Cytometry Procedures

(Excerpt from Becton Dickinson Monoclonal Center Source Book)

Table of Contents

Becton Dickinson Procedures

Preparation of Single Cell Suspensions from Mouse Lymphoid Tissue

Reagents:

I. Medium: Phosphate-Buffered Minimal Essential Medium containing 0.1% sodium azide* and 2% newborn calf serum (for cytotoxicity assays, use 0.5% serum)

Dissolve a liter envelope of Eagle's Minimal Essential Medium (Hank's salts) in —700 ml distilled water.

Add in sequence, with stirring:
100 ml 0.01 M Na_2HPO_4
100 ml 0.01 M $MgCl_2 \cdot 6H_2O$
10 ml 10% (w/v) sodium azide*
20 ml newborn calf serum (5 ml for cytotoxicity assays)

Qs to 1 liter with distilled water; pH should be 7.0 ± 0.05. Store at 2°-8° C.

2. Mouse lymphoid tissue – spleen, lymph node, or thymus

Equipment:

1. Low speed centrifuge with swinging bucket rotor
2. Light microscope
3. Ice bucket
4. Glass microscope slides with frosted ends

* **WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in piping where explosive conditions may develop.

Procedure:

I. Place lymphoid tissue in about 5 ml of cold media in a small petri dish.

2. Gently disrupt the tissue by rubbing it between the frosted ends of two microscope slides.
3. Transfer cells to a 15 ml conical tube, leaving debris from the organ capsule in petri dish.
4. Place conical tube upright in ice bucket and allow debris and larger cell clumps to settle for 2-5 minutes. Decant supernatant containing cells into a clean 15 ml conical tube.
5. Centrifuge this cell suspension at 300 x g for 5 minutes. Aspirate supernatant from the cell pellet.
6. Resuspend the cell pellet in 10-15 ml of cold media and mix. Centrifuge at 300 x g for 5 minutes. Repeat washing and centrifugation.
7. Resuspend cells in 5-10 ml of cold media. Determine cell viability using ethidium bromide/acridine orange.** (See Becton Dickinson Procedure on Viability Staining using Ethidium Bromide and Acridine Orange, Source Book Section 2.3) Cells should be >90% viable.

** **CAUTION:** Ethidium bromide and acridine orange are suspected carcinogens. Handle with care and dispose of properly.

5/89 23-1379-02

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Preparation of Single Cell Suspensions from Human Peripheral Blood

Reagents:

1. Venous blood sample
2. Hank's Balanced Salt Solution (BSS) containing no calcium or magnesium
3. Ficoll-Hypaque (Pharmacia)
4. Phosphate-Buffered Minimal Essential Medium (PMEM) containing 0.1% sodium azide* and 2% newborn calf serum (for cytotoxicity assays, use 0.5% serum)

   Dissolve a 1 liter envelope of Eagle's Minimal Essential Medium (Hank's salts) in ~700 ml distilled water

   Add in sequence, with stirring:
   100 ml 0.01 M Na_2HPO_4
   100 ml **0.01** M $MgCl_2 \cdot 6H_2O$
   10 ml 10% (w/v) sodium azide*
   20 ml newborn calf serum (5 ml for cytotoxicity assays)

   Qs to I liter with distilled water; pH should be 7.0 ± 0.05. Store at 2°-8°C.
5. **RPMI** 1640 containing 50% newborn calf serum (optional: for monocyte depletion)

Equipment:

1. Low speed centrifuge with swinging bucket rotor
2. Heparinized VACUTAINER® Brand blood collection tubes (Becton Dickinson VACUTAINER Systems)
3. CO_2 incubator set at 37° C
4. FALCON® Brand tissue culture flask (Becton Dickinson Labware)

Procedure:

I. Obtain 15-20 ml blood by venipuncture into EDTA VACUTAINER Brand blood collection tubes.

2. Dilute with two volumes of Hank's BSS.
3. Layer 8 ml of diluted cell suspension over 4 ml of Ficoll-Hypaque in a 15 ml centrifuge tube.
4. Centrifuge at room temperature at 400 x g for 20 minutes. Mononuclear cells should form a visible, clean interface between the plasma and Ficoll-Hypaque.
5. Aspirate the plasma and retain the mononuclear cell layer. Place cells in a clean 15 ml centrifuge tube.
6. Fill the tube with Hank's BSS, mix the cells, then spin at 400 x g for 20 minutes. Aspirate and discard supernatant.
7. Resuspend cells in Hank's BSS, mix, and spin at 300 x g for 5 minutes. Aspirate and discard supernatant.
8. Resuspend cells with 10 ml of PMEM, mix gently, and determine cell viability using ethidium bromide/acridine orange** (See Becton Dickinson Procedure on Viability Staining using Ethidium Bromide and Acridine Orange, Source Book Section 2.3). Cells should be >90% viable.

   For Monocyte Depletion:
9. Adjust cell suspension to 1-3 x 10^6 cells/ml with RPMI 1640 containing 50% serum. Add cell suspension to tissue culture flask at a depth of ~1 cm. Incubate at 37°C for 60 minutes in 7% CO_2.
10. Decant non-adherent lymphocytes to a clean centrifuge tube. Rinse culture flask gently with 1/10 volume of Minimal Essential Medium, adding this liquid to the centrifuge tube. Spin at 300 x g for 5 minutes. Aspirate and discard supernatant.
11. Resuspend cells in PM EM, mix gently and determine cell viability using ethidium bromide/acridine orange.** Cells should be >90% viable.

* WARNING: Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in piping where explosive conditions may develop.

** CAUTION: Ethidium bromide and acridine orange are suspected carcinogens. Handle with care and dispose of properly.

5/89 23-1475-02

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Viability Staining Using Ethidium Bromide and Acridine Orange*

Cells:

1. Mouse or human cell suspension (See Becton Dickinson Procedures on Preparation of Single Cell Suspensions, Sections 2.1 and 2.2)

Reagents:

I. Ethidium Bromide/Acridine Orange 100 X Stock Solution

50 mg Ethidium Bromide
15 mg Acridine Orange

Dissolve in 1 ml of 95% ethanol. Add 49 nil distilled water. Mix well; divide into I ml aliquots and freeze.

2. Working Solution of Ethidium Bromide/ Acridine Orange

Thaw a I ml aliquot of the 100X Stock Solution and dilute 1/100 in phosphate-buffered saline. Mix well. Store in an amber bottle at 4°C for up to one month.

Equipment:

1. Fluorescence microscope set up to excite for fluorescein (i.e., with a 495 nm primary filter and a 515 nm secondary filter)
2. Hemocytometer chamber and coverslip

Procedure:

1. Adjust an aliquot of the cell suspension to an estimated 1-5 x 10^6cells/ml in phosphate-buffered Minimal Essential Medium or other isotonic medium.
2. Add equal 25 *Al* volumes of cell suspension and ethidium bromide/acridine orange solution to a tube. Mix gently.
3. Place a small aliquot (-25 *lal)* underneath the coverslip on a hemocytometer slide.
4. Observe cells initially under the microscope using visible light, I00-400X. Adjust the diaphragm to reduce light and still keep the hemocytometer grid visible.
5. Keeping the visible light on, switch to fluorescence and observe the cells. Live cells will fluoresce green (with acridine orange) and dead cells will fluoresce orange (with ethidium bromide).
6. Count cells and calculate viability.

*CAUTION: Ethidium bromide and acridine orange are suspected carcinogens. Handle with care and dispose of properly.

Reference:
Parks, D.R., Bryan, VW, 0i, VW, 0i, VT., and Herzenberg, L.A. (1979) Antigen Specific Identification and Cloning of Hybridomas with a Fluorescence Activated Cell Sorter (FACS). *PNAS 76.* 1962

5/89 23-1476-02

BECTON DICKINSON

Becton Dickinson immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (BOO) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Direct Immunofluorescence Staining of Cell Surfaces

This method is used to detect cells bearing specific membrane antigens by treating a cell population with monoclonal antibodies conjugated to fluorescein (FITC) or phycoerythrin (PE)*. Direct staining is recommended for analysis by flow cytometry or fluorescence microscopy.

Cells:

1. Mouse or human cell suspension (See Becton Dickinson Procedures on Preparation of Single Cell Suspensions, Sections 2.1 and 2.2, respectively).

Reagents:

I. FITC or PE-conjugated monoclonal antibody to human or mouse membrane antigen. Remove aggregates in FITC conjugates by centrifuging at 100,000 x g for 10 minutes immediately prior to use. Do NOT centrifuge PE conjugates.

2. Medium: Dulbecco's Phosphate-Buffered Saline or Phosphate-Buffered Minimal Essential Medium (MEM) containing 0.1% sodium azide** and 2% calf serum. Store at 2°-8°C. For MEM preparation, see Becton Dickinson Procedures on Preparation of Single Cell Suspensions, Source Book Sections 2.1 and 2.2.
3. Mounting medium, protein free — for microscopy: 90% glycerol in phosphate-buffered saline containing 0.1% sodium azide.

Equipment:

1. Centrifuge fitted with plate carrier
2. Round-bottom microtiter plates or 3 ml plastic tubes
3. Ice bath
4. Fluorescence Activated Cell Sorter (FACS® Brand flow cytometer) or fluorescence microscope

Procedure:

1. Dilute 20 Al of FITC or PE conjugated monoclonal anti-human antibody or 4 pl anti-mouse monoclonal antibody in 30 pl medium.

   Add the 50 j,tl of diluted antibody to microtiter wells or plastic tubes.

2. Adjust concentration of cell suspension to 2 x 10^7 cells/ml. Cells should be >90% viable as determined by staining with ethidium bromideiacridine orange*** (See Becton Dickinson Procedure on Viability Staining using Ethidium Bromide and Acridine Orange, Source Book Section 2.3).

   Add 50 AI of the cell suspension (1 x 10^6 cells) to the microtiter wells or plastic tubes and mix gently.

3a. For staining in microliter plates, incubate the mixture for 30-45 minutes on ice. Centrifuge the plate at 100 x g for 3 minutes at 2°-8° C. Carefully aspirate the supernatant from the cell pellet. Wash two times with 100 Fe1 aliquots of cold medium. Centrifuge plate after each washing at 100 x g for 3 minutes. Aspirate supernatant from cell pellet.

3b. For staining in tubes, incubate the mixture for 30-45 minutes on ice. Add 2 ml of cold medium, then centrifuge at 300 x g for 5 minutes at 2°-8° C. Carefully aspirate supernatant from cell pellet.

4a. Flow Cytometric Analysis:
Resuspend cells in 0.5 ml of cold medium, to approximately 1 x 10^6 cells/ml. Keep cold until analyzed. If desired, cells may be fixed with paraformaldehyde prior to analysis. See Becton Dickinson Procedure Source Book Section 2.10.

4b. Fluorescence Microscopy:
Resuspend cells in 1-2 drops of mounting medium. Deposit a portion of the drop (-20 *kil)* on a microscope slide, place a cover slip over the drop, then seal edge of coverslip with clear nail polish.

Alternatively, prepare cytocentrifuge preparations using Becton Dickinson Method on Immunofluorescence Staining of Cell Surfaces — Cytocentrifuge Preparations, Source Book Section 1.4.

For microscope set-up and filter selection, see Handbook of Experimental Immunology, Third Edition (1978) p. 15.18-15.21, ed. Weir, D.M. (Blackwell, Oxford)

* U.S. Patent 4,520,110; European Patent 76,695; and Canadian Patent 1,179,942

** WARNING: Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

*** CAUTION: Ethidium bromide and acridine orange are suspected carcinogens. Handle with care and dispose of properly.

5/89 23-1383-03

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Indirect Immunofluorescence Staining of Cell Surfaces

The indirect method is used to enhance the fluorescence signal and also to facilitate two-color staining of mouse or human cells, when direct conjugated reagents may not be available.i. [2] Becton Dickinson offers two systems for indirect staining:

- biotin-avidin
- conventional "second antibody"

In the biotin-avidin system, cells are incubated first with biotin-conjugated monoclonal antibody and then subsequently incubated with avidin conjugated to fluorescein or phycoerythrin* (F1TC or PE). **In** the "second antibody" technique, cells are incubated with an unconjugated monoclonal antibody followed by goat anti-mouse Ig labeled with fluorescein.

These "sandwich" techniques provide increased fluorescence intensity, which is valuable for microscopy. However, indirect methods may result in the formation of complexes which will artifactually stain Fc receptor-binding cells. The biotin-avidin system gives the least amount of artifactual staining particularly when Streptavidin conjugates are used. Indirect methods will alter the proportionality between the amount of antigen and the fluorescence intensity per cell; therefore, these methods are not recommended for assessing the absolute number of antigenic determinants per cell. However, indirect methods can be used to determine the **relative** quantitative differences between cell populations.

Reagents:

I. Mouse or human cell suspension (See Becton Dickinson Procedures on Preparations of Cell Suspensions, Source Book Sections 2.1 and 2.2). Cells should be washed well if they have been suspended in biotin-containing media; e.g., **RPM1.**

2. Biotin-conjugated monoclonal antibody to human or mouse membrane antigen

   Unconjugated monoclonal antibody

3. Avidin FITC or Streptavidin PE (Catalog Nos. 9011, 9023)

   Goat Anti-Mouse Ig FITC (Catalog No. 9031)

   Remove aggregates by centrifuging at 100,000 x g for 10 minutes immediately prior to use.

4. Medium: Phosphate-Buffered Minimal Essential Medium containing 0.1% sodium azide** and 2% new-born calf serum. Store at 2°-8°C. Do **not** use biotin-containing medium with the biotin-avidin indirect method. For medium preparation, see Becton Dickinson Procedures for Preparation of Single Cell Suspensions, Source Book Section 2.1 and 2.2.

5. Mounting medium, protein free: 90% glycerol in phosphate-buffered saline containing 0.1% sodium azide.

Equipment:

I. Centrifuge fitted with plate carrier

2. Round-bottom microtiter plates or tubes

3. Ice bath

4. fluorescence Activated Cell Sorter, (FACS® Brand flow cytometer) or fluorescence microscope

Procedure:

1. Dilute unconjugated or biotin-conjugated monoclonal antibody by adding 20 Al antibody to 30 ill medium for each test.

   Add the 50 ill of diluted antibody to microtiter wells or plastic tubes.

2. Adjust concentration of the cell suspension to 2 x 10^7 cells/ml. Cells should be >90% viable as determined by staining with ethidium bromide/acridine orange.*** (See Becton Dickinson Procedure on Viability Staining using Ethidium Bromide and Acridine Orange, Source Book Section 2.3).

   Add 50 Ad of the cell suspension (I x 10^6 cells) to the microtiter wells or tubes and mix gently.

3a. For staining in microtiter plates, incubate the mixture for 30-45 minutes on ice. Centrifuge the plate at 200 x g for 3 minutes at 2°-8° C. Carefully aspirate the supernatant from the cell pellet. Wash two times with 100µl aliquots of cold medium. Centrifuge plate after each washing at 200 x g for 3 minutes. Aspirate supernatant from cell pellet.

3b. For staining in tubes, incubate the mixture for 30-45 minutes on ice. Add 2 ml of cold medium, then centrifuge at 300 x g for 5 minutes at 2°-8° C. Carefully aspirate supernatant from cell pellet.

4. Dilute the appropriate second step (fluorochrome-conjugated avidin or goat anti-mouse 1g) in medium according to the instructions provided on the second step data sheets.

* U.S. Patent 4,520,110; European Patent 76,695; and Canadian Patent 1,179,942

** **WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

*** **CAUTION:** Ethidium bromide and acridine orange are suspected carcinogens. Handle with care and dispose of properly.

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

5a. For staining in microtiter plates, incubate the second step mixture for 30-45 minutes on ice. Centrifuge the plate at 200 x g for 3 minutes at 2°-8° C. Carefully aspirate the supernatant from the cell pellet. Wash two times with 100µl aliquots of cold medium. Centrifuge plate after each washing at 200 x g for 3 minutes. Aspirate supernatant from cell pellet.

5b. For staining in tubes, incubate the second step mixture for 30-45 minutes on ice. Add 2 ml of cold medium, then centrifuge at 300 x g for 5 minutes at 2°-8°C. Carefully aspirate supernatant from cell pellet.

6a. **Flow Cytometric Analysis:**
Resuspend cells in 0.5 ml of cold medium, to approximately 2 x 10^6 cells/ml. Keep cold until analyzed.

6b. **Fluorescence Microscopy:**
Resuspend cells in 1-2 drops of mounting medium. Deposit a portion of the drop (-20 pl) on a microscope slide, place a coverslip over the drop, then seal edge of coverslip with clear nail polish.

Alternatively, prepare cytocentrifuge preparations using Becton Dickinson Method on Immunofluorescence Staining of Cell Surfaces — Cytocentrifuge Preparations, Source Book Section 1.4.

For microscope set-up and filter selection, see Handbook of Experimental Immunology, Third Edition, (1978) p. 15.18-15.21, ed. D.M. Weir (Blackwell, Oxford)

I Parks, D.R., Lamer, L.L., and Herzenberg, L.A. (1986) Flow Cytometry and Fluorescence Activated Cell Sorting (FACS). In Handbook of Experimental Immunology, Chapter 29, eds. D.M. Weir, L.A. Herzenberg, and C. Blackwell (Blackwell Scientific Publications, Oxford)

[2]Jackson, A.L., and Warner, N.L. (1986) Preparation, Staining and Analysis by Flow Cytometry of Peripheral Blood Leucocytes. In Manual of Clinical Laboratory Immunology, Third Edition, p. 226-235, eds. N.R. Rose, M. Friedman, and J.L. Fahey. (American Society for Microbiology, Washington, D.C.)

Becton Dickinson Procedures

Direct Cytotoxicity

This method is used to deplete selected cell types from a cell population by direct complement-dependent cytotoxicity. The direct method applies only to monoclonal antibodies which are of an immunoglobulin subclass that binds complement; e.g., mouse IgG_{2a} or 1gM, but not usually mouse IgG_1

Cells:

I. Mouse or human cell suspension (See Becton Dickinson Procedures on Preparation of Cell Suspensions, Source Book Sections 2.1 and 2.2).

Reagents:

I. Monoclonal antibody to human or mouse membrane antigen. Remove aggregates by centrifuging at100,000 x g for 10 minutes immediately prior to use.

2. Medium: Phosphate-Buffered Minimal Essential Medium containing 0.1% sodium azide* and 0.5% newborn calf serum (Note: Higher amounts of serum may inhibit complement activity). For medium preparation, see Becton Dickinson Procedures for Preparation of Single Cell Suspensions, Sections 2.1 and 2.2.

3. Complement: Serum from 3-week old rabbits

a. Collect blood from 3-week old rabbits by heart puncture.

b. Allow blood to clot on ice for 3 to 4 hours.

c. Centrifuge at 4°C at 1500 x g for 15 minutes and decant serum.

d. Aliquot serum immediately and freeze at -85°C.

e. Pretiter an aliquot of this rabbit serum for specific antibody-dependent cytotoxicity and for non-specific background cytotoxicity against the desired cell type. Some rabbit sera may be unusable due to their nonspecific cytotoxicity for cells. For experiments which employ functional assays, additional screening is required.

f. Do not refreeze thawed aliquots. Use within 4 hours of thawing.

4. Ethidium bromide/acridine orange solution** (See Becton Dickinson Procedure on Viability Staining using Ethidium Bromide and Acridine Orange, Source Book Section 2.3).

Equipment:

I. Centrifuge fitted with plate carrier

2. Falcone Brand Round-bottom microtiter plates (Becton Dickinson Labware)

3. Ice bath

4. Fluorescence microscope

5. Incubator set at 37°C

Procedure:

I. In microtiter well, add sufficient monoclonal antibody to medium to obtain the required amount for cytotoxicity in -50 pi volume.

The antibody should be tested in duplicate and at several dilutions to determine the concentration for optimal killing under the conditions which prevail in the individual laboratory. For controls (complement and "cells only"), add 50 ,21 of medium to each well.

2. Adjust concentration of cell suspension to 6 x 10^6cells/ml. Cells should be >90% viable as determined by staining with ethidium bromide/acridine orange.**

Add 50µl of the cell suspension (3 x 10^5cells) to all microtiter wells and mix gently.

3. Incubate the microtiter plate for 45 minutes on ice.*** Centrifuge the plate at 500 x g for 3 minutes. Remove supernate.

4. Add 100 pl of medium containing the appropriate amount of baby rabbit complement as determined by pretitration. For "cells only" control, add 100 pi of medium. Gently resuspend the pellet.

5. Incubate the microtiter plate at 37° C for 45-60 minutes. After incubation, immediately move the plate to an ice bath to inhibit further killing.

6. Gently resuspend cells in each well. Remove a 40 'Al aliquot and mix with 40 pl of ethidium/acridine orange solution** immediately before counting.

7. Determine cell viability by counting live and dead cells with a fluorescence microscope. Counting of all wells should be complete within one to two hours.

NOTE: Cells with low densities of antigenic determinants are generally more difficult to kill) Therefore, immuno-fluorescence staining may indicate a higher percentage of positive cells in a population than the percentage killed by antibody and complement.

* **WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

** **CAUTION:** Ethidium bromide and acridine orange are suspected carcinogens. Handle with care and dispose of properly.

*** Some monoclonal antibodies require initial incubation at room temperature for optimal cytotoxicity (e.g., Anti-Leu-M1).

I Ledbetter, J.A., Rouse, R.V., Micklem, H.S., and Herzenberg, L.A. (1980) T Cell Subsets Defined by Expression of Lyt-1, 2, 3 and Thy-1 Antigens. *J. Exp. Med. 152,* 280

5/89 23-1518-02

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Enzyme-Linked Immunoabsorbent Assay (ELISA): Alkaline Phosphatase

The indirect enzyme-linked immunoabsorbent assay (ELISA) is used to detect and/or measure monoclonal antibodies reactive with soluble antigens. The four-step procedure involves: I.) Coating microtiter plates with a target antigen such as purified human immunoglobulin; 2.) Incubating test samples (which contain monoclonal antibody) in the target and control wells; 3.) Adding an alkaline phosphatase-labeled second step reagent; and 4.) Adding enzyme substrate to detect positive reactions.

Reagents:

1. Carbonate buffer, pH 9.6

   1.59 g NA_2CO_J
   2.93 g $NaHCO_3$

   Dissolve in I liter of distilled water
   Store at **4°C** for not more than two weeks.

2. PBS • BSA solution
   500 ml phosphate-buffered saline, pH 7.2-7.4 (PBS)
   0.5 g bovine serum albumin (BSA)

3. PBS • Triton solution
   500 ml PBS
   0.25 ml Triton" X-100 (Sigma Chemical Co., St. Louis, **MO)**

4. Antigen coat solution

   Dilute antigen in carbonate buffer to a concentration of 1-10 pg/m1

5. Test sample containing mouse monoclonal antibody
   a. Culture supernatant
   b. Ascites fluid or serum
   c. Purified antibody
   d. Biotin-conjugated antibody

6. Alkaline phosphatase-labeled second-step reagent
   a. For purified or non-purified antibody, use enzyme conjugate of goat anti-mouse immunoglobulin.
   b. For biotin-conjugated antibody, use enzyme-labeled avidin or streptavidin.

   For preparation of enzyme conjugates, see Voller, A., Bidwell, D., and Bartlett, A. (1976) Microplate Enzyme Immunoassays for the Immunodiagnosis of Virus Infections. In Manual of Clinical Immunology, 1st Ed., p. 506-512, eds. N.R. Rose and H. Friedman (American Society for Microbiology, Washington, D.C.)

7. Substrate
   a. 10% Diethanolamine buffer
      97 ml Diethanolamine
      800 ml H_2O
      100 mg $MgC1_2 \cdot 6H_2O$
      0.2 g sodium azide*

      Add I M HC1 until pH = 9.8. Qs to 1 liter.
      Store at 4°C in the dark. Stable for 6 months.
   b. Just prior to use, dissolve one phosphatase substrate tablet (Sigma) per 5 ml of diethanolamine buffer (at room temperature).

8. Stopping reagent - 3N NaOH

Equipment:

1. 96 well microtiter plate with flat, optically clear bottom (for ELISA)
2. Multi-channel pipetter, 50-250 pl
3. Single pipetter, 50-250 pl

Procedure:

I. Using a single or multi-channel pipetter, dispense 100 pi** per well of carbonate buffer to at least 4 wells of the microtiter plate to serve as reagent blanks. Fill the remaining wells with 100 pl each of the antigen coat solution.

   Incubate the plates for 2 hours at room temperature or for 18 hours at 4°C.

2. Remove coat solution by turning the plate upside-down and shaking with a few quick jerks. Quickly rinse once with tap water by placing the plate under running water. Shake water out of the plate.

   Wash all wells two times with PBS • Triton solution.

3. Prepare appropriate dilution(s) of test samples containing mouse monoclonal antibody in PBS • BSA solution.

4. Add 90 pl per well of PBS • BSA solution to the non-coated wells and also to a row of coated wells (that will serve as background controls). Next add 90 pl of test sample per well. (Four duplicate wells per test sample are generally used.)

   Incubate 1-2 hours at room temperature.

5. Wash plate once with tap water. Wash two times with PBS • Triton solution.

6. Dilute the alkaline phosphatase-labeled antibody (or enzyme-labeled avidin) in PBS • BSA solution to the appropriate concentration. (Optimal concentration can be obtained by titration.)

7. Using a multi-channel pipetter, add 100 Al of diluted enzyme-labeled antibody (or enzyme-labeled avidin) per well to all wells except the non-coated wells, which receive an equal volume of PBS • BSA solution. Incubate 1-2 hours at room temperature in the dark.

8. Prepare the alkaline phosphatase substrate solution.

9. Wash plate once with tap water, then two times with PBS • Triton solution. Do **NOT** allow wells to dry out before adding enzyme substrate solution.

10. Add 100 pl per well of enzyme substrate solution to the entire plate, and incubate the plate at room temperature in the dark. Note the time that the substrate is added. Check the plates periodically for color development. Color should become apparent in 15-30 minutes. For quantitation, read plates periodically in an automatic ELISA microtiter plate reader and measure the enzyme concentration by determining the rate of color development. For qualitative results, note the positive (colored) wells, or remove the content of each well and determine optical density at 405 nm using a spectrophotometer.

I I. The reaction can be stopped by adding 100 pi per well of stopping reagent if plates need to be saved.

* **WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in piping where explosive conditions may develop.

** The volume added per well can be reduced to 50 microliters if it is necessary to conserve reagent. Evenly cover the bottom of each well with fluid.

7/89 231380-02

BECTON DICKINSON

Becton Dickinson Immunocytomeiry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226: In Calif. (800) 821-9796

Becton Dickinson Procedures

Enzyme-Linked Immunoabsorbent Assay (ELISA): Horseradish Peroxidase

This indirect enzyme-linked immunoabsorbent assay (ELISA) is used to detect and/or measure monoclonal antibodies **reactive** with soluble antigens. The four step procedure involves: 1.) Coating microtiter plates with a target antigen such as purified human immunoglobulin; 2.) Incubating test samples (which contain monoclonal antibody) in the target and control wells; 3.) Adding a horseradish peroxidase-labeled second step reagent; and 4.) Adding substrate and indicator dye to detect positive reactions.

Reagents:

1. Carbonate buffer, pH 9.6

   1.59 g Na_2CO_3
   2.93 g $Na\ HCO_3$

   Dissolve in 1 liter of distilled water
   Store at 4°C for not more than two weeks.

2. PBS • BSA solution
   500 ml phosphate-buffered saline, pH 7.2-7.4 (PBS)
   0.5 g bovine serum albumin (BSA)

3. PBS • Triton solution
   500 ml PBS
   0.25 ml Triton X-100 (Sigma)

4. Antigen coat solution

   Dilute antigen in carbonate buffer to a concentration of 1-10 pg/m1

5. Test sample containing monoclonal antibody
   a. Culture supernatant
   b. Ascites fluid or serum
   c. Purified antibody
   d. Biotin conjugated antibody

6. Horseradish peroxidase-labeled second-step reagent
   a. For purified or nonpurified antibody, use enzyme conjugate of goat anti-mouse immunoglobulin.
   b. For biotin-conjugated antibody, use enzyme-labeled avidin or streptavidin

   For preparation of enzyme conjugates, see Nakane, **P** (1979) Preparation and Standardization of Enzyme-labeled Conjugates. In Immunoassays in the Clinical Laboratory, ed. R.M. Nakamura (Alan R. Liss, New York)

7. Substrate
   a. Citrate buffer - prepare fresh daily

      525 mg citric acid, monohydrate
      50 ml distilled water

      Adjust to pH 4.0 ± 0.1 with 3N NaOH

   b. ABTS" dye solution:
      15 mg/ml ABTS [2,2'-azino-bis-(3-ethyl-benz-thiazoline-sulfonate)] (Sigma Chemical Co., St. Louis, MO, Catalog No. A1888 or A4798) in water. Store at 4°C in the dark. Prepare fresh monthly.

   c. Substrate solution - prepare just prior to use

      0.1 ml ABTS dye solution
      0.33 ml 0.3% $\mathbf{H_2O_2}$
      10.0 ml citrate buffer

Equipment:

I. 96 well microtiter plate with flat, optically clear bottom (for ELISA)

2. Multi-channel pipetter, 50-250 gl

3. Single pipetter, 50-250 1.d

Procedure:

1. Using a single or multi-channel pipetter, dispense 100 p.1* per well of carbonate buffer to at least 4 wells of the microtiter plate to serve as reagent blanks. Fill the remaining wells with 100 gl each of the antigen coat solution.

   Incubate the plates for 2 hours at room temperature or for 18 hours at 4°C.

2. Remove coat solution by turning the plate upside-down and shaking with few quick jerks. Quickly rinse once with tap water by placing the plate under running water. Shake water out of the plate.

   Wash all wells two times with PBS • Triton solution.

3. Prepare appropriate dilution(s) of test samples containing mouse monoclonal antibody in PBS • BSA solution.

4. Add 90 gl per well of PBS • BSA solution to the uncoated wells and also to a row of coated wells (that will serve as background controls). Next add 90 gl of test sample per well. (Four duplicate wells per test sample are generally used.)

   Incubate 1-2 hours at room temperature.

5. Wash plate once with tap water. Wash two times with PBS • Triton solution.

6. Dilute the horseradish peroxidase-labeled antibody (or enzyme-labeled avidin) in PBS • BSA solution to the appropriate concentration. (Optimal concentration can be obtained by titration.)

7. Using a multi-channel pipetter, add 100 Ad of diluted enzyme-labeled antibody (or enzyme-labeled avidin) per well to all wells except the non-coated wells, which receive an equal volume of PBS • BSA solution. Incubate 1-2 hours at room temperature in the dark.

8. Prepare the horseradish peroxidase substrate solution.

9. Wash plate with tap water, then two times with PBS • Triton solution. Do **NOT** allow wells to dry out before adding enzyme substrate solution.

10. Add 100 gl per well of enzyme substrate solution to the entire plate, and incubate the plate at room temperature in the dark. Note the time that the substrate is added. Check the plates periodically for color development. Color should become apparent in 15-30 minutes. For quantitation, read plates periodically in an automatic ELISA microtiter plate reader and measure the enzyme concentration by determining the rate of color development. For qualitative results, note the positive (colored) wells, or remove the content of each well and determine optical density at 414 nm using a spectrophotometer.

1 I. The reaction can be stopped by adding 100 gl per well of stopping reagent if plates need to be saved.

* The volume added per well can be reduced to 50 microliters if it is necessary to conserve reagent. Evenly cover the bottom of each well with fluid.

5/89 23-1381-02

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Paraformaldehyde Fixation of Hematopoietic Cells Stained with Fluorochromes

This procedure is used to preserve hematopoietic cells stained with fluorochrome-conjugated monoclonal antibodies for subsequent analysis by fluorescence microscopy or flow cytometry. The fixed cells may be stored for at least one week at 4°C in the dark. This method of fixation does not significantly alter the volume, light scatter, or fluorescence properties of mouse, rat, or human lymphoid cells, or transformed cell lines.[1]

Cells:

1. Mouse, rat or human cells stained by a direct or indirect immunofluorescence technique (see Becton Dickinson Procedures on Direct Immunofluorescence Staining of Cell Surfaces, Source Book Section 2.4, and Indirect Immunofluorescence Staining of Cell Surfaces, Source Book Section 2.5).

Reagents:

I. Phosphate-Buffered Saline (PBS) Solutions:

25X Stock Solution:

Dissolve in 1 liter of distilled water:
188 g K_2HPO_4
33 g $NaH_2PO_4 \cdot H_2O$
180 g NaCI

IX Working Solution:

Add 40 ml 25X Stock Solution to 960 ml of distilled water. Add 1 g sodium azide. Mix well.

WARNING: Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

2. 2% Paraformaldehyde Stock Solution:

Add 2 g paraformaldehyde (Eastman Kodak) to 100 ml Phosphate buffer. Heat to 70°C in fume hood or in a 56°C water bath until the paraformaldehyde goes into solution (approximately 60 minutes). Allow the solution to cool to room temperature. Adjust to pH 7.4 using 0.1 M NaOH or 0.1 M HCl as needed. Store at 4°C.

0.5% Working Solution:

Add 10 ml 2% solution to 30 ml PBS (IX). Store at 4°C. Stable 1 week.

Equipment:

I. Refrigerated centrifuge fitted with swinging bucket rotor (4°C)

2. Ice bucket

Procedure:*

1. Following the last incubation with antibody or fluorescent second-step reagent, centrifuge the cells at 250 x g for 10 minutes. Remove the supernatant.
2. Add 1 ml of cold 1X PBS per 10^6 stained cells. Gently vortex the cells and centrifuge at 250 x g for 10 minutes. Remove the supernatant.
3. Repeat Step 2. Loosen pellet gently.
4. Add 0.3 to 1.0 ml of cold 0.5% paraformaldehyde solution to the pellet. Vortex the cell suspension **immediately.**
5. Store the fixed cells at 4°C in the dark. These cells may be stored for at least one week prior to FACs analysis.

* Note: Cells may also be fixed following staining in microtiter plates using these modifications:

I. Following the last staining step, centrifuge the cells (-10^6/well) in microtiter plates at 250 x g for 5 minutes. Carefully remove the supernatants.

2. Add 100 pl PBS to each well. Centrifuge at 250 x g for 5 minutes. Carefully remove the supernatants.
3. Repeat Step 2.
4. Add 100 pl of cold 0.5% paraformaldehyde solution to each well and **mix thoroughly at once.**
5. Store covered in the dark as above. Transfer into tubes for analysis. Dilute to proper volume with 0.5% paraformaldehyde. Cells fixed at least 4 hours may be diluted in PBS. If cells are analyzed with the FACS Analyzer, cells fixed at least 4 hours may be diluted in sheath fluid, not PBS.

References:

I Lanier, L.L., and Warner, N.L. **(1981)** Paraformaldehyde Fixation of Hematopoietic Cells for Quantitative Flow Cytometry (FACS) Analysis. *J. Immunol. Meth.* 47, 25

5789 23-1457-03

BECTON DICKINSON

Becton Dickinson immunocylometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

Direct Immunofluorescence Staining of Cell Surface Antigens In Unseparated Blood

This method is used to detect cells bearing specific membrane antigens by treating blood cells with monoclonal antibodies conjugated to fluorescein (FITC) or phycoerythrin* (PE). With this method, the blood sample is first treated with the red blood cell lysing solution; cells are then washed, then stained with the monoclonal antibody. Lysing followed by staining reduces possible interference by red blood cells and cellular debris.

Direct staining is followed by analysis with a FACS® Brand flow cytometer. **Note:** When the FACS Analyzer is used for analysis, the FACS Lysing Solution (Catalog No. 92-0002) is the recommended lysing agent. Refer to this product's package insert for instructions on staining and lysing.

Reagents:

I. F1TC or PE conjugated monoclonal antibody to human cell surface antigen.

2. Lysing Solution [Note: This is **not** FACS Lysing Solution]

   10X Stock Solution:

   Dissolve in I liter of glass distilled water

   89.9 g NH_4CI
   10.0 g $KHCO_3$
   370.0 mg tetrasodium EDTA

   Adjust to pH 7.3. Store at 2° to 8°C in a tightly closed bottle.

   IX Working Solution:
   To 90 ml glass distilled water, add 10 ml 10X Lysing Stock Solution. Mix well. **Store at room temperature.** Discard after one week.

3. Phosphate Buffered Saline (PBS) Solution or modified Dulbecco's Buffered Saline Solution containing no calcium or magnesium (mDBSS) [CIRCO, Grand Island, NY].

   25X PBS Stock Solution:

   Dissolve in I liter of glass distilled water

   188.0 g K_2HPO_4
   33.0 g $NaH_2PO_4 \cdot H_2O$
   180.0 g NaCI

   Store at room temperature.

   1X PBS Working Solution:
   To 960 ml glass distilled water, add 40 ml of 25X PBS Stock Solution. Add 1.0 g sodium azide. Mix well. The pH should be 7.2 ± 0.2. Store at 2° to 8°C. **Use cold buffer for diluting and washing cells.**

   IX PBS with Protein Carrier (2% wt/vol)

   Add 2 ml of newborn or fetal calf serum to 98 ml 1X **PBS** working solution. Serum Albumin may also be substituted at 2 g/100 ml IX PBS or as dilutions of 30-33% sterile buffered solutions. **Use cold buffer for diluting and washing cells.**

4. 0.5% Paraformaldehyde

   Dissolve 0.5 g paraformaldehyde (Baker S-898-7) in 100 ml of appropriate dilutent recommended for the flow cytometer or in IX **PBS.** (See Source Book Section 2.10).

Equipment and Materials:

I. EDTA VACUTAINER® Brand blood collection tubes (Becton Dickinson VACUTAINER Systems)

2. 12 x 75 mm test tubes (FALCON® Brand 2052 or 2058, Becton Dickinson Labware)

3. Low speed refrigerated centrifuge with swinging bucket rotor

4. Ice bucket with cover

5. Flow cytometer (with wide angle light scatter detector)

Specimen Collection and Preparation **of Whole Blood:**
Using VACUTAINER tubes containing EDTA, obtain 5 ml of blood by venipuncture.

Lysing Method for Unseparated Blood:

1. To 14 ml of 1X Lysing Solution at room temperature, add 1 ml of blood. Cap tube and mix immediately by inverting. (**Do not** use FACS Lysing Solution for this procedure. See Cat. No. 92-0002 for directions for use of this reagent.)

2. Let mixture stand at room temperature for 3 to 5 minutes. **Note:** Do not extend incubation beyond this time.

3. Centrifuge at 300 x g for 5 minutes at room temperature.

4. Remove supernatant by aspiration, leaving approximately 50 ;Al of fluid to avoid disturbing the cell pellet.

5. Mix gently and add 5 ml of 1 X cold PBS. Mix again and centrifuge at 300 x g for 5 minutes at 2° to 8°C.

6. Aspirate and discard supernatant. Resuspend pellet in I ml of IX PBS with protein carrier.

7. Test for viability — see Limitations No. 2.

Staining Method for Unseparated Blood on reverse side

* U.S. Patent 4,520,110; European Patent 76,695; and Canadian Patent 1,179,942

BECTON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Staining Method for Unseparated Blood:

1. a) For single color staining, add 20 Al of the FITC or PE labelled antibody to a 12 x 75 mm test tube.

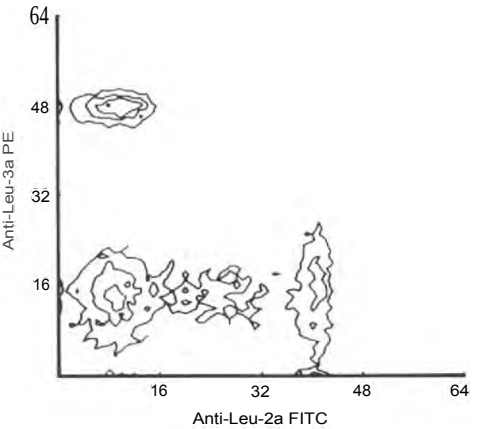
   b) For two color staining, add the specified amount of each FITC and PE labelled antibody per tube.
2. Transfer 50 *ill* aliquots of the cell preparation to each tube. If the white cell count is above or below normal, the volume may be adjusted. Total volume should not exceed 100 Al.
3. Incubate the mixture in the dark in an ice bath for 30 minutes.
4. After incubation, add 2-3 ml of IX PBS. Mix gently.
5. Centrifuge at 200 x g for 5 minutes.
6. Remove the supernatant by aspiration, leaving approximately 50 n1 of fluid.
7. Add 0.3-1.0 ml of appropriate diluent recommended for the flow cytometer. Mix and add an equal volume of cold 0.5% paraformaldehyde in IX PBS. Gently mix each tube immediately and thoroughly. Store cold in the dark, and analyze within 96 hours.

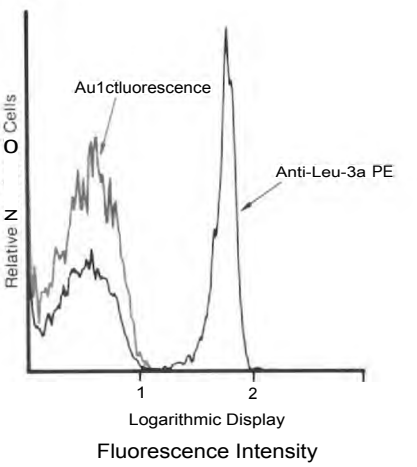
Results and **Interpretation:**
Representative data obtained with the FACS 440 cell sorter are shown below.

Limitations:

1. Each research laboratory should establish normal ranges using its own test conditions.
2. The specimen should contain >90% viable cells. The cytoplasm of dead cells stains nonspecifically, especially with FITC antibodies.
3. Avoid excessive centrifuge speeds and vigorous mixing of cells. Cell damage may cause non-specific staining, cell clumping, and excessive debris.
4. The amounts of reagents recommended for use are based on studies of normal human blood.
5. Monoclonal reagents which react with granulocytes (e.g., Anti-CR_3or Anti-Leu-1la and Anti-Leu-11c) may require additional amounts of reagent to saturate all binding sites.
6. Donors with nucleated red blood cells may show incomplete lysis of the red blood cell population.

Displays of Peripheral Blood Lymphocytes Analyzed with a FACS Brand Flow Cytometer
(Logarithmic Fluorescence Intensity)





Flow Cytometric Analysis: Performed on lysed whole blood with gates set on lymphocytes

Becton Dickinson Procedures

Two Step Indirect Immunoperoxidase Staining of Human Lymphoid Tissue

This procedure is used for immunoperoxidase staining of human lymphoid tissue with monoclonal antibodies detecting cell surface antigens. The staining technique consists of two stages. First, the tissue section is incubated with the unconjugated primary monoclonal antibody. The secondary antibody, Goat Anti-Mouse lgG conjugated with horseradish peroxidase, is then applied, binding to the primary antibody. The enzyme-antibody complex is visualized by development with a substrate chromogen.

Tissue:
Human cryostat section (4-5 pm) on chrom alum coated slide (see Reagents, item 10) or paraffin-embedded (4-5 pm) tissue section.

Reagents:

1. Monoclonal antibody to human cell surface antigen*

   Dilute to 10 to 25 pg/ml in IX mPBS immediately prior to use.

2. Goat Anti-Mouse lgG, Horseradish Peroxidase Conjugate (GAM Ig Peroxidase)

   Titer to determine appropriate dilution. Dilute in I X mPBS with 0.1% thimerosal prior to use. (0.2% gelatin is optional but may be added for increased reagent stability.)

3. 0.5% Copper sulfate 0.9% NaCI
4. Gill's Progressive Hematoxylin:

   To 730 ml distilled water, add 250 ml ethylene glycol
   4.0 g hematoxylin (Sigma)
   0.40 g $Na10_3$
   70.40 g $Al_c(SO_4)_3 \cdot 18H_2O$

   Mix well. Add 20 ml glacial acetic acid. Stir until solution contains no undissolved particles. Store at 2° to 8°C in a tightly sealed container.

5. 3,3 Diaminobenzidine (DAB),* 3 mg/vial (Sigma Chemical Co., St. Louis, MO)

   Reconstitute by adding 1 ml of IX mPBS to 3 mg of DAB. Add 10 ***pl*** of 30% H_2O_2. Prepare immediately prior to use. Do not store this solution. **Note:** It is essential to use the formula for modified phosphate-buffered saline (below).

6. Modified Phosphate Buffered Saline (mPBS), 0.1 M, pH 7.2 10X Stock Solution

   Dissolve in 1 liter of distilled water

   75.2 g K_2HPO_4
   13.2 g $NaH_2PO_4 \cdot H_2O$
   72.0 g NaCI

   I X Working Solution (1 X mPBS)

   To 900 ml of distilled water, add 100 ml of 10X Stock Solution. Mix well. Final pH should be 7.2 ± 0.2. Do not attempt to modify pH with acid or base. **(Note: Do not** add sodium azide.) Thimerosol (0.1%) (Sigma Chemical Co., St. Louis, MO, Catalog No. T5125) may be added to inhibit bacterial growth.

7. Anhydrous reagent grade acetone (frozen sections only)
8. Ammonia water

   Immediately before use, add I to 3 drops concentrated ammonium hydroxide (NH_4OH) to 250 ml of tap water. **(Paraffin Sections only)**

9. Slide Mounting Media -
   aqueous such as Aqua-mount" (Lerner Laboratories)
   or
   resinous type such as Protexx" (Lerner Laboratories)
10. Chrom Alum Solution (frozen sections)

    Slides are pre-coated with this solution to prevent sections from floating off during staining. Heat I liter of glass distilled water to 55°C. Do not overheat. Add 5.0 g gelatin and mix to dissolve. When gelatin is completely dissolved, add 0.5 g chrom alum (chromium potassium sulfate, Baker 1621-1), and mix to dissolve. While the solution is warm, filter with Nalge filter (0.45 micron) and store at 2° to 8°C. Do not add sodium azide or other bactericidal reagents. If contamination occurs, make up fresh solution. Wipe slides free of dust and dip in Chrom Alum coating solution. Drain off excess solution and stand slides vertically to dry.

Equipment:

1. Humidified chamber
2. Staining dishes
3. Coplin jars
4. Wash bottles
5. Light microscope

Frozen Section Preparation:
Cut 4 to 5 *jAm* cryostat sections of the frozen human tissue specimen. Air dry at room temperature 2 hours - overnight. (This will be determined by the relative humidity in your area.) Sections must be thoroughly dry. Fix in room temperature acetone for 10 minutes and then air dry. The slides may be stained immediately. For prolonged storage, keep slides at -20°C to -70°C in the presence of a dessicant. Allow only the slides you will use to equilibrate to room temperature 20-30 minutes in the presence of dessicant prior to staining.

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Paraffin Section Preparation:

1. Deparaffinize the sections by placing them for 5 minutes each in 2 changes of xylene, 1 minute each in 2 changes of 100% ethanol, 1 change of 95% ethanol, I change of 80% ethanol, and then water and mPBS.
2. If blocking of endogenous peroxidase is desired, place sections in 3% aqueous hydrogen peroxide (5 ml 30% H_2O_2 + 45 ml distilled water) for 5 minutes. Rinse slides in distilled H_2O.
3. Rinse slides in mPBS for 3-5 minutes.
4. Blocking serum may also be used if desired: After incubation with the mouse monoclonal antibody and mPBS rinse, apply a 1/10 dilution of normal serum (goat serum is recommended) to the section for 20-40 minutes. do not rinse in mPBS. Tip slide to remove excess serum, wipe around section leaving a square of moist film over the tissue and immediately add Working Solution of GAM Ig Peroxidase.

Staining Procedure:

I. Place the slide in a humidified chamber. Do not allow tissue sections to dry during the staining procedure. Add one drop of an appropriate dilution of monoclonal antibody to each section, carefully covering the entire section. Incubate at room temperature for 45 minutes. Rinse the section gently with IX mPBS, then immerse in IX mPBS for 5 minutes. Carefully wipe around the rinsed section, leaving a square of moist film over the tissue. Repeat this procedure for GAMIg-Peroxidase, etc., according to the schedule below.

a. Monoclonal antibody* **mPBS**	45 minutes
b. Blocking serum (if desired) (See Step #4 in Paraffin Section Preparation)	20-40 minutes
c. GAMIg-Peroxidase (at determined dilution)	45 minutes
d. I to 2 drops DAB Solution (prepare immediately before use)	5 minutes
e. Wash with mPBS; then rinse with distilled water	
f. Copper sulfate solution	5 minutes
g. Dip slide several times in distilled water	

2. Counterstain section by adding 1 drop of Hematoxylin. Incubate slide for 3 minutes in humidifying chamber at room temperature.
3. Rinse in running tap water until slide is clean (about 5 minutes).
4. Dip in ammonia water 3-5 times until blue brightens **(Paraffin Sections only).**
5. Rinse slides in distilled water.
6. Slides may be coverslipped in an aqueous mounting media or they may be dehydrated through alcohol and xylene and coverslipped with a resin-type mounting media.
7. Examine tissue section using a light microscope.

*** WARNINGS:**

1. Becton Dickinson monoclonal antibody reagents contain sodium azide as a preservative. Under acidic conditions sodium azide yields hydrazoid acid, an extremely toxic compound. Azide compounds should be diluted with running water before disposal, to avoid deposits in plumbing, where explosive conditions may develop.
2. Diaminobenzidine is listed as a carcinogen in California. Handle with care and dispose of properly. Rubber gloves are suggested. Do not store reconstituted DAB.

Becton Dickinson Procedures

Chicken Erythrocyte Nuclei Suspensions for Establishing Flow Cytometric Performance

INTRODUCTION

This procedure prepares chicken erythrocyte nuclei (CEN) suspensions. Stained CEN suspensions can be used to set up a FACS® 440, FACScanTM, FACStarTM, FACStarPIUSTM, or other flow cytometers for DNA analysis. The suspension will contain single nuclei, plus doublets, triplets, and some larger aggregates, stained with propidium iodide. This suspension will produce 4 or more reference peaks in the DNA content histogram, when it is analyzed on a flow cytometer.

We recommend analyzing this suspension at least once a day to obtain a consistent record of flow cytometer performance. Keep a log of mean peak channels of all peaks and the coefficient of variation of the singlet peak.

MATERIALS

Equipment

- Aspiration system, low vacuum
- Centrifuge at room temperature, with a swinging bucket rotor
- Hemacytometer
- Micropipettor, adjustable to 100 **Ail**
- Microscope for counting nuclei
- Shaker, Lab Quake, Labindustries No. 400-110'
- Vortex mixer

Supplies

- Aluminum foil
- Bottles, brown glass, **100** and 1000 ml
- Centrifuge tubes, polystyrene, 50 and 15 ml, Falcon Nos. 2098 and 2099[2]
- Pasteur pipettes, disposable, 14.6 cm^3
- Pipette bulbs, I ml, for Pasteur pipettes[3]
- Pipettes, 5 and 10 m1[3]
- Test tubes, polystyrene, 12 x 75 mm, Falcon No. 2058[2]

Media and Chemicals

- Ethanol, absolute
- Calcium chloride (dihydrate), Sigma No. C3881[4]
- Chicken blood, fresh (use within 3 days), Micro-biological Media[5]
- Dulbecco's phosphate buffered saline (PBS), calcium and magnesium free, Gibco No. 310-4190[6]
- Nonidet **P40** (NP40), US Biochemical Corp. No. 19628[7]
- Propidium iodide (PI), Calbiochem No. 537059[8]
- Saponin, Sigma No. S1252[4]
- Sodium chloride, Sigma No. S9625[4]
- Water, MilliQ or distilled

STOCK SOLUTIONS

Saline (0.85% NaCI, 3mM $CaCl_2$)	In 900 ml water, dissolve 8.5 g sodium chloride and 0.33 g $CaCl_2$ (dihydrate). Adjust to 1,000 ml. Store in stoppered bottle at room temperature.
Saponin§ (0.05% saponin, 0.85% NaCl, 3 mM $CaC1_2$)	In 900 ml water, dissolve 8.5 g sodium chloride, 0.33 g $CaCl_2$ (dihydrate), and 0.5 g saponin. Adjust to 1,000 ml. Store in stoppered bottle at room temperature.

§ WARNING: Saponin powder is poisonous. Do not inhale and handle with care.

Stain§ (50 µg/m1 PI, 0.6% NP40 in PBS)	To 90 ml PBS, add 5 mg propidium iodide and 0.6 ml NP40. Protect from light, Stir gently to dissolve completely. Adjust volume to 100 ml with **PBS.** Store protected from light at 4°C for up to 3 months.

§ WARNING: Propidium iodide is a suspected carcinogen. Handle with care.

PROCEDURE

I. Measure 10 ml of chicken blood (well mixed) into a 50 nil centrifuge tube. Add saline to a total volume of 50 ml.

2. Centrifuge 15 minutes at 800 x g.
3. Aspirate the supernatant through a Pasteur pipette.
4. Add 20 ml saponin solution and resuspend the pellet by gently vortexing. Shake the tube for 10 minutes at room temperature on the Lab Quake shaker.
5. Add 30 ml saline to the tube (total volume of 50 ml). Mix gently. Centrifuge 15 minutes at 800 x g.
6. Wash nuclei at least three times with saline to remove all traces of red color in the supernatant, as follows:
   a. Aspirate the supernatant. Visually confirm the removal of red color.
   b. Resuspend the pellet by pipetting 2 ml saline into the tube with a Pasteur pipette. Draw the suspension into the pipette several times to disperse aggregates. Add 48 ml more saline and mix gently.
   c. Centrifuge 15 minutes at 800 x g. Aspirate the supernatant.
7. Add 20 ml saline to the nuclear pellet and vortex to resuspend. Divide the suspension into five 15 ml centrifuge tubes, 4 ml per tube. Vortex each tube vigorously to disperse clumps of nuclei.
8. To each tube, add drop-by-drop 4 ml of absolute ethanol. Vortex vigorously while adding the ethanol.
9. Pool the suspensions of nuclei and count in a hema-cytometer. Record the count. Store at 4°C for up to 3 months.
10. To stain nuclei, add a well-mixed volume containing 8 x 10^6 nuclei to I ml of stain solution in a 12 x 75 mm tube. Vortex to mix.

II. Incubate for 10 minutes at room temperature, protected from light. Hold the stained sample at 4°C before analysis. Use within 6 hours.

I. Labindustries, Berkeley, CA, (415) 843-0220
2. Becton Dickinson, Lincoln Park, NJ, (800) 235-5953
3. Baxter Scientific Products, McGaw Park, IL (800) 325-4515
4. Sigma Chemical Co., St. Louis, MO, (800) 325-3010
5. Microbiological Media, Concord, CA, (415) 746-6616
6. Gibco Laboratories, Grand Island, NY, (800) 626-6666
7. US Biochemical Corporation, Cleveland, OH, (800) 321-9322
8. Calbiochem - Behring Diagnostics, San Diego, CA, (800) 854-9256

NOTE: These are suggested sources. You may use other suppliers that provide comparable products.

BECTON DICKINSON

Becton Dickinson I mmunocylometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

DISCUSSION

This procedure produces nuclei, stained with propidium iodide for verifying the performance of a flow cytometer for DNA Analysis. Use only fresh, unfixed chicken red blood cells for this procedure. Frozen of lyophilized cells are not suitable. The unstained nuclei or step 9 of the procedure are stable for at least three months when stored at 4°C.

When stained with propidium iodide, these nuclei give four or more peaks in the orange or red fluorescence histogram upon flow cytometer analysis (Figure I). The peak at the far left represents single nuclei. The next three peaks to the right have positions (mean channel values) approximately 2, 3, and 4 times greater than the single nuclei peak. The actual numbers you obtain indicate the linearity of the flow cytometer when analyzing particles of this size. The coefficient of variation (CV) of the first peak should be less than 3%.

NOTE: This procedure is for research use only.

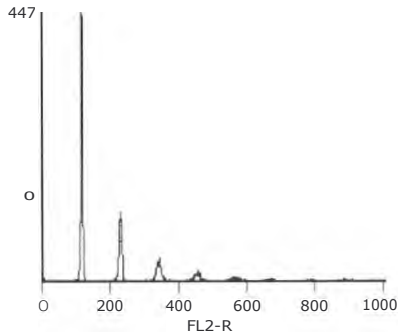


Figure 1.

A typical fluorescence histogram of chicken red blood cell nuclei, prepared by this procedure and stained with propidium iodide. This is ungated integrated fluorescence data from a FACScanTM flow cytometer obtained with CellFIT" software. The peak at channels 110-120 represents single nuclei. The ratios of the channel positions (mean values) for the doublet, triplet, and quadruplet peaks to the singlet peak shown here are 1.99, 2.97, and 3.95. The CV of the singlet peak is 2.0%

REFERENCES

Braylan, R.C., Attributes and Applications of Flow Cytomctry. *ANN Clin Lab Sri.* 13(5): 379-384, 1983.

[2]Thornthwaite, J.T., Sugerbaker, E.V., and Temple, WI, Preparation of Tissues for DNA Flow Cytometric Analysis. *C•tometry,* 1:229-237, 1980.

Becton Dickinson Procedures

Blockage of Cytophilic Ig-Binding to Lymphocytes

This procedure may be used to block binding of serum cytophilic Ig to lymphocytes (such as NK cells) which possess Fc receptors for human immunoglobutin. Fc binding of cytophilic Ig to these cells may cause problems with reagents such as Simultest Kappa/Lambda. In the case of this reagent, serum Ig which contains both Kappa chains and Lambda chains is bound to Fc receptor-bearing lymphocytes (primarily NK cells). These cells, which have bound human Ig, will then stain with both the Anti-Kappa FITC and Anti-Lambda PE antibodies in this reagent, and will appear as a double-stained population in a fluorescence contour plot. Preparation of PBMC's using this procedure will reduce this type of staining. If lymphocytes bearing Fc receptors to human Ig are of interest, simply separate the PBMC's following BD Procedure for Preparation of Single Cell Suspensions from Human Peripheral Blood, Section 2.2, and stain per BD Procedure for Direct lmmunofluorescence Staining of Cell Surfaces, Section 2.4.

Reagents and Supplies:

I. IX PBS (Dulbecco's) + 0.1% NaN_3*
2. LeucoPREP tubes (Becton Dickinson) or Ficoll-Hypaque (Pharmacia)
3. Normal rabbit serum
4. 0.5% Paraformaldehyde in PBS
5. EDTA Vacutainer Brand blood collection tubes (Becton Dickinson)
6. Ethidium Bromide and Acridine Orange**
7. Pipets
8. 15 ml centrifuge tubes
9. Disposable test tubes or 96 well-microtiter plates.

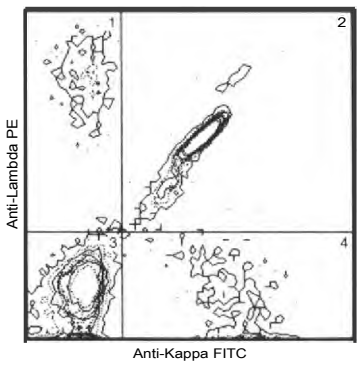
Equipment:

1. Refrigerated centrifuge with swinging bucket rotor
2. 37°C water bath

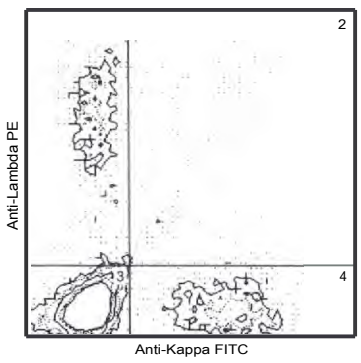
Procedure:

1. Obtain venous blood sample by venipuncture into blood collection tubes.
2. Separate PBMCs using LeucoPREP tubes, following instructions in the product insert. (Ficoll-Hypaque may be used.) Remove the plasma and place the remaining PBMC's into a clean 15 ml centrifuge tube.
3. Fill the tube with room temperature I X PBS + 0.1% NaN_3, mix the cells, and centrifuge at 300 x g for 7-10 minutes. Aspirate and discard the supernatant.
4. Repeat the wash procedure in Step 3, but centrifuge at 200-250 x g.
5. Resuspend the pellet in room temperature IX PBS + 0.1% NaN_3, mix gently, and determine viability using ethidium bromide/acridine orange (see BD procedure on Viability Staining Using Ethidium Bromide and Acridine Orange, section 2.3.) Cells should be >90% viable. Determine cell concentration.
6. Centrifuge cells for 5 minutes at 200-250 x g. Aspirate and discard the supernatant.
7. Resuspend the pellet in IX PBS + 0.1% NaN_3 containing normal rabbit serum. (Recommended dilution is 1:5 - 1:10. Optimal dilution should be determined by each laboratory.) Resuspend cells to a concentration of 2 x $l0^7$ cells/ml.
8. Incubate cells in a 37°C water bath for 30 minutes.
9. Remove cells from the water bath, mix gently, and add 1 x 10^6 cells to each test tube or microtiter well containing antibody. Mix well.
10. Incubate on ice for 30 minutes, in the dark.
11. Wash cells with cold IX PBS + 0.1% NaN_3. Centrifuge at 4°C for 5 minutes at 250 x g. Aspirate and discard the supernatant. Repeat the wash procedure.
12. Add 0.5 ml of cold 0.5% paraformaldehyde to each test tube, and mix well. (If stained in microtiter plates, add 0.1 ml, mix, and transfer to a test tube containing an additional 0.4 ml of paraformaldehyde. See BD procedure for Paraformaldehyde Fixation of Cells, section 2.10.)
13. Store cells at 2-8°C in the dark, until ready for analysis.

Two Parameter Display of Peripheral Blood Lymphocytes Analyzed with a FACS Brand Flow Cytometer
(Logarithmic Fluorescence Intensity)



Cells (PBMC) prepared per BD Procedure for Direct Immunofluorescence Staining of Cell Surfaces, Source Book Section 2.4.
Note double-stained NK cells in Quadrant 2.



Cells prepared per BD Procedure for Blockage of Cytophilic lg-Binding to Lymphocytes, Source Book Section 2.15.

* **WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

** **CAUTION:** Ethidium bromide and acridine orange are suspected carcinogens. Handle with care.

1/89 23-1756-00

BECTON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Becton Dickinson Procedures

For DNA Analysis: Preparation of Nuclei from Paraffin-Embedded Tissues

This procedure produces a nuclear suspension from paraffin-embedded tissues that has been optimized for DNA analysis using a FACScanTM flow cytometer equipped with the Doublet Discrimination Module and CellFIT Software. Use the Alternate Instrument Setup in the Becton Dickinson Procedure "Setting Up the FACScan flow cytometer with Doublet Discrimination for DNA Analysis", Source Book Section 2.16. Starting with paraffin-embedded microtome sections, you remove the paraffin with AmeriClearTM and release the nuclei by pepsin digestion and mechanical disruption. Staining the nuclear suspension with propidium iodide completes the preparation.

Materials

Equipment

- Aspiration system, low vacuum
- Centrifuge, refrigerated at 4°C
- Fluorescence microscope with epi-illumination
- Hemacytometer
- Micropipettors, 25 and 100 ill
- Microtome to cut 50-100 *p* sections
- Offset-tipped Forceps
- Paper clips
- Scissors, dissecting, to reach to the bottom of a 5 ml centrifuge tube
- Stapler, standard office type
- Staple remover, standard office type
- Vortex mixer
- Water bath at 37°C

Supplies

- Biopsy Nylon Bag, 35 x 40 mm, Shandon, Inc. No. 67740014[7]
- Centrifuge tubes, polystyrene, 5 ml (12 x 75 mm test tube) and 15 ml, Becton Dickinson Falcon® Nos. 2058 and 2099[1]
- Coplin Jars (8 per specimen)
- Glass stirring rods, thin, to fit 5 ml centrifuge tubes
- Hypodermic needles, 18G, 1 1/2 inch, Becton Dickinson No. 5196[2].
- Labeling tape
- Luer Stubs, I8G, Becton Dickinson No. 7563[2]
- Microcentrifuge tubes, 1.5 ml, Scientific Products No. C35 I5-10A[3]
- Micropipettor tips
- Microscope slides, frosted end
- Nylon cloth, 35 p mesh, Tetco No. 3-35/ 22[4]
- Pasteur pipettes, disposable, 14.6 cm
- Pipette bulbs, 1 ml, for Pasteur pipettes
- Syringes, plastic, disposable, 1 ml Tuberculin, Becton Dickinson No. 5602[2]
- Transfer pipettes, non-sterile, Becton Dickinson Falcon No. 7524[1]

Media and Chemicals

- AmeriClear histology clearing solvent, Scientific Products No. C4200-1[3]
- Citric acid, trisodium salt, Sigma No. C8532[5]
- Ethanol, 100%, 95%, 70%, and 50%
- Hanks balanced salt solution (HBSS), Sigma No. H8264[5]
- HEPES, Sigma No. 117006[5]
- HCI, Scientific Products No. 2611-500*NY[3]
- Pepsin, Sigma No. P6887[5]
- Propidium Iodide (PI), Calbiochem No. 537059[6]
- Polyethylene Glycol (PEG) 8000, Sigma No. P2139[5]
- Ribonuclease (RNAse), Sigma No. R4875[5]
- Sodium Chloride (NaCI), Sigma No. S9625[5]
- Sodium Azide, Sigma. 52002[5]
- Triton® X-100, Sigma No. T6878[5]
- Water, high quality, Milli-Q® or glass distilled

Stock Solutions

Solution	Dissolve	Final Volume and Storage
Sodium chloride, 0.9%, with 3% Poly-ethylene Glycol 8000, and HCI to pH 1.5	9 g NaCI, 30g PEG 8000 in 900 ml distilled water, adjust pH to 1.5 with HC1	1,000 ml**
Sodium chloride, 3M	17.5 g NaCI in 90 ml distilled water	100 ml**
Sodium azidet 10%	10 g Sodium Azide in 90 ml distilled water	100 ml**
Sodium citrate, 1 M	29.4 g Sodium Citrate in 90 ml distilled water	100 ml**
Sodium citrate, 4mM	0.4 ml 1M sodium citrate in 99.6 ml distilled water	100 ml**
Propidium iodide§ (P1), 500 tig/m1	50 mg in 100 ml distilled water	100 ml. Store in dark at 4°C, up to 6 months
Ribonuclease A (RNAse), 10 mg/ml	100 mg in 10 ml 4mM sodium citrate. Use 15 ml tube; vortex. Heat 30 mins. at 75° C. Cool to room temperature.	Store 1 ml aliquots frozen in micro-centrifuge tubes for up to 6 months, at -20° C

•* Store in stoppered, labeled bottle at 4°C up to three months.

t WARNING: Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Handle with care. Dilute sodium azide solutions with running water before discarding to avoid deposits in plumbing where explosive conditions may develop.

§ **CAUTION:** Propidium iodide is a **known** mutagen **and a suspected carcinogen. Handle** with care.

For research use only. Not for use in diagnostic or therapeutic procedures.

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

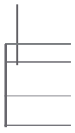
Working Solutions

Pepsin (0.5% pepsin, 3% Polyethylene Glycol 8000 in 0.9% NaCl, pH 1.5)	To 30 ml NaCl/ PEG/ HC1 add 0.15 g pepsin; check pH for 1.5. Store at 4°C. Prepare fresh daily. (3 ml needed for each sample)
Wash (10mM HEPES, 0.02% NaN_3, 3% PEG in HBSS)	In 80 ml of HBSS, dissolve 3 g PEG 8000, 0.26 g HEPES, and add 2 ml 10% sodium azide solution. Adjust volume to 100 ml with HBSS. Store at 4°C for up to three months.
Stain (50 µg/ml PI, 0.1% Triton X-100 in 4mM sodium citrate)	To 80 ml 4mM sodium citrate, add 10 ml propidium iodide stock solution and 0.1 ml Triton X-I00. Adjust to 100 ml with 4mM sodium citrate. Protect from light. Store at 4°C for up to 3 months. (pH 7.0 ± 2)

Procedure

1. Turn on the water bath and set to 37° C.
2. For each specimen,
   fill 8 Coplin jars: #1: AmeriClear
   #2: 100% ethanol
   #3: 100% ethanol
   #4: 95% ethanol
   #5: 70% ethanol
   #6: 50% ethanol
   #7: water
   #8: water
3. Cut 50-100 *um* tissue sections from each paraffin-embedded specimen. Start with three sections per paraffin block. If the block contains little tissue, use up to ten sections per specimen in this procedure.
4. Place up to three sections from each block in a separate mesh bag. Close and label bags as shown in Figure 1.

A. An open biopsy bag is approximately 2 inches long by 1-3/8 inches wide.



B. Insert tissue sections. Fold top edge twice and staple. Attach a paper clip and label as shown.

Figure 1.
Preparation of nylon mesh bag for treating tissue sections.

CAUTION: Avoid breathing in vapors or getting AmeriClear on your skin. AmeriClear dissolves plastic items and vinyl gloves.

5. Use offset-tipped forceps to break the paraffin away from the tissue section by squeezing the nylon bag at separation point. Dip nylon bags in Coplin jar #1 of AmeriClear for 20 minutes or until sections are transparent and the paraffin is dissolved, maximum time: 3 hours.
6. Rehydrate sections by placing bags sequentially in each Coplin jar (#2 - #8) for 10 minutes per jar. NOTE: One set of Coplin jars should be used per specimen.
7. Remove sections from bags by peeling bag open and scraping the section off with forceps. Transfer to a 5 ml centrifuge tube.
8. Add 0.1 ml pepsin solution to bottom of tube. Mince the pellet with dissecting scissors to dissociate the tissue, then mash gently with a glass rod. If the tissue is gelatinous, mince before pushing to bottom of tube. Mincing well enhances cell yield. Use a separate rod and scissors for each specimen.
9. Add 1.0 ml of pepsin solution to each tube and vortex on medium speed for I minute to resuspend the tissue.
10. Incubate tube for 1 hour in 37°C water bath. Vortex at 10 minute intervals. NOTE: With some tissues, a longer incubation may increase recovery.
11. At room temperature, using a Pasteur pipette, resuspend the tissue suspension 5-10 times. this increases the yield of single nuclei.
12. Use a 1 ml syringe with an 18G luer stub to hold the 35 um nylon mesh in place. (Fig. 2). Filter the nuclear suspension by removing the plunger, adding the nuclear suspension, replacing the plunger and forcing the suspension slowly through the cloth and into a 5 ml test tube. If mesh becomes clogged, hold syringe horizontally, carefully remove luer tip, displace the mesh to clean area, re-attach the luer tip and continue filtering nuclei into 5 ml test tube.

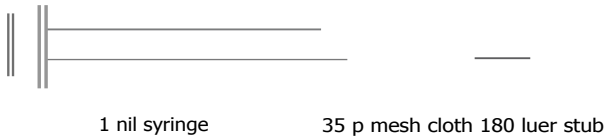


Figure 2.
A device to remove debris from the nuclear suspension. Use the 18G luer stub to fasten the nylon cloth to the end of a 1 ml Tuberculin syringe.

13. Add wash solution to 5 mi total volume per tube.
14. Centrifuge suspension at 400 x g for 10 minutes at 4°C. Carefully take off the supernatant with a Pasteur pipette. The thin pellet may be difficult to see.
15. Gently resuspend the pellet (by tapping) in 2 ml wash solution plus 0.5 ml stain solution.

    Caution: Propidium Iodide is a known mutagen and suspected carcinogen. Handle with care.
16. Count the nuclei in a hemacytometer, using a fluorescence microscope. The nuclei concentration should be at least 200,000/ml. (Lower concentrations result in long flow cytometer data collection periods.)

    NOTE: You can now store the samples overnight at 4°C, in the dark.
17. Centrifuge at 400 x g for 10 minutes at 4°C. With a Pasteur pipette, aspirate off all but about 100 ul of supernatant.
18. Gently vortex for 5 seconds to resuspend the pellet in the remaining supernatant.
19. Add stain solution. If the concentration of nuclei at step 16 was greater than 10^6 nuclei/ml add 2 ml stain and protect from light. Otherwise, add 0.5 ml stain and protect from light.
20. Add 0.2 ml RNAse (thawed). Vortex for 5 seconds.
21. Incubate for 10 minutes at 37° C, protected from light.

22. Add 25 ill of 3M NaC1 to 0.5 ml of nuclear suspension, or 100 IA1 of 3M NaC1 to 2 ml of nuclear suspension. Vortex for 2 seconds. Keep suspension at 4°C, protected from light. Analyze immediately or within 6 hours.
23. Set up your FACScan with the alternate procedure (Section G.) of the Becton Dickinson Procedure "Setting Up the FACScan Flow Cytometer with Doublet Discrimination for DNA Analysis", Source Book Section 2.16. You may find it helpful to modify the settings for "Peak Channel for Automatic PMT Adjustment" to the following values, SSC Average: 450, FL2-A Average: 200. When staining the set up particles using the Stain solution from this Paraffin-Embedded Tissues procedure, add 50 Ail of 3M NaCI per 1.0 ml of Stain solution. Addition of NaCI should be the final step in the staining procedure.

Discussion

This procedure produces nuclei, stained with propidium iodide, suitable for DNA analysis in a flow cytometer. Propidium iodide staining provides an approximate, relative measure of nuclear DNA content. Sample preparation factors which affect the accuracy of this relative measurement include: the presence of undigested double stranded RNA, differences in chromatin structure between different cell types, type of fixative, length of time in fixative, amount of debris in the sample, sample storage conditions before and after fixation, and the extent of chromatin protein digestion by pepsin during the release of nuclei.

Because of the effects of fixation and treatment on paraffin-embedded tissues, it is not possible to use cells from other sources, such as peripheral blood lymphocytes, as internal and external controls for this procedure. However, normal cells in the same paraffin block are appropriate as controls.

Expected Results

The flow cytometer data shows a typical DNA distribution for human tonsil nuclei, extracted by this procedure (Figure 3). The major peak represents nuclei in GO/ GI phases of the cell cycle. The coefficient of variation is typically 8%.

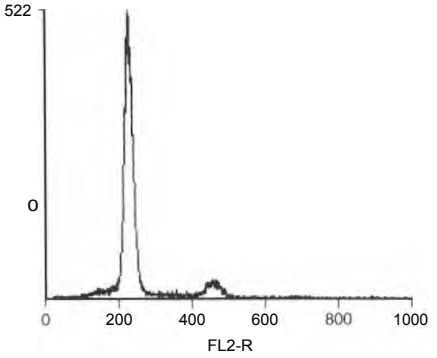


Figure 3.
A histogram showing propidium iodide fluorescence of nuclei isolated by this procedure from human tonsil tissue. The data in this histogram is from the gated region of the FSC vs. SSC dot plot shown in Figure 4 (A). The data in this histogram may be analyzed for percentage of cells in the G0/01, S, and G2/ M phases of the cell cycle.

Dot plots, or two dimensional histograms, of the same data (Figure 4) may reveal the presence of minor populations or contaminants. The forward scatter (FSC) versus side scatter (SSC) dot plot shows one primary size population of nuclei. The forward scatter (FSC) versus PI fluorescence (FL2-A) dot plot shows the presence of particles with FL2 values less than GI cells and low FSC values. These particles may be nuclear fragments created during sectioning or isolation of nuclei.

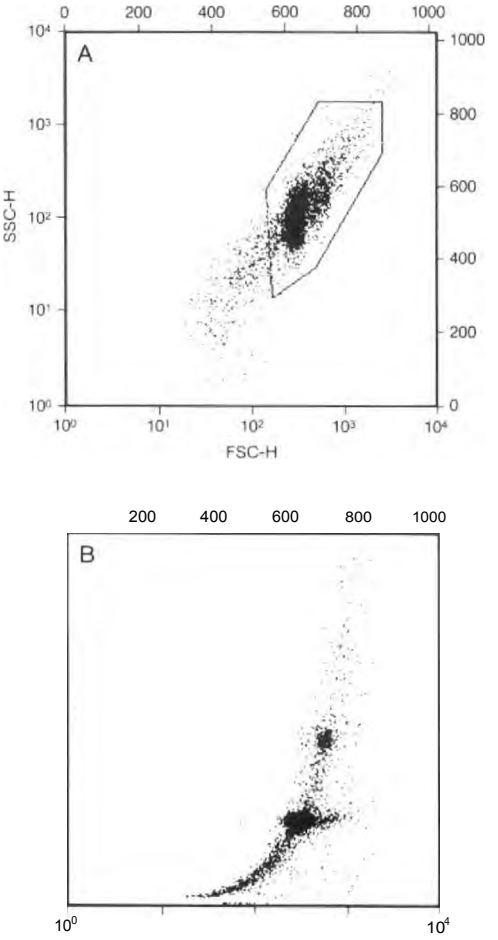


Figure 4.
Dot plots from a Cell FIT data analysis of human tonsil nuclei. A: Typical dot plot of forward scatter (FSC) versus side scatter (SSC) parameters. The polygon is a gate, created with software, that partially isolates nuclear data from debris and aggregates. B: Typical dot plot of FSC versus propidium iodide fluorescence (FL2-A).

Limitations

Fresh tissue is generally preferable to paraffin-embedded material for most routine clinical research purposes. This technique for extracting and staining nuclei from paraffin-embedded tissues is intended for use in retrospective studies in which DNA content can be evaluated in cohorts of patients where the outcome is already known.

Limitations of working with paraffin-embedded materials include:

a. The procedure is time consuming. Five to seven hours are required compared with one hour or less for procedures beginning with fresh tissue.
b. The histograms are of poorer quality than one obtains from fresh tissue preparations. The amount of debris is greater, making measurements of % S phase less certain. Also, the GO/GI peaks are wider, so that abnormal near-diploid peaks may be obscured.
c. Some commonly used fixatives, such as Bouins, or mercury containing fixatives interfere with staining.
d. Formalin is the most appropriate fixative. However, since formalin penetration of tissues is slow, fixation may be uneven and autolysis may have occured in central regions of larger samples.

Users adapting this method should not be too discouraged by poor initial results. Unsuitable starting material is often a problem. We have found that paraffin embedded tonsil tissue is suitable starting material for anyone learning the procedure.

Sources

1. Becton Dickinson, Lincoln Park, NJ (800) 235-5953
2. Becton Dickinson, Rutherford, NJ (201) 460-4900
3. Scientific Products, McGaw Park, IL (800) 325-4515
4. Tetco, Inc., Monterey Park, CA (818) 289-9153
5. Sigma Chemicals Co., St. Louis, MO (800) 325-3010
6. Calbiochem-Behring Diagnostics, San Diego, CA (800) 854-9256
7. Shandon, Inc., Pittsburg, PA (800) 245-6212

NOTE: These are suggested sources. You may use other suppliers that provide comparable products.

References

Bauer, K.D., Clevenger, C.V., Endow, R.K., Murad, T, Epstein, A.L., and Scarpelli, D.G., Simultaneous Nuclear Antigen and DNA Content Quantitation Using Paraffin Embedded Colonic Tissue and Multiparameter Flow Cytometery, *Cancer Res.*, 46:2428-2434, 1986.

Hedley, D.W., Friedlander, M.L., Taylor, I.VV., Rugg, C.A., and Musgrove, E.A., Method for Analysis of Cellular DNA Content of Paraffin-Embedded Pathological Material Using Flow Cytometry, *J. Histochem. Cytochem., 31 (11):1333-1335,* 1983.

Hedley, D.W., Friedlander, M.L., and Taylor, Application of DNA Flow Cytometry to Paraffin-Embedded Archival Material For Study of Aneuploidy and Its Clinical Significance, *Cytometry,* 6:327-333, 1985.

Hedley, D.W., Flow Cytometry Using Paraffin-Embedded Tissue: Five Years On, *Cytometry,* 10:229-241, 1989.

Schulte, B., Reynders, M.M.J., Bosman, FT, and Bijham, G.H., Flow Cytometric Determination of DNA Ploidy Level in Nuclei Isolated From Paraffin-Embedded Tissue, *Cytomerry,* 6:26-30, 1985.

Methods | Isolation of Human T Cell Populations by Panning

Method provided by Claudia Benike and Edgar G. Engleman, M.D., Stanford University School of Medicine, Department of Pathology.

This method is used to positively select specific lymphocyte subpopulations based on their cell surface markers. Total peripheral blood mononuclear cells or E-rosette positive (T) cells are incubated with monoclonal antibody, then poured into petri plates precoated with anti-mouse immunoglobulin. Cells positive (adherent) and negative (nonadherent) for the specific monoclonal antibody are collected after incubation.

Reagents:

Ia. Peripheral blood mononuclear cells which have undergone a plastic adherence for monocyte depletion (I hour at 37°C in RPMl 1640 with 5% human or fetal calf serum.) Cells are decanted following incubation.

or

Ib. Sheep erythrocyte (E) rosetted cells of human peripheral blood mononuclear cells (<3cii, surface immunoglobulin positive cells, <3% alphanaphthyl acetate positive cells). Centrifuge cells just prior to use, then remove supernatant.

2. Monoclonal antibody to human T lymphocyte subpopulaion (e.g., anti-Leu-2a for T cytotoxic/ suppressor cells; anti-Leu-3a for T helper inducer cells)
3. Diluent: Fetal Calf Serum/ Phosphate-Buffered Saline (FCSI PBS)
   a. Dulbecco's Phosphate-Buffered Saline (PBS) with calcium and magnesium
   b. 5% FCSI PBS
      5 ml fetal calf serum:100 ml **PBS**
   c. l % **FCS/ PBS**
      l ml fetal calf serum.' 100 ml PBS
4. Goat anti-mouse immunoglobulin G (IgG), affinity purified (or use anti-isotype as appropriate)
5. 0.05M Tris Buffer, pH 9.5

Equipment:

1. Plastic petri dishes, bacteriological grade (not treated for tissue culture), 15 x 100 mm
2. Refrigerator (2°-8°C)

Procedure:

I. Dilute goat anti-mouse IgG to a concentration of 10 pig ml in 0.05 M Tris.

2. Incubate each petri dish with 10 ml of diluted goat anti-mouse IgG at room temperature for 40 minutes. Remo).e unbound antibody by washing dish three times with PBS and one time with I% FCS. PBS.
3. Dilute monoclonal antibody to 10 Agf ml in PBS.
4. Mix cell pellet (2-3 x 10' cells) with 20 mg (2 ml) of monoclonal antibody. Incubate at room temperature for 20 minutes. Centrifuge cells, remove supernatant. then wash with 5% FCS PBS. Repeat centrifugation and washing. Resuspend x 10" cells in 3 ml ()I FCS. PBS and pour antibody-treated cells onto one coated plate. Incubate at 2°-8°C for 2 hours.
5. Collect nonadherent cells by decanting. Wash plate gently with 5-7 ml of I% FCS: PBS. Decant. Repeat gentle washing four times, pooling washes. (Negative population)
6. Add 15-20 ml of I% FCS PBS to plate. Pipet vigorously to remove bound cells (positive population). Check plate tier cells using an inverted microscope. If necessary, wash plate again x`ith V i **FCS, PBS.**

Comments:

Results using anti-Leu-2a as the first stage antibody, as determined by flow cytometric analysis:

Bound cells	>98%	Leu-2a+
Unbound cells	< 5%	Leu-2a+

Results using anti-Leu-3a:

Bound cells	>98%	Leu-3a+
Unbound cells	< 5%	Leu-3a*

Reference:

Engleman, E.G., Benike, C.J., Grumet, EC.. and Evans. R.L. (1981) Activation of Human T Lymphocyte Subsets: Helper and Suppressor Cytotoxic T Cells Recognize and Respond to Distinct Histocompatability Antigens. *J. Immunol. 127.* 2124

5/89 23-1375-02

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Methods

Immunofluorescence Staining of Cell Surfaces — Cytocentrifuge Preparations

Method provided by William Gathings, Ph.D., Southern Biotechnology Associates, Inc., Birmingham, AL.

This method uses monoclonal antibodies to membrane antigens for direct or indirect immunofluorescence staining of single cell suspensions which are then fixed to slides using a cytocentrifuge.

For direct immunofluorescence, cells are incubated with fluorochrome-conjugated monoclonal antibodies. For indirect immunofluorescence, cells are incubated with antibody that is unconjugated or labeled with a hapten such as biotin, followed by the appropriate fluorochrome-conjugated second step reagent (anti-mouse Ig or avidin/streptavidin). The stained cells are centrifuged onto slides and then fixed. When stored at 2° to 8°C in the dark, the fixed suspensions maintain fluorescence for up to one year.

Cells:

I. Human or mouse single cell suspension

Reagents:

For direct immunofluorescence:

I. Fluorescein (FITC) or phycoerythrin (PE) labeled mouse monoclonal antibody specific for membrane antigen of interest.

For indirect immunofluorescence:

Ia. Unconjugated or biotin-labeled mouse monoclonal antibody specific for membrane antigen

lb. Anti-mouse Ig MC/PE, or avidin F1TC/Streptavidin PE

2. Hank's balanced salt solution containing 5% fetal bovine serum (BSS/ FBS)

3. Phosphate-buffered saline containing 5% fetal bovine serum and 0.1% sodium azide* (PBS/ FBS)

4. Phosphate-buffered saline (PBS), pH 7.4

5. Fixative: 95% ethanol/5% glacial acetic acid

6. Slide mounting medium:
   Phosphate-buffered Fluoromount-G

   Dissolve 10 g Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, **AL)** in 70 ml PBS at 80°C. Cool to room temperature. Add 30 ml Spectral Grade glycerol (non-fluorescent). Mix well and adjust pH to 7.4. Add sodium azide* to a final concentration of 0.1% (w/v). Filter through Whatman #1 paper.

Equipment:

I. Refrigerated centrifuge with swinging bucket rotor

2. Cytospin® cytocentrifuge (Shandon Inc., Pittsburgh, PA)

3. 6 x 50 mm glass test tubes

4. 12 x 75 mm glass test tubes

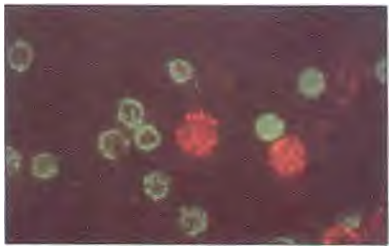
5. Bellco stainless steel staining rack

6. Wheaton staining dishes with racks

7. Fluorescence microscope

Two-Color Indirect Immunofluorescence with Cytocentrifuge Preparations of Human Peripheral Blood. T cells (green) are detected by Anti-Leu-4 and monocytes (red) are detected by a monclonal antibody, clone H83-2.

Phase



Double Exposure

Photos courtesty of John Kearney. Ph.D.
Division of Developmental and Clinical Immunology
University of Alabama.

Procedure:

1. Prepare cell suspension, wash thoroughly, then determine cell concentration and viability (>90%). Dilute to approximately 2 x 10^6/ml in BSS/ FBS or PBS/ FBS. Incubate in a culture dish or flask at 37°C for one hour. The adherence step will decrease background due to cytophilic 1g.
2. Wash cells 2X in BSS/ FBS, resuspend cells in 2-3 ml and determine cell count and viability.
3. Transfer 5 x 10^5 cells to each 6 x 50 mm tube in which staining will be done; spin cells at 800 rpm for 5 minutes at 4°C.
4. Aspirate supernatant using a fine bore Pasteur pipet, leaving the cell pellet as dry as possible. Add 10 pi of appropriately diluted monoclonal antibody to each tube, vortex and incubate on ice for 20 minutes (vortex 2X during incubation period).
5. Wash cells 2X in PBS/ FBS, then aspirate supernatant.

   For indirect immunofiuorescence, add 10 *pi* of appropriately diluted second-step reagent and incubate on ice for 20 minutes.
6. Wash stained cells 2-3X in PBS/ FBS.
7. Resuspend cells in 0.4 ml PBS/ FBS, transfer to 12 x 75 mm tubes and dilute to a final volume of 0.7 ml (-7.5 x 10^5 cells/nil).
8. Place 0.1 ml of diluted cells in each cytospin block and centrifuge at 800 rpm for 5 minutes.
9. Allow preps to air dry for 5 minutes, then check cell density (20-30 cells/field is optimal). Place slides in rack of staining dish and fix in ethanol-glacial acetic acid for 20 minutes at -20° C.
10. Transfer rack containing slides to a staining dish containing fresh PBS; rehydrate and wash in 3-4 changes of PBS.
11. Remove one slide at a time from staining dish, add one drop of Fluoromount-G mounting medium to cell prep, mount cover slip and blot with a 4 x 4 gauze sponge. Allow 10 minutes for preps to dry.
12. Stores prep at 4°C until they are examined. Prep can be stored in this state for up to one year without loss of fluorescence staining.

Reference:

Gathings, WE., Lawton, A.R. and Cooper, M.D. (1977) Immunofluorescent Studies of the Development of Pre-B Cells, B Lymphocytes and lmmunoglobulin Isotype Diversity in Humans. *Eur. J. Immunol.* 7, 804

*** WARNING:** Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be diluted with running water before being discarded. These conditions are recommended to avoid deposits in plumbing where explosive conditions may develop.

Methods

Two Color Immunofluorescence Staining of Human Lymphoid Tissue and Skin

Method provided by Gary S. Wood, M.D. and Roger A. Warnke, M.D., Stanford University, Department of Pathology.

This method is used for the detection of two distinct surface antigens by immunofluorescence staining of human lymphoid tissue and skin with monoclonal antibodies.' The technique employs the biotin-avidin system, a "sandwich" method for enhancing fluorescence intensity **and** for labeling cells in tissue section with two different fluorochromes. The staining procedure consists of five stages: (1) Biotin-conjugated monoclonal antibody to one cell surface marker; (2) Avidin-fluorescein isothiocyanate (FITC); (3) Biotin - to saturate any available biotin-binding sites on the avidin FITC'; (4) Biotin-conjugated monoclonal antibody to a second cell surface marker; (5) Avidin-rhodamine isothiocyanate (RITC).

Reagents:

I. Human cryostat section

2. Acetone

3. Phosphate-Buffered Saline (PBS) Solutions

25X Stock Solution:
Dissolve in I liter of distilled water
188 gm K_2HPO_4
33 gm NaH,PO_4
180 gm NaCI

I X Working Solution:
To 960 ml distilled water, add 40 ml of 25X Stock Solution and 20 mg merthiolate. Mix well.

4. Biotin-conjugated monoclonal antibodies to cell surface antigens. Dilute to 10-20 Agjrn1 in IX PBS immediately prior to use.

5. Avidin conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson Catalog No. 9011). Dilute to 25 µg/ml in PBS. Centrifuge at 100,000 x g for 10 minutes immediately prior to use.

6. Biotin (Sigma): 100 ,ug ml in PBS; to saturate excess Avidin FITC.

7. Avidin conjugated with rhodamine isothiocyanate (RITC). Dilute to 25 mg/m1 in PBS. Centrifuge at 100,000 x g for 10 minutes immediately prior to use.

8. Glycerol mounting media

Equipment:

I. Fluorescence microscope

Procedure:

I. Fix 4 to 6 gm cryostat sections in acetone (room temperature) for 5 seconds, dry, and store at -20C.

2. Immediately before staining, fix frozen sections in cold acetone at 4°C for 10 minutes, then dry slides at room temperature.

3. Incubate sections with IX PBS for 3 minutes at room temperature.

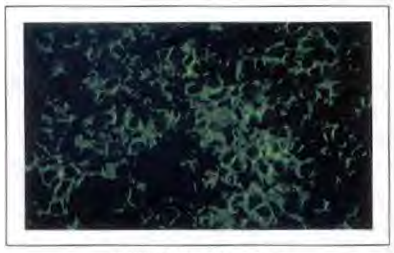
4. Incubate with diluted reagents in the order given below, adding sufficient amount to cover the section (20-50 ill). Wash once with IX **PBS** as indicated.

a. Biotin-labeled monoclonal antibody #1	15 minutes
PBS	3 minutes
b. Avidin FITC	15 minutes
PBS	3 minutes
c. Biotin	15 minutes
PBS	3 minutes
d. Biotin-labeled monoclonal antibody #2	15 minutes
PBS	3 minutes
e. Avidin RITC	15 minutes
PBS	3 minutes

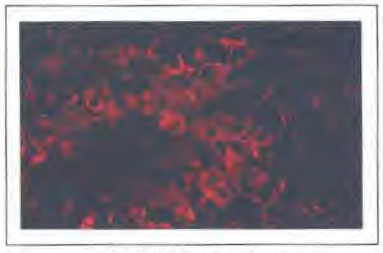
5. Add glycerol mounting media and place coverslip over section. Sections are examined using a microscope equipped with exciter-barrier filter combinations for rhodamine and fluorescein and with vertical illuminations by an HBO 50W direct current mercury vapor lamp. Double-labeled tissue sections are recorded by photographing separately for fluorescein and rhodamine. For two-color photomicrographs, single frames of film are double exposed for both fluorescein and rhodamine.[2] In the resulting photomicrographs rhodamine-labeled cells are red, fluorescein-labeled cells are green, and double-labeled cells are orange-yellow (see illustrations on the reverse side).

Two-Color Immunofluorescence on Tissue Sections Using Anti-Leu-1 (Green) and Anti-HLA-DR **(Red): Case Studies of Mycosis Fungoides**

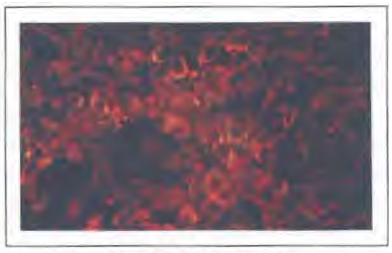
Case 1: Dermal tumor nodule showing all tumor cells labeled with FITC and **RITC,** indicating coexpression of Leu-1 and la.



F1TC Excitation

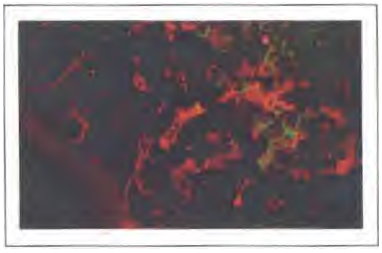


RITC Excitation



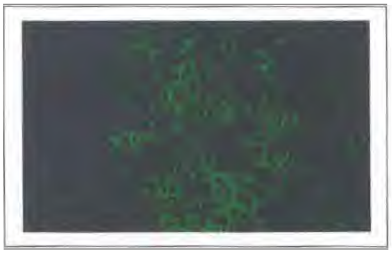
Double Exposure

Case 2: Section of epidermis showing FITC-labeled cutaneous T-cell lymphoma cells and RITC-labeled la' dendritic cells (Langerhans cells). Tumor cells express Leu-1 but not la.



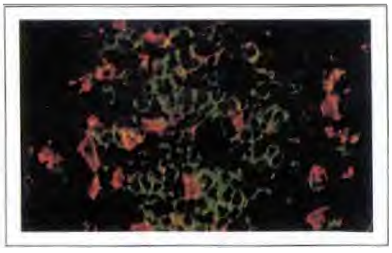
Double Exposure

Case 3: Dermal tumor nodule showing FITC-labeled neoplastic T cells and RITC-labeled macrophages. Tumor cells express Leu-I but not la.



FITC Excitation

RITC Excitation



Double Exposure

References:

Wood, G. and Warnke, **R.** (1981) Suppression of Endogenous Avidin-binding Activity in Tissues and Its Relevance to Biotin-Avidin Detection Sstems. *J. Histochem. Cytochem.* 29. 1196

[2] Seymour, G.J., Greaves, M.F. and Janossy, G. (1980) Identification of Cells Expressing T and p2833 (la-like) Antigens in Sections of Human Lymphoid Tissue. *Clin. Exit, Immunol. 39,* 66

Methods

Immunohistochemical Staining of Human Imprint Preparations

Method provided by Peter M. Banks, M.D. and B. Lynn Caron, B.S., H.T. (ASCII, Department of Surgical Pathology. Mayo Clinic.

Human leucocyte subpopulations can be identified in tissue using monoclonal antibodies in an indirect immunoperoxidase technique.[1-2] This technique has been applied to imprint preparations of lymphoid tissue. Air-dried imprints of lymphoid tissues are easy to prepare, consume no tissue, and can be transported. Immunoreactivity is retained for 7-10 days at room temperature. Storage cannot be prolonged with fixation or refrigeration.

Reagents and Materials:

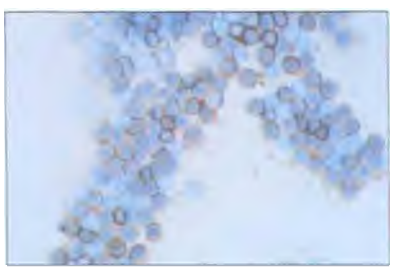
1. **Human** lymphoid tissue, e.g., lymph node, tonsil. spleen, bone marrow
2. Monoclonal antibodies to human cell surface antigens
3. Peroxidase-conjugated goat anti-mouse 1g (GAM Ig peroxidase) RAGO, **Inc.]. Dilute 1 30 just** before use in PBS.
4. Phosphate-Buffered Saline (PBS) solution. pH 7.0-7.2
5. 0.1 M Acetate buffer, pH 5.2

   Dissolve 10.75 g sodium acetate • 3 **FLO in 21 ml of 1.0** N acetic acid. Add one liter of water. Store at 4°C.
6. AEC Solution. Prepare immediately before use.

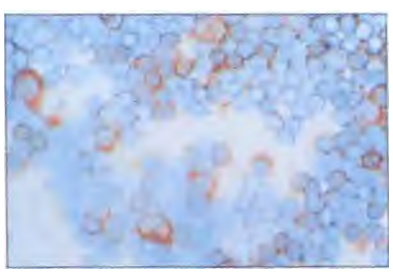
   Dissolve 10 mg of 3-amino-9-ethvl carbazole (AEC) in 2.5 ml N. N-dimethyltOrmamide. Add 47.5 ml of 0.1 M acetate buffer. Add 0.5 ml of 3ri **H2Q.**

   CAUTION: 3-amino-9-ethyl carbazole is a suspected carcinogen. Handle with care and dispose of properly.

Peroxidase Staining of Imprints of Normal Human Lymph Node



Anti-Leu-1 on lymph node imprint (X400)



Anti-HLA-DR on lymph node imprint (X400)

Note that Anti-Leu-I marks T cells (predominantly small cells), whereas Anti-HLA-DR marks **B** cells as well as histocytes (predominantly large cells).

7. Non-alcoholic progressive hematoxylin (e.g., Mayer's)
8. Kaiser's glycerin jelly mounting medium'
9. Acetone

Equipment:

1. Light microscope

Procedure:

I. Cut a fresh tissue surface and immediately touch the tissue to a clean glass slide, avoiding any sideways shearing motion. Make a series of slides to obtain a uniform monolaver of intact cells.

For immunohistochemical staining, use the last (thinnest) in a series of "touch preps". Earlier (thicker) preparations are best suited for cvtochemical methods.

Air dry imprints. Store at room temperature for 7-10 days, if desired.

2. Immediately prior to processing, fix imprints in cold (4°C) acetone for 5 minutes, then allow slides to dry fully at room temperature.
3. Incubate imprints with PBS for 3 minutes.
4. Incubate imprints with diluted reagents in the order given below. adding sufficient amount to cover the section (20-50 Al).

a. Monoclonal antibody Anti-Leu-2a, Anti-Leu-3a - dilute to 10 µg: m1 with PBS Anti-141.A-DR, Anti-Kappa, Anti-Lambda - - dilute to 1 µg; ml with PBS	15 minutes
h. PBS (3 times)	3 minutes
c. GA M Ig-peroxidase. dilute at 1 30 in PBS	15 minutes
d. PBS (3 times)	3 minutes
e. AEC solution	15 minutes
f. Distilled water	3 minutes

5. Counterstain with a progressive hematoxylin for 1-3 minutes.
6. Wash in running tap water until clear.
7. Coverslip with glycerin jelly.

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

References:

' Banks, P.M., Caron, L., and Morgan, TW. (1982) Application of Monoclonal Hybridoma Antibodies to Imprint Preparations of Lymphoid Tissue for the Identification of Immunologic Cell Types. *Am. J. Clin. Path. 78,* 266

[2] Banks, P.M., Caron, B.L., and Morgan, TW (1983) Use of Imprints For Monoclonal Antibody Studies: Suitability of Air-Dried Preparations from Lymphoid Tissues with an Immunohistochemical Method. *Ant. J. Clint Path. 79,* 438

[3] Theory and Practice of Histotechnology, Second Edition (1980). p. 204. Sheehan. D.C. and lirapchak. B.B. (C.B. Mosby, St. Louis)

Methods

T Cell Depletion of Human Peripheral Blood

Method provided by Anthony S. Fauci, M.D. and Gail Whalen, National Institute of Allergy and Infectious Diseases, Laboratory of Immunoregulation.

This method is used to deplete T cells from preparations of E-rosette negative peripheral blood mononuclear cells. The resulting cell population is enriched for B cells and monocytes.'

Peripheral blood mononuclear cells (PMBC) are first E-rosette depleted, then incubated with Anti-Leu-1 monoclonal antibody. Rabbit serum is added as a complement source to lyse cells which have bound Anti-Leu-l. The enriched population is valuable in studies of B cell activation and immunoregulation.[2-4]

Cells:
Peripheral blood mononuclear cells, depleted of cells which form rosettes with AET-treated sheep erythrocytes (E-rosette "negative" PBMC)'. Wash the cells 2-3X in **RPM! 1640.**

Reagents:

1. Anti-Leu-1 monoclonal antibody (pan T), Becton Dickinson Catalog No. 6300
2. RPM! 1640
3. RPM! 1640 containing 10% fetal calf serum (RPMI/FCS)
4. Pooled young rabbit sera (complement source)

   Prescreen sera for:
   a. Nonspecific cytotoxicity to T and B cells by trypan blue exclusion
   b. No effect on the proliferative responses of unfractionated PBMC to *S. aureus* Cowan, Pokeweed Mitogen (PWM), Phytohemagglutinin, and Concanavalin A
   c. No toxicity toward spontaneous or PWM-generated 1g-secreting cells by Protein A reverse hemolytic plaque-forming cell assay
   d. Ability to induce T cell lysis in the presence of Anti-Leu-1
5. Trypan blue or ethidium bromidefacridine orange for viability staining

Equipment:

1. Low speed centrifuge **(4°C)** with swinging bucket rotor
2. Ice bucket
3. Light microscope (or fluorescence microscope for ethidium bromide/acridine orange method)
4. Capped test tubes, tissue culture grade (e.g. Falcon 2057 tubes)
5. Water bath, 37°C

Procedure:

1. Pipet 10^6 to 6 x E-rosette negative PBMC into a test tube.
2. Centrifuge the cells at 600 x g for 5 minutes. Aspirate supernatant.
3. Add 0.2 lig of Anti-Leu-I per 10^6 cells in the pellet (i.e., for 10^7 cells, add 2 mg of undiluted Anti-Leu-1). Vortex gently.
4. Incubate cells in an ice bucket for 1 hour.
5. Adjust concentrations to 10^7 cells/ml by adding 1 ml of 25-50% rabbit serum (depending upon the source) in RPM! 1640 per 10^7 cells (based on initial cell count).
6. Incubate the cells at 37°C on a rotator for 1 hour.
7. Gently vortex the cells. Place the tube on ice for 5 minutes to allow clumps to settle. Clumps contain lysed cells.
8. Transfer cell suspension to a clean tube. Fill tube with fresh medium. Centrifuge at 600 x g for 5 minutes.
9. Wash non-clumped cells 3 times in RPM! 1640. Centrifuge at 600 x g for 5 minutes.
10. Resuspend cells in R PM I/ FCS.
11. Determine cell viability using trypan blue or ethidium bromide/acridine orange.

Results	**Analysis**
% of Total PBMC	—20% Coulter Counter
Viability	90-95% Trypan blue exclusion
of Viable Cells	
B Cells	30-55% Surface Ig
T Cells	SI% E-rosetting; Mitogen (PHA, Con A) treatment, uptake of ^{3}H-thymidine
Monocytes	30-50% Non-specific esterase staining; morphology

References:

Falkoff, R.M., Peters, M., and Fauci, A.S. (1982) T Cell Enrichment and Depletion of Human Peripheral Blood Mononuclear Cell Preparations. Unexpected Findings in the Study of the Separated Populations. *J. Immunol. Meth. 50.* 39-49

[2] Lane, H.C., Volkman, D.J., Whalen, G. and Fauci, A.S. (1981) In Vitro Antigen-Induced, Antigen-Specific Antibody Production in Man. Specific and Polyclonal Components, Kinetics, and Cellular Requirements. *J. Exp. Med. 154.* 1043-1057

[3] Falkoff, R.J.M., Zhu, L.P., and Fauci, A.S. (1982) Separate Signals for Human B Cell Proliferation and Differentiation in Response to *Staphylococcus aureus:* Evidence for a Two Signal Model of B Cell Activation. *J, Immunol. 129.* 97-102

[4] Volkman, D.J., Allyn, S.P., and Fauci, A.S. (1982) Antigen-Induced In Vitro Antibody Production in Humans: Tetanus Toxoid-Specific Antibody Synthesis. *J. Immunot 129,* 107-112

5/89 23-1488-02

BECTON DICKINSON

Becton Dickinson immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Methods

Immunoperoxidase Staining of Cell Surfaces - Cytocentrifuge Preparations

Method provided by Florence M. Hofman, Ph.D., University of Southern California, Department of Pathology.

This method is used for immunoperoxidase staining of cytocentrifuge preparations of cell suspensions. Fixed cell preparations may be examined simultaneously for morphology and staining by light microscopy. The stained preparations provide a permanent record which can be stored at room temperature.

Cells:
Human single cell suspensions from peripheral blood, tonsil, bone marrow, and tumor masses.

Reagents:

1. PBS pH 7.4 containing 10% fetal calf serum (PBS,' FCS). Add 10 ml of fetal calf serum to 90 ml of PBS.
2. Mouse monoclonal antibodies specific for human membrane antigens
3. Biotin-conjugated horse anti-mouse Ig reactive with all immunoglobulin subclasses (HAMIg-biotin) [Vector Laboratories, Inc., Burlingame, CA]
4. Avidin-biotin-horseradish peroxidase complex (ABC) [Vector Laboratories, Inc.]. Mix solutions A and B prior to use according to the manufacturer's instructions.
5. Modified Phosphate-Buffered Saline (mPBS), pH 7.4

   25X Stock Solution:
   37.6 g K_2HPO_4
   6.6 g NaH_2PO_4
   36.0 g NaCI
   Dissolve in water and Qs to 200 ml.

   I X Working Solution (I X PBS):
   To 960 ml of distilled water, add 40 ml of 25X Stock Solution and 20 mg of thimerosal. Mix well.
6. 0.1 M Acetate Buffer, pH 5.2

   Add 79 ml of 0.1 M sodium acetate • 3 H_2O (13.61 g/1) to 21 ml of 0.1 N acetic acid (5.75 m1/1).
7. 3-amino-9-ethylcarbazole (AEC), 120 mg in 15 ml N,N-dimethyl formamide
8. AEC Solution: mix 0.5 ml of AEC, 9.5 ml of 0.1 M Acetate Buffer, and 50 Fel of 3% H_2O_2. Prepare immediately before use to avoid precipitate formation.
9. Mayer's hematoxylin (Counterstain)
10. Aqua-mount' mounting medium (Lerner Laboratories, Pittsburgh, PA). Do not use organic mounting media such as Permount" with AEC; the reaction product will dissolve.

Equipment:

I. Cytospin cytocentrifuge (Shandon Inc., Pittsburgh, PA)
2. Light microscope
3. Humidity chamber (25°C)

Procedure:

1. Prepare human cells in suspensions at 1-5 x 10^5 cells per ml in PBS/ FCS
2. Place 0.2 ml (10^5 cells) in each Cytospin block and centrifuge at 800 rpm for 5 minutes
3. Air dry slides. Dry cell preparations may be stained immediately or within 3-4 days.
4. Place slides in humidity chamber for remainder of procedure. Dilute reagents as indicated, add 20-50 pi to cover cells, and wash in the order given below:

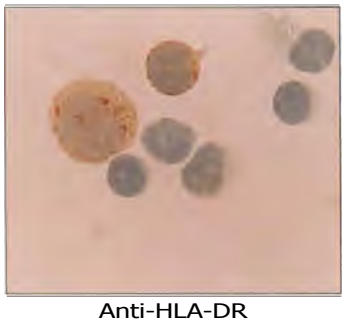
a.	Modified PBS (mPBS)	3 minutes
b.	Monoclonal antibody, diluted to 10-20 ug/ml in mPBS	15 minutes
	mPBS	8 minutes
c.	HAM1g Biotin, diluted to 50 µg//ml in mPBS	15 minutes
	mPBS	8 minutes
d.	ABS Solution	15 minutes
	mPBS	8 minutes
e.	AEC Solution	10 minutes
	Rinse in tap water.	
f.	Tap water	10 minutes
g.	Mayer's hematoxylin	10-15 minutes
	Tap water	10 minutes

   h. Add Aqua-mount and a cover slip to the slide. Examine cells using a light microscope.

Peroxidase Staining of Cytocentrifuge Preparations of Normal Human Peripheral Blood

Cells which bear the antigen recognized by the monoclonal antibody display reddish-brown staining whereas cells that do not bear that antigen have a light blue color.

Anti-Leu-I/CD5



Anti-HLA-DR

Photographs by Andrew Gero, USC.

Reference:
Hofman, FM., Billing, R.J., Parker, J.W., and Taylor, C.R. (1982) Cytoplasmic as Opposed to Surface la Antigens Expressed on Human Peripheral Blood Lymphocytes and Monocytes. *Clin. Exp. lmmunol. 49,* 355-363

6/89 23-1489-02

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive. San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Methods

Immunoperoxidase Staining of Human Lymphoid Cells and Tissue Sections ABC Method'

Method provided by Su Ming Hsu, M.D., Laboratory of Pathology, National Cancer Institute, National Institutes of Health.

This method uses monoclonal antibodies to membrane antigens for indirect immunoperoxidase staining of either single cell suspensions fixed on slides by a cytocentrifuge or frozen tissue sections. The method employs an avidin-biotin-peroxidase complex (ABC) which is a highly sensitive and specific detection system." The staining procedure consists of three stages: (1) monoclonal antibody to a cell surface antigen; (2) biotin-conjugated horse anti-mouse (or rat) IgG; (3) ABC.

Reagents and Materials:

1. Cytocentrifuge smear or thoroughly dried 4-6 nm cryostat section
2. Acetone, anhydrous
3. 0.05 M Tris buffer, pH 7.6
4. Normal horse serum (Vector)

   Dilute 1/100 in Tris buffer prior to use.
5. Monoclonal antibodies to cell surface antigens. Dilute to **0.5-1 mg/m1** (for cytocentrifuge smears) or 2-5 µg/ml (for cryostat sections) in Tris buffer immediately prior to use.
6. Horse Anti-Mouse IgG, Biotin Conjugate (HAMIg-Biotin) [Vector]

   Dilute 1/200 in Tris buffer prior to use.
7. ABC solution (Vector)

   Prepare ABC solution according to the manufacturer's instructions. Add two drops of Reagent A (Avidin DH) to 2 ml of Tris buffer. Then add two drops of Reagent B (biotinylated horseradish peroxidase) to diluted Reagent A. Mix immediately. Allow ABC solution to stand 5 minutes before use.
8. 8% $NiC1_2$

   Dissolve 8 g in 100 ml of distilled water.
9. DAB-NiH_2O_2Solution

   Add 10 mg diaminobenzidine HCI (DAB) [Sigma], 0.1 ml 8% $NiC1_2$and 5 ,u1 of 3% $11_2O_2$to 20 ml of Tris buffer.

   CAUTION: Diaminobenzidine is a listed carcinogen in California. Handle with care and dispose of properly.
10. Methyl Green Solution

    Dissolve I g methyl green in 100 ml absolute methanol.
11. Absolute ethanol
12. Xylene
13. PermountTM (Fischer)

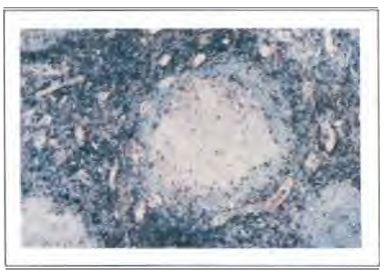
Equipment:

1. Light microscope

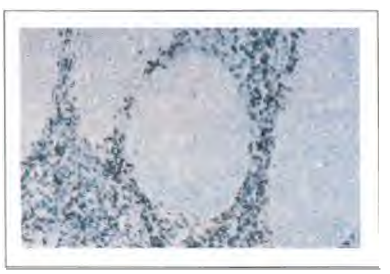
Procedure:

I. Prepare cytocentrifuge smears or cryostat sections on gelatin-coated slides. Do not fix. Allow the slides to dry at 4°C overnight. Slides may be stored for 2-3 days at 4°C.

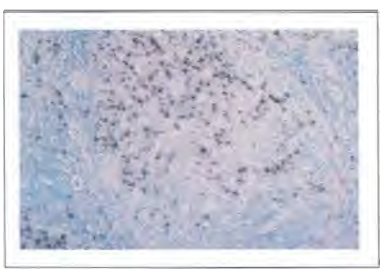
Peroxidase Staining of Sections of Human Tonsil



Anti-Leu-1



Anti-Leu-2a



Anti-Leu-7

2. Immediately before staining, fix the cytocentrifuge smears or tissue sections in acetone (room temperature) for 5 minutes.
3. Immediately transfer slides into Tris buffer. Do not allow slides to dry.
4. Wash slides in 2-3 changes of Tris buffer. Do not allow slides to dry.
5. Incubate slides for 5 minutes in diluted normal serum.
6. Blot excess fluid from slides.

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

7. Incubate slides with diluted reagents in the order given below, adding sufficient amount to cover the section (20-50 pi). Wash with Tris buffer as indicated.

a. Monoclonal antibody	30-60 minutes
Tris	5 minutes
b. HAMIg-Biotin	30 minutes
Tris	3 minutes
c. ABC Solution	30-60 minutes
Tris	3 minutes
d. DAB-NiH_2O_2	2-5 minutes
Tris	3 minutes

Observe the color development under light microscopy. When the reaction appears optimal, transfer the slides to Tris buffer.

CAUTION: Diaminobenzidine is a listed carcinogen in California. Handle with care and dispose of properly.

8. Counterstain the slides with Methyl Green Solution for 5 seconds. Place slides in 3 consecutive changes of absolute ethanol, then in 3 consecutive changes of xylene. Mount slide with Permount.

Comments:

I. The use of some biotin-labeled monoclonal antibodies (e.g., Anti-Kappa, Anti-Lambda) results in excellent staining. The staining procedure is also considerably shortened. For these cases, omit the normal horse serum and horse anti-mouse Ig-biotin.

2. Blocking of endogenous peroxidase is not required for most cells and tissues. If it is necessary to block endogenous peroxidase, a CH_3OH-H_2O_2 method may be used, but is not always adequate and may destroy some antigens. Other blocking reagents such as periodic acid should then be tried. For CH_3OH-H_2O_2 blocking, incubate slide in a solution of 3 ml 30% H_2O_2 in 200 nil methanol for 30 minutes prior to step 7c.

References:

Hsu, S.M., Raine, L., and Fanger, H. (1981) Use of Avidin-Biotin-Peroxidase Complex (ABC) in Immunoperoxidase Techniques: A Comparison Between ABC and Unlabeled Antibody (PAP) Procedures. *J. Histochem. Cvtochem. 29,* 577

[2]**Hsu, S.M.,** Raine, L., and Fanger, H. (1981) A Comparative Study of the Peroxidase-Anti-Peroxidase Method and an Avidin-Biotin Complex Method in Studying Polypeptide Hormones with Radioimmunoassay Antibodies. *Am. J. Clin. Path. 75,* 734

[3] Hsu, S.M. and Raine, L. (1981) Protein A, Avidin and Biotin in Immunohistochemistry../. *Histochem. Crtochem. 29,* 1349

[4]**Hsu, S.M. (1983)** Distribution of **T** Cell Subsets in Human Lymphoid Tissues: An lmmunohistochemical Study. *Am. J. Clin. Path. 80,* 21

[5] Hsu, S.M., and Soban, E. (1983) Color Modification of Diaminobenzidine (DAB), Precipitation by Metallic Ions and its Application for Double Immunohistochemistry../. *Histochem. Cytochent. 10,* **1079**

Methods

Differential Immunoenzymatic Staining of Mononuclear Cells in Lavage Samples

Method provided by Gary W. Hunninghake, M.D., Martha Monick, and Mary Stalp Brady, University of Iowa, Department of Internal Medicine, Director of Pulmonary Disease Division.

This method is used for the differential staining of mononuclear cell suspensions obtained from bronchoalveolar lavage.' Lymphocytes/monocytes reactive with the selected monoclonal antibody stain red, while peroxidase-positive monocytes or macrophages stain brown to black.

Cells:

I. Human single cell suspensions from bronchoalveolar lavage.

Reagents:

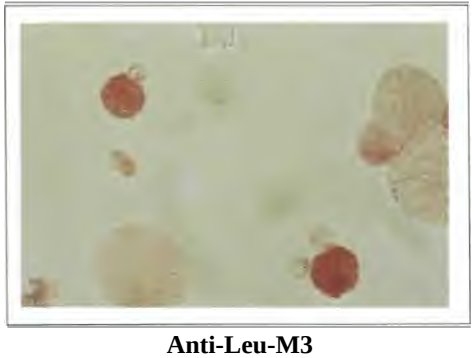
I. Mouse monoclonal antibodies specific for human membrane antigens (e.g., Anti-Leu-2a for T cytotoxic/ suppressor cells, Anti-Leu-3 for T helper/inducer cells, Anti-Leu-M3 for monocytes).

2. Goat anti-mouse immunoglobulin-alkaline phosphatase (GAM lg-AP) [Cappel]

3. Trizma' buffer solution, pH 7.6 (Sigma)

Differential Staining of Bronchoalveolar Lavage in Hypersensitivity Pneumonitis

Anti-Leu-2a



Anti-Leu-M3

Lymphocytes (top) or monocytes (bottom) reactive with the selected monoclonal antibody stain red. Negative lymphocytes are clear. In the top panel, peroxidase-positive monocytes/macrophages stain brownish-black.

4. Alkaline phosphatase indicator, histochemical (Sigma). Contains 0.03% fast violet B salts and 0.1% napthol AS-TR phosphoric acid (phosphatase substrate) when reconstituted according to manufacturer's instructions.

   Phosphatase indicator solution: Add 2 ml Trizma buffer to 1 vial of indicator 5 minutes before use.

5. Peroxidase indicator (Sigma), 0.67% 2,7-Diamino-fluorene in ethylene glycol monomethyl ether.

   Caution: 2,7-Diaminofluorene is a possible carcinogen. Handle with care and dispose of properly.

6. 0.12% H_20, Solution:
   Add 0.40 ml of H_2O_2 (30%) to 99.6 ml of distilled water.

7. RPMI 1640 containing 5% fetal calf serum (RPMI/FCS).

   Add 5 ml of fetal calf serum to 95 ml of RPMI 1640.

Equipment:

I. Refrigerated centrifuge (4°C) with swinging bucket rotor

2. 12 x 75 mm plastic test tubes, tissue culture grade

3. M icropi pettors

4. Light microscope with 40 X objective

5. Incubator with 5% CO_2 (37°C)

6. Microscope slides and coverslips

Procedure:

1. Prepare human cells in suspension at 5 x 10^6 cells per ml of RPMI/FCS. Use bronchial lavage cells within 1 hour of collection.

2. Place 0.2 ml (I x 10^6) cells and 5µl of monoclonal antibody in a 12 x 75 mm test tube. Incubate the test tube at 4°C for 30 minutes.

3. Add 2 ml of RPMI/ FCS. Centrifuge at 400 x g for 5 minutes at 4°C. Remove the supernatant. Repeat washing step.

4. Add 30 ***ul*** of GAM Ig-alkaline phosphatase to the cell pellet. Incubate the test tube at 4°C for 30 minutes.

5. Wash the cells twice as directed in step 3.

6. Add 0.5 ml of phosphatase indicator solution to each pellet. Incubate the tube at 37°C for 30 minutes.

7. Centrifuge the tube at 400 x g for 5 minutes. Remove the supernatant.

8. Add I ml of RPMI/FCS, 50 ill of 0.12% H_2O_2 and 25 *pl* of peroxidase indicator to the tube. Incubate at 22°C for 1 minute.

   Caution: Peroxidase indicator contains 2,7-Diamino-fluorene, a suspected carcinogen. Handle with care and dispose of properly.

9. Centrifuge the tube at 400 x g for 5 minutes. Remove the supernatant.
10. Add 25 ill of RPMI/FCS to the pellet. Resuspend the cells. The cells may be stored at 4°C for up to 3 days before they are examined; however, staining is brightest during the first 24 hours.
11. Place 7 Al of the cell suspension on a slide, add a cover-slip, and examine with a light microscope.

NOTE: When the monoclonal antibody is specific for monocytes, omit Steps 8 and 9 (peroxidase indicator).

References:

I Hunninghake, G.W., Gadek, J.E., Kawanami, 0., Ferrans, VI, and Crystal, R.G. (1979) Inflammatory and Immune Processin in the Human Lung on Health and Disease: Evaluation by Bronchoalveolar Lavage. *American Journal at' Pathology 97,* 149

Method | Preparation of Alcohol-Fixed Whole Cells From Suspensions For DNA Analysis

This method was provided by Dr. Anne Hurley of cytyc Corporation, Marlborough, MA. This is a simple, reproducible procedure for fixing suspended cells in methanol and staining them for DNA content with propidium iodide. It is suitable for cells derived from peripheral blood, from other body fluids, and from suspension cultures. The stained cells may be analyzed for DNA content on a FACScanTM brand or other flow cytometer with either laser or arc lamp excitation.

To analyze cells derived from human tissue for the "DNA Index", this procedure recommends the use of a tissue-specific normal diploid population of cells as a control. This method includes the use of an internal control.

Equipment

- Aspiration system, low vacuum
- Centrifuge, room temperature with swinging bucket
- Hemacytometer
- Micropipettors, 500
- Microscope
- Stirring plate
- Vortex

Supplies

- Centrifuge tubes, polystyrene, 5 ml and 15 ml, Becton Dickinson Falcon® No. 2052 and 2095[1]
- Microcentrifuge tubes, 1.5 ml, Fisher No. 05-407-5[2]
- Micropipettor tips, 500 Al
- Pasteur Pipettes, disposable, 14.6 cm
- Marking Pen resistant to absolute methanol
- Nylon cloth, Nitex, 35 pm mesh, TETCO No. 3-35/22[6]

Reagents

- Methanol, acetone-free, absolute, Fisher No. A412[2]
- Ribonuclease, 200 units/ml (RNAse)
  10 mg RNAse, Sigma R9005[5]
  5 ml PBS

Combine and place in a 15 ml tube. Heat to 75°C for 20 minutes; cool to room temperature. Store in 1 ml aliquots frozen in microcentrifuge tubes for up to 6 months.

- Propidium iodide Stain **(PI)**
  10 mg propidium iodide, Calbiochem No. 537059[3]
  0.1 ml Triton X-100, Sigma T6878[5]
  3.7 mg EDTA, Sigma E5134[5]
  90 ml PBS, Gibco 310-4190[4]

Combine and dissolve. Protect from light. Stir gently to dissolve completely. Adjust volume to 100 ml with PBS. Store at 4°C.

Caution: Propidium iodide is a known mutagen and suspected carcinogen. Handle with care.

Flow Cytometer Configuration

DNA studies of cells or nuclei stained with propidium iodide require a flow cytometer equipped with a light source providing excitation in the UV to green range. For laser excitation, an argon laser emitting at 488 nm to **514** nm is optimal. Instruments with arc lamps may use either UV, blue, or green excitation since propidium iodide has absorption peaks in each of these regions.

Propidium iodide emits fluorescence primarily at wavelengths above 610 nm. For flow cytometers requiring filter selection, use a red or orange, long-pass emission filter (for example, 00570, RG6I0, or RG630). With the FACScan flow cytometer use the FL2 parameter to analyze cells prepared according to this method. The appropriate filter is factory-installed.

Procedure

Caution: Use standard biohazard precautions when handling any biological sample.

I. Chill PBS, methanol, test and control samples on ice.

2. Prepare single cell suspensions of test and control samples in ice cold PBS.

   Note: If normal, tissue-specific cells are not available, use fibroblasts, normal fresh lymphocytes or chicken erythrocyte nuclei (CEN) as a control. However, prepared CEN function as an instrument control; they do not control for all the steps in this method. (See references).

3. Gently mix each cell suspension and count in a hemacytometer. Adjust the concentration of cells in the suspension to 1-2 x 10^6 cells/nil by adding ice cold PBS.

4. Transfer 0.5 ml of cells from the control sample to a 12 x 75 mm test tube marked "Control".

5. Add 0.5 ml cells from the test tissue sample to the test tube marked "Control".

6. Transfer 1 ml cells from the test tissue sample to the test tube marked "Test".

7. Add methanol dropwise from a pasteur pipette while mixing the cells on a vortex mixer. Add total of 2 ml methanol to 1 ml of the cell suspensions.

8. Incubate the tube for a minimum of 30 minutes on ice. At this point, some samples may remain stable for up to one week, when held in methanol solution at **4°C.**

9. Centrifuge the tube at 300 x g for 5 minutes. Aspirate the supernatant using a pasteur pipette.

10. Add 500 ill of stain solution to each and vortex gently to resuspend. Add 500 Al of thawed RNAse solution to each tube (Final concentration of 100 units/m1) and vortex to mix. Once thawed, discard unused portion of RNAse.

11. Incubate in the dark, at room temperature for 30 minutes.

12. Filter samples through 35 Am nylon mesh.

13. Analyze samples within an hour on a flow cytometer. Keep samples stored in the dark at 4°C until ready to analyze.

Caution: Use proper disposal methods for **biohazardous materials.**

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Oume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Discussion

This method provides, with a minimum of manipulation, propidium iodide-stained cells for flow cytometric analysis.

This method is effective for blood cells. However, it is possible to use it to determine the DNA content of many different cell types. It is advisable to test this and other methods for optimal staining of cell types. Examination of stained cells under a fluorescence microscope is recommended.

Sources

1. Becton Dickinson, Lincoln Park, NJ (201) 628-1144
2. Fisher Scientific, Pittsburgh, PA (800) 242-3772
3. Calbiochem Corporation, San Diego, CA (800) 854-3417
4. Gibco Laboratories, Grand Island, NY (800) 828-6686
5. Sigma Chemical Co., St. Louis, MO (800) 325-3010
6. TETCO, Inc., Monterey Park, CA (818) 289-9153

Note: These are suggested sources. You may use other suppliers that provide comparable products.

References

[1] Braylan, R.C. et. al., Correlated Analysis of Cellular DNA Membrane Antigens and Light Scatter of Human Lymphoid Cells. *Cytometry 2.* 337-343,1982.

[2] Crissman, H.A. and Steinkamp, J.A., Rapid Simultaneous Measurement of DNA, Protein and Cell Volume in Single Cells From Large Mammalian Cell Populations. *J Cell Biol. 59,* 766-771, 1973

[3] Krishan, A., Rapid Flow Cytometric Analysis of Mammalian Cell Cycle by Propidium Iodide Staining. *J Cell Biol.* 66, 188-193, 1975

[4] Dietch, A.D., Law, H. and White, R.D., A Stable Propidium Iodide Staining Procedure for Flow Cytometry. *J Histochem and Cytochem 30.* 967-972, 1982.

[5] Tate, E.H., Wilder, M.E., Cram, L.S., and Wharton, W. A Method for Staining 3T3 Cell Nuclei With Propidium Iodide in Hypotonic Solution. *Cytometry 4,* 211-215, 1983,

Methods

Preparation of Nuclei From Solid Tissue For DNA Analysis

This method was provided by Dr. Raul Braylan at the University of Florida, Gainesville. It produces nuclei from fresh solid tissues, such as tumor samples, for DNA content analysis by flow cytometry. This analysis is used to detect cell populations with quantitative changes in DNA content (aneuploid cells), and to estimate cell cycle fractions.

Equipment

- Analytical Balance
- Centrifuge
- Forceps
- Freezer, -20°C
- Heating block or waterbath at 75°C
- Hemacytometer or cell counter
- Adjustable Micropipettors
- Microscope, fluorescence with epi-illumination
- Refrigerator, 4°C
- Scissors, curved, 5-1/2", stainless steel
- Aspiration system for low vacuum
- Vortex

Supplies

- Biohazard gloves
- Bottle, brown, 2 x 200 ml
- Centrifuge tubes, 15 ml, polystyrene, Becton Dickinson Falcon® No. 2099[1]
- Filter units, 0.2 mm, sterile, (Nalgene No. 127-0020), Baxter No. T4216-3[2]
- Petri dish, 100 x 15 mm, Falcon No. 1029'
- Pipettes, disposable transfer, Baxter No. P5214-10[2]
- Pipette tips, 200 Al, 1.0 ml
- Microcentrifuge tubes, 1.5 ml, Fisher No. 05-407-5[3]
- Nylon mesh, Nitex, 40 um, TETCO No. 3-40/26[4]
- Standard mesh kit, stainless steel, (100 mesh screen), VWR No. 62-399-930[5]
- Tissue sieve, 85 ml, stainless steel, VWR No.62-399-918[5]
- Aluminum foil
- Propidium Iodide, Calbiochem No. 537059[6]

Reagents

- Chicken Erythrocyte Nuclei (CEN), prepared according to previously published method' or the Becton Dickinson Procedure: Chicken Erythrocyte Nuclei as a Flow Cytometer Internal Reference Standard (Optional reagent; see Discussion and Reference Sections)
- Ficoll-Paque®, Pharmacia No. 17-0840-02[7] or LeucoPREP" brand cell separation tubes, 13 x 100 mm, Becton Dickinson No. 96-2750 or 96-2751[1]
- Sodium hydroxide, IN solution
- Human blood from a normal donor, collected in a VACUTAINER® brand blood collection tube' with EDTA, 5 ml
- PBS, Dulbecco's phosphate buffered saline, calcium and magnesium free, Gibco No. 310-4190[8]
- 1.12% Sodium Citrate Stock
  5.6 gm Sodium Citrate

Dissolve in 500 ml MilliQTM or distilled water. Store at 4°C for up to 4 months.

- Nuclear Staining Solutions:

10X Nuclear Staining Solution (IOXNSS)
100 mg propidium iodide.

Dissolve in 200 ml 1.12% Sodium Citrate stock. Store in brown bottle at 4°C for up to 4 months.

Nuclear Staining Solution (NSS)
20 ml 10XNSS
0.6 ml Triton X-100, Sigma No. T6878[9]

Combine and dilute 1:10 by adding 180 ml MilliQ or distilled water. Mix well. Store in a brown bottle at 4°C for up to 4 months.

- Ribonuclease A (RNAse), Sigma No. R5503[9]

Prepare 100 ml solution of RNAse at 6.5 Kunitz units/ml in 1.12% Sodium Citrate. Adjust pH to 8.2 ± 0.2 using IN NaOH. Cap the flask with aluminum foil and heat at 75°C in a waterbath for 30 minutes to inactivate any DNAse. Aliquot in 1 ml portions into microcentrifuge tubes and store at -20°C. Once thawed, discard unused portion.

Caution: Propidium Iodide is a known mutagen and a suspected carcinogen. Handle with care.

Procedures

Caution: Use **standard biohazard precautions when handling any biological sample.**

Preparation of Funnel Filters

Cut a plastic disposable transfer pipette into five sections as shown in Figure 1. Retain sections 2 and 4. Cut a 2.5 cm x 2.5 cm **(I"** x 1") square of 40µm Nitex mesh. Place mesh across the top of the narrow opening of section 4 and wedge the mesh in place. The completed filter should appear as shown in Figure 2.

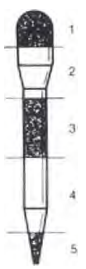


Figure 1
Transfer Pipet

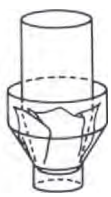


Figure 2
Preparation of Filter

BECTON DICKINSON

Becton Dickinson Immunocytometry Systems
2350 Qume Drive, San Jose, California 95131-1807
Telephone (800) 223-8226; In Calif. (800) 821-9796

Preparation of a Diploid Peripheral Blood Mononuclear Cell (PBMC) Control

1. In advance, have a funnel filter prepared for each tube to be analyzed.
2. Thaw one aliquot of RNAse at room temperature for every 2 tubes to be analyzed.
3. Using Ficoll-Paque or LeucoPREP, separate the PBMC's from the whole blood. Recover the PBMC layer to a clean 15 ml centrifuge tube. Add PBS to a final volume of 10 ml.
4. Centrifuge at 300 x g for 5 minutes at room temperature. Aspirate supernatant.
5. Resuspend cells in 2.0 ml PBS. Vortex to mix.
6. Determine PBMC concentration by counting using a hemacytometer or a cell counter.
7. Adjust the PBMC concentration to I x 10^6/m1 in PBS.
8. Aliquot 1 x 10^6 PBMC and I x 10^5 CEN in a 12 x 75 mm test tube labelled "PBMC + CEN". Hold the remaining PBMC's at 4°C for use with the tissue samples.
9. Centrifuge at 300 x g for 5 minutes at room temperature.
10. Aspirate and discard supernatant.
11. Add 500 µl NSS. Wait 15 minutes.
12. Add 500 µl RNAse solution and vortex. Incubate at room temperature for 15 minutes. If held longer, store at 4°C protected from light for up to 2 hours.
13. Using the funnel filters and a plastic transfer pipette, filter the PBMC + CEN sample into a clean, labelled 12 x 75 mm test tube. Filter just prior to analysis. Discard used filters.
14. Analyze samples on a FACScanTM flow cytometer.

Preparation of Tissues:

1. In advance, have a funnel filter prepared for each tube to be analyzed. Also, install a 100 mesh screen in the tissue sieve.
2. Thaw one aliquot of RNAse at room temperature for every 2 tubes to be analyzed.
3. Place the tissue sieve with the 100 mesh wire screen into the petri dish. Place a representative piece of tissue 3 mm x 3 mm x 3 mm on the wire screen. Using a plastic transfer pipette, dispense about 1.0 ml of NSS over the tissue.
4. Mince tissue using the scissors repeatedly until fine fragments are obtained. With the scissors, rub the minced tissue very gently against the screen. Make sure that the tissue remains moistened in NSS throughout the procedure. As the nuclei are released, the NSS in the petri dish will cloud.
5. Tilt the petri dish, collect the NSS and flush it over the remaining tissue fragments on the screen several times using a plastic transfer pipette. With a clean pipette, pour fresh NSS (not more than 2 ml) over remaining tissue and screen.
6. To wash the sieve and screen for subsequent use, place it in 10% bleach for 30 minutes. Wash with copious amounts of water and air dry.
7. Transfer the nuclear suspension to a 12 x 75 mm test tube. Centrifuge at 1000 x g for I minute at room temperature. Aspirate and discard supernatant. There should be a red pellet of nuclei at the bottom of the tube.
8. Quickly resuspend pellet in 1.0 ml NSS and vortex. Using a plastic transfer pipette, resuspend sample further by aspirating and expelling into the 12 x 75 mm test tube. Repeat 5 times.
9. Determine nuclear concentration in a hemacytometer, preferably using fluorescence microscopy.
10. Aliquot 1 x 10^6 nuclei into a 12 x 75 mm test tube labelled with patient identification.
11. If enough nuclei are available, prepare two additional 12 x 75 mm test tubes. To one tube labelled with patient identification + CEN, add 1 x 10^6 nuclei plus 1 x 10^5 CEN. To the second tube labelled with patient identification + PBMC, add 5 x 10' nuclei plus 5 x 10^5 PBMC. Vortex both tubes and add NSS to a final volume of 500 Al in each tube.
12. Add 500 µl thawed RNAse to each tube and vortex.
13. Incubate for 15 minutes at room temperature. If held for longer, store at 4°C protected from light. Samples should be run within 2 hours.
14. Using the funnel filters and a plastic transfer pipette, filter each sample into a clean 12 x 75 mm test tube. Filter just prior to analysis. Discard used filters.
15. Analyze samples on a FACScan flow cytometer.

Caution: Use proper disposal methods for biohazardous materials.

Discussion

It is recommended that cell cycle phase percentage analysis be performed on the sample without internal standards added. The optional sample containing CEN would be used primarily for monitoring instrument performance during analysis. The optional sample containing PBMC would be used for "DNA ploidy" determination (DNA Index Calculation).

Sources

1. Becton Dickinson, Lincoln Park, NJ (201) 628-1144
2. Baxter Scientific Products, McGaw Park, IL (312) 689-8410
3. Fisher Scientific, Pittsburgh, PA (800) 242-3772
4. TETCO, Inc., Monterey Park, CA (818) 289-9153
5. VWR Scientific, San Francisco, CA (415) 467-6202
6. Calbiochem Corporation, San Diego, CA (800) 854-3417
7. Pharmacia LK B, Piscataway, NJ (800) 526-3593
8. Gibco Laboratories, Grand Island, NY (800) 828-6686
9. Sigma Chemical Co., St. Louis, MO (800) 325-3010

Note: These are suggested sources. You may use other suppliers that provide comparable products.

Reference

Diamond, LW and Braylan, RC. Flow Analysis of DNA Content and Cell Size in Non-Hodgkin's Lymphoma. *Cancer Research 40,* 703-712, 1980

Methods | Immunohistochemical Staining of Human Lymphoid Tissue and Skin

Method provided by Roger Warnke M.D., Stanford University, Department of Pathology

This method is used for immunoperoxidase or immunofluorescence staining of human lymphoid tissue and skin with monoclonal antibodies detecting cell surface antigens. The technique for staining human tissue consists of three stages: **1.)** Monoclonal antibody; 2.) Biotin-conjugated horse anti-mouse IgG; and 3.) Avidin horseradish peroxidase or avidin conjugated with fluorescein.

Reagents:

1. Human cryostat section
2. Monoclonal antibody to human cell surface antigen
3. Biotin-conjugated horse anti-mouse IgG reactive with all subclasses (Vector Labs, Inc.)
4. Avidin conjugated with horseradish peroxidase, (Vector Labs, Inc.) or fluorescein isothiocyanate (FITC) (Becton Dickinson Catalog No. 9011)
5. Phosphate-Buffered Saline (PBS) Solutions

   25X Stock Solution:
   Dissolve in 1 liter of distilled water

   188 gm K_2HPO_4
   33 gm $NaH_2PO_4 \cdot H_2O$
   188 gm NaCI

   1X Working Solution:
   To 960 ml distilled water, add 40 ml 25X Stock Solution and 20 mg merthiolate. Mix well.
6. Diaminobenzidine (DAB) Solution: 1-3 mg/ml 3, 3 diaminobenzidine in 0.3% H_2O_2 in PBS (or 10 pi 30% **H_2O_2/m1)**

   CAUTION: Diaminobenzidine is a listed carcinogen in California. Handle with care.
7. Copper sulfate solution: 0.5% in normal saline
8. Methylene blue solution: 1% in distilled water
9. Acetone
10. 100% Ethanol
11. Xylene
12. Glycerol or Pro-Texx mounting media

Equipment:

Light or fluorescent microscope

Procedure:

I. Fix 4 to 6 pm thoroughly dried cryostat sections in acetone (room temperature) for less than 5 seconds, dry, and store at -20° C.

2. Immediately before staining, fix frozen sections in cold acetone at 4°C for 10 minutes, then dry slide at room temperature.
3. Incubate with appropriate dilution (10-20 pg/m1) of monoclonal antibody for 30 minutes, adding sufficient amount to cover section (20-50 pl). Wash with PBS for 1 minute.
4. Incubate with appropriate dilution (10-20 pg/m1) of biotin-conjugated anti-mouse IgG for 30 minutes, adding sufficient amount to cover section (20-50 pl). Wash with PBS for 1 minute.

Immunoperoxidase:

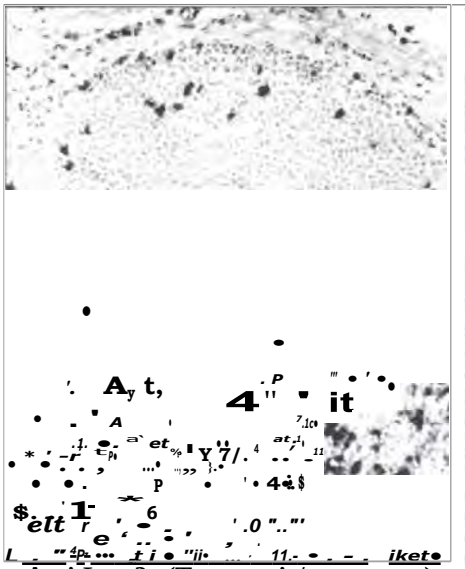
5. Incubate with avidin-horseradish peroxidase for 30 minutes. Wash two times with PBS, 1 minute each.
6. Incubate with DAB for 5 minutes. Wash two times with PBS, 3 minutes each. Rinse by dipping 10 times in distilled water.
7. Incubate with copper sulfate solution for 5 minutes. Dip in distilled water several times.
8. Counterstain with methylene blue for 10 minutes. Rinse with 100% ethanol, then xylene. Place coverslip over section. Sections are examined using a light microscope.

Immunofluorescence:

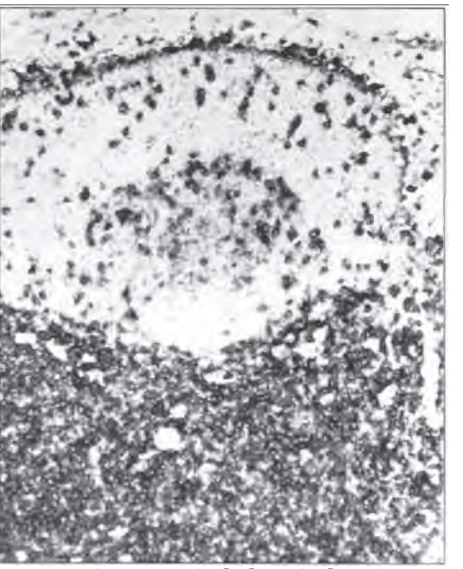
5. Centrifuge avidin FITC at 100,000 x g for 10 minutes immediately prior to use. Add appropriate dilution (10-20 iig/ml) of avidin FITC, using sufficient amount to cover section (20-50 pl). Incubate for 15 minutes. Wash two times with PBS, 3 minutes each.
6. Add glycerol mounting media (or aquamount) and place coverslip over section.

   Sections are examined using a Zeiss microscope equipped with exciter-barrier filter combinations for rhodamine or fluorescein and with vertical illumination by an **HBO** 50W direct current mercury vapor lamp.

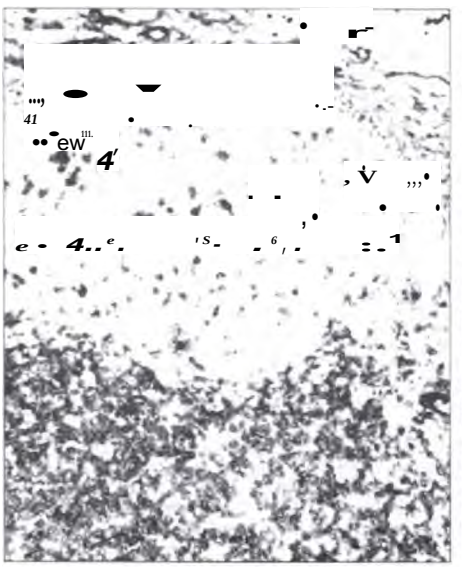
Peroxidase Staining of Serial Sections of a Normal Human Lymph Node - Secondary Follicle (Upper) and Adjacent Paracortex (Lower)'



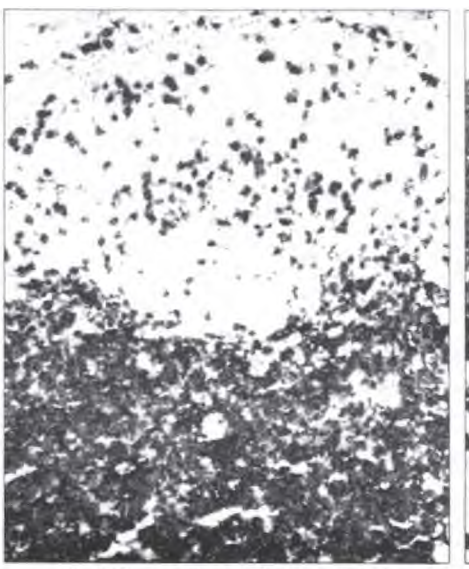
Anti-Leu-2a (T cytotoxic/suppressor)



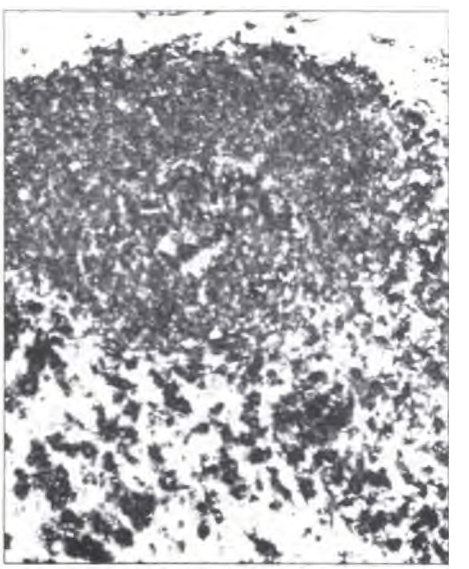
Anti-Leu-3a (T helper/inducer)



Anti-Leu-1 (pan T)



Anti-Leu-4 (pan T, mitogenic)



Anti-HLA-DR (Ia)

I Rouse, R.V., Weissman, I.L., Ledbetter, J.A., Warnke, R.A. (1982) Expression of T Cell Antigens by Cells in Mouse and Human Primary and Secondary Follicles. In: Nieuwenhuis P, van den Broek, A.A., Hanna, M.G., eds.: *In Vivo Immunology*. New York: Plenum; pp. 751-756.