Advances in cell culture for chromaffin cells and related cell types.

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Cell Biology of the Chromaffin Cell
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Studies making use of chromaffin cells (CCs), PC12 cells and enterochromaffin cell cultures continue to contribute significantly to our knowledge about basic mechanisms of neurotransmitter release and action[1-6]. These cells are derived embryologically from the neural crest[1,5] and serve as a convenient source of neural cells (paraneurons) for neurobiologists interested in exploring molecular and cellular mechanisms of neuronal processes including neuronal development and signal transduction. The combined use of techniques from biophysics and molecular biology has provided insight into synaptic transmission, in particular the molecular mechanisms of Ca^{2+}-triggered exocytosis[7].

Technical aspects of the isolation and culture of these cells are often lost in the fine print of Methods sections of published papers. Nevertheless this information is essential to anyone practicing the art of cell culture of these and related neuronal cells, including PC12 cells and enterochromaffin cells[6,7]. CCs have been succesfully maintained as primary monolayer cultures[2] and as suspension cultures[8]. In the latter, flow cytometry appears to be a useful technique to characterize noradrenaline and adrenaline cell subtypes[9] and to follow their phenotypic changes in response to growth factors[5,8].

Protocols have been refined in different labs but not officially published. CCs have been isolated primarily from bovine adrenal medullae but also from sheep, pig, cat, rat, mice, and even deer! Primary monolayer cultures of CCs and PC12 cells and suspension cultures of these and related cell types demand different protocols: specialized enzymes and plasticware, growth factors, substratum[10] and optimized culture media[2,5]. Catecholamine secretion and firing frequency can be analyzed at the single-cell level by means of perforated patch-clamp and carbon fiber electrochemical detection[11,12]. Tools such as electophysiology[7] and use of terrestrial and marine neurotoxins have been used to excite[13] and to inhibit[14,15] catecholamine secretion from CCs and to provide information about the subunit composition of the functional neuronal-type nicotinic receptors on these cells[14,16].

Aims. The aims of this Workshop were fourfold:

1. To review and update protocols for the isolation and maintenance of chromaffin and related cells.
2. To share our experience about technical aspects of chromaffin cell culture.
3. To provide working protocols for isolation and culture of chromaffin and related cells, and
4. To encourage on-going update of these protocols placed on the web.

Participants. The Discussion Leaders for this Workshop were Manuela G. López (Madrid), José-María Trifaró (Ottawa), Jakob Sørensen (Gottingen), Juan Antonio Gilabert (Madrid), Arthur Tischler (Boston) and Christian Prinz (Munich).

A brief history. To set the scene, Bruce Livett presented an outline of the history of development of techniques for isolation of chromaffin, PC12 and related cell types (see Table 1).

It is now timely to review and update the information gained by hundreds researchers who have used these cultures and related cell culture systems. The six Discusión Leaders addressed a number of important technical issues summarized below.

Experience in cell culture of chromaffin cells from bovine, mice and rat. *Manuela G. López* drew attention to enzymatic digestion as a limiting factor affecting yield, function and viability of isolated CCs. Gradient fractionation of NA and Ad cells on Urograffin[9] and Percoll provides a means of obtaining relatively pure populations of Ad cells but not as yet pure NA cells. Another important factor in isolation of CCs is the media. Ideally, the media should be at pH 7.2 – 7.4. Whether the serum is heat-inactivated or not and whether supplemented by serum, and for how long (24–48 h) affect the outcome.

Table 1. History of Cell Culture Techniques for Chromaffin and related cells.

Year	Advances in Cell Culture Techniques	Investigators
1967	Gerbil adrenal CCs, first isolated using 'Viokase'	*Kanno, Sampson, Douglas*
1970s	Bovine adrenal CCs first isolated by retrograde perfusion of bovine adrenal glands with collagenase	*Fenwick, Livett / Brookes / Zinder, Pollard / Viveros / Trifaro / Aunis / Garcia*
	PC12 cells	*Greene / Tischler*
	Rat chromaffin cells isolated	*Unsicker / Tischler*
1980s	Monolayer cultures bovine chromaffin cells	*Livett / Trifaro / Viveros /Aunis / Garcia*
	Pharmacology / patch-clamp electrophysiology of secretion	as above / Neher
	Suspension and 'flow-through' cultures of bovine chromaffin cells	*Westhead / Perlman*
	Electroporation *"Leaky Cells"*	*Baker, Knight / Kirshner /Schneider/ Holz*
	Fluorescence Calcium imaging	*Burgoyne / Morgan / Cheek*
1990s	NA and Ad cells 'separated'	*Moro / Krause*
	Cytometry on suspension cultures	Garcia / *Gilabert*
	Amperometry of released catecholamines	*Westerlink /Borges*
	Enterochromaffin cells	*Prinz*
2000s	Molecular mechanisms of Ca^{++} depdt. exocytosis	*Rettig, Neher*

Labelling catecholamine stores in chromaffin cells in culture. *José-María Trifaró* discussed optimal conditions for [^{3}H]NA labelling of amine stores in CCs as this affects release results[10]. Incubation with 10^{-7}-$5x10^{-7}$ M [^{3}H]NA for 5-10 min followed by 6 washes of 10 min. duration were found to label the stores are evenly. There was no activation of uptake 2, very little cytosolic or extra [^{3}H]NA, and importantly parallel release of endogenous amines and [^{3}H]NA. Moreover, there was a good correlation between the % total [^{3}H]NA released and the number of vesicles released.

A strategy for structure-function studies of exocytotic proteins using chromaffin cells from knockout mice and viral rescue techniques. *Jakob Sørensen* discussed ways of overcoming problems of working with transgenic mice. Problems include the peri-natal lethal phenotype, problems of not enough cells and lack of techniques for isolating and culturing mouse CCs for up to 3 days, suitable for single-cell measurements of exocytosis. Of particular interest, he provided a description of protocol for isolating CCs from K/O mice.

Conditions for maintaining rat adrenal chromaffin cells in primary culture. *Juan Antonio Gilabert* discussed conditions that affect yield and long-term survival of rat CCs in culture[8]. Dr. Gilabert is conducting and on-going survey of choice of rat strains, age, yield and tests of viability and encourages others to contribute. He has also devised an automated method for isolation of pig CCs.

Culturing pheochromocytoma cells. *Arthur Tischler* commented that "establishment of pheochromocytoma cell (PC) lines is challenging" because PCs from humans, rats and mice cease proliferating in primary culture. Only a proportion undergo spontaneous neuronal differentiation[5]. As a rapid index of success, he makes use of I.H. for TH and BrdU after BrdU pulse-labeling. He discussed the advantages and disadvantages of MPC lines from neurofibromatosis knockout mice, including precautions and cautions (including use of horse serum, prior irradiation, plating density) and cautioned that growth factors may give opposite responses in CCs and their corresponding tumours.

The art of culturing enterochromaffin cells. *Christian Prinz* asked "why do it?". Well, histamine release controls GI functions (acid secretion, motility, intestinal secretion), and isolated cells could prove useful both for basic research on mechanisms and as replacement therapy[6]. A protocol for cell isolation was described in which excised rat

stomach is enzymatically dispersed and fractionated by counterflow elutriation and density gradients.

Working protocols for isolation and culture of chromaffin and related cells. An outcome from this Workshop will be the posting of working protocols on the web, enabling sharing of techniques and on-going updates of these protocols.

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