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3D Culturing of Organoids from the Intestinal Villi Epithelium Undergoing Dedifferentiation.

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TITLE:

3D Culturing of organoids from the intestinal villi epithelium undergoing dedifferentiation.

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KEYWORDS:

Villi, organoids, intestinal epithelium, stem cells, dedifferentiation, cell fate

SUMMARY:

The procedure describes isolation of the villi from the mouse intestinal epithelium undergoing dedifferentiation to determine their organoid forming potential.

ABSTRACT:

Clonogenicity of organoids from the intestinal epithelium is attributed to the presence of stem cells therein. The mouse small intestinal epithelium is compartmentalized into crypts and villi: the stem and proliferating cells are confined to the crypts, whereas the villi epithelium contains only differentiated cells. Hence, the normal intestinal crypts, but not the villi, can give rise to organoids in 3D cultures. The procedure described here is applicable only to villus epithelium undergoing dedifferentiation leading to stemness. The method described uses the Smad4-loss-of-function:β-catenin gain-of-function ($Smad4^{KO}$:$\beta\text{-catenin}^{GOF}$) conditional mutant mouse.The mutation causes the intestinal villi to dedifferentiate and generate stem cells in the villi. Intestinal villi undergoing dedifferentiation are scraped off the intestine using glass slides, placed in a 70 µm strainer and washed several times to filter out any loose cells or crypts prior to plating in BME-R1 matrix to determine their organoid-forming potential. Two main criteria were used to ensure that the resulting organoids were developed from the dedifferentiating villus compartment and not from the crypts: 1) microscopically evaluating the isolated villi to ensure absence of any tethered crypts, both before and after plating in the 3D matrix, and 2) monitoring the time course of organoid development from the villi. Organoid initiation from the villi occurs only two to five days after plating and appears irregularly shaped, whereas the crypt-derived organoids from the same

intestinal epithelium are apparent within sixteen hours of plating and appear spherical. The limitation of the method, however, is that the number of organoids formed, and the time required for organoid initiation from the villi vary depending on the degree of dedifferentiation. Hence, depending upon the specificity of the mutation or the insult causing the dedifferentiation, the optimal stage at which villi can be harvested to assay their organoid forming potential, must be determined empirically.

INTRODUCTION:

The intestinal crypts, but not villi, form organoids when cultured in Matrigel or BME-R1 matrix. These organoids are self-organizing structures, often referred to as the "mini-gut", owing to the presence of the various differentiated lineages, progenitor, and stem cells present in the intestinal epithelium *in vivo*. The potential to form organoids from crypts is attributed to the presence of stem cells[1]. The intestinal villi on the other hand consist only of differentiated cells, and hence cannot form organoids. However, mutations[2] or conditions that permit dedifferentiation of the villus epithelium may lead to stem cells in the villi[2, 3]. This fate change resulting in stemness in the villi epithelium can be confirmed by plating the dedifferentiating villus epithelium in 3D matrix to determine their organoid-forming potential as an indicator of *de-novo* stemness in the villus epithelium. Hence, the critical aspect of this procedure is to ensure absence of crypt contamination.

The $Smad4^{KO}$:β-$catenin^{GOF}$ conditional mutation causes dedifferentiation in the intestinal epithelium, marked by the expression of proliferation and stem cell markers in the villi, and eventually the formation of crypt-like structures in the villi, referred to as ectopic crypts. The presence of stem cells in these dedifferentiated villi was determined by the expression of stem cell markers in the ectopic crypts (*in vivo*) and the ability of the mutant villi to form organoids when plated in Matrigel[3]. The below mentioned procedure elaborates the methodology used to confirm the stemness of the dedifferentiating intestinal epithelium in the $Smad4^{KO}$:β-$catenin^{GOF}$ mutant mice. A key feature of this methodology for isolating villi was the use of scraping of the intestinal lumen, as opposed to the EDTA chelation method[4]. Unlike in the EDTA chelation method, villi isolation by scraping retains majority of the underlying mesenchyme and allows to adjust the pressure of scraping to yield villi without tethered crypts. The pressure of scraping is subjective to the operator, hence, the optimal pressure to yield villi without crypts tethered must be determined empirically by the operator. The critical aspect of this procedure is to ensure the absence of crypt contamination by microscopic examination of the villi both before and after plating in the BME-R1 matrix.

Intestinal villi are scraped off the intestinal lumen with glass slides and placed in a 70 µm filter and washed with PBS to get rid of loose cells or crypts, if any, prior to plating in BME-R1 matrix. The method stresses on the following criteria to avoid crypt contamination: a) confining the villi harvest to the proximal half of the duodenum where the villi are the longest, b) minimizing the number of villi-yielding scrapes, c) washing the filter containing the villi through a series of PBS in a six-well dish, and d) confirming the absence of crypt contamination by microscopic examination prior to and after plating in BME-R1 matrix. Villi isolation by scraping, rather than by EDTA chelation, prevents the complete loss of the underlying mesenchyme that may provide the niche signals[5, 6, 7, 8], if required, for organoid initiation from the villus epithelium.

PROTOCOL:

All the mouse experiments conducted, including the use of Tamoxifen and euthanasia by cervical dislocation, had the approval of the Institutional Animal Care and Use Committee at Stevens Institute of Technology.

1. Mice

NOTE: The generation of *Smad4$^{f/f}$; Catnb$^{lox(ex3)/+}$; Villin-Cre^{ERT2}* mice have been previously described[3]. Adult female mice between eight to twelve weeks of age were used.

1.1. Induce the Smad4^{KO}:β-catenin^{GOF} mutation in the *Smad4$^{f/f}$; Catnb$^{lox(ex3)/+}$; Villin-Cre^{ERT2}* with intraperitoneal injection of Tamoxifen in corn oil for four consecutive days.

1.2. Sacrifice the mice by cervical dislocation ten days after the first tamoxifen injection.

1.3. Spray the abdomen with 70% ethanol to prevent mouse fur getting into the peritoneal cavity.

1.4. Open the abdominal cavity with dissection scissors to expose the intestine. Isolate the intestine with the aid of the scissors and forceps.

NOTE: Concomitant loss of Smad4 along with gain of function mutation of β-catenin *(Smad4^{KO};β-catenin^{GOF})* in intestinal epithelium was attained by injecting *Smad4$^{f/f}$; Catnb$^{lox(ex3)/+}$; Villin-Cre^{ERT2}* mice every day for four consecutive days with 0.05 g Tamoxifen/kg body weight in corn oil. These mice are harvested ten days after the first tamoxifen injection to ensure the presence of cells expressing stem cell-associated markers in the dedifferentiating villi.

2. Duodenum isolation and preparation

2.1. Dissect out the proximal half of the duodenum.

2.2. Flush the duodenum with 5 mL of ice-cold PBS in a 10 mL syringe to clear the luminal content.

2.3. Open the duodenum longitudinally with an angled scissor and lay the duodenum flat on a 15 cm Petri plate on ice with the lumen of the duodenum facing the operator.

3. Villi isolation by scraping

3.1. Prior to beginning the scraping, place a 70 µm mesh strainer in one of the wells of a 6-well tissue culture plate. Fill all the wells with 4 mL of 1x PBS and place the 6-well tissue culture plate on ice (**Figure 1**).

3.2. Scrape the villi as follows using two microscopic glass slides: one to hold the duodenum down and the other to scrape (**Figure 1B1**).

3.2.1. Scrape the luminal side of the duodenum superficially to-and-fro twice to remove the mucus. Apply pressure such that this step removes the mucus layer, but not the villi.

3.2.2. Scrape the duodenum again, to-and-fro twice applying the same pressure as in 3.2.1, during which villi can be seen collecting on the slides (**Figure 1B2**). This is the optimal pressure (which must be determined empirically by the operator) to yield the villi without the crypts tethered.

3.3. Use a 1 mL transfer pipet containing PBS to transfer the villi (that are collected on the slide from step 3.2.2.) to a 70 µm mesh strainer placed in a 6-well dish. The villi are collected thus, after every scrape (**Figure 1B2**).

3.4. Wash the villi collected in the 70 µm strainer (from step 3.3) by transferring the strainer (with the villi) through a series of wells in a 6-well dish containing cold PBS (~ 4 mL/well). This is to remove loose crypts, if any.

3.5. Using a p1000 pipet, transfer the villi suspension in PBS (~ 3 mL) from the 70 µm strainer to a new 15 mL tube on ice.

3.6. Use a 0.1% BSA coated blunt-ended p200 pipet tip to aspirate 50 µL volume of the villi suspension onto a glass slide. Count the number of villi in the 50 µL droplet under 4x magnification to determine the concentration of villi in the PBS suspension. For example, if there are 10 villi in the 50 µL suspension examined, then the villi concentration is 0.2 villi/µL. This is also the time to confirm the absence of tethered crypts.

3.7. Calculate the volume of villi suspension required to plate at a concentration of 0.5 villi/µL of BME-R1 matrix. Add an extra 100 µL to account for pipetting error and the volume required for microscopic examination required to ensure the purity of the villi.

CAUTION: The pressure used in the first two scrapes, that removes the mucus but not the villi, should be used in the subsequent scrapes during which villi will be released. Limit the number of to-and-fro scrapings to two after the villi release is first observed. This measure avoids release of villi with the crypts tethered. It is essential to microscopically evaluate the villi to ensure the absence of the tethered crypts.

4. Plating of villi on BME-R1 matrix

4.1. Using a 0.1% BSA coated p200 blunt-ended pipet tip, transfer to a microcentrifuge tube the volume of villi (from step 3.6) required to plate at a density of ~ 6 villi per well in 12.5 µL of BME-R1 matrix. The use of BSA-coated blunt-ended tip at this point ensures that the villi, which are too large for a pointed tip are aspirated without being blocked or lost from being stuck to the sides of the tip.

4.2. Spin down the villi for 2 min at 200 x g in a refrigerated centrifuge (4 °C) and remove the supernatant.

4.3. Repeat step 4.2 to remove any residual PBS and proceed to the next step under a laminar flow-hood.

4.4. Resuspend the villi pellet gently in the required amount of cold BME-R1 thawed on ice.

4.5. Using a p20 pipet, plate 12.5 µL/well of the villi in BME-R1 matrix in 3D in a pre-warmed 96-well U-bottom plate.

4.6. Incubate the plate in a tissue culture incubator at 37 °C for 15 minutes to allow solidification of the BME-R1 matrix.

4.7. Add 125 µL/well of pre-warmed ENR (Epidermal growth factor/Noggin/R-spondin1) media: advanced DMEM F-12 media, supplemented with 1x Penicillin: Streptomycin, 10 mM HEPES, Glutamax, 50 ng/mL EGF, 100 ng/mL Noggin, 5% conditioned media from HEK293-T cells expressing R-Spondin1, 1x N2, 1x B27, 1 mM N-acetyl cysteine, and 0.05 mg/mL Primocin.

4.8. Incubate the plated villi in a tissue culture incubator maintained at 37 °C with 5% CO_2, and change the media every other day.

4.9. Discard any well in which organoids appear before two days of plating, since the earliest time point at which villi-derived organoids are expected is after two days.

NOTE: Any villi that has half or more than half the length of villi are counted as one villus. Unlike crypt-derived organoids, villi-derived organoids are not expected within 24 hours after plating. The organoid-initiating villi appear to be darkening and shrinking prior to formation of organoids. Hence, any wells with organoids developing within a day of plating should be discarded to avoid the plausibility of having derived from plausible crypt contamination. The methodology used to produce the conditioned media is available upon request.

REPRESENTATIVE RESULTS:

The determining factor for the success of the procedure is preventing crypt contamination. Organoid development from the villi (and not from any contaminating crypts) is ensured by confirming four major criteria: 1) ensuring the purity of the harvested villi by microscopic examination before and after plating the villi in BME-R1, 2) plating limited number of villi per well to allow visualization of all the plated villi individually, 3) monitoring the development of organoid daily; images of the time course show development of organoid from villi (**Figure 3**), and 4) monitoring the kinetics and morphological appearance of the organoids initiating from villi; the organoids initiating from villi appear irregularly shaped at first and take two to five days before they can be seen under 4x magnification, as opposed to the crypt-derived organoids (cultured by isolating crypts using the EDTA/PBS chelation method[1,4]) that appear overnight as spherical structures with well-defined borders (**Figure 2**).

The optimal time for sacrificing the mice for testing the organoid-forming potential of the villi is after the dedifferentiating villi-epithelium express stem cell markers and begin the development of ectopic crypts in the villi *in vivo* (around 10 days after Tamoxifen injection of the *Smad4$^{f/f}$; Catnb$^{lox(ex3)/+}$; Villin-Cre^{ERT2}* mice). These mice have a tamoxifen inducible cre-recombinase that

causes Smad4 loss concomitant with β-catenin activation with tamoxifen. The ectopic crypts develop fully within two weeks of induction of the mutation, while the stem cells in the villus epithelium appear within a week after the induction of mutation *in vivo*. The number of villi that form organoids when plated in Matrigel has been reported to vary between 2% to 12%[3]. Harvesting the mutant mouse for plating the villi around the time when stem cells are present (around a week after the cre-recombinase induction) will prolong the time required for villi-derived organoids appear, whereas delaying the harvest to later than ten days after the cre-induction increases the risk crypt of contamination.

Figure 1: Experimental set-up and villi scraping from intestinal epithelium. A) PBS-filled syringe (syrg) with tubing and filter (Fltr) in PBS. **B**) Villi isolation by scraping (**B1**) and transferring to a filter (**B2**). The arrow points the villi collected on the slide.

Figure 2: Distinction in the appearance of the organoids emerging from the crypts versus the dedifferentiated villi epithelium from the same mouse intestine: A) Cartoon showing villus and crypts. **B**) Whole mount (in PBS) of villi prepared by scraping (top panel) and crypts prepared by EDTA-chelation (lower panel). **C**) Villi-derived (top panel) and crypt-derived (lower panel) organoids from the same mouse intestinal epithelium in BME-R1 (scale bars, 500 um).

Figure 3: Time course of organoid formation from the dedifferentiating villi. Organoid initiation from two different villi is shown (the boxed regions are enlarged in the middle and bottom panels). The villi with organoid-forming potential appears dense, possibly due to the retention of the underlying mesenchyme. Organoid initiation from the villus is apparent at day two (solid arrow) from one of the villi (box with broken boundary), while in the other villus (box with solid boundary) the organoid appears at day four (arrow). The upper box in the day 6 panel shows a developed villi-derived organoid. Scale bar, 100 μm.

DISCUSSION:

This method can confirm the self-renewal capacity of dedifferentiating villi epithelium that acquire stem cell markers *in vivo.* The normal intestinal epithelium can give rise to organoids from the crypt, but not the villi compartment, when cultured in 3D because of the presence of stem cells in the crypts[1]. Thus, organoid formation from the dedifferentiated villi epithelium cultured in 3D cultures confirms stem cell formation from cell fate reversal. Reports on cell fate reversal in the intestinal epithelium arising from mutations and/or injury are increasing[2, 3, 9, 10, 11, 12]. Hence, this method is applicable in similar scenarios where the dedifferentiating villi epithelium expresses stem cell markers *in vivo*.

We confirmed the development of the organoids from the villi of the $Smad4^{KO}$:$\beta\text{-catenin}^{GOF}$ mutant through the time course showing development of organoid from the villi (Figure 3). The most critical aspect is taking precautionary measures against crypt contamination, which would lead to false positives. The following measures were taken to ensure the purity of the villi preparation before and after plating: a) isolation of villi from the proximal half of the duodenum where the villi are the longest, b) minimizing the number of scrapes and adjusting the pressure of scraping to yield villi without tethered crypts, c) washing the villi with PBS after placing them in a 70 μm mesh strainer to exclude any loose crypts or cells, d) microscopic examination of the

harvested villi prior to and after plating, and e) minimizing the number of villi plated per well so that they can be visualized individually in the plated matrix.

Several considerations were made to optimize the procedure. Firstly, we harvested the villi from the proximal duodenum where the villi are the longest. The physical delineation between the differentiation and the proliferative compartments in the small intestine allows us to adopt scraping to mechanically separate the villi from the crypts. This method can thus be adopted only in the cases where the compartmentalization between the differentiation and proliferative compartment is strictly delineated - as is the case in the small intestine but not in the colon. Secondly, we optimized the time of villi isolation after induction of the mutation based on the degree of dedifferentiation observed *in vivo.* Dedifferentiation in the conditional $Smad4^{KO}$:β-$catenin^{GOF}$ mutant is marked by the appearance *in vivo* of stem cell markers and ectopic crypts in the villus epithelium within seven and ten days respectively[3] . Thus, the time required to initiate organoids from the mutant villi decreases as the time elapsed after induction of the mutation increases. Therefore, while adapting this method to other dedifferentiation models of the intestine, the optimal time for sacrificing the mice for villi isolation should be determined empirically depending upon the kind of mutation or injury and the degree of ensuing dedifferentiation *in vivo*. Thirdly, we opted to isolate the villi by scraping rather than by the EDTA-chelation method in order to retain the underlying mesenchyme, as epithelial-mesenchymal cross-talks have been implicated in intestinal stem cell function in the intestine[5, 6, 7, 8, 13]. Villi that initiate the organoids mainly appear dark possibly due to the presence of the underlying mesenchyme (**Figure 2B and 3**). We verified the expression of the stem cell associated marker CD44[14, 15], and the intestine specific transcription factor Cdx2[16, 17, 18, 19, 20] confirming the epithelial origin of the villi-derived organoids (**Figure S1**).

Compared to *in vivo* approaches, organoids are more amenable to genetic manipulation and screening. Since we observe phenotypic differences between the organoids emerging from the crypts and the villi of the same $Smad4^{KO}$:β-$catenin^{GOF}$ intestinal epithelium (F**igure 2B**), we speculate molecular differences between the two, which is currently being investigated. Thus, this method is useful in investigating the implications of dedifferentiation-causing mutations and its differential effects in the progenitor versus differentiation compartment.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

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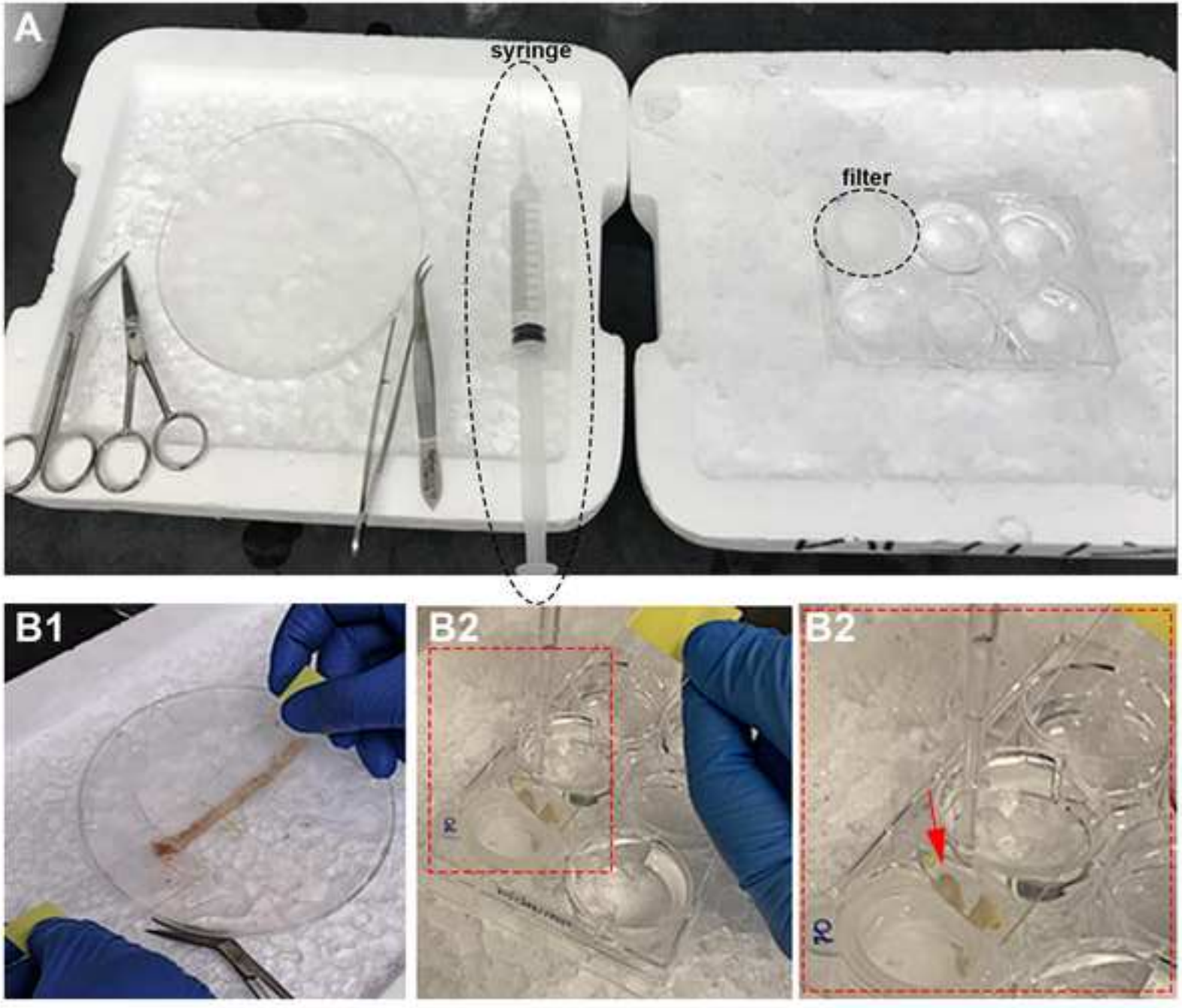

Figure 2

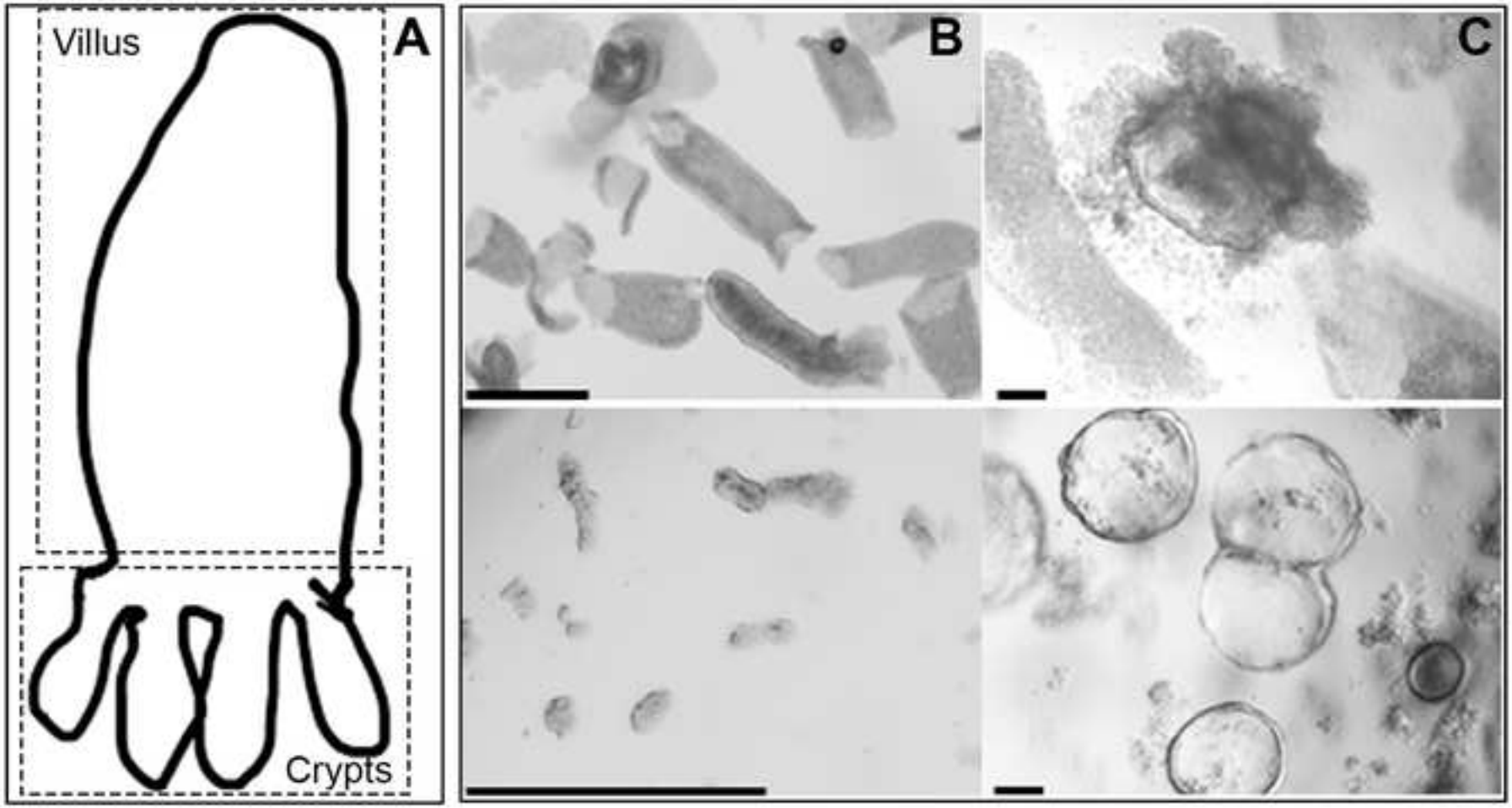

Figure 3

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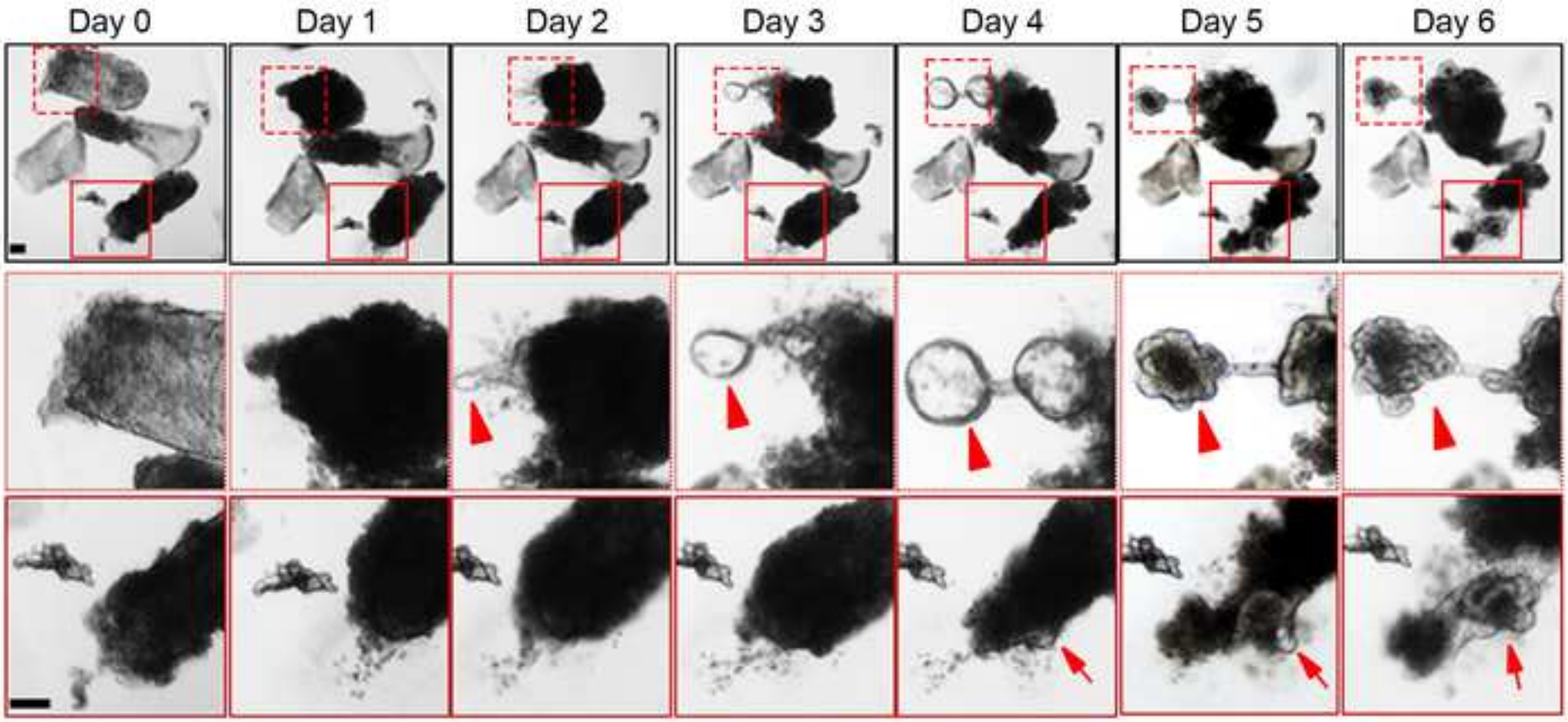

Name of Material/ Equipment	Company	Catalog Number
Advanced DMEM F-12 media	Gibco	12634010
3,3-diaminobenzidine	Vector Labs	SK-4105
96 well U-bottom plate	Fisher Scientific	FB012932
ABC kit	Vector Labs	PK4001
Angled scissor	Fisher Scientific	11-999
Animal-Free Recombinant Human EGF	Peprotech	AF-100-15
B-27 Supplement (50X), minus vitamin A	Gibco	12587010
Bovine Serum Albumin (BSA) Protease-free Powder	Fisher Scientific	BP9703100
CD44 antibody	BioLegend	1030001
Cdx2 antibody	Cell Signaling	12306
Corn oil	Sigma-Aldrich	C8267-500ML
Corning 70-micron cell strainer	Life Sciences	431751
Cultrex Reduced Growth Factor Basement Membrane Extract, Type R1	R&D	3433-005-R1

Dissection scissors	Fisher Scientific	22-079-747
Forceps	Fisher Scientific	17-456-209
Glutamax (100X)	Gibco	35050-061
HEK 293-T cells expressing RSPO-1	Gift from Dr. Michael Verzi	
HEPES (1M)	Gibco	15630-080
Histogel	Thermoscientific	HG-4000-012
Mesh filter	Fisher Scientific	07-201-431
Micrscope glass slide	VWR	89218-844
N-2 Supplement (100X)	Gibco	17502048
N-acetyl cysteine	Sigma-Aldrich	A9165
p200 Blunt tips	VWR	46620-642
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140-122
Primocin (50mg/mL)	Invivogen	ant-pm-1
Quality Biological Inc PBS (10X)	Fisher Scientific	50-146-770

Recombinant Murine Noggin	Peprotech	250-38
Signal diluent	Cell Signaling	8112L
Tamoxifen	Sigma-Aldrich	T5648-1G
6-well tissue culture plate	Fisher Scientific	50-146-770

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February 25, 2020

Dear Editor and Reviewers:

Thank you for the peer review and the required revisions. Our manuscript "3D Culturing of organoids from the intestinal villi epithelium undergoing dedifferentiation" is now very clear. We appreciate the feedback very much as it has tremendously improved the quality of manuscript. The concerns and suggestions by the editors and reviewers (in black) are *addressed in green italicized font*. We have attached two version of the manuscript: the first with all the tracked changes, and the second has all the changes accepted and without the tracking.

Ansu Perekatt, Ph.D.
Assistant Professor

Editorial comments:

Changes to be made by the Author(s):

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

No grammatical and spelling errors could be located.

2. Please remove commercial language from the written manuscript and place this information in the Table of Materials instead.

We have removed all the commercial language from the text. Material list has all the materials used, and the company information and catalogue numbers.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have satisfactorily addressed the majority of my concerns, particularly including the revision of several figures to include time course analysis (Fig.3), which is my major concern. Moreover, they have explained the method more detail how they care the contamination of

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crypts. In light of the authors' revisions, this manuscript highly merits publication in the JOVE.

Minor Concerns:
However, the revised manuscript still remains several errors in their revisions.
Please note that:
1. Figure1 legend: "The arrow points…" There is no arrow in the Fig.1
2. Fig.3: The upper box at Day6 (at top panel) should be described in the dash line.
3. Fig.3: Could you please put the information of scale bars at the top columns because those are different magnified images compared with lower's one.

Thank you again for the detailed observation. All the three points have been addressed.

Reviewer #2:
Manuscript Summary:
Perekatt and coworkers resubmitted the revised manuscript in titled "3D culturing of organoids from the intestinal villi epithelium undergoing dedifferentiation". The revised manuscript is getting better than the manuscript ver-1. However, there are some minor concerns as follows.
Thank you again for the detailed observation. All the four points have been addressed.

Minor Concerns:
Line 113: "Note" is repeated. Please delete one.
Repetition is removed
Line131, 134, 140, 145, 214, 219, 262, 289, 296, and figure legends: Which is correct, Fig and Figure? Please unify it.
This is now revised to Figure
Line 162: Please delete a part of bracket.
Corrected
4. Plating of villi on BME-R1 matrix; Line185-198: The number of the part is correct? 4.6, 4.5, 4.6, 4.7, and 4.8?
Corrected

Reviewer #3:
It is satisfying to see that the requested minor issues were properly addressed. The current version of the manuscript clearly describes a way to generate organoids from intestinal villi. It

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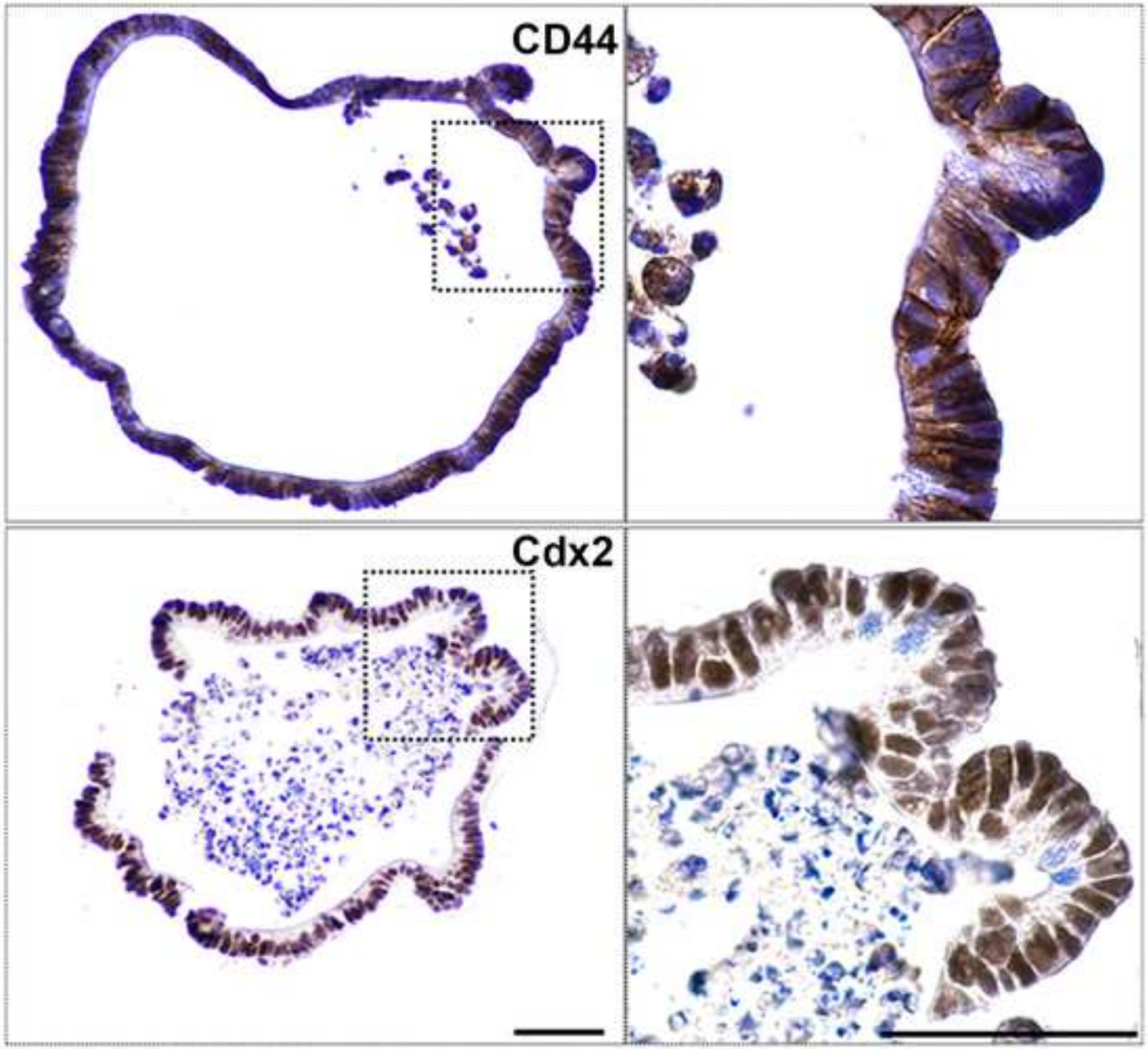
still falls short on providing reliable way to estimate amounts of contaminating crypts. As outlined before, the described procedures with proper optimizations might work best when tissues are derived from Smad4f/f; Catnblox(ex3)/+; Villin-CreERT2 mice. There may be some issues if the approach is used in a different intestinal segment or in a different mouse strain. The authors do point out which issues to focus on, which is certainly helpful if such problems arise.
Thank you, for the acknowledgement. We agree with this point. Since the issues are going to vary depending upon the combination of mouse strain and the intestinal segment used, it would be premature to comment on it. However, the principal criteria, which is preventing contamination from the endogenous proliferative compartment, have been addressed.

Reviewer #4:
Major Concerns:
This is a very specific model and whether the plasticity is relevant to other models is not known and thus whether this model described in this JOVE submission has relevance beyond the specific mouse is not established. In spite of the author's previous publication in which the relevant data is shown in a single figure (Fig 7) this model is poorly characterized as to state of differentiation in the villus or crypt compartment. Importantly, comparison with wild type mouse villus and crypt should be presented using the same methodology to separate what changes are present.

The issue of crypt contamination by the method is argue based on appearance but contamination by TA or base of crypt cells needs to be proven using biochemical or molecular approaches
We agree with this point, and the issue is discussed in the discussion section.

Figure S1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1_ Revised_1_21_21.psd



Supplementary methods

Organoid fixation and embedding: The organoids were fixed in 2% PFA in PBS for 2 hours at room temperature, resuspended in Histogel (Thermo Scientific, HG-4000-012) followed by standard paraffin embedding.

Immunostaining: 5-micron sections were processed for immunostaining and antigen retrieval performed with 10 mM Sodium citrate. For CD44, sections were blocked in 5% goat serum, and incubated with diluted antibody (1:1000) overnight at 4^0C. For Cdx2, the antibody was diluted (1:100) in signal diluent. Secondary antibodies were used according to the manufacturer's protocol (ABC kit, Vector Labs, PK4001), and development of the color reaction after addition of 3,3-diaminobenzidine (Vector Labs, SK-4105) was monitored under a light microscope.

Supplementary Figures

Figure S1. Immunostaining showing the expression of the intestine specific transcription factor Cdx2, and stem cell associated marker CD44 in the villi-derived organoids. Top panel shows nuclear staining (in brown) for Cdx2 and bottom panel shows membrane staining of CD44. The boxed region is shown enlarged on the right. Scale bar, 100 μm.