Title
Cellular Composition and Fractional β-Cell Viability Assay Protocol at the Diabetes Research Institute – University of Miami

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Cellular Composition and Fractional β-Cell Viability Assay

PURPOSE: To outline the procedure for assessment of cellular composition and fractional beta cell viability for purified islets of Langerhans.

I. INTRODUCTION

The following procedure describes the use of image and flow cytometric analysis to determine beta cell content and fractional viability in the final islet cell preparation designated for transplant. The samples are examined through the use of Laser Scanning Cytometry (LSC) for cell composition, while β-cell-specific apoptosis at the mitochondrial level is identified through FACS analysis.

II. EQUIPMENT AND MATERIALS

A. Equipment

1. FACSCalibur, which uses Cell Quest Pro software (Becton Dickson)
2. Laser Scanning Cytometer (Compucyte, Cambrige, MA).
3. Waterbath @ 37^0C
4. Centrifuge @ 4^0C (Allegra 6R Centrifuge, Beckman Coulter)
5. Micro pipettors, 1-20, 20-200, 200-1000 uL (Pipettman, Gilson or equivalent)

B. Supplies and Materials

1. Disposable conical tubes, 15 ml (Corning, Cat # 430055)
2. Disposable FACS tubes, 4.5 ml (Falcon, Cat #-352054)
3. Nylon filter, 50 μm
4. Glass slide (VWR, Cat # 48311-703)
5. Cover slip (Corning, Cat # 48371-045)
6. Pap pen (Molecular Probes, Cat # 2377821-1E)
7. Disposable micro-pipette tips, 1-20, 20-200, 200-1000 μL

C. Reagents

1. Accutase (Innovative Cell Technologies, Inc. Cat # AT104)
2. Paraformaldehyde 16% (Electron Microscopy Sciences, Cat # 15710)
3. OptiMax washing solution (BioGenex, Cat # HK583-5K)
4. Fluorescent Dyes:
   - Newport Green (NG) (Cat# 24191 - Molecular Probes)
   - Tetramethylrhodamine TMRE) (Molecular Probes, Cat# T669)
   - 7-Aminoactomycin D (7-AAD) (Molecular Probes, Cat# A1310)
5. Antibodies:
   - Mouse α-Insulin antibody (Neomarker, Cat# RB-038-A)

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

- Rabbit α-Somatostatin antibody (DakoCytomation, Cat#A0566)
- Mouse α-Glucagon antibody (Sigma-Aldrich, Cat# 62654)
- Rabbit α-pancreatic polypeptide antibody (DakoCytomation Cat#M0619)
- Goat α-mouse 2° antibody conjugated with Alexa Flour 488 (Molecular Probes, Cat# A-11029)
- Gout α-rabbit 2° antibody conjugated with Alexa Flour 647 (Molecular Probes Cat# A-212245)

6. DPBS, without Ca^{++} and Mg^{++}
7. Fetal Calf Serum (Hyclone, Cat#SH30088.03)
8. Anti-fade solution (Molecular Probes, Ca# P7481)
9. 4',6-diamidino-2-phenylindole (DAPI) (DakoCytomation, Cat# D1306)
10. Protein Block (Bio-Genex, Cat#HK112-9K)
11. Common AB Diluent (Bio-Genex Cat#HK156-5K)

III. LIMITATIONS OR SPECIAL CONSIDERATIONS

A. These procedures must be performed by properly trained FACS and LSC users.

B. To allow for the best possible outcome, FACS acquisition must be performed immediately after samples are stained.

IV. PROCEDURE

A. Islet Cell Preparation:

1 Collect islets into a 15mL conical tube as follows:

NOTE: for islets of high purity, 90% collect 2,000 IEQ; for islets that are 70% pure, collect 1,500 IEQ; for islets with a purity of ≤ 50%, collect 1,000 IEQ. (This is to try and keep the final tissue volume similar regardless of islet purity, so that the enzymatic treatment can be performed with identical volumes of reagents).

Centrifuge at 280xg for 3 minutes at 4°C, and remove the supernatant. Wash islets twice with DPBS using the same centrifuge speed.

2. Dissociation:

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

- Add 1 mL of Accutase pre-warmed to 37°C. Mix well and incubate in a 37°C water-bath for 10 minutes.

- To the same tube, add 1 mL of undiluted cold FCS (4°C) to stop the enzymatic action of Accutase. Vortex quickly once or twice to dissociate the islets.

- Cover a 4.5 ml FACS tube with a Nylon filter. Using micropipette, transfer the sample, passing it through the Nylon filter, from the 15 ml conical where dissociation took place, into the prepared FACS tube.

- Use 1 mL of DPBS to rinse the Nylon filter in order to make sure that there are no cells left on the filter.

- Centrifuge the suspension at 280xg for 5 min at 4°C. Remove the supernatant, resuspend islet cells with 70-80 micromole DPBS to get concentration of approximately 10,000 cells per microliter (10×10^6).

- Transfer two aliquots of 10 microliters (1×10^5 cells each) to two fresh FACS tubes (labeled as no. 1-2). Save the remaining cells for LSC analysis adjusting the concentration to 10,000 cells per microliter by diluting them in DPBS.

B. Media Preparation for FACS:

1. **Newport Green (NG):**
   - Stock solution of NG is prepared @ 1 mM in DMSO and stored in -20^0C freezer.
   - Working solution (1 µ M) is prepared by diluting stock 1:1,000 in DPBS.

2. **TMRE:**
   - Stock solution of TMRE is prepared @ 10 mM in DMSO and stored at -20^0C freezer.
   - Working solution (100 nM) is prepared by diluting stock 1:100,000 in DPBS (stock is diluted twice: first to 1:1,000, followed by 1:100).

3. **NG/TMRE mixture:**
   - Prepare 1 ml of NG/TMRE mixture by adding 1 µl of NG stock (1 mM) to 1 ml of TMRE working solution (100 nM).

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

4. **7-AAD:**
   - 7-AAD stock solution is prepared @ 1 mg/ml in 10% DMSO in DPBS. Working solution is prepared by diluting stock 1:200 in DPBS.

C. FACS Sample Preparation:

1. Add 300 μL of NG 1 μM solution to FACS tube #1
2. Add 300 μL of TMRE (100 nM) solution to FACS tube #2
3. Add 300 μl of a NG/TMRE mixture (1 μM/100 nM) respectively to FACS tubes #3 and 4.
4. Mix well and incubate for 45 hour at 37°C.
5. Wash all (1-4) tubes twice, with 1 mL of DPBS by centrifuging at 500xg for 5 min, and remove the supernatant.
6. Add 300 μL of 7-AAD (5-10 μg/ml) and incubate for 5 min at room temperature.
7. Analyze stained samples as soon as it is possible.

C. Analysis of samples by FACS: Determination of fractional β-cell viability.

FACS analysis requires training that is beyond the scope of this SOP.
We have here listed some highlights of the analytical approach that is peculiar to this protocol. Care should be used in setting the acquisition parameters so that all events are acquired, and color compensation should be performed by using the single-stained samples (NG only and TMRE only), to prevent false readings due to the leaking of one fluorochrome in the other acquisition channel.

<u>Compensation and detection level adjustment</u>

Utilize the two tubes labeled 1 and 2
1. NG and 7AAD

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

2. TMRE and 7AAD,

2. Click on set up and acquire cells in tube2. Adjust forward scatter and SSC level to see whole cells within dot plot opened with X axis-forward scatter, Y axis-SSC.

3. Adjust Fl3 detection level to set 7AAD positive population around 10^2 level and negative population @ less than 10^2 level in dot plot opened with X axis-Fl2, Y axis-Fl3.

4. Adjust Fl2 detection level to set TMRE positive population around the level of 10^2 in a dot plot opened with Fl1 on X axis, Fl2 on the Y axis.

5. Acquire tube1. Adjust Fl1 detection level to set NG bright positive population above the level of 10^2. Adjust Fl2-Fl1 compensation to set Fl2 level of NG bright positive population below 10^1.

Acquisition

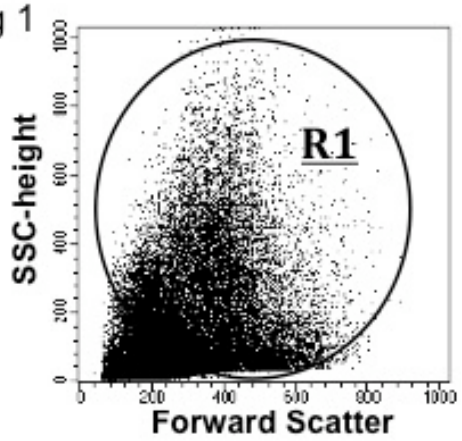
Events are acquired by standard techniques recording side scatter and forward scatter in a two dimensional plot. Both SSC AND FS are in linear (not Log) scale.
Additionally, each event is scored for its emission in FL1, FL2 and FL3, channels, which allow the measure of fluorochromes with diverse spectra.
NG emits in the FL1 detection spectrum, TMRE in the FL2 and 7-AAD in the FL 3.
Note that we do not gate out any event at this time, since gating is performed after acquisition, during analysis.

Analysis

1. Acquire at least 50,000 events, open dot plot (X axis-Forward Scatter, Y axis-SSC).
Remove debris from further analysis with R1 gate (Figure 1).

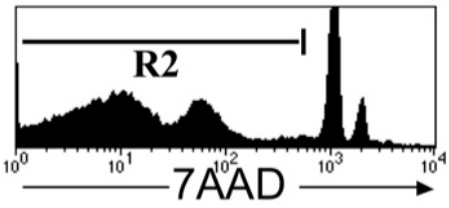
CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

Fig 1



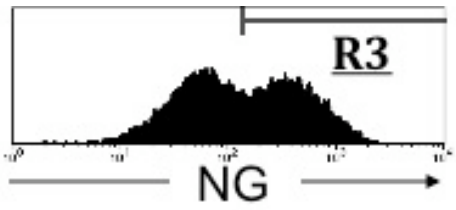
2. Open histogram plot (prameter-FI3, Gate-R1).
Dead cells can be evaluated by 7-AAD positivity.
Gate 7AAD negative cells like shown in Figure 2 for further analysis.

Fig 2



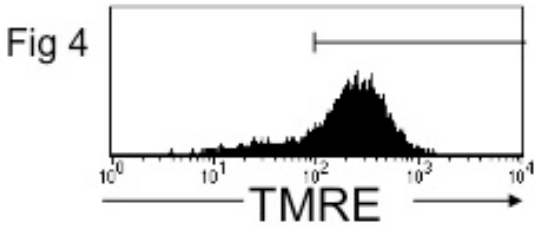
3. Open histogram plot (prameter-FI1, Gate-R1 and R2). Beta cell content (%) can be evaluated by Newport green(NG) bright positive event count.
Gate NG bright positive population like shown in Figure 3.

Fig 3



4. Open histogram plot (prameter-FI2, Gate-R1, R2 and R3). Non-apoptotic beta-cell (%) can be evaluated by TMRE positive event analysis (Figure 4).

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042



D. Sample Preparation for LSC

1. Clearly label each slide with the following information:
   a. HP#
   b. Layer#
   c. Length of Culture
   d. Date of Sample

2. Using a pap pen, make two circles on a glass slide (at least 1 slide is required for the assessment of cellular composition).

3. The optimal final cell concentration of the sample, is 10,000/μL (dilute the sample after counting and removing the aliquots for FACS analysis)). Place two ~3 μL samples on a glass slide, each within a circle made with a pap pen, and spread each sample to a diameter of ~ 0.5 cm using the pipette tip.

3. Allow the cells to dry (5-10 minutes), and **immediately** cover the area with 2.5% Paraformaldehyde, and incubate for 10 minutes at room T, to fix the cells. If cells are left on the slide for longer that 10 minutes after air drying and prior to paraformaldehyde fixation, results can be unreliable.

4. Aspirate the excess Paraformaldehyde, ensure that the fixed cells are not scraped and/or washed off.

5. Prepare the Optimax working solution by diluting the stock 1:20 in distilled water. Wash the slides three times (1 min each time) with Optimax working solution.

6. At this point the slides may be stored at 4°C before further analysis (to be performed within a week). If longer time is expected to pass before analysis, it is advisable to store samples at least at -20°C.

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

E. Sample Staining for LSC analysis

1. Incubate the slides once with Optimax working solution for 5min at room T. Aspirate with Pasteur pipette.

2. Incubate with non-diluted Protein Block for 30 min at room T, to reduce non-specific binding. Aspirate.

3. Incubate one of the two samples on a slide for 1-2 hours at room T with the following antibodies:

   A) A combination of:
   - Mouse monoclonal anti-insulin antibody (Ab). Prepare 1:100 dilution, and
   - Rabbit polyclonal anti-somatostatin Ab, 1:500 dilution

4. Incubate the second of the two samples for 1-2 hours at room T with the following antibodies:

   B) A combination of:
   - Mouse monoclonal anti-glucagon Ab, 1:500 dilution and
   - Rabbit polyclonal anti-pancreatic polypeptide Ab, 1:1000 dilution

   NOTE: *All dilutions should be done in Common AB Diluent buffer (Bio-Genex).*

5. Wash the slides three times (1 min each) using Optimax working solution

6. Incubate the slides with a combination of goat α-mouse (Alexa Fluor 488-conjugated goat anti-mouse IgG, 1:200 dilution) and goat α-rabbit (Alexa Fluor 647-conjugated goat anti-rabbit IgG, 1:200 dilution) secondary antibodies. Omission of the primary antibodies serves as a negative control.

7. Wash the slides three times (1 min each).using Optimax working solution

8. Apply 4', 6-diamidino-2-phenylindole (DAPI, 17µg/ml in AB dilution buffer) to the slides to stain cell nuclei for 5 min at room T.

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

DAPI is diluted to the working concentration from a stock @ 5 mg/ml by a 1:300 dilution.

9. Wash slides once with Optimax working solution.
10. Apply anti fade solution as per manufacturer's instructions.
11. Mount coverslip.
12. Analyze the samples using an LSC. (CompuCyte, Cambrige, MA, USA).

E. Analysis of Slides by LSC: Analysis of cellular composition by immunofluorescence.

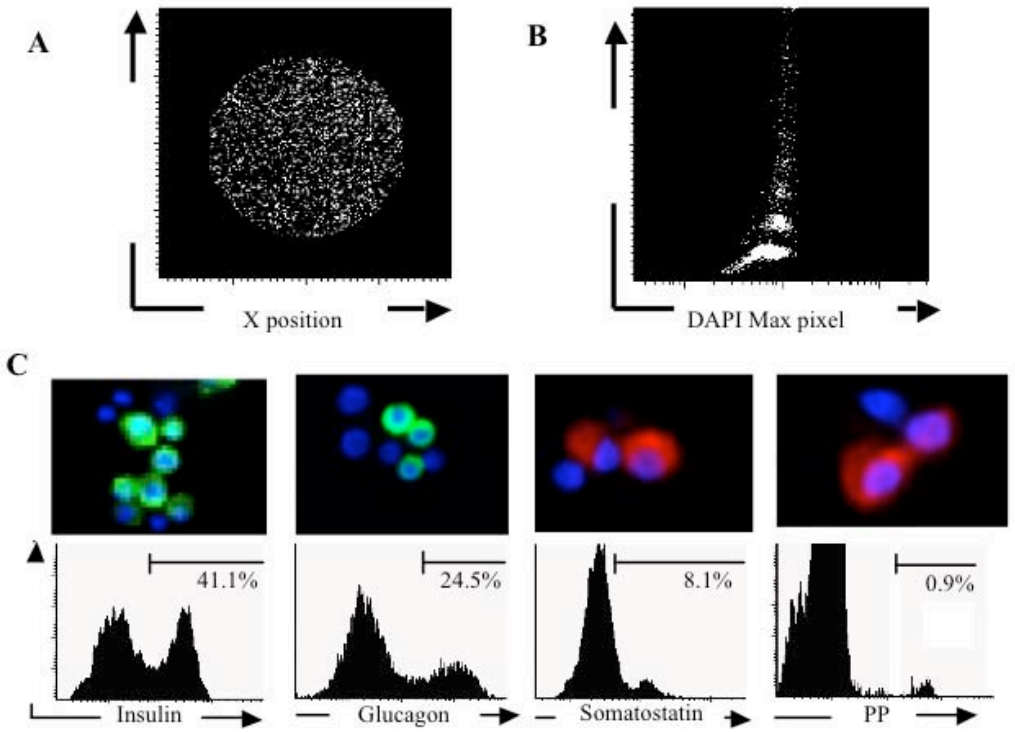
1. The LSC allows for fluorescence-based quantitative measurements on tissue sections or other cellular preparations at single-cell level. An optics/electronics unit coupled to an argon and HeNe laser repeatedly scans along a line as the surface is moved past it on a computer-controlled motorized stage of an Olympus BX50 fluorescent microscope. LSC is used to determine the percentage of each hormone-positive cell on the glass slides. The area to be scanned is visually located, and mapped using the Wincyte software (CompuCyte) (Figure 5A). Slides are scanned at 40x, and nuclei are contoured using UV laser and DAPI detector. Each hormone-positive event is then recorded using the argon laser coupled with the green (Alexa-488) detector, and the HeNe laser coupled with the far-red (Alexa-647) detector. Data that are acquired for all channels are: area, x position, y position, fluorescence integral, and maximal intensity.

   Single cells are identified and gated according to the DAPI staining area (Figure 5B). Fluorescence intensity is recorded on a histogram (Figure 5C). Cells from every subpopulation are visualized directly in the LSC by relocalization to confirm regular morphology. A minimum of 10,000 cells are acquired and analyzed for each sample.
   The LSC allows for fluorescence-based quantitative measurements of tissue sections or other cellular preparations at a single-cell level. In this case, the LSC is used to determine the percentage of each hormone-positive cell on a glass slide. Beta-, α-, δ- and PP-cells

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

were counted and individual cell percentages are calculated by using the DAPI staining on a total number of cells.

Fig 5



2. **Assessment of β-cell content in dissociated islets**

Beta-cell content within islets is calculated based on the analysis of immunostaining for endocrine markers by LSC using the following formula:

$$\frac{\beta\text{-cell}\%}{(\beta + \alpha + \delta + PP\text{–cell})\%} \times 100$$

Where β are beta-cells, α are alpha cells, δ are delta cells, and PP cells are those that secrete pancreatic polypeptide.

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CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

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CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042

CellR4 2014; 2 (4): e1121 – www.cellr4.org – ISSN: 2329-7042