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Title: Quantifying the Brain Metastatic Tumor Micro-Environment using an Organ-On-A Chip 3D Model, Machine Learning, and Confocal Tomography

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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**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonably possible.

Videographer: please film, screen captures not provided

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **Y, ~2.7 mi**

Protocol Length
Number of Shots: **40**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sofia Merajver**: Confocal tomography was developed to obtain the full gamut of cancer cell behaviors as they interact with a cellular barrier and niche. This method provides a basis for studying metastasis **[1]**.

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

REQUIRED:

1.2. **C. Ryan Oliver**: This approach is important for quantifying live cell behaviors and the confocal tomography and machine-learning components are applicable to other lab-on-a-chip systems **[1]**.

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

OPTIONAL:

1.3. **Trisha Westerhof**: Analyzing primary or recurrent tumors or circulating tumor cells can constitute an important diagnostic tool to help predict brain metastasis and to discern brain-metastatic from non-brain metastatic cells **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

Protocol

2. Brain Micro-Environment Endothelial Cell Seeding

2.1. For seeding of the brain micro-environment onto the microfluidic BBN (B-B-N) device, transfer a device from the vacuum desiccator onto a plasma chamber tray with the inlets facing down **[1-TXT]**. Then, place it on a 50 by 75 millimeter glass slide **[2]**.

2.1.1. WIDE: Talent placing device inlet-side-down onto surface *Videographer: Difficult step* **TEXT: BBN: blood brain niche**

2.1.2. Talent placing setup into plasma chamber

2.2. Place the entire setup into a plasma chamber **[1]** and pull a vacuum **[2]** before treating the device with plasma for 30 seconds at 80 watts **[3]**.

2.2.1. Talent placing slide into chamber

2.2.2. Talent pulling vacuum

2.2.3. Device being treated with plasma

2.3. At the end of the treatment, use a guide to quickly place the device inlet-side-up onto the glass side on the lab bench to create a permanent bond between the PDMS (P-D-M-S) of the device and the slide **[1-TXT]**.

2.3.1. Device being placed onto slide *Videographer: Important step* **TEXT: PDMS: polydimethylsiloxane**

2.4. Next, insert 200-microliter pipette tips cut 2-millimeters from the tip into all of the inlets and outlets **[1]** and place the device into the plasma chamber for an 8-minute, 200-watt plasma treatment **[2-TXT]**.

2.4.1. Shot of cut tip, then tip being inserted into device *Videographer: Important step*

2.4.2. Talent placing device into chamber **TEXT: Alternative: Store device in vacuum desiccator until cell seeding**

2.5. After the device has cooled, place the device into a sterile secondary container **[1]** and, within 15 minutes of plasma treatment, transfer 120 microliters of a collagen-astrocyte-microglia solution into the device through the pipette tip for the bottom chamber **[2-TXT]**.

2.5.1. Talent placing device into container

2.5.2. Talent adding cells to chamber *Videographer: Important step* **TEXT: See text for endothelial cell, astrocyte, and collagen preparation details**

2.6. Allow the solution to wick across the chamber into the opposite pipette tip **[1]** and fill the next channel of the device **[2]**.

2.6.1. Solution wicking across chamber *Videographer: Important step*

2.6.2. Next channel being filled

2.7. When all four channels of the device have been filled, place the chip in the cell culture incubator for 1 hour **[1]**.

2.7.1. Talent placing chip into incubator

2.8. When the collagen has set, fill all of the pipette tips feeding the bottom chamber with the appropriate complete cell culture medium **[1]** and coat the upper chamber with 2% growth-factor reduced Matrigel in complete endothelial medium **[2]**.

2.8.1. Bottom chamber tips being filled

2.8.2. Upper chamber tips being filled

2.9. When both chambers have been coated, place the device into the incubator for 1 hour **[1]** before rinsing the upper chamber with the appropriate medium **[2]**.

2.9.1. Talent placing device into incubator

2.9.2. Chamber being rinsed, with medium container visible in frame *Videographer: Important step*

Original:

2.10. Alternating tips, seed 30 microliters of 1 x 10^6 endothelial cells per milliliter of appropriate medium through the tips into the upper chamber every 15 minutes for a total of four aliquots of cells per tip **[1-TXT]**. Incubate the device in between seedings. **[2]**.

2.10.1. Cells being added to tip, with cell solution container visible in frame
Videographer: Important step **TEXT: 15-min application intervals allow cells to settle without overcrowding**

2.10.2. Added shot: Talent placing device into incubator

2.11. When all of the endothelial cells have been seeded, fill all of the tips with the medium **[1]** and return the device to the cell culture incubator for 48 hours **[2-TXT]**.

2.11.1. Talent adding medium to tip, with medium container visible in frame

2.11.2. Talent placing device into incubator **TEXT: Replace medium in both chambers every 12 hours**

3. Cancer Cell Seeding

3.1. When the endothelial layer has matured, replace the medium in the chip to replenish the cell culture medium **[1]** and seed each top chamber channel with 30 microliters of 1 x 10^6 cancer cells per milliliter of appropriate medium **[2-TXT]**.

3.10.1. WIDE: Talent adding medium to chip, with medium container visible in frame

3.10.2. Talent adding cells to tip(s), with cell container visible in frame **TEXT: Seed cancer cells on same side of all 4 top chambers within single device**

3.2. After each 30-microliter aliquot of cells has been seeded **[2]**, return the device to the incubator for 15 minutes **[1].** Then, fill all tips with the appropriate medium **[3-added]** and return the device to the cell culture incubator for 48 hours **[4-added]**.

3.2.1. Talent placing device into incubator

3.2.2. Cells being seeded

3.2.3. Added shot: Talent adding medium to tip, with medium container visible in frame

3.2.4. Added shot: Talent placing device into incubator **TEXT: Replace medium in both chambers every 12 hours**

~~3.3. **[1-TXT]**.~~

~~3.3.1. Talent placing device into incubator **TEXT: Replace medium in both chambers every 12 hours**~~

4. 3D Model Imaging Data Conversion

4.1. After the device has been cultured and imaged, to measure the phenotype of the cancer cells, open the provided software **[1]** and read the confocal image into memory **[2]**. The software will save each color channel from the 3D confocal image into a separate TIFF file **[2]**.

4.1.1. WIDE: Talent at microscope, imaging cells, with monitor visible in frame **TEXT: See text for cell and device imaging details**

4.1.2. SCREEN: 4.1-4.2-4.3-4.4: 00:13-00:18

4.1.3. SCREEN: 4.1-4.2-4.3-4.4: 02:04-02:24 *Video Editor: please speed up*

4.2. Select **Change opacity values for each microscope channel [1]** and adjust the Channel_alpha value for the color channels present in the image such that the background is removed and only the fluorescence within the cells remains **[2-TXT]**.

4.2.1. SCREEN: 4.1-4.2-4.3-4.4: 02:25-02:40 *Video Editor: can speed up* **TEXT: e.g., GFP for cancer and RFP for endothelial cells**

4.3. To visualize the effect, select **View a 3D rendering to verify the threshold are set correctly** and, if the image looks correct, save the opacity values in the Experiment Tracker spreadsheet **[1]**.

4.3.1. SCREEN: 4.1-4.2-4.3-4.4: 02:44-03:08

4.4. Then use **Marching cubes** to convert the volume image into individual 3D triangular meshed objects saved in the VTK (V-T-K) data format **[1-TXT]**.

4.4.1. SCREEN: : 4.1-4.2-4.3-4.4: 03:10-03:20 **TEXT: Use "Convert voxel image into a triangular mesh and save as a VTK file" to repeat conversion for each cell**

5. Plane Fitting

5.1. To fit a plane to the endothelial barrier, first locate the cell centroids **[1]**.

5.1.1. WIDE: Talent locating cell centroids

5.2. Use the **PolyDataConnectivityFilter** to iterate through the list of meshes in the VTK file to extract the regions that are not connected **[1]**.

5.2.1. SCREEN: 5.1-5.2: 00:06-00:20 *Video Editor: please speed up*

5.3. Calculate the centroid of each mesh and add the measurement to a list of centroids filtering for meshes that are too large or too small **[1]**.

5.3.1. SCREEN: 5.1-5.2: 01:08-01:09

5.4. To fit a plane to the list of centroids for the endothelial cells, use a minimization of error method **[1]**, run **Visualize RFP centroids and plane fit** to generate and plot the plane fit **[2]**, and inspect the fit of the plane, adjusting the fit manually as necessary. **[3]**.

5.4.1. SCREEN: 5.3-5.4: 00:00-00:11

5.4.2. SCREEN: 5.3-5.4: 00:12-00:17

5.4.3. SCREEN: 5.3-5.4: 00:18-00:36 *Video Editor: please speed up*

5.5. When the plane has been properly fitted, save the normal of the plane to the Experiment Tracker File **[1]**.

5.5.1. SCREEN: 5.3-5.4: 00:37-01:20 *Video Editor: please speed up* **TEXT: normal: (n1, n2, n3, n4) c and d**

6. Individual Cancer Cell Analysis

6.1. To measure each cancer cells phenotype, after characterizing the endothelial layer with a plane, load the cell_analysis function **[1]** and run **Read in the Experiment_tracker information and analyze the channels that exist** to iterate over and analyze each region in the VTK file **[2]**.

6.1.1. WIDE: Talent loading function data, with monitor visible in frame

6.1.2. SCREEN: 6.1-6.2-6.3: 00:12-00:21

6.2. For each region the function will clip each cell so that the mesh below the membrane is calculated **[1]**.

6.2.1. SCREEN: 6.1-6.2-6.3: 00:25-00:33

6.3. To measure the cellular phenotype, calculate the shape, volume, and position of each cancer cell **[1]**. Then measure the volume and position of each clipped cancer cell to calculate the percentage of cell that extravasated through the endothelial barrier **[2]**.

6.3.1. SCREEN: 6.1-6.2-6.3: 00:40-00:47

6.3.2. SCREEN: 6.1-6.2-6.3: 00:53-01:11 *Video Editor: please speed up*

6.4. After performing these steps for several experiments, run **Export the data as a single .xlsx file** to save the data in a spreadsheet **[1]**.

6.4.1. SCREEN: 6.4: 00:00-00:40 *Video Editor: please speed up*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.3.-2.6., 2.9., 2.10.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.1. Store the devices in desiccator for 48 hours prior to use seems to reduce the probability of failure at this stage by controlling the hydrophobicity of the PDMS channels.

2.10. seeding and maturation of the endothelial layer into a barrier is the most difficult aspect of this procedure. To ensure success the endothelial cells are maintained at low passages and are not grown to confluence prior to use in the device. The 15-minute intervals between endothelial seedings are critical to permit the endothelial cells to flow and settle onto the membrane.

Results

7. Results: Representative Brain Metastatic Tumor Micro-Environment Analyses

7.1. A microfluidic-BBN chip with a confluent endothelial barrier is acceptable for experimentation **[1]**, while a microfluidic-BBN chip with especially poor endothelial coverage is not **[2]**.

7.1.1. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize Figure 3C*

7.1.2. LAB MEDIA: Figures 3B and 3D *Video Editor: please emphasize Figure 3D*

7.2. In this representative analysis, the brain-seeking cancer cell line **[1]** exhibits a subpopulation of cells that extravasates across the endothelial barrier and migrates deep into the brain niche space of the microfluidic-BBN chip **[2]**.

7.2.1. LAB MEDIA: Figure 4A

7.2.2. LAB MEDIA: Figure 4A *Video Editor: please emphasize datapoints within the red boxes*

7.3. At 2 and 9 days after exposure, the parental cancer cells maintained a substantial proportion of cells on top of the barrier away from the brain niche space **[1]**, while the brain-metastatic cancer cell population maintained a proportion of cells that were greater than 100% extravasated **[2]**.

7.3.1. LAB MEDIA: Figure 4B *Video Editor: please emphasize 50% and below green portion of 2- and 9-Day data*

7.3.2. LAB MEDIA: Figure 4B *Video Editor: please emphasize 50% and above blue portion of 1-, 2-, and 9-Day data*

Revised:

7.4. Morphological quantification of the cancer cell shape **[1]** indicated that parental cells demonstrated fewer spherically shaped cells day 1 post seeding **[2]**, while after 2 and 9 days of interaction, both cancer cell lines trended toward decreasing their sphericity **[3]**.

7.4.1. LAB MEDIA: Figure 4C and 4D

7.4.2. LAB MEDIA: Figure 4C and 4D *Video Editor: please emphasize 1-Day data*

7.4.3. LAB MEDIA: Figure 4C and 4D *Video Editor: please emphasize 2- and 9-Day data*

7.5. In addition, the cancer cell subpopulations that extravasated into the astrocytic niche **[1]** were smaller in size **[2]** compared to the cancer cells that remained in interaction with the endothelial barrier without extravasating into the brain **[3]**.

7.5.1. LAB MEDIA: Figures 4E and 4F
7.5.2. LAB MEDIA: Figures 4E and 4F *Video Editor: please emphasize yellow data boxes*
7.5.3. LAB MEDIA: Figures 4E and 4F *Video Editor: please emphasize grey data boxes*

7.6. Brain-seeking cancer cell lines and Patient-Derived Xenografts (P-D-X's) **[1]** exhibit a phenotypic pattern in the microfluidic-BBN chip that could be exploited to differentiate between the brain-metastatic and non-brain metastatic cancer cells by machine learning **[2]**.

7.6.1. LAB MEDIA: Figures 5B and 5C
7.6.2. LAB MEDIA: Figures 5B and 5C *Video Editor: please emphasize colored data lines in both graphs*

Conclusion

9. Conclusion Interview Statements

9.1. **Trisha M. Westerhof**: Cell selection is important. Endothelial cells with a low or high passage number exhibit variable barrier behaviors. In addition, cancer cells and their fluorescence expression may change over time **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.1.)

9.2. **C. Ryan Oliver**: After this procedure, researchers may employ molecular profiling of the secretome and the cells within the device to answer questions about the roles of specific molecular mechanisms during metastasis **[1]**.

9.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*

9.3. **Sofia D. Merajver**: This technique enables the exploration of complex interactions between cancer cells and their micro-environments by combining an engineered micro-environment with the quantitative imaging of niche components over time **[1]**.

9.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can cut for time*