Application Note
Neonatal Cardiomyocytes

Isolation, Quantitation and Viability Analysis of Neonatal Cardiomyocytes using Cellometer

Cellometer®
Cell Viability

Introduction: Neonatal Rat Cardiomyocyte Model

The neonatal rat cardiomyocyte model has been used for many years by researchers studying the heart. In addition to increasing understanding of the morphological, biochemical, and electrophysiological characteristics of the normal heart, neonatal cardiomyocytes have been used to study contraction, ischemia, hypoxia, and the toxicity of different compounds. This model has been used to determine the optimal dosage for certain drugs and to develop and evaluate the efficacy of potential therapeutic agents. Apoptosis, or programmed cell death, has been observed in infarcted and re-perfused myocardium, end stages of heart failure, post-infarction left ventricular remodeling, and diabetes, making apoptosis a key focus in the neonatal rat cardiomyocyte model[1]. In recent years, research into cellular therapy and regenerative medicine has increased. Studies involving the transplant of neonatal rat cardiomyocytes into adult rats following cardiac infarction have generated positive results, yielding improvement in local tissue and overall cardiac function[2].

Increased yield and accurate assessment of cell viability of isolated cardiomyocytes are critical to ongoing cardiac research studies involving the neonatal rat cardiomyocyte model. Nexcelom Bioscience and Worthington Biochemical have demonstrated a simple, consistent procedure for isolation and assessment of viable neonatal cardiomyocytes.

1. "Neonatal Rat Cardiomyocytes - A Model for the Study of Morphological, Biochemical, and Electrophysiological Characteristics of the Heart", S. Chlopčiková, J. Psotová, P. Miketová, Biomed. Papers, Volume 145, Number 2, pages 49-55. 2001

2. "Cell Therapy Enhances Function of Remote Non-Infarcted Myocardium", A. Moreno-Gonzalez, F. S. Korte, J. Dai, K. Chen, B. Ho, H. Reinecke, C. E. Murry, M Regnier, J. Mol. Cell Cardiol., Volume 47, Number 5, pages 603-613. 2009

Experimental Procedure

Primary Reagents:

- Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp., Part# LK003300)
  - CMF HBSS (sterile calcium- and magnesium-free Hank's Balanced Salt Solution)
  - Worthington Trypsin
  - Worthington Soybean Trypsin Inhibitor
  - Worthington Purified Collagenase
  - Leibovitz L-15 Media Powder
  - Cell Strainers
- Trypan Blue Stain or
- Cellometer ViaStain™ AO/PI (acridine orange / propidium iodide) Staining Solution (Nexcelom Bioscience, Part# CS2-0106-5ML)

Equipment:

- Cellometer Vision Cell Analyzer or Cellometer Auto 2000 Cell Viability Counter
- Centrifuge to operate at 50 to 100 x g
- 37°C water bath
- 37°C rocker or rotator
- Oxygen supply

Isolation of Neonatal Cardiomyocytes

The isolation of neonatal cardiomyocytes involves enzyme digestion to dissociate cells from the heart tissue followed by purification steps to remove non-muscle cells and tissue debris. The procedure is designed for maximum dissociation with minimal harm to the cardiomyocyte cells. The Worthington Neonatal Cardiomyocyte Isolation System utilizes purified trypsin and collagenase enzyme preparations to maximize cell dissociation and viability and reduce lot-to-lot variability. Each kit lot undergoes strict functional testing to ensure a consistent yield of viable cells.

Nexcelom Bioscience

Worthington® Biochemical Corporation

Dissociation Procedure

1. Isolate hearts from 5 to 15 rat pups, 1 to 4 days old.
2. Rinse hearts with CMF HBSS reagent
3. Transfer hearts to Petri dish and mince to less than 1mm^3 pieces keeping tissue at 0°C.
4. Add CMF HBSS and Trypsin to Petri dish. Incubate overnight (16-20 hours) at 2-8°C.
5. Transfer tissue and buffer to 50 mL centrifuge tube on ice.
6. Add Trypsin Inhibitor and mix.
7. Oxygenate tissue for 30 seconds to 1 minute
8. Warm tissue and buffer to 30-37°C in water bath for 3-5 minutes maintaining sterility
9. Add Worthington Purified Collagenase and incubate on rocker or rotator at 37°C for 30-45 minutes.
10. In a sterile hood at room temperature, use a 10 mL plastic serological pipette to triturate 10 times to release cells
11. Allow tissue residue to settle for 3 to 4 minutes, then filter through a Cell Strainer into a fresh 50 mL centrifuge tube
12. Add 5mL of culture medium, then repeat trituration step. Filter cells, oxygenate cells for 1 minute, then let sit for 20 min. at room temperature.
13. Sediment cells at 50 to 100 x g for 5 minutes.
14. Suspend final cell pellet in suitable culture medium (1 ml per heart). Routine cell yields are 2-3 x 10^6 cardiomyocytes per heart.
15. Check cell concentration and viability using the procedure below and adjust cell concentration if necessary.
16. Dispense cells into plates or flasks and place in a 37°C incubator. Leave cells undisturbed for 24 hours.

Determination of Cell Concentration and Viability

The Cellometer Vision Cell Analyzer and Cellometer Auto 2000 Cell Viability Counter offer pre-set viability assays for a wide variety of primary cell types, including PBMCs, stem cells, nucleated cells, and splenocytes. For this experiment, we tested viability using a traditional trypan blue method and also created a new assay for analysis of cardiomyocytes dissociated from cardiac tissue and analyzed using a dual-fluorescent AO/PI method. The AO/PI method is highly recommended for samples containing debris. With the Cellometer systems, users can easily create new assays for specific cell types with optimized imaging and counting parameters.

Trypan Blue Viability Staining Procedure

1. Combine 20μl of cardiomyocyte sample and 20μl of trypan blue dye solution and mix well by pipetting up and down
2. Load 20μl of sample into the disposable counting chamber
3. Allow cells to settle in chamber for 1 minute

AO/PI Viability Staining Procedure

1. Combine 20μl of cardiomyocyte sample and 20μl of AO/PI dye solution and mix well by pipetting up and down
2. Load 20μl of sample into the disposable counting chamber
3. Allow cells to settle in chamber for 1 minute

Brightfield Imaging

The Cellometer instrument acquires a brightfield image for each sample tested. The Brightfield image allows researchers to verify cell morphology, evaluate the degree of homogeneity of the sample, and identify the presence of cellular debris. The software detects darkly-shaded cells stained with trypan blue to calculate the number of live and dead cells and the percent viability for cell samples from very clean digestions.

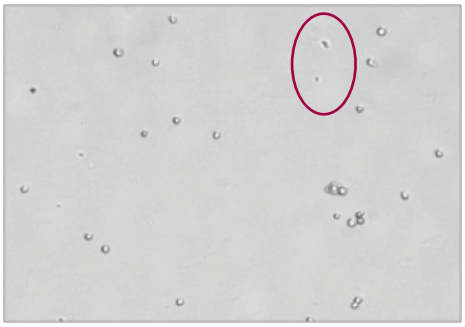

Image 1 Debris is indicated at left in the bright field image. Cell size diameter settings can be adjusted to eliminate smaller debris particles from cell counts.

Fluorescent Imaging

For accurate viability determination in samples containing cellular debris, the cardiomyocyte sample is incubated with an AO/PI dye mixture. The acridine orange dye stains DNA in the cell nucleus of both live and dead cells. Propidium iodide DNA-binding dye is used to determine cell viability. Healthy cells are impermeable to the PI dye. Only dead (non-viable) nucleated cells with compromised membranes are stained. Cells stained with both AO and PI fluoresce red due to quenching, so live nucleated cells fluoresce green and dead nucleated cells fluoresce red. There is no interference from cellular debris or non-nucleated cells, as all Cellometer AO/PI live and dead cell counts are conducted in the fluorescent channels.

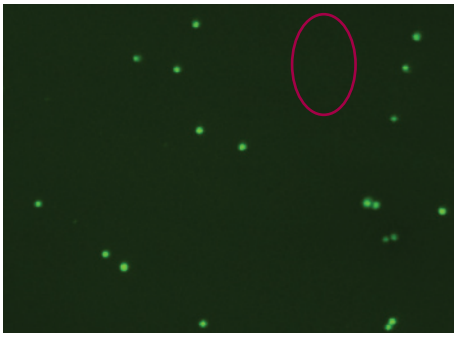

Image 2 Green fluorescent image displaying live nucleated cells. Debris observed in the bright field image (Image 1.) is not visible in the fluorescent channel.

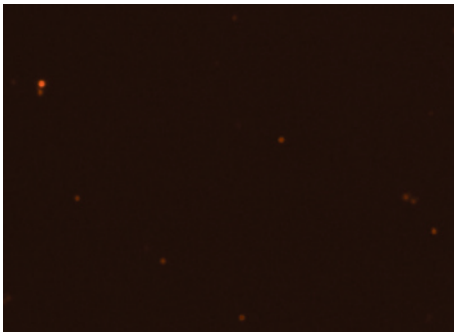

Image 3 Red fluorescent image displaying dead nucleated cells.

The images displayed represent a portion of one of the four areas of the Cellometer counting chamber imaged with the Cellometer instrument. Due to the high viability of the sample tested, there are fewer cells visible in the red fluorescent image.

The fluorescent counted images can be viewed to further verify results. In the image at right, the Cellometer software is correctly counting individual cells within clumps.

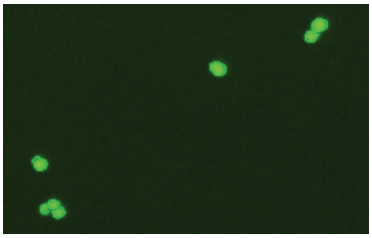

Image 4 Magnified green fluorescent image with live cells circled in green.

Automated Results

The Cellometer system acquires images and automatically calculates live and dead cardiomyocyte cell count, concentration, % viability, and mean cell diameter in <60 seconds. The images, data table, and cell diameter histogram can be easily saved or exported for additional analysis, presentation, or data archiving. Results of the trypan blue analysis of cells isolated from 6 rat neonatal hearts using the Cellometer Vision Cell Analyzer are outlined in the table below.

Trypan Blue Viability Results

Live Cell Count	3,549	cells
Dead Cell Count	604	cells
Total Cell Count	4,153	cells
Mean Live Cell Size	10.4	microns
Mean Dead Cell Size	9.2	microns
Mean Total Cell Size	10.2	microns
Viability	85.5	%
Live Conc.	1.00 x 10^7	cells/ml
Total Conc.	1.17 x 10^7	cells/ml

AO/PI Viability Results

An older cardiomyocyte sample used to demonstrate the AO/PI staining method was 89% viable, but exhibited a reduced number of total cells. Mean cell diameter was 10 microns. Based on observed results, it is strongly suggested that cardiomyocyte samples be analyzed immediately following isolation and transferred to proper culture conditions as soon as possible to preserve the cell population. The results table for the AO / PI analysis is shown below.

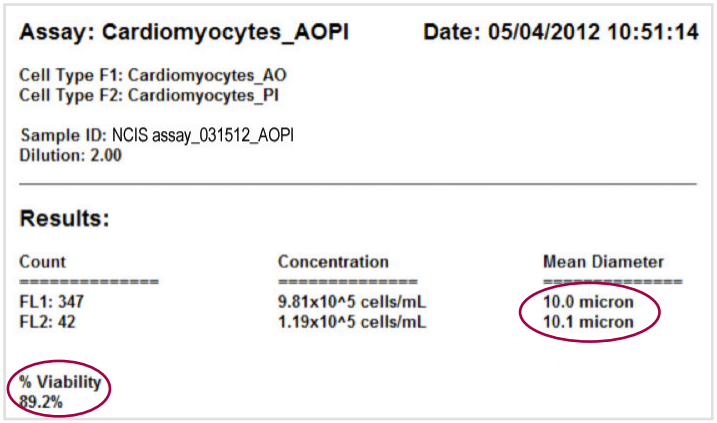

Figure 1 Automated data report from the Cellometer Vision Cell Analyzer for the AO / PI staining displaying live cell count and concentration, dead cell count and concentration, mean diameter and percent viability.

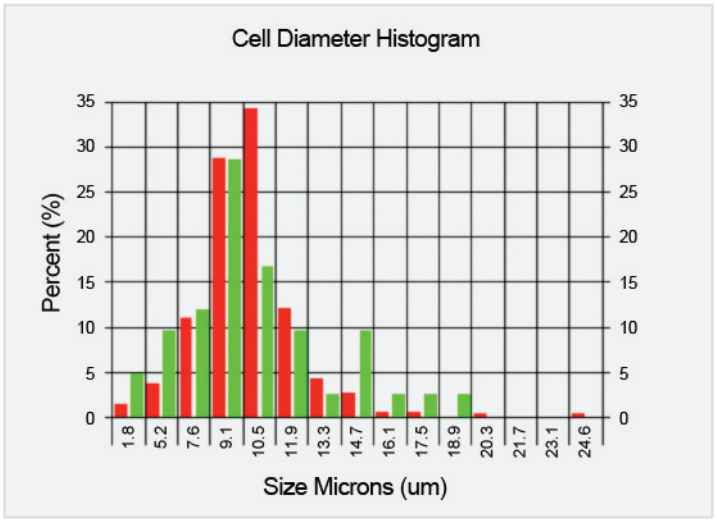

Figure 2 Cell diameter histogram for the cardiomyocyte sample tested. Mean cell diameter calculated by both trypan blue and AO/PI staining methods was 10 microns. The histogram for the AO / PI experiment is displayed at left.

Conclusion

The cardiomyocyte sample generated using the procedure above was >85% viable. Cell concentration and viability determination were completed in <60 seconds.

The Worthington Neonatal Cardiomyocyte Isolation System is a complete, user-friendly system for isolation of viable neonatal cardiomyocytes. The kit includes all of the critical components required for reliable, reproducible cell isolation.

The Cellometer Vision Cell Analyzer and Cellometer Auto 2000 Cell Viability Counter reduce viability analysis time, ensuring the timely transfer of isolated neonatal cardiomyocytes to culture. Using only 20µl of sample, dual-fluorescence staining with Cellometer ViaStain AO/PI Staining Solution offers improved accuracy of viability results with no interference from debris or non-nucleated cells, ensuring a correct cell concentration for in vitro experiments and cell-based assays.

For more information on the Worthington Neonatal Cardiomyocyte Isolation System (LK003300) call Worthington Biochemical at 800-445-9603 or 732-942-1660, e-mail custservice@worthington-biochem.com, or visit www.worthington-biochem.com.

For more information on the Cellometer ViaStain AO/PI Staining Solution (Part# CS2-0106-5ML), the Cellometer Vision Cell Analyzer or the Cellometer Auto 2000 Cell Viability Counter, call Nexcelom at 978-327-5340, e-mail info@nexcelom.com, or visit www.nexcelom.com.

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