Preface

Diabetes mellitus is the collective name for a group of diseases associated with hyperglycemia (high levels of blood glucose) caused by defects in insulin production, insulin action, or both. About 6.2% of the US population (17 million people) have diabetes mellitus. It is the leading cause of kidney failure, blindness, and amputations. It is also a major risk factor for heart diseases, stroke, and birth defects.

Diabetes Mellitus: Methods and Protocols provides a state-of-the-art account of the experimental methodology for studying the molecular defects leading to diabetes mellitus, both at the molecular and biochemical levels. The chapters cover a wide range of topics written by experts in their respective fields and are organized in two sections: Insulin Production and Insulin Action. The detailed experimental protocols presented, including the notes of interest, provide a very useful tool for basic researchers and clinicians for investigating and treating this disease. Each chapter starts with an introduction to a specific technique and explains its application in the field of diabetes research. Following the list of materials, a detailed description of the technique is presented in the methods section in a way that enables the successful execution of the protocol. The "Notes" section at the end discusses the pitfalls of the technique and alternative approaches.

I am grateful to the numerous scientists who have contributed to this volume by writing both highly detailed and understandable chapters. Special thanks also to Prof. John M. Walker, editor of the *Methods in Molecular Medicine* series and Mr. Thomas Lanigan, President of Humana Press, for bringing *Diabetes Mellitus: Methods and Protocols* to fruition.

Sabire Özcan

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Purification of Rat Pancreatic ß-Cells by Fluorescence-Activated Cell Sorting

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1. Introduction

The β-cell is receptive to intricate hormonal, neuronal and nutrient signaling which is key for normal physiology but complicates the study of specific effects of individual factors on β-cell function. To preserve the microenvironment of the β-cell, most studies of β-cell physiology have been performed on in vitro cultured islets of Langerhans. However, whereas islets in the pancreas are highly vascularized and oxygenated, ischemic conditions cannot be avoided in the center of cultured isolated islets, leading to abnormal islet cell function and viability. Moreover, in the absence of blood flow, intercellular communication in islets is likely to change as well. Furthermore, contamination of islets with anatomically associated acinar cells is inevitable during isolation and may have a major influence on the specificity of experiments.

To avoid the above interactions, β-cells need to be investigated at the single-cell level. Much of the analytical information has been obtained by clamping individual islet cells to study their electrophysiology and by reverse hemolytic plaque assay to visualize the insulin release from individual β-cells ***(1)***. However, purification of the individual cell types at the preparative level is necessary to study (sub)cellular mechanisms of hormone synthesis and secretion under normal and pathological conditions. Depending on the available equipment and on the aim of the study, islet cells can be isolated on the basis of differences in cell size ***(2)***, membrane antigens ***(3)*** or metabolic features ***(4,5–6)***. The resulting cell purity and viability will differ according to the method used. This chapter presents a protocol for rat islet cell purification on the basis of dif-

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ferences in light scatter and endogenous fluorescence, thus combining the first and third methods. Increased light scatter, in combination with high levels of the autofluorescent electron carrier flavine–adenine–dinucleotide (FAD) allows the isolation of β-cells at greater than 95% purity ***(4)***. This model has proven very useful for studying regulation of β-cell (dys)function and functional cooperation between islet cells ***(7)***. In addition, acute changes in the redox state of endogenous nicotinamide dinucleotide (phosphate) [NAD(P)H] serve as a basis for further cell separation ***(5)***. This parameter directly correlates to changes in the cellular redox state induced by (glucose) metabolism and allows definition of distinct β-cell populations according to their nutrient responsiveness ***(8,9)***. Moreover, α-cells exhibit stable NAD(P)H pools and can also be purified on the basis of this parameter. The availability of large amounts of pure α- and β-cells that are functionally intact and support long-term, serum-free culture has facilitated detailed studies on the regulation of hormone synthesis and secretion ***(10–13)***, on cell survival and protection of the differentiated phenotype ***(14–17),*** and on the molecular biology of cellular heterogeneity ***(18–20)***.

2. Materials

Adult male Wistar rats (SPF, Han, 6 wk of age and 200–300 g body weight; Elevage Janvier, Le Genest St. Isle, France).

2.1. Reagents

All media are sterilized by filtration through a 0.22-μm filter.

1. Isolation medium: 123 m*M* NaCl, 1.8 m*M* $CaCl_2$, 0.8 m*M* $MgSO_4$, 5.4 m*M* KCl, 1 m*M* NaH_2PO_4, 5.6 m*M* glucose, 4.2 m*M* $NaHCO_3$, 10 m*M* HEPES, 0.5% bovine serum albumin, 0.1 g/L kanamycine (pH 7.4) at room temperature.
2. Dissociation medium: 125 m*M* NaCl, 0.8 m*M* $MgSO_4$, 5.4 m*M* KCl, 1 m*M* NaH2PO4, 5.6 m*M* glucose, 4.2 m*M* $NaHCO_3$, 10 m*M* HEPES, 0.5% bovine serum albumin, 0.1 g/L kanamycine, 7.41 m*M* EGTA (pH 7.4) at 30°C.
3. Cell culture medium: Nutrient mixture Ham's F-10, without glutamine, without glucose (Gibco Laboratories) supplemented with 2 m*M* L-glutamine, 10 m*M* glucose, 0.075 g/L streptomycin, 0.1 g/L penicillin, 0.5 g/L bovine serum albumin (factor V, RIA grade, Sigma), 50 μ*M* 3-isobutyl-1-methylxanthine (Sigma).

2.2. Equipment

Materials are autoclaved or purchased as sterile disposables. Glassware used for collecting islets or cells is treated with silicon solution (Serva, Heidelberg, Germany) for 1 min, followed by three successive washes in distilled water. When dry, the material is sterilized in an oven for 6 h at 180°C.

1. Heated shaker TH25 (Edmond Bühler, Germany).
2. Elutriator JE-X10 X10 (Beckman, Palo Alto, CA).
3. Enterprise II argon laser (Coherent, Santa Clara, CA).
4. FACSTAR Plus (Becton Dickinson, Sunny Vale, CA, USA).
5. Discardit II 10-mL syringe (Becton Dickinson, Heulva, Spain).
6. Catheter tube PTFE, internal diameter of 0.6 mm, external diameter of 0.9 mm (Merck, Darmstadt, Germany).
7. 50-mL propylene conical tube (Becton Dickinson, Franklin Lakes, NJ, USA).

3. Methods

3.1. Dissection of the Rat Pancreata

1. Adult male Wistar rats are intraperitonealy injected with pilocarpine (200 µL per 200 g body weight) 2 h before dissection. Pilocarpine is 4% isoptocarpine.
2. Rats are sedated by treatment with CO_2 and killed by decapitation.
3. After ligation of the pancreatic duct with a Halsted-mosquito forceps, a small incision is made in the pancreatic duct, close to the liver.
4. The pancreata are distended by injection of 10 mL cold isolation medium containing 0.3 mg/mL collagenase (use a 0.6-µm-internal-diameter catheter mounted on an 18-gauge needle placed on a 10-mL syringe).
5. The glands are removed and cleaned from lymph nodes and fat tissue. Four to five pancreata are collected in a 50-mL tube and kept on ice until digestion (*see* **Notes 1** *and* **2**).

3.2. Collagenase Digestion

1. Dissected pancreata are predigested by incubation at 37°C, under continuous shaking (240 strokes/min).
2. After 5–6 min, the supernatant fluid is discarded and the tissue is minced with scissors. Isolation medium is added and after 15 s of sedimentation, the supernatant is discarded.
3. The tissue suspension is then diluted with 1 volume of isolation medium containing 0.3 mg/mL collagenase P and further digested in the air-heated shaker for an additional 15–18 min under continuous shaking at 37°C (*see* **Note 3**).
4. The digested tissue is then gently resuspended and the digestion is stopped by filling the tube with isolation medium with 2% heat-inactivated fetal calf serum.
5. The digest is then filtered trough a 500 µm nylon screen and the filtrate is washed twice by adding 30 ml of isolation medium followed by centrifugation for 2 min at 240*g*.
6. The filter residue is resuspended in isolation medium without collagenase and further dispersed by shaking manually and filtering through a 500 µm nylon screen. The additional filtering of undigested residues is repeated twice. All the digested fractions are then collected and washed in a 50 mL tube.

3.3. Islet Purification

Conditions of centrifugal elutriation allow elimination of particles smaller than 100 μm in diameter. The technique involves the use of a 10X elutriator rotor installed in a JB6 centrifuge.

1. The pancreatic digest is suspended in the mixing chamber that is connected to a flask containing isolation medium.
2. With the elutriator running at 250 rpm, the cellular material is perfused into the elutriation chamber at a rate of 230 mL/min. Particles larger than 100 μm in diameter are retained in the elutriation chamber; smaller fragments leave the rotor and are discarded.
3. After disposal of 800–900 mL eluent, the elutriation chamber is disconnected from the circuit and the centrifugation speed is turned down to zero. While the centrifuge is slowing down, the content of the elutriation chamber is collected (*see* **Note 4**).
4. The elutriation is stopped when 500 mL eluent has been collected. The fraction is examined under an inverted dissection microscope. Clean islets are hand-picked with an elongated Pasteur pipet (*see* **Notes 5** *and* **6**).

3.4. Dissociation

1. The isolated islets are washed twice, by sedimentation in isolation medium, followed by a wash in dissociation medium.
2. Islets are resuspended in 30°C dissociation medium and transferred to a siliconized 100-mL bottle.
3. The islet preparation is transferred to an air-heated shaker at 30°C and incubated for 5 min under continuous shaking (200 rpm).
4. After a brief resuspension of the islets with a siliconized Pasteur pipet, the medium is supplemented with trypsin and DNase at a final concentration of 5 μg/mL and 2 μg/mL, respectively.
5. The degree of dissociation is regularly checked under a phase-contrast microscope and stopped when 50–60% of the cells occur as single-cell units, which is usually the case after 10 min. The dissociation is stopped by adding 2% fetal calf serum (FCS) to the isolation medium.
6. In order to remove cell debris and dead cells, an isotonic Percoll solution with a density of 1.040 g/mL is layered underneath the suspension and the gradient is centrifuged at 800*g* for 6 min (no break).
7. The pellet is collected and suspended in 50 mL isolation medium, which is then filtered through a 63-μm nylon screen to remove the rare, large-cell clumps.
8. The filtrate is washed in Ham's F-10 containing 6 m*M* glucose, 1% bovine serum albumin (fraction V), 2 mmol/L L-glutamine. The cells are cultured in suspension for 30 min at 37°C in 95% air–5% CO_2 prior to fluorescence-activated cell sorting (*see* **Note 7**).

3.5. Purification of Single β-Cells and Non-β-Cells

1. The dispersed islet cells are washed in isolation medium containing 2.8 m*M* glucose and submitted to auto-fluorescence-activated cell sorting (FACS) using a FAC-

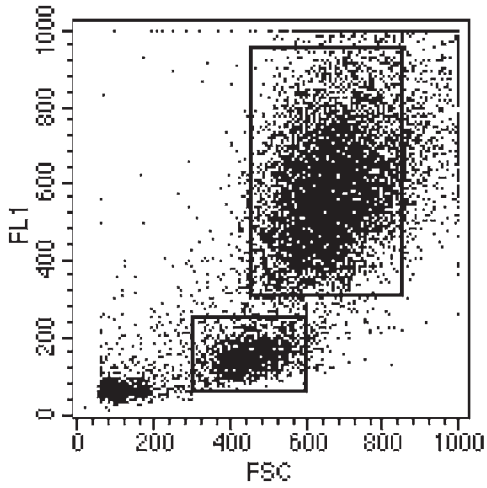

Fig. 1. FACS analysis of unpurified islets cells examined for their FAD fluorescence and FSC intensity at 2.8 mM glucose. The subpopulation with high FAD and high FSC represents the β-cells, whereas the islet non β-cells are lower in FAD content and cause less FSC.

STAR PLUS. Isolation medium is used as sheath fluid. A 0.22-µm filter is put on the sheath tank to remove any particles in the medium.

2. The cells are illuminated with an argon laser (Enterprise II) with 100 mW at 488 nm. The instrument is calibrated according to the manufacturer's guidelines. The fluorescence emission is collected in the FL1 photomultiplier at 510–550 nm (FITC filter, 530-nm bandpass filter). The fluorescence can be taken as a parameter for the cellular FAD content. Rat β-cells have a threefold higher FAD fluorescence than rat non-β-cells at this low glucose concentration. The β-cells are larger than the non-β-cells and have thus a larger forward scatter (FSC). The background signal caused by cell debris is removed by putting a threshold level on FSC. Both FSC and FL1 are linearly amplified.
3. Selection of the appropriate windows allows the simultaneous isolation of single b-cells and single islet non-β-cells. The β-cells are separated on the basis of high FAD fluorescence and high FSC as compared to non-β-cells (**Fig. 1**). The cells in uncharged droplets are collected as well. They constitute the so-called "middle fraction." The middle fraction is collected in a 50-mL tube, spun down (5 min at 500*g*), resuspended in low-glucose-containing isolation medium and re-sorted (*see* **Note 8**).

3.6. Culturing of Purified β-Cells

1. Single rat β-cells do not survive well in suspension. To avoid β-cell losses, FACS-purified rat β-cells are reaggregated in a rotatory shaker for 2 h at 37°C and 5% CO_2, in the presence of Ca^{2+} (*see* **Note 9**).

2. Aggregated β-cell clusters can be kept in suspension in serum-free HAM's F10 medium at 37°C and 5% CO_2. Depending on the experiment, cultures can be maintained in the absence or presence of 50 μ*M* IBMX. The phosphodiesterase inhibitor mimics paracrine hormone actions on the β-cells, stimulating their in vitro survival and function.
3. β-cells can also be cultured as single cells in poly-D-lysine-coated tissue culture plates with good survival rates. Ninety-six-well plates are coated by incubating the wells with 100 μL of poly-D-lysine (Sigma, 10 μg/mL in water) for 30 min at 30°C, followed by three successive washes with Ham's F10 medium.

3.7. Assessment of the Quality of the Purified β-Cells

1. The viability is assessed by the addition of the vital stain neutral red (final concentration 0.01% [w/v] in isolation medium) to a suspension of purified cells. After incubation for 5 min at 37°C, red-stained cells are counted under a light microscope. Immediately after sorting, an average of more than 95% of the purified cells incorporate the dye.
2. The purity of the cell preparation is analyzed by immunocytochemistry by visualizing the islet hormones and by measuring islet hormone levels by utilizing radioimmunoassays.
3. Functional and metabolic activities are evaluated by measuring the glucose response of insulin biosynthesis and secretion, glycolysis, and oxidation.

4. Notes

1. It is important to proceed as soon as possible with the digestion; therefore, the dissection should be done fast. On average, one person should be able to process five rats within 30 min.
2. It is of crucial importance to test different batches of collagenase for their yield and toxicity. Therefore, the number of β-cells that survive the isolation and can be kept in culture without losing their functional responsiveness is determined by the quality of the collagenase. The concentration of the collagenase needs to be adapted according to both parameters (cell survival and functional responsiveness after isolation). For each isolation procedure, the collagenase solution has to be made freshly. The collagenase crystals are dissolved in isolation medium, the pH is adjusted to pH 7.4, and the solution is sterilized by filtration.
3. Progression of the digestion is closely monitored. The optimal duration of digestion varies with different batches of collagenase. An average of digesting for 20 min is achieved by adjusting the concentration of the collagenase solution. Once the digest has a milky appearance, the reaction is stopped.
4. All handling is done in a laminar-flow hood. No visible acinar cell mass should contaminate the hand-picked islets. Islets appear compact, bright, and white, whereas acinar tissue is fluffy and gray.
5. This procedure yields 7000–12,000 islets from 20 rat pancreata within 2–3 h after starting the dissection. Using this technique, only the larger-size islets of more than

100 μm are selected. This islet fraction represents more than 50% of the total insulin content of the adult rat pancreas.

6. Instead of being discarded, the fraction that is smaller than 100 μm is very suitable for use as a preparation enriched in acinar cells and small islets. Cellular composition: The "smaller than 100 μm" elutriation fraction contains less than 2% endocrine material, whereas the "larger than 100 μm" fraction is enriched in endocrine material up to 10%. After the islets have been hand-picked, this endocrine fraction contains 70–80% endocrine cells and less than 10% exocrine cells. Approximately 30% of the insulin hormone content is recovered in the "smaller than 100 μm" fraction and 60% in the "larger than 100 μm" fraction. The islet-enriched fraction contains 50% of the total insulin content.
7. The final cell suspension usually contains 5×10^5 to 1×10^6 cells per pancreas when starting from 20 rat pancreata.
8. The β-cell population consists of more than 95% insulin-containing cells and comprises less than 3% of glucagon-, somatostatin-, or pancreatic polypeptide-containing cells. Between 92% and 100% of the cells are single. The non-β-cell subpopulation consists of 75–85% glucagon-, 2–5% insulin-, 5–10% somatostatin, and 5–10% pancreatic polypeptide-expressing cells.
9. Cultured aggregates of β-cells display much less central necrosis, as compared to cultured islets, probably because of increased oxygen and nutrient diffusion.

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