Journal of Visualized Experiments
Surgery and Sample Processing for Correlative Imaging of the Murine Pulmonary Valve
--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62581R2
Full Title:	Surgery and Sample Processing for Correlative Imaging of the Murine Pulmonary Valve
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Additional Information:	

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Please specify the section of the submitted manuscript.	Engineering
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1 **TITLE:**
2 Surgery and Sample Processing for Correlative Imaging of the Murine Pulmonary Valve
3
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26 **KEYWORDS:**
27 murine pulmonary valve, heart valve, collagen, extracellular matrix, correlative imaging, serial
28 block face scanning electron microscopy, micro-computed tomography, heart valve disease
29

30 **SUMMARY:**
31 Here, we describe a correlative workflow for the excision, pressurization, fixation, and imaging
32 of the murine pulmonary valve to determine the gross conformation and local extracellular
33 matrix structures.
34

35 **ABSTRACT:**
36 The underlying causes of the heart valve related disease (HVD) are elusive. Murine animal models
37 provide an excellent tool for studying HVD, however, the surgical and instrumental expertise
38 required to accurately quantify the structure and organization across multiple length scales have
39 stunted its advancement. This work provides a detailed description of the murine dissection, en
40 bloc staining, sample processing, and correlative imaging procedures for depicting the heart valve
41 at different length scales. Hydrostatic transvalvular pressure was used to control the temporal
42 heterogeneity by chemically fixing the heart valve conformation. Micro-computed tomography
43 (μCT) was used to confirm the geometry of the heart valve and provide a reference for the
44 downstream sample processing needed for the serial block face scanning electron microscopy

45 (SBF-SEM). High-resolution serial SEM images of the extracellular matrix (ECM) were taken and
46 reconstructed to provide a local 3D representation of its organization. µCT and SBF-SEM imaging
47 methods were then correlated to overcome the spatial variation across the pulmonary valve.
48 Though the work presented is exclusively on the pulmonary valve, this methodology could be
49 adopted for describing the hierarchical organization in biological systems and is pivotal for the
50 structural characterization across multiple length scales.
51
52 **INTRODUCTION:**
53 The pulmonary valve (PV) serves to ensure unidirectional blood flow between the right ventricle
54 and the pulmonary artery. Pulmonary valve malformations are associated with several forms of
55 congenital heart disease. The current treatment for congenital heart valve disease (HVD) is
56 valvular repair or valve replacement, which can necessitate multiple invasive surgeries
57 throughout a patient's lifetime[1]. It has been widely accepted that the function of the heart valve
58 is derived from its structure, often referred to as the structure-function correlate. More
59 specifically, the geometric and biomechanical properties of the heart dictate its function. The
60 mechanical properties, in turn, are determined by the composition and organization of the ECM.
61 By developing a method for determining the biomechanical properties of murine heart valves,
62 transgenic animal models can be used to interrogate the role of the ECM on heart valve function
63 and dysfunction[2–5].
64
65 The murine animal model has long been regarded as the standard for molecular studies because
66 transgenic models are more readily available in mice compared to other species. Murine
67 transgenic models provide a versatile platform for researching heart valve related diseases[6].
68 However, the surgical expertise and instrumentation requirements to characterize both the
69 geometry and ECM organization have been a major hurdle in progressing HVD research.
70 Histological data in literature provides a picture of the content of the murine heart valve
71 extracellular matrix, but only in the form of 2D images, and are unable to describe its 3D
72 architecture[7,8]. Additionally, the heart valve is both spatially and temporally heterogeneous,
73 making it difficult to draw conclusions across experiments regarding ECM organization if the
74 sampling and conformation are not fixed. Conventional 3D characterization methods, such as
75 MRI or 3D echocardiography, do not provide the resolution necessary to resolve ECM
76 components[9,10].
77
78 This work details a fully correlative workflow where the temporal heterogeneity due to the
79 cardiac cycle was addressed by fixing the conformation of the murine PV with hydrostatic
80 transvalvular pressure. The spatial heterogeneity was controlled precisely by sampling regions of
81 interest and registering data sets from different imaging modalities, specifically µCT and serial
82 block face scanning electron microscopy, across different length scales. This method of scouting
83 with µCT for guiding downstream sampling has been proposed previously, but because the
84 pulmonary valve exhibits temporal variation, an additional level of control was needed on the
85 surgical level[11].
86
87 *In vivo* studies describing murine heart valve biomechanics are sparse and, instead, rely on
88 computational models when describing the deformation behavior. It is of critical importance that

89 local extracellular data on the nanometer length scale be related to the geometry and location
90 of the heart valve. This, in turn, provides quantifiable, spatially mapped distributions of
91 mechanically contributing ECM proteins, which can be used to reinforce existing biomechanical
92 heart valve models[12–14].
93
94 **PROTOCOL:**
95 The use of animals in this study was in accordance with Nationwide Children's Hospital
96 institutional animal care and use committee under protocol AR13-00030.
97
98 **1. Pulmonary valve excision**
99
100 1.1. Autoclave the necessary tools needed for the mouse dissection. This includes fine scissors,
101 micro forceps, micro vascular clamps, clamp applying forceps, microneedle holders, spring
102 scissors, and retractors.
103
104 1.2. Acclimate all mice for a minimum of 2 weeks before the operation. Remove C57BL/6 mice,
105 approximately 1 year of age, from their cages and weigh, then euthanize with a
106 ketamine/xylazine cocktail (3:1 ketamine:xylazine, 0.01 mL per g) overdose.
107
108 1.3. Place the mouse in a dorsal recumbence position on a tray and secure its limbs with tape.
109 Once secured, perform the thoracotomy.
110
111 1.3.1. Expose the heart by removing any excess adipose tissue and fascia.
112
113 1.3.2. Remove the right atrium and perfuse through the left ventricle with room temperature
114 saline solution (0.9% NaCl). The perfusion should take approximately 20 mL over 30 s. This results
115 in the exsanguination of the mouse.
116
117 1.4. Remove the entire heart by severing the superior vena cava, inferior vena cava, pulmonary
118 artery, and aorta. Special care should be taken for the pulmonary artery. Cut approximately 2
119 mm above the ventriculo-arterial junction, as this will serve as the conduit for pressurization.
120
121 1.5. Remove the left and right ventricles to expose chambers to atmospheric pressure. Ensure
122 that the structure of the pulmonary trunk should be unaffected by the removal of the ventricles.
123
124 **2. Pressure fixation of pulmonary valve**
125
126 2.1. Anastomose pressurization tubing with pulmonary artery, leaving approximately 1 mm
127 distance between the sino-tubular junction and the end of the tubing to accommodate for large
128 movements of the leaflets and pulmonary trunk.
129
130 2.2. Elevate the reservoir to an analogous physiological pressure and fill it with the saline
131 solution. Test the flow-through system to ensure there are no blockages or air bubbles.
132

133 2.3. Attach a stopcock to the anastomosed pulmonary valve and ensure adequate flow through
134 the tubing (i.e., no air bubbles) by switching the outflow tract. Once the flow is adequate, switch
135 the outflow to the anastomosed pulmonary valve and ensure pressurization of the pulmonary
136 trunk. This is identified by pulmonary trunk distention.

137

138 2.4. Once the pressurization of the pulmonary trunk is confirmed, gradually incorporate primary
139 fixative solution (1.25% glutaraldehyde, 1.0% paraformaldehyde in 0.15 M cacodylate) until the
140 saline solution is purged. This is done by removing a portion, approximately 25% of the reservoir
141 capacity, of the saline solution and replacing it with the primary fixative.

142

143 CAUTION: The fixatives used (paraformaldehyde and glutaraldehyde) are highly toxic and
144 appropriate personal protective equipment (PPE) should be worn to ensure safety.

145

146 2.5. Place a fixative-soaked gauze over the tissue sample to prevent drying.

147

148 2.6. Perfuse the fixative for 3 h, refilling the reservoir as needed to maintain a constant pressure.
149 Throughout fixation, it is not uncommon for the pulmonary valve to shrink because of the
150 chemical fixation. If this is the case, constantly replenish the reservoir with primary fixative to
151 maintain physiological pressure.

152

153 2.7. Store the heart valve in the fixative solution at 4 °C until use. Samples were stored for up to
154 1 week without any discernable difference.

155

156 **3. En bloc sample staining and embedment**[15,16]

157

158 CAUTION: The staining reagents used in this section (potassium ferrocyanide, osmium tetroxide,
159 thiocarbohydrazide, lead aspartate, and uranyl acetate) are highly toxic and should be handled
160 with extreme care. Use of a fume hood and proper PPE is advised.

161

162 3.1. Staining

163

164 3.1.1. Wash the fixed heart valve sample for 5 min with cold 0.15 M cacodylate buffer. Repeat
165 the wash two more times.

166

167 3.1.2. Completely submerge the heart valve in a solution of 1.5% potassium ferrocyanide, 0.15
168 M cacodylate, 2 mM calcium chloride, and 2% osmium tetroxide, on ice for 1 h.

169

170 3.1.3. While the sample is incubating, prepare thiocarbohydrazide (TCH) solution by dissolving
171 0.1 g of TCH in 10 mL of ddH$_2$O. Place the solution in a 60 °C oven for 1 h. Gently agitate
172 periodically to ensure TCH is dissolved completely. Filter the solution through a 0.22 μm syringe
173 filter immediately before use.

174

175 3.1.4. Wash the samples with room temperature ddH$_2$O by placing them in a tube of ddH$_2$O for
176 5 min and slightly agitate it by shaking the container. Repeat this process three times.

3.1.5. Place it in filtered TCH solution for 20 min at room temperature. Perform the washing step three times (5 min each) with room temperature ddH$_2$O as described in step 3.1.4.

3.1.6. Once done, place the sample in 2% osmium tetroxide for 30 min at room temperature. Then wash it again for a total of three times for 5 min each with room temperature ddH$_2$O.

3.1.7. Incubate the sample in 1% uranyl acetate overnight at 4 °C.

3.1.8. During this time, make a solution of 0.066 g of lead nitrate in 10 mL of aspartic acid stock solution. Adjust the pH to 5.5 with 1 N KOH. Place the solution in a 60 °C oven to dissolve lead nitrate.

3.1.9. After the overnight incubation, perform the washing step as described in step 3.1.4. Repeat three times. Then, incubate the heart valve tissue in lead aspartate solution from step 3.1.8 in a 60 °C oven for 30 min.

3.2. Dehydration

3.2.1. Wash the tissues for 5 min at room temperature with ddH$_2$O. Repeat three times.

3.2.2. Make fresh solutions of 20%, 50%, 70%, 90%, and 100% ethanol in ddH$_2$O. Keep on ice until use.

3.2.3. To dehydrate the heart valve tissue. Perform subsequent treatments of 20%, 50%, 70%, and 90% ethanol on ice for 5 min each. Then perform two subsequent treatments of 100% ethanol on ice for 5 min.

3.2.4. Move the tissue to ice-cold acetone for 10 min. Then place in fresh acetone at room temperature for 10 min.

3.3. Embedding

3.3.1. Make the resin mixture (see **Table of Materials**) according to the manufacturer's specifications: 11.4 g of component A, 10 g of component B, 0.3 g of component C, and 0.05–0.1 g of component D. Initially mix components A and B by heating each of the components to 60 °C before adding components C and D. The mixture will turn into an amber color upon adding components C and D. It will be uniform when properly mixed.

3.3.2. Make mixtures with volume ratios of 25:75, 50:50, and 75:25 resin:acetone. Mix thoroughly.

3.3.3. Place tissues in subsequent treatments of the 25:75, 50:50, and 75:25 resin:acetone mixture for 2 h each at room temperature.

221

222 3.3.4. Place tissues in 100% resin overnight at room temperature.

223

224 3.3.5. The following day, place tissues in fresh 100% resin for 2 h at room temperature.

225

226 3.3.6. Transfer the heart valve tissues to an embedding capsule, substitute with fresh 100% resin,
227 and place in a 60 °C oven for 48 h to cure.

228

229 3.4 Perform correlative imaging as shown below.

230

231 **4. Micro-computed tomography imaging**

232

233 4.1. Mount the resin sample block onto a µCT sample holder with an adhesive (glue or double-
234 sided tape works well).

235

236 4.2. Place the holder into µCT chamber and fix it onto the stage by screwing the collet such that
237 there is no motion of the holder. Sample movement during the scan will decrease the image
238 quality.

239

240 4.3. Open the µCT user interface by double-clicking on the icon. Add a project by selecting the **+**
241 sign next to **Projects** and fill in the necessary fields.

242

243 4.4. Once this is created, find the created project, and select it by clicking on it. This will open up
244 a second column **Acquisitions**. Select the **+** next to **Acquisitions** and fill out the necessary fields.

245

246 4.5. Close the chamber doors and arm the system by hitting the arming button on the front panel
247 or the µCT. Warmup x-rays from the user interface by selecting the button indicating **Warmup**.

248

249 NOTE: The system will automatically turn off the x-rays following a successful warm-up
250 procedure. Turn on x-rays by selecting the button.

251

252 4.6. Adjust the stage rotation such that the center of the rotation of the sample does not deviate
253 from the center of the monitor (i.e., the sample is within the field of view for the entire scan). For
254 the system used for this experiment, this involves adjusting the region of interest (ROI) stage
255 under the drop-down tab as detailed below.

256

257 4.6.1. Set the ROI stage adjustment by setting the rotation angle to 0° and marking the edge of
258 the sample. The sample is then rotated to 180° and the edge of the sample is marked again.

259

260 4.6.2. Adjust the ROI stage x-axis position such that the edge of the sample is between these two
261 extremes. Repeat this process for rotation angles of 90° and 270° for the y-position.

262

263 4.6.3. In the case where the rotation of the sample causes the edges to move out of the field of
264 view, decrease the magnification of the µCT needs until the edges of the sample are visible at the

265   above set angles and a coarse ROI stage adjustment can be made.
266
267   4.6.4. Once adjusted at the lower magnification, move the sample or the detector to increase the
268   magnification and the ROI positions can be fine adjusted.
269
270   NOTE: This process may need to be repeated to ensure alignment. Proper alignment results in a
271   sample that shows little to no horizontal movement through all sample rotation angles.
272
273   4.7. Adjust the µCT to desired scanning parameters using the manufacturer's software. These
274   parameters include tube potential, tube current, detector distance, sample distance, exposure
275   time, trajectory, and the number of projections (see **Table S1** for the µCT acquisition parameters
276   used in this study).
277
278   NOTE: Calibration parameters, such as clear field and dark field scans, should be kept at the
279   manufacturer's specifications unless otherwise stated. For example, if a sample is too large and
280   the system cannot move the sample out of the field view, then the sample needs to be removed
281   to perform clear field calibration scans.
282
283   4.8. Once the parameters are set, an approximation of the duration of the scan can be seen by
284   hitting the **Time Estimate** button. Select the **Start** button to begin the scan.
285
286   4.9. Following the scan completion, ensure that the x-rays are turned off, unscrew the collet, and
287   carefully remove the sample from the µCT chamber.
288
289   **5. Sample processing and image correlation**
290
291   5.1. Reconstruct µCT projections using a filtered back projection algorithm with the software
292   provided by the manufacturer (see **Table of Materials**).
293
294   5.1.1. Select the **Recon** tab at the top of the screen. Select the **Project** and **Acquisition** tab.
295
296   5.1.2. Select the recon template associate with filtered back projection. Hit the **Start Recon**
297   button.
298
299   5.2. Identify and segment the pulmonary valve using image processing software. This requires
300   prior knowledge of the anatomy of the pulmonary valve[17]. At elevated arterial pressures, the
301   leaflets coapt and block the lumen of the pulmonary valve.
302
303   5.3. Identify the slicing orientation with respect to the scanning direction and sample. Reorient
304   the sample such that the slicing direction is aligned with the desired axis.
305
306   5.4. Identify regions of interest for high-resolution imaging. In this experiment, the belly (middle)
307   of the leaflet and the arterio-valvular junction was chosen.
308

309 5.5. Remove the excess resin and sample by either razor blade or sanding for large pieces of
310 material, or by microtome for finer pieces.

312 5.6. Once at a location of interest, compare the physical specimen with virtual µCT slices to
313 confirm location. This was done by comparing anatomical features at the cross section.

315 **6. Serial block face scanning electron microscopy**[18]

317 6.1. Reduce the cross-section of the specimen to accommodate SBF-SEM, approximately 2.0 x
318 1.5 x 1.8 mm.

320 6.2. Mount the trimmed sample onto the SBF-SEM aluminum stub using epoxy.

322 6.3. Coat the specimen block with 35 nm gold. Spin the sample on the platform to apply an even
323 layer of coating.

325 6.4. Vent the SBF-SEM chamber and adjust the height of the knife blade to microscope eucentric
326 height.

328 6.5. Insert the sample and level the sample stub.

330 6.6. Image under low vacuum conditions to prevent charging and with backscatter detector (see
331 **Table S2** for SBF-SEM acquisition parameters).

333 6.7. Image and stitch multiple regions of interest at different magnifications using automated
334 software (see **Table of Materials**).

336 **REPRESENTATIVE RESULTS:**
337 Anastomosis of the pulmonary artery to the pressurization tubing is shown in **Figure 1A**.
338 Following the application of hydrostatic pressure, the pulmonary trunk distends radially (**Figure
339 1B**) indicating that the pulmonary valve leaflets are in a closed configuration. Pulmonary valve
340 conformation was confirmed by µCT. In this case, the leaflets were coapt (closed) and the annulus
341 was circular (**Figure 2A**). **Figure 2B,C** shows varying degrees of inadequate pulmonary valve
342 pressurization by either fixation (**Figure 2B**) or arterial collapse (**Figure 2C**).

344 Sample block trimming was guided by the µCT volume rendering. In this case, the plane parallel
345 to the sino-tubular junction was chosen as the slicing direction. Using anatomical landmarks, the
346 µCT volume rendering virtual cross sections was correlated with optical images (**Figure 3**) to
347 confirm the slicing direction and location.

349 Once the specimen block was at the desired location and orientation, high-resolution SBF-SEM
350 images were taken at a local region within a leaflet. Image correlation was done between the
351 µCT volume rendering virtual slice (**Figure 4A**), low-resolution SBF-SEM images (**Figure 4B**), and
352 high-resolution SBF-SEM images (**Figure 4C**). Because of the manual sample mounting, requisite

353 slices of the specimen block were needed to create a flat surface before acquiring images in the
354 SBF-SEM; hence, the different locations between **Figure 3** and **Figure 4**.

355

356 A full image correlation between µCT and SBF-SEM data sets can be seen in **Video 1**. The
357 pulmonary valve specimen in the µCT volume rendering can be easily discerned from the
358 surrounding embedding resin because of the staining of heavy metal atoms. Lengths and angles
359 are measured in the image to guide the slicing. In this example, the plane parallel to the sino-
360 tubular junction was used. A virtual slice through emulates the removal of the material until the
361 depth of interest is reached. High-resolution images taken by SBF-SEM were taken at this cross
362 section and registered to the µCT data set.

363

364 Once acquired, high-resolution images taken by SBF-SEM can be imported into an image
365 processor and compiled into a 3D representation (**Figure 5**) where extracellular components can
366 be identified.

367

368 **FIGURE AND TABLE LEGENDS:**

369

370 **Figure 1: Representative images of anastomosed pulmonary trunk.** The excised pulmonary
371 trunk (**A**) before and (**B**) after hydrostatic pressurization. The dotted line indicates the ventriculo-
372 arterial junction where the annulus of the pulmonary trunk resides. Note the pulmonary trunk
373 distention upon pressurization.

374

375 **Figure 2: Representative µCT volume rendering pulmonary valve.** (**A**) The pulmonary valve is in
376 a closed position with the leaflets adequately stretched and coapt (circle). (**B,C**) Inadequate
377 pressurization of the pulmonary valve. Note that the leaflets are not properly coapt (**B**) and that
378 the annulus is not circular (**C**).

379

380 **Figure 3: Image correlation of pulmonary valve specimen block.** (**A**) µCT volume rendering
381 virtual slice and (**B**) physical specimen block after trimming taken by optical microscopy. Sections
382 of pulmonary valve leaflets are circled in red and were used as landmarks to correlate the two
383 different imaging methods. Scale bar corresponds to 500 µm.

384

385 **Figure 4**: **Image correlation of imaged pulmonary valve cross section.** (**A**) Virtual cross section
386 generated by µCT volume rendering. Red box indicates the region that was imaged using SBF-
387 SEM in (**B**). (**B**) Low-resolution overview images to correlate with µCT cross section. Blue box
388 represents the location of (**C**) high-resolution SBF-SEM imaging. Scale bars correspond to (**B**) 100
389 µm and (**C**) 10 µm.

390

391 **Figure 5: Segmented region of the pulmonary valve taken by SBF-SEM.** Cross-sectional images
392 were stacked and compiled to form a 3D representation of a local pulmonary valve region. Labels
393 were assigned to endothelial cells (green), valvular interstitial cells (blue), and extracellular fibers
394 (yellow). The approximate dimensions of the imaged region are 30 µm x 20 µm x 100 µm.

395

396 **Table S1: Imaging parameters for µCT.**

397
Table S2: Imaging parameters for SBF-SEM.

399
Video 1: Image correction of µCT and SBF-SEM data sets.

401
DISCUSSION:
Removal of the ventricles serves two purposes. First, exposing the ventricle side to the atmospheric pressure, thereby only needing to apply a transvalvular pressure from the arterial side of the pulmonary valve to close, and second, providing a stable base to prevent twisting of the pulmonary trunk. During pressurization, the pulmonary trunk distends radially and inferiorly, making it prone to twisting, causing the collapse of the pulmonary trunk. Preloading the pulmonary valve with a saline solution offers an additional quality check to ensure that the pressurization is adequate and if there are any leaks in the system. The action of the primary fixative is quick, in the order of a few seconds, and without hydrostatic preloading with the saline solution, the pulmonary valve is fixed in a random conformation. Without preloading, the success rate for a closed pulmonary valve was around 10%–20%. With the preloading step, the success rate was above 90%.

The µCT and SBF-SEM imaging conditions were tuned for this application. The pulmonary valve, when fully stretched, can be less than 10 µm thick. As a rule of thumb, a threshold of 3 voxels is required for being able to resolve a feature; so the µCT volume renderings were scanned with a voxel size of 2.9 µm with a field of view of 8.4 x 8.4 x 6.3 mm. Smaller voxel sizes can be achieved in µCT but this requires either sample trimming and/or longer scan times. A smaller sample would allow higher resolution by placing it closer to the x-ray source. Smaller voxels can also be achieved by placing the x-ray detector further back from the sample; however, this will decrease the total flux on the detector and compromise the signal-to-noise ratio. As a reference, our µCT scans were approximately 5–6 h in duration. Specific imaging conditions used in this study are in the **Supplementary Tables S1** and **Supplementary Tables S2**).

There are limitations to this method. The surgical portion of this procedure requires expertise in animal handling to not compromise the pulmonary valve structure while handling. Additionally, the imaging is time-intensive and requires multiple imaging instruments. As a reference, the high-resolution SBF-SEM imaging was approximately 1 week of the continuous imaging for a depth of around 100 µm. This is a demanding task for the instrument to remain stable and consistent for long imaging sessions. A more practical approach would be to devise a sampling strategy to precisely portray the heterogeneity of the pulmonary valve without the time investment. This is yet to be determined. To date, the entire correlative workflow has been done on one mouse but has shown the feasibility and potential of the correlative workflow in investigating the pulmonary valve across length scales.

Future iterations of this correlative approach may involve *in situ* µCT experiments, such that the same sample can be exposed to different transvalvular pressure to remove sample-to-sample variation. This is currently limited by sample and instrument stability for extended scan times, a pressurization apparatus integrated into imaging systems, and contrast due to similar

441 attenuation coefficient of water and tissue. Additionally, though the transvalvular pressures were
442 reflective of physiological conditions, it is not representative of the pulsatile flow that is
443 characteristic of cardiac contraction. However, it has been shown that strain rate has little effect
444 on the conformation of the leaflet. In future iterations, it might prove more relevant to engineer
445 a device capable of administering pulsatile flow[9]. Additionally, much of the work requires manual
446 interrogation of the sample, as currently there is no automated workflow. Locating the
447 pulmonary valve, sample processing toward the region of interest, image correlation, and
448 registration were done manually, but would prove useful in the future to streamline processing
449 and mitigate subjectivity.
450
451 The work presented is a correlative workflow for fixing the conformation of the pulmonary valve
452 and registering imaging in μCT and SBF-SEM. The information obtained using this method will
453 ultimately be utilized to determine the underlying biomechanics of the pulmonary valve in
454 murine animal models, which have yet to be elucidated. Valvular biomechanics can be
455 completely described by its geometry and extracellular matrix, but these are on two different
456 length scales. To do this, precise control of the heterogeneity of the valve and accurate mapping
457 of high-resolution images of the extracellular matrix with respect to its location within the
458 pulmonary valve is needed. This correlative workflow is already being implemented into other
459 experiments to draw comparisons between wild-type and transgenic osteogenesis imperfecta
460 mice to compare fibrillar extracellular matrix differences and can readily be extrapolated to other
461 congenital defects such as bicuspid valve formation[19,20]. This information coupled with possible
462 proteomics will provide a complete picture of how the biomechanics will differ between the two
463 murine animal models.
464
465 Despite this work only portraying the pulmonary valve, this workflow is readily amendable to
466 other heterogeneous, hierarchical biological systems. We utilized 3D imaging techniques to
467 capture the architectural organization of the ECM, but higher resolution techniques, such as
468 transmission electron microscopy or scanning transmission electron microscopy, can be
469 appended depending on the information desired.
470
474
475 **DISCLOSURES:**
476 The authors have nothing to disclose.
477
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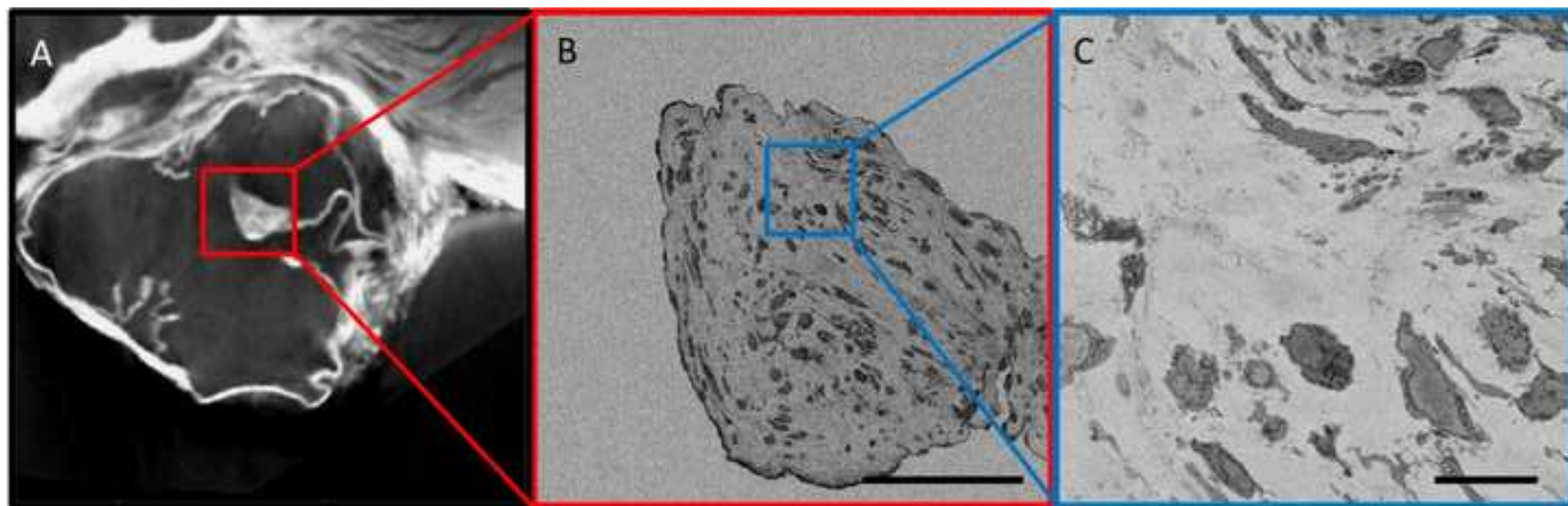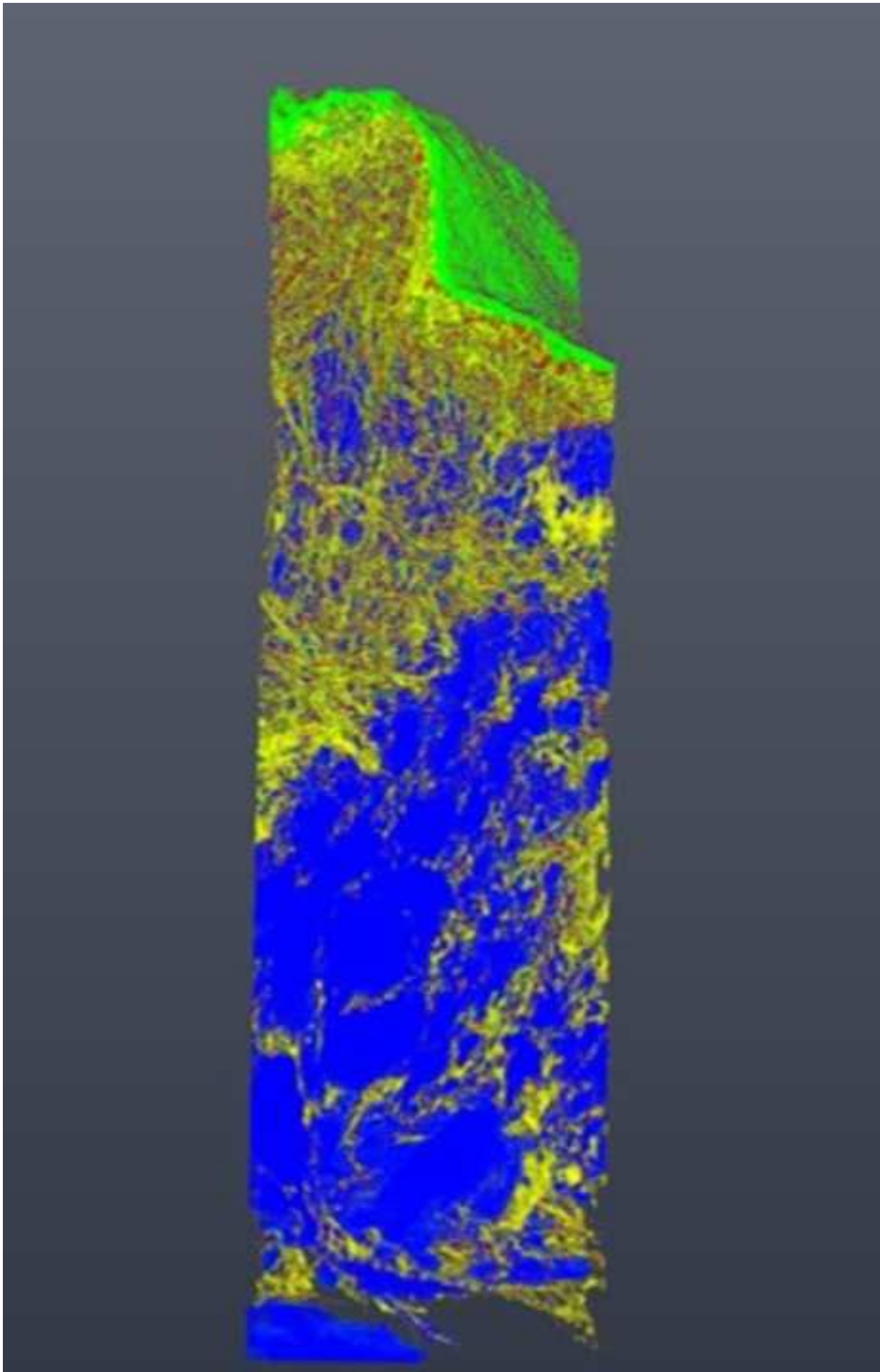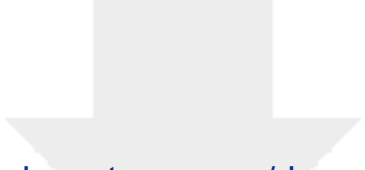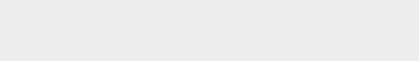
Figure 1

Figure 2
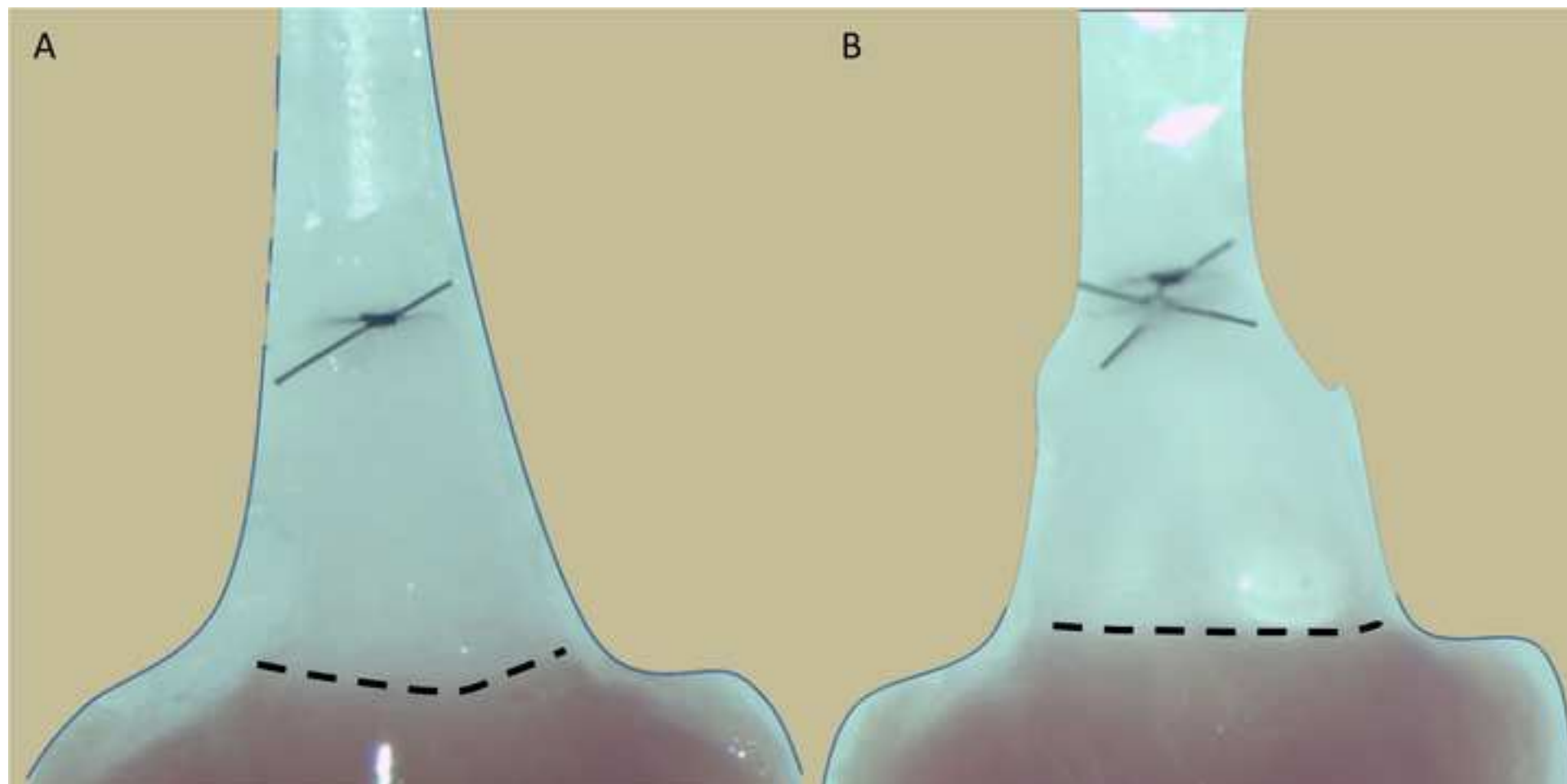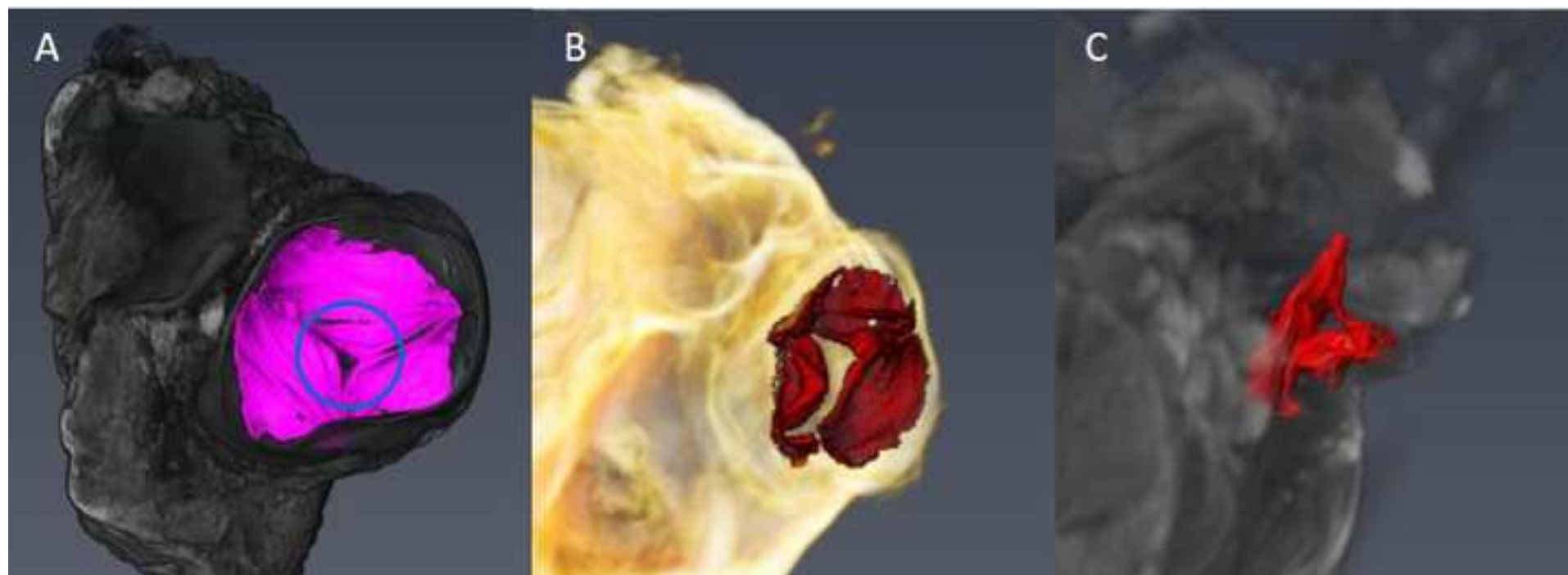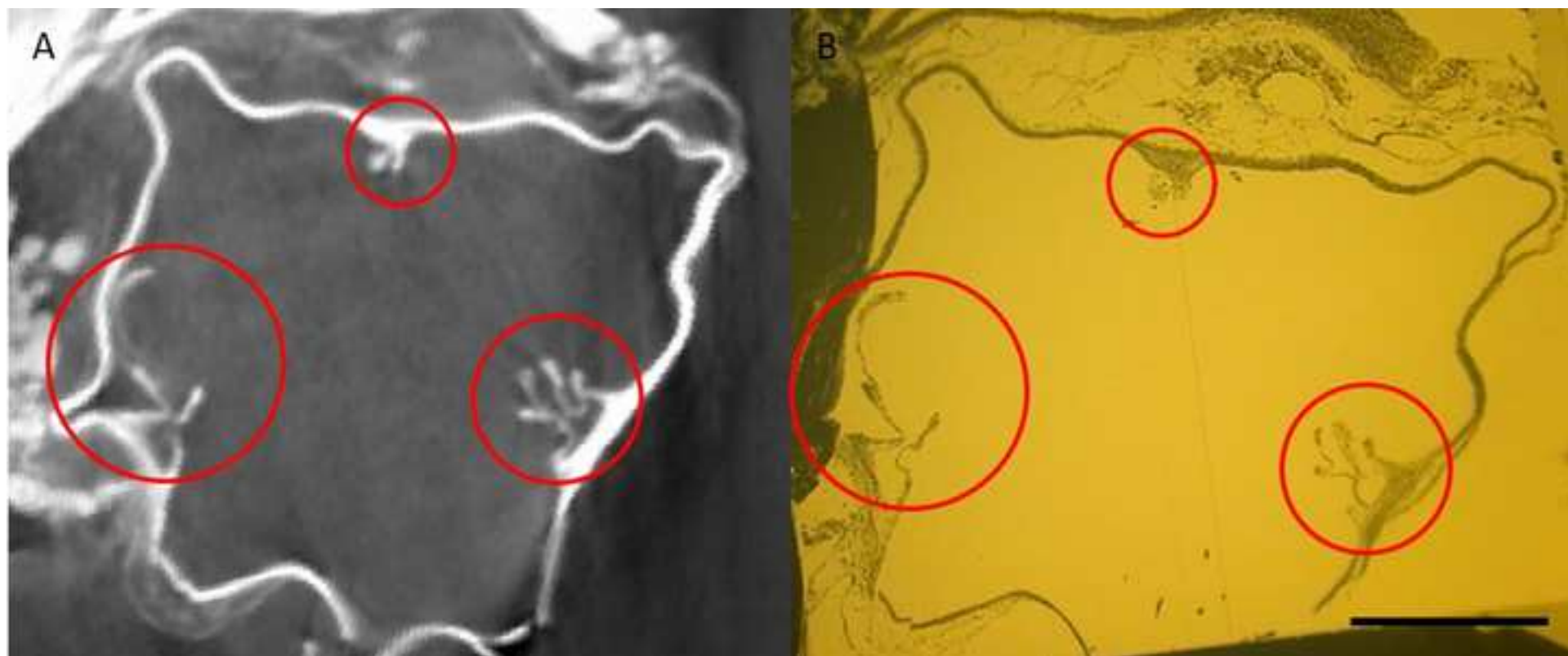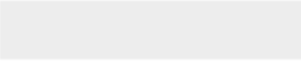
Figure 3

Figure 4

Figure 5

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Media1.mp4

Supplemental
Table S1: Imaging parameters for µCT

Tube potential	70 kV
Tube current	75 µA
Focus mode	M
Trajectory	Circular
Projections/Revolution	2880
Mode	3040 x 3040 px
Averaging	1
Exposure time	1.0 s
Sample-to-gun distance	15 mm
Detector-to-gun distance	725 mm
Voxel size	2.9 µm
Field of view	8.4 x 8.4 x 6.3 mm

Supplmentary

Table S2: Imaging parameters for SBF-SEM

Landing energy	2 - 2.5 kV
Beam current	100 - 400 pA
Working distance	6.5 - 7 mm
Detector	VS-DBS
Dwell time	1 - 2 µs

Table of Materials

Reagent	Company
25% glutaraldehyde (aq)	EMS
0.9% sodium chloride injection	Hospira Inc.
1 mL syringe	BD
10 mL syringe	BD
200 proof ethanol	EMS
22G needle	BD
3 mL syringe	BD
3-way stopcock	Smiths Medical ASD, Inc.
4% osmium tetroxide	EMS
4% paraformaldehyde (aq)	EMS
Absorbable hemostat	Ethicon
Acetone	EMS
Black polyamide monofilament suture, 10-0	AROSurgical instruments Corporation
Black polyamide monofilament suture, 6-0	AROSurgical instruments Corporation
C57BL/6 mice	Jackson Laboratories
Calcium chloride	Sigma-Aldrich
Clamp applying forcep	FST
Cotton tip applicators	Fisher Scientific
DPBS	Gibco
Dumont #5 forcep	FST
Dumont #5/45 forceps	FST
Dumont #7 fine forcep	FST
Durcupan ACM resin	EMS
Fine scissor	FST
Heliscan microCT	Thermo Fisher Scientific
Ketamine hydrochloride injection	Hospira Inc.
L-aspartic acid	Sigma-Aldrich
Lead nitrate	EMS
low-vacuum backscatter detector	Thermo Fisher Scientific
Micro-adson forcep	FST
Millex-GP filter, 0.22 um, PES 33mm, non-sterile	EMD Millipore
Non-woven songes	McKesson Corp.
Potassium hexacyanoferrate(II) trihydrate	Sigma-Aldrich
Potassium hydroxide	Sigma-Aldrich
Pressure monitor line	Smiths Medical ASD, Inc.
Saline solution (sterile 0.9% sodium chloride)	Hospira Inc.
Size 3 BEEM capsule	EMS
Sodium cacodylate trihydrate	Sigma-Aldrich
Solibri retractors	FST
Sputter, carbon and e-beam coater	Leica
Surgical microscope	Leica
Thiocarbohydrazide (TCH)	EMS
Tish needle holder/forcep	Micrins
Trimmer	Wahl
Uranyl acetate	EMS

Volumescope scanning electron microscope	Thermo Fisher Scientific
Xylazine sterile solution	Akorn Inc.

Catalog Number	Comments/Description
16210	Primary fixative component
NDC 0409-4888-10	
309659	
309604	
15055	
305156	
309657	
MX5311L	
19150	Staining component
157-4-100	Primary fixative component
1961	
10012	
TI38402	
SN-1956	
664	Approximately 1 yo
10043-52-4	
00072-14	
23-400-118	
14190-144	
11251-20	
11251-35	
11274-20	
14040	For embedding
14028-10	
	Micro-CT
NDC 0409-2053	
56-84-8	Staining component
17900	Staining component
VSDBS	SEM backscatter detector
11018-12	
SLGP033NS	
94442000	
14459-95-1	Staining component
1310-58-3	
MX562	
NDC 0409-0138-22	
69910-01	Embedding container
6131-99-3	Buffer
17000-04	
EM ACE600	Gold coater
M80	
21900	Staining component
MI1540	
9854-500	
22400	Staining component

VOLUMESCOPESEM   Serial Block Face Scanning Electron Microscope

NADA# 139-236

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

2. Please address all the comments marked in the manuscript.

   Addressed to the best of my ability.

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   Highlighted.

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After speaking with the authors I think it is most convenient if JoVE brings in a production team to film the highlighted portion of the manuscript. However, since this only covers the animal dissection, we would like to demonstrate the image correlation between the microCT and SEM imaging using the submitted video. In this case, would the video need to be referenced in the text?

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