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Corresponding Author:	Hanry Yu National University of Singapore Singapore, SINGAPORE
Corresponding Author's Institution:	National University of Singapore
Corresponding Author E-Mail:	hanry_yu@nuhs.edu.sg
Order of Authors:	Inn Chuan Ng
	Li Zhang
	Narelle Nichola Yi Ying Shen
	Yun Ting Soong
	Chan Way Ng
	Phoebe Kang Sheing Koh
	Yan Zhou
	Hanry Yu

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2 Isolation of Primary Rat Hepatocytes with Multiparameter Perfusion Control
3
4 **AUTHORS AND AFFILIATIONS:**
5 Inn Chuan Ng[1]*, Li Zhang[2]*, Narelle Nichola Yi Ying Shen[3], Yun Ting Soong[4], Chan Way Ng[5],
6 Phoebe Kang Sheing Koh[1], Yan Zhou[3], Hanry Yu[1,3,4,5,6]
7
8 [1]Department of Physiology & The Institute for Digital Medicine (WisDM), Yong Loo Lin School of
9 Medicine, MD9-04-11, 2 Medical Drive, Singapore 117593, Singapore
10 [2]College of Agriculture and Biology, Zhongkai University of Agriculture and Engineering,
11 Guangzhou 510225, China
12 [3]Mechanobiology Institute, National University of Singapore, T-Lab, #05-01, 5A Engineering
13 Drive 1, Singapore 117411, Singapore
14 [4]Institute of Bioengineering and Nanotechnology, A*STAR, The Nanos, #04-01, 31 Biopolis Way,
15 Singapore 138669, Singapore
16 [5]NUS Graduate School for Integrative Sciences and Engineering, Centre for Life Sciences, #05-01,
17 28 Medical Drive, Singapore, 117576, Singapore
18 [6]CAMP, Singapore-MIT Alliance for Research and Technology, 1 CREATE Way, Level 4 Enterprise
19 Wing, Singapore 138602, Singapore
20
21 *These authors contributed equally.
22
23 Email addresses of co-authors:
24 Inn Chuan Ng   (phsnic@nus.edu.sg)
25 Li Zhang   (li_zhang@mail.bnu.edu.cn)
26 Narelle Nichola Yi Ying Shen   (narelleshen97@gmail.com)
27 Yun Ting Soong   (ytsoong@ibn.a-star.edu.sg)
28 Chan Way Ng   (chanwayng@u.nus.edu)
29 Phoebe Kang Sheing Koh   (phskksp@nus.edu.sg)
30 Yan Zhou   (1575773228@qq.com)
31 Hanry Yu   (hanry_yu@nuhs.edu.sg)
32
33 Corresponding author:
34 Hanry Yu   (hanry_yu@nuhs.edu.sg)
35
36 **KEYWORDS:**
37 cell isolation; hepatocyte; two-step; perfusion; cannulation; integrated device
38
39 **SUMMARY:**
40 This protocol details the use of a special intravenous catheter, standardized sterile disposable
41 tubing, temperature control complemented by real-time monitoring, and an alarm system for

42 two-step collagenase perfusion procedure to improve the consistency in the viability, yield, and
43 functionality of isolated primary rat hepatocytes.
44

45 **ABSTRACT:**
46 Primary hepatocytes are widely used in basic research for liver diseases and for toxicity testing
47 *in vitro*. The two-step collagenase perfusion procedure for primary hepatocyte isolation is
48 technically challenging, especially in portal vein cannulation. The procedure is also prone to
49 occasional contamination and variations in perfusion conditions due to difficulties in the
50 assembly, optimization, or maintenance of the perfusion setup. Here, a detailed protocol for an
51 improved two-step collagenase perfusion procedure with multiparameter perfusion control is
52 presented. Primary rat hepatocytes were successfully and reliably isolated by taking the
53 necessary technical precautions at critical steps of the procedure, and by reducing the
54 operational difficulty and mitigating the variability of perfusion parameters through the
55 adoption of a special intravenous catheter, standardized sterile disposable tubing, temperature
56 control, and real-time monitoring and alarm system. The isolated primary rat hepatocytes
57 consistently exhibit high cell viability (85%–95%), yield (2–5 x 10^8 cells per 200–300 g rat) and
58 functionality (albumin, urea and CYP activity). The procedure was complemented by an
59 integrated perfusion system, which is compact enough to be set up in the laminar flow hood to
60 ensure aseptic operation.
61

62 **INTRODUCTION:**
63 Primary hepatocytes are important tools for liver-related basic research, disease treatment, and
64 application such as drug testing. The current gold standard for primary hepatocyte isolation is
65 the two-step collagenase perfusion procedure[1-3] introduced by Seglen in the 1970s[4]. However,
66 this procedure is technically challenging and has a high failure rate when performed by novice
67 surgeons. Even when a perfusion is considered successful, drastic differences in hepatocyte
68 viability (typically 60%–95%) and yield (0.5–5 x 10^8 per 200–300 g rat) may be observed
69 between isolations. This influences the quality and scale of downstream experiments. Apart
70 from the technical procedure, the perfusion setup used for the isolation, either commercially
71 available or custom built, is a contributing factor. Attention must be given to the assembly,
72 optimization, and maintenance of the perfusion setup. The purpose of this protocol is to
73 improve the success rate and stability between isolations of primary rat hepatocytes through
74 multiparameter perfusion control of the technical procedure and perfusion setup of the
75 two-step collagenase perfusion procedure.
76

77 From the technical aspect, the most difficult step in the procedure is the portal vein cannulation.
78 As for the other steps, if good practice is observed and general precautions are taken, the
79 stability of the isolation can be improved. Therefore, understanding of the reasoning for each
80 step is important so that the surgeon could respond to various variables that may occur during
81 the procedure.
82

83   Various protocols for the isolation of hepatocytes and liver non-parenchymal cells from rat and
84   mouse have been published[1,2,5-9]. The perfusion setups used in these protocols had several
85   disadvantages, which include the reuse of perfusion tubing, problems with temperature control,
86   need for routine optimization of perfusion parameters, and/or usage of unsuitable type of
87   intravenous (IV) catheter for portal vein cannulation. The reuse of perfusion tubing will increase
88   the chances of contamination, especially if the tubing was not cleaned and disinfected properly.
89   Reuse of tubing without routine replacement will also expose the perfusion setup to problems
90   such as leaky tubing or connectors, clogged bubble trap and constricted tubing, all which will
91   substantially reduce the perfusate pressure and flow rate, thus, affecting liver digestion
92   efficiency. Without a constant heat source in some setups for temperature control,
93   pre-warmed buffers will cool down over time, leading to low collagenase activity and digestion.
94   Although other setups utilize a jacketed glass condenser connected to a water circulator to
95   warm the buffer, they are bulky and require careful cleaning. Temperature, pressure, and flow
96   rate of buffer exiting the catheter must be measured and optimized before the start of isolation
97   to ensure stable perfusion condition. Even after optimization, the parameters could still change
98   halfway during isolation due to the actions of the operator, thereby leading to suboptimal
99   perfusion and digestion. Most types of IV catheter are not suitable for portal vein cannulation
100   because they do not allow continuous perfusion during cannulation. They are unable to
101   immediately inform the surgeon when the cannulation is successful. Furthermore, it is
102   challenging to secure the portal vein on the soft catheter without deforming it.
103
104   Here, we address these problems using standardized disposable sterile tubing, a silicone heater
105   jacket for precise and stable temperature control, real-time monitoring and alarm system with
106   data storage and management and use of a special IV catheter, which allows continuous
107   perfusion while puncturing portal vein during cannulation. To the best of our knowledge, we
108   are the first group to combine all these features into an integrated perfusion system (IPS) that is
109   compact, making it highly portable and able to be fit into a laminar flow hood to ensure aseptic
110   operation.
111

112   **PROTOCOL:**
113   All procedures and animal housing were carried out under protocol numbers R15-0027 and
114   R19-0669 in accordance with the requirements of the Institutional Animal Care and Use
115   Committee (IACUC) of the National University of Singapore.
116

117   **1.   Preparation of solutions and surgical instruments**
118

119   1.1 Prepare buffers and cell culture media in **Table 1** using ultrapure water.
120

121   1.2 Pre-warm the calcium-free buffer and collagenase buffer to 37 °C in a water bath before
122   use.
123

124 1.3 Autoclave the following surgical instruments and lab equipment: a pair of sharp-blunt
125 surgical scissors, a pair of blunt-blunt surgical scissors, a pair of curved surgical scissors, two
126 pairs of tooth-tissue forceps, two pairs of curved forceps, two vein clips, a 5 cm long 3-0 silk
127 surgical suture, a 100 µm nylon mesh filter, a 400 mL beaker, and a stage (floating
128 microcentrifuge tube rack).

129

130 **2. Setting up the IPS (see Figure 1)**

131

132 2.1 Wipe down the laminar flow hood with 70% ethanol. Wipe down the IPS with 70% ethanol
133 and move it into the laminar flow hood. Sterilize by UV for >15 min before switching on the
134 hood.

135

136 CAUTION: Exposure to UV light can cause painful eyes and skin burns. Ensure that the sash is
137 fully closed when the UV light is turned on.

138

139 2.2 Assemble a new set of disposable tubing onto the IPS. Wrap the tubing downstream of the
140 peristaltic pump in a silicone heater jacket. Assemble the perfusion monitor on the tubing
141 downstream of the silicone heater jacket.

142

143 2.3 Ensure that the roller clamps for both tubing inlets are completely loosened. Connect each
144 tubing inlet to the tapered end of a sterile 2 mL aspiration pipette. Leave both aspiration
145 pipettes (inlets) and the IV catheter (outlet) in a 1 L bottle with calcium-free buffer to allow
146 recirculation of the buffer during subsequent priming steps.

147

148 2.4 Switch on the IPS. Perform the following operations on the touch screen control panel. The
149 software will perform a comprehensive self-test every time it is started.

150

151 CAUTION: The IPS is an electrical equipment which is connected to an external power source.
152 Liquid spills on the IPS or power cables may produce electrical hazards.

153

154 2.4.1   Ensure that self-test status is displayed on the screen (**Figure 2**). Components that have
155 passed the self-test will be shown in green; those that have failed will be shown in red. After
156 rectification, tap the **Testing** icon to repeat the self-test again or tap the **Enter System** icon to
157 enter directly into the operation interface.

158

159 {Place **Figure 2** here}

160

161 2.4.2   Tap anywhere on the screen to log in to the operation interface.

162

163 2.4.3   At the upper-left corner of the operation interface **(Figure 3)**, tap either of the circular
164 arrow icons to set the direction of rotation for the peristaltic pump. At the right panel of the

165 interface, tap the up/down arrow icons to set values for the corresponding parameters. Set the
166 flow to constant flow mode; temperature of the heater jacket to 42 °C (to be adjusted to
167 ensure that the temperature of the perfusate is maintained at 37 °C); and pump speed to 38
168 rpm for a flow rate of ~33 mL/min.

170 2.4.4   At the lower-right panel of the operation interface, check for the status of the bubble
171 alarm, perfusion stop alarm, and mute icon.

173 2.4.5   Check for the temperature, pressure, and flow rate of the perfusate in the left panel.
174 Manually set the intervals by clicking on the up-down arrows at the top and bottom of the
175 columns. Check for the real-time data shown as values at middle of the columns. The color of
176 the column will turn from green to red if the real-time data moves beyond the set range.

178 {Place **Figure 3** here}

180 2.4.6   Locate the icons to start, pause, and stop the perfusion, and the icon to switch from
181 real-time data display to chart mode in the middle panel. Tap the start icon to begin the
182 perfusion and prime the tubing with calcium-free buffer. A new log will be created. Enter the
183 filename and username into the popup window **(Figure 4)**.

185 {Place **Figure 4** here}

187 2.5 Fill the drip chamber to half full. Ensure that there are no trapped air pockets in the tubing
188 during priming.

190 2.6 Remove any bubble that forms downstream of the bubble filter by flicking on the tubing to
191 detach the bubble and allow it to be flushed out.

193 NOTE: Bubbles may form along the tubing as the buffer is warmed up by the heater jacket.

195 2.7 Fill a 400 mL beaker with 200 mL of collagenase buffer. Put the stage on top of the beaker.
196 Without introducing any air into the tubing, completely tighten the roller clamp for one of the
197 tubing inlets and move the aspiration pipette for the inlet into the beaker.

199 **3.   Animal procedure**

201 3.1 Prepare a young adult male Wistar rat around 200–300 g in body weight.

203 NOTE: This protocol was optimized for rats around 200–300 g in body weight. Male rats are
204 preferred since hormonal changes during the estrous cycle in female rats will affect hepatocyte
205 function.

206

207   3.2 Anesthesia

208

209   3.2.1   Draw up the required volume of anti-coagulant heparin (5,000 IU/mL; 0.2 mL/100 g
210   body weight) and rat anesthesia ketamine/xylazine cocktail (37.5 mg/mL ketamine, 5 mg/mL
211   xylazine; 0.2 mL/100 g body weight) into 1 mL syringes with 27 G needle.

212

213   CAUTION: Anesthesia and heparin are harmful substances. Be careful when handling sharps.

214

215   3.2.2   Restrain the rat. Intraperitoneally inject heparin followed by ketamine/xylazine cocktail
216   into the lower-right quadrant of the abdomen.

217

218   NOTE: The caecum has a higher probability of being located at the left. Avoid puncturing the
219   caecum during injection to reduce the risk of contamination.

220

221   3.2.3   After 10 min, check the depth of anesthesia by assessing pedal reflex on both feet of
222   the rat. Continue to check from time to time and wait until the rat no longer responds to toe
223   pinches. Additional anesthesia can be injected, as necessary.

224

225   3.3 Place the rat in a supine position with limbs outstretched on an aluminum foil-covered
226   polystyrene platform on top of a tray. Tape the feet of the rat and pin the tape securely onto
227   the platform with 27 G needles.

228

229   3.4 Disinfect the chest and abdomen by spraying and drenching them with 70% ethanol.
230   Shaving prior to disinfection is optional. Put the rat in the hood. Continue the next step while
231   the fur is still wet.

232

233   NOTE: Drenching with 70% ethanol also keeps dander and fur dust to a minimum.

234

235   3.5 Separate the skin from the muscle.

236

237   3.5.1   With a pair of tooth-tissue forceps in one hand, lift the skin near the base of the
238   abdomen. With a pair of sharp-blunt surgical scissors in the other hand, cut the tented skin.
239   Push the sharp end of the scissors underneath the skin and make a midline incision on the skin
240   from just above the hind legs to just below the fore legs.

241

242   3.5.2   While pulling up and lightly stretching the skin with the forceps, cut any connective
243   tissue holding the skin onto the muscle in the chest and abdomen. To reduce the risk of
244   contamination, prevent loose fur from falling onto the muscle. To remove built-up loose fur on
245   the scissors, wipe them off on the disinfected outer side of skin.

246

247　3.5.3　Make lateral incisions on the skin, from midline to both sides of the rat at slightly
248　above the hind legs and slightly below the fore legs. Push the flaps of skin to the sides to expose
249　the muscle.
250
251　3.6　Cut open the abdominal muscle to expose the organs.
252
253　3.6.1　With a new pair of tooth-tissue forceps in one hand, lift the muscle near the base of
254　the abdomen. With a pair of blunt-blunt surgical scissors in the other hand, carefully cut
255　through the muscle without nicking any of the organs. Make a midline incision on the muscle
256　from slightly above the hind legs to the sternum.
257
258　3.6.2　Make lateral incisions on the muscle, from midline to both sides of the rat at slightly
259　above the hind legs and just below the rib cage, without nicking any of the organs. Push the
260　flaps of muscle to the sides to expose the organs. Ensure that the lateral incisions of the skin
261　and muscle reach the sides of the rat to allow blood and buffers to flow out from the abdominal
262　cavity at later steps.
263
264　3.7　Portal vein cannulation
265
266　3.7.1　With the back of curved forceps, gently push the intestines to the right. Gently flip up
267　the liver lobes to expose the portal vein.
268
269　3.7.2　Use a 3-0 silk surgical suture to make a very loose ligature around the portal vein close
270　to the liver, just before the vein branches left and right into different liver lobes. Do not tighten
271　the ligature to avoid disrupting blood flow through the portal vein. A very thin membrane
272　underneath the bile duct will need to be broken through with the tip of the curved forceps
273　before the suture can pass underneath and be looped around the portal vein (and bile duct).
274
275　3.7.3　Using the tips of two pairs of curved forceps, carefully puncture a hole through the
276　tissue underneath the portal vein, without damaging the portal vein. Do this about 2–3 cm
277　upstream of the first ligature, at just before the gastric vein branches off from the portal vein.
278　The tissue is thinner at this specific location.
279
280　3.7.4　Carefully stretch the hole larger with the forceps. This hole will allow the forceps to
281　support up the portal vein during cannulation.
282
283　3.7.5　Reduce the pump speed to 4 rpm for a flow rate of ~3 mL/min. Ensure that the outflow
284　of calcium-free buffer from the IV catheter is reduced to a slow drip.
285

286   NOTE: Pressure build-up in the liver will cause hepatocyte death. A lower flow rate should slow
287   down the pressure build-up in the liver upon successful insertion of the cannula into the portal
288   vein at a later step.
289

290   3.7.6   Gently support up the portal vein using forceps. With the other hand, hold onto the IV
291   catheter with the bevel of the needle facing up. Direct the needle into the portal vein at a 10–
292   20° angle and slowly advance until the whole bevel is inside the vein. Once the needle is
293   inserted correctly into the portal vein, the liver will start to blanch and lose its dark red color.
294

295   NOTE: The whole bevel of the needle must be inserted into the vein to create a hole large
296   enough for the insertion of the cannula. However, do not insert the needle too deep in to avoid
297   over puncturing the vein.
298

299   3.7.7   Advance the cannula over the needle and into the vein. Then, retract the needle until it
300   is 2–3 mm behind the cannula; just enough so that the sharp tip is safely within the cannula.
301   Use the thumb and middle finger to hold onto the catheter and use the index finger to pull back
302   the wing to retract the needle.
303

304   3.7.8   Quickly secure the portal vein onto the catheter with a vein clip. Do not clip directly on
305   the side hole of the needle to avoid disrupting perfusate flow. Instead, clip below it.
306

307   3.7.9   Immediately cut the infrahepatic inferior vena cava (IVC) to prevent pressure build-up
308   in the liver. By now the infrahepatic IVC and adjacent blood vessels will be obscured by blood
309   from the portal vein. To ensure that the IVC was correctly cut and severed, observe for blood
310   spurting out in pulses; blood will only trickle out from adjacent vessels.
311

312   NOTE: Taking too long to cut the infrahepatic IVC after cannulation or failure to cut it before
313   moving on to the next step will cause hepatocyte death.
314

315   3.7.10   Increase the pump speed to 38 rpm for a flow rate of ~33 mL/min. Flush the liver three
316   times by closing the IVC with forceps for 2–3 s (but not for too long) and re-opening it.
317

318   NOTE: During flushing, the liver will slightly expand before returning to normal. Flushing
319   facilitates the permeation of perfusate throughout the whole liver. After flushing, the liver
320   should be wholly light brown in color.
321

322   3.7.11   Ensure that the cannula tip is placed at before the point where the portal vein
323   branches into different liver lobes, to ensure perfusion of all liver lobes. If necessary, unclip the
324   portal vein and adjust the position of the cannula. Re-clip afterwards.
325

326   <mark>3.7.12   Tighten the loose ligature around the portal vein, at slightly upstream of the branching</mark>
327   <mark>point. Make three knots on where the soft cannula is supported by the hard metal needle, in</mark>
328   <mark>between of the bevel and side hole. Ensure that the knots fix the cannula in position, securing it</mark>
329   <mark>to the portal vein, and prevent backflow of buffers.</mark>
330
331   3.8 Resect the whole liver intact.
332
333   3.8.1   Perfuse the liver with calcium-free buffer at the flow rate of ~33 mL/min for the next
334   12 min (see **Figure 5A**). In the meantime, perform liver resection. Perfusion time can be
335   extended if necessary but ensure that the calcium-free buffer does not run out.
336
337   3.8.2   Using a pair of curved scissors, carefully detach the portal vein from the intestines by
338   cutting the mesentery that connects them together. Do not nick the gastrointestinal tract
339   (esophagus, stomach, and small and large intestines) to ensure that no contamination occurs.
340
341   NOTE: This is to prevent the portal vein from tearing when the liver is moved around during
342   resection.
343
344   3.8.3   Detach the liver from the gastrointestinal tract by cutting connective tissues, pancreas
345   and mesentery that connects the liver to the gastrointestinal tract. To detach, always cut with
346   scissors and do not pull to tear. Again, do not nick or cut the gastrointestinal tract.
347
348   3.8.4   Gently flip down the liver to expose the diaphragm. Cut the diaphragm following the
349   walls of the ribs. Ensure that most of the diaphragm remains connected to the liver. Be careful
350   not to poke or bruise the liver.
351
352   3.8.5   Once the chest cavity is opened, two ducts can be seen: the whitish suprahepatic IVC
353   on the left, and the yellowish esophagus on the right. Clip the suprahepatic IVC and cut it just
354   above the clip. Clipping the suprahepatic IVC is optional. It prevents the chest cavity from
355   getting flooded.
356
357   3.8.6   The esophagus is surrounded by the diaphragm. To isolate the esophagus from the
358   diaphragm, cut through the diaphragm from the right towards the esophagus. Cut any
359   remaining connective tissues that connect the esophagus and stomach to the liver and
360   diaphragm.
361
362   3.8.7   Push away the gastrointestinal tract to the right. Transfer the vein clip from the
363   suprahepatic IVC to the infrahepatic IVC; buffer will now perfuse out from the suprahepatic IVC.
364
365   NOTE: During perfusion, the cannula and infrahepatic IVC will be covered by the liver. Strong
366   buffer outflow from the suprahepatic IVC will help the surgeon to rule out perfusion leakages.

367

368 3.8.8   Cut any remaining diaphragm that connects the liver to the chest cavity. Cut off tissues
369 connecting the liver to the abdominal cavity. Be careful not to cut the infrahepatic IVC above
370 the clip to avoid detaching the clip from the liver.

371

372 3.8.9   To ensure that the liver is completely resected, carefully lift the liver by holding on to
373 the diaphragm with forceps. If there are any remaining tissues connecting the liver to the
374 abdominal cavity, cut them off. If the kidney and spleen are still connected to the liver, detach
375 them.

376

377 **4.   Liver perfusion and digestion**

378

379 4.1 If the resection was completed early, wait until the liver has been perfused with
380 calcium-free buffer for 12 min.

381

382 4.2 Change the perfusion buffer to collagenase buffer. Completely loosen the roller clamp for
383 collagenase buffer before completely tightening the roller clamp for calcium-free buffer (see
384 **Figure 5B**). Once the collagenase buffer reaches the liver through the tubing, flush the liver
385 three times by closing the suprahepatic IVC for 2–3 s and re-opening it.

386

387 4.3 Carefully move the liver to the stage on top of the beaker for recirculation of collagenase
388 buffer. Move the liver by holding onto the diaphragm with forceps while supporting the
389 catheter. Avoid tugging on the catheter to prevent accidental catheter detachment.

390

391 NOTE: If the catheter detaches and the portal vein is too damaged for re-cannulation, cannulate
392 the suprahepatic IVC and allow the buffer to perfuse out from the portal vein. Ensure that the
393 infrahepatic IVC remains clipped.

394

395 4.4 Digest the liver for 12 min. Observe for the appearance of translucent spots/network as the
396 liver starts to lose its smooth brown texture. The liver will get bigger and softer as it gets
397 digested. Once the liver has a mushy consistency, stop the perfusion. Digestion time can be
398 extended, if necessary.

399

400 NOTE: If the Glisson's capsule has been broken, the collagenase buffer in the beaker might turn
401 cloudy due to escaped liver cells.

402

403 **5.   Hepatocyte isolation**

404

405 5.1 Carefully remove the cannula by cutting off the portal vein and transfer the liver to a 150
406 mm dish containing 60 mL of cold Dulbecco's Modified Eagle Medium (DMEM).

407

408   5.2 Gently tap the liver using the backside of the curved forceps to break and peel off the
409   Glisson's capsule. Then, gently sway the liver side to side in DMEM to release liver cells.
410   Continue to do this until liver cells are fully dissociated into the DMEM.

411

412   NOTE: Occasionally the whole liver or certain lobes will only be partially digested. By not
413   scraping the liver which will forcibly remove, and damage undissociated hepatocytes, a higher
414   and more consistent cell viability can be obtained.

415

416   5.3 Gently rock the 150 mm dish until the liver cells are distributed well in DMEM. To remove
417   tissue pieces and cell clumps, pour the cell suspension through a 100 μm pore size nylon mesh
418   filter placed over a new 150 mm dish. Rinse the old dish with 30 mL of DMEM and transfer the
419   suspension to the mesh filter. Gently tap on the mesh filter to allow trapped single liver cells to
420   pass through.

421

422   5.4 Remove the mesh filter and gently rock the new 150 mm dish until the liver cells are
423   distributed well. Divide the suspension equally into 4 x 50 mL tubes. Rinse the dish with 30 mL
424   of DMEM and divide the suspension equally into the same tubes. Make sure each tube has an
425   equal volume of cell suspension.

426

427   5.5 Centrifuge the 4 x 50 mL tubes of cell suspension at 50 x g for 2 min at 4 °C. Carefully
428   aspirate the supernatant without disturbing the loose pellet. Discard the supernatant. Add 20
429   mL of DMEM into each tube and gently rock the tubes to resuspend the cell pellet. Combine the
430   cell suspension from four tubes into two.

431

432   5.6 Centrifuge the 2 x 50 mL tubes at 20 x g for 2 min at 4 °C. Aspirate and discard the
433   supernatant. Add 20 mL of DMEM into each tube and gently rock the tubes to resuspend the
434   cell pellet. Combine cell suspension from two tubes into one. Keep the cells on ice until use.

435

436   5.7 Prepare trypan blue solution by mixing 400 μL of 1x PBS with 50 μL of trypan blue. Add 50
437   μL of hepatocyte suspension into the trypan blue solution. Count the number of viable and
438   dead hepatocytes with a hemocytometer under the light microscope.

439

440   **6.   Hepatocyte culture**

441

442   6.1 Perform all cell culture steps in the hood. Add 1 mL collagen coating solution into a 35 mm
443   dish and incubate for 4 h. Wash the dishes three times with 1x PBS.

444

445   6.2 Dilute hepatocyte suspension in hepatocyte culture medium to a concentration of 0.8
446   million cells/mL. Add 1 mL of diluted hepatocyte suspension into the 35 mm dish.

447

448   NOTE: Hepatocytes are sensitive to shear stress during pipetting. Consider using wide bore
449   pipette tips.

450

451   6.3 Rock the dish to distribute the hepatocytes evenly. Incubate at 37 °C, 5% CO_2 for 3–4 h to
452   allow the hepatocytes to attach.

453

454   6.4 For sandwich culture.

455

456   6.4.1   Remove hepatocyte culture medium. Rinse once with 1 mL hepatocyte culture medium
457   to remove unattached cells. Add 1 mL of collagen overlay solution. Add collagen overlay
458   solution onto the walls of the dish to avoid introducing bubbles into the solution.

459

460   6.4.2   Incubate at 37 °C, 5% CO_2 for overnight to allow collagen gelation.

461

462   6.4.3   Add 1 mL of fresh hepatocyte culture medium. Incubate at 37 °C, 5% CO_2.

463

464   6.5 For monolayer culture.

465

466   6.5.1   After step 6.3, remove hepatocyte culture medium. Rinse once with 1 mL hepatocyte
467   culture medium to remove the unattached cells.

468

469   6.5.2   Add 1 mL of fresh hepatocyte culture medium. Incubate at 37 °C, 5% CO_2.

470

471   6.6 Assess   hepatocyte   purity   and   function   by   performing   immunostaining   for
472   hepatocyte-specific marker and functional assays as described previously[10,11].

473

REPRESENTATIVE RESULTS:
475   A surgeon could tell whether liver perfusion is going on smoothly by observing the outcome
476   after certain steps. The first outcome can be observed upon cannulation, cutting of the
477   infrahepatic IVC, and restoring the perfusion flow rate. The liver should have completely
478   changed color from dark red to brown, while maintaining its volume. If the liver looks slightly
479   deflated and has a reddish tint or blotches of red, it means that the perfusion flow rate was set
480   wrongly (too low), or the portal vein was not cannulated correctly. If the liver not only turns
481   brown but becomes bloated and stiff, it means that the flow rate was set wrongly (too high), or
482   the infrahepatic IVC was not cut properly. If only a few lobes are not being perfused well, it
483   means that the catheter was inserted too deep and is only perfusing one branch of the portal
484   vein. The second outcome can be observed after resection and clipping of the infrahepatic IVC.
485   If the outflow from the suprahepatic IVC is slow and weak, it might mean that the catheter had
486   detached during resection, or that the infrahepatic IVC was not clipped properly. The third
487   outcome can be observed during digestion. On the brown liver, translucent spots/network

should appear and enlarge. The liver should lose its springy consistency and become soft and mushy. We optimize our collagenase concentration so that this state can be reached within 12 min. When trying out a new lot of collagenase, digestion time should be extended until this state is reached. The fourth outcome can be observed when cells are released from the liver. When the cells are released, the media should look cloudy. If a lot of grainy texture is observed, it could mean that the liver was insufficiently digested. If the digestion is complete, only white weblike vessels should be left. Even if the digestion is partial **(Figure 6)**, it is still possible to acquire good cells.

Our protocol described here consistently generate higher cell yield of up to 5×10^8 hepatocytes per isolation from rats weighing 200–300 g and cell viability between 88%–94.8% as determined by trypan blue counting[12-16] (**Table 2**). Hepatocyte purity obtained as determined by immunostaining of albumin was 96.8 ± 2.0%[11] (**Figure 7**). Hepatocytes in sandwich culture formed distinct bile canaliculi and had good cell-cell contact (**Figure 8**). The hepatocytes have high functionality as shown by albumin, urea, and CYP assays[10] (**Table 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the IPS. The perfusate starts in the perfusate bag or bottle (1, 2). Two flow regulators (3, 4) work together to control the passage of perfusate. Perfusate proceeds to a bubble trap (5). Subsequent processes are controlled by the LCD touch screen (6) of the main system. Perfusate is then pulled by a peristaltic pump (7) and passes through a silicone jacketed electronic heater (8), which heats up the perfusate to the desired temperature. The silicone heater jacket (8) is connected to the main system by a connector (9). When necessary, a disposable pressure sensor (10) can be connected to the main system by a connector (11), to allow measurement of the perfusion pressure. The perfusate then passes through a perfusion monitor (12), which measures the temperature of the perfusate and detects the presence of bubbles. The monitor can also determine whether the perfusate has run out. Monitor readings are transmitted to the main system via LoRa. The perfusate then proceeds to a bubble filter (13) and enters the liver through the portal vein cannula (14).

Figure 2: Self-test interface.

Figure 3: Operation interface.

Figure 4: Popup window prompting for filename and username.

Figure 5: Schematic diagram of the two-step collagenase perfusion for primary rat hepatocyte isolation. (**A**) Perfusion of calcium-free buffer. The roller clamp is completely loosened for inlet 1 and completely tightened for inlet 2. (**B**) Liver digestion with collagenase buffer. Resected

528   liver is transferred to the top of a beaker to allow buffer recirculation. The roller clamp is
529   completely loosed for inlet 2 and completely tightened for inlet 1.
530
531   **Figure 6: Liver after collagenase digestion and hepatocyte isolation.** Shown is a partially
532   digested liver lobe. Undigested parts of the liver remained brown. In digested parts of the liver,
533   only white vessels were left behind.
534
535   **Figure 7: Immunostaining of hepatocytic marker for quantification of hepatocyte purity. (A)**
536   Sandwich culture of unpurified liver cell suspension (before differential centrifugation) and
537   purified hepatocyte suspension (after differential centrifugation) seeded at low cell density. **(B)**
538   Representative images show cells stained with DAPI (nucleus) and antibody against albumin
539   (hepatocyte-specific marker). **(C)** Quantification of hepatocyte purity. The purity was
540   determined by counting albumin positive fluorescent stained cells in relation to the total cell
541   number visualized by DAPI nuclear staining. Images in false colors were shown. Albumin (green),
542   nucleus of cells with albumin (blue), nucleus of cells without albumin (red). Scale bars 100 μm.
543
544   **Figure 8: Phase contrast image of isolated primary rat hepatocytes from two-step collagenase**
545   **perfusion in sandwich culture at day 3.** Scale bar 50 μm.
546
547   **Table 1: Recipes for buffers and solutions.**
548
549   **Table 2: Cell viability and yield.** Values ± SD from 18 different experiments are shown.
550
551   **Table 3: Functional levels of isolated primary rat hepatocyte in sandwich culture.** Values ± SD
552   of urea, albumin, CYP1A2, CYP2B1/2, and CYP3B2 assays from three different experiments are
553   shown.
554
555   **DISCUSSION:**
556   There are a few points that are particularly important to observe for two-step collagenase
557   perfusion procedure in general. Firstly, special care must be given when resecting the liver.
558   Ensure that the gastrointestinal tract is not damaged as leakage of the contents will result in
559   bacterial contamination. In addition, avoid damaging the Glisson's capsule, which covers the
560   surface of the liver during the animal procedure. If the tear is large enough, it might allow
561   premature release of disassociated hepatocytes into the collagenase buffer. Secondly, the
562   ability of collagenase to digest the liver while ensuring good hepatocyte viability may vary by lot
563   number. The amount of collagenase used must be optimized for every new lot of collagenase[5].
564   It is advisable to test a few lots of collagenase and select the best lot before buying in bulk.
565   Ensure that the water is ultrapure, and the pH is correct. Impurities in the water such as ferrous
566   and ferric ions and the wrong pH could affect collagenase activity, which will then drastically
567   affect the quality of the isolated cells. Although collagenase buffer recirculation in this protocol
568   can be optional, it is not advisable because the volume of collagenase buffer needed will

569   increase to >400 mL. Lastly, perfusion pressure and flow rate should be in an appropriate range.
570   High pressure and flow rate will lead to liver cell death while low pressure and flow rate will
571   result in insufficient buffer flow through the smaller vessels[10]. During calcium-free buffer
572   perfusion, the pressure will initially rise. However, upon collagenase buffer perfusion, the
573   pressure will then slowly drop over time since digestion by collagenase reduces flow resistance.
574   If the pressure drop was not observed, the digestion time can be extended as required. Since
575   IPS has the optional pressure sensor, it is possible to vary the flow rate of the perfusate to
576   compensate for pressure variations. On another note, although flushing the circuit with an
577   oxygenator was used in some protocols[2,5], we found that was not critical for hepatocyte
578   viability and yield. Thus, our device does not include an oxygenator.
579
580   In contrast to the common practice of liver digestion before resection, this protocol involves
581   resection of the liver prior to digestion. The approach in this protocol holds several advantages
582   compared to the common practice. Firstly, over-digestion of the liver can be avoided.
583   Depending on the surgeon's skill, liver resection may take anywhere from 5–10 min. Following
584   the common practice, even though the perfusion has been stopped, this still equates to an
585   additional 5–10 min where the liver is exposed to collagenase. Over-digestion may decrease cell
586   viability and reduce hepatocyte attachment and function. Secondly, this approach could
587   potentially reduce hepatocyte loss. The Glisson's capsule is less likely to tear when the liver is
588   still springy before digestion, compared to when it is soft and mushy after digestion. Finally,
589   resection of the liver prior to digestion enables the possibility of collagenase recirculation.
590   Collagenase recirculation eliminates the occurrence of failed isolations due to insufficient
591   volume of collagenase being prepared. Our approach allows digestion time to be extended, as
592   necessary. It also has a minor advantage of reducing the amount of collagenase needed per
593   isolation. The main disadvantage of this approach is that it introduces a new problem of
594   catheter detachment halfway during perfusion, but which could be addressed with a few
595   technical precautions. Another difference is that this protocol does not involve the use of
596   density gradient such as Percoll. By removing dead cells, Percoll has been reported to increase
597   the final viability of cells with some loss of yield, from 20%–50%[16]. Our cell viability is high
598   because we test and select collagenase lots. From our experience, some collagenase lots
599   consistently give lower viability. Without density centrifugation, it will be necessary to avoid
600   bad collagenase lots through prior testing. For those who do not have such liberty and must
601   consider density gradient, care should be given during Percoll dilution to avoid selection of
602   hepatocyte subpopulations with a slightly different metabolic profile[17,18].
603
604   Our approach in using an IPS for primary rat hepatocyte isolation resolves several issues with
605   existing perfusion setups[1,2,5-9]. Usage of disposable sterile tubing saves time by removing the
606   need to clean and disinfect perfusion tubing before and after each isolation. It reduces fire
607   hazard since the perfusion tubing need not to be filled with flammable 70% ethanol when not
608   in use to maintain sterility. The risk of contamination due to improper disinfection and storage
609   can also be eliminated. Most importantly, the need for routine replacement of old tubing can

610 be circumvented; this process is essential but is often complicated and time consuming. The
611 addition of a bubble filter to the tubing near the catheter allows bubbles to escape before
612 reaching the catheter and prevents air from passing through when the perfusion buffer runs
613 out. Usage of a silicone heater jacket as the temperature control system allows precise and
614 stable maintenance of perfusate temperature during digestion, in contrast with pre-warmed
615 buffers which will significantly cool down over time. In addition, the silicone heater jacket is
616 compact and can be easily maintained, unlike a glass jacket coupled to a water bath circulator.
617 Usage of monitoring sensors for temperature and pressure replaces the optimization step
618 before isolation. Temperature variation may occur during or between experiments. Pressure
619 (and thus flow rate) may also change due to issues with perfusion tubing. Placement of sensors
620 near the cannula allows more accurate reading of the temperature of buffer perfusing into the
621 liver. Sensor alarms can remind the user to take corrective action. The recorded log also allows
622 for troubleshooting. Usage of a special IV catheter for cannulation simplifies cannulation of the
623 portal vein. Unlike other types of IV catheter, the catheter described here allows fluid
624 communication between the cannula and the tubing in both its puncturing and retracted
625 needle position due to a hole at the side of the needle. Thus, surgeons could use color change
626 of the liver as a sign of successful cannulation. Upon successful cannulation, the needle tip can
627 be retracted behind the catheter tip to prevent the re-piercing of the portal vein. The retracted
628 needle could also act as a foundation for the ligature to secure the portal vein onto the soft
629 cannula and prevent it from being deformed. This catheter can be operated with one hand;
630 therefore, the non-dominant hand can be used to support the portal vein with forceps. The
631 compact integrated design allows the whole perfusion device to be put into the laminar hood,
632 reducing the risk of contamination, and saving space. This is especially important if the animal
633 procedure is to be performed in a dedicated animal facility where space availability can be a
634 challenge.
635
636 In comparison to previously reported rat hepatocyte isolation protocols using the two-step
637 collagenase perfusion procedure[1], the conditions described consistently generate higher cell
638 yield of up to 5 x 10^8 hepatocytes per isolation, cell viability between 88%–94.8% and good
639 hepatocyte-specific function.
640
641 This protocol can be concurrently used to isolate other non-parenchymal liver cells such as
642 Kupffer cells, liver endothelial cells and hepatic stellate cells from the same rat. This protocol
643 can also be modified for the isolation of liver cells from mice by using a smaller gauge IV
644 catheter for cannulation and decreasing the perfusion flow rate to a range suitable for mouse
645 species[6]. The perfusion procedure for mice can be simplified by resecting the liver after
646 digestion.
647
648 The IPS device used in this experiment can be also used for *ex vivo* liver perfusion application
649 such as experiments on liver transplantation as well as decellularization of rodent and
650 discarded human livers[19,20]. The IPS has an advantage where the liver must be perfused for long

651 periods because the IPS can monitor perfusion conditions in real-time and allow
652 discontinuation of the perfusion upon completion either automatically or manually by remote
653 control.
654

671

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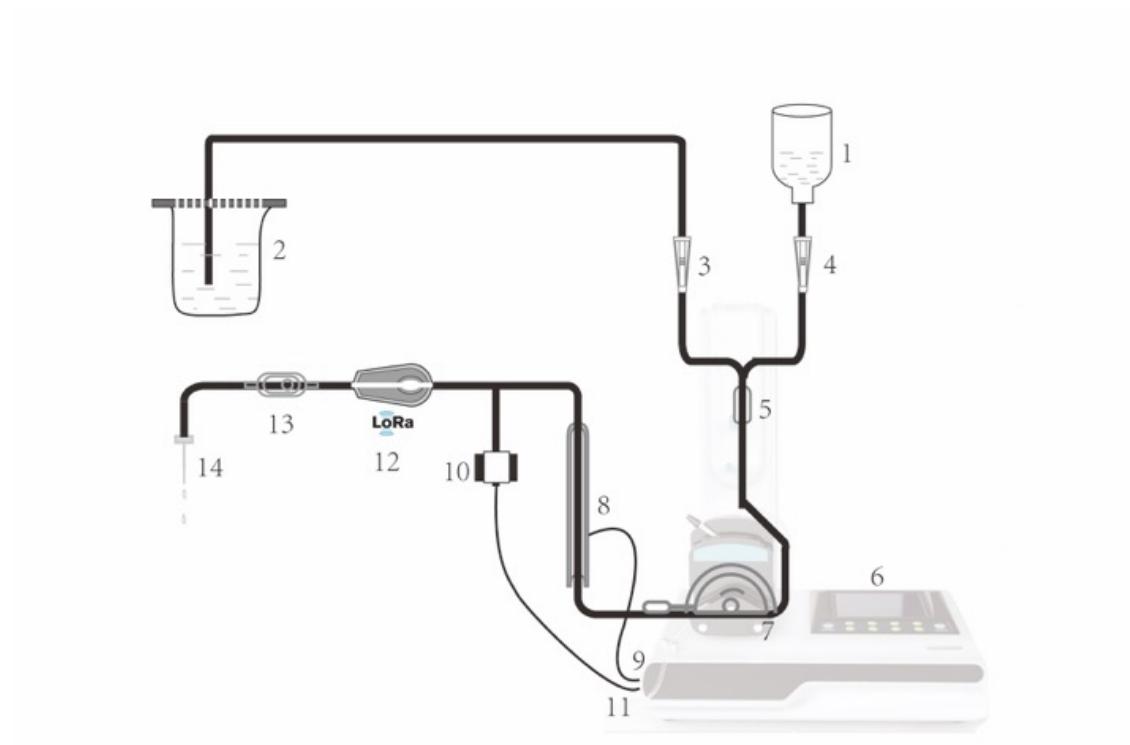718   perfusion device. *Journal of Visualized Experiments: JoVE*. (102), e53029 (2015).
719

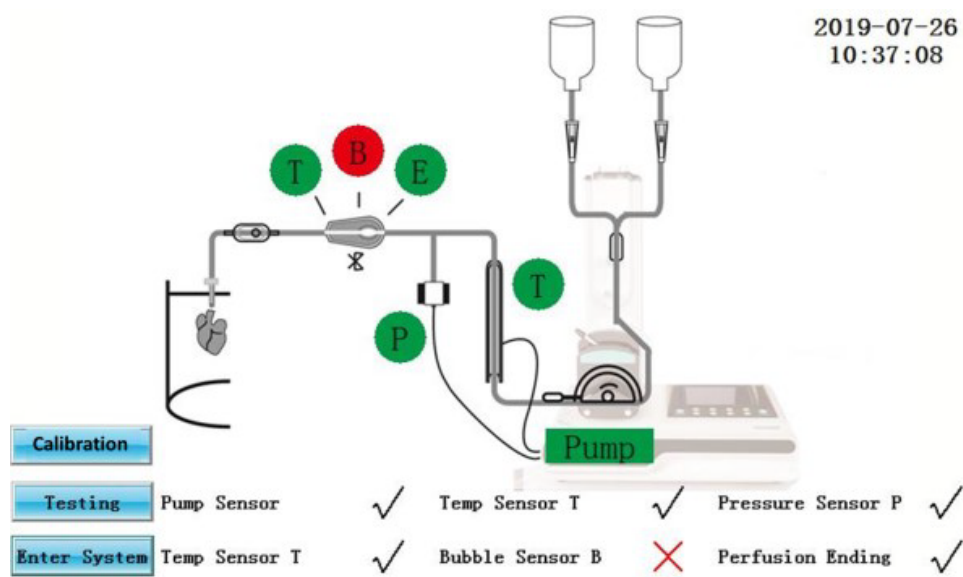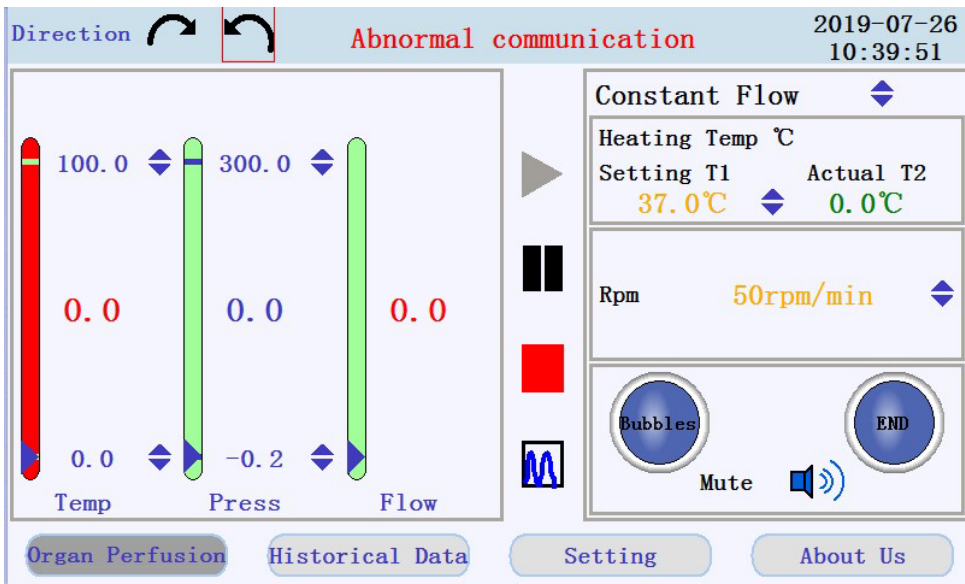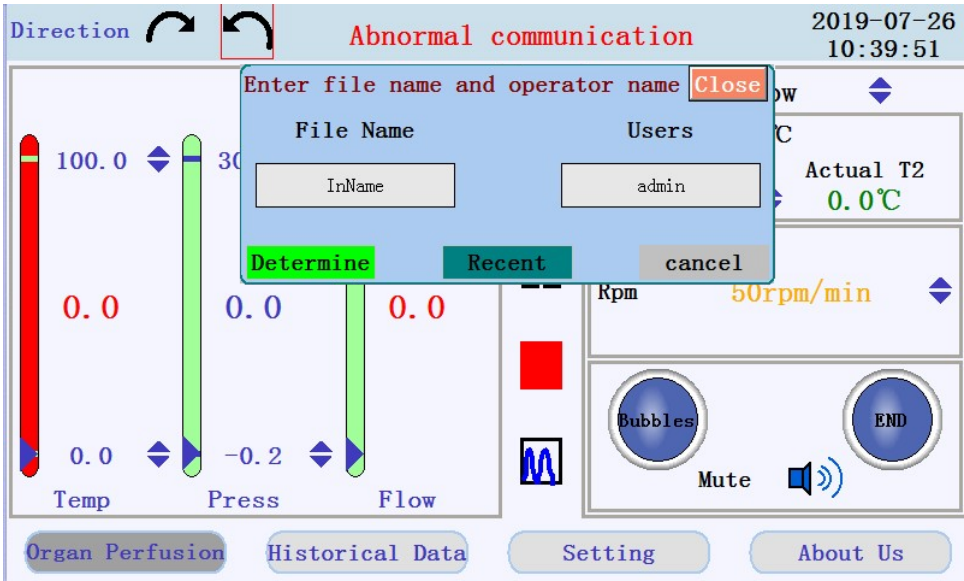Figure 1   Click here to access/download;Figure;Fig1.pdf ±

Figure 2
Click here to access/download;Figure;Fig2.pdf ±

Figure 3

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Figure 4

Figure 4

Figure 5   Click here to access/download;Figure;Fig5.pdf ⬇

A

Inlet 1   Inlet 2

Calcium-free Collagenase
   buffer   buffer

Pump

B

Inlet 1   Inlet 2

Liver

Calcium-free   Collagenase
   buffer   buffer

Pump

Figure 6

Figure 7

A

B

C

Figure 8

Table 1
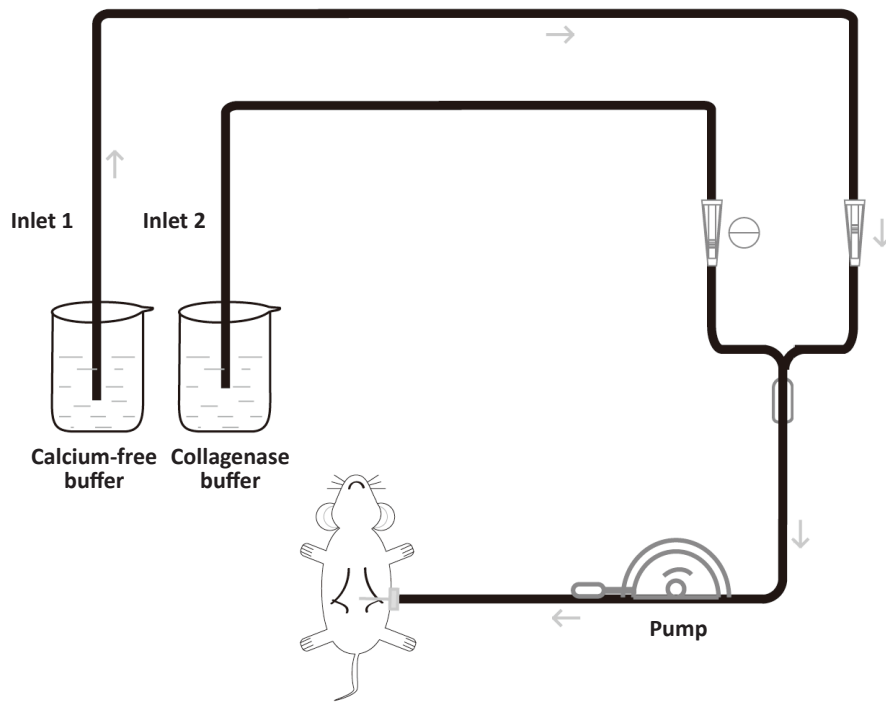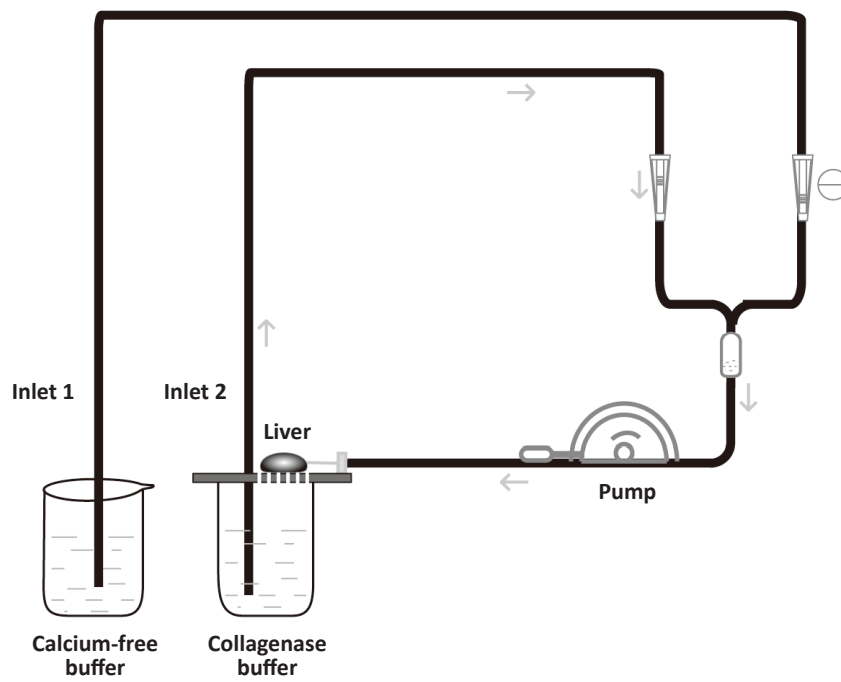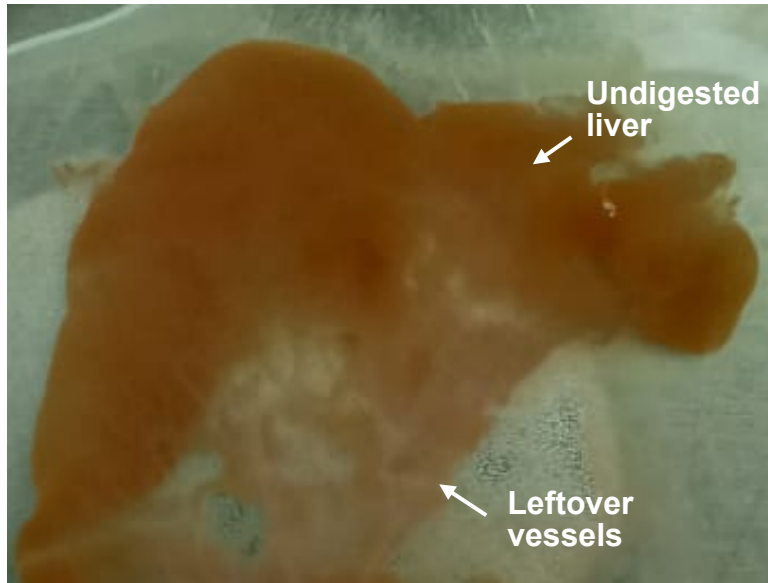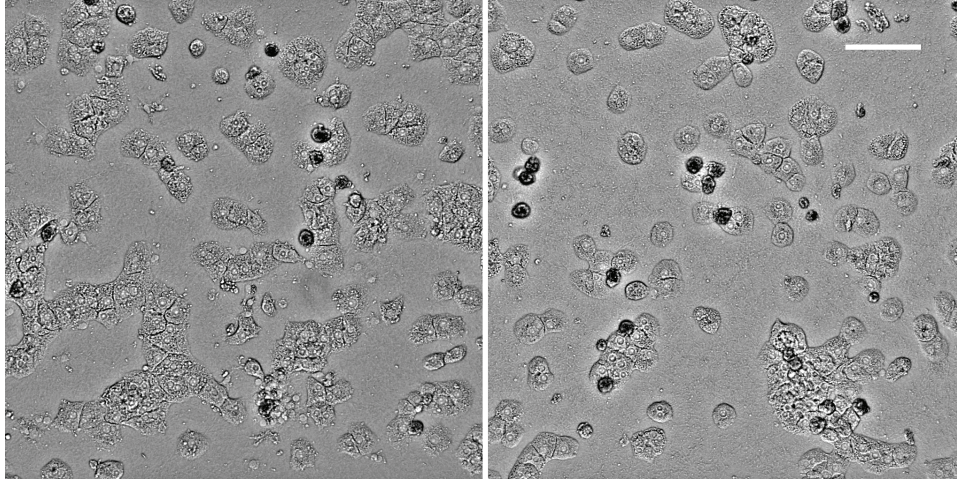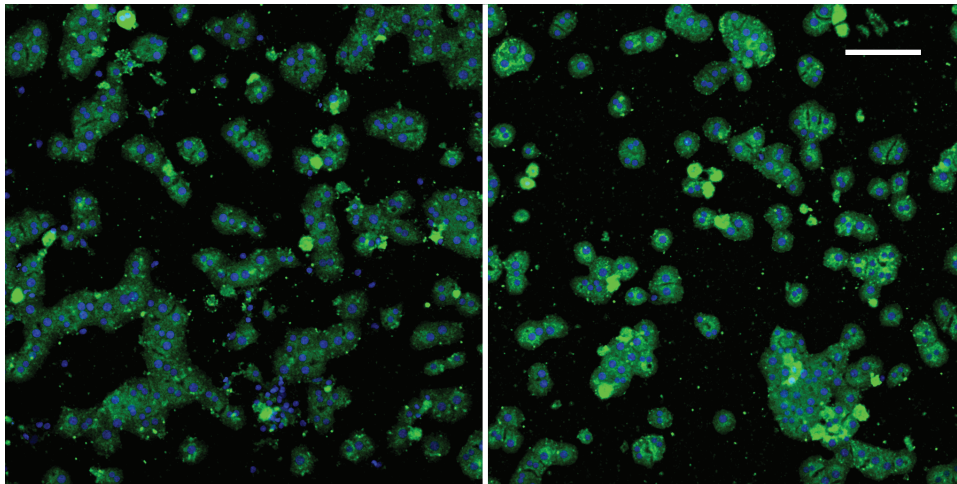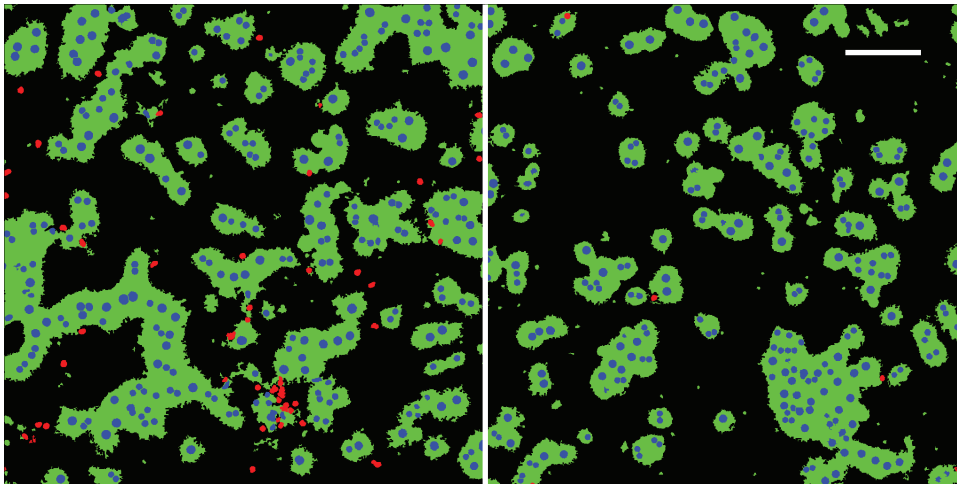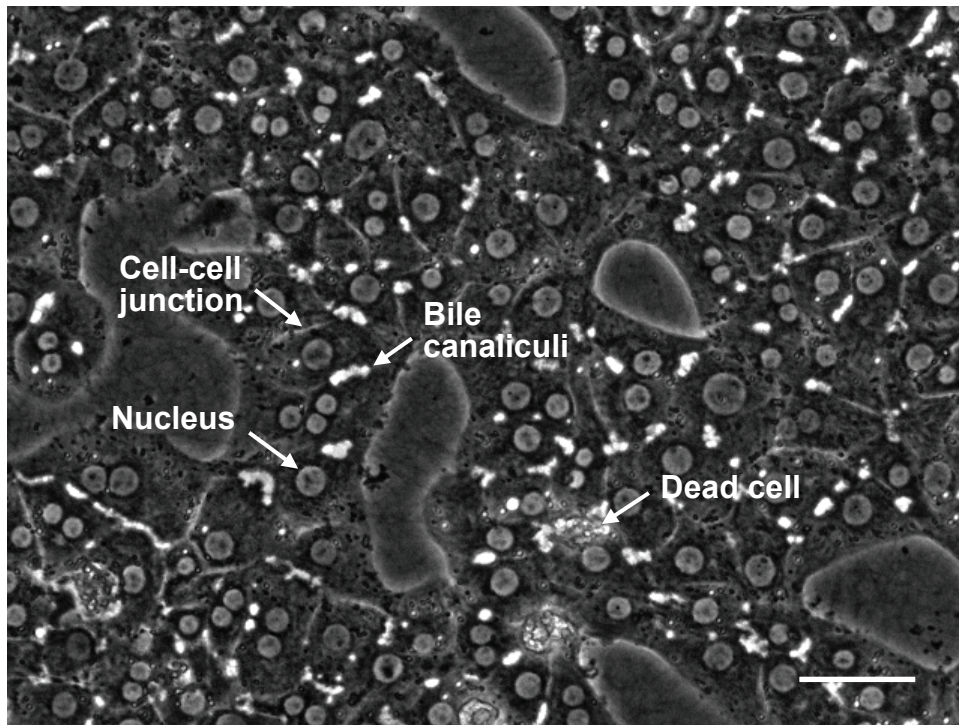
Name of Buffers/Media	Final concentration	Amount	Comments/Description
Hepatocyte culture media			
William's E media		500 mL	Add all reagents into William's E media. Filter through 0.22 μm filter. Store at 4 °C.
BSA	1 mg/mL	0.5 g	
Penicillin-Streptomycin (100X)	1X	5 mL	
Insulin (20 mg/ml)	0.5 μg/mL	12.5 μL	
Dexamethasone (1 mM)	100 nM	50 μL	
Linoleic acid (7.5 μg/mL)	50 ng/mL	3.33 μL	
Glutamax (100X)	1X	5 mL	
Collagenase buffer			
NaCl		0.100 g	Dissolve in 380 mL of ultrapure water. Adjust to pH 7.49 - 7.51 with NaOH. Top up to 400 mL with ultrapure water. Filter through 0.22 μm filter. Store at 4 °C.
KCl		0.789 g	
Collagenase Type IV	80 - 200 U/mL		
$CaCl_2·2H_2O$		0.140 g	
HEPES		4.800 g	
Calcium-free buffer			
KH_2PO_4		0.0815 g	Dissolve in 900 mL of ultrapure water. Adjust to pH 7.49-7.51 with HCl. Top up to 1 L with ultrapure water. Filter through 0.22 μm filter.
$NaHCO_3$		1.0500 g	
NaCl		3.4480 g	
KCl		0.1750 g	
DMEM for cell isolation			
DMEM		1 sachet	Top up to 1 L with ultrapure water. Filter through 0.22 μm filter. Store at 4 °C.
$NaHCO_3$		1.5 g	
Penicillin-Streptomycin (100X)	1X	10 mL	
Collagen overlay (1 mL)			
0.1 M NaOH	1 part	20.8 μL	

10X PBS	1 part	20.8 µL	Prepare fresh. Add collagen last.
1X PBS	6 parts	125 µL	
Hepatocyte culture media	32 parts	667 µL	
Type I bovine collagen	8 parts	167 µL	
Collagen coating solution (1 mL)			
Type I bovine collagen	1 part	0.5 mL	Prepare fresh.
0.01 N HCl	1 part	0.5 mL	

Table 2

Hepatocyte viability (%)	Hepatocyte yield (x 10^8 cells)	Viable hepatocyte yield (x 10^8 cells)
91.4 ± 3.4	4.39 ± 1.58	4.04 ± 1.48

Table 3

	Urea (μg/million cells/day)	Albumin (μg/million cells/day)	CYP1A2 activity (μg/million cells/day)	CYP2B1/2 activity (μg/million cells/day)	CYP3B2 activity (μg/million cells/day)
Day 1	124 ± 2.6	39.0 ± 3.6			
Day 3	65.7 ± 3.8	516.0 ± 95.1	2249 ± 56	188 ± 49	93.7 ± 0.6

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1 mL syringe	Nipro		
27G needle	Nipro		
Black braided silk non-absorbable, non-sterile surgical suture	Look	SP117	
Bochem 18/10 stainless steel forceps, sharp tip contain bent round tip	Bochem	10333511	
Disposable Perfusion Set	Vasinfuse	BPF-112	
Floating circular 1.5 mL microcentrifuge tube rack	Sigma-Aldrich	R3133	
German Standard Tissue Forceps, Serrated / 1×2 teeth , 14.5cm	Walentech		
Greiner Cellstar aspirating pipette	Merck	GN710183	
Haemocytometer			
Integrated Perfusion System	Vasinfuse	IPS-001	
Iris Scissors curved, stainless, 11cm	Optimal Medical Products Pte Ltd	CVD	
Light microscope with 10X lens	Olympus		
Mesh Sheet 100μM Nylon	Spectra-Teknic(s) Pte Ltd	06630-75	
Operating Scissors, BL/BL, 13cm	Optimal Medical Products Pte Ltd	STR – BL/BL	
Operating Scissors, SH/BL, 13cm	Optimal Medical Products Pte Ltd	STR – SH/BL	

Reverse force hemostatic clip	Shanghai Jin Zhong Pte Ltd	XEC230	
Water bath	Grant		

Name of Reagents/Chemicals	Company	Catalog Number	Comments/Description
10X Phosphate buffered saline (PBS)	Sigma-Aldrich		
Bovine serum albumin (BSA)	Sigma-Aldrich	A9056	
$CaCl_2 \cdot 2H_2O$	Merck	137101	
Collagenase Type IV	Gibco	17104019	
Dexamethasone	TCI	D1961	
DMEM	Gibco	31600-034	
Glutamax	Gibco	35050061	
HEPES	Invitrogen	11344-041	
Insulin	Sigma-Aldrich	1-9278	
KCl	VWR	VWRC26764.298	
KH_2PO_4	Sigma-Aldrich	P5379	
Linoleic acid	Sigma-Aldrich	L9530	
NaCl	Sigma-Aldrich	S5886	
$NaHCO_3$	Sigma-Aldrich	S8875	
NaOH	Merck	106462	
Penicillin-Streptomycin	Sigma-Aldrich	P4333	
Type I bovine collagen	Advanced BioMatrix	5005-100ml	
William's E Media	Sigma-Aldrich	W1878	

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Manuscript was proofread for spelling and grammar issues.

2. Please provide an institutional email address for each author.

Institutional email addresses for Yun Ting Soong and Chan Way Ng were added. Institutional email addresses for Narelle Nichola Yi Ying Shen and Yan Zhou have been inactivated after they left National University of Singapore. Therefore, their personal email addresses were provided instead.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text has been revised to remove the use of personal pronouns.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have revised the protocol section and described actions in the imperative tense whenever possible. Text that could not be written in the imperative tense were moved to "Note". "Caution" for safety precautions and procedures were also added.

5. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral, throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

All time units for duration of less than one day have been converted to abbreviated forms.

6. Line 163/317: Please convert speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Revolutions per minute (rpm) in both lines refer to pump rotation speed, not centrifugal speed.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The number of highlighted pages has been reduced to 3.

8. Please expand the Representative Results section. The section should describe representative results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Representative Results have been slightly expanded and a new Figure was added.

Reviewer #1:
Manuscript Summary:
The authors present a nice protocol for isolation of rat hepatocytes with many improvements over existing protocols that could increase efficiency and reproducibility. The major improvements include silicone heating jacket and a more sophisticated peristaltic pump system.

Major Concerns:
- There are many published protocols for isolation of rat and mouse hepatocytes. The authors should cite previous protocols and highlight how their protocol is different.
Citations were added to highlight differences in technical protocols and perfusion setup. Differences in technical protocols were discussed in Discussion. Improvements on perfusion setup were explained in Introduction.

- This protocol involves resection of liver prior to collagenase digestion. This is in sharp contrast to all protocols I know which digest the liver before resection. Because this stands in contrast to common practice, the authors should explain and justify this choice.
Resection of the liver prior to collagenase digestion holds several advantages compared to digestion of the liver before resection. First, over-digestion of the liver can be avoided. In other protocols, liver resection was performed after confirming that the liver is sufficiently digested. Depending on surgeon skill, liver resection may take from 5-10 min. Even though the perfusion has been stopped, this still equates to additional 5-10 min where liver exposed to collagenase. Second, our approach could reduce hepatocyte loss. It will be easier to accidentally break and tear the Glisson capsule when the liver is soft and mushy after digestion, compared to before digestion. Finally, resection of the liver prior to collagenase digestion will enable the possibility of collagenase recirculation. Collagenase recirculation eliminates the problem of failed isolations due to insufficient volume of prepared collagenase. This will allow digestion time to be extended, as necessary. It also has a minor advantage of reducing the amount of collagenase needed per isolation. The main disadvantage of our approach is that it introduces a new problem of cannular detachment halfway during perfusion, but which could be addressed with a few technical precautions. The advantages and disadvantages of resection of liver prior to collagenase digestion have been added into Discussion.

Minor Concerns:
- It is common to use a density gradient (such as Percoll) during isolation in order to improve yield and viability. This protocol does not use a density gradient. If the authors have tried it and found it to be un-needed, this should be mentioned as it can help future users.
We do not use density gradient such as Percoll to improve the viability of our cells. Our cell viability is high enough not to require density gradient. From our experience, some collagenase batches/lots may give lower viability, so it will be better to just avoid these batches/lots. Although density gradient may select subpopulation of hepatocytes with slightly different metabolic profile, for users stuck with a bad batch/lot of collagenase, we will mention the possibility of density gradient in the paper.

- Table 2 lists several measurements that were made. The authors should describe how these measurements were made.
Our protocols for the measurement of albumin, urea and CYP are rather complicated. They will add at least 3 additional pages to the Protocol. To avoid exceeding the 10-page limit for Protocol and distracting readers, we feel that it will be best not to add them. The measurements were made based on: Xia, L., Binte, R., Qu, Y., Hong, X., Zhang, W., Nugraha, B., … Yu, H. (2012). Tethered spheroids as an in vitro hepatocyte model for drug safety screening. Biomaterials, 33(7), 2165–2176. https://doi.org/10.1016/j.biomaterials.2011.12.006

In summary, this is a well-written and detailed protocol which may prove useful to future users

Reviewer #2:
Manuscript Summary:
In this manuscript, Inn Chuan Ng and colleagues Isolation of Primary Rat Hepatocytes with Multiparameter Perfusion Control. The protocol is very interesting. But it is necessary to address several issues.

Minor Concerns:
Please check spelling and grammar. The following are my concerns:
1. At line 68, please change "applications" to "application".
[Line 73 and others] Corrected all "applications" to "application".
2. At lines 118-119: what is the reference for "ionic strength in the water will affect the quality of the isolated hepatocytes"
Confusing statement in Protocol was removed and explanation was provided in Discussion. We were trying to say that impurities in the water such as ferrous and ferric ions could affect collagenase activity, and thus quality of isolated hepatocytes. Reference: Karakiulakis G, Papadimitriu E, Missirlis E, Maragoudakis ME. Effect of divalent metal ions on collagenase from Clostridium histolyticum. Biochem Int. 1991 Jun;24(3):397-404. PMID: 1663343.
3. It would be better to use three-line table format for tables 1 and 2
Tables 1 and 2 were converted to three-line table format.
4. For figure 1 legend, you didn't point out what 15 represent for
Component 15 was a duplicate of component 2. Figure 1 has been modified and corrected. All component number are now represented.
5. For figure 6, it will be better if you can use arrows to point out the site of bile canaliculi.
The site of bile canaliculi in the figure (now Figure 7) was pointed out with arrows. Visually similar structures such as cell-cell junction, the nucleus and dead cell were also pointed out.
6. At line 411, please write the full name then the abbreviation.
[Line 440] Added the full name and abbreviation for Dulbecco's Modified Eagle Medium (DMEM).
7. In 5.5 and 5.6: why is the speed of the centrifuge different since it's used for the same purpose?
$50 \times g$ is the commonly accepted speed used to pellet hepatocytes. We find that using $20 \times g$ for the second centrifugation could reduce the amount of non-parenchymal cells pelleted.
8. In 5.7: the cell count should be under the microscope.
Added that cell count was performed with a haemocytometer under the light microscope.
9. At line 483: the cells availability depends on the table-1 will be between 88-94.8%.
[Line 533] Updated.
10. Is bubble trap (5) necessary to be used, since the heating step (8) may cause bubble, and you already set a bubble filter (13) after heating
The bubble trap acts more like a reservoir to prevent the flow from being disrupted due to accidental introduction of air from the inlets.
11. Do you think the perfusion monitor (12) is better to set after bubble filter (13).
If the perfusion monitor is placed after the bubble filter, it cannot be used to detect air when the perfusion buffer runs out. Therefore, the user will not be notified. The bubble filter will block air from reaching the perfusion monitor.