Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60926R2
Full Title:	Analysis of congenital heart defects in mouse embryos using qualitative and quantitative histological methods
Section/Category:	JoVE Developmental Biology
Keywords:	congenital heart defect, development, mouse, heart, histology, cardiovascular biology, quantitative, microscopy
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Additional Information:	

Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	East Lansing, Michigan, USA

1 **TITLE:**
2 **Analysis of Congenital Heart Defects in Mouse Embryos Using Qualitative and Quantitative**
3 **Histological Methods**
4
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19 **KEYWORDS:**
20 congenital heart defect, development, heart, histology, cardiovascular biology, quantitative,
21 microscopy
22
23 **SUMMARY:**
24 In this protocol, we describe procedures to qualitatively and quantitatively analyze
25 developmental phenotypes in mice associated with congenital heart defects.
26
27 **ABSTRACT:**
28 Congenital heart defects (CHD) are the most common type of birth defect in humans, affecting
29 up to 1% of all live births. However, the underlying causes for CHD are still poorly understood.
30 The developing mouse constitutes a valuable model for the study of CHD, because cardiac
31 developmental programs between mice and humans are highly conserved. The protocol
32 describes in detail how to produce mouse embryos of the desired gestational stage, methods to
33 isolate and preserve the heart for downstream processing, quantitative methods to identify
34 common types of CHD by histology (e.g., ventricular septal defects, atrial septal defects, patent
35 ductus arteriosus), and quantitative histomorphometry methods to measure common muscular
36 compaction phenotypes. These methods articulate all the steps involved in sample preparation,
37 collection, and analysis, allowing scientists to correctly and reproducibly measure CHD.
38
39 **INTRODUCTION:**
40 CHDs are the most common type of birth defect in humans and are the leading cause of birth
41 defect-related deaths[1–6]. Although about 90% of newborn children survive CHD, it is frequently
42 associated with significant morbidity and medical interventions over the years, imposing a heavy
43 burden on the patients' lives and the healthcare system[7–10]. Outside of purely genetic factors,
44 the causes of CHD are poorly understood[4]. Unidentified causes account for ~56–66% of all CHD

cases according to the American Heart Association and other sources[2–4,11]. Well-known factors include genetic mutations, CNVs, de novo single nucleotide variants, and aneuploidy. It is suspected that environmental and dietary factors are also important sources contributing to CHD, as suggested by epidemiological studies linking maternal lifestyle[2,12], economic deprivation, and race[13], and by research into dietary factors such as folic acid[11,14] and the bioactive lipid retinoic acid[15,16]. Investigating the mechanisms and causes of CHD and other cardiovascular defects is important to develop preventive strategies and novel therapeutic options[1,4,17–19].

The developing mouse is a cornerstone model for studying CHD in mammals. However, some of the methods and analyses employed, such as dissections preserving heart morphology, analysis of developmental stages, and identification of CHD-associated defects, can be daunting for scientists that are new to the analysis of murine hearts. The goal of the methods described in this protocol is to offer qualitative and quantitative guidelines for these processes. Thus, in this protocol we explain how to perform timed matings to produce embryos of the desired gestational stage, dissect pregnant females for intact heart recovery (including associated tissues such as the outflow tract), heart fixation and preparation for cryostat sectioning, basic histology methods, quantitative analyses of common heart defects, and qualitative analysis of heart muscle compaction, a common precursor phenotype to some types of CHD.

PROTOCOL:

All animals used in the experiments referenced in this paper were treated using the animal care guidelines of the Michigan State University Institutional Animal Care and Use Committee (IACUC).

1. Timed mating of C57BL6/J mice for embryo production

1.1. Once mice have reached breeding age (6–8 weeks), put them together in harem breeding format (i.e., two females per one male). Set them up for breeding sometime in the afternoon or evening.

NOTE: Mice often breed within 1 h after lights have been turned off within their housing facility. Females should be retired from breeding at about 6–8 months and males should be used no later than 12 months of age.

1.2. If using the weigh-in method to determine fertilization, breed mice in large groups, because the weigh-in method causes timed mating to proceed at a slower pace than methods in which only copulation plugs are monitored.

1.3. The next morning, check for copulation plugs sometime between 8 AM–12 PM. Ensure that this is done at the correct times. If the plug assay is performed earlier than 8 AM, there is a risk that the plug is too deep in the vaginal canal to be observed. If the plug assay is performed later than 12 PM, there is a risk of the plug having fallen out.

1.3.1. Grab the female by the base of the tail and investigate the opening to the vaginal canal

1

89   with the use of a micropipette tip. A copulation plug will look like a mucosal barrier (**Figure 1A**),
90   which might be a bit difficult to see in some cases. Crustiness also is an indication that a
91   copulation plug is or was present. If there is no mucosal barrier (**Figure 1B**) then the female likely
92   has not mated or the copulation plug could have already fallen out.

93

94   1.3.2. Refer to the morning of copulation as e0.5.

95

96   NOTE: With C57BL6/J mice, copulation plugs can be difficult to identify. Therefore, sometimes
97   the mice mated even though no plug was observed. Copulation does not always lead to
98   conception. This is often more likely in C57BL6/J mice. Therefore, monitor weight progression to
99   confirm pregnancy (see step 1.4). The weigh ins can help identify females that are pregnant and
100   confirm that plugs led to conception. Take caution when mating females for consecutive nights.
101   If a particular male is observed to be good at producing a copulation plug, that male can be
102   trusted to mate for consecutive nights with the same female.

103

104   1.3.3. Keep in mind that the chances of pregnancy are higher with mice that are proven breeders,
105   as they have successfully been bred before. To increase the chances of having proven breeders,
106   the males used should not be too old.

107

108   1.4. Following the plug assay, weigh the females. This weighing should be followed up 7–10 days
109   later. If weight gain exceeds 1.73 g, then the mouse is likely pregnant[20]. If weight gain is below
110   1.73 g, it is likely unrelated to pregnancy and the mouse should be reintroduced to the breeding
111   population.

112

113   NOTE: C57BL6/J mice produce small litters (five to six embryos on average). Weigh in methods
114   to determine pregnancy are accurate for the majority. Using this method provides a 12.8% false
115   positive rate and will exclude 3% of females that are actually pregnant[20]. If the researcher is
116   worried that the desired gestational time is later, proceed to step 1.5

117

118   1.5. Optional: The day when the dissection is going to take place, ensure that pregnancy has
119   progressed by physical inspection. Grab the female by the base of the tail and stretch the body
120   out. This is easiest to do by placing the mouse on the wrist and gently pulling the base of the tail.
121   If the female is pregnant, then there likely will be weight gain specifically in the lower torso, and
122   it will appear as small lumps.

123

124   NOTE: Pregnancy can be palpable as early as e12.5 and will be palpable by e14.5 in most cases.
125   C57BL6/J mice have small litter sizes, therefore the female might be pregnant even if there are
126   no physical signs.

127

128   **2. Dissection of females and embryos for heart recovery**

129

130   2.1. Prior to the start of the dissection, prepare the following solutions at room temperature.

131

132   2.1.1. Dissolve ethylenediaminetetraacetic acid (EDTA) into phosphate buffered saline (PBS) at a

133 0.5 mM concentration.

134

135 NOTE: Once prepared, it is preferable to keep the solution on ice and only use it at 4 °C. If
136 contamination is a concern, it should be autoclaved prior to use.

137

138 2.1.2. Dissolve paraformaldehyde (PFA) into PBS at a 4% concentration.

139

140 NOTE: Once prepared, it is preferable to keep the solution on ice and only use it at 4 °C. Store at
141 4 °C or colder for long-term storage. The concentration of the PFA solution can vary depending
142 on the downstream applications of the tissue samples. This percentage is used for hematoxylin
143 and eosin staining, immunohistochemistry, and immunofluorescent staining.

144

145 2.2. Once the female has reached the desired gestational stage, the mouse should be euthanized
146 at the correct time of day according to the approved guidelines of animal use. If timings are on
147 the half day (e.g., e15.5) they should be sacrificed close to 12 PM. If timings are on whole days
148 (e.g., e15.0) they should be sacrificed close to 12 AM.

149

150 NOTE: Conception is assumed to occur near midnight during the night the mice are paired.
151 However, it is difficult to determine the exact time of conception. Due to this, timing is estimated,
152 not exact.

153

154 2.3. After pinning the four limbs down, carefully make an I-shaped incision along the torso,
155 starting from around the urethra up towards the sternum.

156

157 2.4. The uterus will likely be located just above the pelvic region. The mouse uterus is Y-shaped;
158 therefore, it will likely be very long and wrapped around the torso. Remove it by gently lifting it.
159 Do this using the sides of fine forceps in a scooping motion. The superficial tissues of the uterus
160 can be grabbed very carefully by the tips of the fine forceps when initially pulling the uterus out.
161 Use scissors to cut the uterus from the body.

162

163 NOTE: Keeping in mind the unusual Y-shaped of the uterus, make sure the entire uterus is
164 removed.

165

166 2.5. Soak the uterus in ice-cold PBS. Pin one end of the uterus down and stretch it out into a
167 single line.

168

169 2.6. Cut the uterus into sections, each section containing a separate embryo. Gently peel out the
170 embryo with fine forceps. This is easiest to do with a pair of forceps in each hand. Once removed,
171 place the embryo immediately into a new Petri dish filled with PBS-EDTA solution at 4 °C.

172

173 NOTE: Normal PBS solution can also be used, but the EDTA prevents coagulation in the samples.

174

175 2.7. Stage the embryos using Theiler Staging[21]. Because staging can vary between embryos within
176 a single litter, stage each individual embryo.

3

177

178 NOTE: This can also provide foresight into the possible congenital heart defects in the embryos.
179 Typically, symptoms of a congenital heart defect can be observed in the embryonic morphology.
180 Other major defects often associated with certain heart conditions can be present.

181

182 2.8. The heart may be removed in a variety of ways depending on the age of the embryo.

183

184 NOTE: If it is unclear whether or not the outflow tract will remain intact during removal, either
185 remove more tissues than solely the heart (keeping the outflow tract intact and minimizing direct
186 contact between the heart and the forceps) or analyze the whole embryo.

187

188 2.8.1. Using a dissection scope and two pairs of fine forceps, position the embryo on its back and
189 stabilize access to the chest. This can be done by either pinning the arms down with the forceps
190 or removing the arms and holding the torso in position.

191

192 2.8.2. Use the sharp point of a second pair of fine forceps to make an incision along the sternum
193 of the embryo and extend between the clavicles to the navel. Start by making very fine and
194 superficial incisions while gradually working towards the inside of the chest cavity. The heart
195 should just barely become visible. Do not contact it with the forceps during these superficial
196 incisions.

197

198 2.8.3. Peel the rib cage open using the first pair of forceps to gently squeeze on the torso of the
199 embryo, slightly caudal to the rib cage. The heart should pop out or become apparent. The heart
200 might require some gentle coaxing to come out. Use the side of the forceps, making sure that the
201 point of the forceps never comes in contact with the heart. To keep the workspace and forceps
202 clean, keep a lint-free wipe nearby.

203

204 2.8.4. Gently grab the pulmonary blood vessels with the sides of the forceps, making sure to not
205 sever the tissue, and pull the heart out of the chest cavity. Then, clean the heart.

206

207 NOTE: For embryos e13.5 and younger the heart is covered by the pericardium. This must be
208 removed in order to reach the heart.

209

210 2.9. Using a stereoscope, take a photograph of the heart for later reference.

211

212 2.10. Rinse the hearts in PBS-EDTA solution for 1–2 min, and then place them into 4% PFA
213 solution for approximately 45–60 min to fix them. If analysis of the whole embryo is desired, soak
214 overnight. The volume of the PFA solution should be 5–6x the volume of the tissue being fixed.

215

216 NOTE: This incubation time can vary depending on the later studies. This incubation time is used
217 for hematoxylin and eosin staining, immunohistochemistry, and immunofluorescent staining.

218

219 2.11. Wash 5–10 min with PBS-glycine 3x and place back in PBS. Sodium azide or
220 penicillin/streptomycin can also be added to minimize bacterial growth and preserve intact

4

221 tissues. The hearts can now be stored short-term at 4 °C.
222
223 **3. Tissue preparation**
224
225 NOTE: Tissues can either be prepared using OCT (optimal cutting temperature) embedding or
226 paraffin embedding. There are advantages and disadvantages to either method and the goal of
227 the analysis should be considered when deciding which embedding method should be used.
228
229 3.1. OCT embedding
230
231 NOTE: This method may be preferable to formalin/paraffin embedding because cryosections
232 preserve antigen reactivity, can still be used for hematoxylin and eosin staining, and produce
233 slices faster.
234
235 3.1.1. Prepare a solution of sucrose dissolved into PBS at a 30% (w/v) concentration.
236
237 3.1.2. Transfer the tissues to a new 15 mL conical tube or 1.5 mL microcentrifuge tube containing
238 PBS-30% sucrose. Initially, the tissue will float.
239
240 3.1.3. Place it at 4 °C in the fridge. The tissue will be ready for OCT embedding when it sinks to
241 the bottom of the tube, usually in 24–40 h.
242
243 NOTE: Muscle tissues suffer significantly during freeze/thaw cycles and lose their native
244 morphology. Sucrose is absorbed by the tissue and works as a cryopreserving agent that allows
245 improved preservation of the morphology. Make sure there is enough solution in the tube for
246 the floating of the tissues to be sufficiently monitored. PBS-sucrose is easily contaminated with
247 bacteria, so check the solution frequently for cloudiness, which indicates either fungal or
248 bacterial overgrowth. To minimize the risk of contamination either penicillin/streptomycin or
249 sodium azide can be added to the PBS-sucrose solution.
250
251 3.1.4. Remove the hearts with a Pasteur pipette, make sure the opening of the pipette is wide
252 enough to not tear the tissue. Cut the pipette with scissors if necessary. If working with whole
253 embryos, use forceps to retrieve the sample.
254
255 NOTE: Do this gently. Even if the pipette opening is wide enough to retrieve the hearts, the edges
256 of the pipette can still tear the tissues. Forceps can indent tissue samples if used too aggressively.
257
258 3.1.5. Briefly allow the tissue to air dry on a lint-free wipe. Place the sample in an OCT mold in
259 the desired position for cutting (i.e., sagittal, transverse, coronal). Add OCT compound until the
260 sample is completely submerged, making sure there are no bubbles in contact with the tissue.
261
262 3.1.6. Place the mold at -20 °C overnight or several days and then move the mold to -80 °C. After
263 24 h the mold is ready for cryosectioning. Tissues in this format can be stored long-term.
264

5

265 3.2. Paraffin Embedding

266

267 3.2.1. Using ethanol, dehydrate the tissues in this succession: 50% ethanol for 10 min, 70%
268 ethanol for 10 min, 80% ethanol for 10 min, 95% ethanol for 10 min, 100% ethanol for 10 min
269 (do this 3x).

270

271 3.2.2. Soak the tissues in a series of ethanol/xylene solutions in this succession: 2:1
272 ethanol/xylene solution for 10–15 min, 1:1 ethanol/xylene solution for 10–15 min, 1:2
273 ethanol/xylene solution for 10–15 min, 100% xylene for 10–15 min (do this 3x).

274

275 3.2.3. To replace the xylene with paraffin, soak the tissues in a vacuum oven (set between 54–58
276 °C) in this succession: 2:1 xylene/paraffin solution for 30 min, 1:1 xylene/paraffin solution for 30
277 min, 1:2 xylene/paraffin solution for 30 min, 100% paraffin for 1–2 h, 100% paraffin for 1–2 h or
278 overnight.

279

280 NOTE: Heating the tissues beyond 60 °C for extended periods of time will cause the paraffin
281 polymers to degrade and the tissue to become brittle.

282

283 3.2.4. Using fresh paraffin, embed the tissues in the desired position.

284

285 **4. Cryostat sectioning for OCT embedded tissues**

286

287 4.1. At the cryostat, place all samples inside for a minimum of 1 h if the samples were previously
288 stored in the -80 °C freezer. They should remain there until all of the sections have been collected.

289

290 4.2. Make sure the cryostat is set at the correct temperature settings. The environmental
291 temperature inside the cryostat chamber should be at about -20 °C and the arm of the cryostat
292 should be at about -24 °C.

293

294 NOTE: This temperature can vary depending on how the block responds to slicing. If inconsistent
295 slicing is an issue, the temperature of the environment could be lowered.

296

297 4.3. Remove the OCT block from its mold by pulling at the edges of the open side of the mold to
298 loosen it and then pinching the block at the closed end, where the mold narrows.

299

300 4.4. To mount the OCT block, place the room temperature OCT on the chuck, starting from the
301 middle and working to the edge. The entire chuck does not need to be covered.

302

303 4.5. Place the OCT block (see step 3.1) on the mold. The edge of the block that was against the
304 closed side of the mold should be facing up on the chuck. Allow the OCT on the chuck to freeze
305 until it is opaque white, about 5–10 min.

306

307 4.6. Place the chuck on the arm for another 5–10 min for the block to come to the arm
308 temperature. To get a parallel slice, adjust the arm until the edge of the block is parallel to the

309　stage and there is an even distance between the stage and the bottom edge of the block is the
310　same as the stage and the top edge of the block.
311
312　4.7. Insert the blade and adjust the distance of the block from the blade to take sections.
313
314　4.8. Take slices at a thickness of 10 µm. Keep slicing until the point of interest is reached either
315　manually or using the trim function.
316
317　4.9. To collect slices, use a small flat paintbrush and a detail brush. When the mold begins to be
318　cut, use the flat brush to gently pull the slice against the stand. When cutting through the sample,
319　the movement must be continuous and without pause, although the speed can depend on the
320　firmness of the sample.
321
322　4.10. While slicing, use the flat brush to gently guide the slice as it is made. The slice does not
323　need to be flush with the stage at this point, so allow it to billow to not cause tears or pulls and
324　affect histology.
325
326　NOTE: When slicing whole embryos, this part can be especially difficult, especially when changing
327　tissue types. Make sure the slices are made with no pauses and the brush does not pull on the
328　slices too aggressively. When tissue types change, the slice can break, so keeping the
329　temperature cold is key. If breaking is an issue, decrease the temperature or increase the
330　thickness of the sample.
331
332　4.11. Pause the movement of the block once the sample is completely cut through, but the slice
333　is not free from the block yet. Without moving the flat brush from the slice, use the detail brush
334　to pat the slice down to make it flat and freeze against the stage.
335
336　4.12. Hold the slice there for ~10 s before removing the detail brush and finishing the slice. Use
337　the two paint brushes to gently flatten the slice against the stage, prevent any rolling, and hold
338　it against the stage for ~20–30 s.
339
340　4.13. Flip the slice and flatten against the stage again. Move an electrostatically charged slide
341　close enough for the slice to be attracted and adhere to the slide without having to touch the
342　slide to the stage. Label the slides with a pencil.
343
344　4.14. Store the slides in an airtight box to protect the tissues from ice buildup during storage. For
345　short-term storage, slides should be stored at -20 °C before staining. For long-term storage keep
346　them at -80 °C.
347
348　**5. Microtome sectioning for paraffin embedded tissues**
349
350　5.1. Place the blocks on ice prior to sectioning in order to chill the samples. When cool, paraffin
351　slices more easily and can produce thinner slices.
352

7

353   5.2. Prepare a 40–45 °C water bath using ultrapure water.

354

355   5.3. Put the blade in the holder within the microtome. Ensure it is secure and then set the
356   clearance angle. Make sure the clearance angle is correct. This can be checked following the
357   manufacturer's instructions.

358

359   5.4. Place the paraffin block into the microtome. Make sure it is correctly oriented so that the
360   blade will cut straight across the block.

361

362   5.5. Ensure the blade is oriented correctly with the block by making a couple of slices. Do this
363   carefully so that adjustments can be made.

364

365   5.6. Trim the block until the point of interest is reached.

366

367   5.7. Make slices at the desired thickness. Take into account that the first few slices will likely be
368   discarded.

369

370   5.8. Pick up the sections and move them into the water bath using forceps. This should make the
371   sections flatten out. Use the forceps to adjust them as needed.

372

373   5.9. Move an electrostatically charged slide close enough for the slice to be attracted and adhere
374   to the slide. Label the slides with a pencil.

375

376   5.10. Place all collected slides into a slide rack. Allow the slides to dry overnight at 37 °C.

377

378   **6. Deparaffinization of tissues**

379

380   6.1. Wash the slides in the following sequence: 3 min in xylene (2x), 3 min in a 1:1 xylene/100%
381   ethanol solution, 3 min in 100% ethanol (2x), 3 min in 95% ethanol, 3 min in 70% ethanol, 3 min
382   in 50% ethanol.

383

384   **7. Hematoxylin and eosin staining**

385

386   7.1. Prepare the slides for staining.

387

388   7.1.1. If using OCT prepared tissues, soak in distilled water for ~4 min until OCT is dissolved and
389   let dry.

390

391   7.1.2. If using paraffin embedded tissues, they must be deparaffinized. See step 6.1.

392

393   7.2. Place the slide in filtered 0.1% hematoxylin for 10 min. Place the slide in distilled water,
394   changing the water 1x immediately and then again after 3 min.

395

396   7.3. Place the slide in 0.5% eosin for 10 s or dip 12x. Dip the slide in distilled water until the eosin

8

397 no longer streaks, about 2–3 dips.

399 7.4. Dehydrate the slide by soaking in 50% ethanol for 10 s, 75% ethanol for 10 s, 95% ethanol
400 for 30 s, and 100% ethanol for 1 min. Dip in xylene until xylene comes out smooth, ~5-7 dips.

402 7.5. Allow xylene to evaporate and mount with a quick drying mounting medium that has a
403 refractive index close to glass.

405 **8. Qualitative analysis of common heart defects**

407 8.1. Take images of all samples using an inverted or stereo microscope and its respective camera.
408 Take macroscopic and microscopic images, depending on the types of defects being analyzed.
409 Macroscopic images can be taken with any magnification below 10x. Microscopic images should
410 be taken at 40x magnification or higher. The photos taken in this paper were taken with 5x
411 magnification using a stereo microscope and a 40x air objective (lens N.A. of 0.55) using an
412 inverted microscope.

414 NOTE: Macroscopic images are a good starting point because they provide a view to the entire
415 heart (**Figure 3**). As patterns become identified, specific areas can be focused on and further
416 investigated.

418 8.2. Line up all of the images side by side on a computer screen. Have all of the images of control
419 samples on one side of the screen and all of the images of experimental samples on the other
420 side of the screen. It is best to analyze one treatment group at a time.

422 8.3. Look for differences in phenotypes between treatment groups, starting at key areas where
423 common heart defects take place. This will vary depending on whether the images are
424 macroscopic, which portray gross anatomy of the heart, or microscopic, which portray
425 microscopic anatomy of the heart tissue.

427 8.4. Begin searching for common macroscopic heart defects, such as ventricular septal defects
428 (**Figure 2A**), atrial septal defects (**Figure 2B**), and patent ductus arteriosus (**Figure 2C**). All of these
429 defects are most easily seen in the transverse view of the heart.

431 8.5. To identify septal defects, consider looking at multiple slices, because they might be
432 observed in one plane of the tissue and not another.

434 NOTE: Sometimes a defect is not the lack of a septum, but a hole in the septum. Therefore, pay
435 careful attention to not mistake a tear for a septal defect. Looking for consistencies between
436 nearby slices in a specific region can confirm if this is actually a defect or a tear from slicing.

438 8.6. To identify defects in the outflow tract, such as the patent ductus arteriosus, use multiple
439 slices to follow the major blood vessels as they leave the heart. Create an image of the major
440 blood vessels relative to one another. An example is featured in **Figure 2C**.

9

441

NOTE: Some irregularities may be due to an embryo's developmental stage (e.g., the patent ductus arteriosus closes postnatally, soon after birth; the ventricular septum does not completely close until ~e14.5). Some other common macroscopic heart defects that are not included in the figures include persistent truncus atereriosus, which can be detected most easily at e16.5 and later; double outlet right ventricle (DORV), which can be detected in slices in which the heart enters the outflow tract; and an overriding aorta[22]. A common microscopic heart defect includes decreased muscle compaction (**Figure 4**).

9. Quantitative analysis of cardiac muscle compaction using hematoxylin and eosin stained tissues

9.1. Using the macroscopic view of the stained tissue samples (**Figure 4A**), identify a region of interest to image at a 40x magnification (**Figure 4B**). For this paper, the wall of the left ventricle was used. Save the image in the desired format. For this paper, images were saved as.tif files.

9.2. Open the image in ImageJ software.

9.3. Set the image to 8-bit. This allows the image to utilize the threshold tool in the next step[23,24]. To do this, select "**Image | Type | 8-bit**".

9.4. Set the threshold of the photo. The purpose of this step is to select only the pixels that represent the background, excluding the pixels that represent the tissues[23,24].

NOTE: This surface area will be subtracted later on. A correct threshold will select the pixels that the researcher wants to exclude from muscle tissue surface area. Therefore, the ending result should include the tissue in grayscale and the background will be selected.

9.4.1. Click on "**ImageJ | Image | Adjust | Threshold**". Move the top bar to make threshold adjustments. Close out the threshold adjustments window without making any other changes once the desired sections are selected[21].

9.5. Use the **Polygon Selections** tool to select the space that the tissue occupies. Carefully make sure the outlines trace the tissue borders, because loose tracings will provide false measurements.

9.6. Adjust the measurement settings on ImageJ so that the software only measures the area of pixels that were selected using the threshold tool in step 9.4[23]. "**ImageJ | Analyze | Set Measurements | *Area* and *Limit to Threshold***". Select only ***Area* and *Limit to Threshold.***

9.7. Measure the area of the highlighted pixels. "**ImageJ | Analyze | Measure**". This value represents the area of the negative space.

9.8. Measure the area of the entire region of interest. This is the area that was selected using the

10

485 **Polygon Selections** tool. "**Image J | Analyze | Set Measurements | *Area***. Select only ***Area*** and
486 deselect ***Limit to Threshold***. Then measure, selecting "**Image J | Analyze | Measure**".

487

488 9.9. Calculate the area the actual muscle tissue is occupying within the region of interest.
489 Subtract the area of the negative space from the area of the entire region of interest. This value
490 is the area of the muscle tissue.

491

492 9.10. Calculate the Muscle Compaction Index (MCI). Divide the area of the muscle tissue by the
493 area of the region of interest.

494

498
$$Muscle\ Compaction\ Index\ (MCI) = \frac{Area\ of\ Muscle\ Tissue}{Area\ of\ the\ Region\ of\ Interest}$$

499

495 The larger the MCI value, the more compacted the muscle is. These MCI values can then be used
496 for statistical analysis to test for significant changes in muscle compaction between treatment
497 groups (**Figure 4C**).

500

501 **REPRESENTATIVE RESULTS:**
502 The muscle compaction index was compared between hearts developing under two different
503 environments, a control and an experimental group. These protocols were used to analyze the
504 compaction of muscle tissue quantitatively, which permitted statistical analysis. Muscle
505 compaction was shown to be significantly reduced in the experimental hearts relative to the
506 embryos that developed in nonexperimental conditions.

507

508 Embryos were discarded if the observation timing seemed inaccurate. Although stunted growth
509 of embryos could be a result of the treatment of the experiment, and there is a range in
510 developmental progress, breeding was spaced out sufficiently to ensure the timing of embryo
511 development. Staging of the embryos was done using Theiler Staging[21]. Samples were considered
512 to be poorly sliced if the slices did not reflect consistency or if they had obvious tears or folds.
513 Staining provided enough contrast to make out the tissue edges, but was not so dark as to make
514 cell features indistinguishable. Slides were then imaged with an inverted microscope with a 40x
515 objective. MCI values were calculated using ImageJ software and Microsoft Excel. These MCI
516 values were analyzed using a T-test.

517

518 **FIGURE AND TABLE LEGENDS:**

519

520 **Figure 1: Plug assay results in C57BL6/J mice.** (**A**) A mouse with an easily identifiable copulation
521 plug. (**B**) A mouse with no copulation plug.

522

523 **Figure 2: Common heart defects.** Schematics of the true anticipated morphology of a congenital
524 heart defect. (**A**) Transverse view of a ventricular septal defect. (**B**) Transverse view of an atrial
525 septal defect. (**C**) Patent ductus arteriosus.

526

527 **Figure 3: Hematoxylin and eosin stain of e15.5 hearts.** (**A**) A stained heart that developed under

11

528 normal conditions. Scale bar = 750 μm. (**B**) A stained heart that developed under experimental
529 conditions. Scale bar = 750 μm.
530
531 **Figure 4: Quantitative analysis of muscle compaction between hearts that developed under**
532 **normal maternal diets and hearts that developed under essential fatty acid deficient maternal**
533 **diets.** (**A**) Hematoxylin and eosin stained hearts at a macroscopic (5x) view. Scale bars = 1,000
534 μm. (**B**) Hematoxylin and eosin stained hearts at microscopic view (40x). Scale bars = 75 μm. (**C**)
535 The resultant data of Muscle Compaction Index measurements. The values were analyzed using
536 a T-test (n = 4 per treatment group).
537
538 **DISCUSSION:**
539 This protocol explores the techniques involved in the analysis of cardiac development in
540 embryonic hearts. Some limitations of this method are the required physical dexterity for the
541 preparatory techniques, which may require practice, and skill with microscope imaging. If the
542 slices obtained at the cryostat are messy, the hematoxylin and eosin staining will not be clear, or
543 if the images taken at the microscope have poor lighting, then the method used with ImageJ will
544 not work. A limitation of the threshold feature of ImageJ software is that it selects pixels located
545 on one end of a threshold dependent on the pixel value. Therefore, any pixels that exist on the
546 wrong side of the threshold will be incorrectly included or excluded in the calculation. Ultimately,
547 troubleshooting will be necessary for adoption of the protocols for use in a study.
548
549 In the event that heart extraction is too difficult, consider skipping to step 2.8. Additionally,
550 consider removing more than just the heart and lungs from the chest cavity. The target then
551 becomes larger and easier to manipulate, and direct contact between the heart and the fine
552 forceps is minimized. When cryostat sectioning, the experimental samples will always be directly
553 compared to control samples. This allows some room for user error so long as the error is
554 consistent in all samples. To minimize user error and avoid false results, make sure comparable
555 sections are obtained from both treatment groups. For example, if more than one person is
556 producing sections, make sure that one individual produces an even number of sections for
557 controls and all treatment groups, and not just one subgroup. Lastly, when thresholding images
558 on ImageJ, the threshold tool is used to allow maximum freedom in selecting pixels that
559 represent tissue and pixels that do not. If necessary, adjust the contrast of the images to further
560 intensify the pixel value of the background from the pixel value of the tissue. Although there are
561 troubleshooting options, the techniques still require a high degree of skill. However, performing
562 this protocol provides access to data that are not typically measured in other cardiac
563 developmental research studies. Thus, utilizing features of this protocol might make a significant
564 contribution to a scientific investigation.
565
566 Identifying the impacts of experimental treatments in cardiology research often is investigated
567 through morphology assessment. This is especially the case in developmental biology, in which
568 the placenta makes it difficult for physiological features of developing hearts to be measured.
569 Therefore, morphological analysis that allows insight into the function of the heart dramatically
570 shifts the trajectory of developmental biology research. Although most papers analyze the
571 thickness of the cardiac muscle to determine the strength of the heart, direct measurement of

12

muscle compaction could provide further insight into the physiology of the developing mammalian heart[25–27].

DISCLOSURES:
The authors have no disclosures to report.

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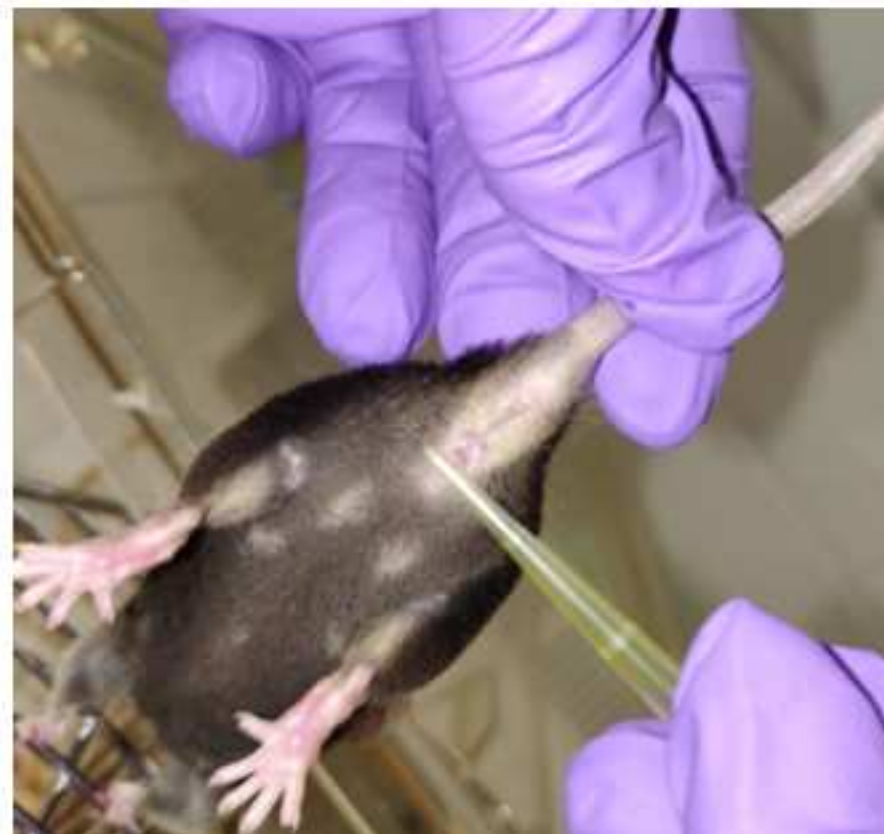
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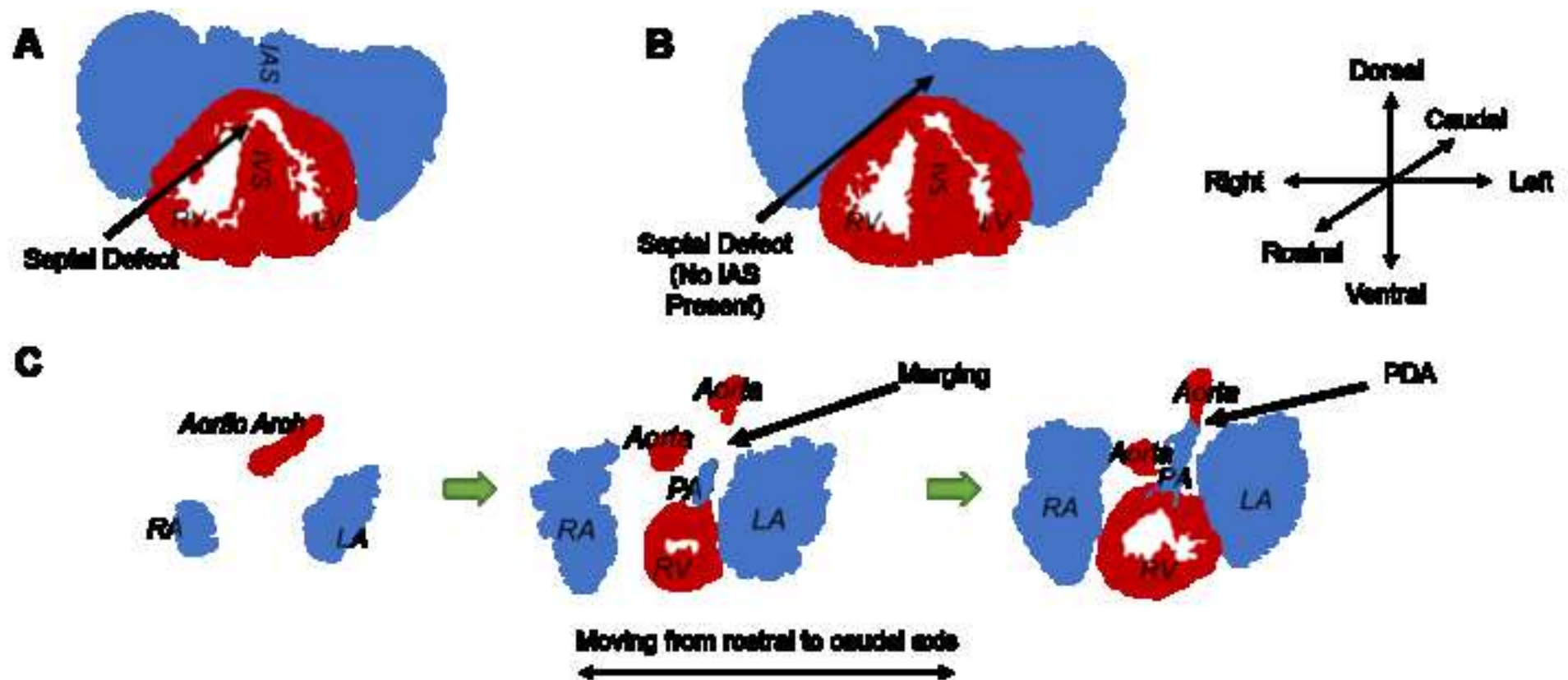636   24.   Reinking,   L.   *Examples   of   Image   Analysis   Using   ImageJ*.
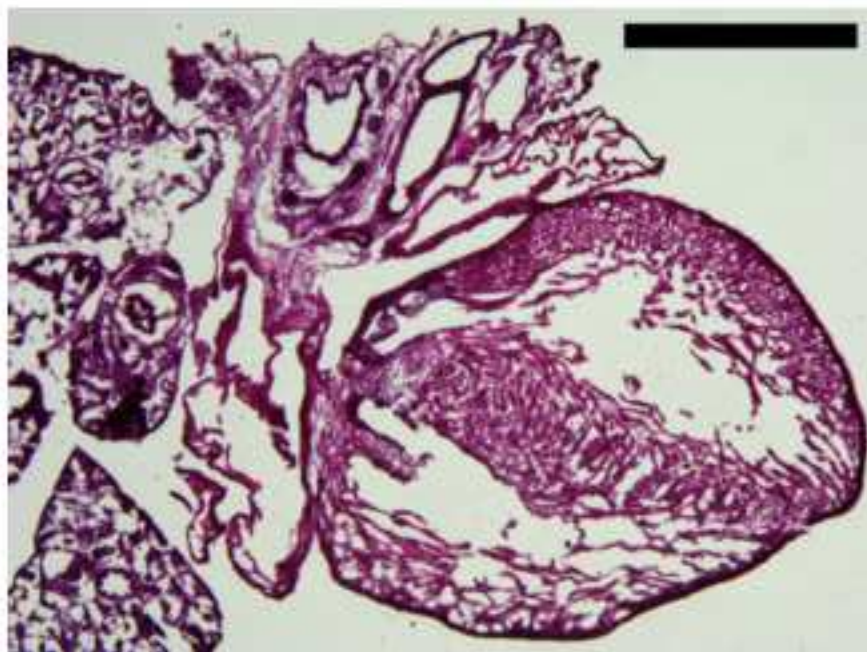637   https://imagej.nih.gov/ij/docs/pdfs/examples.pdf (2007).

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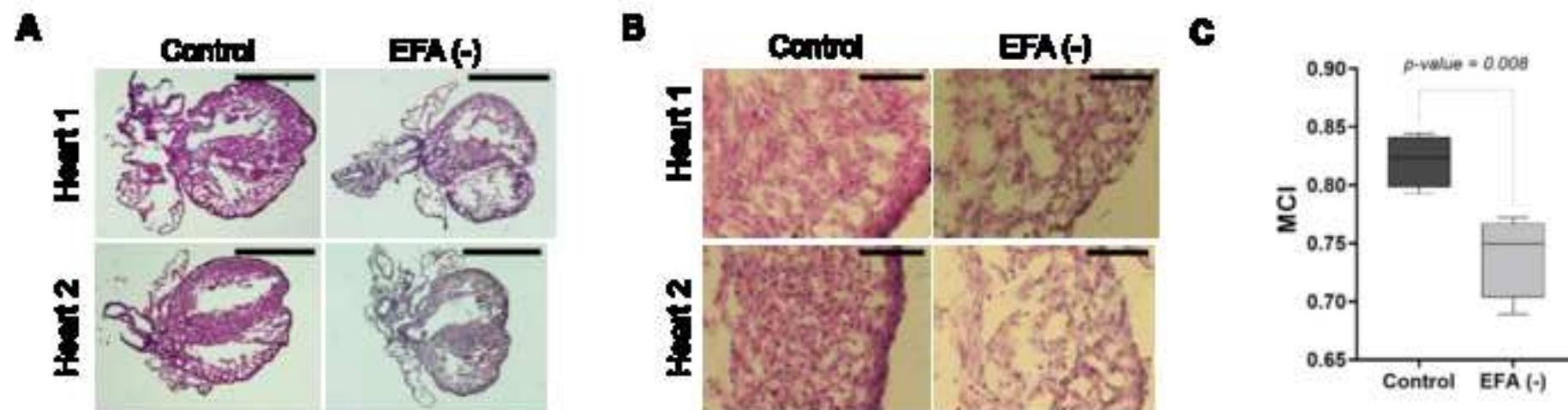640   (2015).

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14

Figure1

Click here to access/download;Figure;Figure1.tif ⬇

Figure2

Figure 2

Figure3

A

B

Figure4

Click here to access/download;Figure;Figure4.tif

A

Control   EFA (-)

Heart 1

Heart 2

B

Control   EFA (-)

Heart 1

Heart 2

C

p-value = 0.008

MCI

0.90

0.85

0.80

0.75

0.70

0.65

Control   EFA (-)

Name of Material/Equipment	Company	Catalog Number	Comments/Description
15 mL Conical Tube(s)	Fisher Scientific	# 1495970C	
C57BL/6J Mice	Jackson Labs	C57BL/6J - stock 000664	
Coplin Staining Jars (x6)	VWR Scientific	# 25457-006	
Coverslips 24X50MM #1.5	VWR Scientific	# 48393-241	
Cryostat - Leica CM3050S	Leica	N/A	
Dissecting Dish(s)	Fisher Scientific	# 50930381	
Dumont #5 - Fine Forceps (x2)	Fine Science Tools	# 11254-20	
Eosin Y Solution	Millipore Sigma	# HT110116-500ML	
Ethyl Alcohol (Pure, 200 proof)	Fisher Scientific	# BP2818-500	
Ethylenediaminetetraacetic acid (EDTA)	Millipore Sigma	# E9884-100G	
Eukitt	Millipore Sigma	# 03989-100ML	
Fine Scissors	Fine Science Tools	# 14060-10	
Fluorescent Stereo Microscope Leica M165 FC	Leica	N/A	
Glycine	Millipore Sigma	# 410225-250G	
Graefe Forceps	Fine Science Tools	# 11052-10	
Graphpad Prism 8 Software	Graphpad		
ImageJ Software	ImageJ		
Kimwipes	Fisher Scientific	# 06666A	
Mayer's hematoxylin solution	Millipore Sigma	# MHS16-500ML	
Micropipette tip(s) - p200	Fisher Scientific	# 02707448	
Microsoft Excel Software	Microsoft		
OCT Compound	VWR Scientific	# 102094-106	
Olympus CkX53 Microscope	Olympus		
Paint Brushes (at least 2)			
Paraformaldehyde	VWR Scientific	# 0215014601	Make into 4% solution (dissolved in PBS)
Pasteur pipette(s)	Fisher Scientific	# 13-711-7M	

Penicillin-Streptomycin	ThermoFisher Scientific	# 15140122	
Phosphate Buffered Saline (PBS)	ThermoFisher Scientific	# 70011044	Dilute from 10x to 1x before using
Scale	Mettler Toledo	# MS1602TS	
Scale	Mettler Toledo	# MS105	
Scalpel Handle #3	VWR Scientific	# 10161-918	
Scapel Blades	VWR Scientific	# 21909-612	
Square Mold	VWR Scientific	# 100500-224	For OCT molds
Sucrose	Millipore Sigma	# S9378-500G	
Superfrost Plus Slides	Fisher Scientific	# 1255015	
Surgical Scissors	Fine Science Tools	# 14002-14	
Tissue-Tek Accu-Edge Disposable Microtome Blades	VWR Scientific	# 25608-964	
Travel Scale	Acculab	VIC 5101	
Xylene	Millipore Sigma	214736-1L	

Please thoroughly revise the language and grammar. Several phrasing errors and errors in verb usage are present.
Trim the title to " analysis of congenital heart defects in mouse embryos" or make it more specific by mentioning the kind of analysis methods.

- The title has been both trimmed to be similar to the suggested title, has described the types of methods the reader can anticipate, and corrected grammar

I have edited the protocol structure to match jove's format

- We have taken note of this revision

Please avoid or limit the use of pronouns her/him/he/she throughout.

- We have changed all instances of her/him/he/she throughout the paper

Update to match the current numbering.

- The text has been revised to reflect these comments throughout the paper.

What temperature is this maintained and used at?

- We have changed both instances in which this comment in this paper.

Lift?

- We have changed the text with this comment in mind. The text now reads as: "Remove it by gently lifting it, making sure to not disturb the uterus."

At what temperature?

- We have revised the text to specify the ideal temperature to use this at.

Needs a reference citation

- We have included a citation at this part of the paper.

I have highlighted some steps for continuity. These are marked in green highlight.

- We have taken note of this revision

Needs grammatical corrections.

- This sentence has been grammatically corrected.

I'm not sure why you say this when the tissue is ready only after Oct embedding.

- This step has been reworded to more clearly represent the intensions of the authors' instructions.

Unclear what reaches the cryostat. Please revise grammar.

- This step has been revised to more clearly represent the intensions of the authors' instructions.

Grammar errors.

- The text has been revised to be grammatically correct.

Grammar error

- The text has been revised to be grammatically correct.

Grammar error

- The text has been revised to be grammatically correct.

Grammar error

- The text has been revised to be grammatically correct.

Replace the commercial term with a generic alternative.

- The text has been changed to avoid referencing commercial names.

Mention specific step numbers

- The text has been revised, the steps are now listed.

Replace the commercial term with a generic alternative.

- The text has been changed to avoid referencing commercial names.

What imaging tool is used? A camera?

Mention all relevant settings e.g., magnification, lens N.A. etc.

- The text has been revised to include more detail. The types of microscopes used, magnifications, and lens N.A. are listed.
- The magnifications for macroscopic and microscopic analysis have also been described.

Needs to be rewritten in the imperative voice like the above steps

- We have revised the text to correct the tone used at this part of the text. We have done so for all three times this comment was mentioned.

Which part of the tissue is this?

- We have revised the text to clarify this confusion.

Remove the commercial name.

- The text has been changed to avoid referencing commercial names.

Remove the commercial name.

- The text has been changed to avoid referencing commercial names.

The scale bars are too small to be seen. Please enlarge them.

Define EFA

- The text has been revised to satisfy the editor's concerns. The scale bars at this part of the figure have been revised so that they are more legible. EFA is now defined in the figure description.

Mention sample sizes.

- The text has been revised in the figure description to include sample sizes used for MCI calculation.

Update.

- The text has been revised to reflect the correct step number.

References?

- The text has been revised to include references for this claim.

Needs grammatical corrections. Unclear what the intention is.

- This portion of the text has been removed due to the editor's concern of the reason for this sentence.