Journal of Visualized Experiments

Detergent Free Decellularization of the Human Pancreas for Soluble Extracellular Matrix (ECM) Production
--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61663R2
Full Title:	Detergent Free Decellularization of the Human Pancreas for Soluble Extracellular Matrix (ECM) Production
Keywords:	Extracellular Matrix; ECM; decellularization; Pancreas; Islets; regenerative medicine; bioengineering; tissue engineering
Corresponding Author:	GIUSEPPE ORLANDO Wake Forest Baptist Medical Center WINSTON SALEM, NC UNITED STATES
Corresponding Author's Institution:	Wake Forest Baptist Medical Center
Corresponding Author E-Mail:	gorlando@wakehealth.edu
Order of Authors:	Riccardo Tamburrini
	Deborah Chaimov
	Amish Asthana
	Kevin Enck
	Sean M. Muir
	Justine Mariam Aziz
	Sandrine Lablanche
	Emily Tubbs
	Alice A. Tomei
	Mark Van Dyke
	Shay Soker
	Emmanuel C Opara
	Giuseppe Orlando
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.	Winston Salem, North Carolina, United Sates

1   **TITLE:**
2   Detergent-Free Decellularization of the Human Pancreas for Soluble Extracellular Matrix (ECM)
3   Production
4
5   **AUTHORS AND AFFILIATIONS:**

6   Riccardo Tamburrini[1,2,3], Deborah Chaimov[1,3], Amish Asthana[1,3], Kevin Enck[3], Sean M. Muir[4],
7   Justine Mariam Aziz[5], Sandrine Lablanche[6], Emily Tubbs[6], Alice A. Tomei[7,8], Mark Van Dyke[9], Shay
8   Soker[3], Emmanuel C Opara[3], Giuseppe Orlando[1,3]
9
10   [1]Department of Surgery, Wake Forest Baptist Medical Center, Medical Center Boulevard,
11   Winston Salem, NC, USA
12   [2]Department of General Surgery, PhD Program in Experimental Medicine, University of Pavia,
13   Pavia, Italy
14   [3]Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical
15   Center Boulevard, Winston-Salem, NC, USA
16   [4]Wake Forest University College of Arts and Science, Winston Salem, NC, USA
17   [5]Wake Forest University School of Medicine, Winston-Salem, NC, USA
18   [6]Grenoble Alps University, Laboratory of Fundamental and Applied Bioenergetics (LBFA), and
19   Environmental and System Biology (BEeSy), Inserm, Grenoble, France
20   [7]Department of Biomedical Engineering, University of Miami, Coral Gables, FL, USA
21   [8]Diabetes Research Institute, University of Miami Miller School of Medicine, Miami, FL, USA
22   [9]Department of Biomedical Engineering and Mechanics, Virginia Polytechnic Institute and State
23   University, School of Biomedical Engineering and Sciences, Blacksburg, VA, USA
24
25   Corresponding author:
26   Giuseppe Orlando   (gorlando@wakehealth.edu)
27
28   Email addresses of co-authors:
29   Riccardo Tamburrini   (rtamburr@wakehealth.edu)
30   Deborah Chaimov   (dchaimov@wakehealth.edu)
31   Kevin Enck   (kenck@wakehealth.edu)
32   Sean M. Muir   (smuir@wakehealth.edu)
33   Amish Asthana   (aasthana@wakehealth.edu)
34   Justine Mariam Aziz   (jaziz@wakehealth.edu)
35   Sandrine Lablanche   (slablanche@chu-grenoble.fr)
36   Emily Tubbs   (Emily.tubbs@univ-grenoble-alpes.fr)
37   Alice A. Tomei   (atomei@med.miami.edu)
38   Mark Van Dyke   (mvandyk5@vt.edu)
39   Shay Soker   (ssoker@wakehealth.edu)
40   Emmanuel C Opara   (eopara@wakehealth.edu)
41   Giuseppe Orlando   (gorlando@wakehealth.edu)
42
43   **KEYWORDS:**
44   extracellular matrix, ECM, decellularization, pancreas, islets, regenerative medicine

45

SUMMARY:

The protocol described in this manuscript explains the steps for the fabrication of a soluble extracellular matrix (ECM) from the human pancreas. The solubilized ECM powder obtained through this protocol may be used for the recapitulation of pancreatic islets' microenvironment in vitro and, potentially, in vivo settings.

ABSTRACT:

Islet transplantation (ITx) has the potential to become the standard of care in beta cell replacement medicine but its results remain inferior to those obtained with whole pancreas transplantation. The protocols currently used for human islet isolation are under scrutiny because they are based on the enzymatic digestion of the organ, whereby the pancreas is demolished, its connections to the body are lost and islets are irreversibly damaged. Islet damage is characterized by critical factors such as the destruction of the extracellular matrix (ECM), which represents the 3D framework of the islet niche and whose loss is incompatible with islet euphysiology. Researchers are proposing the use of ECM-based scaffolds derived from the mammalian pancreas to address this problem and ultimately improve islet viability, function, and lifespan. Currently available methods to obtain such scaffolds are harsh because they are largely detergent based. Thus, we propose a new, detergent-free method that creates less ECM damage and can preserve critical components of pancreatic ECM. The results show that the newly developed decellularization protocol allowed the achievement of complete DNA clearance while the ECM components were retained. The ECM obtained was tested for cytotoxicity and encapsulated with human pancreatic islets which showed a positive cellular behavior with insulin secretion when stimulated with glucose challenge. Collectively, we propose a new method for the decellularization of the human pancreas without the use of conventional ionic and non-ionic chemical detergents. This protocol and the ECM obtained with it could be of use for both in vitro and in vivo applications.

INTRODUCTION:

The isolation of pancreatic islets is a meticulous process carried out through the enzymatic digestion of the connections between islets and their extracellular surrounding supportive structure. This destruction of the extracellular matrix (ECM) is one of the critical factors in characterizing islets' damage taking place during isolation processes[1-4]. The peri-islet ECM is an essential acellular component of the endocrine pancreas. It is composed of proteins and polysaccharides, which interact and cross-link to form a three-dimensional net that structurally and biochemically supports the physiological homeostasis, and helps in the recreation of this in vitro microenvironment[2,5,6]. The loss of fundamental signaling processes between islets and ECM is recognized as one of the contributing factors, which limits islets' survival in vitro and in vivo[2,7-10].

Animal- and human-derived ECM have been widely used for the recapitulation of the pancreatic islets' microenvironment[11-19]. Since the ability of the ECM to enhance rat islet cells attachment, proliferation, and long-term culture maintenance was first reported in ref.[20], many other studies have provided strong evidence that the restoration of native ECM interaction with human islets

89   enhances islet function[21,22]. For instance, recent data has shown that islets encapsulated with
90   ECM significantly improved glycemic control in diabetic mice, enhancing and facilitating the
91   delivery of insulin in a novel cell-based insulin delivery platform[23]. Furthermore, studies have
92   demonstrated that incorporation of critical components of pancreatic ECM can significantly
93   improve the endocrine function of β-cells[24-27].

94

95   ECM manufacturing protocols present in the literature are based on the application of chemical
96   detergents, e.g., Triton X-100 or sodium dodecyl sulfate (SDS). Despite providing excellent DNA
97   clearance, chemicals used in decellularization processes are cytotoxic, expensive, and residues
98   on the decellularized tissue bring concerns in view of potential clinical application.

99

100   Based on these observations, the objectives of this study were three-fold: First, to develop a
101   decellularization method for the human pancreas with minimal use of ionic or non-ionic chemical
102   detergents; Second, to produce a soluble ECM scaffold for tissue culture; Third, to characterize
103   the pancreatic ECM in order to assess its cytotoxicity and impact on islet cell function. The
104   characterization is necessary for all the cell-culture-based applications, as it demonstrates that
105   solubilized pancreatic ECM could be beneficial in recapitulating the pancreatic microenvironment
106   for isolated islets. Described herein is an effective, detergent-free decellularization method for
107   the human pancreas, characterization of the ECM, and the effect of ECM on viability and function
108   of encapsulated isolated human pancreatic islets.

109

110   **PROTOCOL:**
111   This research study was approved by the human research committee of Wake Forest Baptist
112   Medical Center. Human pancreases were ethically obtained from organ donors through Carolina
113   Donor Services. Organ donors were screened for infectious diseases relevant to humans,
114   according to UNOS regulations. Organs were received in sterile preservation solution where they
115   were kept until use. Upon delivery to the lab, all organs were inspected, and samples of the native
116   pancreas were collected for histological purposes. The organs were then frozen and stored in
117   sterile conditions at -20 °C until further use.

118

119   **1. Surgical preparation of the human pancreas**

120

121   NOTE: Frozen pancreases were thawed overnight at 4 °C.

122

123   1.1.   Before initiating surgical dissection of the human pancreas, prepare 1 L of washing
124   solution, consisting of 950 mL of deionized water (dH$_2$O), 50 mL of iodine-based reagent and 1%
125   Pen/Strep solution.

126

127   1.2.   Wear sterile gloves and a lab coat during the surgical dissection. Perform all surgeries
128   under a cell-culture-grade hood to avoid possible contamination.

129

130   1.3.   Transfer the pancreas from the sterile transport bag into the sterile placenta basin. Ensure
131   the pancreas is oriented with the head of the organ to the left and the tail to the right.

132

133 1.4.   Carefully dissect the duodenum from the head of the pancreas using sterile scissors and
134 non-toothed forceps. Be careful not to make a hole in the duodenum, as this increases the
135 chances of contamination.
136
137 NOTE: If a hole is made in the gastrointestinal tract, quickly clamp the hole, and proceed with the
138 dissection.
139
140 1.5.   Once the dissection of the head of the pancreas is completed, dispose of the duodenum
141 into an appropriate biohazard bag and follow institutional guidelines for its disposal.
142
143 1.6.   Dissect and discard all extra-pancreatic tissue remaining around the head of the pancreas,
144 including major blood vessels and the common bile duct, which are usually marked at the time
145 of organ procurement with non-absorbable sutures. Dispose of the tissue following institutional
146 guidelines.
147
148 1.7.   Dissect and discard all extra-pancreatic tissue and peripancreatic adipose tissue
149 surrounding the tail of the pancreas, exposing the splenic blood vessels.
150
151 1.8.   Once the vascular pedicle of the spleen is exposed, remove the spleen, and dissect the
152 splenic blood vessels in their entirety, including those attached to the posterior surface of the
153 pancreas (the splenic artery and vein). Dispose of the spleen and remove tissue following
154 institutional guidelines.
155
156 1.9.   Appropriately dissect and dispose any extra-pancreatic fat clearly visible, especially
157 peripancreatic fat.
158
159 NOTE: At the level of the tail of the pancreas, the retroperitoneum may be visible. This should be
160 lifted with non-toothed forceps, removed accordingly with surgical forceps, and disposed of
161 properly. The pancreas should now be free of any extra-pancreatic tissue.
162
163 1.10.   Dissect the pancreas to obtain 1 cm^3 cubes of pancreatic tissue using surgical scissors,
164 sterilized histological blades, or surgical scalpels. Pancreatic tissue cubes obtained after this
165 dissection will undergo the decellularization process. The average number of pancreatic cubes
166 that undergo decellularization depends on intrinsic characteristics of the organ. On an average,
167 between 40–60 pancreatic cubes underwent decellularization in the presented study.
168
169 1.11.   Proceed to step 2.1
170
171 **2. Decellularization of the pancreatic tissue**
172
173 NOTE: This protocol was used with pancreas with an average weight of 100 g; therefore, it is not
174 recommended to exceed this weight when using this decellularization technique.
175
176 2.1.   Place all the pancreatic tissue previously dissected in step 1.10 in a sterile plastic container

(see **Table of Materials**) with the washing solution and incubate for 15 min at room temperature (RT).

2.2.   Remove the pancreatic tissue with the help of sterile forceps and place it in a new sterile container that contains sterile, endotoxin-free deionized water. Firmly close the cap of the container and place it on a refrigerated orbital shaker with a set temperature of 4 °C and a shaking speed between 180–200 rpm for 24 h.

2.3.   Remove the container with the pancreatic tissue and clean the outside appropriately before placing it into the hood for continuation of the process.

2.4.   Remove the pancreatic tissue using surgical forceps and place it into a new sterile container, along with 1 L of deionized water, 50 mg of DNase I, and 0.0025% magnesium chloride. Firmly close the cap of the container and place it in an orbital shaker, with a preset temperature of 37 °C and shaking speed of 100 rpm for 6 h.

2.5.   Prepare the EDTA-Trizma solution

2.5.1.   Gather a new sterile 1 L container and clean it appropriately before introducing it into the hood. To prepare 1 L of EDTA-Trizma solution, add the following: 985 mL of sterile deionized water, 7.5 mL of Tris-HCl Buffer, and 7.5 mL of EDTA solution. Store it at 4 °C until use.

2.6.   Remove the container enclosing the pancreatic tissue and clean the outside appropriately before introducing it into the hood for the continuation of the process.

2.7.   Remove the pancreatic tissue with the help of surgical forceps and place it into a new sterile container with 1 L of EDTA-Trizma-based solution. Firmly close the cap of the container and place it in an orbital shaker, with a preset temperature of 4 °C and a shaking speed between 180–200 rpm for 18 h.

2.8.   Remove the container containing the pancreatic tissue and clean the outside appropriately before introducing it into the hood for the process.

2.9.   Remove the pancreatic tissue with the help of sterile forceps and place it in a new sterile container with sterile endotoxin-free deionized water. Firmly close the cap of the container and place it on a refrigerated orbital shaker, with a set temperature of 4 °C and shaking speed between 180 and 200 rpm for 24 h.

NOTE: During this processing period, there will be a noticeable change in color, in which the tissue will appear brighter.

2.10.   Collect the pancreatic tissue and store it in sterile 50 mL centrifugation tubes, reaching a maximum of 25 mL per tube for subsequent freezing and lyophilization. The number of centrifugation tubes varies depending on the size of the pancreas. Store samples at -80 °C.

221

3. Production of pancreatic ECM powder – lyophilization and cryo-milling

223

3.1.   After a minimum of 24–40 h of storage of the decellularized material at -80 °C, transfer the pancreatic ECM into a freeze dryer.

226

3.2.   Transfer the frozen material into a freeze dryer, preventing the material from thawing.

228

NOTE: The lyophilization step takes approximately 3–5 days depending on several factors, such as the amount of material in each tube undergoing the process and the surface exposed for lyophilization. It is recommended that the decellularized tissue does not exceed the level of 25 mL in each tube and that the tubes are frozen at a 45° angle, allowing more surface to be readily exposed once the lyophilization starts. Remove the lids from the tubes.

234

3.3.   After lyophilization, transfer the materials into a cell-culture-grade hood.

236

3.4.   Cut each piece of the dried ECM into half of its size using sterile scissors. These dried ECM pieces will be subjected to an automated milling process to produce an ECM powder. Use a cryo-milled automatic processor to avoid a rise in temperature and subsequent denaturation of the biological properties of the ECM. Make sure to adjust the setting of the cryo-milling machine to obtain a fine powder after the milling process.

242

NOTE: In this study, pancreas from donors with a BMI < 30 were used. Pancreata from donors with a BMI > 30 showed a tendency to contain more intraparenchymal fat. The process of lyophilization is not considered optimal and may be inhibited by fat content. If after lyophilization the tissue is visibly oleaginous, it must be discarded as it will not be optimal for manufacturing the ECM powder.

248

4. Solubilization of the pancreatic ECM

250

NOTE: At this point, from an average size pancreas (100 g), the yield of the decellularized pancreatic powder is 1–2 g.

253

4.1.   Weigh 1 g of ECM powder with the aid of a sterile plastic spatula.

255

4.2.   Achieve solubilization following protocols previously described: Solubilize 1 g of ECM in 100 mL of 0.01 M HCl and 100 mg of pepsin for 48 h at RT with constant stirring[28].

258

4.3.   After 48 h, use NaOH to neutralize the pH (pH = 7) of the acidic ECM while keeping the beaker containing the acidic ECM on ice to avoid gelation.

261

NOTE: Gelation kinetics varies according to the temperature, collagen content, pH, and neutralizing solution. In this research study, 0.01 M of NaOH was used. The volume of NaOH added for pH neutralization was approximately 100 mL. Consider measuring the pH multiple

265 times while neutralizing the acidic ECM solution.

266

267 4.4.   Transfer the neutral ECM solution into sterile 50 mL centrifuge tubes. Close the tubes and
268 place them into a centrifuge. Centrifuge the tubes containing the solubilized ECM at maximum
269 speed (12,000 *x g*) for 15 min at 4 °C. Following centrifugation, transfer the tubes into a cell
270 culture hood.

271

272 NOTE: Following centrifugation of the sample, the insoluble component of the ECM will be
273 deposited at the bottom of the tube, while the solubilized ECM-solution will be found at the top
274 in the supernatant.

275

276 4.5.   Using sterile techniques, collect the supernatant of the tube containing the solubilized
277 ECM solution and transfer it into fresh 50 mL tubes, reaching a maximum of 25 mL per tube.

278

279 4.6.   Store the ECM-solution tubes at -80 °C.

280

281 **5. Production of pancreatic ECM powder – lyophilization and storage**

282

283 5.1.   After at least 24–40 h of storage of the soluble ECM-solution at -80 °C, transfer the tubes
284 into a freeze dryer.

285

286 5.2.   Transfer the frozen material into a freeze dryer, ensuring that the material does not thaw.

287

288 NOTE: The lyophilization step takes approximately 3–5 days depending on several factors, such
289 as the amount of material in each tube undergoing the process and the surface exposed for
290 lyophilization. It is recommended that the solubilized ECM solution does not exceed the level of
291 25 mL in each tube and that the tubes are frozen at a 45° angle to allow more surface to be readily
292 exposed once the lyophilization starts. Remove the lids from the falcon tube before
293 lyophilization.

294

295 5.3.   After lyophilization of the solubilized ECM-solution, transfer the material into a cell
296 culture-grade hood. After complete lyophilization, a fine ECM powder will be visible.

297

298 NOTE: The obtained yield is 700 mg from 1 g of decellularized tissue.

299

300 5.4.   Store the solubilized ECM powder or use for the cell culture purposes.

301

302 NOTE: It is advised to store the ECM at 4 °C for immediate use, at -20 °C for later use, and at -80
303 °C for long-term storage.

304

305 **6. Characterization of the pancreatic ECM with histological staining**

306

307 6.1.   Collect a piece of native pancreas soon after the delivery of the organ and before the
308 decellularization. Fix this for 24 h in 10% formalin. Wash the samples in deionized water.

309   Dehydrate it in graded alcohol before embedding it in paraffin and slice into 5 mm sections.
310
311   6.2.   At the end of the decellularization process, collect a sample of the decellularized material
312   and fix it for 24 h in 10% formalin. Wash the sample in deionized water. Dehydrate it in graded
313   alcohol before embedding it in paraffin and slice into 5 mm sections.
314
315   6.3.   To verify a successful decellularization, perform H&E and DAPI staining on both the native
316   pancreas and the decellularized counterpart.
317
318   6.4.   For histological characterization, perform Masson's Trichrome (MT) and Alcian Blue (AB)
319   stainings, which will highlight the collagenous and GAGs structures, respectively.
320
321   **7. Characterization of the soluble ECM powder**
322
323   7.1.   Perform DNA content and total collagen analysis of the native pancreatic tissue and the
324   soluble ECM powder as described previously[29].
325
326   7.2.   Quantify sulfated glycosaminoglycans (sGAG) based on the protocol provided by a
327   commercially available kit (see **Table of Materials**). Use a microplate spectrophotometer to
328   measure sulfated sGAG content in the pancreatic ECM at a wavelength of 595 nm[30].
329
330   NOTE: Report DNA content as ng/mg of dry tissue and the collagen and GAGs content as μg/mg
331   of dry tissue.
332
333   **8. Human Islets encapsulation with the ECM and culture**
334
335   NOTE: Human pancreatic Islets were commercially obtained (see **Table of Materials**).
336
337   8.1.   Upon arrival, culture the human pancreatic islets in non-tissue culture treated plates for
338   24 h under standard condition with an approximate density of 2,000 IEQ in each 10 cm plate.
339
340   8.2.   Manipulate the human islets to test the effect of the ECM on the islets' functionality and
341   viability with the following experimental groups: Free Islets, Islets encapsulated in alginate, and
342   Islets encapsulated in alginate-ECM.
343
344   NOTE: The above-mentioned groups of islets were cultured in vitro under standard condition for
345   up to 8 days to assess the effect of the ECM on islets functionality and viability.
346
347   8.3.   Weigh alginate on a scale and suspend it with HBSS into a centrifugation tube in order to
348   obtain a ratio of 1.5% (w/v). Place the centrifuge tube on a rotator overnight at 4 ˚C.
349
350   NOTE: A molecular weight between 75–200 kDA and a G/M ratio of ≤ 1 were reported by the
351   manufacturer for the alginate.
352

353 8.4.   Weigh 0.1 mg of ECM and suspend it in 1 mL of the alginate previously prepared in order
354 to have ECM concentration of 0.1 mg/mL.
355
356 8.5.   Encapsulate human pancreatic islets in alginate and alginate-ECM according to a
357 previously described protocol[31]. The procedure is described in brief below.
358
359 8.5.1. Gently collect and mix 3,000 IEQ in 1 mL of alginate and 3,000 IEQ in 1 mL of alginate-ECM
360 at an ECM concentration of 0.1 mg/mL previously prepared in step 8.4.
361
362 8.5.2. Pump the obtained suspensions through a microfluidic encapsulation device set to 0.2
363 mL/min and flow rate and air pressure of 2.0 Pa, respectively.
364
365 8.5.3. Upon production, collect the microcapsules in 100 mM of $CaCl_2$ bath with 10 mM of
366 HEPES. Allow alginate to crosslink for 10 min.
367
368 8.5.4. Prior to being cultured, wash the encapsulated islets with HBSS under standard condition,
369 for 2–3 min at 37 ˚C, with 5% CO_2.
370
371 8.5.5. Add the culture media (see **Table of Materials**) and change two-third of the media every
372 other day until the end of the experiment.
373
374 NOTE: The above steps must be performed both for the Islets in alginate and Islets in alginate-
375 ECM.
376
377 8.6.   On day 8, post-encapsulation, perform glucose-stimulated insulin secretion (GSIS) and
378 DNA analysis to assess the production of insulin and islets viability, respectively.
379
380 8.7.   Obtain brightfield images and Live/Dead staining of free and encapsulated islets to assess
381 cellular morphology and viability.
382
383 NOTE: Qualitative assessment of the images was performed to assess the islets' health.
384 Parameters taken into consideration include shape, border, integrity, and diameter of the islets,
385 as well as the presence of single cells in culture.
386
387 **9. Glucose-stimulated insulin release (GSIS) and DNA measurement**
388
389 NOTE: On day 8, post-encapsulation, human pancreatic islets were collected and incubated with
390 Kreb's buffer, containing low (2.8 mM) and high (16.8 mM) glucose concentrations, followed by
391 KCl depolarization solution (25 mM). The glucose challenge was performed with modification of
392 a protocol previously described[32].
393
394 9.1.   Insert gel filtration resin in polypropylene columns.
395
396 9.2.   Insert treatment groups (free islets or encapsulated islets) in the middle portion of the gel

397   filtration resin within the polypropylene columns.

398

399   9.3.   Pipette glucose and depolarizing solutions into the polypropylene columns described
400   above and incubate for 1 h, in the following order: perform a pre-incubation period with low
401   molarity glucose solution, then proceed with a series of incubations with low, high, low, and
402   depolarizing glucose solution as mentioned in the NOTE at the beginning of section 9.

403

404   9.4.   Collect the medium from each incubation phase and store it at -80 °C for subsequent
405   analysis.

406

407   9.5.   Following incubation and collection of the depolarizing solution, incubate human
408   pancreatic islets with 1 mL of DNA extraction buffer and store it until further analysis.

409

410   9.6.   Measure the insulin content from the glucose and the depolarizing solution incubations
411   with an Insulin ELISA assay, following the manufacturer's protocol.

412

413   9.7.   Measure the DNA content from the extraction buffer collected in step 9.5 using a DNA
414   assay kit following the manufacturer's protocol.

415

416   9.8.   Normalize the insulin measurements according to the DNA content.

417

418   **10. Statistical analysis**

419

420   NOTE: Group comparisons refer to the same batch of human islets with n = 3 independent
421   assessment conducted for each assay described in this manuscript. Values are expressed as Mean
422     $\pm$ SD.

423

424   10.1.   Use a statistical software to perform the Mann-Whitney test for the assessment of
425   decellularization and DNA remnants analyses, compare the native, the decellularized pancreas,
426   and the solubilized ECM.

427

428   10.2.   Perform an unpaired t-test for the assessment of the glycosaminoglycans and collagen
429   between the native, the decellularized pancreas, and the solubilized ECM.

430

431   10.3.   Perform a 2-way ANOVA with post-hoc Turkey's multiple comparisons for the Glucose
432   Stimulation Test in the assessment of the islets stimulation on day 8.

433

434   10.4.   Consider statistical significance at $p < 0.05$ with designation of *$p < 0.05$, **$p<0.01$, ***p
435   < 0.001, and ****$p < 0.0001$.

436

437   **REPRESENTATIVE RESULTS:**
438   Native and acellular pancreatic samples were processed for histological staining with H&E, MT,
439   and AB. The H&E staining showed complete absence of nuclear material and cells, confirming

440   successful decellularization. MT and AB stainings showed the framework of the ECM, highlighting
441   qualitatively collagenous and stromal components, respectively (**Figure 1**).
442
443   This method enabled the consistent generation of an ECM powder from the human pancreas.
444   DNA quantification tests confirmed satisfactory cell clearance[33]. Native, acellular, and solubilized
445   pancreatic ECM were biochemically characterized in order to assess basic biological composition.
446   The pancreatic ECM was determined to be acellular and DNA-free (DNA < 50 ng.mg^{-1} of dry
447   tissue), with consistent preservation of collagen and glycosaminoglycans as reported by
448   others[11,15]. DNA analysis demonstrated clearance of deoxyribonucleic acid in both acellular and
449   solubilized pancreatic ECM (from 4.56 µg/mg ± 3.42 µg/mg to 30.05 ng/mg ± 22.89 ng/mg for
450   the acellular pancreas p = 0.0001; from 4.56 µg/mg ± 3.42 µg/mg to 22.81 ng/mg ± 11.31 ng/mg
451   for the solubilized pancreatic ECM). (**Figure 2**).
452
453   Total collagen was quantified in the native pancreas, in the acellular and in the solubilized
454   pancreatic ECM. Results showed a statistically significant increase in collagen in the acellular
455   pancreas and in the soluble pancreatic ECM compared to the native tissue (from 7.35 µg/mg ±
456   1.68 µg/mg to 27.74 µg/mg ± 2.35 µg/mg for the acellular pancreas p < 0.0001; from 7.35 µg/mg
457   ± 1.68 µg/mg to 26.08 µg/mg ± 3.63 µg/mg for the solubilized pancreatic ECM p < 0.001). This
458   increase in collagen content is due to the isolation and purification of the ECM from the cellular
459   components, which depict an overall enrichment in collagen and sGAG in the ECM compared to
460   the native organ (**Figure 2**).
461
462   Quantification of glycosaminoglycans showed a statistically significant decrease of GAG content
463   in the acellular tissue and solubilized pancreatic ECM comparable to our previous study[11,15], with
464   a decrease from 15.08 µg/mg ± 3.03 µg/mg to 4.87 µg/mg ± 1.20 µg/mg for the acellular pancreas
465   p < 0.001 and from 15.08 µg/mg ± 3.03 µg/mg to 3.45 µg/mg ± 0.20 µg/mg for the solubilized
466   pancreatic ECM (p < 0.01) (**Figure 2**).
467
468   Encapsulated islets cultured in non-tissue culture treated plates were viable 8 days post-
469   encapsulation. Both live and dead staining showed that islets cultured in the three different
470   conditions were viable; however, there was an increased presence of dead cells when un-
471   encapsulated (**Figure 3**). Encapsulated islets maintained their spherical shape with a well-
472   rounded border, a stable diameter, and almost no single cells in culture. At the time point
473   analyzed, free islets showed a tendency to aggregate, to develop irregularities at the borders and
474   shapes, and to exhibit a darker core, suggestive of a necrotic event.
475
476   Both free and encapsulated islets were found to be glucose responsive at the time point analyzed
477   (**Figure 3**). Islets encapsulated in ECM-alginate showed a significant increase in insulin secretion
478   following high glucose stimulation and KCl depolarization compared both to free and alginate-
479   only encapsulated islets.
480
481   **FIGURE AND TABLE LEGENDS:**
482

483 **Figure 1: Representative H&E, Masson's Trichrome and Alcian Blue histological images.** Native
484 human pancreas (**A,C,E**) and human pancreatic ECM (**B,D,F**) decellularized with the newly
485 developed experimental protocol. H&E panel demonstrated a complete loss of nuclear structures
486 compared to the native pancreatic tissue. Masson's Trichrome staining highlight the framework
487 of the extracellular matrix and Alcian Blue staining highlight the connective tissue framework of
488 the extracellular matrix. Scale bar = 100 μm for panels A,C,D,E,F; Scale bar = 25 μm for panel B.
489
490 **Figure 2: Biochemical characterization of native pancreas, acellular, and solubilized pancreatic**
491 **ECM.** (**A**) Satisfactory removal of DNA in the acellular pancreas and in the solubilized pancreatic
492 ECM was confirmed. (**B,C**) Glycosaminoglycans and collagen quantification performed in the
493 native pancreas compared to the acellular and solubilized ECM. Statistical analysis was
494 performed by the t-test of native versus decellularized pancreas and native vs solubilized ECM;
495 **** = $p < 0.0001$; ***$p < 0.001$; **$p < 0.01$.
496
497 **Figure 3: Qualitative assessment of viability of human islets and quantitative assessment of**
498 **insulin secretion.** (**A**) Brightfield and Live/dead images of human isolated islets cultured as free,
499 in alginate capsules and in alginate-ECM capsules on day 6 post-encapsulation. (**B**) Glucose
500 stimulation assessment of human isolated islets cultured on non-tissue culture treated plate in
501 three different settings: free, in alginate capsules and in alginate-ECM capsules on day 8 post-
502 encapsulation. Values of the insulin secretion were reported after DNA normalization. Statistical
503 comparisons of the insulin secretion are made between the three culture conditions in high
504 glucose and after KCl depolarizing solution, respectively; *$p < 0.0$ and ***$p < 0.001$.
505
506 **DISCUSSION:**
507 The aim of this work was to develop a gentler, detergent-free decellularization protocol to
508 produce pancreatic ECM. Attention was paid to the preservation of ECM components of the
509 pancreatic parenchyma and the avoidance of a lengthy tissue exposure to conventional ionic or
510 non-ionic chemical detergents during the decellularization process.
511
512 The most innovative aspect of the developed decellularization method is the avoidance of classic
513 ionic and non-ionic chemical detergents. Our previous experience with the production of human
514 pancreatic ECM [11-13,22,34,35] set the baseline for the production of acellular pancreatic supportive
515 media. Nevertheless, the infusion with hundreds of liters of chemical detergents through the
516 pancreatic duct, the superior mesenteric artery, and the splenic artery[11,13], requires specific
517 surgical techniques and is not always feasible in large-scale production. Most importantly,
518 decellularizing the tissue in an orbital shaker rather than infusing detergents through the
519 vasculature represents a paradigm shift in methodology, contributing enormously to technical
520 ease, consistency, and feasibility of decellularization, which enhance ECM production for
521 translation. Moreover, organs procured for research purposes are often damaged due to
522 inconsistencies in the procurement process, sometimes attributable to abnormal anatomy that
523 frustrates cannulation. Therefore, our method would allow for all organs to be processed for
524 decellularization. We infer that this method could be of interest for the decellularization and the
525 production of ECM from various organs and potentially for both in vitro and in vivo applications.
526

527 Development of the present decellularization method was influenced by our previous experience
528 using Triton X-100 as the main chemical detergent[11,13,36]. Being unable to quantify the remnant
529 Triton X-100 on ECM post-decellularization and the lack of feasibility in scaling up the
530 manufacturing process in a cGMP environment led our group to investigate the feasibility of
531 obtaining decellularization by mechanical shaking rather than a perfusion of the whole pancreas.
532 We hypothesized that utilization of a hypotonic solution would be efficient in precipitating
533 osmotic damage in residing cells and exposing cellular nuclear material for a subsequent
534 enzymatic treatment, a process aimed at the clearance of deoxyribonucleic acid residues.
535 Following initial approaches to decellularize human pancreases by shaking diced tissue in
536 chemical detergents for 48 h, we noticed that deionized water was effective in providing the
537 cellular damage needed for the subsequent enzymatic step and cellular removals. We then
538 explored the option of reducing the detergent-free phase at 24 h and confirmed that cellular
539 mechanical damage due to the hypotonic solution, deionized water, was consistently able to
540 provide a successful decellularization while avoiding the use of potential cytotoxic agents. Our
541 data on the quantification of collagen and glycosaminoglycans showed consistent trends with
542 our previous experiences and other research groups' recent observations of the human
543 pancreas[11,15]. Moreover, we found that a solubilization step did not reduce the quantity of critical
544 components of the ECM, as their preservation is critical for ECM scaffolds to exert the
545 function[15,21,24,36]. Pancreas decellularization methods currently discussed in literature[37] are
546 characterized by the use of ionic or non-ionic detergents and the consequent loss of the innate
547 ECM composition, allowing us to infer that a more gentle method, possibly detergent-free, would
548 better preserve the pancreatic ECM basic components.
549
550 The ECM is the 3D framework that provides structural and biochemical support to the cells in
551 multicellular organisms. ECM proteins and structures play vital roles in the determination,
552 differentiation, proliferation, survival, polarity, and migration of cells[6]. The rationale for
553 incorporating the pancreatic ECM in both in vitro and in vivo applications is based on the evidence
554 that: in mature islets, interactions with ECM or other matrix materials regulate cell survival,
555 proliferation, and insulin secretion, and aid in the preservation and restoration of the spherical
556 islet morphology[3,21,22,38]; partially digested islets that retain some of their native ECM
557 connections display markedly reduced apoptosis rates and significantly higher GSIS functionality
558 compared to highly purified and ECM-free islets[38-40]; and ECM enhances islet function by
559 preventing leukocyte infiltration and up-regulating the expression of α3 integrin and focal
560 adhesion kinase, a crucial characteristic for beta cell-ECM attachment and interaction[41-43].
561
562 Glucose-stimulated insulin secretion following the encapsulation of human islets in alginate
563 microcapsules shows how the reconstitution of a tridimensional setting provides a healthier
564 environment for islets in comparison to an in vitro culture on non-tissue culture treated plates.
565 Encapsulation with alginate provides maintenance of the initial structure of the islets, avoiding
566 cellular clumping and dispersion of single cells, therefore constituting the physiological structure
567 of human islets. It is well known that islet function gradually declines over time when cultured in
568 vitro under normal conditions, and alginate, an inert biomaterial, does not provide sufficient
569 biochemical signaling for the maintenance of optimal islet function. Our ECM biomaterial proved
570 to have a positive in vitro impact on the viability and the functionality of the islets when

571 encapsulated with alginate compared to alginate only. Further investigation is necessary to prove
572 whether the addition of this biomaterial when solubilized could serve as a step toward the
573 improvement of islet-based technologies for in vitro culture systems and for potential in vivo
574 applications[44,45].
575
576 Some critical steps can be highlighted in the presented decellularization protocol: (1) a non-
577 optimal surgical dissection of the human pancreas can lead to an excessive presence of adipose
578 tissue, which will drastically impair the decellularization process; (2) an effective lyophilization
579 may not be achieved when excessive adipose tissue is present; (3) this will inevitably affect the
580 cryo-milling step, which will be non-efficient.
581
582 Several limitations were found using the presented protocol: (1) human-to-human organ
583 variability before and after decellularization was observed; (2) there were also some non-optimal
584 outcomes of decellularization in organs derived from donors with history of alcohol abuse,
585 patients with BMI > 30 and excessive peri- and intra-pancreatic adipose tissue accumulation. We
586 concluded that human pancreata from donors with BMI < 30 were generally deemed suitable for
587 decellularization.
588
589 The results of this study show that the human pancreas can be decellularized with an osmotic-
590 based, detergent-free process, to obtain ECM scaffolds for application in beta cell replacement
591 medicine, and islet manipulation in vitro. The manufacturing process presents a feasible and
592 reproducible approach for translational purposes and the avoidance of both chemical detergents
593 and the use of a perfusion-based system represent efficient and cost-effective solutions to
594 produce an ECM-based biomaterial, which when solubilized could be used in research and
595 potentially in clinical settings. There is potential in assessing the efficacy of this biomaterial
596 through recapitulating an optimal niche for the pancreatic islets and for insulin-producing cells
597 to improve long-term cell maintenance, viability, and cellular differentiation.
598

603

604 **DISCLOSURE**
605 There are no competing financial interests to declare by any of the coauthors.
606

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715

Figure1 Multipanel

Hematoxylin & Eosin
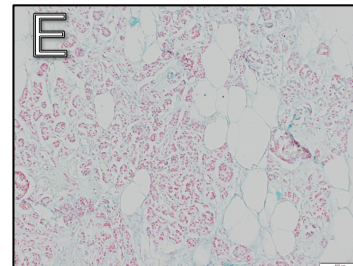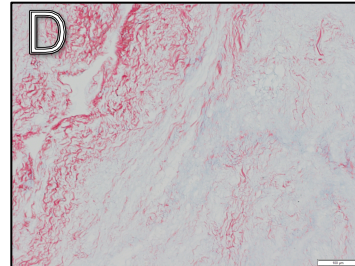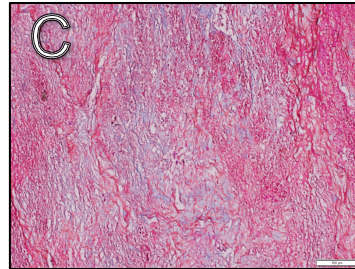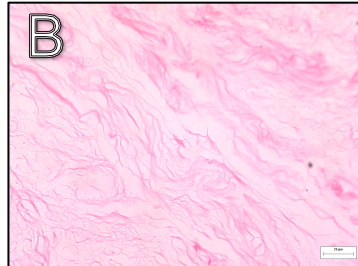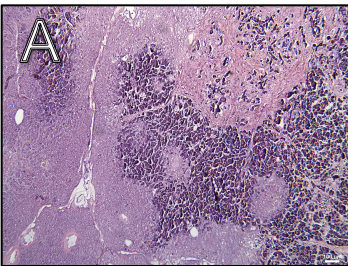
Masson's Trichrome

Alcian Blue

Figure2 Multipanel

A DNA quantification
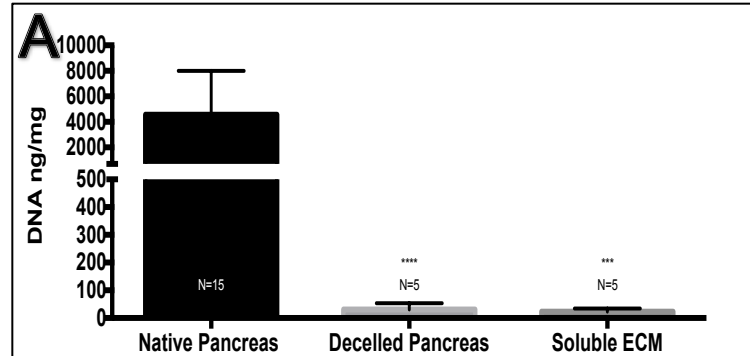
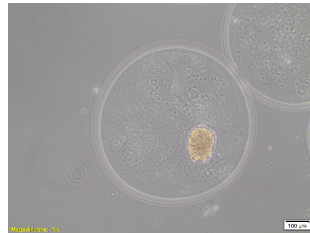**B** Glycosaminoglycans quantification
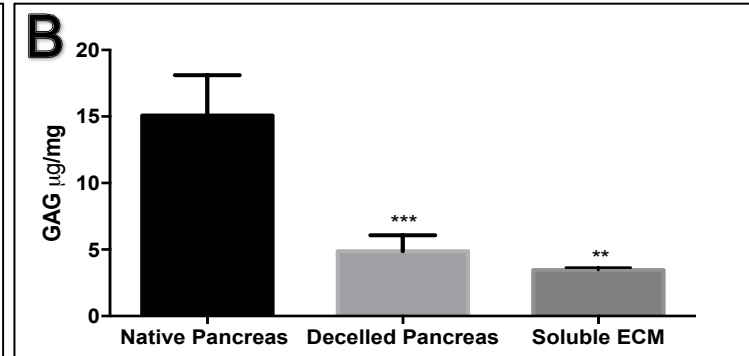
C Collagen quantification

Figure3 Multipanel

A
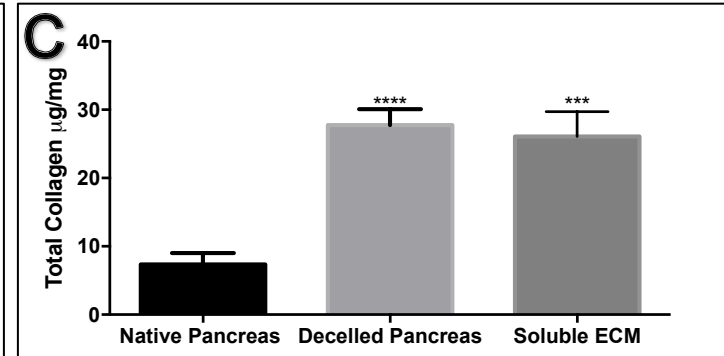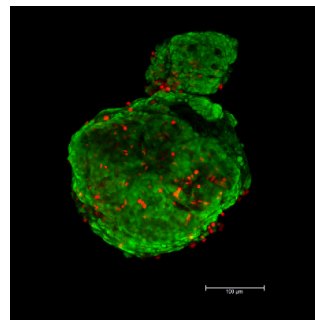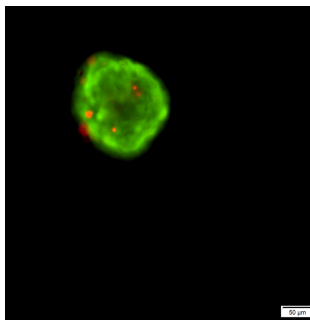
Free Islets | Alginate Capsules | Alginate-ECM Capsules

Brightfield

Live/Dead

B

Glucose Stimulation Test

Insulin Secretion (mU/ng*1000 DNA)

Low   High   Low   KCl

Free Islets
Alginate Capsules
Alginate-ECM Capsules
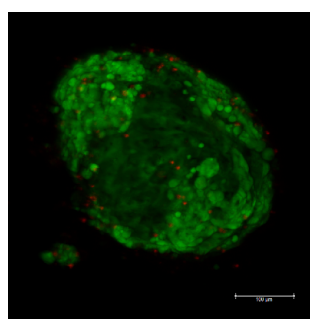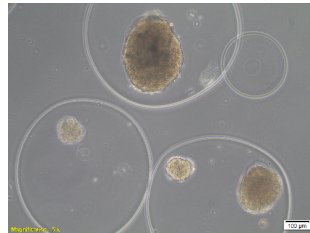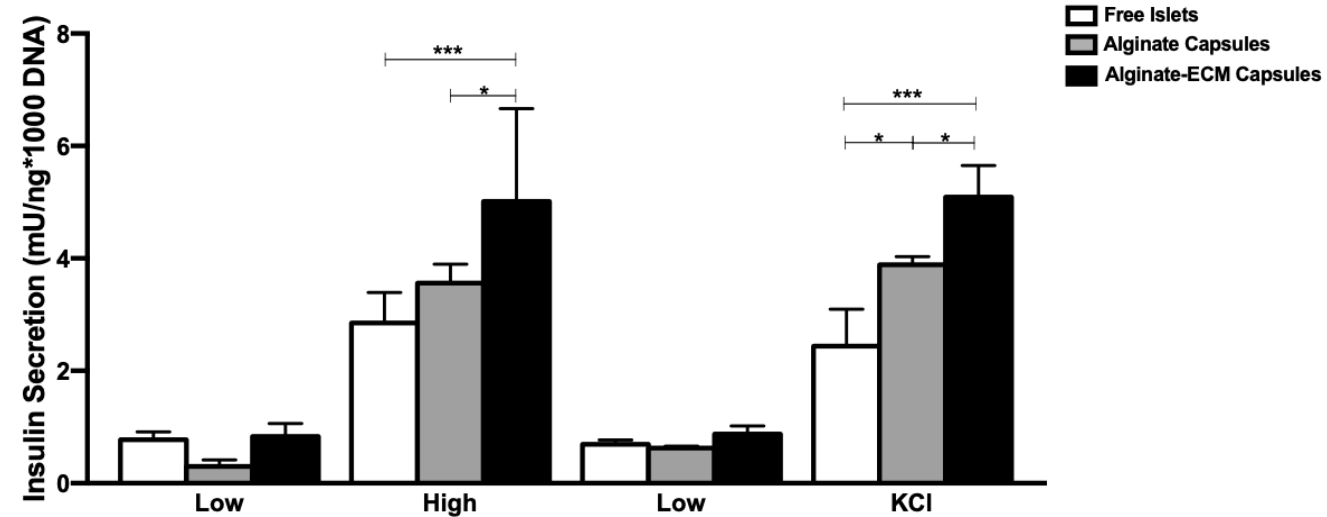
Name of Material/ Equipment	Company
Corning 1L Easy Grip Polystyrene Storage Bottles with 45mm Caps	ThermoFisher
Cryogenic Mill	SPEX Certiprep
Deoxyribonuclease I from bovine pancreas	Sigma Aldrich
Distilled Water	ThermoFisher
Falcon 50mL Conical Centrifuge Tubes	Corning
Human Pancreas	na
Insulin-Elisa	Mercodia
Magnesium Chloride, 1M, Sterile	Bio-World
Pepsin from porcine gastric mucosa	Sigma Aldrich
Placenta Basin w/o Lid, Sterile	DeRoyal
Polypre chromatography tubes	Bio-rad
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen
SamplePrep Large-Capacity Freezer/Mill Accessory	SPEX
Sephadex G-10 beads	Cytiva
Surgical kit	na
UltraPur 0.5M EDTA, pH 8.0	ThermoFisher
UltraPur 1 M Tris-HCl Buffer, pH 7.5	ThermoFisher
UltraPure DNase/RNase-Free Distilled Water	ThermoFisher

Catalog Number	Comments/Description
430518	Container use for decellularization
6870-230	
DN25	
15230147	
352070	
na	
10-1113-01	
41320004-1	
P7012-5G	
32-881	
731-1500	Polypropylene columns
P7589	DNA Kit
6801	Large Grinding Vial Set
17001001	Gel Filtration Resin
na	
15575020	
15567027	
10977023	

TITLE:

Detergent Free Decellularization of the Human Pancreas for Soluble Extracellular Matrix (ECM) Production

AUTHORS AND AFFILIATIONS:

Riccardo Tamburrini[1,2,3], Deborah Chaimov[1,3], Amish Asthana[1,3], Kevin Enck[3], Sean M. Muir[4], Justine Mariam Aziz[5], Sandrine Lablanche[6], Emily Tubbs[6], Alice A. Tomei[7,8], Mark Van Dyke[9], Shay Soker[3], Emmanuel C Opara[3], Giuseppe Orlando[1,3]

[1]Department of Surgery, Wake Forest Baptist Medical Center, Medical Center Boulevard, Winston Salem, NC, USA.

[2]Department of General Surgery, PhD Program in Experimental Medicine, University of Pavia , Pavia, Italy .

[3]Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC, USA.

[4]Wake Forest University College of Arts and Science, Winston Salem, NC, USA.

[5]Wake Forest University School of Medicine, Winston-Salem, NC, USA.

[6]Grenoble Alps University, Laboratory of Fundamental and Applied Bioenergetics (LBFA), and Environmental and System Biology (BEeSy), Grenoble, France; Inserm, U1055, Grenoble, France.

[7]Department of Biomedical Engineering, University of Miami, 1251 Memorial Dr, Coral Gables, FL, USA

[8]Diabetes Research Institute, University of Miami Miller School of Medicine, 1450 NW 10th Ave, Miami, FL, USA

[9]Department of Biomedical Engineering and Mechanics, Virginia Polytechnic Institute and State University, School of Biomedical Engineering and Sciences, Blacksburg, VA, USA.

Email addresses of co-authors:
Riccardo Tamburrini   (rtamburr@wakehealth.edu)
Deborah Chaimov   (dchaimov@wakehealth.edu)
Kevin Enck   (kenck@wakehealth.edu)
Sean M. Muir   (smuir@wakehealth.edu)
Amish Asthana   (aasthana@wakehealth.edu)
Justine Mariam Aziz   (jaziz@wakehealth.edu)
Sandrine Lablanche   (slablanche@chu-grenoble.fr)
Emily Tubbs   (Emily.tubbs@univ-grenoble-alpes.fr)
Alice A. Tomei   (atomei@med.miami.edu)
Mark Van Dyke   (mvandyk5@vt.edu)
Shay Soker   (ssoker@wakehealth.edu)
Emmanuel C Opara   (eopara@wakehealth.edu)
Giuseppe Orlando   (gorlando@wakehealth.edu)

Corresponding author:
Giuseppe Orlando   (gorlando@wakehealth.edu)

Dear Review Editor,

On behalf of the coauthors, we are re-submitting the revised version of the manuscript entitled:

"Detergent Free Decellularization of the Human Pancreas for Soluble Extracellular Matrix (ECM) Production"

for the JoVE collection on Experimental and Analysis Methods for Biomaterials and Biomechanics

We are very grateful for the comments received from the Review Editor. We believe that addressing the comments received we are able to improve to meet the high standard of the journal and that the EIC will seem it suitable for publication.

Sincerely

Giuseppe Orlando, MD, PhD, Marie Curie Fellow
Associate Professor
Department of Surgery, Section of Transplantation
Director of Translational Research
Wake Forest University School of Medicine
Wake Forest University Baptist Medical Center
Medical Center Boulevard
Winston Salem, NC 27157-1095
Email: gorlando@wakehealth.edu

Dear Editor,

We are really appreciative of your comments and formatting to the article we submitted to your attention.

As requested we addressed individually the editorial comments in the manuscript. You will be able to find the track changes. Please find below the comments addressing the modifications requested:

- An ethical statement has been added as requested.

- We included details regarding the surgical dissection and clarified details of the decellularization step as requested

- Regarding the number of centrifugation tubes used per pancreas decellularization: per our experience the number of centrifugation tubes depends on the size of the pancreas. We observed optimal freezing and lyophilization when the amount of tissue does not exceed 25ml of volume in each centrifugation tube.

- Regarding the volume of the lyophilized material: we noted that the volume of the lyophilized material depended on specific properties of the organ therefore it was variable on specific weight and characteristics of the pancreas. We have not recorded the volume of dried decellularized tissue before cryo-milling.

- We included details regarding lyophilization, cryo-milling and solubilization as requested

- A paragraph included in the protocol was deleted as not appropriate per journal guidelines as noted by the editor.

- Details regarding the histological process and stainings were added

- We expanded and added details regarding the sections "Human Islets encapsulation with

the ECM and culture" and "Glucose Stimulated Insulin Release and DNA measurement"

- We updated the section regarding the Statistical analysis as requested

- Details in the figure's legends were updated as requested.

- The Discussion section has been revised addressing the Editor comment and requests.

- The Acknowledgement section was updated.

- A Disclosure section was added as requested.

- The Table of Materials has been updated accordingly after removal of brand names from the manuscript.

- The article has been proofread and clarity has been brought where needed.

- As requested part of the protocol was highlighted for the video production