Chapter 29

Flow Sorting for Isolating CFU-E

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1. Introduction

Erythroid progenitor cells have been classified into three groups of increasing maturity: the primitive burst forming unit (p-BFU-E), the mature burst forming unit (m-BFU-E), and the erythropoietin responsive colony forming unit (CFU-E). This classification is based on their time of maturation in vitro, their proliferative capacity, and their responsiveness to growth factors (*1*). The CFU-E can be distinguished from the more primitive erythroid progenitors by their ability to proliferate and mature in response to a single growth factor, erythropoietin. In clonal assays in vitro, the CFU-E form single or double clusters characteristically containing 8–64 mature or maturing erythroid cells 2 d after cultures have been initiated with mouse bone marrow or fetal liver (*2,3*).

The availability of enriched populations of CFU-E is important to our understanding of the function and mode of action of growth factor receptors in normal cells, the influence and regulation of molecules, genes and viruses that are specific to the erythroid lineage, and as a baseline for understanding errors in gene regulation or function that govern the development of leukemic or preleukemic states. One approach to purifying CFU-E has relied on the use of fluorescently tagged monoclonal antibodies as probes to cell surface molecules together with flow cytometry (*4,5*). Al-

though probes that specifically identify CFU-E are not available, phenotypic analysis has revealed that CFU-E can be segregated from more primitive erythroid precursors, from morphologically recognizable erythroid cells, and from mature myelomonocytic cells and their progenitors with a series of rat monoclonal antibodies (*4–6*). Hemopoietic tissues vary in their content of different types of hemopoietic progenitor cells and of maturing cells of particular lineages (*6*). Since mouse fetal liver is a major site of erythropoiesis and contains high numbers of CFU-E, the strategy for isolating CFU-E described here relies on the fractionation of low density fetal liver cells on the basis of their forward light scatter characteristics and differential reactivity with two rat monoclonal antibodies, YBM 42.2.2 and YBM 10.14.9 using flow cytometry (*4–6*). The anti-T200 antibody YBM 42.2.2 does not react with the CFU-E or more mature erythroid cells (*4*) but reacts with all leukocytes, thus allowing segregation of CFU-E from both myelomonocytic and lymphoid cells and from all other hemopoietic precursors. The YBM 10.14.9 antibody is then used to separate CFU-E from more mature erythroid cells (*5*). This approach yields cell populations containing at least 60% CFU-E, whereas 80% of the cells have the morphology of early erythroid blast cells and do not stain with benzidine, which identifies hemoglobin containing cells (*5–7; see* Note 1).

2. Materials

2.1. Reagents for Media Preparation

1. Powdered Iscove's Modified Dulbecco's Medium (IMDM) containing (per L) 3.024 g $NaHCO_3$, 60 mg penicillin, 100 mg streptomycin, 5 x 10^{-5}*M* 2-mercaptoethanol. After preparation, do not adjust pH. Store at 4°C. Light sensitive. This should be prepared at 1x and 2x strength.
2. IMDM without bicarbonate but with all other additions. Adjust to pH 7.3 with 5*M* NaOH or pH 5.1 with HCl.
3. Powdered modified Eagle's minimal essential medium with Earle's salts, but without phenol red containing 20 g/L BSA and sodium azide (0.02%).
4. Sodium bicarbonate ($NaHCO_3$).
5. Sodium benzylpenicillin.
6. Streptomycin sulphate (745 U/mg).
7. 2-Mercaptoethanol.
8. IM HEPES buffer pH 7.3 (commercially available).

9. 10x PBS: 0.2*M* sodium phosphate buffer with 1.48*M* sodium chloride pH 7.3.
10. 4% (w/v) sodium azide.

2.2. In Vitro Culture Reagents

1. Methylcellulose (65 HG, 4000 mPa, Fluka, AG, Buchs, Switzerland) prescreened to support CFU-E growth as described below and in Note 2.
2. Bovine serum albumin (BSA). Store at 4°C.
3. Lipids
   a. Cholesterol (5-cholesterol-3-β-ol).
   b. Oleic acid (*cis*-9-octadecanoic acid).
   c. L-α-phosphatidyl choline dipalmitoyl.
4. Human transferrin.
5. Erythropoietin (Epo). Epo step 1 (1000 U/mg) or pure Epo (80,000 U/mg) obtained from Terry Fox Laboratory Media Preparation Service, Vancouver, Canada or commercial recombinant Epo. Store in 200-µL aliquots at –70°C in 0.1% BSA or prescreened FCS. Do not refreeze after thawing.
6. Commercial Ficoll-Hypaque (density 1.077 g/cc). Light sensitive. Store at room temperature.
7. Fetal Calf Serum (FCS). Prescreened for supporting CFU-E growth. Store in 10-mL aliquots at –20°C.

2.3. Animals

Day 12–13 pregnant CBAf/CaH mice 8–12 wk of age. Day 0 of pregnancy is taken as the day of appearance of the vaginal plug.

2.4. Hybridomas

1. YBM 42.2.2 (Rat IgG2a antibody). This does not bind protein A at neutral pH.
2. YBM 10.14.9 (Rat IgG2c antibody). This antibody binds protein A at neutral pH.

2.5. Antibody Purification

1. Commercial rabbit anti-rat Ig.
2. Commerical Protein A-Sepharose CL-4B.

3. 0.1*M* phosphate buffer pH 8.
4. 0.1*M* glycine-HCl buffer pH 3.
5. 2*M* Trizma base in water.
6. PBS: 0.02*M* phosphate buffer with 0.148*M* sodium chloride pH 7.3.
7. Saturated ammonium sulfate solution pH 6.8.
8. 0.1*M* borate buffer pH 8.2.
9. 0.2*M* triethanolamine pH 8.2.
10. 20 m*M* dimethylpimelimidate dihydrochloride in 0.2*M* triethanolamine pH 8.2.

2.6. Antibody Labeling

1. Commercial fluorescein isothiocyanate (FITC) coupled protein A. Store at 4°C.
2. Fluorescein isothiocyanate (Isomer 1). Store dessicated at 4°C.

2.7. Stains

1. Commercial May-Grunwald Stain prefiltered through a Whatman 1MM filter paper.
2. Commercial Giemsa R66 improved stain.
3. Benzidine stock solution: 0.2% (w/v) benzidine hydrochloride in 0.5*M* acetic acid. This can be stored in the dark at 4°C for 3–4 wk. Caution, benzidine is a carcinogen.
4. 30% (w/w) H_2O_2 solution.

2.8. In Vitro Culture Reagents

2.8.1. 2% Methylcellulose

1. Add 20 g methylcellulose to 500 g sterile glass freshly double-distilled deionized boiling water.
2. Boil for 2 min with great care to avoid excess foaming. Control the level of heating.
3. Add sterile double-distilled deionized water to 520 g to correct for water loss resulting from evaporation.
4. Cool to approximately 37°C.
5. Add 500 mL of double-strength IMDM. Keep covered with foil.
6. Cool on ice with mixing for 2–3 h.
7. Stir on a magnetic stirrer at 4°C overnight to allow the methylcellulose solution to clear.
8. Store in 50-mL aliquots protected from light at –20°C for up to 4 wk.

2.8.2. Deionized and Delipidated BSA

1. Dissolve 400 mg of Dextran T40 in 400 mL of glass double-distilled deionized water.
2. Add 4 g of activated charcoal (Norit A or SX-1) and leave at room temperature for 30 min with occasional mixing.
3. Add 20 g BSA to the surface of the dextran-coated charcoal solution and leave for 2–3 h at 4°C to dissolve without mixing.
4. Titrate to pH 3.0 with concentrated hydrochloric acid to inhibit heat-induced polymerization of the albumin.
5. Incubate for 30 min at 56°C in a shaking water bath.
6. Centrifuge at 10,000 rpm for 20 min and Millipore filter the supernatant.
7. Adjust the pH to 5.5 with 2*M* NaOH.
8. Deionize the BSA solution overnight at 4°C over 40 cm^3 of Amberlite MB-1 mixed in exchange resin.
9. Concentrate the solution to 150 mL on an Amicon UM10 membrane at 4°C.
10. Adjust the pH to 7 with 2*M* hydrochloric acid.
11. Millipore filter and store in 10-mL aliquots indefinitely at –20°C or 4°C.

2.8.3. Lipids

1. Dissolve 4 mg L-α-phosphatidyl choline dipalmitoyl, 3.9 mg cholesterol, and 2.8 mg oleic acid in a few drops of chloroform at room temperature or ethanol at 50°C in a 25-mL glass beaker. Completely evaporate the solvent under a stream of nitrogen, leaving the film of lipid on the bottom of the beaker.
2. Add 10 mL of bicarbonate-free IMDM pH 5.1 containing 1% of the deionized and delipidated BSA.
3. Immerse the beaker containing the lipids in ice and sonicate under air for 10 min at maximum energy just below the foaming point so that the lipids form small micelles.
4. Millipore filter through 1.2 μm and then 0.45 μm filters. Store indefinitely at 4°C.

2.8.4. Other Reagents

1. $5 \times 10^{-2}M$ 2-mercaptoethanol in double-glass distilled deionized water. Millipore filter. Prepare fresh stocks before use.
2. Ferric chloride stock ($FeCl_3$). Since ferric chloride is hygroscopic,

weigh out a large piece of $FeCl_3 \cdot 6H_2O$ and immediately dissolve in $10^{-3}M$ hydrochloric acid (HCl). Dilute to a $7.9 \times 10^{-3}M$ stock solution in $10^{-3}M$ HCl. Store at –20°C.

3. Transferrin: Dissolve 360 mg of human transferrin in 4 mL of bicarbonate-free IMDM pH 7.4 and 1.15 mL of $7.9 \times 10^{-3}M$ $FeCl_3$ in $10^{-3}M$ HCl. Millipore filter and store indefinitely at 4°C.

3. Methods

3.1. Antibody Preparation

1. Collect the supernatant from the hybridoma cell lines grown in IMDM with 1% FCS, 24 h after cells reach confluency.
2. Precipitate the immunoglobulin (Ig) by adding an equal volume of saturated ammonium sulfate solution (pH 6.8). Mix for 1 h at 4°C.
3. Centrifuge at 10,000*g* for 10 min at 4°C.
4. Resuspend the pellet to one-tenth the original volume in PBS and dialyze against 0.1*M* phosphate buffer pH 8.0 at 4°C.
5. Estimate the protein concentration by measuring the absorbance at 280 nm.

3.2. Antibody Purification

1. Mix purified rabbit antirat Ig with Protein A-Sepharose to a final concentration of 11 mg Ig/mL of Sepharose beads in 0.1*M* borate buffer pH 8.2 for 30 min at room temperature.
2. Wash the beads in excess borate buffer and then in 0.2*M* triethanolamine pH 8.2.
3. Resuspend the Sepharose in 20 vol of 20 m*M* dimethyl pimelimidate dihydrochloride freshly made in 0.2*M* triethanolamine pH 8.2. Mix for 45 min at room temperature. This will covalently crosslink the Ig to the protein-A and prevent it from leaching from the column.
4. Spin the beads at 500*g* for 1 min and resuspend in an equal volume of 20 m*M* ethanolamine pH 8.2 for 5 min at room temperature.
5. Wash the beads three times in 0.1*M* borate buffer pH 8.2.
6. At the same time, equilibrate Protein A-Sepharose with 0.1*M* borate buffer pH 8.2. YBM 10.14.9 will bind to Protein A-Sepharose at neutral pH, whereas YBM 42.2.2 will not.
7. Apply the concentrated YBM 10.14.9 sample to a Protein A-Sepharose column and the YBM 42.2.2 to the rabbit anti-rat Ig-Protein A Sepharose column; 10–20 mg of Ig can be applied/mL of beads.

8. Elute the bound Ig with 0.1*M* glycine-HCl buffer pH 3, and neutralize the eluted material immediately with Trizma base.
9. Dialyze the antibodies against PBS and store in small aliquots with 0.1% BSA.
10. If the purified antibodies are to be coupled to fluorescein isothiocyanate (FITC), dialyze against 0.1*M* bicarbonate buffer pH 9.3 instead of the PBS, and couple with FITC immediately. Do not store antibodies for an extended period of time in the bicarbonate buffer.

3.3. Fluorescein Labeling of Antibodies

1. Dialyze the antibodies against 0.1*M* bicarbonate buffer pH 9.3 for 2–5 h at 4°C.
2. Dissolve the fluorescein isothiocyanate isomer 1 (FITC) at 1 mg/mL in DMSO. Add 25 µg of FITC/mg of purified YBM 42.2.2 Ig for 2 h at room temperature with constant rotation to give a fluorescein to protein ratio of approximately 3:1.
3. Separate the FITC conjugated Ig from the free FITC by passing through a 5-mL Sephadex G-25 column equilibrated with PBS. Collect the Ig fraction and store in small aliquots containing 1% BSA and 0.02% NaN_3 at –20°C.

3.4. Cell Preparation and Labeling

1. Dissect the livers from d 12–13 mouse fetuses using cataract knives and place in chilled bicarbonate-free IMDM containing 10% FCS.
2. Prepare a single-cell suspension by gently syringing the fetal livers through 19-, 21-, and 25-gage needles sequentially attached to a 2-mL syringe.
3. Place the cells in a 10-mL centrifuge tube and allow cell clumps to settle at 4°C for 5 min. Pass the supernatant through sterile nylon gauze to remove smaller clumps.
4. Centrifuge 5 mL of cells (10^7 cells/mL) over 4 mL of Ficoll-Hypaque (density 1.077 g/mL) at 1600 rpm for 30 min at room temperature.
5. Collect the low density cells from the Ficoll-Hypaque interface and wash three times in Eagle's-HEPES medium with BSA and sodium azide. Approximately 10^5 cells are recovered/fetal liver processed.
6. Add normal mouse serum, heat inactivated at 56°C for 30 min to the cell suspension at a final concentration of 10% to block Fc receptors. Incubate the cells for 20 min at room temperature, and wash the cells in Eagle's-HEPES medium containing BSA and sodium azide.

7. Centrifuge the antibodies in a Beckman airfuge at 26 lb/in^2 for 10 min at room temperature to remove aggregrates and further minimize Fc binding.
8. Label cells (10^7/mL) with saturating levels of FITC-tagged YBM 42.2.2 (approximately 400 μg/mL) at 4°C for 30 min. Include propidium iodide (50 μg/mL) during the incubation.
9. Wash and resuspend the cells to 2 x 10^6 cells/mL in Eagle's-HEPES medium containing BSA and sodium azide at 4°C.

3.5. *Cell Sorting* (See *Note 1*)

1. Separate the labeled cells on a fluorescence-activated cell sorter by the two parameters of forward light scatter and fluorescein fluorescence.
2. For a FACS-II cell sorter, cellular excitation is achieved with an argon ion laser at an output power of 0.3 W and an emission wavelength of 488 nm. Set the light scatter gain at approximately 4. For fluorescein fluorescence, set the photomultiplier voltage at 650 V with an amplifier gain of 8–16. These voltages and gains will vary with the instrument used. The fluorescein fluorescence is detected by placing a 530-nm long pass interference filter and a 530-nm long pass filter in front of the appropriate photomultiplier tube.
3. Sterilize the tubing and nozzle by passing ethanol through the cell sorter for 30 min. Wash out the ethanol with sterile distilled water, and pass sterile distilled water through the tubing and nozzle for at least 1 h, following this with a 0.9% saline wash for 30 min prior to sorting.
4. Run the cells at 4°C through the cell sorter, collecting cells with intermediate to high forward light scatter characteristics that are negative for YBM 42.2.2 labeling when compared to control cells labeled with an irrelevant FITC-tagged rat monoclonal antibody of the same subclass. *See* Fig. 1A and 1B.
5. Collect the sorted cells in bicarbonate-free IMDM pH 7.3 containing 20–50% FCS at 4°C in earthed siliconized glass tubes. Note: CFU-E will die if the collection medium becomes alkaline.
6. Spin the sorted cells at 800 rpm for 15 min at 4°C.
7. Resuspend the cells to 2 x 10^6 cells/mL in Eagle's-HEPES medium with BSA and sodium azide.
8. Label with FITC-tagged YBM 10.14.9 or with unlabeled YBM 10.14.9 (using 400 μg/10^7 cells) for 30 min at 4°C.
9. Wash the cells twice with Eagle's-HEPES medium with BSA and sodium azide. Resuspend to 2 x 10^6 cells/mL in the same medium.

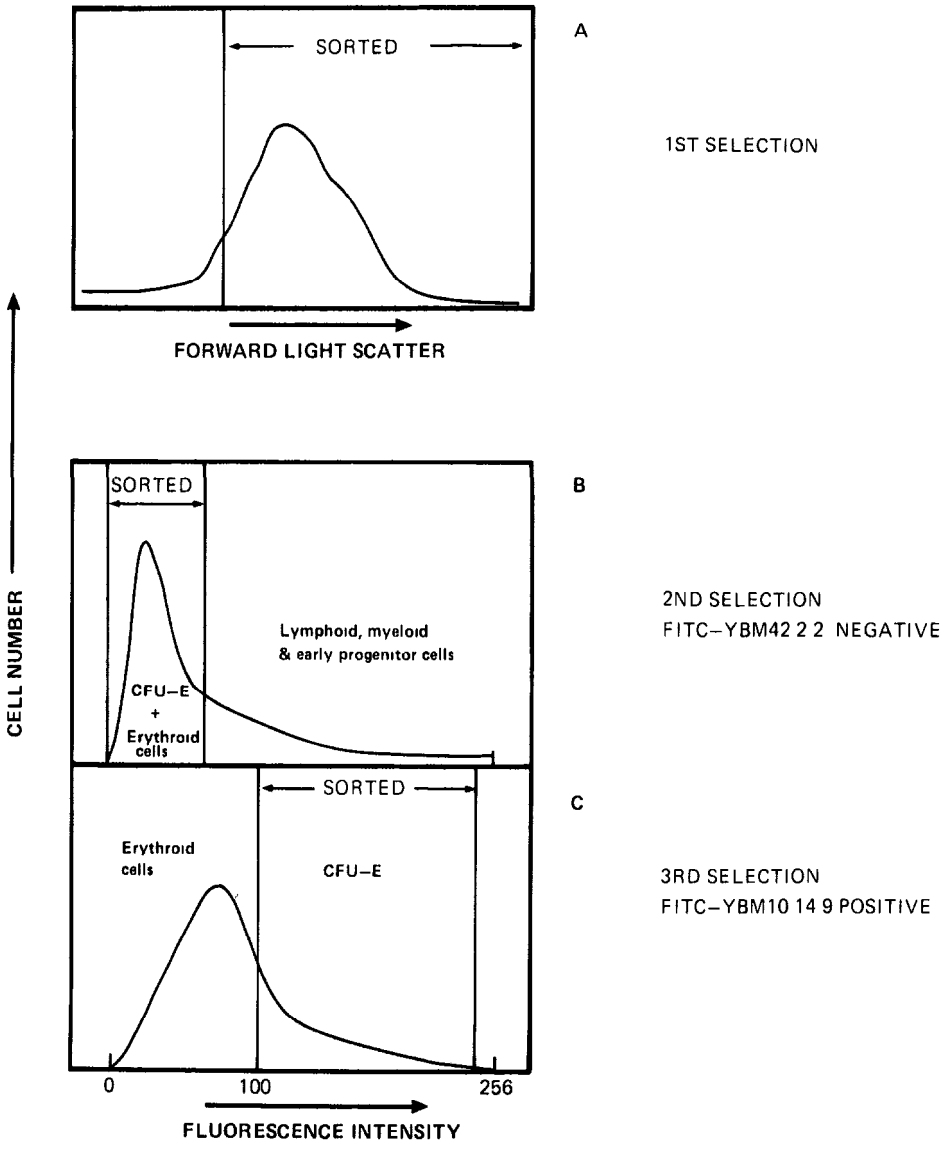

Fig. 1. Typical histograms showing the regions selected for isolating CFU-E. The fetal liver cells are first selected on the basis of their intermediate to high forward light scatter characteristics (A) and the YBM 42.2.2 negative cells (B) are sorted. The isolated cells are labeled with FITC-tagged YBM 10.14.9, and the positive cells (C) are collected. Care is taken to exclude the highly positive cells that are stained with propidium iodide and represent the nonviable cell fraction.

10. When using the unlabeled YBM 10.14.9, a two-stage indirect labeling procedure is necessary. For this, incubate the cells with FITC-Protein A (20 μg/mL final concentration) for 30 min at 4°C prior to washing, and resuspension in Eagle's-HEPES medium with BSA and sodium

azide. Include propidium iodide (50 µg/mL) in the final incubation step. Dead cells labeled with propidium iodide give a very high fluorescence signal in the fluorescein channel and can be gated out when only two parameters are available for sorting.

11. Sort the labeled cells selecting the YBM 10.14.9 positive cells (avoiding the very highly propidium iodide labeled nonviable cells) using conditions described for the first sort and shown in Fig. 1C.
12. Centrifuge the sorted cells at 800 rpm for 10 min at 4°C. Resuspend in bicarbonate-free IMDM containing 10% FCS at 10^5 cells/mL. Keep at 4°C until cultured.

3.6. *CFU-E Culture* (See *Note 2*)

1. Thaw the 2% methylcellulose stock at room temperature.
2. Set up triplicate cultures in 35-mm plastic Petri dishes in a final vol of 1 mL containing: 0.8% (w/v) methylcellulose, 10% (w/v) FCS, 1% (w/v) deionized and delipidated BSA, 0.3 mg transferrin saturated with $FeCl_3$, 5×10^{-5} *M* 2-mercaptoethanol, 0.05 U erythropoietin, 8 µg L-α-phosphatidylcholine dipalmitoyl, 7.8 µg cholesterol, 5.6 µg oleic acid, and 10^2–10^3 sorted fetal liver cells or 10^4 unsorted fetal liver cells all in single-strength IMDM pH 7.3.
3. Incubate the cultures at 37°C in a humidified incubator gassed with 5% CO_2 in air for 2 d.
4. Score single- or double-cell clusters containing 8–64 mature or maturing erythroid cells using an inverted microscope with a 100-fold magnification.

3.7. *Morphology*

1. Cytocentrifuge sorted cells and air dry.
2. Fix the cells with methanol for 10 min at room temperature.
3. Incubate in May-Grunwald stain for 20 min at room temperature and then in 3% Giemsa in tap water for 20 min at room temperature.
4. Wash the slides in tap water. Air dry and mount the coverslips with DPX.

3.8. *Benzidine Staining*

1. Suspend the cells in tissue culture medium (without NaN_3) to a concentration of 1.5×10^6 cells/mL.

2. Pipet 150 µL of the cell suspension into the well of a 96-well-flat bottom microtiter plate.
3. Prepare the staining solution; add 10 µL of 30% H_2O_2 to 1 mL of benzidine stock solution. Mix and use within 30 min.
4. Add 50 µL of staining solution to cells, and mix quickly by pipeting up and down several times.
5. Wait 5 min. At this time, cells containing at least 10% hemoglobin will have stained a dark blue. Estimation of the number of hemoglobin containing cells is best achieved by photographing the cells at this stage, since both the color of the stain and the number of stained cells will change with time.
6. The early erythroid blast cells represent the most immature and largest erythropoietic precursors recognizable, containing a prominent nucleolus, basophilic cytoplasm, and loose chromatin pattern, and are negative for benzidine staining. More mature erythroid cells containing hemoglobin will stain with benzidine.

4. Notes

1. The method describes the isolation of CFU-E using sequential sorting with two monoclonal antibodies, YBM 42.2.2 and YBM 10.14.9. Relatively high recoveries of CFU-E (40%) can be achieved in this way. These studies could be done equally well using a single multiparameter sort with antiisotype reagents labeled with fluorescein and phycoerythrin or directly conjugated reagents. Substantial enrichment for CFU-E from both normal fetal liver and bone marrow can also be achieved with a set of monoclonal antibodies listed in reference 7. Indeed, more efficient purification may be obtained for CFU-E by combining three probes, such as YBM 10.14.9, YBM 42.2.2, and YW 13.1.1, since all these antibodies exhibit different patterns of reactivity with normal hemopoietic cells. Studies using simultaneous two- and three-color sorting with a variety of antibodies to human erythroid precursors show the potential benefit of such approaches to cell fractionation (*8*). Other procedures that allow substantial enrichment for CFU-E include multiparameter sorting using fluorescein-conjugated pokeweed mitogen and rhodamine-conjugated antineutrophil/monocyte antibodies (*9*). In addition, Nijhof and Wierenga (*10*) have obtained sufficient numbers of highly purified CFU-E for biochemical analysis

in a relatively short time by using density separation and elutriation of spleen cells from thiamphenol-treated mice.

2. The isolated cells are analyzed for CFU-E by their growth in methylcellulose (3). Details of methylcellulose preparation are also given in reference *11*. Fetal calf serum can be omitted from the cultures, since the BSA, lipid, transferrin, and erythropoietin additives have been designed to allow CFU-E growth in serum-free conditions (3). The approximate concentrations of each additive are described, but it is essential to test and titrate each additive in order to obtain the best conditions for CFU-E growth.
3. Details of antibody purification are given in reference *12*. The benzidine staining technique described here was adapted from reference *13*. Single cells from the purified CFU-E can also be sorted directly into 150 µL of the methylcellulose-supplemented culture medium in a microtiter tray. The colonies are allowed to develop for 2 d at 37°C, and the maturing erythroid cells can be stained after cellulase digestion of the methylcellulose (*14*). For this, 75 µL of FCS containing 1.08 mg/mL cellulase (1943 cellulase U/g) is added to each well, and the cultures are incubated overnight at 37°C. The following day, the contents of each well are transferred as drops to a glass microscope slide and allowed to air dry. The cells are fixed and stained with May-Grunwald/Giemsa stain. Alternatively, the cells may be stained *in situ* with benzidine with or without digestion of the methylcellulose with cellulase.

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