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Corresponding Author:	Yue Zhao University Hospital Cologne: Uniklinik Koln Cologe, NRW GERMANY
Corresponding Author's Institution:	University Hospital Cologne: Uniklinik Koln
Corresponding Author E-Mail:	yue.zhao@uk-koeln.de
Order of Authors:	Ningbo Fan
	Lisa Raatz
	Seung-Hun Chon
	Alexander Quaas
	Christiane Bruns
	Yue Zhao
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TITLE:

Subculture and Cryopreservation of Esophageal Adenocarcinoma Organoids: Pros and Cons for Single Cell Digestion

AUTHORS AND AFFILIATIONS:

Ningbo Fan[1#], Lisa Raatz[1#], Seung-Hun Chon[1], Alexander Quaas[2], Christiane Bruns[1], Yue Zhao[1*]

[1]Department of General, Visceral, Cancer and Transplantation Surgery, University Hospital Cologne, 50937 Cologne, Germany

[2]Institute of Pathology, University Hospital Cologne, 50937 Cologne, Germany

[#]These authors contributed equally

*Corresponding Author

Yue Zhao (yue.zhao@uk-koeln.de)

Email addresses of co-authors:

Ningbo Fan (ningbo.fan@uk-koeln.de)

Lisa Raatz (lisa.raatz@uk-koeln.de)

Seung-Hun Chon (seung-hun.chon@uk-koeln.de)

Alexander Quaas (alexander.quaas@uk-koeln.de)

Christiane Bruns (christiane.bruns@uk-koeln.de)

Yue Zhao (yue.zhao@uk-koeln.de)

SUMMARY:

This protocol describes the methods of subculture and cryopreservation of esophageal adenocarcinoma organoids with and without single cell digestion to enable researchers to choose appropriate strategies based on their experimental design.

ABSTRACT:

The lack of suitable translational research models reflecting primary disease to explore tumorigenesis and therapeutic strategies is a major obstacle in esophageal adenocarcinoma (EAC). Patient-derived organoids (PDOs) have recently emerged as a remarkable preclinical model in a variety of cancers. However, there are still limited protocols available for developing EAC PDOs. Once the PDOs are established, the propagation and cryopreservation are essential for further downstream analyses. Here, two different methods have been standardized for EAC PDOs subculture and cryopreservation, i.e., with and without single cell digestion. Both methods can reliably obtain appropriate cell viability and are applicable for a diverse experimental setup. The current study demonstrated that subculturing EAC PDOs with single cell digestion is suitable for most experiments requiring cell number control, uniform density, and a hollow structure that facilitates size tracking. However, the single cell-based method shows slower growth in culture as well as after re-cultivation from frozen stocks. Besides, subculturing with single cell digestion is characterized by forming hollow structures with a hollow core. In contrast, processing EAC PDOs without single cell digestion is favorable for cryopreservation, expansion, and histological

characterization. In this protocol, the advantages and disadvantages of subculturing and cryopreservation of EAC PDOs with and without single cell digestion are described to enable researchers to choose an appropriate method to process and investigate their organoids.

INTRODUCTION:

Esophageal cancer (EC) is the tenth most common and the sixth leading cause of death from cancer worldwide[1]. Esophageal adenocarcinoma (EAC) is one of the major histologic subtypes of EC and mainly occurs in western countries[2]. In the recent decade, the EAC incidence has significantly increased in many developed countries, including Germany[3]. Due to the aggressiveness of cancer and the lack of symptoms during the early stage of tumor development, the overall prognosis in EAC patients is poor, showing a 5-year survival rate of about 20%[2,4,5].

Since the late twentieth century, several models have been established for the biomedical research of EAC. The classic human EAC cell lines that were established in the 1990s[6], extend our knowledge of EAC tumor biology, tumor genetics as well as anti-tumor strategies, and are commonly used in EAC research. Besides, some research groups have successfully developed animal models of EAC or Barrett's esophagus by exposing the animals to known risk factors such as gastroesophageal reflux through surgical or inflammatory approaches[7–9]. In addition, patient-derived xenograft (PDX) models that engraft EAC primary cancer tissues subcutaneously or orthotopically into immunodeficient mice, were developed to simulate human EAC tumor biological behavior and tumor environment[10–12]. However, despite these models improving clinical applications and our understanding of molecular mechanisms behind EAC tumorigenesis and progression, there is still a major challenge to extrapolate results from these research models to humans.

Patient-derived tumor organoids (PDOs) are grown in a 3D culture system that mimics human development and organ regeneration *in vitro*. Generated from patients' primary tissue, PDOs recapitulate the molecular and phenotypic characteristics of the human tumor and have shown promising applications in drug development and personalized cancer treatment[13,14]. By comparing ten cases of EAC PDOs with their paired tumor tissue, EAC PDOs are reported to share similar histopathological features and genomic landscape with the primary tumor, retain intra-tumor heterogeneity and facilitate efficient drug screening *in vitro*[15]. EAC PDOs were also used in studying the interaction of EAC tumor cells with patient-derived cancer-associated fibroblasts (CAFs), indicating a powerful application in the field of tumor microenvironment research[16]. Unfortunately, there have been limited protocols available for developing and propagating EAC PDOs. Here, two different methods are described for subculturing and preserving EAC PDOs in detail: with and without single cell digestion. The standardized methods for maintenance of EAC PDOs and their applications can support researchers to choose appropriate methods for different purposes in their EAC PDO research.

PROTOCOL:

An established and well-growing PDO culture represents the basis for a successful subculture and cryopreservation described in this protocol. Here, EAC PDOs were generated from EAC patients' primary tumor tissue using the protocol described by Karakasheva T. A. et al[17]. EAC tissues were

collected from biobank under the approval of BioMaSOTA (approved by the Ethics Committee of the University of Cologne, ID: 13-091).

NOTE: EAC PDOs have been cultured in a humidified incubator at 37 °C and 5% CO_2 using a PDO culture medium (**Table 1**). In the following steps, two methods of the subculture are described in detail. A 12-well plate is recommended for subculturing the PDOs with a seeding density of three extracellular matrix (ECM) gel domes per well, as it allows flexible use of each well and appropriate quantity of PDOs for different purposes. An aseptic technique is compulsory while handling the PDOs.

1. Preparations in advance

1.1. Pre-warm a 12-well plate by placing it into a 37 °C CO_2 incubator overnight before subculture to ensure complete warming of the plate. If available, use empty wells from a plate with the current PDO culture.

NOTE: Continuous storage of 1–2 fresh plates at 37 °C is recommended for flexible subculture planning.

1.2. Pre-cool 1,000 µL and 200 µL tips with a wide orifice at -20 °C (continuous storage recommended). Pre-cool centrifuge at 4 °C.

1.3. Set up the temperature of the rotating incubator to 37 °C (if single cell digestion is performed).

1.4. Incubate an appropriate volume of ECM gel for 1 h on ice to liquefy. Place cell recovery solution on ice.

2. Harvesting organoids

2.1. Remove the plate with growing PDOs from the CO_2 incubator.

2.2. Aspirate old medium using a vacuum pump.

NOTE: Avoid touching the domes.

2.3. Add an appropriate volume of ice-cold cell recovery solution (500 µL/dome) into the well.

2.4. Disintegrate the ECM gel by pipetting up and down several times to fragment ECM gel domes into small pieces using 1,000 µL tips with a wide orifice.

2.5. Combine the mixture of PDO, ECM gel and cell recovery solution from a maximum of two wells (six domes) and transfer it into a 5 mL low bind tube (use a second tube in case more wells are used for subculture).

NOTE: Optionally, if ECM gel was not dissolved completely, add an additional 1.5 mL of cell recovery solution to the mixture of PDO, ECM gel, and cell recovery solution.

2.6. Incubate the tube containing the mixture in step 2.5 on ice for 20 min, mix every 5 min by inverting the tube five times to ensure the liquefaction of the ECM gel.

2.7. Centrifuge at 500 x *g* for 4 min at 4° C.

2.8. If there is a visible and stable pellet after centrifugation, proceed with step 2.10. Otherwise, continue with step 2.9.

2.9. If there is no visible pellet and the PDOs still seem to be stuck in a gel phase, carefully remove the supernatant with a vacuum pump until the phase containing ECM gel-PDO-Solution is reached and add 3 mL of ice-cold cell recovery solution.

2.9.1. Invert the tube a few times and incubate on ice for another 10 min. Mix by inverting the tube from time to time.

2.9.2. Centrifuge at 500 x *g* for 4 min at 4 °C and continue with step 2.10.

2.10. Discard the supernatant carefully using a vacuum pump or a 1,000 µL pipette. Try to remove the supernatant as much as possible.

NOTE: Due to the low bind surface of the tube, the pellet will not be as stable as usual.

2.11. Store the PDO pellet on ice and proceed with step 3 (without digestion) or step 4 (with single cell digestion) depending on the different purposes.

3. Subculturing without digestion

NOTE: This method aims to increase the PDOs' size and density. The larger size and higher density facilitate the embedding process, histological characterization, and PDO expansion. Depending on the PDO split ratios (based on the density of PDOs, a ratio between 1:3 and 1:6 is recommended), resuspend the pellet from step 2.8 in an appropriate volume of liquid ECM gel.

3.1. Remove pre-cooled 200 µL and 1,000 µL tips with a wide orifice from the -20 °C freezer and place them onto a clean bench.

3.2. Resuspend the pellet from step 2.11 in ECM gel using pre-cooled 1,000 µL tips. Mix by pipetting up and down about 10 times to make sure PDOs are not clumping and are evenly distributed in the ECM gel.

NOTE: Use 50 µL ECM gel/dome. Always calculate for one dome more than required (e.g., for

nine domes (i.e., three wells), resuspend the pellet in 500 µL of liquid ECM gel (450 + 50 µL extra). Try to avoid producing bubbles during resuspension!

3.3. Remove the pre-warmed 12-well plate from the incubator right before seeding the domes.

3.4. Seed domes containing 50 µL ECM gel into the warm plate (three domes/well). Avoid pipetting bubbles into the ECM gel domes.

3.5. Put the plate back into the 37 °C and 5% CO_2 incubator and incubate for 20–30 min to solidify the ECM gel.

3.6. Add pre-warmed PDO medium (**Table 1**) carefully without disturbing the domes.

3.7. Culture the PDOs for 7–14 days until the required density and morphology occur.

4. Subculturing with single cell digestion

NOTE: The following steps aim to increase the number of PDOs per dome. The single cell digestion facilitates cell number control and PDO expansion.

4.1. Prepare digestion medium by mixing 2 mL of 0.25% Trypsin-EDTA and 20 µL DNase I (for digestion of three domes).

4.2. Resuspend the pellet from step 2.11 with an appropriate volume of pre-warmed 0.25% Trypsin-EDTA + DNase I and mix it about 10 times by pipetting up and down using a 1,000 µL pipette (use normal 1,000 µL tips).

4.3. Incubate for 10 min at 37 °C in a rotating incubator with a rotation speed of a minimum 28 rpm.

4.4. Prepare a 15 mL tube containing 6 mL of Soybean Trypsin Inhibitor (STI, **Table 2**) solution (per 2 mL of 0.25% Trypsin-EDTA).

4.5. After digestion, mix the digested PDOs thoroughly a few times with a 1,000 µL pipette to disrupt the PDOs.

4.6. Transfer the digested PDOs to the 15 mL tube containing STI solution to stop the digestion process.

4.7. Centrifuge at 500 x *g* for 4 min at 4 °C. Discard the supernatant carefully using a vacuum pump or a 1,000 µL pipette. Resuspend the pellet in 1 mL of basal medium (**Table 3**).

4.8. Determine cell concentration and viability using an automated cell counter or a Hemocytometer.

4.9. Seed digested PDO into a 12-well plate with 2 x 10^4 cells per dome.

4.9.1. Calculate the cell number according to the domes planned for seeding and transfer them to a fresh 1.5 mL low bind tube.

NOTE: Calculate for one dome more (+ 2 x 10^4 cells extra). For example, for seeding three domes into one well, take 8 x 10^4 (2 x 10^4 * 3 + 2 x 10^4 extra) cells.

4.9.2. Centrifuge at 500 x *g* for 4 min at 4 °C.

4.9.3. In case there is no visible pellet, remember the orientation of the tube inside the centrifuge to know where the pellet is located.

4.9.4. Carefully discard the supernatant using a 1,000 µL pipette. Remove the supernatant as much as possible without disturbing the pellet.

4.9.5. Add appropriate volume of ECM gel to the pellet using a 1,000 µL pipette with pre-cooled 1,000 µL wide orifice tip (50 µL/dome + 50 µL extra).

4.9.6. Follow steps 3.3–3.7.

5. Cryopreservation of the digested and undigested PDOs

NOTE: Single cell digested and undigested PDOs are suitable for the preparation of frozen backup stocks. Note that re-cultivated PDOs from the single cell frozen stocks require a longer time to recover and to reach a certain size.

5.1. Cryopreservation of the undigested PDOs.

5.1.1. Start cryopreservation process with the pellet from step 2.8. Use 500 µL of cold freezing medium to resuspend the pellet and transfer it to a cryogenic vial.

NOTE: Store two domes per vial.

5.1.2. Freeze PDOs overnight in a -80 °C freezer using an appropriate cell freezing container.

5.2. Cryopreservation of the single cell digested PDOs

5.2.1. After harvesting and digesting PDOs, start cryopreservation from step 4.8.

5.2.2. For storing one cryogenic vial, transfer 4–5 x 10^5 cells into a fresh 1.5 mL low bind tube.

NOTE: Store three domes/vial.

5.2.3. Centrifuge at 500 x *g* for 4 min at 4 °C. Discard the supernatant carefully using a 1,000 µL pipette. Remove the supernatant as much as possible without disturbing the pellet.

5.2.4. Resuspend the pellet in an appropriate volume of freezing medium (500 µL/vial) and transfer it to a cryogenic vial.

5.2.5. Freeze PDOs overnight in a -80 °C freezer using an appropriate cell freezing container and transfer them into a -150 °C freezer or liquid nitrogen for long-term storage.

REPRESENTATIVE RESULTS:

This protocol presents the procedures including subculture and cryopreservation of EAC PDOs with and without single cell digestion.

Figure 1 shows representative phase-contrast pictures of the two different subculture strategies. EAC PDOs reached appropriate density for subculturing (**Figure 1**, left). Subculturing without single cell digestion takes less time to reach comparable density and mainly leads to compact structures (**Figure 1**, top row). In contrast, the single cell digested PDOs show hollow structures with a hollow core (**Figure 1**, bottom row). **Figure 2** shows the Hematoxylin-Eosin (H&E) staining and immunohistochemistry (IHC) staining of paraffin-embedded EAC PDOs with compact and hollow structures. The pan-cytokeratin (Pan-CK) enables the identification of epithelial tumor cells[18]. The cytokeratin 7 (CK7) highlights the glandular differentiated tumor cells[19]. The compact structure (top row) predominantly exists in the undigested culture, while the hollow structure (bottom row) is dominant in the culture that underwent single cell digestion.

Figure 3 shows the immunofluorescence (IHC) staining of paired EAC tissue and PDOs with compact structure and hollow structure. The Ki67 highlights the cell populations with higher cellular proliferation[20]. The Ki67 (red) and Pan-CK (green) were similarly distributed among EAC primary tissue, EAC PDO compact structure, and EAC PDO hollow structure. **Figure 4** shows the morphological characteristics of EAC PDOs on the first day of recovery from frozen stock with single cell-based cryopreservation (left) and undigested PDO-based cryopreservation (right).

Figure 5 summarizes a flow chart of the subculture process of EAC PDOs with and without single cell digestion. Briefly, a well growing EAC PDO is ready to be passaged. EAC PDOs were harvested and pelleted. For single cell digestion, PDOs were enzymatically digested for 5–10 min to get single cells, which were likely to grow into hollow structures that facilitate experiments requiring cell number control, uniform density, and size tracking. For undigested subculture, PDOs were split to gain more growing space without enzymatically disrupting, which were likely to grow into compact structures that facilitate histological analyses, quick expansion, and faster recovery from cryopreservation.

FIGURE AND TABLE LEGENDS:

Figure 1: Morphological characteristics of EAC PDOs subculture with and without single cell digestion under a phase-contrast microscope. EAC PDOs grow to a certain density prior to

subculture (left). Upon subculturing EAC PDOs without single cell digestion, PDOs gradually grow from hollow structures to compact structures (right, top row), whereas PDOs grown from single cells show predominantly hollow structures (right, bottom row). Pictures were taken with inverted light microscope using a 5x objective. Scale bar: 100 µm.

Figure 2: Histological characteristics of EAC PDOs' compact structure and hollow structure. The H&E staining (left), Pan-CK staining (middle), and CK7 staining (right) of the compact structure (top row) and hollow structure (bottom row). Pictures were taken with inverted light microscope using a 20x objective. Scale bar: 50 µm.

Figure 3: Immunohistochemistry staining of paired EAC tissue and PDOs. The immunofluorescence (IF) staining of paired EAC tissue (top row), compact structure (middle row) and hollow structure (bottom row) with Pan-CK (green), Ki67 (red), and DAPI (blue). Pictures were taken with inverted automated fluorescence microscope using a 20x objective. Scale bar: 50 µm.

Figure 4: Morphological characteristics of EAC PDOs on the first day of recovery from frozen stock. Phase-contrast pictures of recultivation from single cell-based cryopreservation (left) and undigested PDO-based cryopreservation (right) on the first day of recovery. Pictures were taken with inverted light microscope using a 5x objective. Scale bar: 100 µm.

Figure 5: The flow chart of the subculture process of EAC PDOs with and without single cell digestion.

Table 1: Preparation of EAC PDO culture medium.

Table 2: Preparation of Soybean Trypsin Inhibitor (STI) solution.

Table 3: Preparation of basal medium.

Table 4: Pros and cons for subculturing EAC PDOs with and without single cell digestion.

DISCUSSION:

In this protocol, two different subculture and cryopreservation methods of EAC PDOs are described, i.e, with and without single cell digestion. Several studies recommended passaging EAC PDOs with single cell digestion[15,17], which is beneficial to most experiments that require cell number control, uniform density, and a hollow structure that facilitates size tracking. However, the single cell-based method is characterized by slower growth after recultivation from frozen stocks and less compact morphology during the culture period. Experience indicates 2–3 weeks for single cell-based recultivation to reach applicable density for the subculture process. In contrast, frozen EAC PDOs without single cell digestion can reach the same size in a shorter period (about 1 week) after recultivation. One reason could be the extra stress from the trypsin digestion for a relatively long time (10 min). Therefore, it is recommended to preserve undigested EAC PDOs in a ratio of 1:1.5 (freezing two domes of undigested EAC PDOs and seeding back into three domes for the recultivation). In addition, using undigested EAC PDOs is recommended for quick

expansion and histological characterization by IHC or IF staining due to the compact structure. The pros and cons of the two subculture methods are summarized in **Table 4**.

Several critical steps require attention in this protocol. Firstly, the plates for PDO culture need to be pre-warmed overnight in a 37 °C incubator to ensure the solidifying process of freshly seeded ECM gel domes. It is recommended to use a hot plate for keeping the plate at 37 °C while dealing with extended seeding duration. Secondly, low bind tubes are required during the subculture process to avoid significant PDO loss. To prevent ECM gel loss, tips with a wide bore opening can be pre-cooled in the -20 °C freezer before use. Here, the wide opening of the tips avoids the damage of PDO structures during the harvesting step. Next, it is recommended to incubate PDOs for 20 min on ice before the first centrifugation step, to ensure complete liquefying of the ECM gel. Note that the centrifuge needs to be set at 4 °C during centrifugation steps to keep residual ECM gel in the liquid state. In addition, for the single-cell method, it is recommended to thoroughly mix the PDOs after trypsin incubation using a normal 1,000 µL tip to break cell clumps before adding the STI, rather than directly filtering the cell suspension with cell strainers, to avoid cell loss.

Some modifications can be made in this protocol. The cell recovery solution can be replaced by ice-cold DPBS for dissolving the ECM gel in the harvesting step. However, experiences showed a better ability to dissolve the ECM gel using the cell recovery solution. Therefore, ice-cold DPBS is rather recommended only as an alternative backup method. If the laboratory is not equipped with a rotating incubator, EAC PDOs can be incubated with trypsin in a 37 °C water bath along with mixing by inverting the tube every 2–3 min. 10% DMSO with fetal bovine serum (FBS) can be used as an alternative for freezing medium to prepare frozen PDO stocks. However, a commercial freezing medium with lower or no serum is preferred due to a better PDO recovery.

Some limitations need to be addressed in this protocol. Since these methods have been tested only in EAC PDOs, the application of this protocol to other types of PDOs is not clear. Although procedures for passaging PDOs with and without single cell digestion are standardized for most organoid types[21,22], there is still a need to attempt current protocols on other cancer types to ensure reproducibility. In addition, a 10 min 0.25% trypsin incubation may stress the cells during digestion; therefore, the incubation time could vary based on the pre-subculture PDO condition and the individual PDO diversity. During early attempts, it is suggested to set different trypsin incubation times for each EAC PDO.

In conclusion, this is the first protocol describing and discussing subculture and cryopreservation of EAC PDOs with and without single cell digestion. Subculturing EAC PDOs with single cell digestion is applicable for comparison experiments between groups while undigested EAC PDOs are beneficial for histological characterization, cryopreservation, and quick expansion. Here, the routine maintenance of EAC PDOs is standardized, providing a guide for researchers to choose appropriate methods for EAC organoid generation.

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DISCLOSURES:

The authors declare no conflicts of interest in this work.

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Figure 1

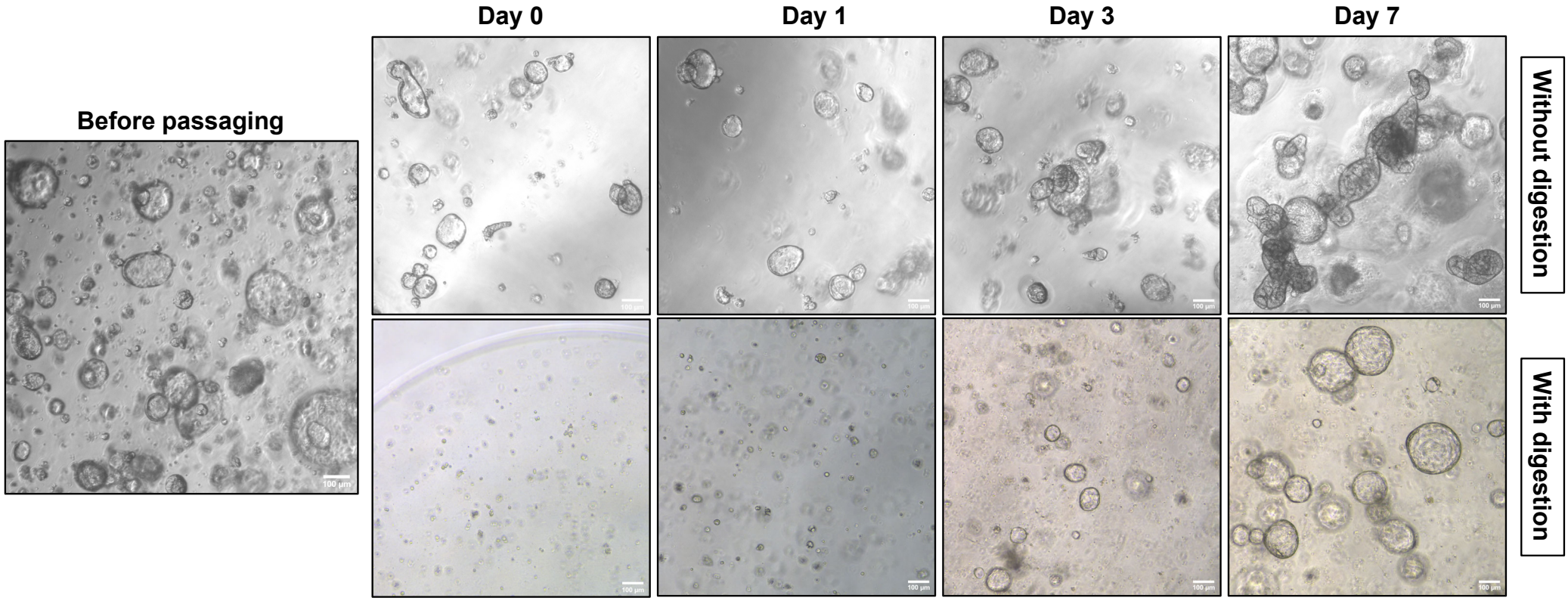

Figure 2

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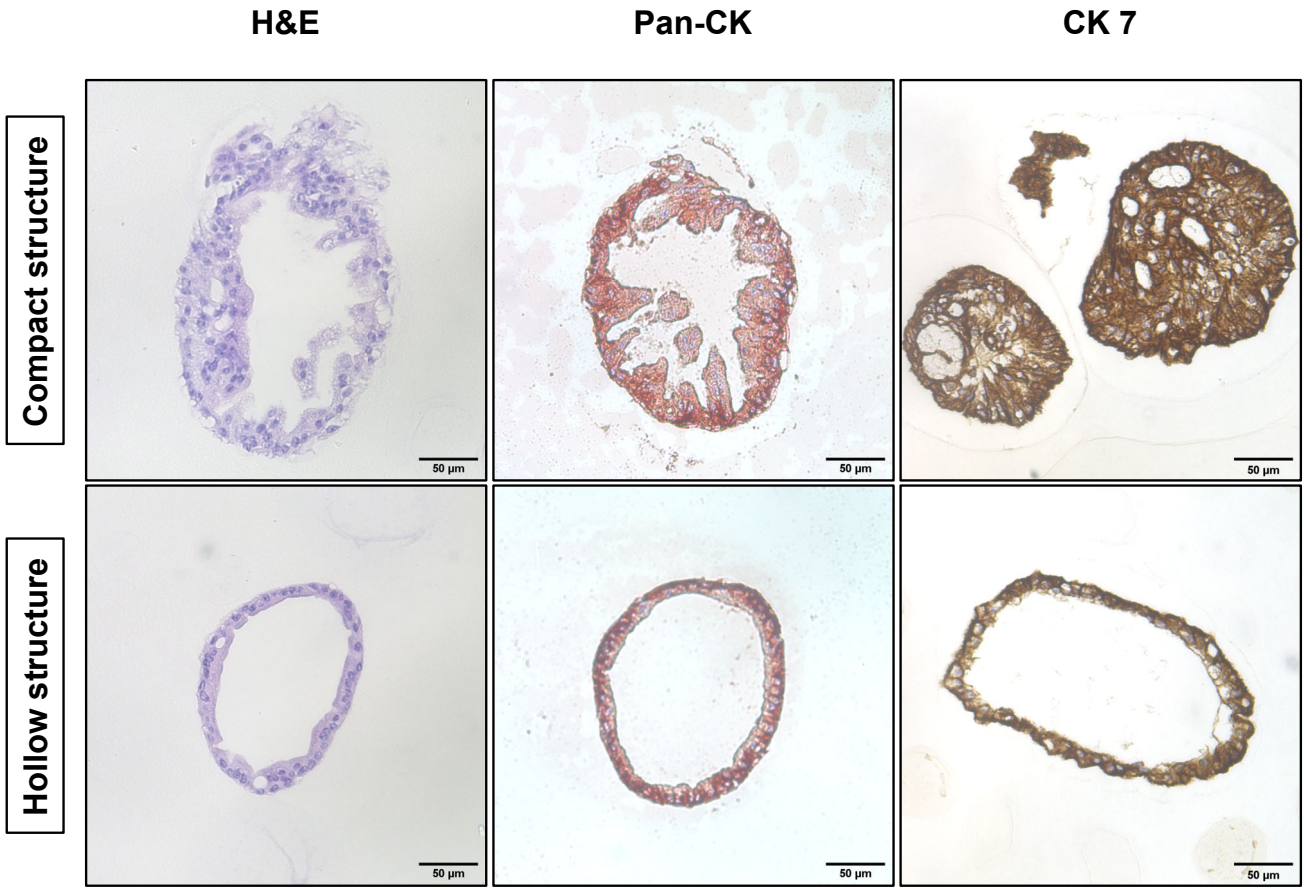

Figure 3

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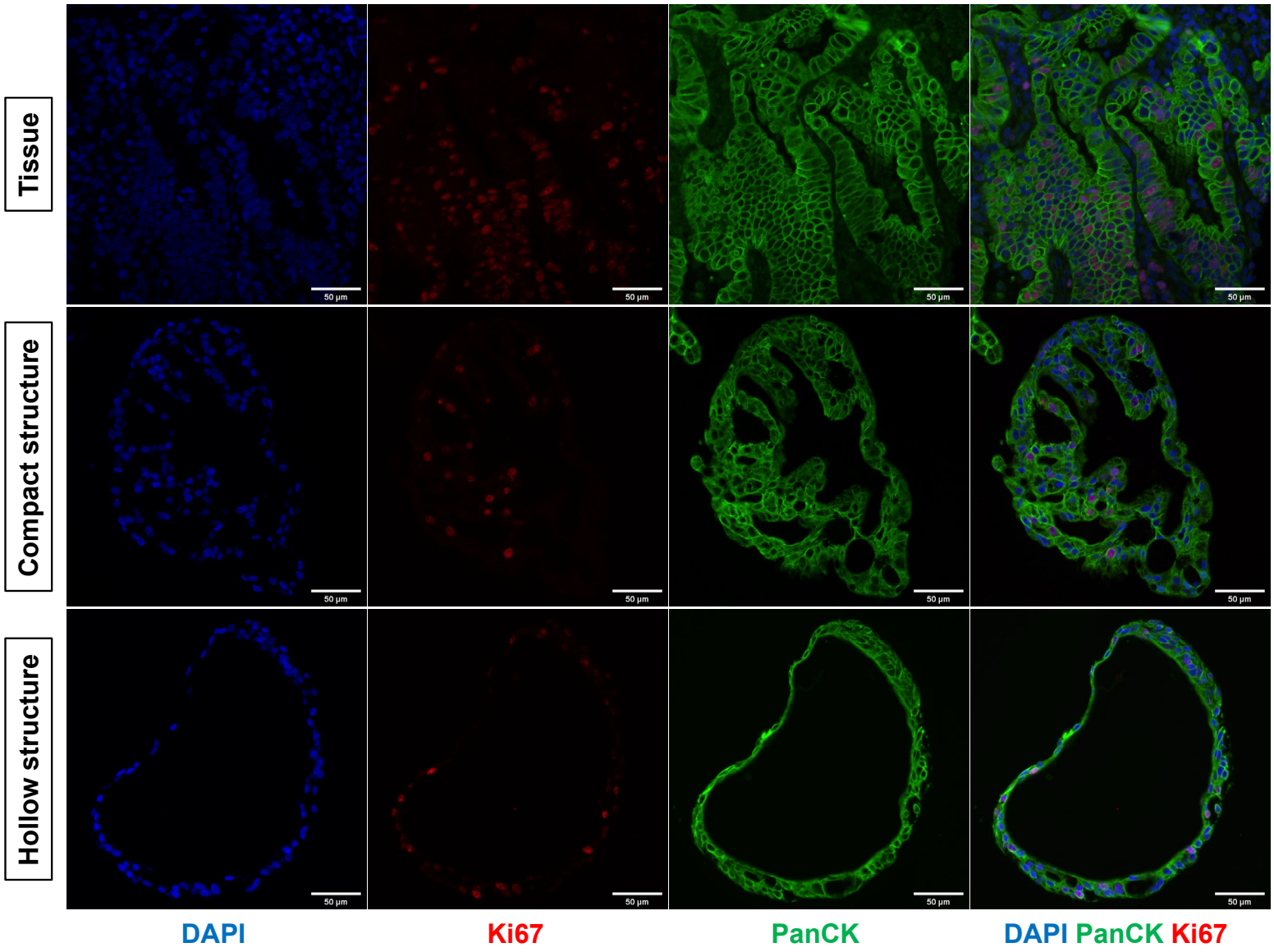

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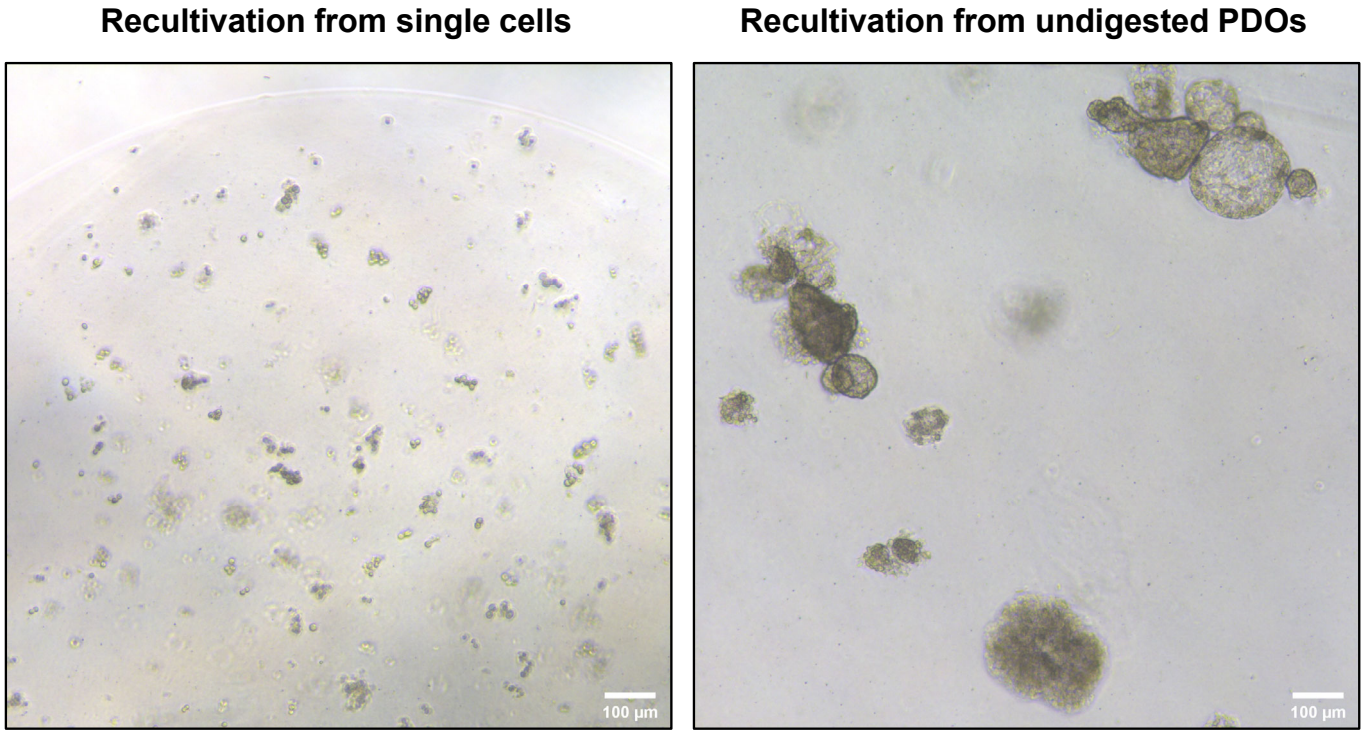

Figure 5

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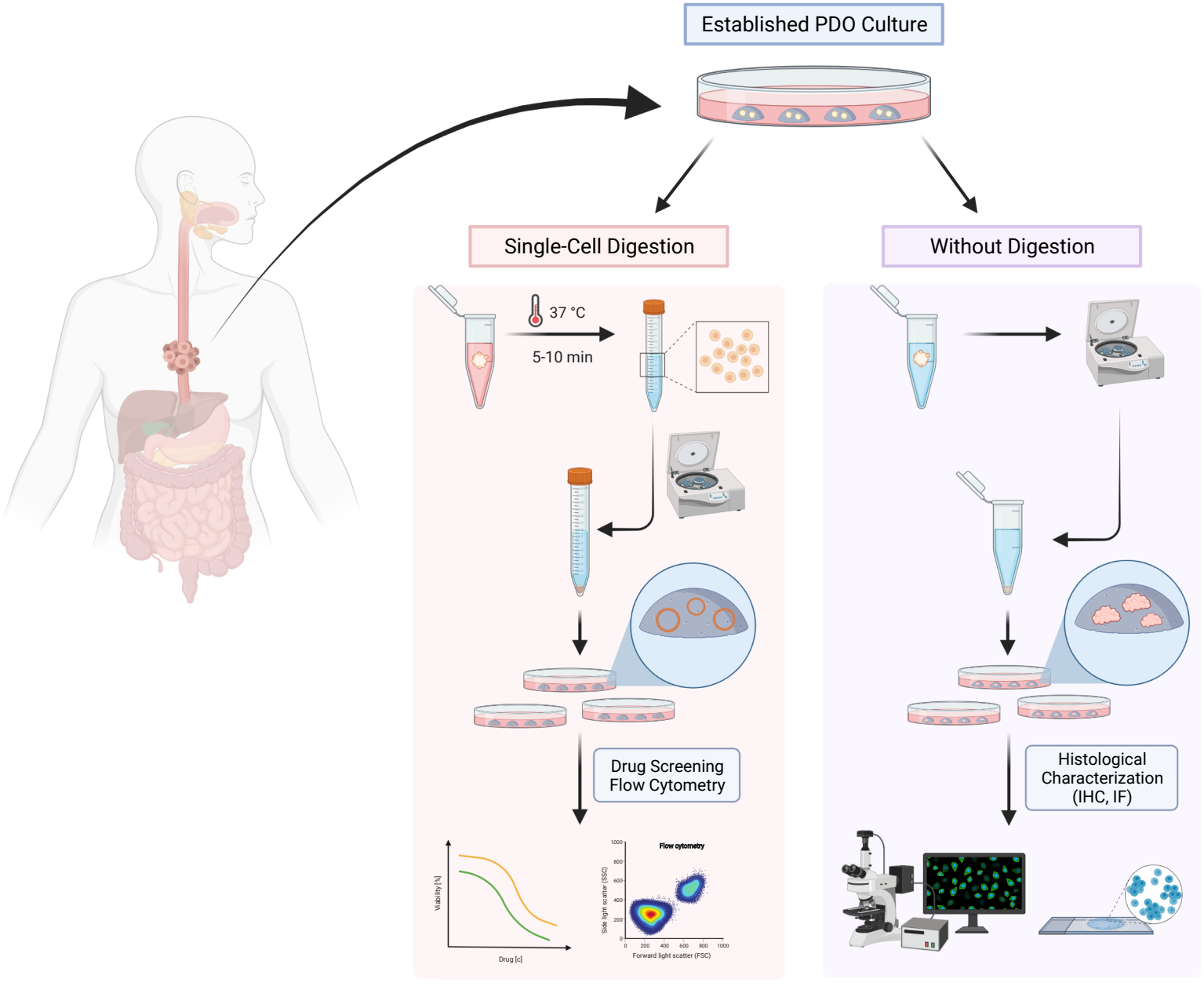

Table 1

	Stock	Final Concentration
Basal medium (see Table 3)		
Wnt-3A conditioned medium		
R-Spondin1 conditioned medium from Cultrex R-Spondin Cells		
N-2	100x	1x
B-27	50x	1x
N-Acetylcysteine	0.5 M	1 mM
CHIR-99021	5 mM	0.5 µM
Recombinant human epidermal growth factor (EGF)	100 µg/mL	250 ng/mL
A83-01	25 mM	0.5 µM
SB202190	10 mM	1 µM
Gastrin	100 µM	0.1 µM
Nicotinamide	1 M	20 µM
Gentamicin	50 mg/mL	10 µM
Penicillin/Streptomycin	100x	1x
Amphotericin B	250 µg/mL	0.60%
Add freshly into well:		
Noggin	100 µg/mL	
Y-27632	10.5 mM	
Add when establishing new PDOs from primary tissue or recovering from frozen stocks		
FGF-10a	100 µg/mL	100 ng/mL

50 mL
24 mL
12 mL
12 mL
500 µL
1 mL
100 µL
5 µL
125 µL
1 µL
5 µL
50 µL
1 mL
5 µL
500 µL
300 µL
50 µL
50 µL
50 µL

Soybean Trypsin Inhibitor (STI)	**12.5 mg**
Adjust to 50 mL with DPBS	
Filter through 0.2 µm sterile filter	

Table 3

Reagent	Volume	Final concentration
Advanced DMEM/F-12	48.2 mL	
HEPES (1 M)	500 µL	10 mM
L-Glutamine (100X)	500 µL	1X
Penicillin-Streptomycin (100X)	500 µL	1X
Amphotericin B	300 µL	0.60%
Gentamicin (50 mg/mL)	5 µL	5 µg/mL

Table 4

Single cell digestion	
Pros	**Cons**
Cell number control	Fragile during embedding
Viability check	Longer time needed between passages
Applicable for e.g., Drug Screening, Flow Cytometry	Longer recovery time from frozen stocks
Without single cell digestion	
Pros	**Cons**
Morphology is beneficial for histological analyses	Expansion of PDOs more in size than in number
Higher stability in embedding process	Not applicable for analyses where single cell suspension is mandatory
Quick recovery from frozen stocks	Lack of cell number control and size tracking

Response to editor and reviewers:

Dear editor,

Please accept our sincere gratitude to you and the reviewers for the in-depth review and constructive comments.

The replies to each reviewer are structured into 3 parts: 1. the comment, 2. the response to each comment and 3. the changes made in the manuscript wherever necessary and appropriate. All the revised contents were highlighted with a yellow background in the 'marked' manuscript.

Through this process we have learnt a lot more about the strengths and weaknesses of our manuscript and hope that this revised manuscript meets the standard of your esteemed journal.

In addition, during the revision process, we have invited Prof. Alexander Quaas from institute of pathology at our hospital for histological support and manuscript revision. All the authors agree to the update of current authorship.

We hope to hear from you soon.

Yours sincerely,
Yue Zhao
Department of General, Visceral, Cancer and Transplantation Surgery, University Hospital Cologne, 50937 Cologne, Germany

Editorial comments

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Answer to comment 1: As reminded, we have invited a native speaker to thoroughly check the manuscript for spelling or grammar issues. Besides, we confirm that all abbreviations are defined at the first use.

Comment 2: JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Matrigel; Cell Recovery Solution; Recovery Cell Freezing Medium etc.

Answer to comment 2: Thank you for the reminder, we have changed "Matrigel" to "extracellular matrix (ECM) gel", "Cell Recovery Solution" to "cell recovery solution", and "Recovery Cell Freezing Medium" to "freezing medium" in the manuscript.

Changes to comment 2: We have changed commercial languages "Matrigel" to "extracellular matrix (ECM) gel", "Cell Recovery Solution" to "cell recovery solution", and "Recovery Cell Freezing Medium" to "freezing medium" in the manuscript.

Comment 3: Please note that your protocol will be used to generate the script for the video and must

contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Answer to comment 3: We confirm that our update version contains enough details in each step to supplement the actions which will be shown in the video.

Comment 4: Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
Answer to comment 4: We have revised the manuscript to match the required format.

Comment 5: Please combine Table 1 and Table 2 and upload one .xls or .xlsx files to your Editorial Manager account. This table can then be referenced in the protocol text.
Answer to comment 5: We confirmed that we have added tables into one .xlsx file.

Comment 6: Please cite Figure 3 in the text (after Figure 1 and Figure 2).
Answer to comment 6: We confirm all the figures and tables were cited in the manuscript.
Changes to comment 6: We have cited all the figures in the representative results section, and tables in the main body of the manuscript.

Comment 7: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
Answer to comment 7: We confirmed that all the relevant figures have scare bars. As requested, we have added information of microscope with magnification and defined the scale bar in the figure legends.
Changes to comment 7: We have added information of microscope with magnification and defined the scale bar in the figure legends**.**

Comment 8: Please add all items (plastic and glassware, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.
Answer to comment 8: As requested, we have revised the table of materials to include all the items and we have sorted the Materials Table alphabetically by the name of the material.
Changes to comment 8: We have revised the table of materials to include all the items and we have sorted the Materials Table alphabetically by the name of the material.

Reviewer comments

To reviewer #1:

Dear reviewer,

We would like to thank you for your kind words and helpful suggestions. Please find below the detailed response to each comment and the respective changes.

Comment 1: Is the protocol restricted to organoids already in culture or can it also be performed directly with fresh primary material of which organoids should be established? This is not completely clear.

Answer to comment 1: The protocol only focused on the subculture and cryopreservation of the organoids and is restricted to organoids already in culture. The description can be found in line 84-86: "An established and well growing PDO culture is representing the basis for a successful subculture and cryopreservation described in this protocol.". Here, we have revised the description of this sentence and added a citation to make the content clearer.

Change to comment 1: "An established and well growing PDO culture is representing the basis for a successful sub-culture and all following downstream analyses" was revised to "An established and well growing PDO culture is representing the basis for a successful subculture and cryopreservation described in this protocol. Our EAC PDOs were generated from EAC patients' primary tumor tissue referring to the protocol described by Karakasheva T.A. et al1[1]": ***line 84-86***.

Comment 2: p. 7, line 224: should be contrast

Answer to comment 2: We apologize for the typo we have corrected in the manuscript, and we are grateful for the reviewer's kind comment. Besides, we have invited a native speaker to thoroughly check our manuscript to improve the language.

Change to comment 2: The typo "contract" was corrected to "contrast" in the manuscript: ***page 7, line 279***.

Comment 3: it would be nice if the pros and cons for single cell digestion mentioned in the abstract and the discussion were summarized in a table which facilitates quick visualization of the advantages and disadvantages of both methods.

Answer to comment 3: We thank the reviewer for this valuable suggestion, and we have added a table 4 to summarize the pros and cons for single cell digestion/ undigested PDOs in the manuscript.

Changes to comment 3: We have added a table to summarize the pros and cons for single cell digestion/ undigested PDOs in the manuscript: table 4.

Once more, we would like to thank the reviewer for your time and considerations and hope to hear favorable feedback from you soon.

Best Regards,

Authors of JoVE63281

To reviewer #2:

Dear reviewer,

We would like to express our utmost gratitude to the reviewer for the constructive suggestions to improve our protocol. It has led us to better understand the needs of the readers and the strength and weakness of our manuscript. We have made our best to answer to each of your comments and made appropriate changes.

We sincerely hope that the revisions are agreeable to you. Please find our answer to each of your comments with appropriate changes as below:

Comment 1:

The two different methods of passaging the organoids is intriguing. From the images provided in Figure 1, it seems the two methods might result or select for different types of organoids. Have the authors assessed these differences in more depth? This is important to ascertain whether both methods can be used interchangeably or need to be done in parallel to ensure that all representative organoid types remain available. On that note, in Figure 3, the authors suggest that the single-cell digestion protocol may be used to grow organoids for drug screenings and flow cytometry, while organoids grown without digestion shall be used for histological characterization. It remains unclear what the rationale behind this distinction is. Evidently all these subsequent methods can be used from organoids generated by either strategy (as the authors have done themselves for the histological analysis presented in Figure 2).

Answer to comment 1: We thank the reviewer for this detailed remark on our protocol. Firstly, we do not think that the two different sub-culture methods are selecting different types of organoids. As shown in figure 2 and figure 3, the staining shows similar expressions between two different structures. Therefore, we hold a view that the compact structure is the result of long-term culturing of hollow structures that leads to higher density. Secondly, we agree with the reviewer that both methods can be used for further analyses such as flow cytometry, histological characterization, and drug screening. Nevertheless, the two methods show different advantages towards different experiments.

For example, as mentioned in the discussion section, our experience showed that the compact structures are easier to embed as intact organoids while the hollow structures are likely to get tattered during the embedding process. In contrast, for comparing cell viability after different drug treatments between different organoids, it is more convenient to grow organoids from single cells because of the need of cell number control at the seeding stage.

Taken together, we did not advocate to select PDOs for different experiments, we want to point out the advantages of the two methods and suggest that it might be more beneficial to use certain methods for certain experiments accordingly, so that researchers could perform their downstream analyses more efficiently.

Comment 2: It remains unclear how the EAC organoids were initially generated from patient tissue. Could the authors please provide a reference for the protocol that has been used here? Also, the actual EAC growth medium is not detailed in the protocol (see step 3.7). Please provide the compounds used and its recipe as a Table in the revised manuscript.

Answer to comment 2: In this protocol, we focused on the sub-culture and cryopreservation of EAC PDOs but not on the establishment of PDOs from EAC patients' tissue. We thank the reviewer for the

kind reminder. As suggested, we have added a citation of the protocol that we have used to establish EAC PDOs in the manuscript. Besides, we have added a table of our EAC PDO growth medium in the revised manuscript, and we hope the reviewer find the changes appropriate.

Changes to comment 2:

1. A citation of EAC PDO establishment was added in the manuscript: "Our EAC PDOs were generated from EAC patients' primary tumor tissue referring to the protocol described by Karakasheva T.A. et al1[1]." : ***page 2, line 85-86***.
2. A table was added to the manuscript describing the EAC PDO growth medium we used in our protocol: ***table 1***.

Comment 3: Could the authors please clarify why they invert the tube instead of using a pipet to separate the organoids from the dissolved Matrigel in step 2.9.1?

Answer to comment 3: We do pipet the Matrigel-organoids up and down several times to fragment Matrigel domes into small pieces at step 2.4. Here, we invert the tube in the incubation step on ice to ensure the liquefaction of the Matrigel in the cell recovery solution. As reminded, we have revised the description in step 2.4 and 2.6 to be more precise.

Changes to comment 3:

1. The description in step 2.4 was revised to "2.4. Disintegrate the ECM gel by pipetting up and down for several times to fragment ECM gel domes into small pieces using 1000 µL tips with wide orifice.": ***page 3***, ***line 125-126***.
2. The description in step 2.6 was revised to "Incubate the tube containing ECM gel-PDO-Solution on ice for 20 min, mix every 5 min by inverting the tube 5 times to ensure the liquefaction of the ECM gel.": ***page 4, line 133-134.***

Comment 4: When organoids are not enzymatically digested into single cells or smaller clumps, almost all published protocols shear the organoids using a pipet tips or siliconized Pasteur pipets ('phyical disruption'). In part 3, do the authors at all separate the organoids into smaller pieces or how are the organoids expanded here?

Answer to comment 4: In our group, we expand the organoids in two different ways. One way is to expand organoids by increasing the size and density. Therefore, we subculture the organoids without digestion as described in the part 3.

The intension of the subculture is to optimize the PDOs growth condition by decreasing the confluency inside the domes so that the PDOs can grow in bigger size and reach a higher density. The other way is to expand organoids by increasing the total number of PDOs. Therefore, we digested the PDOs into single cells by using trypsin as described in the part 4. Then we seed the single cell suspension into a high number of domes to increase the amount of the PDOs.

In summary, we did not expand PDOs by physically disrupting them into smaller clumps via pipets. Here, we have added short descriptions in the protocol regarding the aims of step 3 and step 4.

Changes to comment 4:

1. Add a description regarding the aims of step 3: ***Page 4, line 159-160***.
2. Add a description regarding the aims of step 4: ***Page 5, line 190-191***.

Comment 5: Could the authors provide representative images of (and describe) typical density, morphology, and size of the organoids on the day of passaging? Please provide images of an entire Matrigel drop if possible.

Answer to comment 5: In figure 1, the picture "before passaging" was taken on the day of passaging. Unfortunately, our group seed PDOs into 50 ul Matrigel so that it is not possible to capture the whole dome under the microscope. Here, we have added two pictures right after passaging (day 0) into the figure 1 to better show the morphology and size of PDOs.

We hope the reviewer find the answer and changes appropriate.

Changes to comment 5: Two pictures showing PDOs right after passaging (day 0) were added in the figure 1: ***Figure 1***.

Comment 6: The enzymatic digestion using 0.25% Trypsin-EDTA for 10 minutes seems very harsh considering the EAC organoids are non-stratifying (steps 4.1 to 4.3). Did the authors test lower Trypsin-EDTA concentrations and/or shorter incubation times?

Answer to comment 6: We have tested our PDOs with 0.25% Trypsin-EDTA in 5 minutes, 7 minutes, and 10 minutes. We did not try lower Trypsin-EDTA. There are no obvious differences regarding cell viability from 5-,7- and 10-minutes' incubation. The observed cell viability varies from 70% - 90% even after 10 minutes' 0.25% Trypsin-EDTA incubation, so we used 10 minutes as our routine passaging procedure.

However, as we described in discussion, similar to the 2D cells handling, we recommend the readers check the incubation time of trypsin for each PDO at the first culturing.

Comment 7: What is the expected cell viability in the single-cell suspension after the Trypsin-EDTA digestion (step 4.10).

Answer to comment 7: The observed cell viability varies from 70% - 90% after 10 minutes' 0.25% Trypsin-EDTA incubation, and it also depends on the tissues from each single cases.

Comment 8: In part 5, what is the efficiency of recovering organoids following single-cell digestion. Could please be more specific how much longer (on average, in comparison to standard cryopreservation) it takes to get these organoids into a state that requires passaging? Most protocols recommend to not freeze organoid cultures as single cells, as this greatly reduces cell viability and organoids recovery. Therefore, what is the relevance of freezing single cells? Please provide images how typical organoid cultures appear right after recovery (day 0).

Answers to comment 8: As requested, we have added a figure 4 with the images of PDOs right after recultivating from the frozen stock. From our experience, PDOs recultivated from single cells usually need at least one week more to reach the passaging density compared with undigested PDOs.

When we first started to establish EAC PDOs, our group mostly referred to the protocol from Karakasheva TA et al.[1], where they recommended to freeze 1 $\times 10^5$ single cells (but not undigested PDOs) per vial for cryopreservation.

However, our experience showed there was significant cell loss during the recultivation process, so we increased the cryopreservation cell numbers to 4-5 $\times 10^5$/vial, showing better recultivation efficiency.

Besides, we also learned from other protocols to freeze undigested PDOs [2–4].

Taken together, we have tried both methods and they both work at our laboratory, so that we described them together in our protocol.
As presented in the discussion section, we also prefer to freeze undigested PDOs, which is consistent with the most protocols.
Changes to comment 8: We have added a figure 4 with the images of PDOs right after recultivating from the frozen stock though single cell-based cryopreservation and undigested PDOs cryopreservation: ***Figure 4***.

Comment 9: Line 231, pan-cytokeratin and Ki67 are not only tumour markers but are readily expressed by normal epithelial (basal) cells. Could the authors please amend their statements here?
Answer to comment 9: We highly appreciated the valuable advice; we have amended the inappropriate statements (tumor markers) in the representative results as well as figure legend.
Changes to comment 9: The incorrect statement "tumor marker" was amended in the representative results and the figure legend: ***page 7 and 8.***

Comment 10: Lines 232-234, Figure 1 and its legend, what is meant with tissue-like structures? Please rephrase, as this term appears very unspecific and confusing.
Answer to comment 10: We thank the reviewer for the comment, we have rephrased all terms of "tissue-like structures" to "compact structures" in the manuscript. Besides, we have rephrased all terms of "sphere-like structures" to "hollow structure" to assure the consistency.
Changes to comment 10: All the previous terms of "tissue-like structures" in the manuscript were rephrased to "compact structures". All the previous terms of "sphere-like structures" in the manuscript were rephrased to "hollow structures".

Comment 11: In Figure 2, the organoids seem to be devoid of Ki67-positive cells. On what day were the organoids collected here? It might be better to replace both images with those showing Ki67-positive cells. Also, could the authors provide images from similar staining of reference tissue?
Answer to comment 11: The organoids were embedded into paraffin three weeks after subculture without single cell digestion. The organoids do have Ki67-positive cells, but the color was covered. Therefore, we have optimized the figure 2 into 2 different figures representing IHC results (figure 2) as well as IF staining (figure 3) of the EAC PDOs. We hope the reviewer find it appropriate.
Changes to comment 11:

1. Optimized figure 2 into figure 2 and figure 3.
2. Changed previous figure 3 to figure 5.
3. Relative descriptions in **representative results** and **figure and table legends** sections were revised according to the figures.

Comment 12: Lines 279-281, could the authors please clarify why filtering the single-cell preparations through a cell strainer would result in (significant) cell loss? This seems illogical to some extent when the single cells are properly resuspended and do not clump.
Answers to comment 12: We apologize for the confusion we made here. We aim to point out that even after trypsin digestion, the cells are still sticking together (like 2D cells), so that we need to break the clumps into single cells by thoroughly mixing with pipette.

At the beginning, we directly filtered the cells with cell strainers after digestion without pipetting them first, which caused significant cell loss. Here, we have revised the confusing description to be more concise.

Changes to comment 12: The description "we strongly recommend thoroughly mixing the PDOs after trypsin digestion using a normal 1000 µL tip, rather than directly filtering the cells with cell strainers, to avoid cell loss" was revised to "we recommend thoroughly mixing the PDOs after trypsin incubation using a normal 1000 µL tip to break cell clumps before adding the STI, rather than directly filtering the cell suspension with cell strainers, to avoid cell loss": ***page9 line 362-364***.

Comment 13: Lines 284-286, DPBS cannot serve to replace Cell Recovery Solution, as PBS does not digest the Matrigel. Therefore, please clarify that ice-cold DPBS may allow to better separate the organoids from the Matrigel. PBS is not a proper alternative for Cell Recovery Solution.

Answer to comment 13: The aim of using ice-old DPBS is not to 'digest' the Matrigel, but we used ice-old DPBS here to dilute the Matrigel and liquidity Matrigel by incubating Matrigel-PBS-PDOs suspension on ice for 10- 20 minutes. As we described in line 368-369, we agree with the reviewer that Cell Recovery Solution is better than ice-cold DPBS: "However, our experiences showed a better ability to dissolve the Matrigel using the cell recovery solution".

Here, we only recommend using ice-cold DPB as an alternative backup method for readers. As reminded by the reviewer, we have added a description in this section to make our statement clearer.

Changes to comment 13: We have added a description to make our statement clearer: "Therefore, we only recommend using ice-cold DPBs as an alternative backup method for readers." : ***page 9, line 369-370***.

Comment 14: Lines 289-290, have the authors noticed any differences in organoid recovery when cryopreserving in DMSO+FCS? Many organoid types are sensitive to FCS and therefore FCS-free commercial freezing medium is often preferred due to better recovery.

Answer to comment 14: The commercial Recovery Cell Freezing Medium that we use in our protocol also contains 10% serum. For Recovery Cell Freezing medium, we did not wash PDOs after recultivation from frozen stock. However, when used 10%DMSO+90%FCS(FBS), we wash PDOs at least 2 times with basal medium and then seed them into Matrigel, which undoubtedly cause cell loss especially for single cell-based cryopreservation.

We agree with the author that commercial freezing medium is preferred due to better recovery, and we have added this description in the manuscript.

Changes to comment 14: We have added a description of "However, commercial freezing medium with lower or no serum is preferred due to a better PDO recovery" in the manuscript: ***page 9, line 374-375***.

Comment 15: Line 294-295, there are many papers describing organoid cultures that are passaged with and without single-cell digestion. This is standard procedure for most non-stratifying organoid types. Here, the preferred method is dependent on the ease of shearing organoids or when organoid expansion based on single-cell cloning is required for specific applications (e.g. validation of successful gene editing via sequencing). Please clarify and add sensible references here.

Answer to comment 15: Thank you for the nice advice. When we started to establish EAC organoids, there were few published protocols focusing on esophageal cancer. Although there are several

publications reporting EC PDOs, the subculture methods in their methods section were not detailed described. Even for protocols reporting other types of PDOs, they usually only described one subculture method (mostly with single cell digestion). We are grateful for the reviewer' reminder. After deliberations among authors, we still decided to keep the statement "Since we have only tested these methods in EAC PDOs, the application of this protocol to other types of PDOs is not clear" in the manuscript, given that detailed steps in our protocol are not tested in other types of PDOs. Besides, following your kind suggestions, we have made a description with citations of the two standard procedures, and we hope the reviewer find the changes appropriate.

Changes to comment 15: We have added a statement with citations "Despite passaging PDOs with and without single cell digestion are both standard procedures for most organoid types[5, 6], we still need to attempt our protocols on other cancer types to ensure reproducibility." in the manuscript: ***page 9, line 378-380***.

Comment 16: In general, the discussion would benefit from a more in-depth assessment of the pros and cons of the two methods (with and without digestion).

Answer to comment 16: We appreciate the reviewer's comment, we have summarized the pros and cons in table 4 for better visualization.

Changes to comment 16: Table 4 was added to the manuscript to summarize pros and cons of the two methods.

Comment 17: There are a few typos here and there, e.g. line 224 (phase contract pictures). Please carefully review the manuscript for such errors.

Answer to comment 17: We apologize for the typo we have made, and we are grateful for the reviewer's kind comment. Besides, we have invited a native speaker to check our manuscript thoroughly and optimize the language.

Changes to comment 17: The typo "contract" was corrected to "contrast" in the manuscript: ***page 7, line 279***.

Finally, we would like to thank the reviewer for the nice guidance and advice that helps us improve our work.

Best regards,
Authors of JoVE63281

Reference:

1. Karakasheva, T.A. *et al.* Generation and Characterization of Patient- Derived Head and Neck, Oral, and Esophageal Cancer Organoids. *Current Protocols in Stem Cell Biology*. **53** (1), doi: 10.1002/cpsc.109 (2020).
2. Souster, E. *et al.* Cryopreservation of organoid cultures. *protocols.io*. at <https://www.protocols.io/view/cryopreservation-of-organoid-cultures-bh4ij8ue> (2020).
3. How to Freeze Mouse Intestinal Organoids. at <https://www.stemcell.com/how-to-cryopreserve-

intestinal-organoids.html>.

4. Organoid-Cryopreservation-Protocol.pdf. at <https://resources.amsbio.com/Protocol/Organoid-Cryopreservation-Protocol.pdf>.
5. Seidlitz, T. *et al.* Human gastric cancer modelling using organoids. *Gut*. **68** (2), 207–217, doi: 10.1136/gutjnl-2017-314549 (2019).
6. Driehuis, E., Kretzschmar, K., Clevers, H. Establishment of patient-derived cancer organoids for drug-screening applications. *Nature Protocols*. **15** (10), 3380–3409, doi: 10.1038/s41596-020-0379-4 (2020).

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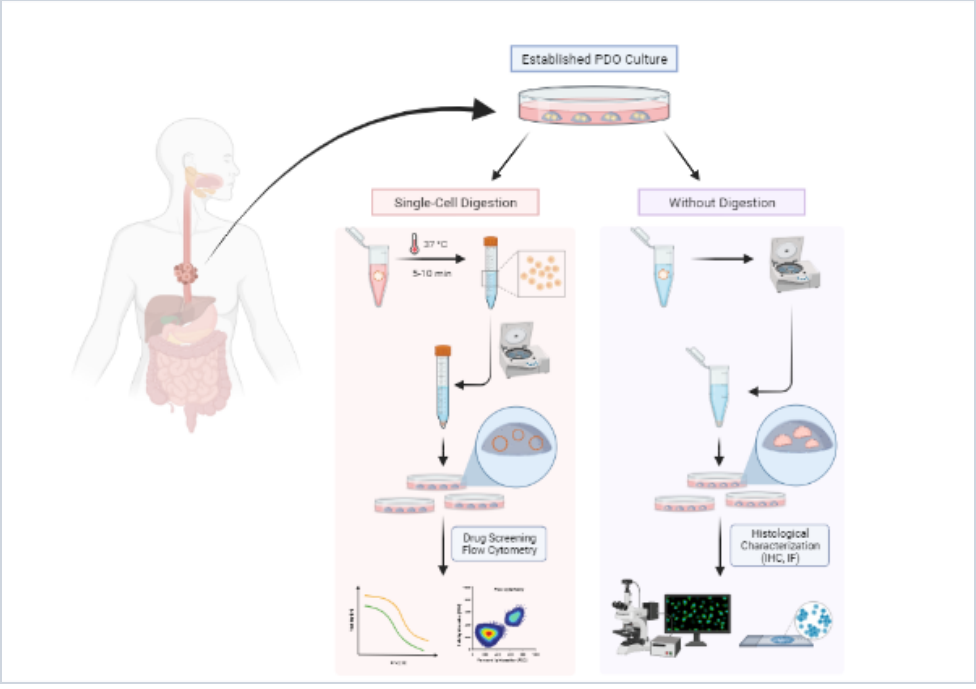

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