Methods for Preparing Sorted Cells as Monolayer Specimens

ROBERT C. LEIF

Introduction

The major reason for sorting or separating Cells is to make a subsequent measurement on a purified population(s). Since, the quantity of cells separated by sorting and other techniques is often quite small, the conventional technique of making a smear on a glass slide produces a very sparse, essentially unusable, dispersion. In fact, the Centrifugal Cytology technique for preparing cells on a microscope slide, described below, was invented to permit conventional morphological analysis of separated cells. Initially, this was for density gradient separation of cells. Subsequently, the fluorescence activated cell sorter was invented, and Centrifugal Cytology was employed to determine the cellular composition of sorted fractions.

One of the best quality control techniques for both flow cytometry and cell sorting is to observe the cells with a microscope. Even prior to sorting, it very often pays to look at the cells. Centrifugal Cytology permits samples prepared and stained for flow cytometry to be deposited on a microscope slide for viewing. Since, many of the laboratories which perform cell sorting analyze and sort other groups' samples, it is very useful to be able to show the individuals supplying a sample what they really have. Although sorters and other cell separation techniques are often capable of separating a few good cells from many that are dead or in very poor condition, the old aphorism, garbage in equals garbage out, often still holds.

Conventional microscopic imaging of cells requires that they be supported on microscope slides. Current histochemical technique involves the application of stains to the cells after they have been placed on the slide. As stated above, this need not be the case for cells prepared for flow or sorting. Two requirements for

cell preparation on slides are 1) the cells remain on the slides during staining or other processing and 2) the number of cells per unit area be in a range which is high enough to permit the visualization of a significant number of cells, yet low enough to minimize the proportion of cells layered over each other. Overlaid cells interfere with human visual or machine analysis.

ADVANTAGES OF MONOLAYERS VERSUS CONVENTIONAL SMEARS

The conventional smear has been the traditional way to place cells on a microscope slide. Since the distribution of cells in fresh or mixed stored blood is approximately random and the concentration of cells is approximately between 25 and 50%, monolayers have been made by smearing a drop on a slide. The drop is pulled across the slide, usually by a second slide. Air drying in the plasma ensures that the cells will remain glued to the slide. The surface tension and other drying effects reduce the thickness of the cells and spread them out. This significantly increases the visibility of the leukocyte granules and the thin strands of chromatin which connect the segmented parts of neutrophil nuclei. It often destroys the structure of both lymphocyte and monocyte chromatin. Most other cytology samples have a lower cell concentration than blood, and the cells are not homogeneously distributed. For instance, it has already been demonstrated that standard Pap smears do **not** provide a representative sample of exfoliated cells.[1,2]

Sorted cells and other samples where the cells are suspended in solution have significant advantages over traditional clinical cytological samples: 1) their concentration can be adjusted to produce dispersions approaching the optimum number of cells per unit area. 2) After the solution is mixed, a representative sample of the cells will be available for transfer to the slide. 3) The number of cells required to make an individual dispersion is minimized. 4) The cells can be processed and stained in suspension in a similar manner to present flow cytometry techniques. And 5) The capacity to have multiple preparations, each containing a representative sample of cells, permits the scientific method to be applied to optimize the staining and other parts of the specimen preparation procedure.

Subprotocol 1
Coating Methods for Glass Slides to Prevent Cell Loss

Materials

Cleaning Solutions

- Liquinox detergent
- 1% Ammonium bifluoride

Adhesive Solutions

- 50-65% Mayers' albumin fixative in DH_2O
- 1 mg/ml Poly-Lysine
- 2% Aminopropyltriethoxysilane

Procedure

Slide Cleaning

1. First clean them in conventional laboratory detergent such as Liquinox (Alconox inc.) in a small ultrasonic bath for about ten minutes. Ethanol also works.
2. Rinse thoroughly with distilled water.
3. Immerse in one percent ammonium bifluoride for two minutes (periods up to 12 min. have been used)

Note: Excess ammonium bifluoride can visibly etch the surface of a glass slide; however, if you do not see it, you have no need to worry about it.

4. Rinse and store in distilled water.
5. At time of use dry with lens tissue.

Note: A clean slide like any piece of clean glassware wets evenly with water without drop formation.

Adhesives

Note: For maximum adherence of centrifuged cells to glass slides, the slides must be coated.

6a. Mayers' albumin fixative. The slides must be coated by momentarily dipping them in a Coplin Jar which contains Mayers' albumin fixative solution (Harleco) diluted in distilled water to achieve a concentration between 50 and 65%.[3,4] Bake the slide, vertically, in a 70 °C oven for 10 minutes to insure a thin, even, tacky surface.

Note: The use of an albumin or other protein based adhesive has the advantage that the cells are literally glued to the slide. This is particularly important for cells with reduced adhesive properties, which often result from prior stabilization or fixation for shipping or the use of dissociation protocols. The use of Mayers' albumin has the very significant disadvantage of providing a very large number of sites for nonspecific stain binding.

6b. High molecular weight Poly-Lysine. Dip slides in 1 mg/ml Poly-Lysine and air dry.

Note: A high molecular weight Poly-Lysine solution (M.W. $>$70,000)[5] may be substituted for albumin.

Note: The use of this technique may reduce the number of cells which adhere to the slide, but it will diminish the amount of background debris. The homogenous D or L polymers should be used. Precoated slides are available from Sigma (Cat. # P 0425).

6c. Aminopropyltriethoxysilane.

Note: Rentrop et al,[6] have reported on the effect of various coating materials on the adhesion of frozen sections to glass slides. They found that 2% aminopropyltriethoxysilane in dry acetone was superior to: egg white, gelatin, collagen, and Elmer's White Glue. These authors speculate that the aminopropyltriethoxysilane forms covalent bonds with the aldehydes and ketones present in the tissues. Precoated slides are available from Sigma (Cat. # S 4651)

Subprotocol 2
Methods for Preparing Sorted Cells as Monolayer Specimens: Centrifugal Cytology

Preparation of Specimens for Image Cytometry or Human Observation

The three major factors controlling recovery of cells from a suspension onto a slide are 1) Cell losses in the apparatus; 2) The adhesiveness of the cell binding surface; and 3) the force pushing the cells onto the surface. The two basic methods for preparing monolayers are 1) pressure transfer where the cells are initially placed on a nonadhesive substrate and transferred by pressure to a slide which binds them.[7] And, 2) centrifuging the cells onto a slide. One variation of centrifugation is to have the cells settle at unit gravity.[8]

Monolayer Apparatus

Centrifugal Cytology

Centrifugal Cytology is the process where cells in suspension are centrifuged onto a substrate and then fixed concurrently with the application of centrifugal force. Thus, while the cells are being hardened by the fixative, they are simultaneously being pushed against the support. This fixation during centrifugation results in a very high recovery of the cells.[9] In order to ensure maximum recovery, the apparatus is configured to prevent fluid loss.[10]

Although presently, Leif Centrifugal Cytology Buckets or similar apparatus[11] are used for Centrifugal Cytology, a distinction must be made between the apparatus and the process. The latter is of scientific significance and is a direct outgrowth of modern cell biology. The Centrifugal Cytology process was originally based on electron microscopic technique and subsequently was evolved to follow Papanicolaou's wet fixation methodology.[12] Centrifugal Cytology can be used clinically to prepare cells for human screening[13] and shows great potential for automated clinical cytology.[14] It also is an extremely useful tool for monitoring the cell suspensions employed for flow cytometry or sorting.

One of the major uses of the Centrifugal Cytology Buckets is a generic protocol for viewing suspension preparations. The flexibility of the centrifugal cytology procedure and the Centrifugal Cytology Buckets, as well as the heterogeneity of biological cells precludes the absolute specification of many procedures. However, it is possible to provide rational starting points for development of optimized procedures.

Cells that have been prepared for flow analysis or subsequent to sorting can be monitored by centrifuging them onto a standard microscope slide, adding a coverslip, and viewing the wet mount with a microscope. Thus, virtually all flow preparation procedures are also centrifugal cytology procedures. Often, the flow staining procedures result in the chromophores being bound with sufficient strength to the cells, that they will remain localized during fixation, dehydration, and permanent mounting. In the text below, the rationale behind the selection of fixatives and staining procedures will be given with a few specific examples.

The Leif Centrifugal Cytology Bucket (Leif Bucket) is based on a swinging bucket rotor. The original aluminum bucket is a replacement for the standard swing-out cup of a swinging bucket rotor. A fluid tight chamber is formed by pressing and sealing an elastomeric sample block against a standard 3 by 1 inch microscope slide.[10] The slide serves as the base of the pyramidal sample chambers present in the block. The incline of the slanted chamber walls follows the radius emanating from the center of the centrifuge. Since cells and other particles follow a radial trajectory, this prevents their deposition on the chamber walls. The cell containing suspension is first placed in a chamber and then the cells are centrifuged onto the slide. Most of the supernatant is removed and a fixative is added in a manner that does not dislodge the cells. During fixation, the cells are pressed onto the slide by centrifugal force. After fixation, the slide is separated from the sample block and can then be processed by conventional staining techniques. The Centrifugal Cytology Bucket was designed to facilitate the cytological examination of cells from dilute biological fluids.

The first commercial embodiment of the Leif Buckets was used in conjunction with any universal laboratory centrifuge with a standard 1.875 inch rotor spacing. As described below, subsequent models have been designed for other centrifuges.

The Leif Bucket has been used to prepare the following tissues and body fluids for cytological examination: blood[9,15,16] bone marrow,[17] cervical scrapes,[18] body fluids including cerebral spinal fluid, nipple aspirate,[19] sputum,[20] urine,[21] eye fluids including tears and vitreous humor.[22] Centrifugal Cytology has been employed to quantitate biologically active lymphocytes: Jerne Plaque and rosette forming cells,[23] as well as natural killer cells.[24] Detailed information about these Centrifugal Cytology applications are given in the references including their use to collect sorted cells and prepare them for human observation.[25]

Description

The Centrifugal Cytology Bucket consists of the following components:

A. Carriers. Aluminum carriers (Fig. 1) for use in a centrifuge with a 1.875 inch rotor spacing. These carriers replace the rotor's sample cups and thus, are required to maintain dimensional stability at 500 times gravity. Many centrifuges now have swing out rotors which have cups that hold 500 ml. Since microscope slides are 3 by 1 inch, special plastic carriers (See also Fig. 5) have been designed for cups which have inner dimensions greater than 3 inches. The design of these plastic carriers was simplified because during centrifugation they are supported by the standard centrifuge rotor cup. Both the aluminum and plastic carriers support a foam pad, microscope slide, and the sample block during centrifugation. The hold downs and screws are used to press the sample block against the slide with sufficient pressure to form a fluid tight seal.

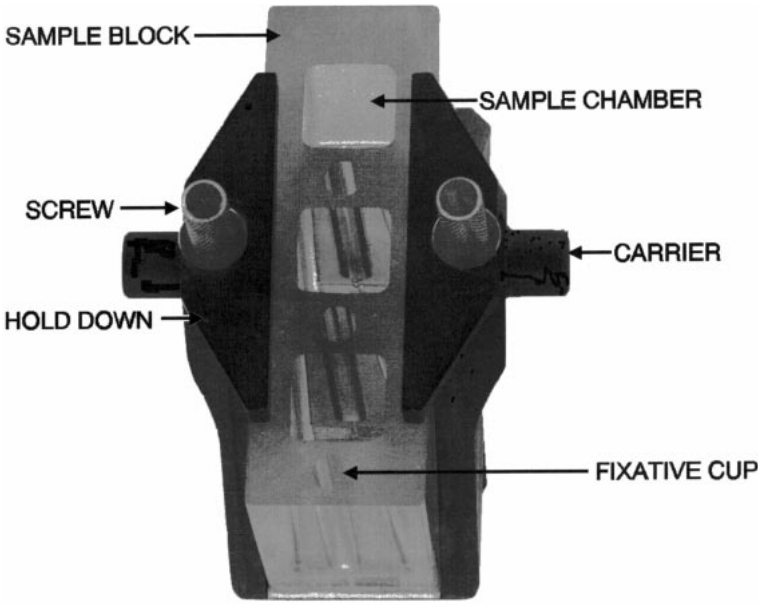

Fig. 1. Aluminum carrier version of the Centrifugal Cytology bucket. The carrier is supported by and pivots on the two cylindrical pins. Loosening of the two screws permits both of the hold downs to be rotated 180°. This permits insertion or removal of the sample block, microscope slide, and foam pad

B. Sample and Sorter Blocks. Presently, both three and four chamber sample blocks (Fig. 2) are available. Eight chamber and special sorter blocks (Fig. 3) have also been fabricated. Each sample chamber has an interconnected fixative cup. A plastic frit at the bottom of the fixative cup at unit gravity retards the flow of the fixative solution, while permitting the flow of fixative into the sample chamber during centrifugation. Rapid flow of any solution onto unfixed cells could dislodge them from the microscope slide. The sample blocks are molded from an elastomeric plastic, that is sufficiently hard to withstand the centrifugal force, yet soft enough to deform and thus seal to the surface of the microscope slide. For some work where the supernatant fluid must be removed with absolutely minimal disruption of the cell monolayer, a capillary (not shown in Fig. 2) which connects the top of the sample block to 1 mm above the slide has been added.

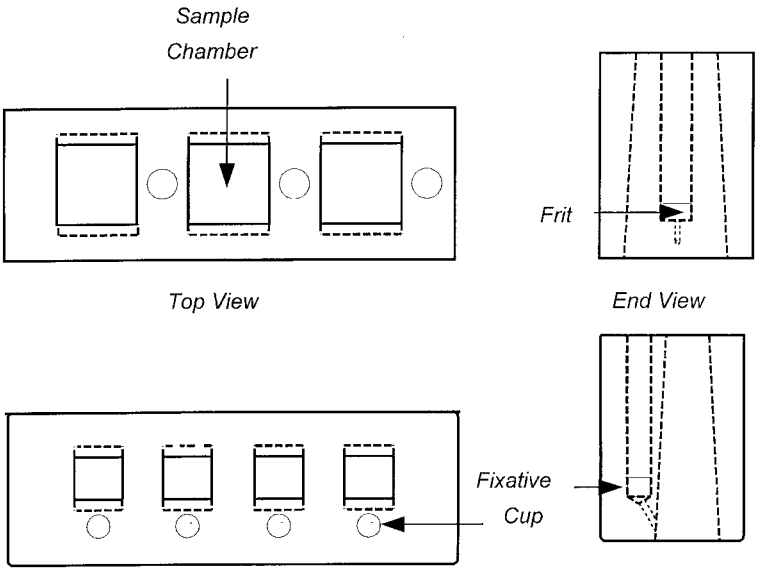

Fig. 2. Sample blocks. The top and end views of the 3 (top) and 4 (bottom) chamber sample blocks are shown. The hatched cylinder at the bottom of each fixative cup is fabricated out of a plastic frit, which retards the flow of solution at unit gravity. The capillaries, dashed lines, beneath the frit convey the fixative to about 1 mm above the slide. The chambers are sector shaped. In the top views, the dashed lines in the three chamber insert (top) and the four chamber insert (bottom) show the outline of the chamber at the base of the sample block, which is in contact with the microscope slide and delineates the area of the cellular dispersion

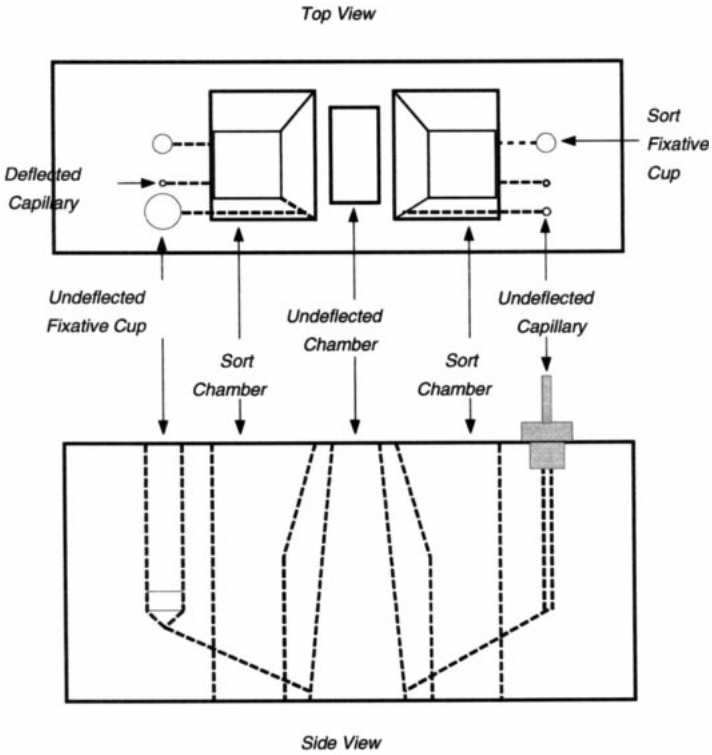

Fig. 3. Special Sample Block for Sorting. The top and side views of a special 3 chamber sorter sample block are shown. A chamber to collected the undeflected drops is located between the two sort chambers. The internal walls of the chambers and the capillaries in the sample blocks are shown by the dashed lines. The fluid retarding plastic frits at the bottom of each fixative cup are shown as hatched cylinders. The side view shows only the fixative cup, frit and capillaries for the undeflected chamber. The top view shows all three fixative cups, frits, and capillaries. The sort deflected fixative cup on the left and the deflected capillary on the right of the top view are located at the same height as their labelled counterparts. The undeflected fluid waste connector, which is shown at the top right in the side view, connects with the undeflected capillary and can also be connected to the sorter vacuum line

Each of the two sort chambers consists of two flattened pyramids. In order to provide the largest area to collect the sorted droplets, the top of the chamber consists of an inverted pyramid. This pyramid connects just above the level of the frits with a short conventional sector shape chamber. Any fluid that collects on the walls of the inverted pyramid will be driven by the centrifugal force into the standard sector shaped chamber. These standard sector shaped chambers are offset towards the back of the sample block in order to permit the undeflected fixative and fluid removal capillaries to connect with the undeflected chamber. The undeflected chamber is a sector shaped flattened pyramid.

C. Foam Pads. Three inch long by one inch wide, disposable foam pads are placed on the flat surface of carriers. These deformable pads support and cushion the microscope slides.

Materials

Equipment

Leif Centrifugal Cytology Buckets

Procedure

Operating Instructions

Bucket Assembly

The Centrifugal Cytology Bucket is assembled (Fig. 4-5) as follows:

1. Place a 3 X 1 in. foam pad, backing-side down, on the flat surface of the aluminum or plastic carrier. Do **not** remove the backing from the pad; since, the adhesive on the pad will glue the gasket to the carrier.
2. Place a plain, clean, unfrosted glass slide on top of the pad in the carrier (Fig. 4a).
3. Place a transparent sample block on top of the slide (Fig. 4b). The deformability of the sample block creates the seal between slide and sample block.

4a. For the single slide aluminum carriers, rotate the hold-downs 180° and tighten the thumbscrews by hand (Fig. 4c). Do not overtighten the screws.

Seal the sample block to the slide

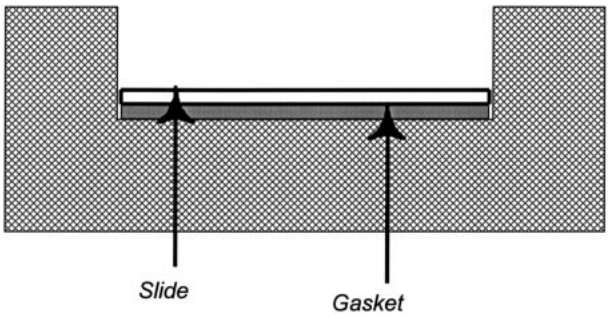

Fig. 4a. The gasket is first placed in the carrier followed by a cleaned slide

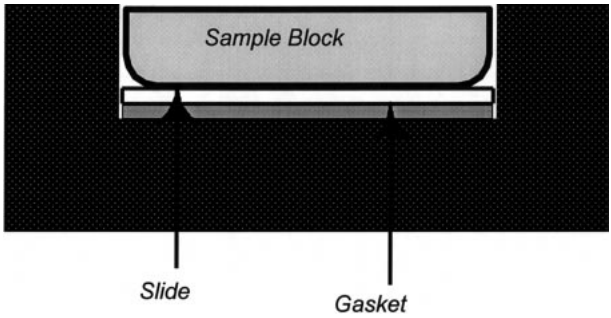

Fig. 4b. Compression of the sample block seals it to the slide, which is in turn supported by the gasket and carrier. The tops of the carrier and sample block have been removed

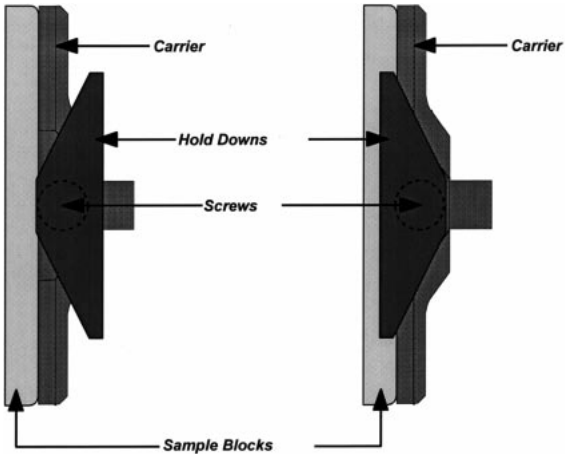

Fig. 4c. At the left, the hold down is shown rotated, which permits the sample block to be inserted or removed. At the right, the hold down is shown rotated 180°, which locks the sample block into place and seals the block to the slide

4 b. For the two slide plastic carriers (Fig. 5), place the transparent top over the sample block. Check that the drill holes in the top are aligned with the sample chambers and the fixative cups. Tighten the single central screw by rotating the knob. This results in the transparent cover compressing the two sample blocks, each against its underlying slide.

Note: If necessary, cover the sample chambers with tape to avoid airborne contamination. The use of volatile buffers, such as bicarbonate, can result in unacceptably large pH changes.

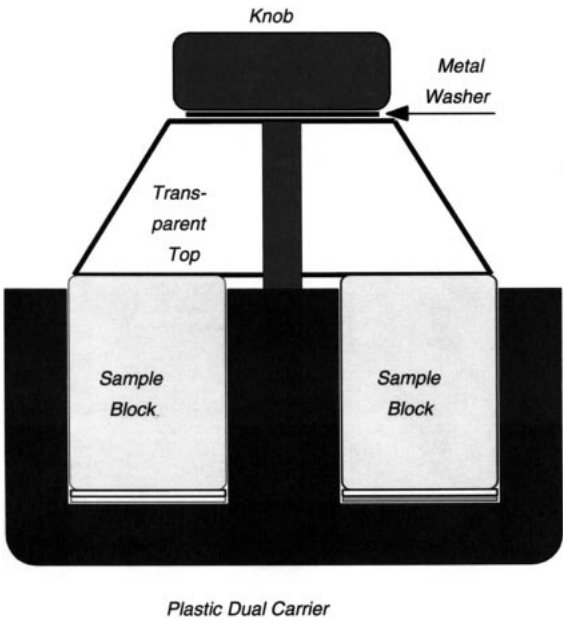

Fig. 5. The knob is attached to a threaded rod. The threaded rod is screwed into a metal insert (not shown), which is located in the center of the plastic carrier. Thus, rotation of the knob results in pressure on the flat metal washer, which distributes the force across the transparent top. The top, in turn, compresses the sample blocks, which seals them to the microscope slides. As before with the aluminium carrier, the slides are supported on gaskets. Removal of the top permits the sample blocks to be removed. Access to the sample chambers is through holes (not shown) in the transparent top

Sample Processing

5. Take up the sample with a syringe, or pipette, and deliver it into one of the sample chambers.

6. Balance a pair of buckets according to the centrifuge manufactures' specifications.

Note: If it is necessary to increase the weight of one bucket, suspending fluid should be added to the center chamber of a three-port block, or to the two center chambers of a four-port block. Suspending fluid added to the outer chambers should be added equally to keep the slide truly vertical during centrifugation. Always balance a three-chamber sample block against another three-chamber sample block, and a four-chamber block against another four-chamber block.

7. Centrifuge the samples at 250 to 500 x gravity for 10 min.

8. After the centrifuge has stopped, gently tilt the bucket assembly and aspirate the supernatant from each sample chamber with a Pasteur pipette.

Note: Retighten the screws after the initial centrifugation; the pads can become irreversibly compressed.

Note: From step 9 on, fluid is removed from or overlaid onto a cell monolayer. A 21 gauge syringe needle or Pasteur pipette can be used or preferably a bidirectional auto-pipettor with a speed control should be employed to slowly add and remove fluid, so as to minimize disruption of the cell monolayer and maximize ist contact with fixatives.

9. Add fixative to the fixative cup with a syringe or pipette.

10. Recentrifuge the samples at 250 to 500 x gravity for a time appropriate to the fixation protocol you are using.

11. After centrifugation with fixative, draw off the supernatant as described in step 8.

12. Disassemble the bucket in the reverse order of its assembly.

13 a. For the single slide aluminum carriers, loosen the screws, rotate the hold-downs 180°, and lift the sample block straight up and out.

13 b. For the two slide plastic carriers, unscrew the single central screw from the two slide plastic carrier and remove the

transparent cover, and lift the two sample blocks straight up and out.

Note: Should the slide remain attached to the sample block, break the seal by inserting the edge of a thin metal spatula between the slide and the sample block.

Cleaning Procedures

14. To avoid carryover between samples, clean the sample block in a 5% solution of household bleach, in an ultrasonic bath (Branson B220 or equivalent) for 12 min. Remove the bleach from the buckets by rinsing them for 5 min. in tap water, followed by a 5-min. rinse in distilled water.

Note: Avoid the use of organic solvents other than ethanol with the sample block. Use ethanol only as a fixative.

Sterilization

15. Should sterilization be necessary, sterilize the sample block in either 70% ethanol or 10% formalin for no longer than 2 hours.

Note: ***Do not autoclave the sample block!***

Storage and Handling

16. The sample blocks should be stored with their bottom side facing up. Handle the bottom surface with care; it serves as a seal to prevent the escape of the cell suspension during centrifugation.

Bucket Malfunction

17. Any time malfunctioning of the bucket is suspected, perform the following tests.

17 a. Assemble the bucket and fill all chambers with water. Centrifuge the bucket for 10 min. at 250 x gravity. Inspect for water loss, and empty the chambers.

17 b. Fill every other sample chamber with water and centrifuge at 250 x gravity for 10 min. Check for water loss from the filled chambers and water gain in the empty ones. This is a test of the isolation of the fluid chambers from each other.

Note: Make sure that the bucket is evenly balanced.

17 c. To check the proper functioning of the fixative cups, place water in the cups and allow the sample block to stand at unit gravity on a level surface. It should take approximately 2 minutes for the cups to drain into their respective sample

chambers. Fill the cups again and centrifuge at 250 x gravity for 2 minutes to determine if the cups empty when centrifugal force is applied to them.

Subprotocol 3
Methods for Preparing sorted Cells as Monolayer Specimens: Fixation and Staining

Fixation

The selection of fixatives is based on the requirements of the procedure. For either transmission or scanning electron microscopy, the fixative of choice is glutaraldehyde. For flow cytometry fluorescence measurements, it is glyoxal. For fluorescence image cytometry where morphological integrity or comparison with flow cytometry are important, it is glyoxal. For absorbance staining where granular structures need to be emphasized or previous tradition must be adhered to, 95% ethanol with 5% polyethylene glycol average molecular weight 1,450 is a good choice.

Proper fixation for centrifugal cytology is significantly facilitated by employing fixatives that are denser than the fluid originally present above the cell monolayer. A very simple test to determine relative density is to add a small amount of an absorbance dye (usually Phenol Red or food coloring) to the fixative and then test whether it is denser than the original fluid. This test is also a very good training procedure for performing centrifugal cytology. One or more mLs of the supernatant fluid should be added to a small test tube. Then, the colored fixative should be delivered slowly to the bottom of the tube. The results of too fast delivery will be readily apparent. The colored fixative ricochets off of the tube bottom and flows up. If after successfully layering the fixative under the supernatant, the delivery device is removed too quickly, there will be a colored tail extending upwards.

If the aqueous fixative floats or does not effectively sink, then the density of the fixative can be increased by the addition of 5 to 10.0% (v/v) DMSO, the replacement of the water by 20% deuterium oxide, or both.[26]

Ethanol

Since ethanol is the fixative commonly employed for exfoliative cytology, it has been extensively used. The addition of polyethylene glycol with a Mol. Wt. of Ca. 1,450 helps stabilize the morphology of the cells. In fact, if one is very careful and has a strong hair drier, it is possible to air dry 95% ethanol 5% polyethylene glycol fixed blood leukocyte centrifugal cytology preparations (J. Hudson, unpublished results). For routine fixation with ethanol, 15 minute centrifugation at 250 x gravity appears to be adequate.

Scanning electron microscope studies of centrifugal cytology preparations which were air dried from xylene were essentially identical with conventional critical point dried preparations. The explanation is the surface tension of organic solvents is much lower than that of aqueous solutions.

Aldehydes

Three aldehyde fixatives are shown in Fig. 6. Aldehydes primarily fix by forming inter and intra molecular crosslinks. They also form adducts with amino groups, which eliminate the latter's capability of being positively charged at neutral pH. This decrease in charge decreases both the solubility of proteins and the binding of negatively charged dyes.

Formaldehyde

Formaldehyde, since it is a monoaldehyde, is a poor crosslinking agent. It can only crosslink by forming an aminal by condensing with two separate, but very close amino groups. A major advantage of formaldehyde is that it is a sufficiently poor fixative, that its reaction with the tissue can be reversed.[27,28] Optimal results of centrifugal cytology studies of erythrocytes were achieved with formaldehyde as the fixative. Note, a trace of bovine serum albumin must be present for most manipulations of erythrocytes.

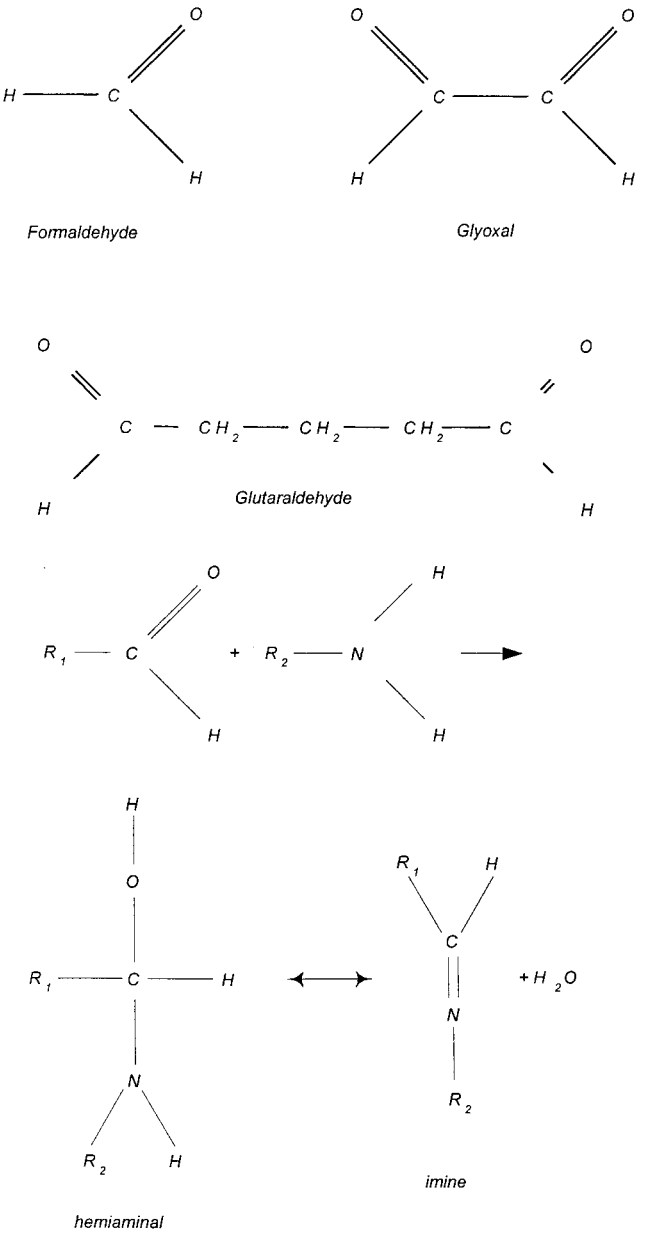

Fig. 6. Structure of three aldehyde fixatives. When the aldehyde with the R_1 functional group reacts with the amine with the R_2 functional group, the hemiaminal is formed. The hemiaminal loses water to form the imine, with which it is in equilibrium

Glutaraldehyde

Dialdehydes, such as glutaraldehyde, can form two hemiaminals by reacting with two amino groups that can easily be located on separate macromolecules. Glutaraldehyde has one very significant disadvantage; it forms fluorescent adducts, which provide significant background fluorescence.

Glyoxal

The crosslinking activity of glyoxal, which also is a dialdehyde, is between formaldehyde and glutaraldehyde. Glyoxal has the major advantage of not forming fluorescent adducts which produce detectable background. Golomb and Monnier[29] demonstrated that in the case of glyoxal, the hemiaminals convert to imines (Schiff bases). They demonstrated that for twenty mM glyoxal, the time for maximum reaction with BSA was 30 minutes. Dimer formation with reduced RNase was strongly evident at the minimum time, 5 hours, as shown by these authors. Ueno et al.[30] demonstrated single strand breaks in rat liver DNA 2 hours after a single oral dose of 200 mg/kg body weight. After 9 hours, the DNA extracted from the other organs, except possibly for the spleen appeared to be unaffected. No crosslinking of the DNA was observed. Thus, Ueno et al. concluded that, glyoxal must be genotoxic in man.

Most of the work[16] on glyoxal was for flow analysis of fluorescent antibody stained leukocytes obtained after lysis of whole blood at neutral pH. Selective lysis of the erythrocytes at neutral pH does not have the problem of acid induced desorbtion of the antibody from the remaining leukocytes.[31] Examples 5 and 6 of the Leif et al. Patent[16] describe a procedure for multiparameter flow analysis. Example 7 of the same patent describes a preparation employing a Centrifugal Cytology Bucket. The prior exposure to saponin required very rapid stabilization of the leukocytes. This was achieved with 14 g/L (0.24 M/L) glyoxal and 146.7 g/L DMSO (1.88 M/L) DMSO, and Polyethylene glycol MW 1450 was also present at 16.7 g/L. For more conventional work, the DMSO concentration should be reduced to 50 to 100 g/L.

DMSO

Dimethylsulfoxide, DMSO, is an excellent fixation and stain enhancing agent, which facilitates the entry of the fixative and often of dyes, such as DAPI, into cells. It also stabilizes the hydrophobic bonds in cell membranes. Another major advantage of DMSO is it has a density of 1.10. The addition of 5 to 10% DMSO (v/v) to a fixative containing solution results in a density significantly greater than that of the original cell suspending solution. Thus the fixative, which underlays the cell suspending solution, comes into intimate contact with the cells, which are located on the surface of the microscope slide. Concentrations of DMSO greater than 10% (v/v) have been employed to freeze cells.

Evaluation of Fixatives

There are three excellent tests for fixation protocols. The first is to perform electron microscopy on the fixed cells. The second is to measure the electronic cell volume distribution prior to and after fixation. And, the third is to perform image analysis on the cells.[32] The goals of the automated cytology procedure will significantly influence the criteria for fixation quality. Procedures which are useful for fluorescence in situ hybridization nuclear spot counting may be very poor for automated analysis of exfoliated cytology specimens. For morphological studies and/or textural analysis of nuclear features, a reasonable starting place is a supravital DNA dye, such as Hoechst 33342.[33]

Thornthwaite et al.[15] employed the Automated Multiparameter Analyzer for Cells II focused flow electronic cell volume transducer to determine optimum fixation conditions. They established that even blood leukocytes from the same individual do not have the same osmotic properties. "The optimum concentration of glutaraldehyde (v/v) at a fixed concentration of 0.05 M. cacodylate buffer (pH 7.4), based on the position of the peak channel of the electronic cell volume distributions, was 3.8% for mastocytoma cells, 4.9% for human lymphocytes and 4.0% for human granulocytes and monocytes. The existence of a significant osmotic effect of glutaraldehyde indicates that a significant amount of this fixative is excluded during the period

of fixation when the initial cross-linking takes place. Unfortunately, focused flow impedance cell volume instruments for the research market presently are not commercially available. Several of the Coulter clinical hematology instruments do employ focused flow for their special purpose electro-optical leukocyte transducers.

Staining

Slides may be stained with your choice of stain or other labeling agent. For surface immuno-labeling of cells, start with a procedure for flow analysis where the antibodies are applied in suspension prior to fixation. Standard, routine PAP stain protocols may be used. Hematoxylin and eosin stains are also appropriate. Air drying must be avoided at all stages where information concerning chromatin fine structure is to be acquired.

In order to avoid the presence of water droplets, which are immiscible in mounting media, aqueous staining procedures require that the cells be dehydrated through graded alcohols (all v/v): 50%, 70%, 95% and 100% ethanol. Usually, an immersion in 50% ethanol and 50% xylene or xylene substitute is included prior to immersion in xylene or xylene substitute, which is followed by the final step permanent mounting. The use of nonaqueous mounting media has the obvious advantage of eliminating nonradiative energy loses to water.

Procedure

Glutaraldehyde

Two to 4% (v/v) glutaraldehyde phosphate buffer (pH 7.2) or 0.05M/L Na-cacodylate brought to pH 7.4 with 2M HCl and containing up to 5 to 12% DMSO (v/v).

Note: The use of electron microscope grade of glutaraldehyde is highly recommended. In fact, the use of glutaraldehyde fixing solutions which have recently been used successfully by your local electron microscopist is highly recommended.

Note: For routine fixation with glutaraldehyde, 15 minute centrifugation at 250 x gravity appears to be adequate. For ultrastructural studies with scanning or transmission electron micro-

scopy, 45 minute exposure to glutaraldehyde is required for proper fixation.

Glyoxal Concentrate

Note: This formula is preliminary.1. Dissolve 10 grams Glyoxal trimer dihydrate (Cat. # G5754 Sigma) in 20 ml of distilled water.

Note: Heating in a 37°C bath probably will be needed to solubilize the glyoxal. Glyoxal and other aldehydes slowly transform into an organic acid(s), which can adversely effect the morphology of the cells.

2. Remove the acid components by adding 0.5 grams of a large bead such as, Mixed Bed Resin TMD-8 (Cat. # M 8157 Sigma) to remove the acid components, let sit for 30 minutes or until resin changes color from blue green to yellow. Since the resin beads are large the solution can be either filtered, centrifuged or centrifuged and decanted through filter paper. If the beads change color, repeat the addition of the resin.

Note: This resin treatment should also work with the commercially available 40% aqueous solution. Unfortunately, present commercial glyoxal often has a significant acid contamination.

0.1M Tricine Buffer

1. Dissolve 1.79 g (0.01 mole) of tricine (Cat.# 3077T Research Organics) and 0.10 g of NaN3 in 100 ml volumetric flask about a third full of deionized/filtered water.
2. Heat polyethylene glycol average Mol. Wt. 1450 to liquefy most of the contents of the container. A microwave oven works.
3. Add 5 g of the polyethylene glycol to the tricine solution.
4. Add 7.5 g DMSO to the tricine solution.

Note: With a viscous liquid, it is often easier to take the liquid up in a wide bore disposable transfer pipette and drip it into a volumetric flask which is placed on a top-loading balance.

5. Add deionized/filtered water to the 100 ml line on the flask.

Note: The 100 ml of solution can now be transferred to a polyethylene bottle.

6. Check the pH of the solution; it is usually slightly acid.

7. Add 10 M. NaOH to bring the pH to about 7.4-7.6. It is advisable to add small drops of the NaOH in successive small portions, checking the pH after each portion has dissolved.

8. Add 1 ml of the Glyoxal Concentrate to 9 ml of the 0.1 M Tricine buffer.

Glyoxal Fixative Solution

Subprotocol 4
Plaque Cytogram Assays

Centrifugal Cytology has been employed to enumerate biologically active cells. Thornthwaite and Leif[23] were able to enumerate and morphologically characterize both plaque-forming and rosette-forming cells. The technique described below can be modified to permit the visualization of any type of cell that can be effected by its neighbors in a manner that produces a result that can be made visible either by cytochemical or special optical techniques.

Materials

Equipment

– Centrifugal Cytology Buckets

Note: For these studies, special 12 chamber Centrifugal Cytology Buckets were employed. Since at present, these are not commercially available, the volumes have been scaled up by a factor of 2, which is approximately the ratio of the dispersion area of the presently commercially availabe 4 chamber bucket (56 mm^2) to that of the previous 12 chamber bucket (25 mm^2).

– Multi-pipetting device

Note: When this procedure was developed, a special crank driven multi-pipetting machine was employed for fluid transfer beginning with step 6. This should be replaced by a suitably configured commercially available auto-pipettor.

– Centrifuge

Note: Almost any laboratory centrifuge that has a rotor for the Centrifugal Cytology buckets shown in Fig. 1 or a centrifuge with a swing out rotors with cups suitable for the buckets shown in Fig. 5.

Solutions

– Sheep red blood cells
– 4% glutaraldehyde in 0.05 cacodylate buffer (pH 7.4)

Note: The glutaraldehyde fixation provided excellent morphology. The addition of 5 to 7.5% DMSO (v/v) to the glutaraldehyde solution will both increase the rate of fixation and facilitate the over-layering. However, if fluorescence measurements are to be made, the glutaraldehyde should be replaced by glyoxal.

Procedure

Prepare the spleen cells

1. Dilute the spleen cells to 2 x 10^5/ml.
2. Transfer 0.2 ml into a bucket chamber. Traditionally a 1 or 0.5 ml disposable syringe has been used.
3. Sediment the cells by centrifuging at 400 x gravity at 4°C for 5 min.
4. Slowly overlay the spleen cell monolayer with 0.2 ml of 4 x 10^7/ml sheep red blood cells SRBC.
5. Sediment the RBCs by centrifuging at 400 x gravity at 4°C for 5 min.
6. Remove the supernatant down to 1 mm above the cell surface

Add guinea pig complement

7. Slowly, (over a period of 5 min) layer 0.2 ml of 10% guinea pig complement into the bucket champer.

Incubate

8. Incubate for 20 min. in a level, 37°C incubator.
9. Remove supernatant down to 1 mm above the cell monolayer.

10. Very slowly (15 min), add 0.2 ml of 4% glutaraldehyde in 0.05 cacodylate buffer (pH 7.4). The end of the delivery system should be at about 1 mm from the surface of the slide. **Fix**

11. Let fix for 1 hr. at room temperature. The cells overlaid with fixative can be stored overnight at 4°C.

12. The intact erythrocytes can stained with benzidine[17] and the slides subsequently processed with Papanicolaou or any other absorbance stain. **Staining**

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