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## **Title: Generation and Single-cell Transcriptomic Analysis of Hepatocellular Carcinoma Organoids Following Drug Treatment**

### **Authors and Affiliations:**

Dan He<sup>1#</sup>, Zhong Liang<sup>1,2,3#</sup>, Cheng-Yang Zhang<sup>1,2,3#</sup>, Jie-Shen Xian<sup>4#</sup>, Yuan-Xiang Yang<sup>5</sup>, Yuan-Feng Gong<sup>3</sup>, Hui Tang<sup>3</sup>, Guang-Ding Zhou<sup>1,2,3</sup>, Chen-Yu Jiang<sup>1,2,3</sup>, Le Xing<sup>1,2,3</sup>, Dong-Mei Li<sup>1,2,3</sup>, Ying-Chen Hou<sup>1,2,3</sup>, Chen Yue<sup>1,2,3</sup>, Wei Cheng<sup>1,2</sup>, Yun-Qiang Tang<sup>3</sup>, Xiao-Feng Zhang<sup>1,2,3,6</sup>, Ming Liu<sup>1,2,3</sup>

# These authors contributed equally

<sup>1</sup> School of Basic Medical Science, Guangzhou Medical University

<sup>2</sup> Guangzhou Municipal and Guangdong Provincial Key Laboratory of Protein Modification and Degradation, Guangzhou Medical University

<sup>3</sup> Affiliated Cancer Hospital and Institute, Guangzhou Medical University

<sup>4</sup> The First Clinical School of Guangzhou Medical University, Guangzhou Medical University

<sup>5</sup> Department of Obstetrics, Guangzhou Eighth People's Hospital, Guangzhou Medical University

<sup>6</sup> Department of Pediatric Surgery, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University

### **Corresponding Authors:**

|                 |                          |
|-----------------|--------------------------|
| Xiao-Feng Zhang | ZhangXF_GZMU@hotmail.com |
| Ming Liu        | liuming@gzhmu.edu.cn     |

### **Email Addresses for All Authors:**

|                  |                               |
|------------------|-------------------------------|
| Dan He           | 2022125020@stu.gzhmu.edu.cn   |
| Zhong Liang      | liangzhong@stu.gzhmu.edu.cn   |
| Cheng-Yang Zhang | zhangcygzhmu@stu.gzhmu.edu.cn |

|                 |                             |
|-----------------|-----------------------------|
| Jie-Shen Xian   | 2021163003@stu.gzhmu.edu.cn |
| Yuan-Xiang Yang | yuanxiangyang2010@163.com   |
| Yuan-Feng Gong  | gongyuanfeng@gzhmu.edu.cn   |
| Hui Tang        | 343061924@qq.com            |
| Guang-Ding Zhou | 2025210232@stu.gzhmu.edu.cn |
| Chen-Yu Jiang   | 2025210203@stu.gzhmu.edu.cn |
| Le Xing         | 2023311312@stu.gzhmu.edu.cn |
| Dong-Mei Li     | 2023210075@stu.gzhmu.edu.cn |
| Ying-Chen Hou   | 2023210465@stu.gzhmu.edu.cn |
| Chen Yue        | 2024210050@stu.gzhmu.edu.cn |
| Wei Cheng       | chanwei1101@163.com         |
| Yun-Qiang Tang  | 13822220488@163.com         |
| Xiao-Feng Zhang | ZhangXF_GZMU@hotmail.com    |
| Ming Liu        | liuming@gzhmu.edu.cn        |

## **Author Questionnaire**

**1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

**Leica DM IL LED 383908**

**2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

**3. Filming location:** Will the filming need to take place in multiple locations? **No**

**4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

### **Current Protocol Length**

Number of Steps: 24

Number of Shots: 43

# Introduction

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*Videographer: Obtain headshots for all authors available at the filming location.*

**Authors:** Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

## INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Xiao-Feng Zhang:** We focus on how HCC organoids respond to drug treatment and how their transcriptional states change afterward.
  - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Dan He:** This protocol is useful for HCC organoids and can also be adapted to other tumor organoid systems.
  - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

## CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Dan He:** This protocol allows us to study changes in cell states, cellular composition, and gene expression after treatment.
  - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

1.4. **Zhong Liang:** The biggest challenge is preserving cell viability and keeping sample processing consistent across all groups.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

1.5. **Cheng-Yang Zhang:** Several downstream analyses can be conducted following this procedure, including clustering, differential gene expression analysis, pathway enrichment analysis, and trajectory inference.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

***Videographer: Obtain headshots for all authors available at the filming location.***

**Ethics Title Card**

This research has been approved by the Committee for Ethical Review of Research of Guangzhou Medical University. Written informed consent was obtained from all patients

# Protocol

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***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

## **2. Drug Treatment of Hepatocellular Carcinoma (HCC) Organoids**

**Demonstrator:** Dan He

- 2.1. To begin, establish hepatocellular carcinoma or HCC (*H-C-C*) patient-derived organoids and prepare them for therapeutic perturbation [1].
  - 2.1.1. Establishing shot of the talent with the prepared organoid containing plate in front of him.
- 2.2. Prepare the lenvatinib stock solution in dimethyl sulfoxide or DMSO (*D-M-S-O*) according to the manufacturer's instructions [1]. Dilute the stock solution in pre-warmed culture medium immediately before use to the predefined working concentration [2]. Prepare enough solution to achieve equal final volumes in all wells, ensuring the same DMSO concentration across each well [3].
  - 2.2.1. Talent preparing the lenvatinib stock solution in dimethyl sulfoxide.
  - 2.2.2. Talent diluting the stock solution in pre-warmed culture medium.
  - 2.2.3. Talent preparing a sufficient volume of treatment medium for equal final volumes in each well.
- 2.3. Next, add lenvatinib-containing medium at 20 micromolar along the wall of each well to avoid disturbing the matrix domes [1]. Include a vehicle control with the same final DMSO concentration [2].
  - 2.3.1. WIDE: Talent adding lenvatinib-containing medium along the wall of each well.
  - 2.3.2. Talent adding the vehicle control with the same final dimethyl sulfoxide concentration.
- 2.4. Incubate the organoids under standard culture conditions for the predefined treatment period [1]. For treatments exceeding 72 hours, replace the drug-containing medium every 48 to 72 hours, ensuring a consistent replacement schedule across all wells [2].
  - 2.4.1. Talent placing the organoids for incubation.
  - 2.4.2. Talent replacing the drug-containing medium.
- 2.5. Record baseline bright-field images before treatment [1]. Acquire images at fixed intervals during treatment using identical microscope settings, magnification, and field positions [2].
  - 2.5.1. SCOPE: Shot of the baseline bright-field images before treatment.

*Videographer: Please film shots labelled as "SCOPE" using the scope kit*

- 2.5.2. SCOPE: Images during treatment using identical microscope settings, magnification, and field positions.
- 2.6. For morphology-based evaluation of the treatment response, use identical exposure settings, magnification, and analysis thresholds for all images [1].
  - 2.6.1. Talent setting exposure settings, magnification, and analysis thresholds.
- 2.7. Measure the organoid area growth curve by calculating the total organoid area per well or field at each time point and normalize the values to baseline [1].
  - 2.7.1. **SCREEN**: Talent calculating the total organoid area per well or field at each time point and normalizing the total organoid area values to baseline.

*Videographer: Please film the computer screen for 2.7.1*

- 2.8. To measure the mean organoid diameter, determine the diameter of individual intact organoids and calculate the mean value per well [1].
  - 2.8.1. **SCREEN**: Talent determining the diameter of individual intact organoids and calculating the mean organoid diameter value per well.

*Videographer: Please film the computer screen for 2.8.1*

- 2.9. Next, determine the surviving organoid count by counting morphologically identifiable intact organoids while excluding any debris or collapsed fragments [1].
  - 2.9.1. **SCREEN**: Talent counting morphologically identifiable intact organoids.

*Videographer: Please film the computer screen for 2.9.1*

- 2.10. Perform a three-dimensional luminescence viability assay at the treatment endpoint if additional bulk viability assessment is required following the manufacturer's instructions [1].

- 2.10.1. Shot of the talent with all the required reagents in the frame.

- 2.11. Then, plot the organoid area growth curves over time [1]. Compare the mean organoid diameter between vehicle-treated and lenvatinib-treated groups [2]. Also, compare surviving organoid counts across treatment groups. Ensure using consistent imaging schedules, inclusion criteria, and analysis parameters to ensure reproducibility.[3].

- 2.11.1. **SCREEN**: Show the organoid area growth curves being plotted over time.

*Videographer: Please film the computer screen for 2.11.1.-2.11.3*

- 2.11.2. **SCREEN**: Show the mean organoid diameter values for vehicle-treated and lenvatinib-treated groups being compared.

- 2.11.3. **SCREEN**: Show the surviving organoid counts for the treatment groups being compared.

### **3. Preparation of Organoids for Single-cell RNA Sequencing**

- 3.1. Select organoid wells with intact 3D morphology, sufficient material for single-cell capture, and no visible contamination [1]. Collect organoids at matched time points across control and treated groups while maintaining identical plating density, treatment schedule, and medium replacement conditions across all samples.[2].
  - 3.1.1. SCOPE: Shot of the organoid wells with intact three-dimensional morphology and sufficient material with no contamination.
  - 3.1.2. Talent collecting organoids from control and treated samples at matched experimental time points.
- 3.2. Record bright-field images of each selected well before dissociation using identical microscope settings, magnification, and field selection criteria [1]. Aspirate the culture medium completely from each well [2].
  - 3.2.1. SCOPE: Bright-field images of each selected well before dissociation.
  - 3.2.2. Talent aspirating the culture medium completely from each well.
- 3.3. Wash each well twice with ice-cold PBS to remove residual medium and debris [1]. Add 1 milliliter of ice-cold cell recovery solution to each well [2] and incubate on ice for 20 to 30 minutes [3]. Pipette gently every 5 to 7 minutes during incubation to facilitate matrix dissolution [4].
  - 3.3.1. Talent washing each well twice with ice-cold phosphate-buffered saline.
  - 3.3.2. Talent adding 1 milliliter of ice-cold cell recovery solution to each well.
  - 3.3.3. Talent placing the sample on ice for incubation.
  - 3.3.4. Talent pipetting the sample gently during incubation.
- 3.4. Transfer the dissolved material into pre-chilled tubes using a wide-bore or cut pipette tip to minimize shear stress [1].
  - 3.4.1. Talent transferring the dissolved material into pre-chilled tubes using a wide-bore or cut pipette tip.
- 3.5. Proceed to enzymatic dissociation only after the matrix is largely dissolved and minimal visible gel residue remains [1].
  - 3.5.1. Shot of the sample after the matrix is largely dissolved and minimal visible gel residue remains. Videographer's NOTE: Sections 3.5.1(1) and 3.5.1(2) refer to wide-angle and close-up shots, respectively
- 3.6. Centrifuge the recovered organoid suspension at 300 g for 5 minutes at 4 degrees Celsius [1]. Remove the supernatant carefully without disturbing the pellet [2]. Resuspend the pellet in 1 milliliter of recombinant cell dissociation enzyme [3] and incubate at 37 degrees Celsius for 5 to 10 minutes [4].
  - 3.6.1. Talent placing the recovered organoid suspension in the centrifuge.

- 3.6.2. Talent removing the supernatant carefully without disturbing the pellet.
- 3.6.3. Talent resuspending the pellet in 1 milliliter of recombinant cell dissociation enzyme.
- 3.6.4. Talent placing the sample for incubation.
- 3.7. Pipette gently 8 to 10 times every 2 to 3 minutes using a wide-bore P1000 tip to promote dissociation [1]. Stop the digestion when most organoid fragments have dispersed into single cells and only small residual clusters remain [2].
  - 3.7.1. Talent pipetting the sample gently with a wide-bore P1000 tip.
  - 3.7.2. Shot of most organoid fragments dispersed into single cells.
- 3.8. Next, add 4 milliliters of ice-cold PBS containing 2 percent fetal bovine serum to stop the digestion [1]. Then, filter the suspension through a 40 micrometer cell strainer [2]. Wash once with PBS to remove residual enzyme, aggregates, and debris [3].
  - 3.8.1. Talent adding 4 milliliters of ice-cold phosphate-buffered saline containing 2 percent fetal bovine serum to the suspension.
  - 3.8.2. Talent filtering the suspension through a 40 micrometer cell strainer.
  - 3.8.3. Talent washing the sample once with phosphate-buffered saline.
- 3.9. Count the cells using trypan blue exclusion [1].
  - 3.9.1. SCOPE: Shot of the cells after trypan blue staining.
- 3.10. After ensuring the cell viability of 85 percent or above, adjust the final cell concentration to 700 to 1,200 cells per microliter [1].
  - 3.10.1. Talent adjusting the cell concentration to 700 to 1,200 cells per microliter.
- 3.11. Exclude samples with excessive debris, abundant dead cells, large visible aggregates, or incomplete dissociation [1].
  - 3.11.1. Shot of samples with excessive debris, dead cells, aggregates, or incomplete dissociation.
- 3.12. Repeat filtration before loading if aggregates are present [1].
  - 3.12.1. Talent repeating filtration of the sample before loading.
- 3.13. Process control and treated samples under identical conditions, including the dissociation enzyme, digestion time, pipetting frequency, filtration method, cell concentration, and loading strategy [1]. Finally, after single-cell library amplification, perform single-cell sequencing [2]
  - 3.13.1. Talent pipetting the cells of both control and treated groups.
  - 3.13.2. Talent showing the computer screen with the sequencing data.

## Results

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### 4. Results

4.1. Organoids survived and expanded under standard three-dimensional culture conditions from day 1 to day 6 post-recovery [1].

4.1.1. LAB MEDIA: Figure 1B.

4.2. At day 1, organoids appeared as small, compact structures [1], whereas by day 6 they exhibited increased size and well-defined morphology [2].

4.2.1. LAB MEDIA: Figure 1B. *Video editor: Zoom in on the small, tightly clustered round shapes in the “1-day incubation” image*

4.2.2. LAB MEDIA: Figure 1B. *Video editor: Zoom in on the larger, more clearly shaped structures in the “6-day incubation” image*

4.3. Lenvatinib-treated organoids were smaller and displayed altered morphology compared to DMSO-treated controls [1].

4.3.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the smaller and more irregular shapes under “Lenvatinib”*

4.4. Quantitative analysis showed a reduction in mean organoid diameter following lenvatinib treatment compared to DMSO controls [1].

4.4.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the lower distribution for “Lenvatinib”*

### Pronunciation guides:

1. Hepatocellular

Pronunciation link: <https://www.merriam-webster.com/dictionary/hepatocellular>

IPA: /həˌpɑ-tō-'se-l(y)ə-lər/

Phonetic Spelling: heh·puh·toe·seh·lyuh·ler

2. Carcinoma

Pronunciation link: <https://www.merriam-webster.com/dictionary/carcinoma>

IPA: /ˌkär-sə-'nō-mə/

Phonetic Spelling: kar·suh·noh·muh

3. Organoids

Pronunciation link: <https://www.merriam-webster.com/dictionary/organoid>

IPA: /'ör-gə-ˌnòid/

Phonetic Spelling: or-guh·noyd

4. Therapeutic

Pronunciation link: <https://www.merriam-webster.com/dictionary/therapeutic>

IPA: /ˌther-ə-ˈpyü-tik/

Phonetic Spelling: theh·ruh·pyoo·tik

5. Perturbation

Pronunciation link: <https://www.merriam-webster.com/dictionary/perturbation>

IPA: /ˌpər-tər-ˈbā-shən/

Phonetic Spelling: per·ter·bay·shuhn

6. Lenvatinib

Pronunciation link: <https://www.howtopronounce.com/lenvatinib>

IPA: /ˌlen-və-ˈti-nib/

Phonetic Spelling: len·vuh·tee·nib

7. Dimethyl

Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl>

IPA: /dī-ˈme-thəl/

Phonetic Spelling: dye·meh·thuhl

8. Sulfoxide

Pronunciation link: <https://www.merriam-webster.com/dictionary/sulfoxide>

IPA: /səl-ˈfāk-ˌsīd/

Phonetic Spelling: sul·fok·side

9. Micromolar

Pronunciation link: <https://www.merriam-webster.com/medical/micromolar>

IPA: /ˌmī-krō-ˈmō-lər/

Phonetic Spelling: my·kroh·moh·ler

10. Morphology

Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>

IPA: /môr-ˈfä-lə-jē/

Phonetic Spelling: mor·fol·uh·jee

11. Luminescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/luminescence>

IPA: /ˌlü-mə-ˈne-sən(t)s/

Phonetic Spelling: loo·muh·neh·suhnts

12. Viability

Pronunciation link: <https://www.merriam-webster.com/dictionary/viability>

IPA: /ˌvī-ə-ˈbi-lə-tē/

Phonetic Spelling: vy·uh·bil·uh·tee

13. Dissociation

Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>

IPA: /di-ˌsō-sē-ˈā-shən/

Phonetic Spelling: dih·soh·see·ay·shuhn

14. Centrifuge  
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>  
IPA: /ˈsen-trə-ˌfyūj/  
Phonetic Spelling: sen-truh-fyooj
15. Supernatant  
Pronunciation link: <https://www.merriam-webster.com/medical/supernatant>  
IPA: /ˌsü-pər-ˈnā-tənt/  
Phonetic Spelling: soo-per-nay-tuhnt
16. Recombinant  
Pronunciation link: <https://www.merriam-webster.com/dictionary/recombinant>  
IPA: /ri-ˈkām-bə-nənt/  
Phonetic Spelling: ree-kom-buh-nuhnt
17. Enzymatic  
Pronunciation link: <https://www.merriam-webster.com/dictionary/enzymatic>  
IPA: /ˌen-zī-ˈma-tik/  
Phonetic Spelling: en-zye-mat-ik
18. Microliter  
Pronunciation link: <https://www.merriam-webster.com/medical/microliter>  
IPA: /ˈmī-krō-ˌlē-tər/  
Phonetic Spelling: my-kroh-lee-ter
19. Sequencing  
Pronunciation link: <https://www.merriam-webster.com/dictionary/sequencing>  
IPA: /ˈsē-kwən-sɪŋ/  
Phonetic Spelling: see-kwuhn-sing
20. Amplification  
Pronunciation link: <https://www.merriam-webster.com/dictionary/amplification>  
IPA: /ˌam-plə-fə-ˈkā-shən/  
Phonetic Spelling: am-pluh-fuh-kay-shuhn
21. Phosphate-buffered  
Pronunciation link: <https://www.howtopronounce.com/phosphate-buffered>  
IPA: /ˈfäs-ˌfāt ˈbə-fərd/  
Phonetic Spelling: fos-fayt buh-ferd
22. Trypan  
Pronunciation link: <https://www.howtopronounce.com/trypan>  
IPA: /ˈtrī-pæn/  
Phonetic Spelling: try-pan
23. Filtration  
Pronunciation link: <https://www.merriam-webster.com/dictionary/filtration>  
IPA: /fil-ˈtrā-shən/  
Phonetic Spelling: פִּיל-tray-shuhn
24. Debris  
Pronunciation link: <https://www.merriam-webster.com/dictionary/debris>

IPA: /də-'brē/

Phonetic Spelling: duh·bree

25. Magnification

Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>

IPA: /,mag-nə-fə-'kā-shən/

Phonetic Spelling: mag·nuh·fuh·kay·shuhn