Video Article

Three-dimensional Alginate-bead Culture of Human Pituitary Adenoma Cells

Dulce Avila-Rodríguez¹, Karina Paisano-Cerón¹, Irene Valdovinos- Ramírez¹, Carmen Solano-Agama¹, Alma Ortiz-Plata², María E. Mendoza-Garrido¹

¹Department of Physiology, Biophysics and Neurosciences, Center for Research and Advanced Studies (CINVESTAV-IPN)

²Department of Experimental Pathology, Nacional Institute of Neurology and Neurosurgery

Correspondence to: María E. Mendoza-Garrido at mmendoza@fisio.cinvestav.mx

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Abstract

A three-dimensional culture method is described in which primary pituitary adenoma cells are grown in alginate beads. Alginate is a polymer derived from brown sea algae. Briefly, the tumor tissue is cut into small pieces and submitted to an enzymatic digestion with collagenase and trypsin. Next, a cell suspension is obtained. The tumor cell suspension is mixed with 1.2% sodium alginate and dropped into a CaCl₂ solution, and the alginate/cell suspension is gelled on contact with the CaCl₂ to form spherical beads. The cells embedded in the alginate beads are supplied with nutrients provided by the culture media enriched with 20% FBS. Three-dimensional culture in alginate beads maintains the viability of adenoma cells for long periods of time, up to four months. Moreover, the cells can be liberated from the alginate by washing the beads with sodium citrate and seeded on glass coverslips for further immunocytochemical analyses. The use of a cell culture model allows for the fixation and visualization of the actin cytoskeleton with minimal disorganization. In summary, alginate beads provide a reliable culture system for the maintenance of pituitary adenoma cells.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53637/>

Introduction

Three-dimensional scaffolds have been extensively used in cell culturing because they provide a three-dimensional structural support for cultured cells and the maintenance of cell-to-cell communication¹. Naturally derived polymer materials have been used to make three-dimensional scaffolds including type I collagen, chitosan and alginate. Alginate is a natural polymer derived from the brown sea algae *Macrocystispyrifera* (Kelp). This polymer is composed of repeated units of β-D-mannuronic acid (M) and α-L-guluronic acid (G)² and forms stable gels in the presence of certain divalent cations, such as calcium and barium. Calcium chloride (CaCl₂) solutions are commonly used as the crosslinking reagent to form alginate beads. Alginate solutions dropped into CaCl₂ immediately form three-dimensional spherical gels. When the alginate solution is mixed with cells, the cells are encapsulated into the alginate beads³.

Alginate has properties that have enabled it to be used as a matrix for the encapsulation of a variety of cells, including chondrocytes⁴, skeletal myoblasts⁵ and neural stem cells⁶. It is an inert material and permits the diffusion of nutrients, oxygen and metabolic products that maintain cell survival and function; moreover, unlike other gel-based culture systems, cells cultured within alginate beads can also be liberated from the scaffold using calcium chelators, such as sodium citrate, and the cells can then be harvested for further investigations⁷.

Pituitary adenomas are typically benign tumors with low proliferation rates. Rat pituitary adenoma cell lines have been successfully cultured in a two-dimensional system⁸. However, this culture of secretory human pituitary cell tumors is not an efficient development; dispersed tumor pituitary cells grow poorly in culture, the cells exhibit limited attachment and spreading, and the cells typically form floating aggregates in the culture dish^{9,10}.

Several attempts have been made to obtain a tumor pituitary cell suspension, including an enzymatic and mechanical dispersion approach¹¹, a solely mechanical dispersion approach¹² and the culturing of tumor explants¹⁰. With these approaches, different authors have obtained viable adherent cultures for different periods of time. The capacities of the tumor secretory cells to survive in these two-dimensional systems depend on the tumor type and their proliferation rates⁹. However, in long-term cultures, cells with fibroblast phenotypes predominate^{11,13}. This paper describes a method for obtaining a primary culture from pituitary adenoma cells encapsulated in alginate beads that further liberates them from the alginate scaffold that enables detailed analyses of aspects of their cell biology, e.g., their cytoskeletal arrangements.

Protocol

This study was approved for the use of human material by the local ethical committees of the Medical Institution and the Center of Research and Advance Studies of the National Polytechnic Institute.

1. Tumor Sample Acquisition

1. Obtain the pituitary adenoma tissue biopsy from a patient through a trans-sphenoidal surgical procedure¹⁴.
2. Collect the pituitary adenoma sample and place it into a sterile glass bottle containing 50 ml of fresh 199 culture medium (M199) enriched with 10% fetal bovine serum (FBS), 0.6 mg/ml sodium bicarbonate, 2.4 mg/ml HEPES and 1% antibiotics (penicillin/streptomycin) at pH 7.4. Maintain the sample in a glass bottle on ice.
Note: The tumor tissue can be processed several hr after the surgery.
3. Transport the tumor sample to the laboratory in the glass bottle on ice.

2. Preparation of the Enzymatic Tissue Dissociation Solutions, the Trypsin Inhibitor Solution and the Alginate Solutions

Note: Prior to the cell culture procedure prepare the following solutions. Filter all the solutions using a 0.22 μ m membrane filter, before use.

1. Prepare collagenase 1% solution by dissolving 5 mg of collagenase (type I) in 5 ml of M199-serum free.
2. Prepare trypsin 0.01% solution by dissolving 0.01 g of trypsin in 10 ml of PBS-EDTA 0.3%.
3. Prepare 0.1% trypsin inhibitor solution by dissolving 2 mg of soy trypsin inhibitor in 20 ml of M199-serum free.
4. Prepare DNase solution by dissolving 20 μ l of DNase I 134 U/ml in 4 ml of the trypsin inhibitor solution.
5. Prepare EGTA solution (1.2 M) by dissolving 22.8 mg of EGTA in 40 ml of 155 mM NaCl. Adjust the pH to 7.4. Continue to a final volume of 50 ml.
6. Prepare HEPES solution (20 mM) by dissolving 238 mg HEPES in 50 ml of 155 mM NaCl.
7. Prepare 1.2% alginate solution by dissolving 1.2 g sodium alginate in 100 ml of the HEPES solution. Autoclave the alginate solution.
8. Prepare calcium solution (102 mM) by dissolving 750 mg of CaCl_2 in 50 ml HEPES solution, adjust pH to 7.4

3. Primary Cell Culture and Alginate Encapsulation

1. Inside a laminar flow cabinet, place the tumor tissue in a 35 mm Petri dish containing approximately 20 ml of PBS Ca^{2+} and Mg^{2+} free, pH 7.4 (PBS*).
2. Take the tissue with a surgical tweezer and place it in another petri dish containing fresh PBS*, gently wash the tissue. Repeat this step until the red blood cells and debris are removed.
3. Using a surgical tweezer, hold the pituitary adenoma tissue, and with a small surgical scissor, cut it into small pieces. With a Pasteur pipette with rounded edges, transfer the tissue fragments and the PBS* into a 15 ml tube, Centrifuge at 68 x g for 10 min at RT.
Note: To round the edges of a Pasteur pipette, slightly flame the tip of the pipette in a Bunsen burner.
4. Remove the PBS* using a Pasteur pipette and add 3-5 ml of the collagenase solution (approximately 5 ml for 65 mm³ of tissue), mix two to three times by inverting. Incubate the tissue in the collagenase for 30 min at 37 °C in constant rotation.
5. Centrifuge the tube at 68 x g for 10 min at RT and remove the collagenase with a Pasteur pipette.
6. Add 5 ml of DNase and mix two to three times by inverting, then centrifuge the tube at 68 x g for 10 min at RT.
Note: If the tissue is not completely dissociated, perform a second enzymatic digestion with trypsin for 3 min at 37 °C. Stop the digestion by the addition of 2 volumes of trypsin inhibitor solution.
7. Aspirate the supernatant with a Pasteur pipette and discard. Re-suspend the pellet in 1 volume of EGTA solution and mix with 2 volumes of alginate solution.
8. Gently mix the cell suspension and alginate by pipetting with a Pasteur pipette. Take a sterile syringe with a 21 G needle, remove the plunger; use a pipette to load the syringe with the alginate and cell solution.
9. Gently insert the plunger into the syringe and carefully dispense the alginate-cell solution drop-by-drop into a glass beaker containing approximately 30 ml of the calcium-rich solution.
10. Place the needle of the syringe approximately 5 cm up to the CaCl_2 solution. The alginate-cell suspension gels on contact with the CaCl_2 to form spherical beads. Keep the alginate beads in the calcium solution for 5 min.
Note: Stir the glass beaker in which the beads are dropped. Move it slowly with the hand in circular motions, to prevent them from sticking to one another.
11. Carefully remove the calcium solution with a Pasteur pipette, and wash the beads twice with 3-5 ml of M199 enriched with 20 % of FBS.
12. Transfer the alginate beads into a regular T25 tissue culture flask using a sterile spatula, add 4-5 ml of M199 enriched with 20% FBS and incubated at 37 °C in a humidified incubator with 5% CO_2 . Change the culture medium every third day.

4. Liberate Cells from the Alginate

Note: Prior to liberating the cells from the alginate beads, coat 13 mm diameter coverslips with poly-D-lysine.

CAUTION: SULPHURIC ACID IS A HIGHLY CORROSIVE ACID, AND APTS CAN CAUSE EYE AND SKIN IRRITATION. USE LATEX GLOVES TO HANDLE THESE REAGENTS.

1. In a fume hood, with a point tweezer immerse the coverslips in a glass petri dish containing 20% aqueous sulphuric acid for 1 hr at RT.
2. Decant the sulphuric acid, and wash the coverslips five times with distilled water for 5 min each. Using a point tweezer place the clean coverslips in a petri dish containing 0.1 M NaOH for 5 min at RT¹⁵.
3. Decant the NaOH and wash one time with distilled water, aspirate the water and dry the coverslips.
4. Use a micropipette to coat the coverslips with APTS (aminopropyl-triethoxysilane) for 4 min (use 500 µl for 6 coverslips) flood the Petri dish with distilled water. Quickly aspirate the water and continue to wash with distilled water, moving the coverslips with point tweezers to allow the water to penetrate beneath.
5. Aspirate the water and dry the coverslips, using a point tweezer place the coverslips into a clean Petri dish and sterilize them inside the microwave during 3 times, 45 sec each. Add 1,000 µl of sterile water to 100 µg of poly-D-lysine (0.1 mg/ml).
6. Incubate the coverslips with approximately 50 µl of poly-D-lysine for 5 min at RT. Aspirate the poly-D-lysine completely.
Note: This is important because soluble poly-D-Lysine in the culture medium can inhibit cell proliferation.
7. Rinse the coverslips five times with sterile water for 5 min each. Aspirate the sterile water and allow coverslips to dry inside a laminar flow cabinet.
8. To analyze the actin cytoskeleton, liberate the pituitary cells from the alginate. Take some of the alginate beads from the culture flask using a 1 ml pipette tip and place them into a 15 ml tube.
9. Add 5 ml of 55 mM sodium citrate (dissolve 323.6 mg of Na₃C₆H₅O₇, 2 H₂O in 20 ml HEPES solution) and centrifuge at 68 x g for 10 min at RT. Aspirate the supernatant and discard.
10. Re-suspend the pellet in 1 ml of warm M199 enriched with 20% FSB; gently mix the cell suspension with a Pasteur pipette.
CAUTION: PHALLOIDIN AND DAPI ARE TOXIC. USE GLOVES TO HANDLE THESE REAGENTS.
11. To count the cells, transfer 125 µl of 0.4% Trypan blue solution to a 1.5 ml tube. Add 75 µl of M199 and 50 µl of the cell suspension obtained in the last step (dilution factor = 5). Mix thoroughly.
12. Use a micropipette tip to place approximately 10 µl of the Trypan blue-cell suspension in the hemocytometer chamber.
13. Count the cells in the hemocytometer counter using an inverted microscope with phase-contrast illumination. Count all the cells in the 1 mm center square and four 1 mm corner squares. Obtain the cell density per milliliter by multiplying the average count per square by the dilution factor - 10⁴. For example if the average counts per square is 45 cells x 5 x 10⁴ = 2.25 x 10⁶ cells/ml.
Note: Count the cells in ten squares of the hemocytometer chamber. Do not count cells touching the middle line at bottom and right sides.
14. Seed 35,000 cells on 13 mm diameter glass coverslips covered with poly-D-Lysine.

5. Actin Cytoskeleton Arrangement

1. After 48 hr in culture, remove the culture medium with a Pasteur pipette and quickly add 500 µl per coverslip of PBS, immediately remove the PBS.
2. Fix and permeabilize the cells with 500 µl per coverslip of fixation buffer (0.1 M PIPES, pH 6.75, 4% PEG-6000, 1 mM EGTA, 1 mM MgSO₄, 0.5 % Triton X-100 and 2% formaldehyde) for 10 min at 37 °C
3. Remove the fixation buffer and add 500 µl per coverslip of 3.5% paraformaldehyde in PBS for 30 min at RT. Remove the paraformaldehyde solution.
4. Wash the coverslips three times by adding 500 µl per coverslip of PBS for 5 min each. Rinse the coverslips at RT, in constant agitation.
5. Incubate the cells with 50 mM ammonium chloride for 10 min, aspirate the ammonium chloride solution and repeat step 5.4.
6. Incubate the cells with 1% BSA (IgG-free, proteases-free) in PBS for 30 min, remove the BSA solution and repeat step 5.4.
7. Incubate the cells with 50 µM of rhodamine-conjugated phalloidin for 7 min at RT, repeat step 5.4.
8. Incubate the cells with 255 µM of DAPI diluted in PBS for 5 min at RT, repeat step 5.4. Finally mount the coverslips with a mounting medium and seal them with nail polish.
9. Acquire images of the actin cytoskeletal arrangements using a confocal laser scanning microscope with a 63X objective, at 541 nm excitation wavelength.

Representative Results

This protocol has been applied successfully in the culture and maintenance of adenoma pituitary cells in alginate beads for different periods of time. **Figure 1** shows embedded cells in alginate beads after three months; these cells exhibit bi-refractive rounded shapes under an inverted light microscope (**Figure 1**). **Figure 2** shows the localization of N-cadherin in rat pituitary adenoma cells embedded in alginate. The N-cadherin is localized at cell-cell contacts (**Figure 2A** and **2C**) and the nucleus showed the chromatin extended (**Figure 2B** and **2C**).

Tumor cells in alginate beads remain viable for up to 4 months in culture; therefore, the cells can be liberated from the alginate beads at different times, which allows for the analyses of different aspects of the cell biology in the same cell culture. For example, the proliferation index was obtained from ten non-functioning human pituitary adenomas using the immune-reactivity for Ki-67, and a mean labeling index of 19.2 ± 1.5% (mean ± S.E.M) was obtained. **Figure 3** shows cultured human pituitary adenoma cells immunostained for Ki-67.

The actin cytoskeletons of cells of a non-invasive were stained according to the immunocytochemical protocol describe above. Culture cells exhibited elongated shapes with small actin stress fibers (**Figure 4A**).The morphologies and actin filament arrangements varied with the pituitary adenoma independent of the culture system; for example, in a non-functioning invasive adenoma, the predominant shape among these cells was rounded with an arrangement of their actin filaments in discontinuous cortical rings. (**Figure 4B**).

Figure 1. Cells embedded in alginate beads for 3 months. Cells exhibit bi-refractive rounded shapes under an inverted light microscope. In the right panel invasive macroadenoma cells embedded in alginate are shown, and in the left panel non-invasive macroadenoma cells are shown. Scale bar = 15 μ m [Please click here to view a larger version of this figure.](#)

Figure 2. Immuno-cytochemical analysis of N-cadherin in Rat pituitary adenoma cells (GH3) culture in alginate beads. GH3 cells were cultured in alginate, fixed embedded in the alginate system stained for N-cadherin (red) and the nucleus (blue). Cells are arranged in a cumulus showing N-cadherin at the cell-cell borders (A and C). The nucleus (B and C) are shown with the chromatin extended. Scale bar = 15 μ m [Please click here to view a larger version of this figure.](#)

Figure 3. Immunostaining of nuclear antigen ki-67 cultured human pituitary adenoma cells. After 2.5 months in alginate bead culture, invasive non-functioning adenoma cells were fixed and then immunostained for Ki-67 (Red) and the nucleus (blue). Some adenoma cells show positive immunostaining for Ki-67. Scale bar = 15 μ m [Please click here to view a larger version of this figure.](#)

Figure 4. Actin cytoskeleton arrangements of pituitary adenoma cells liberated from alginate beads. Cells were fixed and stained for actin filaments with TRITC-phalloidin. Cells from a non-invasive macroadenoma were extended over the substrate, showing small actin stress fibers (A). Cells from an invasive macroadenoma showed a rounded shape with discontinuous actin rings (B). Scale bar = 15 μ m [Please click here to view a larger version of this figure.](#)

Discussion

This protocol has several critical steps. The first is bead size homogeneity, which is required to maintain the same conditions of diffusion of the nutrients and gases in all of the cell culture. In our experience, the use of a 21 G needle to make the alginate beads allows for the acquisition of an efficient culture of uniform bead size. The second important factor is the distance from the needle; this distance must be no more than 5 cm from the calcium solution to avoid deformed pearls and dead cells due to the collision of the stream of the alginate/cell solution with the surface

of the calcium solution. A third critical step is the glass stirring in which the beads are dropped to prevent them from sticking to one another. Sticking results in uneven cell encapsulation.

Some modifications to this protocol can be performed depending of the research question, for example, in our experience the solution of sodium alginate can be mixed with a basement membrane protein like type IV collagen, and following the protocol previously described, obtain a three-dimensional alginate scaffold. Another possibility is to make floating alginate cushions in 15 mm diameter wells; inside these gels, cells can be seeded above or inside them. Troubleshooting when working with the alginate could be necessary if any particular experiment needs modifications in their Ca^{2+} or Mg^{2+} concentrations. This is because the hydrogel stability can be altered. Ba^{2+} - and Cu^{2+} -crosslinked alginate gels are relatively stable in aqueous solutions, unfortunately, these cations are often cytotoxic³.

When working with tumor tissues such as pituitary adenoma tissues, one limitation of this technique is the small tissue sample provided. Therefore, the number of alginate beads obtained depends of the size of the tissue sample.

Previously, a system to maintain pituitary adenoma cells in a three-dimensional culture has been described. The authors submitted the cells to gyratory shaking to form aggregates and maintained the cells in this condition for different periods¹⁶. The use of this cell suspension culture requires gyratory equipment inside the culture chamber. One advantage of the protocol described here is that it allows for the culturing of cells in a three-dimensional system without the need for extra laboratory equipment. The cells embedded in the three-dimensional alginate system are maintained in a regular incubator inside a regular flask. Another advantage is that the alginate scaffold, in contrast with other three-dimensional systems, permits the cells to be liberated from the hydrogel for further investigation⁷. Furthermore, an important aspect of culturing cells in alginate beads is that the cells embedded in this system are able to synthesize *de novo* extracellular matrix. An alternative method for the study of cells embedded in alginate is the fixation of the alginate beads followed by their dehydration with increasing concentrations of polyethylene glycol for subsequent sectioning and Immuno-histochemical analyses¹⁷.

We are interested in the future to apply the three-dimensional alginate beads system to embedded primary culture normal pituitary rat cells in order to analyze their interactions in a three-dimensional environment, and also to culture other type of pituitary adenomas.

Disclosures

The authors have nothing to disclose.

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