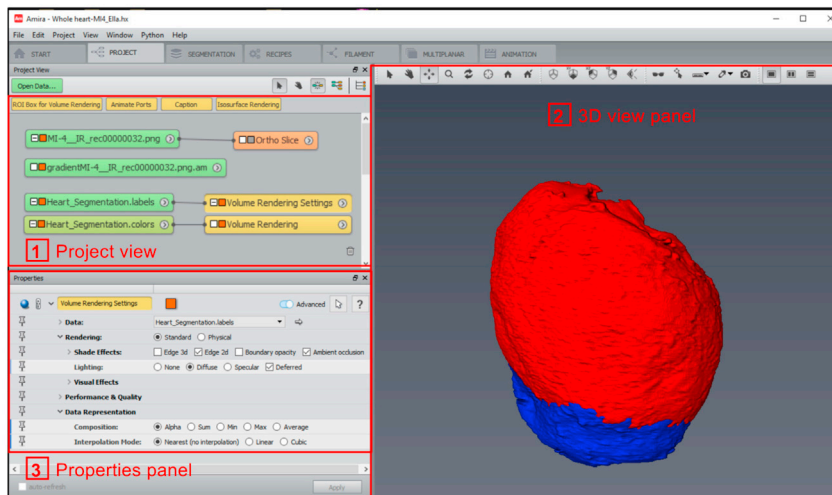**B** **Material statistics**

C **Volume rendering**

Figure 5. Manual curation and data acquisition using Amira
(A) Identification, selection and correction of inaccuracies.
(B) "Material statistics" window.
(C) Volume rendering and surface reconstruction.

EXPECTED OUTCOMES

This protocol for soft-tissue contrast enhancement has been adapted and modified from previously published protocols (Martin et al., 2021; Wong et al., 2013). To our knowledge, this is the first study presenting a hierarchical 3D (micro-CT-based) approach to segment and quantify post-infarction scar and myocardial volume in 3D reconstructed data from the infarcted mouse heart. Cardiac infarct size is often characterized by conventional 2D histology, but this approach incorrectly assumes that there is a consistent relationship between scar and myocardium in 3 dimensions. Our method also

overcomes challenges faced by the 2D approach associated with sample handling, specimen variability, variation in scar size and non-uniformity of tissue sectioning. Moreover, our volume-based method is capable of robust quantification of volume differences not detectable by conventional 2D analysis. This quantification approach generates more spatially accurate and objective measures and represents an important step toward improving the reliability of morphological comparisons. Minimizing user-based decisions and relying more heavily on semi-automated computational approaches further improves objectivity. While we have used this protocol extensively for studies mainly in infarcted mouse hearts, researchers could choose to include other parameters (e.g., lumen volume, thickness of the compact LV/RV myocardium, atrial parameters) in the analysis for further comparison and statistical analysis. We also anticipate that the approach could be applied to other tissues with similar results. Thus, the method described here may serve as a basis for the accurate 3D measurement of various tissues and properties.

QUANTIFICATION AND STATISTICAL ANALYSIS

The output file can be imported in most of the commonly used data analysis software such as Excel and GraphPad Prism. Statistical analysis can be performed on the absolute or relative volume of myocardium and scar as required. Samples with missing parts (because of damage and/or incomplete segmentation) should be excluded from the analysis.

LIMITATIONS

This protocol is optimized for quantifying scar volume in infarcted murine hearts. Hence, the protocol is applicable for replacement fibrosis and is not optimized to quantify interstitial fibrosis. Furthermore, this method of scar quantification is based on "negative staining" i.e., there is no reaction between the stain (Lugol's solution) and the scar tissue. Our attempts to "positively" stain scar with phosphotungstic acid (PTA) were unsuccessful, possibly due to inadequate penetration into highly cross-linked, dense fibrous network of ECM within the scar since PTA is a larger molecule containing tungsten ions (Lesciotto et al., 2020). This protocol can be used to quantify fibrosis in other tissues if differential contrast can be achieved between fibrotic/nonviable and normal/healthy/surviving sections of the tissue using Lugol's solution as a contrasting agent. Additionally, the 3D image analysis protocol is based on manual handling (manual curation) and it is therefore not feasible to apply common settings to all samples.

TROUBLESHOOTING

Problem 1
Bright spots/line are visible at the surface of the sample during reconstruction.

Potential solution
Hydrogel pieces attached to the sample (Figure 6A, arrow) absorb Lugol's solution and therefore appear very bright during image analysis (Figure 6B, arrow). To avoid this, remaining hydrogel pieces should be removed completely after polymerization (step 5). If needed, perform additional washes in PBS and use lint-free tissue paper to ensure complete removal of hydrogel from the sample.

Problem 2
Rings appear around the sample (Figure 6C).

Potential solution
Eppendorf tube will absorb some of Lugol's solution over time and appear as a ring around the sample during image analysis (Figure 6C); this needs to be considered while scanning multiple samples at the same time using a sample changer. Do not scan more than 5 samples at one time. After staining with Lugol's solution, place the stained heart on lint-free tissue to remove residual Lugol's solution before placing it in the Eppendorf tube (step 7).
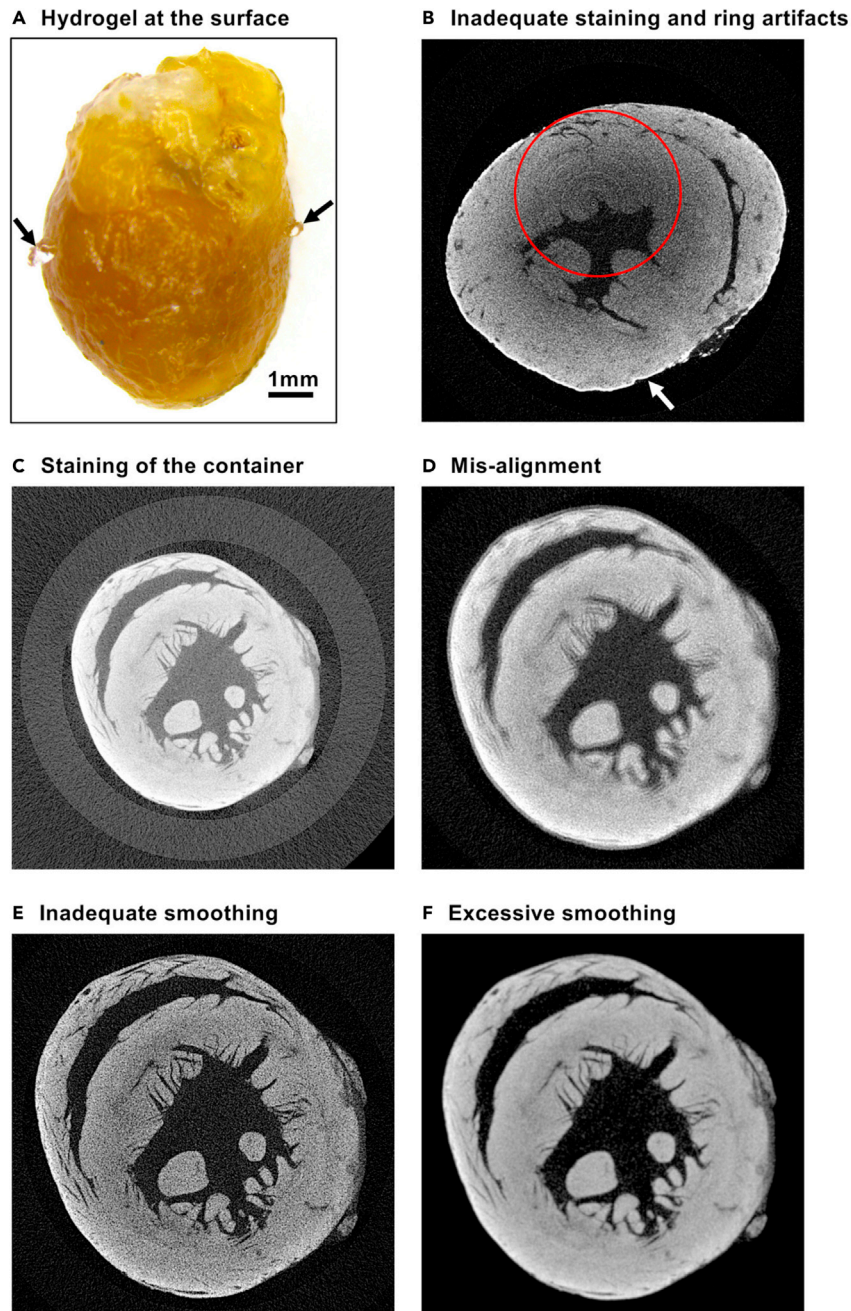
Figure 6. Troubleshooting

(A–F) Images show commonly encountered problems during sample processing and 3D reconstruction: (A) hydrogel pieces sticking to the sample. Scale bar = 1 mm; (B) inadequate staining, bright line at the surface (arrow) and ring artifacts during 3D reconstruction (red circle); (C) staining of the tube due to absorption of Lugol's solution; (D) misalignment of 2D images; and (E and F) inadequate and excessive smoothing during 3D reconstruction.

Problem 3

Non-uniform or inadequate staining (Figure 6B).

Potential solution

Always use fresh Lugol's solution and do not re-use. If enough contrast is not achieved, replace the Lugol's solution every day and incubate samples longer (3–5 days) (step 6).

Problem 4

Inability to remove doubling and blurring from the reconstruction by fine-tuning (Figure 6D).

Potential solution

If Post-Alignment fine-tuning (step 14) does not remove image doubling, the sample has probably moved during image acquisition. Sample movement during scanning will result in a dataset that cannot be properly reconstructed. Ensure ample dental wax is used to secure the Eppendorf containing the sample (the X-ray will warm up the wax during scanning, allowing it to become softer) (step 7). Dehydration of the sample during scanning can also cause movement so parafilm should be used to seal the lid to avoid this (step 7).

Problem 5

Slow loading and/or processing in Amira (step 16).

Potential solution

Since the reconstructed dataset is large, steps can be taken to reduce its size. At the reconstruction stage, the NRecon ROI function (step 15) can be used where a box is drawn around the region of interest. The area inside the box will be reconstructed while the rest is omitted. After reconstruction, DataViewer (Bruker Corporation) can be used to reduce the image size by a factor of 2 in each axis. In the DataViewer 'Load image/dataset' dialog box, tick resize with a value of 2 and tick 'averaging in 3D'. The dataset will be resized upon opening and can then be saved at this reduced size.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Richard P. Harvey (r.harvey@victorchang.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any data set or code. Janbandhu et al. (2021) contains representative datasets as would typically be obtained using this protocol.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, and Investigation, V.J., E.M.M.A.M., and G.C.; Writing – Original Draft, V.J., E.M.M.A.M., and G.C.; Writing – Review & Editing, All Authors; Funding Acquisition and Supervision, V.J., R.P.H., G.C., and S.L.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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