A simple and fast method for cell recovery and DNA content analysis from various mouse tissues by flow cytometry

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Abstract Cell division in tissues can be investigated in various ways. We present here a method for improving cell recovery and cell cycle analysis for a wide range of mouse tissues. This strategy combines a cell isolation procedure for various mouse tissues based on intracardiac perfusion and subsequent treatment followed by flow cytometry. This easy and reproducible method allows a rapid analysis of nuclear DNA content, providing an estimate of the cell number at different phases of the cell cycle. This combined procedure could also be used for the isolation of specific cell subpopulations from different mouse tissues by fluorescence activated cell sorting.

Keywords Cell cycle · Cell isolation · Flow Cytometry · Mouse · Tissue dissociation

Introduction

One of the main aims of biology is to determine how tissues can co-ordinate single cells to work together in a specific function. Studies of single cells can provide information about the distribution of roles to each cell subpopulation in a tissue. Such analyses are often performed in cell culture systems and provide useful data. However, these observations are made outside the physiological context, and it is therefore difficult to extrapolate them to *in vivo* function. One major problem remains the isolation of sufficient numbers of viable single cells from tissues for qualitative (characterisation of cell types), quantitative (number of cells in a tissue) or functional studies.

Cell cycle analyses reflect tissue proliferation capacities at a given time. In recent decades, various approaches have been used to assess the proliferation properties of tissues. Tritiated thymidine incorporation has been successfully used to draw an overview of the proliferation and migration capacities of different tissues (Messier and Leblond 1960). The development of antibodies directed against modified nucleotides, such as bromodeoxyuridine (BrdU), has made it possible to observe DNA replication *in situ* with limited handling care (deFazio et al. 1987). Although these techniques can provide an estimate of cell cycle activity in a tissue based on direct observation, accurate quantification of the number of cells

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engaged in the various phases of the cell cycle remains difficult and time-consuming.

Flow cytometry has been extensively used for monitoring the cell cycle in cell cultures (Krishan 1975). It can be used for the rapid, accurate quantification of cells and for the analysis of cell populations or cellular components (Cram 2002).

Technologies for the isolation of single cells from tissues would be extremely useful for estimating the proliferation properties of organs. We present here a simple, rapid method combining a cell isolation strategy suitable for use in various mouse tissues and flow cytometry analysis of the DNA content of each organ, providing an estimate of the number of cells that entered S phase. Extended applications of this methodology are discussed.

Materials

Animals

C57Bl/6 mice were maintained in a conventional animal facility at 23°C, with a 12-h light period. Mice were housed and handled according to French government ethical guidelines.

Equipment

- (1) Stepdos Pump. Cat. No. w-89717¹
- (2) 0.5 × 16 mm needles. Cat. No. 050102²
- (3) 1 ml syringe. Cat. No. 050001²
- (4) 70 µm filter. Cat. No. 352350²
- (5) Becton Dickinson LSR Benchtop³
- (6) Heraeus, Megafuge 1.0, rotor 2150

Chemicals

- (1) DMEM. Cat. No. 31885-023⁴
- (2) Foetal Calf Serum. Lot 40F6130K, Cat. No. 10270-106⁴
- (3) Hepes⁵

- (4) NaCl. Cat. No. 479687⁶
- (5) NaCl 0.9%. Cat. No.
- (6) KCl. Cat. No. 4933⁵
- (7) Na₂HPO₄·2H₂O. Cat. No. 6580⁵
- (8) EGTA. Cat. No. E-4278 Sigma-Aldrich,⁷
- (9) CaCl₂. Cat. No. 2382⁵
- (10) Collagenase IV. Cat. No. C5138⁶
- (11) 1× PBS. Cat. No. 14190-094⁴
- (12) 10× PBS. Cat. No. 14200-067⁴
- (13) Triton X-100. Cat. No. X-100⁶
- (14) RNase A, DNase-free. Cat. No. 0109169⁸
- (15) Propidium iodide. Cat. No. P4170⁶
- (16) Rompun 2% Bayer,⁹
- (17) Imalgene 1000¹⁰
- (18) Heparin 25,000 IU/ml.

Methods

Solution preparation

Buffer 1 contains 140 mM NaCl, 5.4 mM KCl, 0.8 mM Na₂HPO₄·2H₂O and 25 mM Hepes. Dissolve the various component molecules in H₂O and autoclave before storage. Prepare buffers 2 and 3 on the day of the experiment. Buffer 2 contains 0.25 mM of EGTA diluted in buffer 1. Buffer 3 contains 7.5 mg of collagenase IV dissolved in 30 ml of Buffer 1 supplemented with 2 mM CaCl₂. Prepare a complete medium containing DMEM supplemented with 15% foetal calf serum was prepared. All solutions must be warmed at 37°C before injection into mice.

Anaesthesia of the mice

Mice are anaesthetised by the intraperitoneal injection of 3.5 µl/g of anaesthetic solution, which is prepared by mixing 50 µl of 2% Rompun with 100 µl of Imalgene 1000 in a final volume of 350 µl of 0.9% NaCl. Mice are then injected with

¹ Fisher Bioblock, Illkirch, France

² Dominique Dutscher, Brumath, France

³ Becton Dickinson, Le Pont de Claix, France

⁴ Invitrogen, Cergy-Pontoise, France

⁵ Merck, Darmstadt, Germany

⁶ CarloErba, Val de Reuil, France

⁷ Saint-Quentin Fallavier, France

⁸ Roche, Meylan, France

⁹ Puteaux, France

¹⁰ Merial, Lyons, France

250 IU of heparin to ensure that the blood remained sufficiently fluid.

Perfusion of the mice

The dissection of the mice is initiated by cutting the skin and the peritonea from the bottom of the ventral face. The thoracic cage is then opened to provide access to the heart. Care must be taken to avoid cutting too many vessels during these steps, as intact blood vessels are required to ensure that the solution spreads throughout the body during perfusion. Mice are perfused via the apex of the left ventricle. It is important to make a small incision with a needle in the right ventricle, to serve as an outlet. Mice are perfused with prewarmed solutions as follows: 30 ml of buffer 1 at a flow rate of 3 ml/min, 10 ml of buffer 2 at 3 ml/min and 25 ml of buffer 3 at 2.5 ml/min.

Tissue dissociation and cell isolation

Collect and mince pre-dissociated tissues in a 35 × 10 mm Petri dish containing 5 ml of prewarmed buffer 3, using forceps and scissors. Transfer pieces of tissue in buffer 3 to a 15 ml conical tube and incubate for 1 h at 37°C. Tubes are vigorously shaken every five minutes to facilitate tissue dissociation. Once the tissue is dissociated into a homogeneous suspension, the tubes are centrifuged for 3' at 174 × *g* at room temperature. Discard the supernatant and resuspend the pellet in 5 ml of complete medium. Eliminate the small residual aggregates by filtering the solution through a 70-µm filter. The filtered solution is centrifuged for 3' at 174 × *g* at room temperature. Remove the supernatant and resuspend the pellet in 5 ml of complete medium. Cells are counted three times in a Malassez hemocytometer. Eliminate the residual medium by centrifugation and wash the cells once in 5 ml of 1× PBS. The cells are collected by an additional centrifugation and resuspended in 2 ml of 1× PBS and 5 ml of cold 100% ethanol for long-term fixation. Cells were stored at -20°C. Experiments can be successfully performed in the six-month period following cell isolation.

Flow cytometry analysis of cells isolated from tissue

Wash 1×10^6 fixed cells twice in 2 ml of 1× PBS. Cells are then resuspended in 750 µl of labelling buffer (20 µg/ml propidium iodide (PI), 0.2 mg/ml RNase A, 0.1% Triton X-100 in 1× PBS) and incubated for 15' at 37°C. Care must be taken to protect solutions containing PI from light. Following the labelling of DNA with PI, the cells are diluted in 5 ml of 1× PBS. DNA content was quantified by flow cytometry, using an argon ion laser at 488 nm, according to the manufacturer's protocol.

Statistical analyses

Comparison of percentages between mouse and tissues were analysed by Fisher's exact test. The observed differences were considered statistically significant only if *P* values were <0.05.

Results

Experimental design

Tissues were dissociated using collagenase type IV, a weak digestive enzyme, to isolate sufficient numbers of viable cells. Intracardiac collagenase perfusion has been successfully used to isolate functional mouse hepatocytes for metabolic experiments (Edstrom et al. 1983). We extended this technique to other tissues. Intracardiac perfusion leads to the complete diffusion of the dissociation solution to all organs via the veins and vessels. High levels of tissue disintegration are therefore possible, facilitating the recovery of a maximum number of cells. An additional collagenase type IV treatment was administered to ensure the complete dissociation of tissues for the isolation of single cells. Dissociated tissues were filtered to eliminate debris (such as the fibrous connective tissue supporting the cells) that might cause artefacts in flow cytometry. We then analysed the DNA content of single cells. Experiments were carried out on two-month-old and 10-month-old mice, for comparison of the cell cycle at two different ages.

Analysis of the DNA content of cells

The thymus, lung, heart and brain had different cell cycle profiles (Fig. 1). Cells that have entered the S and G2/M phases of cell cycle were highest (~ 20%) in the lung and heart. Most of the cells in the brain (~ 99%) were not actively dividing, as expected. (Fig. 2)

Cells isolated from the thymus of two-month-old mice had a cell cycle profile significantly different from that of heart ($P = 0.03095$) and brain ($P = 0.000161$) cells. Indeed, in two-month-old mice, the thymus, the lung, the heart, and the brain were found to contain 9.43%, 11.44%, 4.69% and 0.33% of cells in S phase respectively. The brain had a very high proportion of non-replicating cells (99%), significantly different from that in the thymus ($P = 0.000161$), lung ($P = 2.953e-07$) and heart ($P = 1.515e-06$).

A comparison of percentages between cells isolated from tissues collected from two-month-old and 10-month-old mice revealed no significant difference between these two ages (thymus: $P = 0.772$; lung: $P = 1$; heart: $P = 0.8387$; brain: $P = 1$). The lung and thymus of 10-month-old mice displayed the highest levels of cell in S phase, with, respectively 6.67% and 10.57% versus only 3.59% for the heart. Very low percentages of S-phase (0.75% respectively) were

detected in brain of 10-month-old mice. The proportion of 4n (G2/M, tetraploid), S and G0/G1 cells observed in brain is significantly different from the proportion observed for the thymus ($P = 0.001766$), lung ($P = 9.029e-06$) and heart ($P = 6.48e-05$).

Discussion

The combination of intracardiac perfusion, allowing collagenase to penetrate deep into the tissues, followed by additional treatment with a collagenase-containing buffer and flow cytometry made it possible to estimate the DNA content in different cells of various mouse tissues. The results obtained were reproducible for different mice of the same age. Identical observations were made for all studies carried out in the six-month period following cell recovery (data not shown), highlighting the possibility of long-term storage for fixed cells. Such storage is useful, as it makes possible the analysis of a large number of cell populations by flow cytometry.

Burns et al. published an extensive analysis of the cell cycle in 16 different mouse tissues, using three different isolation procedures (Burns et al. 1983). We observed some differences in cell cycle phase percentages in our study. Lungs and hearts

Fig. 1 Percentage of cells in the different phases of the cell cycle. Cells in the G0/G1 phase are shown in black and cells in S phase are shown in white. Cells in the G2/M phase or tetraploid cells (4n), that cannot be distinguished from G2/M cells are shown in grey. Data are presented for

cells isolated from the thymus (Th), lung (Lu), heart (He) and brain (Br) of two-month-old (2 m) and 10-month-old (10 m) mice perfused with collagenase. The numbers in brackets are the standard deviations of three independent experiments

Fig. 2 Example of flow cytometry analysis of cells isolated from lung and brain of 2-month-old mouse. The top-panel graphs present cell analysis according to their size (x-axis) and their granularity (y-axis) for lung (**A**) and brain (**C**). The DNA histograms present the cell population isolated from lung (**B**) and brain (**D**) in which the relative DNA content (estimated by fluorescence intensity after PI staining) was plotted against cell number

isolated from two-month-old mice had higher proportions of cells in S phase than reported for the mice studied by Burns et al. (1983) (lung: 11.44% vs. 8.48%, heart: 4.69% vs. 13.46%). In contrast, the thymus contained a lower percentage of cells that entered in S phase (9.43% vs. 16.74%). There may be many reasons for these variations. First, the genetic backgrounds of the mice used in the two studies are different (C57Bl/6 vs. CD1). Second, we used a different cell isolation procedure. Burns et al. (1983) prepared their cells by mincing tissues with forceps. Thus, many cells may have been incompletely isolated, resulting in their elimination at the filtration step. Burns et al. (1983) also used chemical or enzymatic dissociation for the isolation of cells from some tissues. This procedure enabled them to estimate the percentages of cells in each phase of the cell cycle, but the possibility of that treatment with a strongly digestive enzyme, such as pepsin, damaged some cells cannot be ruled out.

Our approach combined the use of a weak digestive enzyme, to ensure the safe dissociation

of cells, with intracardiac perfusion to ensure the diffusion of the dissociation solution deep into the tissues. The use of an additional enzymatic treatment made it possible to maximise the number of cells recovered. The method described here therefore provides a simple, rapid procedure for isolating single cells from various tissues.

Several applications of this method may be considered. First, the use of a flow cytometer with cell sorting functions would allow selecting a specific cell population, based on position in the cell cycle, from a tissue. In this study, we fixed the cells after tissue dissociation for long-term storage. Cell sorting based on DNA content could be used also to set up synchronised cultures of living cells. This could be achieved using a viable DNA-intercalating agent such as Hoechst 33342, analysed with a UV laser at 460 nm. Cell cultures have been successfully prepared with cells isolated from the brain, cerebellum and thymus, using the method described here (data not shown).

This method, combining cell isolation by perfusion and flow cytometry analysis, could also

be used after pulse labelling with BrdU in living mice. Several papers have described methods for feeding mice with BrdU. This should allow monitoring the dynamics of the cell cycle accurately *in vivo*.

Finally, this method could also be applied for the isolation of specific cell subpopulations from tissues by fluorescence-activated cell sorting, using antibodies directed against specific proteins of the cell population concerned. Freshly isolated single cells from various subpopulations could then be used for molecular or cellular methods, such as PCR or immunocytochemistry.

The simple, rapid method described in this paper, combining efficient cell isolation from mouse tissue by perfusion and analysis by flow cytometry, is of greater potential value for use in studies of small numbers of very specific cells, making it possible to avoid the bias introduced by *in vitro* systems.

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