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GBM-39 Flat and Spheroid Culture with Imaging

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Lexi Simar¹, Irma Torres-Vazquez¹, Eduardo Rosa-Molinar^{1,2}

¹The University of Kansas;

²The Washington University in Saint Louis School of Medicine, Washington University Center for Cellular Imaging



Lexi Simar

Columbia University

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We use this protocol and it's working

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Abstract

GBM-39 cells are cultured into a spheroid or flat 2D culture to examine the mitochondrial movement in tubual structures built by the cells. These mitochondria move across the tubes and can be seen at various points in the developing and established tubes.

Attachments



[332022_IHC_gliospher...](#)

19KB

Materials

Laminin- (EMD Millipore, Cat. number: CC095, Lot: 29722820)

PBS 1x (calcium and Magnesium Free), sterile

TrypLE™ Express Enzyme (Gibco, Cat. number: 12605-010, Lot number: 2381446)

HBSS 10X no phenol red, (Gibco, cat. number: 14065056)

PFA 16X (Sigma, cat. number: P6148-1KG)

Sucrose (Fisher Chemical, cat. number: S5-500)

Hemocytometer

Trypan blue

DMEM/F12 (Gibco, catalog number: 11330-032)

B27 with Vitamin A (ThermoFisher, catalog number: 17504044)

Glutamax, 100X (ThermoFisher, catalog number: 35050-061)

Penicillin/Streptomycin, 10,000U/ml (ThermoFisher, catalog number: 151440122)

Heparin 5 mg/ml (Sigma, catalog number: H3149)

Recombinant human EGF (hEGF), 0.1mg/ml (Peprotech, catalog number: AF-100-15).

Recombinant human FGF-basic (bFGF), 10 µg/ml (Peprotech, catalog number: 100-18B, Lot number: 052108J1221)

8-well plate (ibidi, cat number: 80807)

35 mm µ-dish (ibidi, cat. number: 81158)

Hoechst 33342 (Invitrogen , cat number: H3570, lot number: 1156367)

Mitotracker Red CMXros (Invitrogen, cat. number: M7512, lot number: 1884975)

water bath

10-100 uL, 100-1000 uL micropipette and pipette tips

aluminum foil

parafilm

ITO coated conductive coverslip, 18 X 18 mm, 70-100Ω, # 1.5 (SPI, Cat. num: 06478-A)

PELCO Tabs™, Carbon Conductive Tabs, 12mm OD (Ted Pella, product num: 16084-1)

Standard SEM Pin Stub Mount, Ø12.7mm x 8mm pin height (Ted Pella, product num: 16111)

Vectashield Plus (Vector Labs, cat num: H-1900)

ICC reagents in attached file

Karnovsky's fixative (2% PFA + 2.5% Glutaraldehyde diluted in Cacodylate buffer 0.2M/CaCl)

2% osmium tetroxide

Pelco Biowave

Hitachi Scanning Electron Microscope (Model S-4700 Type II) (The University of Kansas)

3i/Olympus Epifluorescence Inverted Microscope (The University of Kansas)

20X/0.50 NA Olympus UPlan FL N (The University of Kansas)

40X/0.95 NA Olympus UPlanSApo (The University of Kansas)

60X/0.90 NA Olympus UPlanApo objective (The University of Kansas)

Before start

All steps preparing and plating the cells must be done under aseptic conditions in a BSC certified room.



GBM-39 Media

- 1 Preparation of gliosphere medium (not complete), typically prepared 50 mL at a time.
Mix DMEM/F12 (48 mL) with:
2% B27 with Vitamin A = 1 mL
1% Glutamax = 0.5 mL
1% Penicillin/Streptomycin = 0.5 mL
- 1.1 Store at 4°C.
- 2 Preparation of complete gliosphere medium- always use freshly prepared gliosphere medium
Mix the Gliosphere medium with (measurements for 25 mL are in step 2.1):
5 µg/ml of Heparin
50 ng/ml of EGF
20 ng/ml of bFGF.
- 2.1 For 25 ml of complete gliosphere medium add to the Gliosphere medium:

25 µl of Heparin
50 µl bFGF
12.5 µl EGF
- 3 For live **imaging and staining**, prepare fresh imaging media following steps 1 and 2 but use DMEM/F12 **without** phenol red. The amount prepared can and should be adjusted according to the size of the experiment.

Thawing and Plating Cells from Cryo

- 4 Thaw in 2 ml of complete glioblastoma medium
- 5 Centrifuge at 400 x g for 4 minutes
- 6 Count the cells (follow steps in the Counting Cells for Culture section below).
- 7 Plate at 100,000 cells/mL in 6 well plates (2 mL per well) or in a T75 culture flask. This is day 0. Use the GBM media (not complete).



7.1 Complete media will be used on day 5 to feed or change the media.

8 Cells will need to be subcultured on day 7.

Counting Cells for Culture

9 Warm GBM media and TrypLE in water bath.

10 Collect the cell suspension in a centrifuge tube.

11 Centrifuge the cells at 400g for 4 min.

12 Remove the supernatant and add 1 ml of TrypLE. Gently tap the tube to disrupt the pellet.

13 Incubate at room temperature for five minutes.

13.1 If the pellet is still large and "solid" after gently tapping the tube, place the tube in the incubator for another 3-5 minutes.

14 Add 2 ml of GBM medium; mix by gently shaking the tube

15 Centrifuge at 400g for 4 minutes. Aspirate the supernatant.

16 Resuspend the cells by pipetting multiple times in 1- 2ml of Complete GBM medium.

17 Count the cells with Trypan blue in the hemocytometer.

17.1 Seeding density will depend on intended experimental culture as described in the remaining sections.

- 18 See either flat or spheroid culture section for plating steps.

Coatings for Flat Experimental Culture

- 19 Prepare the appropriate volume of laminin (final concentration of 5 ug/mL) for the chosen culture dish.
8 well plate will use 200 uL per well
35mm plate will use 400 uL per plate

Note

If not controlling for aggregates, coating is unnecessary. If maintaining the culture as close to a monolayer as possible is important?, then coating is necessary.

- 20 Dispense appropriate amount into culture dish.
- 21 Incubate for 2 hours at 37°C with 5% CO₂ or overnight at 4°C.
- 21.1 If incubating overnight, wrap dish in parafilm.

Flat Experimental Culture for 8-well or 35 mm μ -dish

- 22 For the 8-well plate, a density of 40-50,000 cells/well is required.
For the 35mm μ -dish, there can be a minimum of 300,000 cells/dish or 1 mL of spun down cells from culture flask.
- 22.1 For the 35mm μ -dish, if choosing the 1 mL measurement follow the steps in the previous section but stop after step 8 and plate into the coated 35mm μ -dish when done.
- 23 Remove the laminin (if needed) from the dish. Seed the cells with the complete GBM media.

**Note**

This is day 0. Media should not need to be changed until day 3. If media needs to be changed before day 3, take note of the condition of the cells. The cells could be growing faster than anticipated, or contamination might have occurred.

- 24 On day 2, check the cells under a microscope. Note how many tubes/connections have grown between the cells.
- 25 On day 3, change the media with the complete GBM media. If connections have increased experiment can be run on day 4 or day 5.
- 25.1 Experimental labeling can be performed on day 3 or day 4.

Spheroid Culture for Round Bottom 96-well plate

- 26 The round bottom well plate does not need to be coated. Can seed anywhere from 2-3,000 cells/well.

Note

This is day 0. The spheres should not need to be fed until day 3.

- 27 On day 3, check the health of the sphere and change the media (use complete GBM media).
- 28 On day 5, one more feeding could be necessary, depending on growth seen by day 3.
- 28.1 If the spheres look fine (no contamination, smooth in appearance), experimental labeling can be performed on day 6.
- 28.2 At day 6, if more time is needed for the spheres to grow, a feeding schedule of every other day or every two days can be used.

**Note**

Spheroids used in experiment can be mounted in 35mm μ -dish or depression slide.

Mitochondria Visualization

- 29 Warm complete media in water bath at 37°C

Note

This labelling procedure will work for either the flat or the spheroid preparation.

- 30 Remove media from cells.

- 31 Stain with Mitotracker and Hoechst cocktail. Cocktail should be made with the complete GBM media without phenol red.

Note

Mitotracker working dilution is 250 nM.
Hoechst working dilution is 1.6 μ g/mL.

For 8-well plate, the working solution is 200 μ L/well.
For 35 mm μ -dish, the working solution is 400 μ L/plate.

- 31.1 Incubate for 30 minutes at 37°C with 5% CO₂.

Note

At this stage (incubating with Mitotracker and Hoechst) cell exposure to external light should be limited while working or imaging the sample.

- 32 Remove the cocktail media.

- 32.1 For live imaging of cells, go to step 25; otherwise continue to 24.2.

- 32.2 To fix cells, remove cocktail media and rinse with warmed (37°C) 1X HBSS for 5 minutes.
- 32.3 Fix with 2% PFA solution for 10 minutes.
- 32.4 Rinse with HBSS:Su solution (0.01%) for 5 mins. Do this twice and image in fresh HBSS:Su.
- 33 Add fresh complete GBM media (no phenol red). Cells are ready to image at 37°C with 5% CO₂.
- 33.1 If storing, transferring, or using over multiple days, wrap culture dish in aluminum foil.

Live Imaging: Epifluorescent Microscope

- 34 Turn on the microscope and the software, but not the lamp.
- 35 Wipe down the microscope with 70% ethanol, including the incubation chamber.
- 36 Close the incubation chamber, and turn on the humidifier and open the CO₂ gas line.

Note

Watch the CO₂ meter until it levels around 5%.

- 37 Turn on the lamp and allow it to warm up for 5 minutes prior to imaging.
- 38 Remove the labelled 2-D culture (8-well ibidi, or 35-mm μ -dish) from the incubator.
- 39 Spray down the culture dish with 70% ethanol and place in the prepared incubator on the microscope.



- 40 Open imaging software (Slidebook), and save the new slide for the experiment.
- 41 In Slidebook, while the lamp is still shuttered and open the filter for the eyepieces.
- 42 Using the DAPI filter (DAPI eyes), use the eye pieces to locate a region of interest (ROI) and bring that into focus.
- 43 Shutter the eyepieces filter, and open the lamp shutter.
- 44 Bring the sample into focus on the 20X objective. If this is hard to see (no large aggregates in the culture), increase magnification to the 40X objective.
- 45 Once the sample is in focus, using the capture window, test the filters (that match the Mitotracker CMXros and Hoechst) for exposure time.
- 46 Select the desired objective (20X, 40X, 60X).
- 47 Once the exposure times are set, an image can be captured.
- 48 If tracking mitochondrial movement, this first image will be time 0. Images for this experiment were taken at 15, and 30 minute intervals for 2 different experiments.

Note

For the 30 minute interval experiment, the lamp was shuttered and not turned off.

- 49 Repeat 44-47 for each well and each ROI. Also repeat with different magnifications as desired.

Note

The exposure times should remain the same across the sample and same objective used, regardless of well. If changing the objective to capture the same ROI, new exposure times will be needed. Then those new exposure times can be used for the remaining images needed using that new objective.

- 50 If performing a timelapse study, steps 44-47 can be repeated in 20 minute intervals.

Note

20 minutes was chosen to allow the cells to recover from the light exposure, in addition to allowing the mitochondria to move or TNTs to adapt (grow, break down, etc.).

ICC for Gliosphere

- 51 Gently remove media and for 5 minutes (per wash), wash twice with 200 μ l of warm sterile HBSS at room temperature.
- 52 Fix the gliospheres with 2% PFA diluted in HBSS with 0.01% sucrose for 10 minutes at room temperature.
- 52.1 The washes, blocking, and antibody incubations steps can be performed inside the Pelco Biowave (See attached document) . This can be done in 96 round bottom well plate or the Ibidi 8-well plate.
- 52.2 Solutions need to be changed after each step.
If spheres are in the ibidi, replace the final buffer with mounting media (Vectashield) ~150-200 μ l.
- 53 If spheres are not in the 8-well ibidi, transfer individual gliospheres to glass depression slides.
- 53.1 Fill the depression with VectaShield mounting medium.
- 53.2 Place the cover slip and seal with transparent nail enamel.
- 53.3 Image of gliosphere ICC.

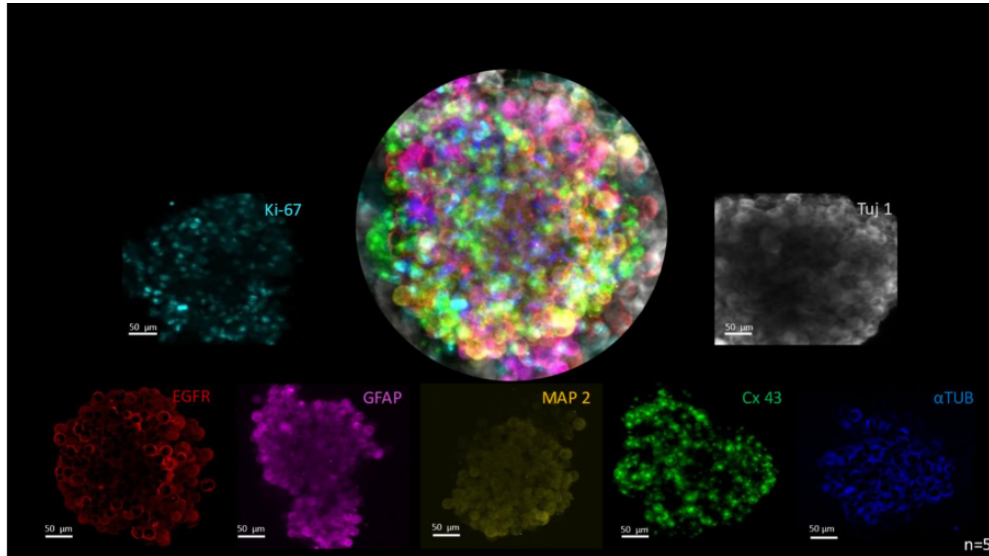


Figure 1. Image of gliospheres, spheres generated from cells of a brain cancer. The image results from merging 7 different antibodies and using Image J. The secondary antibody in all preparations was AF 555. We assigned one color to each protein marker and merged them. All images were acquired in a Leica Scanning Confocal Microscope with a 40X objective. Image courtesy of Irma Torres-Vasquez, Ph.D

SEM Visualization of Gliospheres

- 54 Transfer cultured spheres into individual low retention snap cap (1 or 0.6 mL) falcon tube.

Note

After initial fixation with PFA, all steps must be performed at room temperature inside a chemical hood.

- 55 Fix with 2% PFA diluted in HBSS (1X diluted with DI H₂O) for 30 minutes.
- 56 Rinse with 1x HBSS for 5 minutes (two times).
- 57 Fix in Karnovsky's fixative (2% PFA + 2.5% Glutaraldehyde diluted in Cacodylate buffer 0.2M/CaCl) for 20 minutes.
- 58 Rinse with 1x HBSS for 5 minutes (two times).

- 59 Fix with 2% osmium tetroxide (diluted in HBSS) for 1 hour.
- 60 Each dehydration step is performed for 5 minutes per occurrence.
- 50% EtOH
 - 50% EtOH
 - 70% EtOH
 - 95% EtOH
 - 100% EtOH
 - 100% EtOH
- 61 Infiltration steps, each step is performed for 5 minutes per occurrence.
- 2:1 100% EtOH:100% HMDS
 - 1:1 100% EtOH:100% HMDS
 - 1:2 100% EtOH:100% HMDS
 - 100% HMDS
 - 100% HMDS
- 62 Remove excess HMDS. With less than 1 mL of HMDS, transfer the gliosphere onto an aluminum stub with carbon tape. Place stub into a stub box. Let the sample dry in a vacuum chamber (or dessicator) overnight before imaging.
- 63 An example image of a gliosphere having gone through the SEM preparation.



Figure 2. A low magnification (LM) image of a broad surface view of a GBM-39 gliosphere at 250 Volts (V) with a 12.4 mm working distance. Image taken with SEM for morphology. (OTOTO coated)

- 64 Remove stub box from the desiccator.
- 65 Log onto the computer and create a new file.
- 66 Set the beam to 5.0 kV and 15 μ A.
- 67 Load the stub onto the holder into position 1 using stub tweezers.
- 68 Lock the screw that holds the sample stub in place using the allen wrench.
- 69 Open the sample chamber.



- 70 Load the stub holder onto the screw of the sample extender.
- 71 Close the sample chamber.
- 72 Lock the extender at the decreased position.
- 73 Put the sample chamber back under vacuum.
- 74 Once the lights show the system is ready, open the divider and use the extender to move the holder towards the stage.
- 75 Remove the extender from the holder.
- 76 Retract the holder to the sample chamber.
- 77 Close the divider.
- 78 Turn on the beam.
- 79 Find the stub in position one on the stage.
- 80 Increase the magnification.

Note

High magnification may be needed to see the pollen grains.
- 81 Bring the grains into view and use the coarse and fine knobs to bring the grains into focus.



- 82 Use different scan speeds to assist in bringing the sample into better focus.

Note

This can take a few minutes to get the best resolution possible.

- 83 Once the grains are in focus, return the scan speed to the initial speed and capture the image.

- 84 Increase the magnification to get higher magnified images of the spidkes or other surface features.

- 85 Repeat steps 81 and 82; then capture the structures at a higher magnified view.

Protocol references

Simar, L. (2025). *Imaging Phantoms for Calibration and Performance Assessment for Multiscale Microscopy* [Unpublished doctoral dissertation]. The University of Kansas.