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<tr><td colspan="3">OSUCCC LTB Laboratories Procedure
Non-enzymatic Single-Cell Dispersion of normal or tumor</td><td>Effective:
5/1/15</td></tr>
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<tr><td>Version 1.0</td><td></td><td>5/1/2015</td><td>5</td></tr>
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1.0 PRINCIPLE

Tissue dissociation/primary cell isolation and cell harvesting are principal applications for enzymes in tissue culture research and cell biology studies. However, despite the widespread use of enzymes for these applications over the years, their mechanisms of action in dissociation and harvesting are not well understood. Nor are the effects of enzymes on antigen expression. As a result, the choice of one technique over another is often arbitrary and based more on past experience than on an understanding of why the method works and what modifications could lead to even better results. The goal of a cell isolation procedure is to maximize the yield of functionally viable, dissociated cells, and there are a number of parameters, which affect the outcome of any particular procedure. However non-enzymatic methods are appropriate for a variety of tissue types, including fresh lymph node.[1]

2.0 SPECIMEN

A Pathologist, or Pathologist Assistant will prepare fresh tissue. For research purposes only residual tissue, not needed for any diagnostic purpose should be obtained. Upon excision and gross dissection, tissue should be placed in sterile D-PBS + 2% FBS, non-complete sterile media or other sterile solution such as Hank's Balanced Salt Solution, either in sterile soaked gauze in a sterile culture dish or in sterile 50cc conical tube for transport to the laboratory. Specimens should be procured as soon as they are received by the lab and preferably within 24 hours of being procured from the patient/subject. Alliance or OSU treatment protocols may specify collection methods for samples requiring acute cell isolation. Samples for all Alliance studies should be collected into the appropriate collection tube indicated in the study protocol. Any problems or comments regarding sample collection, shipment and receipt will be noted by the technician on the sample procurement form.

3.0 MATERIALS AND REAGENTS

Sterile 15 or 50cc conical tube
Sterile pipets (5ml, 10ml)
Sterile Dulbecco's PBS (Invitrogen # 19140-144) or other buffer solution of choice
Sterile Fetal Bovine Serum (Invitrogen#/16140-071)
Cell freezing Medium (see cell freezing medium protocol) Sterile 4x4 gauze
Sterile "Cellector" cell separator with glass pestle. The plunger of a sterile syringe may be substituted if necessary.
Sterile forceps and fine dissection scissors
Sterile cell culture dish (100mm x 100mm)

[1] Freshney, R. (1987) Culture of animal cells: A Manual of Basic Technique. Alan R. Liss, Inc., New York.

Sterile pipet tips (200µl and 1000 µl)
Micropipetters (20-200µl and 1000 µl) ViCell
specimen cup
Beckman Coulter – Isoton Solution
Zapoglobin solution (if using Beckman Coulter®Counter)
70% isopropyl alcohol
Wexcide solution (for surface decontamination)

4.0 EQUIPMENT

Biosafety cabinet
Benchtop centrifuge with swinging bucket rotors to hold 15 and 50cc conical tubes

5.0 QUALITY CONTROL AND SAFETY

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-T2. No known test sample can offer complete assurance that human blood samples will not transmit infection. Therefore, all derivatives are potentially infectious. Always spray alcohol on the caps before opening solutions. The alcohol can be dried off using gauze. If you have been out of the hood for a while and are wearing the same pair of gloves, use a new pair of gloves. Remember not to touch the sides (inside or outside) of any bottles with your pipette – if you do, dispose of the pipette and start again.

6.0 PROCEDURE

6.0.1 Record all necessary sample information, carefully and clearly, in the written sample record including: patient name, protocol number, accession number, diagnosis, sample type, sample period, and the number tubes received. If possible estimate weight of tissue or record if known.

6.0.2 In biosafety cabinet, transfer samples into a sterile cell culture dish. If processing multiple samples, be sure to label dish with sample ID.

6.0.3 Using sterile fine dissection scissors and forceps, carefully remove any non-lymph node tissue present in the sample such as capsule, fat or other connective tissue. Care must be taken not to over dissect tissue and lose valuable lymph node. Sterile gauze may also be used to remove capsule or fat from the tissue by gently rolling tissue over gauze.

6.0.4 Place sterile Cellector (containing fine mesh) in a new sterile culture dish. Pour tissue in sterile media or buffer into Cellector. Using glass pestle, carefully disperse tissue through mesh into sterile dish below.

6.0.5 Make several passes with glass pestle until most of the tissue has been expressed through mesh. There may be some residual, extraneous tissue that will not pass through the mess. This can be removed with pipet or sterile forceps.

6.0.6 Using a sterile 5 or 10ml pipet, carefully wash cell suspension through mess several times. Transfer cell suspension to sterile 50cc conical tube labeled with sample ID.

6.0.7 With a sterile pipet, wash mesh using fresh sterile buffer or media. Transfer this wash through to same 50cc conical tube.

6.0.8 Dilute cell suspension to 40cc and centrifuge for 10 min at 1100 rpm (with full braking).

6.0.9 Pour off, pipet or aspirate supernatant being careful not to disturb pellet. Resuspend pellet in 2 to 20ml of D-PBS depending on the pellet size. (If the pellet is very large resuspend pellet in a larger volume of D-PBS to obtain accurate cell count.)

6.1 SAMPLE EVALUATION – TRYPAN BLUE EXCLUSION STAIN WITH HEMACYTOMETER COUNT

6.1.1 It is important not to overload the chamber, as doing so will give an inaccurate count. The same is true if the cover slip is moved after the sample is loaded (Figure 1).

6.1.2 The sample is allowed to settle for 2 or 3 minutes so that the cells stop drifting around the chamber and most will be in the same plane of focus (Figure 2). It is important not to allow the sample to settle too long or it will dry out, concentrating the cells over the grid. To avoid drying, the hemacytometer can be placed on straws within a Petri dish containing a moistened filter paper.

6.1.3 The full grid on a hemacytometer contains nine squares, each of which is 1 mm square.

6.1.4 The central counting area of the hemacytometer (as it will be called here) contains 25 large squares and each large square has 16 smaller squares.

6.1.5 When counting, count only those cells on the lines of two sides of the large square to avoid counting cells twice.

6.1.6 All 25 large squares can be counted (Figure 3), or a counting pattern using fewer squares can be used like the ones below. It is important to distribute the counting areas in a non-biased manner since cells can be more concentrated on one side of the chamber.

6.1.7 If you count over only 5 of the 25 large squares, then multiply that value by 5 to obtain the number of cells per central counting area (Figure 5).

6.1.8 Each of the nine squares on the grid, including the central counting area of 25 large squares, has an area of 1 square mm, and the cover glass rests 0.1 mm above the floor of the chamber. Thus, the volume over the central counting area is 0.1 mm^3 or 0.1 microliter. You can thus multiply the average number of cells over each central counting area by 10,000 to obtain the number of cells per ml *of diluted sample*.

6.1.9 In other words, to calculate the number of cells per ml of original sample: Calculate the mean number of cells counted for each chamber (i.e. for each of the central counting areas of each chamber).

6.1.10 Multiply the mean obtained in (1) by 10,000 to obtain the number of cells per ml of diluted sample.

6.1.11 Multiply the count obtained in (2) by the dilution factor.

Example: Assume that you dilute the original sample by adding 0.1 ml of cell suspension to 9.9 ml of diluent (1:100 dilution factor). You then count the number of cells in 5 of the 25 large squares within the central counting area of two chambers, obtaining counts of 132 and 128 cells.

1. The mean number of cells per chamber is thus 130 x 5 or 650 cells per counting area (650 cells per 0.1 microliter).
2. Multiply the 650 cells per counting area by 10,000 to obtain the number of cells per ml of diluted sample (answer = 6,500,000)

Multiply 6,500,000 cells per ml of diluted sample by 100 (the dilution factor) to obtain 650,000,000 per ml of original sample.

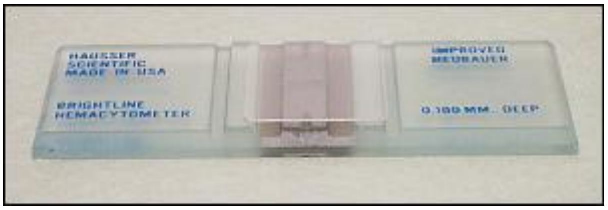

Figure 1. Neubauer Hemacytometer

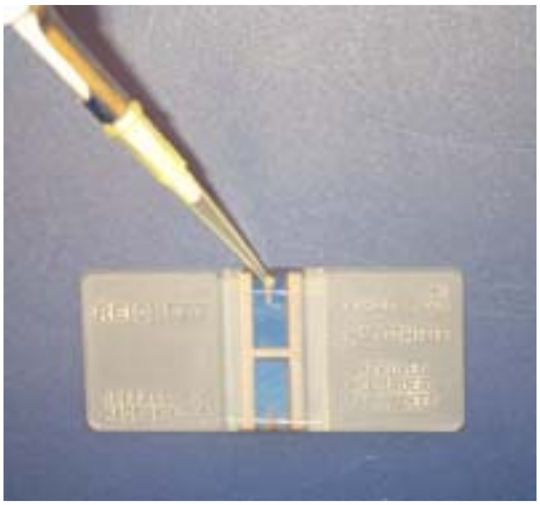
Figure 2.Adding sample to hemacytometer

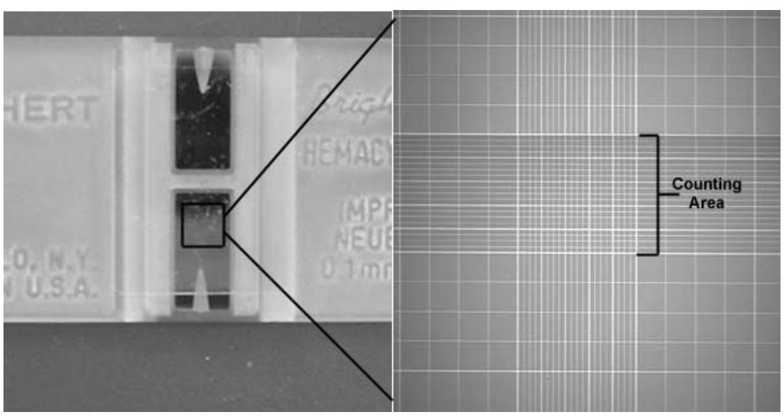

Figure 3. Counting area

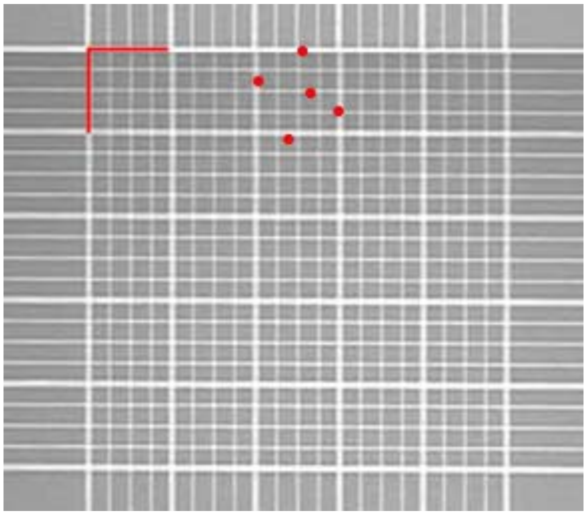

Figure 4.Close up of counting area

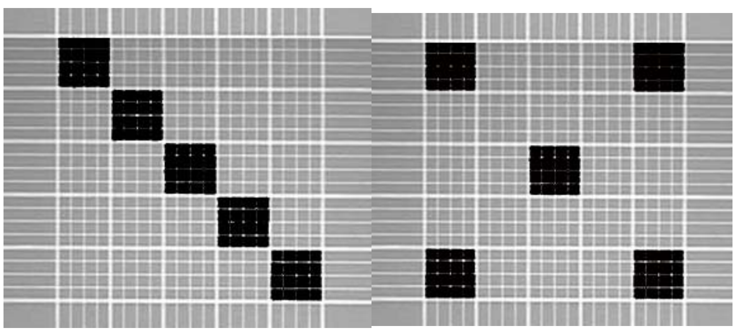

Figure 5.Detail showing possible counting schema

7.0 LIMITATIONS OF THE PROCEDURE

Dye exclusion is a simple and rapid technique measuring cell viability but it is subject to the problem that viability is being determined indirectly from cell membrane integrity. Thus, it is possible that a cell's viability may have been compromised (as measured by capacity to grow or function) even though its membrane integrity is (at least transiently) maintained. Conversely, cell membrane integrity may be abnormal yet the cell may be able to repair itself and become fully viable. Another potential problem is that because dye uptake is assessed subjectively, small amounts of dye uptake indicative of cell injury may go unnoticed. In this regard, dye exclusion performed with a fluorescent dye using a fluorescence microscope routinely results in the scoring of more nonviable cells with dye uptake than tests performed with Trypan blue using a transmission microscope. A more sophisticated method of measuring cell viability is to determine the cell's light scatter characteristics or propidium uptake.[2] However, this technique is far more time consuming and is necessary only when precise measurements on the number of dead cells in a cell mixture must be obtained. Trypan blue exclusion, as described in the above protocol, can be performed in 5 to 10 minutes.

8.0 REFERENCES

1. Strober, W. Commonly user immunological techniques. Current Protocols in Immunology, Appendix 3. 2007.).
2. Shapiro, H.M. 1988. Practical Flow Cytometry, 2nd ed., p. 129. John Wiley & Sons, New York.