# Islet Viability Assessment by Single Cell Flow Cytometry

**Purpose:** To comprehensively assess the viability of the islet cell preparation prior to transplantation.

## Tissue Samples:

- A sample containing ~ 5-10,000 IEQ from the islet preparation intended for transplant.
- A sample of INS-1 cells (any cell line can serve this function) as a "viable cell" control.

## General Materials:

Dithizone, counting dishes, inverted microscope
12 x 75 mm BD Falcon tubes
50 ml conical centrifuge tubes
BD 50 ml conical tube 40 µm cell strainer filters (Ref.# 352340)
37$^0$C 5% $CO_2$ incubator
p1000 pipettor with standard precision tips
p20 pipettor with standard precision tips

## Reagents:

Trypsin (0.05%) EDTA (0.53 mM) for islet/acinar dispersion (Cellgro Product # 25-052-CI)
Culture media (CMRL 1066 with HSA or FBS) used for Trypsin inactivation)

Recommended buffer for islet cell staining:

1 x Modified Kreb's Ringer Bicarbonate (Base Solution) with 3.3 mM glucose

**1 x MKRB Stock Solution**

| Chemical | F.W. | [mM] | g/L |
|---|---|---|---|
| NaCl | 58.44 | 137 | 8.0 |
| KCl | 74.55 | 4.7 | 0.44 |
| $KH_2PO_4$ | 136.1 | 1.2 | 0.16 |
| $MgSO_4$-$7H_2O$ | 246.48 | 1.2 | 0.3 |
| $CaCl_2$-$2H_2O$ | 147 | 2.5 | 0.37 |
| $NaHCO_3$ | 84.01 | 25 | 2.1 |
| HEPES | 1M stock | 10 | 10.0 ml |

- pH to 7.4 with HCL or NaOH if necessary, **do not charge with 95% $O_2$/5% $CO_2$** as this will increase the ROS background.
- Sterilize by filtration
- Shelf life is ~ 1 month

**1x MKRB (working solution) – PREPARE ON DAY OF USE!**

| MKRB | Glucose [1.65 M] | BSA (optional) |
|---|---|---|
| 100 ml | 200 µl | .25 g |

pre-warm to 37$^0$C for use in islet cell staining and flow analysis

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## Fluorescent probes (supplier):

***ToPro-3* (viability dye)** (Invitrogen) – use at ~20 nM in the final sample just prior to analysis on the FACS Vantage

- Stock is 1 mM in DMSO.
- Dilute 1 µl of stock in 999 µl of 1x PBS [1:1000, 1 µM].
- Use 10 µl of 1 µM ToPro3 stock per 0.5 ml of sample just prior to analysis.

***Caspace FITC* (apoptosis)** (Promega)– use at 1 µM for cell loading prior to analysis

- Stock is 1 mM in DMSO.
- Use 1 µl per 1.0 ml of cell suspension for a final concentration of 1 µM.
- Incubate cells for 30 minutes at $37^0$C in the $CO_2$ incubator.

***Annexin V PE (apoptosis)*** (Invitrogen) – use 3-4 µl per 1.0 ml islet cell sample.

**Dihydroethidine (reactive oxygen species)** (Invitrogen) – 5 mM stock, use at 5 µM for 5-7 min of staining at room temp. No need to wash out. Visualize using a 550 LP with a 575/26 for emission detection. The fluorescent product that is created upon reaction with superoxide anion emits at 567nm. It is critical that DHE staining NOT be performed in oxygenated media.

**JC-1 (mitochondrial membrane potential** (Invitrogen**)** – use 10 µl per 1.0 ml islet cell sample. Visualize using the 530/30 (FL1 Green) and 575/25 or 585/42 (FL2 Orange) PMTs for emission detection. Cells with healthy polarized mitochondria should have a greater JC-1 Red>Green fluorescence ratio. CCCP is a mitochondrial membrane uncoupler which induces rapid depolarization and is a useful control.

**CCCP** [50 mM] – use 2 µl per 1.0 ml islet cell sample.

**FDA (viability)** (Invitrogen) – stock is 5 mg/ml in acetone. Final working concentration for single cell staining is 10 ng/ml. Dilute the stock 1:100 dilution into 1x PBS, then dilute the 1:100 stock 1:10 into 1x PBS to make the working solution, use 2 µl per 1.0 ml of islet cell sample. Incubate cells at $37^o$C for 25-30 minutes.

**7-AAD (necrosis)** – (BD Biosciences) – ready to use solution. Use 10-20 µl per sample and incubate for 10 – 20 minutes prior to analysis. No washing is necessary. For use on FACScalibur flow cytometers without a 635nm diode laser. Excitation can be achieved with the 488 nm argon laser with detection at 650 nm.

**Propidium Iodide – (necrosis)** (Invitrogen**)** [50 µg/ml] use 10 µl/ 1 ml sample, add just before analysis (ex 488 nm, em 670 nm)

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### Staining Protocol:

1. Trypsinize a sufficient quantity of islets (2000 – 5000 IEQ) and INS-1 cells (optional) to produce ~ 1.4 x $10^6$ cells.
    a. Incubation with trypsin should not exceed 5 minutes at 37$^o$C. After the 5 minute incubation, the cells should be pipeted through a p1000 tip and then a p200 tip. INS-1 cells come free from the flask easily after only 3-5 minutes of trypsin exposure.
    b. 2000 IEQ yields ~ 1.0 x $10^6$ cells.
2. Add an equal volume of culture media to quench the trypsin.
3. Filter the cell suspensions through a 40 µm BD filter into a 50 ml tube.
4. Remove a 10 µl sample for counting using a hemocytometer.
5. Pellet the cells by centrifugation (5 minutes, 1000 rpm, room temp, with brake).
6. Decant the supernatant, tap the tube to resuspend the cells, add 10 ml of islet staining buffer (MKRB and 3.3 mM glucose, pH 7.4).
7. Pellet the cells by centrifugation (5 minutes, 1000 rpm, room temp, with brake).
8. Decant the supernatant, tap the tube to resuspend the cells, add 12 ml of islet staining buffer, aliquot 1ml into each of the fourteen (14) 12 x 75 mm snap cap tubes.
    a. Target cell concentration per tube is 1.0 x $10^5$ to 1.0 x $10^6$ cells
9. Add the appropriate staining reagents (**DO NOT ADD THE HYDROXY ETHIDINE**).
10. Incubate at 37$^o$C for a minimum of 25 minutes in the dark (a tissue culture incubator works well).
11. Wash all of the tubes with 1.0 ml of staining buffer.
12. Pellet the cells by centrifugation (5 minutes, 1000 rpm, room temp, with brake).
13. Decant the supernatant, tap the tubes to resuspend the cells.
14. Repeat steps 11 – 13.
15. Resuspend the cells in 0.5 ml of islet staining buffer and place in a covered container ***without ice***!
16. Proceed to the flow cytometer immediately!
17. Add the Hydroxyethidine within 10 minutes of running the samples on the flow cytometer. No washing is necessary. Be sure to collect the 488 excited light emission using a 575/25 filter not a 615/25.

## Islet Viability Assessment by Single Cell Flow Cytometry

### Sample Staining Guide :

#### For flow cytometers with 325, 488 and 633 nm excitation lasers

| | |
|---|---|
| Tube # 1. | Unstained |
| Tube # 2. | ToPro3 alone control |
| Tube # 3. | FDA alone control |
| Tube # 4. | VADFMK FITC alone control |
| Tube # 5. | Annexin V PE alone control |

| | |
|---|---|
| Tube # 6. | FDA + ToPro3 |
| Tube # 7. | FDA + ToPro3 |
| Tube # 8. | FDA + ToPro3 |

| | |
|---|---|
| Tube # 9. | VADFMK FITC + Annexin V PE + ToPro3 |
| Tube # 10. | VADFMK FITC + Annexin V PE + ToPro3 |
| Tube # 11. | VADFMK FITC + Annexin V PE + ToPro3 |

| | |
|---|---|
| Tube # 12. | JC-1 + ToPro3 |
| Tube # 13. | JC-1 + ToPro3 |
| Tube # 14. | JC-1 + ToPro3 |
| Tube # 15. | JC-1 + CCCP + ToPro3 |

| | |
|---|---|
| Tube # 16. | DHE + ToPro3 |
| Tube # 17. | DHE + ToPro3 |
| Tube # 18. | DHE + ToPro3 |

| | |
|---|---|
| Tube # 19. | JC-1 alone control (optional) |
| Tube # 20. | DHE alone control (optional) |

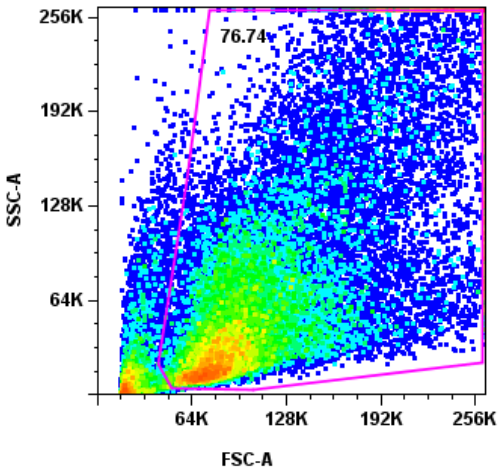
**alternate for ToPro3 is 7-AAD (but do not add to the DHE samples).

*** be sure to appropriately compensate the 670 signal out of the 575 signal when acquiring and analyzing the DHE data.

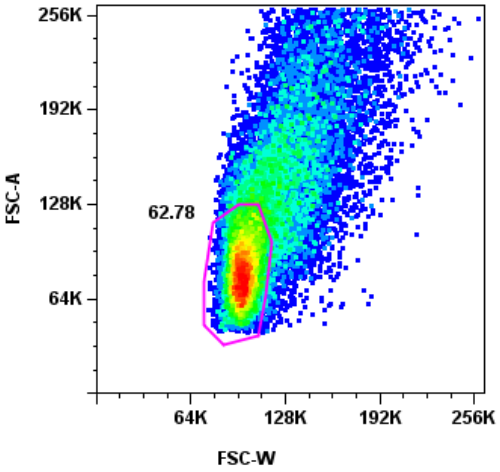
# Islet Viability Assessment by Single Cell Flow Cytometry

## Example Data from a High Quality Human Islet Preparation

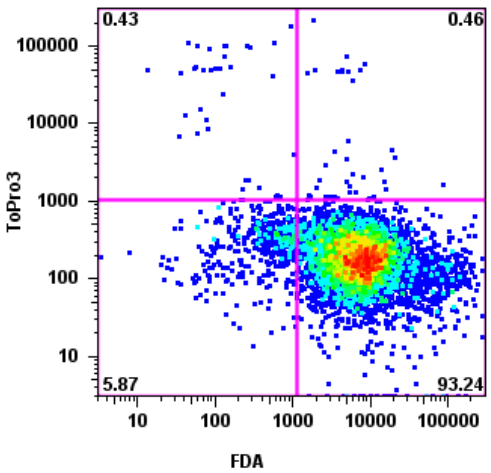
### Intact Cell %



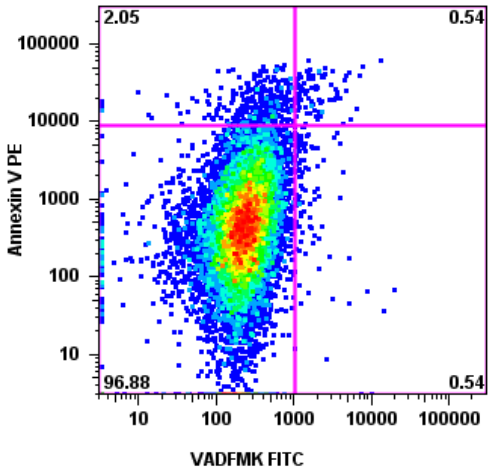

### Gating for Single Cells



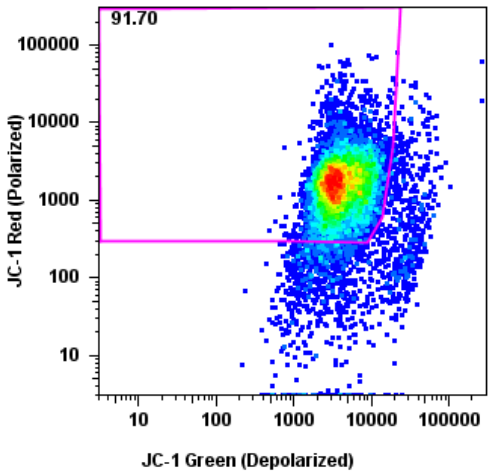
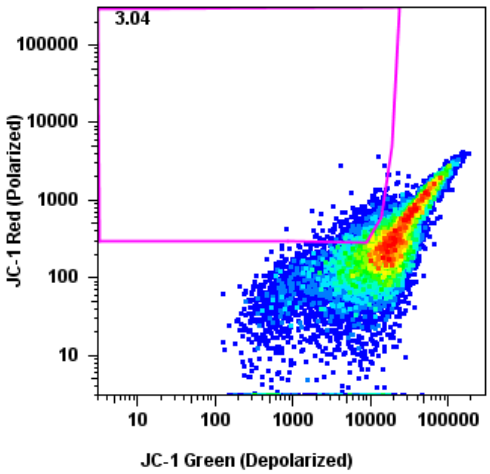

### Viability by FDA x ToPro3




### Apoptosis



## Islet Viability Assessment by Single Cell Flow Cytometry

| Mitochondrial Membrane Potential by JC-1 | CCCP Control for Mito. Membrane Potential |
|---|---|
|  |  |

# Islet Flow Cytometry Data Worksheet

| | |
|---|---|
| **Islet Prep. Code** | |
| **Islet Sample Concentration [IEQ/mL]** | |
| **Sample Volume Used for Assay [mL]** | |
| **Total IEQ Used for Assay** | |

**Comments on Islet Quality:**

**Hemocytometer Count of Trypsinized Islet Sample (Trypan blue is optional):**

| | Live (Trypan blue-) | Dead (Trypan blue+) | Total Count | % Viable (live/total) |
|---|---|---|---|---|
| Grid 1: | _____ | _____ | _____ | _____ |
| Grid 2: | _____ | _____ | _____ | _____ |
| Grid 3: | _____ | _____ | _____ | _____ |
| Grid 4: | _____ | _____ | _____ | _____ |
| **Average:** | _____ | _____ | _____ | _____ |

**Average Total Count:** _____ x $10^4$ = _____/mL

**Total Cell Count:** _____/mL x _____ mL (total sample volume) = _____

**Islet Cells Aliquoted Per Flow Sample**: Total Cell Count/16 = _____

**Number of Islet Cells Obtained per IEQ:**

Total Cell Count/IEQ trypsinized = _____/_____ =

# Islet Flow Cytometry Data Worksheet

**DATA ANALYSIS:** Isolation Code: _____ Date: _____
Experimentor: _____

1. Gate cells first on FSC x SSC to eliminate debris.
2. Gate cells for single cells using FSC –A vs FSC-W comparison.
3. Determine %viability based upon single cells.
4. Use single color controls to establish correct compensation and gating schemes.

| | | Samples | |
|---|---|---|---|
| **Objective:** | **Stain combination:** | | |
| **Intact cells** | FSC x SSC gated events | | |
| | FSC x SSC gated events | | |
| | FSC x SSC gated events | | |
| | **Mean ± SD** | | |
| **Viability** | % FDA bright ToPro3- | | |
| | % FDA bright ToPro3- | | |
| | % FDA bright ToPro3- | | |
| | **Mean ± SD** | | |
| **Apoptotic% of Viable Cells** | %VADFMK+ToPro3-/dull | | |
| | %Annexin V+ ToPro3-/dull | | |
| | %VADFMK+ and AnnexinV+ ToPro3-/dull | | |
| | %VADFMK+ and AnnexinV+ ToPro3-/dull | | |
| | %VADFMK+ and AnnexinV+ ToPro3-/dull | | |
| | **Mean ± SD** | | |
| **Apoptosis Adjusted Viability** | (% Viable - Apoptotics) x (%ToPro3-/dull ) | | |
| | (% Viable - Apoptotics) x (%ToPro3-/dull ) | | |
| | (% Viable - Apoptotics) x (%ToPro3-/dull ) | | |
| | **Mean ± SD** | | |
| **Mitochondrial Membrane Potential of Viable Cells** | %JC-1 Red>Green ToPro3-/dull | | |
| | %JC-1 Red>Green ToPro3-/dull | | |
| | %JC-1 Red>Green ToPro3-/dull | | |
| | **Mean ± SD** | | |
| **CCCP treated Control** | %JC-1 Red>Green ToPro3-/dull | | |
| **Reactive Oxygen Species (superoxide anion accumulation) in Viable Cells** | %DHE+ ToPro3-/dull | | |
| | %DHE+ ToPro3-/dull | | |
| | %DHE+ ToPro3-/dull | | |
| | **Mean ± SD** | | |
| **Additional Probes Used:** | | | |
| | | | |
| | | | |