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Title: 3D Cell-Printed Hypoxic Cancer-on-a-Chip for Recapitulating Pathologic Progression of Solid Cancer

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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 similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: All screen captures provided, do not film

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **41**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Wonbin Park**: Hypoxia is a key driver of cancer development that induces genomic instability and tumorigenesis. We demonstrate a method for generating central hypoxia in a solid cancer in vitro based on 3D bioprinting technology **[1]**.

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

REQUIRED:

1.2. **Wonbin Park**: Using this method, a radial hypoxic gradient can be reproduced using a simple strategy that combines a 3D-printed gas-permeable barrier and a glass cover **[1]**.

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

OPTIONAL:

1.3. **Mihyeon Bae**: This hypoxic cancer-on-a-chip technology can be used to predict drug efficacy to facilitate patient-specific anticancer drug prescription and is also expected to enable a quick diagnosis of aggressive cancers **[1]**.

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

Protocol

2. Collagen Pre-Gel Solution Preparation

2.1. To assist in the preparation of a 3-milliliter neutralized collagen pre-gel solution, cut 30-milligram collagen sponges into 5- x 5-millimeter-squared pieces **[1]**.

2.1.1. WIDE: Talent cutting sponges

2.2. Place the pieces into a sterile, 10-milliliter glass vial **[1]** and add 2.4 milliliters of 0.2-micron syringe-filtered 0.1-normal hydrochloric acid to the vial for a 3-day incubation at 4 degrees Celsius and 15 revolutions per minute **[2]**.

2.2.1. Talent placing pieces into vial

2.2.2. Talent adding acid to vial, with acid container visible in frame

2.3. After the digestion, strain any undigested collagen particles through a 40-micron cell strainer **[1]** and store the acidic collagen solution at 4 degrees Celsius for up to 7 days **[2]**.

2.3.1. Talent filtering acid through strainer
2.3.2. Talent placing solution at 4 °C

3. 1% Neutralized Collagen Pre-Gel Solution pH Adjustment

3.1. To adjust the pH of the 1% neutralized collagen pre-gel solution, add 30 microliters of phenol red solution to a final concentration of 1% **[1]** and 300 microliters of 10x PBS to a final concentration of 10% **[2]**.

3.1.1. Talent adding phenol red to solution, with phenol red container visible in frame
3.1.2. Talent adding PBS to solution, with PBS container visible in frame

3.2. After mixing and centrifugation, use 1-normal sodium hydroxide to neutralize the pH to 7, verifying the color change **[1-TXT]**, and add distilled water to obtain a total volume of 3 milliliters **[2]**.

3.2.1. Talent adding NaOH to solution, with NaOH container visible in frame **TEXT: 5 min, 516 x g, 4 °C**
3.2.2. Talent adding water

3.3. Then store the pH-adjusted 1% neutralized collagen pre-gel solution at 4 degrees Celsius for use within 3 days **[1]**.

3.3.1. Talent placing solution at 4 °C

3.4. To pre-check the gelation of the neutralized collagen pre-gel solution, use a positive displacement pipette to add 50 microliters of collagen droplets to a small dish **[1]** and incubate the droplets in a 37-degree Celsius incubator for 1 hour **[2]**.

3.4.1. Talent adding droplets to dish
3.4.2. Talent placing dish into incubator

3.5. At the end of the incubation, check whether the collagen has changed from a transparent color to an opaque white **[1]**.

3.5.1. Shot of opaque, white droplet(s) *Videographer: Important step*

3.6. Tilt the container to check confirm that the collagen is adhered to the bottom of the container **[1]** and pour PBS onto the droplet to confirm that the collagen construct does not break in solution **[2]**.

3.6.1. Container being tilted *Videographer: Important step*
3.6.2. PBS being poured into dish *Videographer: Important step*

4. Gas-Permeable Barrier3D Printing

4.1. For 3D printing of a sacrificial PEVA (P-E-V-A) mold, click **File** and **Save-File type as STL** to convert the 3D CAD (cad) file into an STL (S-T-L) file **[1-TXT]** and click **Option** and **Output form as ASCII** (ASS-kee) to generate the G-code **[2]**.

4.1.1. WIDE: Talent clicking File and Save-File type as STL, with monitor visible in frame **TEXT: PEVA: poly (ethylene-vinyl acetate)**
4.1.2. SCREEN: screenshot_1: 00:12-00:29 *Video editor: can speed up*

4.2. To import the generated STL file, click **File** and **Open STL file** and select the saved STL file **[1]**. To automatically generate the G-code of the sacrificial PEVA mold, select the **Slice model** of the STL-CAD exchanger **[2]**.

4.2.1. SCREEN: screenshot_4: 00:07-00:24 *Video editor: can speed up*
4.2.2. SCREEN: screenshot_2: 00:28-00:40 *Video editor: can speed up*

4.3. Then, to generate printing paths for the chip fabrication, use a 50-gauge precision

nozzle at a pneumatic pressure of 500 kilopascals at 110 degrees Celsius to print the sacrificial PEVA mold onto a sterile, adhesive, hydrophilic histology slide **[1]**.

4.3.1. Mold being printed onto slide

5. Polydimethylsiloxane (PDMS) Barrier Casting

5.1. To cast the PDMS barrier, mix 6 milliliters of PDMS (P-D-M-S) base elastomer and 600 microliters of curing agent for 5 minutes in a plastic reservoir **[1]** and load the homogenously blended solution into a 10-milliliter disposable syringe **[2]**.

5.1.1. WIDE: Talent mixing solutions, with elastomer and agent containers visible in frame
5.1.2. Talent loading solution into syringe

5.2. Equip the syringe with a 20-gauge, plastic, tapered, dispense tip **[1]** and fill the sacrificial PEVA mold with the blended PDMS solution **[2]**.

5.2.1. Talent adding tip to syringe
5.2.2. Talent filling mold with solution

5.3. The blended PDMS will fill the sacrificial PEVA mold with a convex surface and the barrier will be higher than that of the mold **[1]**.

5.3.1. PDMS filling mold *Videographer: Important step*

5.4. After curing the PDMS barrier in a 40-degree Celsius oven for over 36 hours to avoid melting the PEVA **[1]**, use a pair of precision tweezers to detach the sacrificial PEVA mold **[2]** and sterilize the gas-permeable barrier at 120 degrees Celsius in an autoclave **[3]**.

5.4.1. Talent placing barrier at 40 °C
5.4.2. Talent detaching mold
5.4.3. Talent placing barrier at 120 °C

6. 1% Neutralized Collagen Pre-Gel Solution Cell Mixing

6.1. To mix the solution with cancer cells, resuspend each type of collected cell pellet in 20 microliters of culture medium **[1-TXT]** and add 1 milliliter of 1% neutralized collagen pre-gel solution into each of the resuspended cell suspensions on wet ice with gentle mixing **[2]**.

6.1.1. WIDE: Talent adding medium to cell(s), with medium container visible in frame

TEXT: See text for cancer and stromal cell preparation details

6.1.2. Talent adding collagen solution to cell(s) *Videographer: Difficult step*

6.2. Use a positive displacement pipette to mix each cell suspension **[1-TXT]**.

6.2.1. Cells being mixed **TEXT: Final concentration: 5 x 10^6 cell type/milliliter 1% neutralized collagen pre-gel solution**

6.3. When homogenous solutions are obtained, use a positive disposable pipette to transfer the cell-encapsulated collagen bioinks into individual 3-milliliter disposable syringes **[1]** and store the syringes at 4 degrees Celsius until 3D cell-printing **[2]**.

6.3.1. Talent adding bioink to syringe
6.3.2. Talent placing syringe(s) at 4 °C

7. Cancer-Stroma Concentric Ring 3D Cell Printing

7.1. For 3D printing of cancer-stroma concentric rings, convert the appropriate 3D CAD file into an STL file format **[1]** and use an STL-CAD exchanger to generate a G-code of the cancer-stroma concentric rings **[2]**.

7.1.1. WIDE: Talent converting file, with monitor visible in frame
7.1.2. SCREEN: screenshot_4: 00:40-01:13 *Video editor: please speed up*

7.2. Load the cell-encapsulated collagen bioinks into the 3D printer head **[1]** and set the temperature of the head and plate to 15 degrees Celsius **[2]**.

7.2.1. Talent loading bioink(s) into head *Videographer: Important step*
7.2.2. Talent setting head and/or plate temperature *Videographer: Important step*

7.3. Load the generated printing path in the control software of the 3D printer **[1]** and click **Start** to print the collagen bioinks onto the gas-permeable barrier according to the loaded G-code with an 18-gauge plastic needle at a pneumatic pressure of approximately 20 kilopascals at 15 degrees Celsius **[2]**.

7.3.1. Talent loading path into software and/or clicking Start, with monitor visible in frame
7.3.2. Bioink(s) being printed onto barrier

7.4. At the end of every printing operation, manually place a sterilized 22- x 50-millimeter glass cover on top of the gas-permeable barrier to generate the hypoxic gradient **[1]**.

7.4.1. Talent loading the glass cover on top of the gas-permeable barrier

7.5. After generating three hypoxic cancer-on-chips, transfer the chips to a 37-degree Celsius incubator 1 hour to cross-link the collagen bioinks **[1]**.

7.5.1. Talent placing chip(s) into incubator

8. Hypoxic Cancer-on-a-Chip Maintenance

8.1. When all of the hypoxic cancer-on-a-chips have been printed, gently rub the cover glasses on top of the gas-permeable barriers with a cell-scraper for tight bonding **[1]**.

8.1.1. WIDE: Talent rubbing cover glass with scraper *Videographer: Important step*
8.1.2. Talent adding medium to chip, with medium container visible in frame *Videographer: Important step*

8.2. To refresh cell culture medium to the chips without detaching the cancer construct, tilt the chips **[1]** and use a pipette to introduce 1.5 milliliters of endothelial cell growth medium to the side of each chip **[2-TXT]**.

8.2.1. Chip being tilted *Videographer: Difficult step*
8.2.2. Medium being added to chip *Videographer: Difficult step* **TEXT: Refresh medium 1x/d/7 d**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
3.5., 3.6., 5.3., 7.2., 8.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
6.1. it is difficult to mix the cell suspension and collagen hydrogel homogeneously without bubble formation. For the step, the user must mix the cell and hydrogel gently by pipetting.
8.2., the user must load media to the chip gently with pipette to prevent a bubble formation in the chip.

Results

9. Results: Representative Solid Pathologic Cancer Progression on a 3D Cell-Printed Hypoxic Cancer-on-a-Chip

9.1. The hypoxic cancer-on-a-chip was designed in the form of concentric rings to mimic the radial oxygen diffusion and depletion observed in cancer tissues **[1]**.

9.1.1. LAB MEDIA: Figure 2A

9.2. After defining the control volume of a space in which oxygen is diffused and consumed by cells **[1]**, an appropriate cellular density for central hypoxia generation can be determined through computational finite element analysis **[2]**.

9.2.1. LAB MEDIA: Figure 2B
9.2.2. LAB MEDIA: Figures 2B and 2C

9.3. Upon 3D cell-printing **[1]**, a compartmentalized cancer-stroma concentric-ring structure can be created to reproduce the anatomical features of the solid cancer **[2]**.

9.3.1. LAB MEDIA: Figure 4B chip image
9.3.2. LAB MEDIA: Figure 4B

9.4. Quantitatively, the post-printing cell viability is typically greater than 96%, confirming that the manufacturing conditions are appropriate for cancer and stromal cells **[1]**.

9.4.1. LAB MEDIA: Figures 4B and 4C

9.5. In this representative analysis, two groups were compared according to the presence **[1]** and absence of the oxygen gradient to verify the effects of a hypoxic gradient on cancer progression **[2]**.

9.5.1. LAB MEDIA: Figure 5A V *Video Editor: please emphasize bottom schematic*
9.5.2. LAB MEDIA: Figure 5A V *Video Editor: please emphasize top schematic*

9.6. Under both conditions, mature CD31 (C-D-thirty-one)-positive endothelial cells were present within the peripheral regions **[1]**, indicating that spatially patterned living constructs were produced using 3D bioprinting technology **[2]**.

9.6.1. LAB MEDIA: Figures 5B and 5C *Video Editor: please emphasize green signal in Figure 5C Peripheral images*

9.6.2. LAB MEDIA: Figures 5B and 5C

9.7. Compared to the oxygen gradient negative condition, the gradient positive condition demonstrated a hypoxic gradient, indicating the gradual expression of HIF1-alpha (heef-one-alpha) **[1]**.

9.7.1. LAB MEDIA: Figures 5B and 5C *Video Editor: please emphasize green signal in Figure 5B GR+ image* **TEXT: HIF1alpha: hypoxia-inducible factor 1-alpha**

9.8. SHMT2 (S-H-M-T-two)-positive pseudopalisading cells **[1-TXT]** and SOX2 (socks-two)-positive pluripotent cells were also observed, indicative of the presence of aggressive pathophysiological features of solid cancer **[2-TXT]**.

9.8.1. LAB MEDIA: Figures 5B and 5C *Video Editor: please emphasize yellow arrowheads in Figure 5C Core GR+ image* **TEXT: SHMT2: serine hydroxymethyltransferase-2**

9.8.2. LAB MEDIA: Figures 5B and 5C *Video Editor: please emphasize red signal in GR+ Peripheral image* **TEXT: SOX2: SRY (sex determining region Y)-box 2**

Conclusion

10. Conclusion Interview Statements

10.1. **Wonbin Park**: The hypoxic cancer-on-a-chip is a useful tool for investigating the pathophysiological characteristics of solid cancers and the dynamic crosstalk between cancer cells and tumorigenesis-promoting microenvironments **[1]**.

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

10.2. **Mihyeon Bae**: As the methodology can be adapted for patient-specific drug design in a reasonable timeframe, this approach is expected to bridge the gap between in vivo and in vitro models of cancer **[1]**.

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