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# Establishment of gastric cancer patient-derived xenograft models and primary cell lines

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| **Corresponding Author:** | Zhaolei Zeng<br>Sun Yat-sen University Cancer Center<br>Guangzhou, Guangdong CHINA |
| **Corresponding Author's Institution:** | Sun Yat-sen University Cancer Center |
| **Corresponding Author E-Mail:** | zengzhl@sysucc.org.cn |
| **Order of Authors:** | Zhaolei Zeng |
| | Jia-huan Lu |
| | Yun Wang |
| | Qi Meng |
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**TITLE:**

# Establishment of Gastric Cancer Patient-Derived Xenograft Models and Primary Cell Lines

**AUTHORS & AFFILIATIONS:**

Jia-huan Lu[1*], Yun Wang[1*], Qi Meng[1*], Zhao-lei Zeng[1]

[1]State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou, China

[*]These authors contributed equally.

Jia-huan Lu: lujiah@sysucc.org.cn
Yun Wang: wangyun1@sysucc.org.cn
Qi Meng: mengqi@sysucc.org.cn

**CORRESPONDING AUTHOR:**

Zhao-lei Zeng
zengzhl@sysucc.org.cn

**KEYWORDS:**

primary tumor cell line, patient-derived xenograft, gastric cancer, heterogeneity, therapeutic strategy, tissue cryopreservation

**SUMMARY:**

The current protocol describes methods to establish patient-derived xenograft (PDX) models and primary cancer cell lines from surgical gastric cancer samples. The methods provide a useful tool for drug development and cancer biology research.

**ABSTRACT:**

The use of preclinical models to advance our understanding of tumor biology and investigate the efficacy of therapeutic agents is key to cancer research. Although there are many established gastric cancer cell lines and many conventional transgenic mouse models for preclinical research, the disadvantages of these in vitro and in vivo models limit their applications. Because the characteristics of these models have changed in culture, they no longer model tumor heterogeneity, and their responses have not been able to predict responses in humans. Thus, alternative models that better represent tumor heterogeneity are being developed. Patient-derived xenograft (PDX) models preserve the histologic appearance of cancer cells, retain intratumoral heterogeneity, and better reflect the relevant human components of the tumor microenvironment. However, it usually takes 4-8 months to develop a PDX model, which is longer than the expected survival of many gastric patients. For this reason, establishing primary cancer cell lines may be an effective complementary method for drug response studies. The current protocol describes methods to establish PDX models and primary cancer cell lines from surgical gastric cancer samples. These methods provide a useful tool for drug development and cancer biology research.

## INTRODUCTION:

Gastric cancer is the fifth-most common cancer worldwide and the third leading cause of cancer death. In 2018, over 1,000,000 new cases of gastric cancer were diagnosed globally, and an estimated 783,000 people were killed by this disease[1]. The incidence and mortality of gastric cancer remain very high in northeastern Asian countries[2,3]. Despite significant progress in the field of cancer therapeutics, the prognosis of patients with advanced gastric cancer remains poor, with a five-year survival rate of approximately 25%[4-7]. Thus, there is an urgent need for the development of new therapeutic strategies for gastric cancer

The treatment of gastric cancer is challenging because of its high heterogeneity[8,9]. Thus, the question of how to address the challenges of tumor heterogeneity to realize precision medicine is central to cancer research. In vitro and in vivo models play crucial roles in elucidating the heterogeneous mechanisms and biology of gastric cancer. However, although there are numerous gastric cancer cell lines and many conventional transgenic mouse models for preclinical research, the disadvantages of these models limit their applications[10]. Because the characteristics of these models have changed in culture, they no longer model tumor heterogeneity, and their responses have not been able to predict responses in humans[11]. These issues severely limit the possibility of identifying subgroups of cancer patients that will respond to targeted drugs. The short-term culture of primary tumors provides a relatively rapid and personalized way to investigate anticancer pharmacological properties, which will likely be the hallmark of personalized cancer treatment.

Patient-derived xenografts (PDXs) are preferred as an alternative preclinical model for drug response profiling[12]. In addition, PDX models offer a powerful tool for studying the initiation and progression of cancer[13,14]. PDX models preserve the histologic appearance of cancer cells, retain intratumoral heterogeneity, and better reflect the relevant human components of the tumor microenvironment[15,16]. However, the limitation of the widely used PDX models is the low success rate for establishing and serially propagating human solid tumors. In this study, decently successful methods for establishing PDX models and primary cell lines are described.

## PROTOCOL:

This human study was approved by the Institutional Ethics Review Board of Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China). The animal study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University.

### 1. Sample preparation

1.1. Obtain gastric cancer tissues (P0 = passage 0) directly from the operation. The tumor specimen should be larger than 0.5 $cm^3$.

1.2. Prepare 3-4 mL of stock solution: for example, RPMI-1640 medium (1x) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin.

1.3. Put the fresh tumor specimen in the stock-solution at 4 °C for no more than 4 hours.

## 2. Establishment of PDX model (Figure 1)

2.1. To ensure a sterile surgical area, disinfect all materials with ultraviolet light for more than 30 min before transfer into the animal laboratory.

2.2. Using forceps and scissors, carefully dissect the tumor tissues and trim them into several small pieces (approximately 1 $mm^3$ cubes) under sterile conditions.

2.3. Anesthetize female 5- to 7-week-old NOD-SCID-IL2rg (NSG) mice by exposure to 1-1.5% isoflurane with an anesthesia machine.

2.3.1. Moisten 3-4 cotton balls with 99.9% isoflurane, and place them in a 50 mL centrifuge tube. Anesthetize the mouse until it stops struggling but maintains even breathing.

NOTE: The 50 mL tube is a simple equipment that functions similar to an anesthesia machine. The use of veterinary eye ointment to prevent dryness is unnecessary due to the short operation time.

2.4. Make a 1 cm incision on both dorsal flanks using sterile scissors, and implant one tumor piece into each flank of the mouse.

2.4.1. To ensure that the tumor piece does not slip out, use sterile forceps to disrupt the subcutaneous tissue. Then, clip a piece of the tumor tissue, and place it into the deep site.

2.5. Close the implant area by subcutaneous suture with surgical suture needles, and mark the mouse ears with labeled ear tags

2.6. Sterilize the wound with iodine.

2.7. Gently place the mice in an empty cage after surgery while maintaining sternal recumbency. Pay close attention to the condition of the mice; they wake up and begin to walk approximately 3-4 min later. Place mice that underwent surgery in another new cage separated from those not subjected to surgery.

2.8. Assess tumor size by palpation of the implantation site. Measure the tumors with a Vernier caliper twice a week.

2.9. Once the tumor reaches 10 mm in diameter, the animal condition worsens, or the tumor ulcerates, euthanize the mouse with an IRB approved method. Reimplant harvested fresh tumor fragments into 2 new mice for passaging, or temporarily store the specimens in PBS on ice for the isolation of primary cell lines.

## 3. Tissue cryopreservation

NOTE: This part primarily references the methods for the Live Tissue Kit Cryo Kit. The main kits and equipment are listed in the **Table of Materials**.

3.1. Euthanize the mice with an IRB approved method when the tumor is greater than 10 mm in diameter.

3.2. Using sterile forceps and scissors, slowly isolate the tumor from the mice.

3.3. Wash the tumor tissues with DPBS in a 10 cm dish. Dissect and remove necrotic areas, fatty tissue, blood clots and connective tissue with forceps and scissors.

3.4. Cut the tumor tissues to a maximum 1 mm thickness with a mold.

3.5. Wash the slices with DPBS in a 10 cm dish.

NOTE: The vitrification process involves the use of tubes labeled V1/V2/V3 in steps 3.6-3.8. The main ingredients are DMSO and sucrose.

3.6. Transfer the slices into tube V1 with forceps, and incubate the tube at 4 °C for 4 min. Roll and invert the tube briefly, and place it at 4 °C for another 4 min.

3.7. Pour the V1 solution and slices into a 10 cm dish. Transfer the slices into tube V2 with forceps, and incubate tube V2 at 4 °C for 4 min. Roll and invert the tube briefly, and place it at 4 °C for another 4 min.

3.8. Pour the V2 solution and slices into a 10 cm dish. Transfer the slices into tube V3 with forceps, and incubate the tube at 4 °C for 5 min. Roll and invert the tube briefly, and place it at 4 °C for at least 5 min. Make sure the slices all sink to the bottom of the tube.

3.8.1. If several slices remain floating, roll and invert the tube briefly, and place the tube at 4 °C again until all slices sink completely; if necessary, discard the floating slices.

3.9. Pour the V3 solution and slices into a 10 cm dish.

3.10. Cut the tissue holders to the proper length, and place them on sterile gauze. Transfer the slices onto the holders. Wrap the holders with the gauze, and place them in liquid nitrogen using forceps, followed by incubation for 5 min.

3.11. Label the cryogenic vials with the tissue information.

3.12. Transfer the holders with tissue slices into cryogenic vials, which are stored in liquid

nitrogen.

## 4. Isolation of primary cells (Figure 2)

4.1. Sterilize forceps and scissors with high pressure steam at 121 °C for 30 min.

4.2. Resect gastric cancer samples from resected specimens or harvested PDX tissues. Place the tissues on ice, and then, transfer the tissues to a 10 cm sterile culture dish.

4.3. Dissect and remove necrotic areas, fatty tissue, blood clots and connective tissue with forceps and scissors.

4.4. Wash the tumor tissues once with DPBS containing 100 U/mL penicillin and 0.1 mg/mL streptomycin in a 10 cm dish.

4.5. Cut the tumor tissue into 1 $cm^3$ pieces on the lid of the dish; the maximum thickness of each piece should be 1 mm.

4.6. Transfer the tissues into a 50 mL centrifuge tube with approximately 7 mL of type 1 collagenase and trypsin (1:14) solution. Vortex the mixture briefly.

4.7. Incubate the tube in a water bath at 37 °C for 30-40 min. Vortex the mixture every 5 min.

4.8. Add an equal volume of RPMI-1640 medium (1x) supplemented with 10% FBS to the tube. Vortex the mixture thoroughly.

4.9. Transfer the mixture into a new 50 mL centrifuge tube by slow filtration through a 40 µm filter.

NOTE: Use 40 µm filters to ensure a higher ratio of cancer cells. If necessary, 100 µm filters can be used to preserve more types of cells, such as immunological cells.

4.10. Centrifuge the filtrates at 113-163 x *g* for 5-7 min at RT. Carefully remove the supernatant.

4.11. Wash the pellet with 5 mL of PBS, and carefully remove the supernatant.

4.12. If the pellet is red, it contains many erythrocytes. Gently resuspend the pellet with 500 µL of red blood cell lysis buffer. After a 5 min incubation, add 5 mL of PBS, and carefully remove the supernatant.

4.13. Resuspend the pellet with culture medium, and transfer the mixture to a sterile 10 cm dish.

4.14. Replace the medium with serum-containing medium every 2-3 days.

4.15. Passage the primary cells using trypsin/EDTA when they reach 50% confluence.

**REPRESENTATIVE RESULTS:**

Here, tumor tissues from an operation were preserved in stock solution until the next step. Within 4 hours, tumor tissues were cut into small pieces and implanted into the dorsal flanks of NSG mice that had been anesthetized using isoflurane-soaked cotton. Tumors larger than 1 $cm^3$ could be resected for implantation into new mice **(Figure 1)** or sliced carefully and preserved in liquid nitrogen following the protocol. In this study, the first-generation tumors grew more slowly than those in later generations, taking 3 weeks or longer to reach the appropriate size. The success rate of first-generation subcutaneous tumor formation was greater than 80%. We confirmed the identity of the cancer cells from PDX models by H&E staining under a microscope **(Figure 3C)**. Finally, the success rate of tumor formation from cryopreserved tumor tissue was approximately 95%.

Primary cancer cells were isolated as another way to investigate the individual tumor. The cells were isolated from either operative specimens or PDX models. The tumor tissues were washed with DPBS and cut into pieces. The tissue fragments were digested thoroughly with type 1 collagenase and trypsin (1:14) before filtration. After removing erythrocytes, the cells were cultured in the same way as other cancer cells. The surviving mesenchymal cells die in subsequent passages **(Figure 2)**. Based on differences in morphology, we can easily recognize tumor cells. Normal cells have a uniform shape and size, but cancer cells come in various sizes and shapes. Additionally, in cancer cells, the nucleus has irregular structures and a relatively small cytoplasm. Therefore, the primary cells were authenticated independently by two pathologists under a microscope **(Figure 3A)**. For further confirmation, H&E staining was used to observe cancer cell morphology after fixation **(Figure 3B)**. The rate of successful isolation of primary cell lines was approximately 40%. The primary cells must be passaged 4 or 5 times after isolation, and pathological authentication is needed. These steps may take approximately 20 days.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Schema for the establishment of patient-derived xenograft (PDX) models.**

To successfully establish PDX models, resect the tumor masses in the operating room for immediate processing. Divide the tumor into several small pieces. Put isoflurane-soaked cotton balls in a 50 mL tube to anesthetize the mice, and cut wounds in dorsal flanks. Blunt dissect subcutaneous tissues with forceps, and use forceps to place one piece of the tumor subcutaneously away from the wound. Suture and sterilize the wounds. The anesthesia process requires careful monitoring of mouse vital signs, such as respiration rate.

**Figure 2. Schema for the isolation of primary cells.**

Obtain tumor specimens from the operating room. Quickly wash the tumor tissues, and cut them into pieces. Digest the tumor specimens with type 1 collagenase and trypsin at 37 °C for

30-40 min, mixing the tube every 5 min. Then, add an equal volume of medium with 10% FBS, centrifuge the mixture, and place the filtrate into a new tube. Remove the supernatant, and wash the pellet. Based on the color of the pellet, decide whether to lyse red blood cells. Transfer the pellet into a new 10 cm sterile dish, and culture the cells for several passages prior to cancer cell screening.

**Figure 3. Authentication of primary cancer cells.**
**(A)** Primary cell images from GC patients. The cells were isolated directly from fresh tissues and passaged more than 5 times. Scale bars: 200 μm (left), 100 μm (middle) and 50 μm (right). (**B**) Pictures of H&E-stained primary gastric cancer cells. Scale bars: 500 μm (left), 200 μm (middle) and 100 μm (right). (**C**) Pictures of H&E-stained PDX tumors. Scale bars: 500 μm (left), 200 μm (middle) and 100 μm (right).

**DISCUSSION:**
Gastric cancer is an aggressive disease with limited therapeutic options; thus, models of gastric cancer have become a critical resource to enable functional research studies with direct translation to the clinic[4,8,17]. Here, we have described the methods and protocol of establishing gastric cancer PDX models and primary cell lines. Importantly, both morphological and biological characteristics of gastric cancer specimens were mostly retained in the PDX models.

There are some critical points in the protocol for establishing PDX models that need to be emphasized to increase the engraftment rate. NSG (NOD-SCID-IL2rg) mice are recommended as the immunodeficient mouse model because these mice are more severely immunosuppressed and are deficient in T, B and NK cells[18-21]. Additionally, due to the severe immunodeficiency of the mice, sterility must be maintained in all experiments. All tools, including forceps and scissors, must be kept sterile. NSG mice should be bred at a low density to avoid infection. In addition, many factors might also affect tumor formation, such as the sample size, proportion of mesenchymal cells in the specimen, and implantation site.

One key aspect in the establishment of primary cancer cells is sterility. Furthermore, because mesenchymal cells usually grow faster than primary cancer cells, cells may need to be passaged for several generations until the mesenchymal cells die out. Finally, the cultured cells need to be authenticated.

We used the vitrified cryopreservation method to preserve the PDX tumor samples. The main merits of this preservation method are as follows: 1) long-term storage after freezing is possible (approximately 10 years); 2) the morphology after thaw is consistent with that of the fresh tissue; 3) primary tumor cells can still be isolated after thawing; 4) the tumor-formation ability of PDXs remains basically unchanged before and after cryopreservation; 5) the tissues can be used to make frozen sections and can be fixed with paraffin; 6) DNA, RNA and protein activity is preserved; and 7) this method is applicable for both surgical specimens and biopsy tissues.

The major limitation of PDX models involves the use of immunocompromised mice, which may attenuate the impact of the tumor microenvironment on tumor growth and drug responses and

thus limit the application of PDX models in screening immunomodulatory agents. Additionally, it typically takes 4-8 months to develop a PDX model, which is longer than the expected survival of many gastric patients. For this reason, establishing primary cancer cell lines may be an effective complementary method for drug response studies.

PDX models and primary cell lines are becoming an integral part of drug development and the initiation and progression of tumor biology research. These models offer more accurate representations of human cancer than traditional cancer cell lines and have the potential to improve the preclinical evaluation of novel anticancer therapies. In addition, these models may be useful in comparing molecular characteristics or tumor signatures between different subgroups of cancer patients. There is no doubt that these tools will eventually play a broader role in drug development and cancer biology research.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



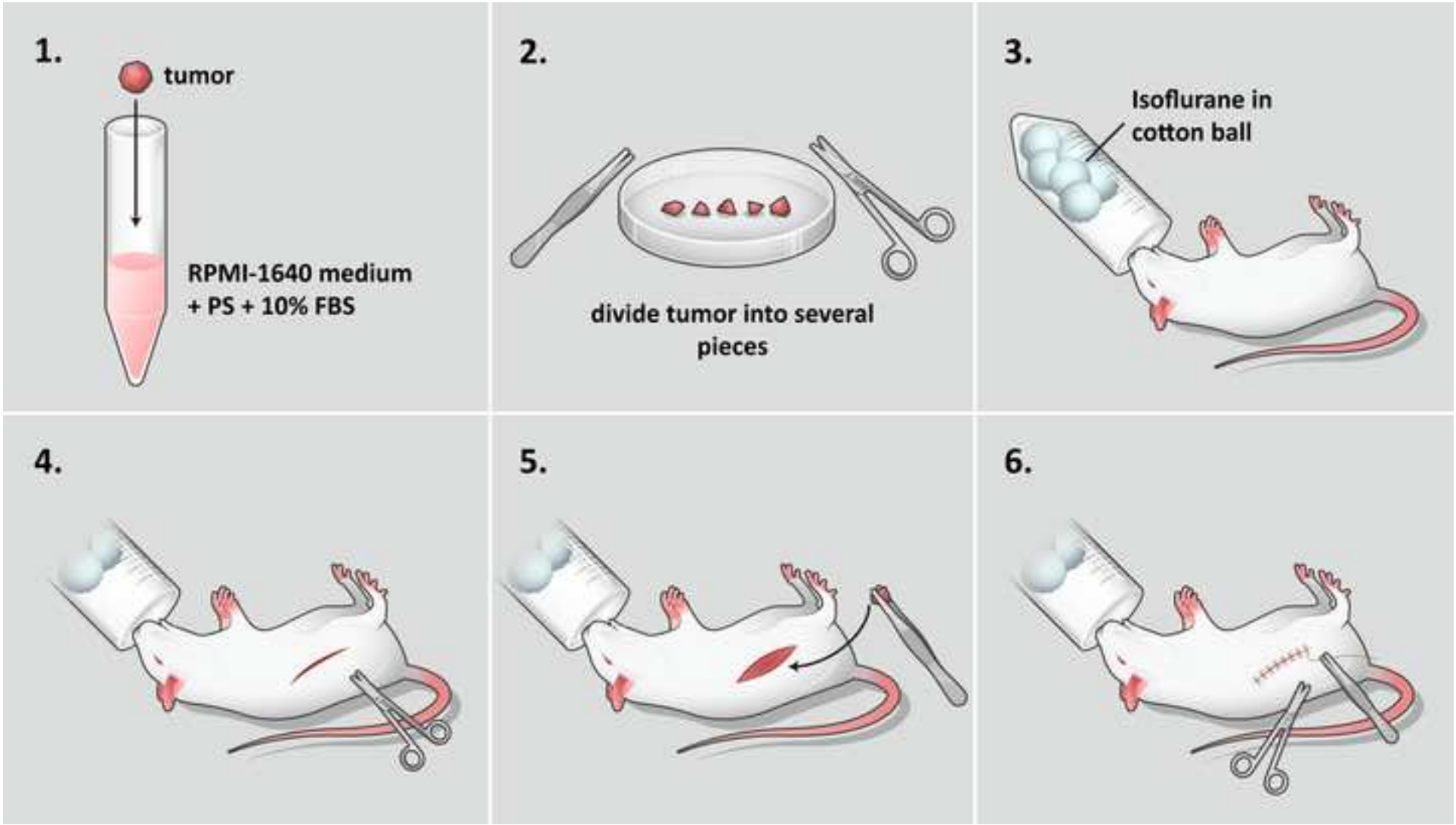


Figure 2



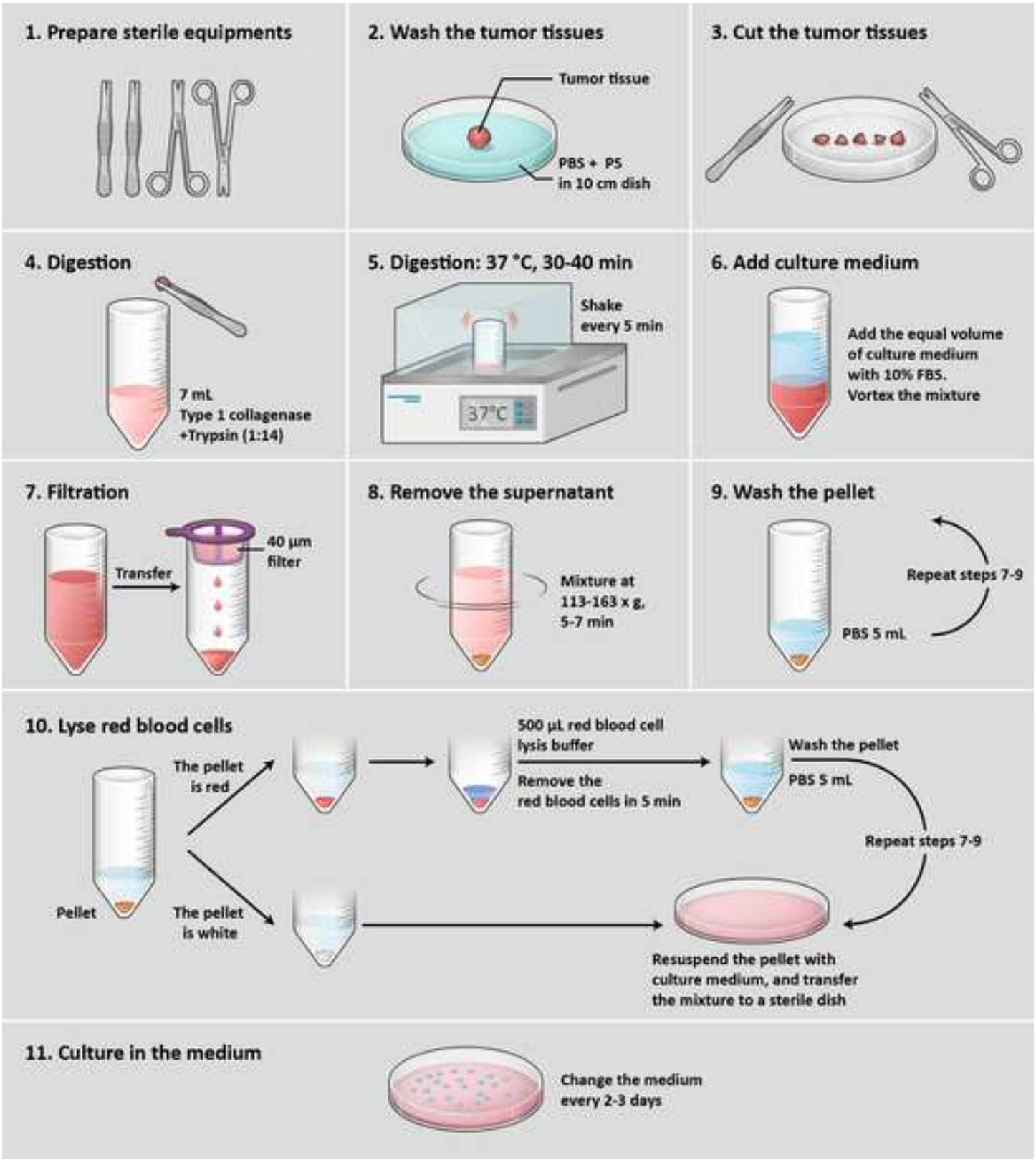
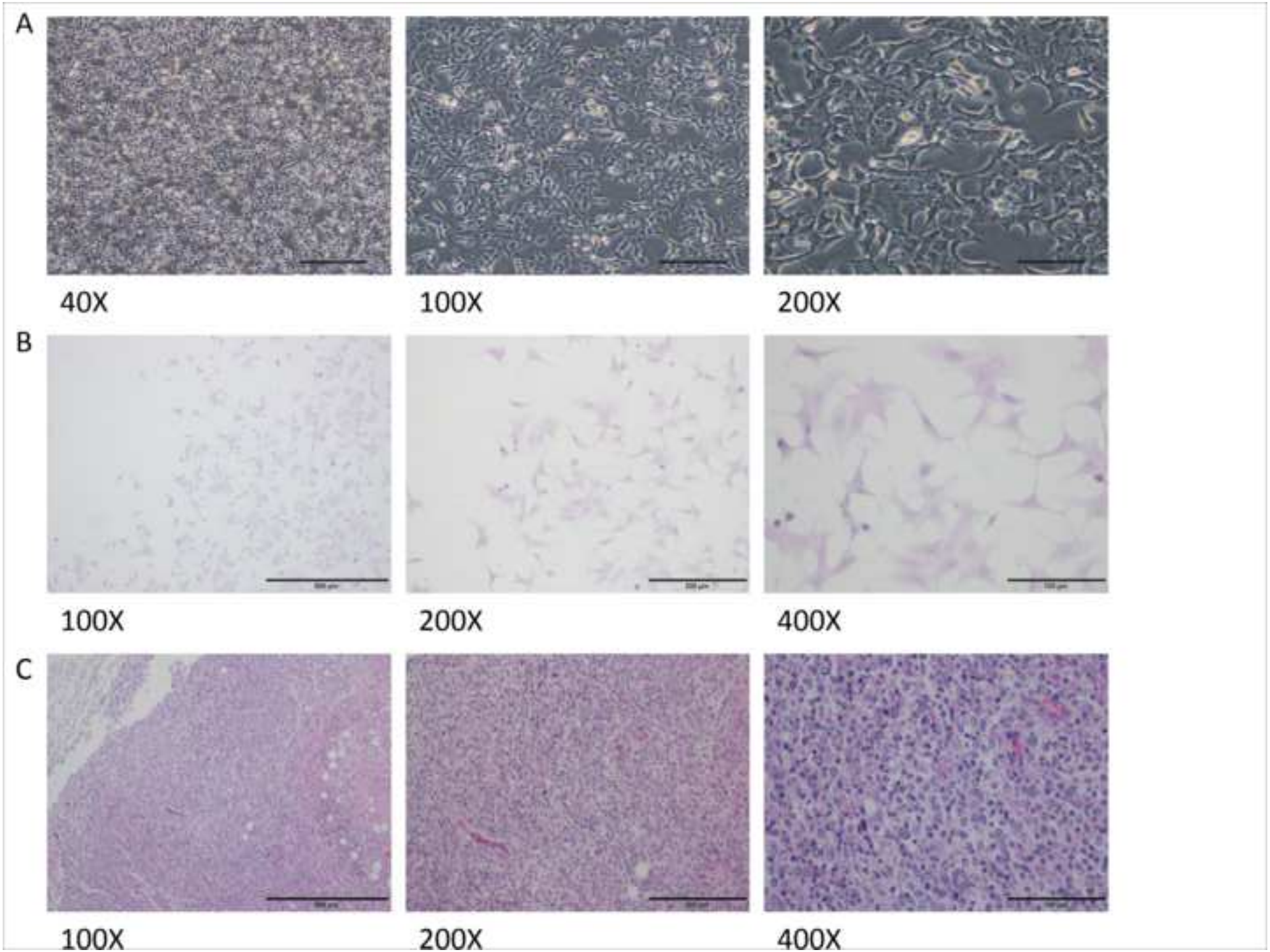


Figure 3





| Name of Material/ Equipment | Company |
|---|---|
| 40 µm Cell Strainer | Biologix, Shandong, C |
| Biological Microscope | OLYMPUS, Tokyo, Jap |
| Centrifuge | Eppendorf, Mittelsacl |
| CO2 Incubator | Thermo Fisher Scienti |
| DPBS | Basalmedia Technolo |
| Electro-Thermostatic Water Cabinet | Yiheng, Shanghai, Chi |
| Fetal bovine serum | Wisent Biotechnology |
| Isoflurane | Baxter, China |
| Live Tissue Kit Cryo Kit | Celliver Biotechnolog |
| Live Tissue Thaw Kit | Celliver Biotechnolog |
| NSG | Biocytogen, Beijing, C |
| Penicilin&streptomycin | Thermo Fisher Scienti |
| Red blood cell lysis buffer | Solarbio, Beijing, Chin |
| RPMI-1640 medium | Thermo Fisher Scienti |
| Surgical Suture Needles with Thread | LingQiao, Ningbo, Chi |
| Tissue-processed molds and auxiliary k | Celliver Biotechnolog |
| Trypsin-EDTA | Thermo Fisher Scienti |
| Type 1 collagenase | Thermo Fisher Scienti |

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| --- | --- |
| 15-1040 | |
| OLYMPUS CKX41 | |
| 5427R | |
| HERACELL 150i | |
| L40601 | |
| DK-8AXX | |
| 86150040 | |
| CN2L9100 | |
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**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Zhao-lei Zeng |
| Department: | Research and Experimental Department |
| Institution: | Sun Yat-sen University Cancer Center |
| Title: | Associate professor |
| Signature: | Zhaolei Zeng |
| Date: | Feb. 18, 2019 |

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March 29, 2019

Dear Dr. Nguyen,

Thank you for your decision letter dated March 15th, 2019, regarding our manuscript JoVE59871, entitled "Establishment of gastric cancer patient-derived xenograft models and primary cell lines". We have taken the points raised by the editors and reviewers and added necessary data as the reviewer suggested. We are now re-submitting a revised manuscript with point-to-point responses to the critiques. I hope that you will find that the current version of our manuscript is suitable for publication in *Journal of Visualized Experiments*.

If you have any question regarding the re-submitted manuscript, please do not hesitate to contact me.

Sincerely yours,

Zhao-lei Zeng, M.D., Ph.D.

State Key Laboratory of Oncology in South China

Sun Yat-sen University Cancer Center

**Response to Editor**

**Point 1:** Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**RESPONSE:** Thank you for the reminder. We have modified several sentences for ensuring no errors in the manuscript.

**Point 2:** Please revise lines 44-46 and 241-242 to avoid previously published text.

**RESPONSE:** Thank you for the reminder. We conscientiously re-wrote these sentences.

**Point 3:** Authors and affiliations: Please provide an email address for each author.

**RESPONSE:** Thanks for the reminder. We have renewed information of each author.

**Point 4:** Keywords: Please provide at least 6 keywords or phrases.

**RESPONSE:** Thank you for the suggestion. We added the keyword of "tissue cryopreserved" in this manuscript.

**Point 5:** Please define all abbreviations before use.

**RESPONSE:** Thank you for the reminder. We defined all abbreviations in the text.

**Point 6:** Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

**RESPONSE:** Thanks for the suggestion. We have revised all wrong usages.

**Point 7:** Please include a space between all numerical values and their corresponding units: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

**RESPONSE:** Thanks for the reminder. We have checked this point all through the text.

**Point 8:** Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

**RESPONSE:** Thanks for the reminder. We added several related statements in 2 steps, such as 1.1, 4.1.

**Point 9:** Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

**RESPONSE:** Thanks for the reminder. We added the ethics statements before the related protocol steps.

**Point 10:** Please remove commercial language (LiveTissue kit (Celliver International Inc.)) from the manuscript text.

**RESPONSE:** Thanks for the suggestion. We have deleted this commercial language.

**Point 11:** Please revise the Protocol to contain only action items that direct

the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

**RESPONSE:** Thanks for the advice. We have revised the protocol according to the advice.

**Point 12:** Please add more details 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. 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. See examples below.

**Point 13:** 1.1, 4.1: Please specify the tumor tissue type.

**RESPONSE:** Thanks for the suggestion. We have compensated the tissue type in 1.1 and 4.1.

**Point 14:** 1.2: Please list an approximate volume to prepare.

**RESPONSE:** Thanks for the advice. We have listed approximate volume (3-4 mL) in the step of 1.2.

**Point 15:** 2.1: Please specify surgical tools used to dissect and trim.

**RESPONSE:** Thanks for the editor's advice. We added the surgical tools in the step of 2.1.

**Point 16:** 2.2: Please specify the concentration of isoflurane and mention how proper anesthetization is confirmed.

**RESPONSE:** Thanks for the editor's reminder. We added a "Note" below the 2.2 step to explain the details.

**Point 17:** 2.3: Is the surgical area sterilized? Please describe the maintenance of sterile conditions during survival surgery. Also please describe what is used to make the incision and how to implant the tumor pieces. How many tumor pieces are implanted?

**RESPONSE:** Thanks for the editor's suggestion. The "Note", below the step of 2.3, has been added to specify the details.

**Point 18:** 2.4: How to suture wounds and what is used? What is marked?

**RESPONSE:** Thanks for the editor's advice. We have specified the needles and ear tags with labels in the step 2.4.

**Point 19:** 2.5: Please describe the post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

**RESPONSE:** Thanks for the editor's advice. We directly described the step 2.6 with details.

**Point 20:** 3.1: Please describe how to isolate the tumor from the mouse and when is this done. Is the mouse scarified and how?

**RESPONSE:** Thank you for the suggestion. We re-wrote this step clearly and supported details.

**Point 21:** 3.3: Are the tissues frozen before cutting?

**RESPONSE:** Thank you for the question. The tissue should be cut into thick slices before frozen.

**Point 22:** 3.5-3.7: What is tube V1/V2/V3? Does it contain any solution?

**RESPONSE:** Thank you for the question. We wrote a "Note" below the step 3.6. Tube V1/V2/V3 mainly contain DMSO and sucrose.

**Point 23:** 3.6: What is tube V2? Does it contain any solution?

**RESPONSE:** Thanks for the question. We have described the ingredients of tube V2 in step 3.6.

**Point 24:** 4.2: Please describe how to sterilize forceps and scissors.

**RESPONSE:** Thanks for the suggestion. We believe that high pressure steam is a useful way to sterilize forceps and scissors in this step.

**Point 25:** 4.4: How many times are the tissues washed? Please specify.

**RESPONSE:** Thanks for the question. We generally washed once with DPBS in this step.

**Point 26:** 4.8: Please provide the composition of culture medium.

**RESPONSE:** Thanks for editor's suggestion. RPMI-1640 medium (1 x) supplemented with 10% FBS, a common culture medium, could be used in this step.

**Point 27:** 4.16: What is considered subconfluence?

**RESPONSE:** Thanks for editor's question. We added the detail in the step. "Subconfluence" means cell density. We think that the primary cells should be passaged when reaching 50% subconfluence.

**Point 28:** Please organize the sections/steps properly so that the protocol can be followed in chronological order.

**RESPONSE:** Thanks for editor's suggestion. We have re-considered the order. The current order is reasonable.

**Point 29:** Please reference Figure 1 and Figure 2 in the Protocol.

**RESPONSE:** Thanks for the reminder. We have added relevant reference in the protocol 2 and 4 respectively.

**Point 30:** Figure 1 legend: Please define all abbreviations in Figure 1.

**RESPONSE:** Thanks for the reminder. We defined the abbreviation of (patients-derived xenografts) in Figure 1 legend.

**Point 31:** Figure 2: Please abbreviate liters to L (L, mL, µL) to avoid confusion. Please change "163 g" to "163 x g"

**RESPONSE:** Thanks for editor's suggestion. We have corrected these mistakes seriously.

**Point 32:** Figure 3: Please define the scale of the scale bar. Please describe the left, middle and right images in the figure legend.

**RESPONSE:** Thanks for editor's reminder. We have defined the scale bar in Figure legend.

**Point 33:** Table of Materials: Please ensure that it has information on all

relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

**RESPONSE:** Thanks for editor's suggestion. We have renewed and supplied information in this part. Then, we re-sorted the items with alphabetical order.

**Point 34:** References: Please do not abbreviate journal titles.

**RESPONSE:** Thanks for editor's reminder. We have updated the references' style.

**Response to Reviewers**

The reviewer raised some questions and suggestions. We wish to take this opportunity to thank the reviewers for their valuable input. To fully address them, we performed additional analyses as well as implementing changes to the manuscript. Below, we summarize the reviewers' comments, and describe point-by-point how we have addressed them.

**Reviewer #1:**

**Point 1:** In the part of tissue cryopreserved, many protocols have been reported only use DMSO and FBS to store live tissues, the author could discuss the advantages and disadvantages respectively about two different methods.

**Response:** Thanks for the reviewer's question. Generally, due to the uncertain composition of fetal bovine serum (FBS) and the poor stability of solution, the success rate is low by using directly DMSO and FBS. Also, it is difficult to retain the original heterogeneity of tumor. Therefore, storing live tissues only with FBS and DMSO reduced the application values and the ability of clinical transformation.

All in all, the vitrified cryopreservation successfully broke through former problems, whose main merits include 1) long-term storage after freezing is available (about 10 years); 2) the morphological function after resuscitation will be consistent with the fresh tissues; 3) primary tumor

cells can still be isolated after resuscitation; 4) the tumor-forming ability of PDX remains basically unchanged before and after cryopreservation; 5) the tissues can replace frozen sections and fixed tissues with paraffin; 6) the preservation of DNA, RNA and protein activities is realizable; 7) both surgical specimens and biopsy tissues are applicable. We added this part in the Discussion.

**Point 2:** many protocols are related to the isolation of primary cell lines online, majority of them use 100 μm filter to isolate cells. The author should explain that why they use 40 μm filter in this paper.

**Response:** Thanks for the reviewer's question. Different filters could isolate various cells of different size. For ensuring higher ratio of cancer cells, we chose 40 μm filters. If necessary, 100 μm filters could be used to preserve more types of cells, such as immunological cells and so on.

**Point 3:** In the schemas of Figure 1 and 2, the initial of each sentence should be capitalized.

**Response:** Thanks for the reviewer's reminder. We have corrected these mistakes.

**Point 4:** In Figure 3, some HE staining of pictures of GC markers for authentication might be provided.

**Response:** Thank you for the suggestion. We provided H&E staining pictures of primary gastric cancer cells and PDX tissues for authentication in Figure 3.

**Reviewer #2:**

**Point:** No results. i.e. How many cultures were successful?

**Response:** Thanks for reviewer's question. The success rates were added in the Results. The success rate of establishment of PDX models is more than 80%; the success rate of resuscitation about 95%; the success rate of primary cell lines isolation was approximately 40%.

**Reviewer #3:**

**Main point 1:** the authors argue that long-term established gastric cancer cell lines change in culture and no longer retain tumor heterogeneity, while gastric cancer is challenging because of its high intratumoral heterogeneity. Although the authors target this issue by providing a protocol to establish PDX models with a better representation of tumor heterogeneity, the same argument could be applied to primary cancer cell lines undergoing selection when growing in culture. Thus it would be desirable to obtain both a PDX model and matched primary cell line from the same patient with the protocols provided in this manuscript, to be able to assess whether heterogeneity is retained either in PDX models or

both models. Therefore, the authors should include the success rates of the protocol ('decent' is currently mentioned) to establish both PDX models and primary cell lines from multiple patients.

**Response:** Thank you very much for review's suggestion. Nowadays, the treatments of gastric cancer are challenged by high intratumoral heterogeneity. Our projects mainly studied gastric cancer, to clarify complex mechanisms and explore unknown targets are major parts in our team. Obviously, these protocols are the first step to establish tools and platforms. Based on long-term researches, we found that our methods retained tumor heterogeneity in every specimen whatever before or after resuscitation, which helped us to build matched primary cell line as soon as possible. Furthermore, we have done exon sequencing in every PDX models and authenticated each matched primary cell line. However, the primary cells may still lose the heterogeneity as time goes by. We will re-check the cells at different time points in the future. The success rate of establishment of PDX models is more than 80%; the success rate of resuscitation about 95%; the success rate of primary cell lines isolation was approximately 40%. We have added these data in the Results.

**Main point 2:** the main argument to establish primary cell lines next to PDX models is that the time frame is shorter for creating primary cell lines. In this way, antitumoral pharmacological properties can be assessed in a short-term manner. However, in this reviewer's experience establishing a

primary cell line from esophagogastric cancer tissue can take several weeks to months to establish before actual pharmacological experiments can be performed. Therefore, the authors should include the average time it takes to establish a gastric primary cancer cell line and give a realistic timeline to use this for the clinic, in order to give a transparent estimation of time and feasibility for users of the protocol.

**Response:** Thank you very much for review's advice. According to our experience, the primary cells need to be passaged 4 or 5 times after isolation and then pathological authentication is needed. All of steps may need about 20 days.

**Main point 3:** the authors briefly mention in the representative results that cells could be isolated from either resected specimens or from PDX models. Please include in the protocol what the steps are (e.g. keeping tissue on ice) of harvesting the PDX tissue until the beginning of the protocol for isolation of primary cells (4.1).

**Response:** Thank you very much for review's suggestion. We compensated several details in the step 2.8 and 4.2.

**Main point 4:** the representation and information regarding Figure 3 is insufficient. The authors should mention whether the visualized cells are derived from specimens directly from the operation or PDX material. In addition to the number of passages the timeframe from the protocol until the images shown would give a better understanding of feasibility of the

procedure. Similarly photos of PDX models established from the protocol should be included.

**Response:** Thanks for reviewer's advice. We have modified Figure 3 legend and added some pictures in it.

**Main point 5:** furthermore, since pathologists have authenticated the primary cells as gastric cells, I would suggest the inclusion of an established gastric cell line as a positive comparison of a gastric phenotype, or show how authentication of the cell lines and PDX specimen was performed.

**Response:** Thanks for reviewer's suggestion. We used H&E staining pictures showing the positive gastric cell phenotype and PDX specimen.

**Minor comments**

Thanks the reviewer very much to mention these flaws, we have corrected them in relevant steps.

**Point 1:** 1.1Mention the minimal size of the tissue.

**Response:** Thanks for the reminder. We consider that the minimal size of the tissue in step 1.1 is 0.5 $cm^3$. However, the success rate depends on whether contain cancer cells.

**Point 2:** In 3.6, 3.7 and 3.8 is mentioned 'pour out the mixture'; while in 3.4 the liquid was removed. Mention in 3.5, 3.6 and 3.7 which mixture was supplemented that should be removed.

**Response:** Thanks for the reminder. we have corrected the information in step 3.6-3.8.

**Point 3:** 4.5 As the minimal amount of tissue can influence the success rate, I would suggest mentioning the exact optimal size of a tumor piece rather than the maximum thickness of the tumor piece, in order to get the best success rate.

**Response:** Thanks for reviewer's suggestion. Based on our experience, 1 mm-thick slices are more likely to be successfully cryopreserved.

**Point 4:** 4.14 Elaborate on the optimal size of the dish in relation to the optimal tissue size to be mentioned in 4.5.

**Response:** Thanks for reviewer's reminder. We replenished the tissue size (1 $cm^3$) in 4.5 and dish size (10 cm) in 4.14.