Development of 3D Glioma Models Based on Cell Spheroids Embedded in Pectin-Collagen Matrix

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Разработка 3D-моделей глиомы на основе клеточных сфероидов, встроенных в пектин-коллагеновую матрицу

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Protocol for the creation of a three-dimensional cell model of gliomas developed on spheroids and pectin-collagen biomaterials and subsequent analysis of cell behavior in culture:

Preparation of agarose coated plates

1. Prepare a 1 % agarose solution by mixing 1 g of agarose powder and 100 ml of saline or phosphate buffer;
2. Autoclave the resulting mixture at 120 °C and a pressure of 0.7 MPa for 15 min.;
3. Cover the bottom of wells of a conical 96-well plate with agarose solution at a temperature not exceeding 60 °C. To do this, use a pipette to add 50–100 µl of the solution to the well, then quickly and carefully remove the excess;
4. Leave to polymerize at room temperature for 15 min.;

Spheroid preparation

5. Remove the medium from cell culture flask and wash the cells using phosphate buffer;
6. Add 0.25 % trypsin solution to the cell flask at a rate of 20 µl per 1 cm^2 of surface area and incubate for 5 min at 37 °C;
7. Add medium to the vial in a volume 5 times greater than the volume of trypsin;
8. Count the concentration of cells in the suspension;
9. If necessary, concentrate the suspension by centrifugation (150 g, 5 min) or dilute to a concentration of 40000 cells per ml;
10. Using a multichannel pipette, add the resulting cell suspension to the wells of the prepared plate in a volume of 100 µl per well;
11. Cultivate cells in plates at 37 °C in a CO_2 incubator for 24–48 hours;
12. The condition of the cells and spheroid formation must be assessed visually using a light microscope.

Preparation of hydrogel components

13. Prepare a 3 % pectin solution: mix 150 μg of dry pectin powder with a degree of esterification close to 30 % and 5 ml of Milli-Q water;

14. Incubate the solution in a water bath for 15 min at 60 °C,

15. Centrifuge the solution at 4000 g for 40 min;

16. Autoclave at 105 °C and 0.7 MPa for 10 min;

17. Prepare a collagen solution with a concentration of 1000 μg/ml by mixing 1 mg of lyophilized type 1 collagen and 1 ml of 30 mM acetic acid and leaving to swell at 4 °C for 12–24 hours;

18. Sterilize the collagen solution by dialysis with 0.5 % chloroform and 30 mM acetic acid;

19. Prepare a gelation initiator solution by mixing 750 μl of 2 M $CaCl_2$, 3000 μl of HEPES, 5000 μl of 5 M NaCl and 1250 μl of Milli-Q water;

20. Sterilize the resulting solution by filtration using a filter with a pore diameter of 0.22 μm.;

Embedding of spheroids in biopolymer hydrogels

21. Prepare a suspension of cell spheroids by transferring them in the volume of the medium from the wells into the prepared tube in the required quantity (we recommend no more than 5–7 spheroids per 100 μl of the final gel) using a pipette with a large opening tip;

22. Mix the gels in a circle outlined with a hydrophobic marker on the bottom of a chilled 35 mm Petri dish. To prepare a 300 μl drop of gel, successively add a chilled solution of collagen I or its solvent (30 μl), a 180 mM NaOH solution to neutralize acetic acid (5 μl), a gelation initiator (8 μl), and a mixture of neutralized pectin (100 μl) and 10–15 cell spheroids in a volume of medium (157 μl), prepared in another tube;

23. Incubate samples for 5 minutes for primary gelation;

24. Quickly and carefully turn the dish upside down and leave in a CO_2 incubator until final gelation for 25 minutes;

25. Turn the Petri dish over again and add 2,5 ml of medium;

26. Cultivate spheroids in gel drops at high humidity, a temperature of 37 °C and a CO_2 level of 5 %, and replace the medium every 2–3 days;

27. We recommend capturing images immediately after the polymerization process and medium replenishment to ensure proper culture maintenance during procedure. Next sequences may be taken at equal intervals of time or at the end of a long-term cultivation period;

28. To process images, use Zeiss ZEN software. To do this, go to “Analysis” and then “Interactive Measurement”. Next, define the “Feature Set” as “Area” and “Diameter”, and use the “Polygon contour” or “Circle (Points)” tool to outline the areas of interest. After this, you can save the results in separate files. Alternatively, you can use FIJI (ImageJ) to process a sequence of photos. To do this, you need to transform a bunch of images into a stack: “Image” > “Stack” > “Images to Stack”. Next, use the appropriate outline tool to make selections. Finally, use “Ctrl+M” to save the measurements in a table;

29. To calculate the “Invasion Index”, you need to divide the values for the diameter or area of the recent temporary points by those of the earliest.

Spheroids visualization and cell viability analysis by laser scanning microscopy

30. Replace the medium with 2,5 ml a solution of 10 µl/ml fluorescein diacetate, 20 µl/ml propidium iodide and 5 µl/ml Hoechst 33342 dyes prepared in a medium according to the manufacturer's protocols;

31. Incubate samples for 40 minutes at 37 °C;

32. Remove the dye and rinse the gel samples thoroughly and gently three times with 3 ml phosphate buffer solution;

33. Add the 2,5 ml medium;

34. Conduct layer-by-layer scanning (Z-stack protocol) of stained samples using a laser scanning microscope;

35. To count live and dead cells, use the 3D reconstructions obtained in Imaris. In the software, select the "Spots" tool, and the "Add new Spots" option. In the algorithm section, use the default settings. Then select the source channel and specify the estimated object size based on the size of your cells. You can measure the size of objects in your image using the "Slice" tool. Then select the filter type and set the signal intensity threshold. The number of recognized objects can be found in the "Statistics" tab;

Spheroids disaggregation and cell viability analysis by flow cytometry

36. Collect the medium;

37. Wash the gel samples thoroughly but gently three times with 3 ml phosphate-buffered saline solution;

38. Add 300 µL 50 mM Na_2EDTA, pH 7,7 to the hydrogel samples;

39. Incubate at room temperature for 5 minutes, mixing by pipetting;

40. Collect the sample in a 15 ml centrifuge tube and centrifuge them at 200 g for 5 minutes and discard supernatant;

41. Wash samples three times with 3 ml phosphate buffer by centrifuging at 200 g for 5 minutes and removing the supernatant after each centrifugation.;

42. Add a 600 µL mixture of 0.25 % trypsin and 100 U/ml collagenase I to the sediment;

43. Incubate the mixture for 25–30 minutes at 37°C and pipette every 7–10 minutes;

44. Inactivate the enzymes by adding 300 µL of 50 mM Na_2EDTA solution;

45. Centrifuge at 200 g for 5 minutes, discard supernatant after centrifugation;

46. Stain the cells with 2,5 ml a mixture of 10 µg/ml FDA and 20 µg/ml PI dyes prepared on a medium for 40 minutes at a temperature of 37 °C;

47. During incubation, prepare a suspension of unstained cells as a negative control (the protocol of cell suspension preparation was described in 5–7 steps);

48. Centrifuge at 200 g for 5 minutes, remove supernatant after centrifugation;

49. Add 1–2 ml phosphate buffered saline;

50. Use 40-µm cell sieve to remove large cell aggregates and collagen, this cell suspension is ready for flow cytometry analysis.