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# Title: Co-Culture of Glioblastoma Stem-Like Cells on Patterned Neurons to Study Migration and Cellular Interactions

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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**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

   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 possible.

   *Videographer: please film screen captures*

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?

Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

**Current Protocol Length**

Number of Steps: 27
Number of Shots: 39

# Introduction

## 1. Introductory Interview Statements

**REQUIRED:**

1.1. **Thomas Daubon:** Glioblastoma are devastating brain cancers with a high invasive capacity. This co-culture system mimics the migration of glioblastoma cells on neurons to recapitulate one of the invasive routes observed in patients **[1]**.

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

1.2. **Vincent Studer:** The geometry and composition of our co-culture model is precisely controlled, facilitating a better reproducibility and straightforward quantification of a complex biological process **[1]**.

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

**OPTIONAL:**

1.3. **Thomas Daubon:** This method can be adapted to clinical diagnosis by co-culturing freshly dissociated patient cells on neurons to define the patient-specific invasive index, which has been shown to be an important indicator of clinical outcome **[1]**.

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

1.4. **Vincent Studer:** This method can also be used to quantify other migrating cells, such as immune cells or fibroblasts **[1]**.

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

**Introduction of Demonstrator on Camera**

1.5. **Thomas Daubon:** Demonstrating the procedure will be Joris Guyon, a PhD student from my laboratory **[1]**.

1.5.1. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

 

# Protocol

## 2. Preparation of Patterned Slides

2.1. To make a substrate for the micropatterning, treat 18-millimeter circular glass coverslips by air or plasma activation for 5 minutes **[1]** before placing the coverslips in a desiccator with 100 microliters of 3-aminopropyl triethoxysilane for 1 hour **[2]**. Then incubate a solution of PEG-SVA *(pronounce 'peg s-v-a')* at 100 milligrams per milliliter for 1 hour **[added-3]**.

2.1.1. WIDE: Talent treating the coverslips *Videographer: This step is important!*
2.1.2. Talent keeping the coverslips in desiccator
2.1.3. Added shot: Talent adding the PEG-SVA solution on the coverslips

2.2. For gel deposition, at the end of the incubation, add 3 microliters of PLPP (P-L-P-P) and 50 microliters of absolute ethanol onto the center of slide and wait until it dries completely **[1-TXT]**.
2.2.1. Talent depositing photoinitiator gel and/or ethanol on slides **TEXT: 4-benzoylbenzyl-trimethylammonium chloride**

2.3. For glass slide micropatterning, mount the coverslip in a Ludin chamber **[1]** and place the chamber onto the stage of a microscope equipped with an auto-focus system **[2]**.
2.3.1. Talent placing coverslip into chamber
2.3.2. Talent placing chamber onto microscope *Videographer: This step is important!*

2.4. After imaging, load the micropattern images into the software **[1-TXT]**. After automatic UV-illumination sequencing, use a pipette to wash the PLPP from the coverslip extensively with PBS **[2]**.
2.4.1. Talent with UV treatment setup. **TEXT: See text for micropattern imaging parameter details**
2.4.2. Talent washing slide with PBS *Videographer: This step is important!*

2.5. Then incubate the coverslip with 50 micrograms/milliliter of laminin for 30 minutes **[1-TXT]** followed by another wash with PBS as demonstrated **[2]**.
2.5.1. Talent keeping the samples for incubation **TEXT: Laminin 50 µg/mL in PBS**
2.5.2. Talent washing slide with PBS *Videographer: This step is important!*

## 3. Preparation of Embryonic Rat Hippocampal Neurons and GBM Cells for Co-Culture

3.1. To set up an embryonic rat hippocampal neuron culture on the micropatterned coverslips, after the last wash, rehydrate the glass slides with neuronal cell culture medium **[1]**.
3.1.1. WIDE: Talent adding neuronal cell culture medium to the micropatterned coverslip

3.2. Seed 5 x $10^4$ rat hippocampal neurons suspended in neurobasal medium enriched with 3% horse serum per square-centimeter onto each micropatterned coverslip **[1-TXT]** for a 24-hour incubation in a 5% carbon dioxide incubator at 37 degree Celsius **[2]**.
3.2.1. Talent seeding the neurons onto coverslips **TEXT: See text for embryonic rat hippocampal neuron preparation details** *Videographer: This step is difficult and important!*
3.2.2. Talent placing coverslip into incubator

3.3. ~~**[1-TXT].**~~
~~3.3.1. Talent adding tube to centrifuge **TEXT: 5 min, 200 × *g*, RT**~~

3.4. Centrifuge dissociated glioblastoma cells for 5 minutes at 1000 rpm **[1]** and resuspend the pellet in Glioblastoma cell culture medium **[2]**.
3.4.1. Talent centrifuging the solution of dissociated GBM cells
3.4.2. Added: Talent resuspending the cell pellet

3.5. Then, deposit 1 x$10^3$ GBM cells over the micropatterned neurons**[1-TXT]** ~~**[2]**~~.
3.5.1. Talent adding to the GBM cells **TEXT: See text for all medium and solution preparation details**
~~3.5.2. Talent seeding the culture *Videographer: This step is difficult and important!*~~

## 4. Live Cell Imaging

4.1. For live cell imaging of the cells, place the co-culture onto the stage of an inverted microscope equipped with a 37-degree Celsius thermostat chamber **[1]** and select the 20x objective **[2]**.
4.1.1. WIDE: Talent placing the samples onto microscope stage
4.1.2. Talent selecting objective

4.2. Then use the multidimensional acquisitions toolbox in the microscope software **[1]** to acquire live brightfield and epifluorescence GFP (G-F-P)-tomato images every 2 minutes for 12 hours in 16 different positions based on the number of patterns with neurons **[2]**.
4.2.1. Talent at microscope, selecting toolbox, with monitor visible in frame
4.2.2. SCREEN: To be provided by Authors: Cells being imaged

## 5. Neuronal Network Analysis

5.1. For neuronal network analysis, after imaging, select one image from the stack **[1]**. Right-click on the **Network** tool to open the corresponding **Options** dialog box and adjust the settings to produce a precise segmentation of the images. Click **OK [2]**.
5.1.1. Talent selecting the image, with monitor visible in frame
5.1.2. SCREEN: 5.Neuronal_Network_Analysis.avi. 0:00 – 0:10

5.2. Left-click on the **Network** tool to duplicate the selected image and **split** the image into the red, grey, and green color channels. Select the grey channel and perform **contrast stretch enhancement** to improve the separation between the different areas **[1]**.
5.2.1. SCREEN: 5.Neuronal_Network_Analysis.avi. 00:10 to 00:12

5.3. Use the **Sobel edge detector** to perform the 2D signal processing convolution as grouped under the **Find Edge** command **[1]**.
5.3.1. SCREEN: 5.Neuronal_Network_Analysis.avi. 00:12 to 00:13

5.4. For **double filtering**, apply Gaussian blur and a median filter to reduce the noise and to smooth the object signal. To **Convert to Mask**, execute adapted threshold algorithms to obtain a binary picture with black and white pixels **[1]**.
5.4.1. SCREEN: 5.Neuronal_Network_Analysis.avi. 00:13 to 00:15

5.5. Next, **Skeletonize** the cell area into a simple network and use **filter particles** to remove small, non-networked particles in the results network **filter particles** in a network image. To obtain red and green channels, perform **double-filtering** and **convert to Mask** as demonstrated, using the adapted thresholding method **[1]**.
5.5.1. SCREEN: 5.Neuronal_Network_Analysis.avi. 00.15 to 00:18

5.6. Use **Analyze Particles** to determine the cell morphology in the binary-green image **[1]**.
5.6.1. SCREEN: 5.Neuronal_Network_Analysis.avi. 00:18 *Video Editor: Emphasize the automatically filling in the results window - top right panel*

5.7. Use the **OR** *(pronounce 'or')* operator to merge all of the channels using their regions of interest and readjust their initial color into a simple RGB (R-G-B) image **[1]**.
5.7.1. SCREEN: 5.Neuronal_Network_Analysis.avi. 00:18 to 00:21

## 6. Single-Cell Motility Analysis

6.1. To perform a single-cell motility analysis, right-click on the **Single Cell Tracking** tool to open the corresponding **Options** dialog box **[1]** and adjust the settings to produce a precise segmentation of images **[2-TXT]**.

6.1.1. WIDE: Talent at computer, right-clicking on Single Cell Tracking tool, with monitor visible in frame
6.1.2. SCREEN: 6.Single_Cell_Motility_Analysis.avi. 00:00 to 00:07

6.2. Click **OK** and left-click on **Single Cell Tracking** to remove the grey channel. To generate an image corresponding to an image stack according to the time, apply **Z projection** and **Double-filter** and **Convert to Mask** the trails left by the cells. Remove the small particles from the binary-red and green image as demonstrated **[1]**.
6.2.1. SCREEN: 6.Single_Cell_Motility_Analysis.avi. 00:07 to 00:13

6.3. Using the **Region of Interest** tool to select each contour of the cell trace and check the **Skip edge detection** box in the **Options** dialog window **[1]**.
6.3.1. SCREEN: 6.Single_Cell_Motility_Analysis.avi. 00:13 to 00:15

6.4. **Isolate** the red channel on the original stack and select one region of interest. **Double filter** all of the images and **Convert to Mask** to allow the centroid X-Y position of each binarized nucleus to be determined **[1]**.
6.4.1. SCREEN: 6.Single_Cell_Motility_Analysis.avi. 00:15 to 00:22

6.5. Then X-Y positions can be used to calculate the mean square displacement, directionality ratio, and average speed for the cell **[1-TXT]**.
6.5.1. SCREEN: 6.Single_Cell_Motility_Analysis.avi. 00:22 to 00:25

## 7. Multiple-Cell Motility Analysis

7.1. For multiple cells tracking analysis, right-click on the **Tracking** tool to open the corresponding **Options** dialog box **[1]** and adjust the settings to produce a precise segmentation of images **[2]**.
7.1.1. WIDE: Talent right-clicking on tool/opening dialog box, with monitor visible in frame
7.1.2. SCREEN: 7.Multiple-cell_Motility_Analysis.avi. 00:00 to 00:05

7.2. Left click on the **Tracking** tool to remove the grey channel. **Split** the red and green channels, **double-filter**, and **Convert to Mask [1]**.
7.2.1. SCREEN: 7.Multiple-cell_Motility_Analysis.avi. 00:05 to 00:53. *Video Editor: Speed up from 00:10 to 00:29 and from 00:35 to 00:51*

7.3. Then use the **Image Calculator** command with the **AND** operator to merge the channels, leaving only the nucleus signal located within the membranes and calculate the trajectory plot, mean square displacement, directionality ratio, and average speed for the cells as demonstrated **[1]**.
7.3.1. SCREEN: 7.Multiple-cell_Motility_Analysis.avi. 00:53 to 00:55

# Results

**8. Results: Representative Analysis of GBM Migration on Patterned Neurons**

8.1. Fluorescent GBM co-cultured with patterned neurons quickly modify their shape **[1]** and show migration along neuronal extensions in a random motion **[2]**.
- 8.1.1. LAB MEDIA: Dissoc_cells
- 8.1.2. LAB MEDIA: Dissoc_cells: 00:00-00:07 *Video Editor: please emphasize at least one green cell moving along a neuron in Neuron's frame of video*

8.2. GBM cells seeded onto neurons display an elongated shape with multiple protrusions that follow the neuron tracts **[1]**, while the cells retain their rounded shape when cultured on laminin **[2]**.
- 8.2.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize Figure 3Ai/green cells in Figure 3Ai*
- 8.2.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize Figure 3Aii/green cells in Figure 3Aii*

8.3. At later stages of culture, thin protrusions linking two cells can be observed in GBM-neuronal co-cultures **[1]**.
- 8.3.1. LAB MEDIA: Dissoc_cells: 00:08-00:15 *Video Editor: please emphasize two neurons left-center that briefly "link"*

8.4. GBM cells seeded onto neurons **[1]** demonstrate a greater migratory capacity **[2]** than GBM cells seeded onto laminin, as observed in these trajectory plots **[3]**.
- 8.4.1. LAB MEDIA: Figure 3B
- 8.4.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize Figure 3Bi/data lines in Figure 3Bi*
- 8.4.3. LAB MEDIA: Figure 3B *Video Editor: please emphasize Figure 3Bii/data lines in Figure 3Bii*

8.5. Analysis of the fluorescent confluence of the cells demonstrates that, over a 500-minute observation period **[1]**, more cell migration is observed when the spheroids are co-cultured with neurons **[2]** than when the cells are cultured-on laminin alone **[3]**.
- 8.5.1. LAB MEDIA: Figure 3Ciii
- 8.5.2. LAB MEDIA: Figure 3Ciii *Video Editor: please emphasize blue data lines*
- 8.5.3. LAB MEDIA: Figure 3Ciii *Video Editor: please emphasize grey data lines*

8.6. Indeed, by the end of the analysis, nearly half of the pattern is covered with GBM cells while the spheroids cultured on laminin remain unadhered to the coverslip **[1]**.

8.6.1. LAB MEDIA: Spheroid *Video Editor: please emphasize green cells in Neurons frame with "nearly … cells" and green cells in Laminin frame with "spheroids … coverslip"*

# Conclusion

**9. Conclusion Interview Statements**

9.1. **Vincent Studer:** Depending on the biological questions you want to answer, take care that the pattern design and cell density are representative of in vivo conditions **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1., 3.5.2.*

9.2. **Vincent Studer:** The cells can be fixed and imaged by confocal microscopy. Live imaging is also possible, since our method does not impair imaging capabilities **[1]**.

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

9.3. **Thomas Daubon:** This technique is well suited for studying molecular stimulations or metabolic exchanges between neurons and glioblastoma cells, allowing the exploration of fine-tuned biological systems and performing high throughput experiments for diagnostic purposes **[1]**.

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