

Necessary conditions to maintain rat adrenal chromaffin cells in primary culture.

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Cell Biology of the Chromaffin Cell
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The first successful attempts to isolate chromaffin cells from small rodents were carried out by Douglas and collaborators in mid-sixties using enzymatic digestion of gerbil adrenal medullae with Viokase (a mixture of pancreatic enzymes)¹. Some years later, Livett and co-workers were able to obtain high yields of viable and pharmacologically responsive cells using retrograde perfusion of a collagenase solution through central lobular vein in bovine adrenal glands² (for a detailed review see ref. 3). Since then, the most popular technique for isolating chromaffin cells has been the use of collagenase, alone or in combination with another enzymes. However, due to the small size of rodents glands comparing with the bovine ones alternative methods to perfusion as the digestion of halved or sliced whole adrenal glands⁴ or medullas, which have been previously separated from the capsule and cortex⁵ have been used.

In spite of culturing rat chromaffin cells is not technically demanding, among the main disadvantages of rat chromaffin cultures are the low yield and short survival of cells in culture. However, rat model offers some advantages comparing to the bovine one. Due to some restrictions in the availability of bovine tissues after bovine spongiform encephalopathy crisis or the need to work with another animal models with an easier breeding or particular scientific interest (as new rat experimental disease models) doing of this preparation a good alternative⁶.

In the next paragraphs, a detailed protocol will be presented pointing out those technical aspects that could be more critical to achieve long-term survival cultures. However, the quality of this culture is highly variable even in the same lab. The explanation could be the human factor and intrinsic variations in the manual procedures but sometimes it is not so clear, doing that a good culture is something almost "magic".

About the animals and the isolation procedure. Any strain of rat can be used to isolate chromaffin cells from adrenal gland but young female animals usually offer better results with the advantage that they are more easily handled. The euthanasia procedure must be fast to avoid excessive stress to the animals, for what a well-trained person is required.

The location of adrenal glands is easy, both can be easily recognised just above the kidneys surrounded by fatty tissue. Once extracted the gland is important keep it always wet with cold saline solution (this preserve the gland and facilitate posterior removal of adipose tissue) and handle the gland with care to avoid damage it. Under a dissection stereomicroscope the adrenal glands are cleaned removing the surrounding adipose tissue using a scalpel blade or a small scissors. Then, the capsule is discarded doing a superficial incision along the gland and removing the capsule rolling the gland with the help of the scalpel blade. Finally, the cortex is also removed with a scalpel blade until the medulla is fully clean (the medulla is easily recognised because is usually clearer than the cortex) and placed in cold saline solution. This method give cultures with a reasonably good yield and enriched in chromaffin cells compared to those using whole adrenal glands.

About the enzymatic digestion. The most popular enzymatic digestion procedure involves the use of collagenase (IUB 3.4.24.3) from *Clostridium histolyticum* alone or in combination with another enzymatic activities (trypsin, hyaluronidase, DNase or proteases). There is not doubt that this is the critical point to obtain good cultures.

One of the main problems with collagenase is that commercial crude collagenases are a blend of different enzymatic activities such as clostripain, neutral proteases and tryptic activities. According to the levels of collagenolytic and proteolytic activities commercial suppliers classify the collagenases in different types. Recently, purified collagenases with a reduced proteolytic activity are also available.

For these reasons, is very important test our enzymatic protocol to find the most suitable enzymatic blend and the best conditions in order to obtain a good yield with the minimum cell damage and keeping as intact as possible the properties of the cells. For it is important to let the enzymes work with minimal mechanical action.

Our digestion solution contains collagenase type I (Worthington Biochemical Co., NJ, USA) which contains caseinase too, bovine serum albumin, DNase type I (which reduces the amount of DNA fibres which favour aggregation and clumping of cells lowering the

yield) and hyaluronidase (often used in conjunction with collagenase to dissociate the extracellular matrix between cells of animal tissue).

The control of the digestion time is very important and the reaction should be stopped by dilution adding cold saline solution and preferably spin at 4°C.

About the culture media. The most common medium used to keep chromaffin cells in culture is Dulbecco's Modified Eagle's Medium (DMEM). However, another media have been used alone (Eagle's medium, RPMI 1640, M-199, etc) or combined in different proportions (DMEM/RPMI, DMEM/Ham's F12). Depending of requirements of your experiments could be important to know the main differences between the formulations of several media available. Thus, DME is a modification of Basal Medium Eagle (BME) that contains four-fold higher concentration of amino acids and vitamins as well as another components.

Another important point for some experiments is to control just as possible the amount of serum or supplements that we are adding to the media. For this reason, culture media with an additional amount of known trophic factors can be prepared to eliminate the serum, which is always a more unspecific solution. Recently, new media formulations from main suppliers are appearing in the market that requires less amount of serum (usually, 2-5 times less). With our protocol (fig. 1) we are able to keep the cells in good conditions during several weeks, usually 1-4 weeks.

New approaches. One proof that the procedure is not technically demanding has been its recent automation. Jacqueline Sagen's group from University of Miami have developed an adaptation of an automated method, previously described for isolation of islets of Langerhans⁷, to isolate chromaffin cells from neonatal porcine adrenal glands⁸. The set-up consists in a digestion chamber where the tissue is placed, connected to a circuit in which a solution of purified collagenase at 37°C is recirculating. When the cells are in small clusters or already isolated the flow permits their way through a metallic mesh being collected in a bottle containing collagenase-free saline solution.

Primary culture of rat chromaffin cells

1. Sacrifice the animals by cervical dislocation after stun them by concussion.
2. The adrenal glands must be immediately extracted after the sacrifice of the animal and placed on ice cold HBBS (Ca^{2+} and Mg^{2+} free) supplemented with antibiotics (Pen+Strep).
3. Under a dissection stereomicroscope, eliminate the surrounding adipose tissue with a stainless steel scalpel blade and decapsulate the adrenal glands, then remove the cortex to isolate the medullar tissue.
4. Place the adrenal medullas in a Falcon-type tube with cold HBBS.
5. Put 3 ml of dissociation solution (2,6 mg/ml collagenase type I (Worthington), 3mg/ml BSA, 0,15mg/ml DNase I, 0,15mg/ml hyaluronidase I-S in HBSS) in the lid of a small culture dish.
6. Put the medullas into the dissociation solution and cut them in four pieces each one with the help of a scalpel blade or two needles. Start to count the time of enzymatic digestion. Recover the medulla chunks and the solution and transfer them to a Falcon tube.
7. Place the tube in a thermostatic bath at 37°C during 30 min approx.
8. Every 10 min the solution must be gently resuspended with a Pasteur pipette with a fire polished broken tip. During the last 5 min the solution is continuously resuspended until the medullary tissue becomes not visible.
9. Fill the tube with cold HBBS until a final volume of 10 ml to stop the enzymatic reaction.
10. Spin at 800 rpm during 10 min in a pre-cooled refrigerated centrifuge at 4°C.
11. Discard the supernatant by decanting and homogenate the pellet with 800 μ l pre-warmed DMEM+10% heat-inactivated FCS supplemented with antibiotics.
12. Put 1 or 2 drops in each poly-D-lysine treated coverslip and leave the cells settle down during 1 h at 37°C.
13. Refill the culture dish with 1.5 ml of DMEM+10% FCS and incubate at 37°C and 5%CO₂.

Figure 1. Our current lab protocol to prepare a primary culture of rat adrenal chromaffin cells.

This system is particularly useful to produce a high number of cells reducing the variability due to manual techniques for enzymatic digestion. This group have made another important contribution to use rat chromaffin cells. They obtained conditionally immortalized chromaffin cells from rat and bovine adrenals⁹. The rat RAD5.2 cell

line was obtained from E17 Sprague-Dawley rat using the temperature sensitive mutant of SV40 large T antigen (tsTag). This cell line is conditionally immortalized and can be later disimmortalized under specific conditions (i.e. temperature). This would particularly useful in cell lines used for neural transplants (in which chromaffin cells are having a renewed interest) because the RAD5.2 cells stops their growth at body temperature avoiding undesired effects.

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