Chapter 20

Preparation and Culture of Adrenal Chromaffin Cells

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Abstract

Cultured chromaffin cells have been used for almost 40 years in the study of different cell functions using biochemical, electrophysiological, pharmacological, and toxicological approaches. Chromaffin cells are essentially secretory cells that are used to model sympathetic neurons or neuroendocrine cells. In this chapter, we describe the most common methods currently used to isolate and culture chromaffin cells from the animals used most commonly: cows, rats, and mice. We also provide some advice on the use of these cells in the laboratory.

Key words: Adrenal, Chromaffin cells, Culture, Collagenase, Papain

1. Introduction

Cell culture techniques are now routinely applied to modern laboratory research, chromaffin cells being no exception. Since the first seminal description of their isolation from mammalian glands (1, 2), the procedure has continually evolved. The improvements in tissue culture procedures and advances in the materials used, notably the highly purified enzymes currently available, has standardized isolation protocols and improved their reproducibility. In this chapter, we provide the "recipes" for culturing cow, rat, and mouse chromaffin cells, describing the standard protocols that we use.

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2. Materials

2.1. General Supplies, Solutions, and Culture Medium

All procedures require standard culture room facilities with a laminar flow cabin, a Bunsen burner, a water bath set at 37°C (preferably with agitation), and a 37°C incubator with a water-saturated atmosphere containing 5% CO_2. An inverted microscope with phase contrast and a low vibration clinical centrifuge are also required. To plate chromaffin cells onto glass coverslips, we strongly recommend high-quality glass Marienfeld (Lauda-Königshofen, Germany). Other required materials and basic solutions include:

1. Sterile plastic Pasteur pipettes.
2. Syringes and 0.22-μm syringe filters.
3. Adjustable volume pipettes with sterile tips.
4. 15-mL conical centrifuge tubes (or 50-mL tubes for culturing cow cells).
5. Hemocytometer (Neubauer).
6. 24-well culture plates.
7. Ca^{2+}- and Mg^{2+}-free Locke's balanced salt solution: 154 mM NaCl, 5 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, and 11 mM glucose.
8. Trypan blue: 4 mg/mL in Locke's solution.
9. Culture medium: We always use standard Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F12 and antibiotics to culture chromaffin cells. The medium is completed by adding 10% fetal calf serum (Lonza, Basel, Switzerland, DE14-801 F, see Note 1).
10. All the solutions used are sterilized by filtering through 0.22-μm syringe filters and are supplemented to contain penicillin G (100 IU/mL) and gentamicin sulfate (40 mg/L).

2.2. Materials for Preparation of Bovine Chromaffin Cells

1. Sterile surgical material required: forceps, scissors, two scalpel blades, and one haemostatic clamp.
2. 2× 100 mm Ø Petri dishes.
3. Glass beakers: 2×≈30 mL, 1×≈100 mL, and 1×≈250 mL (for waste).
4. Two ≈35-mm Ø glass funnels.
5. Standard cotton gauzes, ≈200-μm and ≈90-μm pore-size nylon meshes.
6. 5-mL syringes and 0.22-μm sterilizing syringe filters.
7. For gradient purification, a refrigerated centrifuge capable of reaching ≈8,000×*g* is also required, along with two sterile capped 30-mL transparent tubes suitable for centrifugation.

8. Collagenase IA.
9. Bovine serum albumin fraction V (BSA).
10. Deoxyribonuclease I (DNAase I).
11. Renografin® (ER Squibb & Sons, New Brunswick, NJ, USA) or Urografin® (Schering España, Madrid, Spain) prepared at 15% in sterile pure water (this solution is isotonic).

2.3. Materials for Preparation of Adult Rat Chromaffin Cells

1. Rats over 4 weeks of age.
2. Stereo microscope (×20 magnification).
3. Petri dishes (35 mm Ø).
4. Collagenase IA.
5. BSA.
6. DNAase I.
7. Hyaluronidase I-S.

2.4. Materials for Preparation of Young Rat or Mouse Chromaffin Cells

1. Animals of less than 4 weeks of age.
2. Stereo microscope (×20 magnification).
3. Petri dishes (35 mm Ø).
4. Papain (Worthington Lakewood, NJ).

3. Methods

General Guidelines. Cells are very sensitive to low pressure and as such, strong pipette suction and strong centrifugation should be avoided. Similarly, do not use the brake function on the centrifuge. Standard guidelines for working in culture rooms should be followed, such as wearing gloves and keeping a well-ordered hood to maintain laminar flow conditions. Unnecessary material in the working space will increase air turbulence inside the hood. As we primarily use chromaffin cells for secretion experiments, we have tailored the procedure to produce healthy secretory cells. However, it is possible that the reader may find alternative protocols designed for other purposes, although the maintenance of a healthy secretory machinery usually ensures that the cell is healthy and suitable for any experimental purposes.

3.1. Chromaffin Cell Preparation for Single-Cell Experiments

Any of the methods described below for chromaffin cell isolation and culture are appropriate for amperometry, patch clamping, or fluorescent microscopy. The following is a rule of thumb for amperometric studies using chromaffin cells of any species:

1. The isolation procedure should aim primarily to obtain healthy cells rather than a high yield. We recommend reducing the

concentration of the enzymes used for digestion in the papers cited below by 15% and minimizing the mechanical disruption during tissue digestion. For bovine chromaffin cells, we use a modified version of the original protocol using a bolus of collagenase injected through the adrenal vein, rather than perfusion (3, 4). For rat cells, we adhere to the conditions described by Gilabert (5), and for mouse cells, we use a modified version of the procedure described by Sorensen et al. (6).

2. Cells should be plated at low density (≈20,000–50,000 per cm^2).
3. Replace culture medium every 48 h using serum-free medium.
4. Although cells can be maintained for over a week after plating, optimal secretion usually occurs within 24 h of plating. However, cells may be used within a few hours.

3.2. Coverslips and Adherent Support Sterilization

Glass coverslips can be sterilized by briefly flaming both sides using a Bunsen burner before placing them in culture plates. If it is necessary to coat them with a substrate (see Note 2), place a drop of 0.01% poly-D-lysine solution (Sigma, Catalog P-1024, prepared in water) on each coverslip. Leave it for 20 min and then wash three times with sterile pure water. Sterilize the coverslip by exposing it to UV light in the fume hood for 30 min and use the treated glass within 1 week of preparation.

3.3. Cell Viability and Counting

Although many automatic systems for cell counting now exist, a standard Neubauer hemocytometer is still a valuable and cheap tool to count cells and to get a general idea of their viability. We prepare a 1:9 dilution of cells by mixing 20 μL of cell suspension + 100 μL of Locke's solution + 80 μL of trypan blue staining solution (see above). Guidelines for the correct use of hemocytometers have been published elsewhere (7).

3.4. Procedure for Bovine Chromaffin Cells

Glands may be obtained from the local abattoir. Choose only intact glands to avoid contamination and discard those with visible signs of internal blood coagulation. Do not remove the fat at the abattoir, as it provides protection against contamination. The glands do not require any special care for their transportation to the laboratory if the time from sacrifice to culturing is less than 1 h. In the case of longer intervals, it is advisable to inject 3–4 mL of Locke's solution into the glands and transport them in a plastic bag on ice. Other protocols for bovine cell culture preparation have been published elsewhere (4, 8–14).

Once in the culture room, wearing gloves spray the glands with 60% ethanol under the laminar flow cabinet, making sure that the solution does not enter the adrenal vein, and remove the surrounding fat from the glands. The general procedure to minimize contamination is to proceed progressively from a nonsterile

(transport containers, external fat) to a sterile environment. As such, it is advisable to flame the surgical instruments periodically. Once the glands are clean of surrounding fat and connective tissue, spray again with ethanol and remove all the material used and discard the tissue from the hood. Clean the surface of the hood with ethanol. The procedure described below is intended for two glands but can be easily adapted for more glands. In addition, Fig. 1 can be printed and posted in the culture room as a brief guide to the procedure:

1. Prepare the enzymatic solution. Each gland will require 20 mL of a solution containing 1.5–2 mg/mL of collagenase IA and twice this amount (3–4 mg/mL) of BSA dissolved in Locke's solution. These calculations are aimed for a commercial a collagenase with an activity of 300–400 IU/mg, and should be adjusted when the enzyme has a different activity (see Note 3). The addition of 30 μg/mL of DNAase I increases the yield of the isolation procedure as it prevents cell aggregation that results from the presence of free DNA. However, given the usually high yield of bovine cultures, this step is not critical. Sterilize the solution by filtering through a 0.22-μm syringe filter into a 50-mL conical tube and place it capped in the water bath at 37°C.
2. Place the glands in a sterile Petri dish and inject each gland twice with 3–4 mL of warm (37°C) Locke's solution. Place the glands in a 100-mL glass beaker, keeping the vein orifice facing up. Cover the beaker with a dry sterile Petri dish and incubate for 5–10 min in the water bath.
3. Inject the glands with 3–4 mL of enzyme solution and return the glands to the beaker. Try to avoid accumulation of liquid in the bottom of the beaker which could contaminate the gland (see Fig. 1a).
4. After 20 min, repeat step 3. Exercise care when performing the injections as the gland has been partially digested and may rupture with excessive pressure.
5. After 20 min, the glands should be noticeably softer, indicating the success of the digestion. If this is not the case, repeat the injection and check again after 10 min. It is important to keep the collagenase incubation time to a minimum to avoid overdigestion of the chromaffin tissue.
6. Using a scalpel and clean scissors, open the gland longitudinally (along its length). It should appear as shown in Fig. 1b, with the cream-colored section representing the medulla. Make several cuts along the medulla with small scissors, and use forceps to collect the material and transfer it to a clean Petri dish. Do not collect any purple material that contains the cortical cells. This procedure should be performed swiftly.
7. Using two scalpel blades, mince the tissue into small pieces and transfer the material to a 50-mL conical tube using a plastic

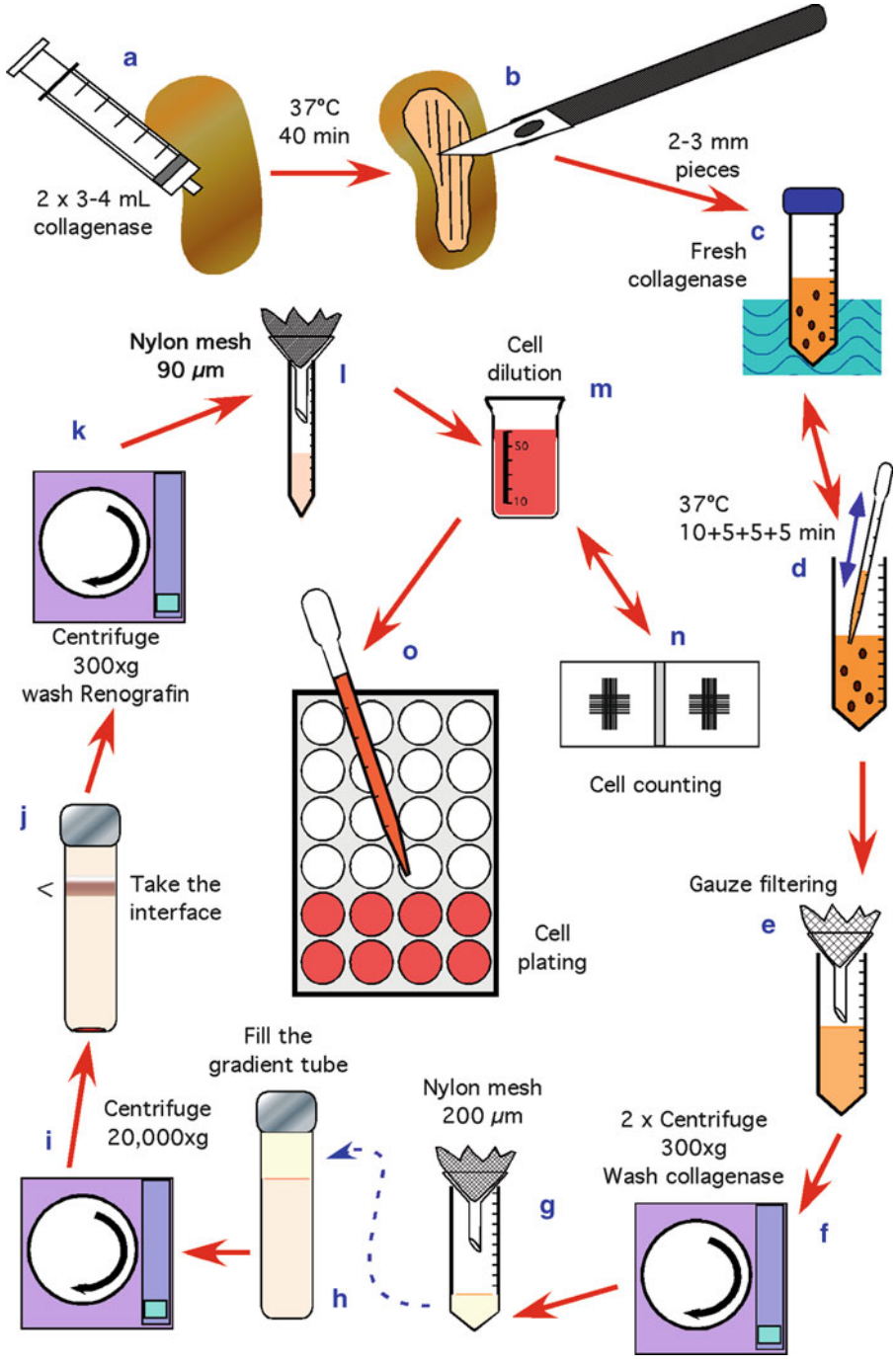

Fig. 1. Procedure to isolate and culture bovine chromaffin cells (see text for guidelines).

Pasteur pipette. Add the rest of the fresh collagenase solution and incubate this tissue at 37°C (see Fig. 1c). Every 5–10 min, carefully dissociate the material by triturating with 5–6 strokes of a Pasteur pipette, always avoiding the production of bubbles (see Fig. 1d). The total incubation time should not exceed 20–25 min.

8. Place a piece of sterile cotton gauze onto a funnel and filter the dissociated material to remove the bulk debris, collecting the cells in two 50-mL conical tubes. Distribute the filtrate between both tubes and wash the gauze with Locke's solution to maximize the yield and to dilute the collagenase (see Fig. 1e).
9. Centrifuge at ≈300 × *g* for 5 min, discard the supernatant, and resuspend the pellet by gently tapping it against the hood bench. Fill the tubes with fresh Locke's solution and repeat the centrifugation.
10. Discard the supernatant, resuspend the cells, and pass them through a 200-μm nylon mesh. Clean the mesh by rinsing with fresh Locke's solution to produce a final volume of 10 mL (see Fig. 1g).
11. The cells obtained are contaminated with debris, ruptured cells, and red and cortical cells. For many purposes, they are fit for culturing and may be diluted into culture medium at this stage. However, we prefer to purify the chromaffin cells, as outlined below (see steps 12–18).
12. Place 20 mL of 15% Renografin solution in two 30-mL centrifuge tubes of (see Note 4).
13. Mix 10 mL of cell suspension with 10 mL of Renografin to produce a final Renografin concentration of 7.5%.
14. Very slowly and carefully, add the cell suspension onto the surface of the 15% Renografin solution using a plastic Pasteur pipette. This is the most critical step! If the interface between the two Renografin concentrations is broken, the discontinuous gradient is compromised, and most of the chromaffin cells will enter the pellet (see Fig. 1h).
15. After equilibrating the tubes by weight, centrifuge at 7,500 × *g* at ≈18°C for 20 min (see Fig. 1i).
16. The chromaffin cells will appear in the interface of the gradient and can be easily recovered with a plastic Pasteur pipette (see Fig. 1j).
17. To remove the Renografin, resuspend the cells in Locke's solution in a 50-mL conical tube and centrifuge at ≈300 × *g* for 5 min (see Fig. 1k).
18. Resuspend the pellet by tapping the tube against the work surface of the hood and add 10 mL of culture medium (see Note 1). As the medium contains calcium, it is important to

avoid unnecessary manipulation of the cell suspension to prevent aggregation.

19. Pass the cell suspension through a 90-μm nylon mesh (see Fig. 1l).
20. Count and plate the cells accordingly (see Fig. 1m, n). Although the described yield in the past was as large as $80–100 \times 10^6$ cells per gland, nowadays it is preferable to reduce this number to obtain healthier cells. Thus, $10–20 \times 10^6$ cells per gland is a suitable yield. As a rule of thumb, we recommend the following densities:
    - For biochemical and secretory studies in 24-well plates: 500,000 cells/well in 1–2 mL of complete medium.
    - For single-cell experiments on 12 mm Ø glass coverslips: 50,000 cells/well in 1 mL of complete medium. Bovine chromaffin cells do not usually require an adherent substrate (collagen, polylysine, etc.).
    - For collecting cells by scraping from 35 mm Ø Petri dishes: $3–4 \times 10^6$ cells/well in 3 mL of complete medium.
21. Change the medium using serum-free medium every 2–3 days, depending on the cell density (see Note 1).

3.5. Procedure for Adult Rat Chromaffin Cells

Animals older than ≈4 weeks produce a lower yield of cells than young rats. This is also observed with mice:

1. Prepare the dissociation solution (3–4 mL) and maintain it at room temperature: collagenase type I (250–350 IU/mL), 3 mg/mL BSA, 0.15 mg/mL DNAase I, and 0.15 mg/mL hyaluronidase I-S in Locke's buffer.
2. Sacrifice the animals according to institutionally approved ethical procedures. Place the animal on its back and spray the abdomen with 70% ethanol.
3. To access the abdominal cavity, perform an incision through the abdominal wall, cutting the skin along the linea alba and pulling it out to either side, using scissors to separate the skin from underlying tissue. The abdominal cavity should tear open, exposing the organs. Locate the two kidneys with the adrenal glands on top, which should be readily visible (see Fig. 2a).
4. Remove the glands with fine-curved forceps and place them in ice-cold Locke's buffer.
5. Under a stereo dissection microscope coupled to a cold-light source, remove the adipose tissue surrounding the gland with a stainless steel scalpel blade and decapsulate the adrenal glands. Next, remove the adrenal cortex to isolate the medullar tissue and cut this into four pieces using a scalpel. Keep the tissue wet throughout with cold Locke's buffer.

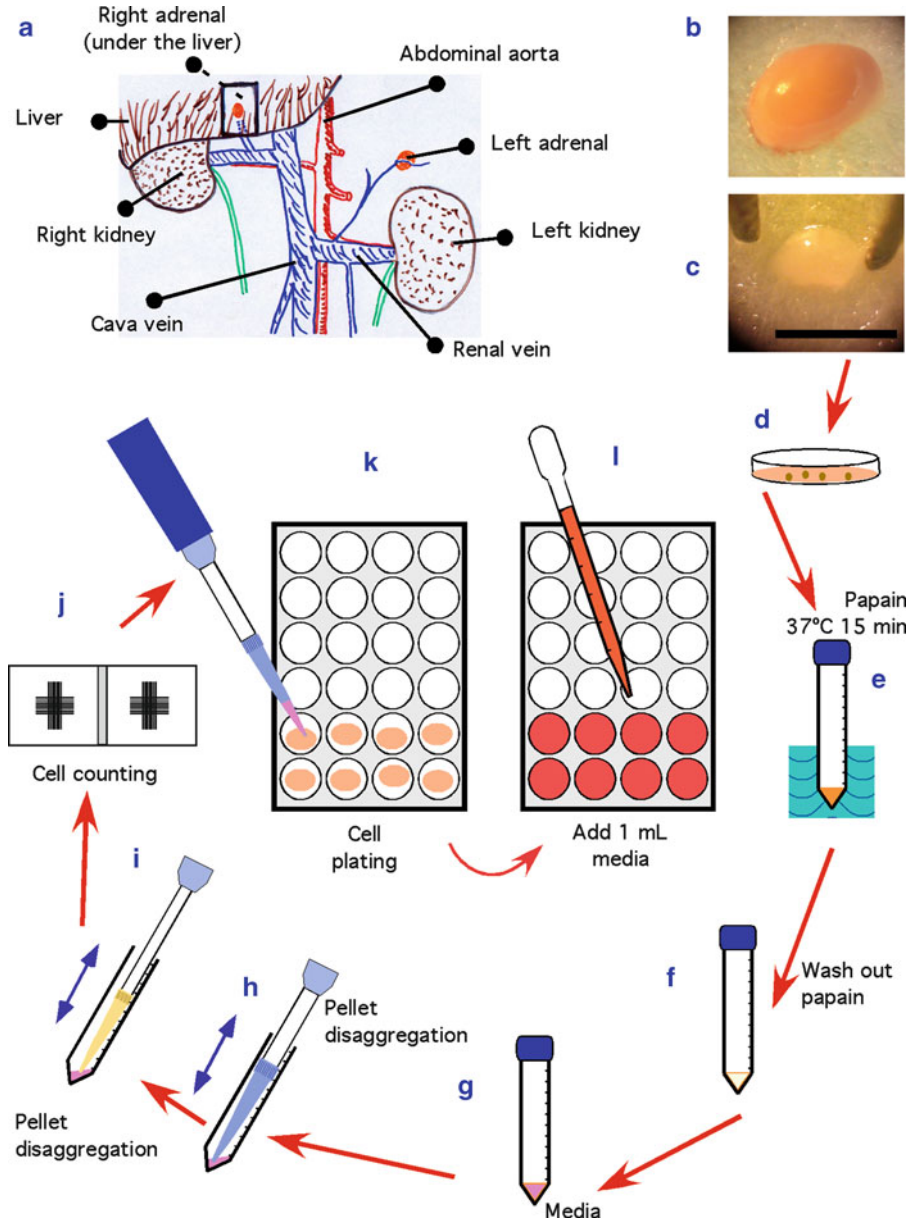

Fig. 2. Procedure to isolate and culture mouse chromaffin cells (see text for guidelines).

6. Recover the adrenal tissue using a sterile plastic pipette and transfer it to a 15-mL conical tube. Add 3 mL of the dissociation solution and incubate for 25–30 min at 37°C. The solution should be gently triturated every 10 min through a 1 mL pipette tip. During the last 5 min, the solution should be continuously triturated using a 100-µL pipette, until it becomes turbid.
7. Add cold Locke's buffer to a final volume of 10 mL to stop the enzyme reaction.

8. Place the tube containing the digested tissue in the rotor of a precooled (4°C) refrigerated centrifuge (where possible) and spin at 100 × *g* for 10 min.
9. Discard the supernatant by decanting and resuspend the pellet with 0.8 mL of prewarmed medium.
10. Add one or two drops on each poly-D-lysine-coated coverslip and place them in the incubator for 1 h to allow the cells to settle.
11. Carefully refill the culture dish with 1–1.5 mL of medium and return the plates to the incubator.
12. Replace the medium after 24 h and every 48 h thereafter (see Note 1).

3.6. Procedure for Young Mouse or Rat Chromaffin Cells

We use animals of 2–4 weeks of age. Given the small amount of collagen in these adrenal tissues, papain is used instead of collagenase, producing a considerably higher cell yield. As the quantity of starting material from the adrenal glands is very small, this procedure is intended for single-cell measurements. We usually use 12-mm Ø glass coverslips placed in 24-well plates, although other sizes can be used. Figure 1 can be printed and posted in the culture room as a brief guide to the procedure:

1. Prepare the papain solution in Locke's buffer (12–18 IU in 200 μL, sufficient for four glands), and store it at 37°C.
2. Sacrifice the animal according to institutionally approved ethical procedures, place it on its back, and spray the abdomen with 70% ethanol. Open the abdomen and remove both adrenal glands by pulling the glands away with an angled forceps (see Fig. 2a).
3. Under a stereo microscope (×20 magnification), carefully remove the adrenal capsule as much as possible of the cortical tissue (see Fig. 2b, c). Work on a sheet of clean filter paper soaked with Locke's solution. This helps to avoid drying and overheating of the tissue by the light. Transfer the medullary tissue to a sterile Petri dish (35 mm Ø) containing 1 mL of sterile ice-cold Locke's solution (see Fig. 2d).
4. Transfer the pieces to a 15-mL conical tube with 200 μL of papain solution and leave them for 15–20 min at 37°C without shaking. The incubation time should be adjusted according to the age of the animals, as younger animals will require shorter digestion.
5. Wash the tissue with 800 μL of fresh Locke's solution.
6. Remove as much of the liquid as possible, taking particular care as the tissue is almost digested, and then add 200 μL of complete medium (see Note 1).
7. Disaggregate the tissue by triturating it several times, first through a 1 mL pipette tip and then through a 100-μL pipette

tip, until the suspension becomes turbid. The degree of cell dissociation can be assessed by taking 10 μL of the suspension and observing under the inverted microscope. It is not recommended to completely dissociate the tissue, as excessive manipulation may damage the cells.

8. Take 20 μL of this cell suspension to count the number of cells in a Neubauer hemocytometer (with experience, cell counting usually becomes unnecessary).
9. Plate the cells by placing a drop of the cell suspension on 12 mm-Ø poly-D-lysine-coated coverslips and allow the cells to settle for 20 min in the incubator before adding 1 mL of medium.
10. Return the plates to the incubator.
11. The cells should be used within the first 3 days after culturing. Where necessary, change the medium (serum-free) every 48 h.

4. Notes

1. The use of a 1:1 mix of Dulbecco's modified Eagle's medium with Ham's F12 medium helps cells to maintain their round shape. This simple trick was suggested by Dr. O. Humberto Viveros and produces excellent results. We prepare 1 L of this mix from powdered medium and after adding antibiotics and filtering, we divide the contents between two bottles. Ten percent (v/v) fetal calf serum is added to one bottle (complete medium), while the second is kept serum-free (incomplete medium). The serum-free medium lasts longer than the complete, and it is used to change the medium after 48 h.
2. Other adherent substrates have been used, including collagen, poly-DL-ornithine, laminin, or fibronectin (3). We use round glass coverslips of 12 mm Ø that fit readily into 24-well culture plates. Cell adhesion improves notably when the glass is pretreated with these substrates, although we do not use any substrate for bovine cells unless they are to be used for immunocytochemistry or electron microscopy.
3. A few decades ago, the quality of commercial enzymes and sera varied considerably from one batch to another. During the 1980s, some particularly active batches of collagenase produced over 100×10^6 cells/gland, possibly due to contamination with other proteases. Although some variation occurs within different batches of these products, the controls applied by manufacturers nowadays have greatly reduced this problem.
4. Several strategies have been described for purifying chromaffin cells from other contaminants and debris. One is differential plating, which is based on the low adherence of chromaffin

cells when compared to fibroblasts or endothelial cells. Although this method gained some popularity, it is currently only performed by few, if any, researchers. Discontinuous gradients of Ficoll® or Percoll® are used by some groups for cell purification, and we have used this system in the past. However, we find that Renografin produces a purer population of chromaffin cells that are enriched in adrenaline.

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