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Coulter Counter use in the enumeration of muscle and fat stem cells

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Abstract. Although the manual counting chamber (hemacytometer) is the gold standard for counting cells, this method is subject to great variability due to the 'human factor'. The automated cell counter (Coulter Counter) can enumerate cells in less time and with greater accuracy than the hemacytometer by removing many of the steps in which errors are made. While the Coulter Counter (and others of its type) has been used for many years in the cell culture field, there have been few studies to validate its use with specific cell types. We conducted several experiments in which we assessed the accuracy of the Coulter Counter over counts made with a hemacytometer as well as validated its use for the counting of satellite cells and preadipocytes.

Key words: Cell culture, counting, satellite cell, adipocyte, adipofibroblast

Abbreviations: DMEM – Dulbecco's modified Eagle medium; FBS – fetal bovine serum; PBS – phosphate buffered saline, pH 7.08; PSG – pigskin gelatin; SC – satellite cell

1. Introduction

The traditional tool for the enumeration of cells has been a manual counting chamber (hemacytometer) and a light microscope. Over the years, other more automated quantification methods have been developed including electronic particle counting, flow cytometry, and magnetic cell sorting [8]. Despite these modern advances, manual counting remains the standard for determining cell numbers in many research settings. Although the automated particle counter has been used for many years for both preadipocyte/adipocyte and satellite cell research [1–7, 9], we know of no study which validates the use of a Coulter Counter for determining cell numbers of satellite cells or preadipocytes for use in cell culture. This study was conducted to determine the validity of using such a particle counter for counting these types of cells.

2. Materials

A. Major equipment
  1. Autoclave, gravity air remover type, model P-89501-091.[1]
  2. Balance, model A-160.[6]
  3. Centrifuge, model TJ-6.[4]
  4. CO_2 water-jacketed incubator, model NU-4500.[14]
  5. Coulter particle counter, model Z1.[3]
  6. Dry heat gravity oven, model 1370 GM.[18]
  7. Hemacytometer, No. 15170-208.[18]
  8. Laminar flow, biological safety cabinet, Labguard Class II, Type A/B3, model NU-425-4000.[14]
  9. Microscope, Diaphot-TMD phase inverted.[13]
  10. Peristaltic pump, model 7015-72.[11]
  11. pH electrode, model 47636.[5]
  12. pH meter, digital, model 430.[5]
  13. Pipet Aid, model 174.[7]
  14. Propane torch, Bernz-o-matic model TS2000.[17]
  15. Water bath stainless steel, model 185.[15]

B. Media, reagents, and chemicals
  1. Dulbecco's modified Eagle's medium (DMEM), product number 12800-017.[8]
  2. Ethylenediamine tetraacetic acid (EDTA), product number 15576-028.[8]
  3. Fetal bovine serum (FBS), product number 26140-079.[8]
  4. Gentamicin, 10 mg/ml gentamicin sulfate, product number 15710-064.[8]
  5. Giemsa stain, product number GS-1L.[16]
  6. KCl, product number P-5405.[16]
  7. KH_2PO_4, product number P-5655.[16]
  8. NaCl, product number S-5886.[16]
  9. $NaHCO_3$, product number S-5761.[16]
  10. NaOH, pellet, product number 3722-I.[9]
  11. Na_2PO_4, product number S-5136.[16]

12. Penicillin-streptomycin (Pen/Strep), liquid; contains 10,000 units of penicillin G (sodium salt) and 10,000 µg of streptomycin sulfate/ml in 0.85% saline, product number 15140-122.[8]
13. Pig skin gelatin (PSG; porcine skin type A), product number G-1890.[16]
14. Trypsin, product number T-4549.[16]

C. Glassware
   1. Media bottles
      a. 125 ml with cap, product number 219715.[19]
      b. 250 ml with cap, product number 219717.[19]
      c. 500 ml with cap, product number 219719.[19]
   2. Pipettes
      a. 2 ml, glass disposable, product number 72120-21100.[10]
      b. 5 ml, glass disposable, product number 53283-774.[18]
      c. 10 ml, glass disposable, product number 53283-776.[18]
      d. Pasteur, non-plugged, glass, 5 $^{3}/_{4}''$ × 1/10″, product number 14672-200.[18]
   3. Volumetric flask, 1000 ml, product number 2813-1000.[10]

D. Plastic supplies
   1. Beakers
      a. 1000 ml, polypropylene, product number 1201-1000.[12]
   2. Centrifuge tubes
      a. 50 ml, product number 352070.[2]
      b. 15 ml, product number 352096.[2]
   3. Tissue culture flask, 75 cm^2, product number 430725.[5]

E. Coulter Counter supplies
   1. Isoton II Diluent, product number 8546719.[3]
   2. Coulter Clenz, product number 8546931.3
   3. Standards Mixed Kit, product number 6601329.[3]
   4. Aperature Instrument Concentration Control, product number PN177495.[3]
   5. 100 µm Aperture Tube, product number 9912786.[3]
   6. Accuvettes II, product number 8320592.[3]

3. Procedures

A. Preparation of reagents and media used in cell culture
   1. Preparation of PBS
      a. Dissolve 10.0 g NaCl, 0.25 g KCl, 1.44 g Na_2HPO_4 in 900 ml of glass distilled H_2O.
      b. Adjust to pH to 7.08 with NaCl/NaOH.
      c. Bring volume to 1 L in a volumetric flask.
      d. Aliquot into 2–500 ml bottles and autoclave 30 minutes at 121 °C at 15 psi.
      e. Store at 4 °C.
   2. Preparation of trypsin solution
      a. Prepare PBS/EDTA by adding 0.168 g $Na_2EDTA \cdot 2H_2O$ to 1 liter of PBS.
      b. Sterilize by autoclaving for 30 minutes at 121 °C at 15 psi.
      c. Add 10 ml sterile trypsin to 90 ml sterile PBS/EDTA.
      d. Store frozen.
   3. Preparation of Pig skin Gelatin
      a. Dissolve 0.5 g PSG in 400 ml glass distilled H_2O. Bring to 500 ml in a volumetric flask.
      b. Aliquot into 5–100 ml glass bottles.
      c. Autoclave for 30 min at 121 °C at 15 psi.
      d. Store at 4 °C.
   4. Preparation of DMEM
      a. Dissolve a 1 liter envelope of DMEM and 3.7 g $NaHCO_3$ in 900 ml of glass distilled H_2O.
      b. Adjust pH to 7.08 with NaCl/NaOH.
      c. Bring the volume to 1 liter in a volumetric flask.
      d. Filter sterilize in the laminar flow hood by passing the medium through a 0.22 µm pore filter into a sterile 1 liter bottle.
      e. Store at 4 °C.
   5. DMEM + 10% FBS
      In the laminar flow hood, prepare 100 ml of serum-containing medium by adding 10 ml of serum to 90 ml of DMEM. Store at 4 °C.

B. Satellite cell and adipofibroblast culture methods
   1. Callipyge sheep satellite cells were isolated from the semimembranosus muscle, expanded in culture, and stored in liquid nitrogen. Bovine adipofibroblasts were isolated from visceral fat depots of an adult steer, expanded in culture, and stored in liquid nitrogen.
   2. Thaw aliquots of the cells and suspend in 20 ml of DMEM + 10% FBS.
   3. Spin the cells down (2700 rpm for 3 min) and re-suspend in 20 ml of DMEM + 10% FBS.
   4. Plate the cells in PSG-coated 75 cm^2 flasks (for satellite cells) or uncoated 75 cm^2 flasks (for adipofibroblasts) and incubate at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h.
   5. At 24 h, remove DMEM + 10% FBS and add fresh DMEM + 10% FBS.
   6. When the cells are 75% confluent (usually 48 h–72 h), detach them from the cultureware with a trypsin/EDTA solution.
   7. Spin the cells down (2700 rpm for 3 min) and re-suspend in 20 ml of DMEM + 10% FBS for counting.

C. Counting methods
1. Hemacytometer: Using a standard hemacytometer and an inverted light microscope, count the cells using the four outside squares in each chamber (total of eight). Determine the number of cells/ml with the following formula: (Number of cells in 8 squares/8) $\times 10^4$ = cells/ml.
2. Coulter counter: Pipet 20 ml of Isoton® diluent into Acuvettes. Add 200 µl of cell suspension to each Acuvette and invert for 30 seconds. Set the lower limit (T*l*) to 15 µm for adipofibroblasts or 10 µm for satellite cells. The dilution factor should be set to 101. Count each sample twice and average the two counts to determine the number of cells/ml.

4. Results and discussion

Satellite cells

Satellite cells were thawed and expanded in T-75 flasks. When needed, the cells were lifted off with 10% trypsin solution and suspended in DMEM + 10% FBS for counting. Hemacytometers (both chambers) were loaded and counted by four trained people. Four squares were counted in each chamber, and the results were averaged. The number of cells/ml was calculated, and this number was noted as the cell count for that person. The results were recorded, and the coefficient of variation was calculated for each sample. The Coulter Counter was calibrated using CC Size Standards to account for the typical diameter of suspended myogenic cells and fat cells (approximately 25 µm), settings were programmed and validated, a background sample was run before each counting session, and duplicate samples of control beads were counted. Four Acuvettes were filled with 20 ml Isoton® solution. The test cell suspension was carefully inverted several times, and 200 µl was removed with a micropipette and carefully added to the Isoton® in the Acuvette. Each Acuvette was then gently inverted for 30 sec to mix the sample. Prior to counting with the Coulter Counter, each sample was inverted twice to ensure a good cell suspension. Each sample was counted twice, and the counts were averaged to determine the actual count for that sample. The results of both the hemacytometer and Coulter Counter counts, as well as the calculated coefficient of variation for each sample counted, are summarized in Table 1. Although the coefficient of variation (C_v) between the two methods of counting was significant ($P < 0.05$), of the ten samples that were counted, nine were not significant between the hemacytometer count and the Coulter Counter count ($P > 0.05$) and one (number 5) was significantly different ($P < 0.05$).

Preadipocytes

Preadipocytes were thawed and expanded in T-75 flasks. When needed, the cells were lifted off with 10% trypsin solution and suspended in DMEM + 10% FBS for counting. Hemacytometers (both chambers) were loaded and counted by four trained people. Four squares were counted in each chamber, and the results were averaged. The number of cells/ml was calculated, and this number was noted as the cell count for that person. The results were recorded, and the coefficient of variation was calculated for each sample. The Coulter Counter was calibrated using CC Size Standards, settings were

Table 1. Satellite cell counts with the hemacytometer and Coulter Counter

	Hemacytometer[a]	C_v[b]	Coulter Counter[c]	C_v
Count 1	4.93 ± 0.33[d]	13.40%	4.67 ± 0.0641	2.75%
Count 2	3.64 ± 0.275	15.12%	2.82 ± 0.0727	5.15%
Count 3	4.71 ± 0.316	13.44%	4.31 ± 0.0437	2.03%
Count 4	4.65 ± 0.319	13.70%	4.23 ± 0.246	11.63%
Count 5[e]	1.35 ± 0.0752	11.14%	1.86 ± 0.0346	3.72%
Count 6	3.60 ± 0.337	18.72%	4.29 ± 0.0288	1.34%
Count 7	2.18 ± 0.201	14.74%	1.94 ± 0.0257	2.13%
Count 8	1.81 ± 0.107	11.78%	1.87 ± 0.0146	1.56%
Count 9	1.80 ± 0.121	13.49%	1.82 ± 0.0357	3.92%
Count 10	1.49 ± 0.098	13.36%	1.34 ± 0.0115	1.72%
Average		**13.89%**		**3.60%**

[a] Each data point was an average of four counts ± SE.
[b] C_v is the coefficient of variation.
[c] Each count was an average of eight separate counts per sample.
[d] All numbers are $\times 10^5$.
[e] The difference between the hemacytometer and the Coulter Counter counts was significant ($P < 0.05$).

programmed, a background was run before each counting session, and duplicate samples of control beads were counted. Four Acuvettes were filled with 20 ml Isoton® solution. The cell suspension was carefully inverted several times, and 200 µl was removed with a micropipette and carefully added to the Isoton® in the Acuvette. Each Acuvette was then gently inverted for 30 sec to mix the sample. Prior to counting with the Coulter Counter, each sample was inverted twice to ensure a good cell suspension. Each sample was counted twice, and the counts were averaged to determine the actual count for that sample. The results of both the hemacytometer and Coulter Counter counts, as well as the calculated coefficient of variation for each sample counted, are summarized in Table 2. Although the coefficient of variation (C_v) between the two methods of counting was significant ($P < 0.05$), of the six samples counted, the differences between the hemacytometer count and the Coulter Counter count of five samples were not significant ($P > 0.05$). There was a significant difference between the two counting methods on one count (number 5; $P < 0.05$).

Our results make evident the wide variability in counts from the same sample between four people experienced with counting cells on the hemacytometer. This inconsistency between counters may stem from many different sources including different methods of filling the hemacytometer chambers, different determination of cell versus debris while counting, and different methods of assuring proper cell suspension prior to aliquoting cells into the hemacytometer. Many of these steps are eliminated when using a Coulter Counter for cell enumeration and, as the results indicate, offer less variability between counts and better reproducibility.

Despite the Coulter Counter's reproducible results, there are two main areas where errors may be introduced. One is the 'human factor' which, with meticulous attention to detail, can be factored out. The second involves the Coulter Counter itself. With proper set-up and maintenance, some of the 'error factors' may be eliminated, but some are flaws in the Coulter Counter's design. Improper mixing of the aliquot of cell suspension into the Isoton® may introduce air into the mixture resulting in a falsely elevated count while insufficient mixing of the aliquot of cell suspension in the Isoton® may result in a non-homogenous mixture resulting in a falsely reduced cell count. Again, these 'human factor' errors may be greatly reduced through diligence to detail.

Areas in which errors might be introduced involving the Coulter Counter are many. A dirty or clogged aperture on the Coulter Counter may result in a reduced cell count. Improper set-up may greatly alter the cell counts. These might include the incorrect dilution factor selected for the samples, incorrect aperture size for selected cells (the overall analysis range for the Coulter Counter Z1 is 1 µm to 120 µm but the actual counting range is based on the chosen aperture size and samples 2–60% of standard aperatures may be detected), and an incorrect lower limit (T*l*) set for the cell size. These types of errors can be corrected through a proper maintenance schedule and an understanding of the set-up procedures of the Coulter Counter.

Unfortunately, there are also intrinsic flaws to the Coulter Counter. It is unable to distinguish between live and dead cells and will count all cells flowing through the aperture. Additionally, the Coulter Counter is unable to distinguish a clump of cells and will count these as one cell thereby resulting in misleadingly low cell counts. These potential areas for count errors are usually not a problem with a trained counter on a hemacytometer as dead cells are not counted and clusters of cells can be counted accordingly. One method to circumvent counting dead cells with the Coulter Counter is to periodically perform a live/dead stain on the cell suspension. A check of the cell suspensions used in these experiments revealed only 0.1% or less of dead cells (data not

Table 2. Adipofibroblasts counts on the hemacytometer and Coulter Counter

	Hemacytometer[a]	C_v[b]	Coulter Counter[c]	C_v
Count 1	1.09 ± 0.048[d]	11.35%	1.00 ± 0.021	3.53%
Count 2	1.79 ± 0.094	10.53%	1.85 ± 0.064	6.86%
Count 3	3.00 ± 0.170	8.83%	3.11 ± 0.055	4.36%
Count 4	1.63 ± 0.189	23.23%	1.81 ± 0.017	1.88%
Count 5[e]	1.58 ± 0.106	13.38%	1.88 ± 0.028	2.95%
Count 6	1.26 ± 0.081	12.87%	1.43 ± 0.026	3.66%
Average		**13.37%**		**3.87%**

[a] Each data point was an average of four counts ± SE.
[b] C_v is the coefficient of variation.
[c] Each count was an average of two separate counts per sample.
[d] All numbers are $\times 10^5$.
[e] The difference between the hemacytometer and the Coulter Counter counts was significant ($P < 0.05$).

shown). Proper vortexing of the cell suspension prior to aliquoting into the Isoton® should alleviate clumping of cells and provide an accurate cell dispersal for cell counting.

While our results did demonstrate a mostly non-significant difference ($P > 0.05$) between the actual cell numbers themselves, it is important to note that it is the average of the four hemacytometer counts that did not differ significantly from the average of four Coulter Counter counts. With the large coefficient of variability (C_v) within the hemacytometer counts, it is likely that any one hemacytometer count (as is practiced in many labs) may differ significantly from any one automated cell count. Despite the seemingly complex nature of the Coulter Counter and its inherent flaws, the results obtained from our experiment show that the Coulter Counter is an efficient, reliable method for counting cells for use in cell culture.

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Notes on suppliers

1. American Sterilizer Company, Erie, PA, USA
2. BD Falcon, Bedford, MA, USA
3. Beckman Coulter Inc., Fullerton, CA, USA
4. Beckman Instruments Inc., Palo Alto, CA, USA
5. Corning Incorporated Life Sciences, Acton, MA, USA
6. Denver Instrument Co., Arvada, CO 80004, USA
7. Drummond Scientific Co., Brownhill, PA, USA
8. Invitrogen/Life Technologies, Carlsbad, CA, USA
9. JT Baker, Paris, KY, USA
10. Kimble/Kontes, Vineland, NJ, USA
11. Millipore Corp., Bedford, MA, USA
12. Nalge Nunc™, Rochester, NY
13. Nikon, Nippon K. K., Tokyo, Japan
14. Nu Aire Inc., Plymouth, MN, USA
15. Precision Scientific, Chicago, IL, USA
16. Sigma Chemical Company, St Louis, MO, USA
17. The Newell Group, Medina, NY, USA
18. VWR Scientific Products Corporation, Chester, PA, USA
19. Wheaton Scientific Products, Millville, NJ, USA

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