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

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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

Interviews

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

***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

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: /həˌpɑːtōˈse-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-səˈnō-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-səns(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