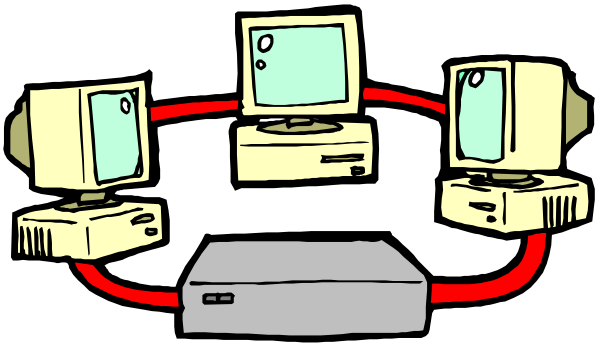

AUS DEM INTERNET

ANNEXIN V

Hi, we have problem in labeling cells with Annexin V. In our protocol the cells are resuspended in an incubation buffer which contains 1.5mM CaCl2,then we add 5 ul of annexin V and we leave on ice for 15'. With this protocol we did not observed any positive cell even in the positive control. Could anybody give us suggestions? thank you
Silvana

Hi Silvana,
Out of curiosity, what cells where you using as your positive controls, and what did you use to induce apoptosis in them? I use the AnnexinV from Pharmingen and their buffer uses 2.5mM CaCl2, and that works well for me. My positive controls are Jurkat cells with anti-CD95 and I get a good positive after 8 hours. Let me know what you use and anything else you use in your buffer. Also you may want to, if you have not already use propidium iodide to just see if you have any death occuring, just as a second control to see if you even have any dead cells.

Jason Cohn
Princess Margaret Hospital
Ontario Cancer Institute
Toronto, Canada

Hi Silvana,
Increase the Ca concentratie up to 2.5 mM. Normally the Ca conc. is between 1.8 - 2.8 mM.There is a possibility that you get a precipitate of Ca-X[phosphate]. Than take another buffer/medium.
Cor

silvana (S.Bonfigli@ipsoe.ss.cnr.it)
I would like to thank for your suggestions, finally we had results with annexin V. The positive control that we use is the 4C Plus Normal Control (human blood preparation obtained from Coulter Corp) and we think that the stupid mistake we did was to resuspend the cells with the sheath fluid.Thank you
Silvana

FLOWEN VON BAKTERIEN

Dear Flow Folks:
I am a chemistry grad student, and my project is to sort genetically engineered libraries of E. coli. I am currently investigating ways to increase the rate of sorting on a commercial droplet sorter. Most of thecommercial machines have a 76um orifice, which is much bigger than coli or yeast. I have noticed a reference from Shapiro's Practical Flow Cytometry(3rd) by Fellner-Feldegg on using a small 25um orifice. Is it realistic tofit a BD Vantage or a Coulter Altra with say a 20um orifice and a 100kHz droplet generation frequency, and thereby increase the effective sort rate? I am guessing that a cycle time of 5.5 microseconds means a maximum of 180,000 decisions/sec. This seems acceptable? I noticed that theCytomation MoFlow uses droplet deflection technology, but would the BD or Coulter drop deflection plates result in acceptable purity and yield? Is jet-in-air required for these kinds of sort rates? Are there other considerations that need to be taken into account? I apologize for the ignorance, and thanks in advance.
Mark Olsen
BI/GG Group
University of Texas at Austin

The orifice size is not of particular relevance here; it is the droplet generation frequency which will determine the maxi-

mum sort rate, up to thepoint at which the instrument electronics can't keep up with the required decision rate. If the processing dead time or cycle time is 5.5 microseconds, the 180,000decisions/second is what would be achieved if you had a steady stream of cells coming one right after the other; in practice, you need to have a lower duty cycle, which among other things, will keep the coincidence abortrate at an acceptably low level. Thus, the practical processing rate of an instrument is generally lower than would be calculated from its cycle time.

But worrying about orifice sizes and droplet generation rates first is going about things ass backwards. You've said that you want to sort genetically engineered libraries of E. coli. What marker(s) will you use to identify the cells you want to sort? What is the frequency of desired cells in the population? What are you going to do next with the sorted cells? Do you need single organisms (neglecting, for the moment, the possibility that two or more bugs might be stuck together and look to the instrument like a single organism)? Does your lab, department, or institution already have the sorter or are you looking to buy one? From your question, it appears that you are considering modifying a B-D or Coulter instrument to run beyond specifications, to achieve performance closer to that specified for Cytomation's system. But, as I said, you need to start with the biological details of what you're doing. Would your project be feasible if you could sort 100,000 cells/second and not if you could only sort 20,000 cells/second, which only takes five times as long (and let's face it, while time may be money, graduate students' time is usually considered to be in the category of small change)? Defining all aspects of the problem precisely will, at least in my opinion, be essential if you are trying to get funding to acquire a high-speed sorter to do this project; there are people who know nothing about flow who can snap their fingers and have the bucks for a high-speed sorter appear, but most of them have also dined with the King of Sweden in December. I hope the further discussion of this subject on the Mailing List doesn't degenerate into "My nozzle has higher pressure than yours", but I can be contacted off the List if necessary. So, let us know more about your problem. - Howard R.Wadley (s9803537@pop3.unsw.edu.au)

Dear Mark,

I manage a facility with a MoFlo high speed cell sorting cytometer. I have been involved in sorting bacteria much smaller than E.coli. I get excellent results using a 100 um tip & about 50kHz drop gen. frequency. I agree that event rates above 25,000/sec a 75 um tip & 100kHz drop gen. would be better. The problem as I understand it is the speed at which the software runs. 180,000 decisions (cells)/sec is fine in theory, but a lot of software (I believe most) cannot cope. The size of the drop is essentially unimportant, its how many cells you get inside the drop that makes a sort good or bad. This is your determination on purity. For a library I assume you are looking for 1 pos. cell/drop, with no other cells (pos. or neg.) present. This is not always best fixed by sorting faster. High speed sorting usually means a high coincidence rate = more pos. cells discarded. How you create your library also determines the rate at which you sort. I can sort directly into a 96 well plate, but the ultimate speed of the sort is dependant on how fast the robotics can move the plate to catch the drop. Mark, no one who wishes to learn is ignorant, only less informed, & nobody knows everything. I too am new to flow cytometry, its whole different game to routine histology & electron microscopy. Regards Rob W.

KAPPA/LAMBDA/KLONALITÄT

1) There should be a simple answer to this question - but I just can't find it !. What is the groups opinion on Kappa/Lambda and Lambda/Kappa ratios which are considered "consistent with monoclonality" ?? - in the clinical setting. ie: K/L ratio >2.0 or 2.5 and how about L/K ?? This question is arising on a day to day basis around here and a reference would be really nice so

that we can settle the arguement and get on with some work !! Peter Chapple Melbourne AUSTRALIA

I would like to recommend to you a paper I wrote which discusses statistical criteria for determining surface immunoglobulin light chain restriction: Surface immunoglobulin light chain restriction in B-cell non-Hodgkin's malignant lymphomas. H. Ratech and S. Litwin. Am J Clin Pathol 91:583-586, 1989. Sincerely, Howard Ratech, M.D.

2) DAHL CHASE DIAGNOSTIC SERVICES (dcdsflow@mint.net) This a question for the clinical Flow people, It is well known that in in some NHL/B lymphomas and other LPD/B the B-cell population may express neither surface kappa or lambda light chains. Would you report this (in your institution) as a "monoclonal population" or as an "abnormal B-cell population" with neither kappa or lambda expression? My second question is if anybody has heard of a condition called "persistent polyclonal B-cell lymphocytosis"? One of our pathologists recently mentioned this and wondered if this condition could be confused with a case which has a definite absolute lymphocytosis with absence of kappa and lambda. I would think, polyclonal means having either kappa or lambda on the B-cells and therefore the absence of both light chains would not make the above mentioned condition part of a differential diagnosis. Is this correct? Thank you for you help Andrea Illingworth Dahl-Chase Diagnostic Services/Flow Cytometry 333 State Street Bangor, Maine 04401 (207)990-4855

"Soll man fehlende Leichtkettenexpression als monoklonal bezeichnen?"

Maryalice Stetler-Stevenson (stetler@box-s.nih.gov)
I usually don't report just "monoclonal B-cell population". I put together the data-flow, morphology and clinical and report a diagnosis. Sometimes the diagnosis is atypical B-cell population if the data is not conclusive but abnormal. I then suggest additional tests to try to reach a diagnosis. I prefer to report "CLL", mantle cell lymphoma, Follicular lymphoma or "monoclonal B-cell leukemia most consistent with..... ". Therefore I may call a light chain negative large cell neoplasm, for example, a large cell lymphoma of B-cell lineage and mention in note the neoplastic cells are light chain negative. An atypical B-cell population is for when I am not sure it is a neoplasm using all data available but I know something is wrong. Polyclonal means that there are lambda and kappa expressing B-cells. If you have polyclonal plasma cells they would be surface light chain negative but have polyclonal intracellular light chains. Polyclonal B-cell lymphocytosis would be part of the differential of a lymphocytosis composed of mature appearing lymphocytes (as might also be seen in a clonal low grade lymphoproliferative disorder). Once you know the light chain expression this can either be ruled in or out of the differential diagnosis. Maryalice

Nebe, Thomas (thomas.nebe@ikc.ma.uni-heidelberg.de)
Dear collegue, let me try to answer. B-cells do not express light chains on their surface in their early (bone marrow) and late (plasma cells) development. In some NHL background due to Fc receptor binding is so high, that light chain restriction is hard to prove. In late stages cytoplasmic light chain restriction can be demonstrated. Monoclonality by light chains could be demostrated by immunofixation methods in the serum as many NHL secrete small amounts of immunoglobulin. Of course a larger panel also depicts clonality but we report the problem with the light chains. Molecular biology methods showing clonality also help. To your second question: Polyclonal B cell lymphocytosis is seen in some HIV positives and are polyclonal by surface light chain restriction. The number of washing steps to remove serum immunoglobulins should be validated in each laboratory. Minimum is three in our hands and four is better. Blocking with serum from the species where the light chain reagent is derived from is a prerequisite. So in expansion of immature B

cells that are kappa/lamda negative will not be recognized. These will be seen in repopulation phases after immunosuppression and regularily in the normal bone marrow. In conclusion monoclonality depends on performance and limitation to flow cytometry. Three colour combinations like kappa/CD19/CD20 or kappa/CD19/CD5 better resolve a subset of abnormal B cells in a mixture with normal B cells (suited eg. for bone marrow, leaky or residual NHL). I already mentioned the autogating software of Hans-Dieter Kleine to pull them out (hans-dieter.kleine@medizin.uni-rostock.de). With kind regards Thomas Nebe

Darf ich hier in Ergänzung der Antworten zur Verwendung von 4-Farben ermuntern. Dann können Sie z.b. Kappa/11c/20/19 und Lambda/5/20/19 analysieren. Ev. auch 10 einbauen. Gerade CD20 teilt ihnen die B-Zellpopulation oft in die normale und die pathologische (z.B. bei CLL meist schwächer, bei FL und HZL meist stärker als die normalen). Mit vier Farben haben sie viel mehr Möglichkeiten und die APC-Antikörper, die wir bis jetzt ausprobiert haben (CD19, CD3, CD34, CD38 BD und CD5 Pharmingen), funktionieren zufriedenstellend. In Beantwortung einer weiter unten gestellten Frage berichtet ein Flower von guten Erfahrungen mit 4 Farben am Coulter XL. Scheinbar lohnt sich also auch am XL der Einsatz von 4 Farben.

Erfahrungen mit 4-Farben und nur einem Laser (488nm) am XL

robert gniadecki (rgniadecki@hotmail.com)
Hello! We are planning immunophenotyping with 4 colours: FITC, PE, Pe-Texas red (ECD) and Pe-Cy5. We have however experienced some severe problems with compensation between PE and PE-TR. Could you share your experience with me? Is that fluorochrome combination possible to use? We have 4 photomultipliers and a 488 nm argon laser.

woodbl (woodbl@u.washington.edu)
Hi, Yes, we have much experience with the system you describe. It took us a while to figure out how to deal with it, but it works well for us now. The issues for us were: 1. Each ECD antibody we have used (Coulter) has a different amount of PE leakage, so the amount of compensation required is different for each ECD antibody. CD8 has had the most leakage (which we initially tried), and results in significant overcompensation of the PE signal when other ECD antibodies are used. CD45 has tended to be next, followed by CD3. CD19 has had the least leakage. CD45, CD3 and CD19 are close enough that one can approximate the comps using any one of them for compensation. Ideally one needs to have a separate compensation matrix set up for each ECD antibody used. 2. Use median values, not arithmetic or geometric means, for compensation. The fluorescence distributions of the population, in particular the negative population, is not normally distributed and incorrect compensation will result. Unfortunately, the Coulter automatic compensation software uses a mean for its calculations and results in suboptimal compensation, most noticeable as marked PE overcompensation. If one attempts to use medians, often the median for negative populations in the higher wavelength PMTs lies very close to the axis and makes accurate determination of the comps difficult. The best solution for us has been to use off-line software compensation with WinList. The resulting comps are consistent and reliable, and the amount of time required to perform the compensation is dramatically reduced. However, one does have to run the samples through the instrument uncompensated. Since we do all our data analysis off-line anyway, this is not a problem. 3. Do not expect to use quadstats to analyze the data. Due to the significant spectral overlap of ECD with both PE and PE-Cy5, the true negative does not lie parallel with the axis (unlike orthogonal quadstats). This effect can be quite marked and can best be seen on histograms displaying PE vs. ECD and ECD vs. PC5. Hope this helps.

Rachel M. Gerstein (Rachel.Gerstein@ummed.edu)

You could use Cy7-PE (using bandpass filter 785+/-50) in the place of PE-TR. Caltag and Pharmingen both sell some Cy7-PE reagents. see also Mario Roederer's site: http://cmgm.stanford.edu/~roederer/abcon/.
A correction: Pharmingen does not sell Cy7-PE reagents. Sorry for any confusion. Rachel

PARAFORMALDEHYDE OR FORMALDEHYDE ?

Juan Luis Castillo Nadevrete (axelyoyi@entelchile.net)

HI. Are differences in the flourescence, scater propieties or antigenic detection if one use formaldehyde for fixed cells ? I know that formaldehyde is a cross-linking fixative, forming reversible methylene bridges between amino, imino, sulfhydryl, and hydroxyl groups within proteins.Also reacts to a lesser extent with lipids and carbohydrates, but, inlike alcoholic fixatives, does not act as a permeabilizing agent. (Clevenger C, Shankey V.: Cytochemistry II: Immunofluorescence measurement of intracellular antigens. In Clinical Flow Cytometry, principles and aplication. Bauer et al ED pp 158.) Also the oxydation of formaldehyde results in the presence of formic acid , and methanol is found as a stabilizing agent in unbuffered solutions of formaldehyde. Does the methanol afect flourescence, scater propieties or antigenic detection ? Does de formic acid afect flourescence, scater propieties or antigenic detection ? Does th formaldehyde afect the satining of intracellular antigens ? I hope yours ideas.

"Ich verwende kein Paraformaldehyd, das älter als 24h ist."

R.Wadley (s9803537@pop3.unsw.edu.au)

Dear Juan, Paraformaldehyde & formaldehyde are the same chemical. You might like to think of them as the solid & gas phases respectively. Formaldehyde solution does contain methanol as a stabilising agent, a preservative. This means that formaldehyde can be prepared & stored in bulk. Over time the formaldehyde molecules polymerise, this gives rise to the white precipitate that can be seen in the bottom of formalin solutions. The methanol in the solution can damage/cover some antigenic sites. Paraformaldehyde is my choice of fixative for immunological work, usually a 2 -4 % solution in buffer. Preferably a buffer that is adjusted for both pH & osmolarity. I prefer PIPES or HEPES buffer for specialist histological or electron microscopy studies, but PBS is far more cost effective especially for flow work. The PBS I run in my flow is not checked for osmolarity. The problem with paraformaldehyde solutions is that they begin to polymerise as soon as you make them up. Always use paraformaldehyde as a fresh solution. I don't use it if it is more than 24 hours old, but some find it is still OK after a week. Methanol has a different fixative action to aldehydes, it is a precipitates proteins. Generally speaking it is an agent that causes shrinking. Therefore, yes it may create altered scatter properties, & yes it may limit some antigenic selection. It should be noted that aldehyde fixatives can also cause modification to cell size & shape through the action (or inaction) of your chosen buffer, & some antigens are vey sensitive to aldehyde fixation. I have never considered the action of formic acid. But, I would never use a fixative without a good buffer. I assume the action of the formic acid would damage some antigenic sites & cellular constituents, so changes to scatter properties may be seen. Hope this helps. Regards Rob W.

ZELLINIE NEGATIV FÜR p53

Joe Dynlacht (jdynlach@iupui.edu)

Joe Trask inquired about a non-adherent cell suspension line that is known to be negative for p53. Well, Joe, I suspect that somebody will respond that the HL-60 promyelocytic leukemia cell line might be a good choice. But

beware, especially if you plan to use the cells for apoptosis studies. Discrepancies have been reported by different labs regarding mode of death after irradiation, and these discrepancies have been suggested to be due in part to slight differences in p53 status among different available strains of cell lines (of course, the assay used to detect apoptosis, and its sensitivity, as well as the time points chosen to examine amount of apoptosis also factor into one's interpretation). My suggestion would be to run a Western to confirm p53 status if you end up going with HL-60 cells. By the way, your e-mail address indicates you are at Lilly and probably work out of Indianapolis. We use HL-60 cells routinely at IUPUI, so if you are interested in acquiring some, give me a call and you can swing by and pick some up next week. Joe Dynlacht Joseph Dynlacht, Ph. D. Indiana University School of Medicine Department of Radiation Oncology Indiana Cancer Pavilion, RT 041 535 Barnhill Drive Indianapolis, IN 46202 phone: (317) 278-3882 FAX: (317) 278-0405 e-mail: jdynlach@iupui.edu

MYCOPLASMEN IN CELL LINES

Kraus, Elizabeth (et.kraus@baylordallas.edu)
This message is being posted for a coworker. Not necessarily flow-related but could be useful to the group: Does anyone have experience in mycoplasma detection in cell lines? Our dilemma: PCR results (ATCC Mycoplasma Detection Kit) are negative while confocal microscopy detects positive DAPI labeling in the cytoplasm of the same cell line. Any help would be appreciated.

Simon_Q_Rice@sbphrd.com
Elizabeth, Assuming the PCR conditions were controlled and you know reagents, method of preparation of the sample etc are ok, I'd be surprised if this technique wasn't the most sensitive of the two you've described-it seems strange then that the least sensitive method should give you a positive signal. My suggestion would be to check conditions of the PCR with a positive control, that cell cultures have been grown in antibiotic free medium for at least three passages (that includes selection agents such as neomycin/geneticin which have anti-microbial properties) and then recheck alongside another technique. We have used the Genprobe detection kit for a number of years and it works very successfully-however the PCR should remain the most sensitive. I hope this is useful. Regards Simon Simon Q.J. Rice Gene Expression Sciences Smithkline Beecham Pharmaceuticals, NFSP-N, Harlow, UK.

DURCHFLUSSZYTOMETRIE UND TRANSPLANTATION

Gyorgy Lustyik (lustyik@apacs.pote.hu)
Greetings, Is any guide-line, review of protocols, or other collection of parctical methods related to the application of flow cytometry in transplantation medicine available? I know there are many publications reviewing such techniques. I am looking rather for an up to date, practical guide-book or other collection listing the currently used, widely accepted flow cytometry methods of such fields like, posttransplantation disorders, cytometric crossmatch procedures in human organ transplantation, etc. Any help is greatly appreciated. Gyorgy Lustyik, Ph.D. Department of Biophysics University Medical School of Pecs

Leider hat Kollege Gyorgy keine Antwort bekommen. Eine gute Übersicht über den Einsatz der Flow-Zytometrie in der Transplantationsdiagnostik und insbesondere dem post-Transplantationsmonitoring würde mich sehr interessieren. Die Einsatzmöglichkeiten der Durchflußzytometrie in diesem Bereich werden an unserem Krankenhaus bei weitem nicht ausgeschöpft und ich nehme an, bei Ihnen ist es ähnlich. Vielleicht hat jemand von Ihnen Informationen zu diesem Thema oder möchte vielleicht eine kleine Review zu dem Thema schreiben.

POSITIVKONTROLLE FÜR APOPTOSE-MESSUNG

Richard Meister [mailto:meister.1@osu.edu] <mailto:[mailto:meister.1@osu.edu] > Apoptosis Hello, everyone: I have two questions re: apoptosis that came up during a recent consult on the subject. 1. What is the best positive control to use; i.e., an easy-to-grow cell line that can be reliably and predictably induced into apoptosis? *Die zweite Frage war unwichtig*

Reece, Lisa (lreece@utmb.edu)
To answer your questions: 1. There is a very reliable cell line called CEM-C7 that is a cloned human leukemic T-cell line. It is sensitive to the cytolytic action of glucocorticoids. You may contact E. Brad Thompson here at the University of Texas Medical Branch Galveston at bthompso@utmb.edu <mailto: bthompso@utmb.edu> or you may reference a few of his articles: "Characterization of a Glucocorticoid-sensitive Human Lymphoid Cell Line", Michael R. Norman and E. Brad Thompson, Cancer Research 37, 3785-3791, October 1977 "'Activation-labile' glucocorticoid-receptor complexes of a steriod-resistant variant of CEM-C7 human lymphoid cells", Thomas J. Schmidt, Jeffrey M. Harmon & E. Brad Thompson, Nature, Vol. 286, No. 5772, pp. 507-510, July 31 1980 "Dexamethasone Induces Irreversible G1 Arrest and Death of a Human Lymphoid Cell Line", Jeffrey M. Harmon, et al., Journal of Cellular Physiology, Vol. 98, No. 2, February 1979 We routinely use this cell line in our apoptosis studies and the cells grow rapidly and are very easy to culture. They label very well with Annexin V, CD95, and CD4.

DARZYNKIEWICZ ZBIGNIEW (DARZYNK@nymc.edu)
The "best" positive control: HL-60 cells treated with >0.15 uM captothecin (CPT) provide a reliable model of apoptosis. Most apoptotc changes (mitochondrial, plasma membrane, DNA fragmentation, nuclear fragmentation) occur during the initial 4 h of the treatment. The advantage of this model is that only S phase cells undergo apoptosis (Del Bino et al., Cancer Res., 51: 1165, 1991). Thus, G1 and G2/M cells, within the same sample may serve as a negative control. The critical point is the cells have to rapidly progress through S phase to be sensitive to CPT. It is a collision between the progressing DNA replication fork and the lesion iduced by CPT that provides the signal inducing apoptosis. Any slowdown in S phase progression, therefore, such as due to higher density of cells in the culture (subconfluency; > 800.000 cells per ml) makes them less sensitive to CPT.

PROGRAMME ZUM EXPORT VON ROHDATEN IN ANDERE PROGRAMME

beisker@GSF.DE
Dear Flow Friends, I have a problem to develop some program for data conversion of FCS2.0 and FCS3.0 files to other statistical packages. Perhaps somebody has a Fortran or C program which reads the FCS format and puts the data in some array or similar. I would need the source code, otherwise I would have to develop it by myself from the described standard. So I hope, somebody has already done the work and is willing to share her/his experiences and codes. It is for a pure scientific project without commercial interest. With best regards from Munich Wolfgang FLOW IS FUN !!!

DAVID JONATHAN NOVO (dnovo@ucla.edu)
Hi Wolfgang, If you are using a PC with windows 95 or better, the program FCS Express can export the FCS data to a text file which can easily be parsed into any format you wish. You can get a free demo copy at http://www.denovosoftware.com -Dave On Tue, 16 Feb 1999 beisker@GSF.DE wrote:

SEPARATION VON MONOZYTEN

Mats Alheim (Mats.Alheim@cnsf.ki.se)
Hello, I am working with immunology of MS, mainly with cytokines. I will study cytokine expression by monocytes. I have problems with monocyte seperation from PBMC. I

will have max 20 ml of blood from each patient and I need around 1 million monocytes. If you can help me with monocyte seperation techniques, I would appriciate. With best regards

smonard@adarc.org
Hi Something key in separating monocytes by FACS is is include some EDTA in your buffer the monoctes are suspended in, 5mM should do. Without EDTA monocytes disappear from the suspension, stikking to tubing, each other or something. You should be able to get 1 million monocytes from 20 ml blood from most individuals. So just ficol your cells, stain with CD14 (purity isn't that bad if you just use scatter) and suspend in PBS with 5mM EDTA and sort Simon Monard

12TH ANNUAL CLINICAL COURSE ON FLOW AND IMAGE CYTOMETRY

Alice L. Givan (Alice.L. Givan@dartmouth.edu)
This is a repeat since the previous message seems to have come through in truncated form. Apologies. Hello Folks, Lebanon, New Hampshire 03756-0001 - Telephone (603) 650-1525. http://www.hitchcock.org/pages/CEB/FlowCyt.html TWELFTH ANNUAL CLINICAL COURSE ON FLOW AND IMAGE CYTOMETRY July 18 - July 23, 1999 Norris Cotton Cancer Center Dartmouth Medical School Dartmouth-Hitchcock Medical Center Lebanon, New Hampshire The Twelfth Annual Clinical Course on Flow and Image Cytometry will be held this year at Dartmouth -Hitchcock Medical Center in New Hampshire, USA. As in past years, the Course will include hands-on laboratory work in small groups, expert lectures by local and visiting faculty, and many opportunities for informal discussion. Emphasis will be on basic theory as well as its practical application in the clinical laboratory. The Course will be limited to approximately 45 participants. It is directed at those individuals with some experience in flow or image analysis. Participants in the Course will gain an understanding of the principles of flow cytometry and image analysis and up-to-date knowledge of the ways in which cytometry can be applied to, among others, the following clinical problems: leukemia and lymphoma phenotyping; analysis of platelets; analysis for cell cycle stage and ploidy; multiparameter assays for DNA with cytoplasmic/nuclear antigens; CD34 stem cell analysis; assays to detect fetal cells in maternal blood; HIV detection and immunophenotyping for AIDS; evaluation of leukocyte function; apoptosis; procedures for quality control. Dartmouth-Hitchcock Medical Center, including both Dartmouth Medical School and the Mary Hitchcock Memorial Hospital, is located on a new campus in Lebanon, New Hampshire, a rural New England town on the Connecticut River. Lectures and laboratories will be held at the Medical Center. Accommodations will be at a nearby hotel. Social events will make use of the neighboring facilities of historic Dartmouth College. Transportation between events will be provided. Alice L. Givan, Ph. D., Course Co-Director and Paul K. Wallace, Ph.D., Course Co-Director ~ PROGRAM ~ SUNDAY, JULY 18 - FRIDAY, JULY 23, 1999 Wednesday afternoon will be free for hiking, swimming, boating, and relaxing. On Thursday evening, there will be a celebratory lobster bake. The Course will conclude after a final session on Friday morning, July 23rd. The Dartmouth-Hitchcock Medical Center is accredited by the Accreditation Council for Continuing Medical Education to sponsor continuing medical education for physicians. DMS/DHMC FACULTY* ALAN EASTMAN, PhD Department of Pharmacology and Toxicology Norris Cotton Cancer Center ALICE GIVAN, PhD^ Department of Physiology Englert Cell Analysis Laboratory of the Norris Cotton Cancer Center NORMAN LEVY, MD Department of Pathology Clinical Flow Cytometry Laboratory PAUL WALLACE, PhD^ Department of Microbiology GUEST FACULTY KENNETH AULT, MD

Maine Medical Center Research Institute, South Portland, Maine KENNETH BAUER, PhD ChromaVision Medical Systems, Inc., San Juan Capistrano, California NANCY BIGELOW, BS, MT(ASCAP)SH William Beaumont Hospital, Royal Oak, Michigan CHARLES CLEVENGER, MD, PhD University of Pennsylvania Hospital, Philadelphia, Pennsylvania LINDA COOK, PhD Lahey Clinic, Burlington, Massachusetts BRUCE DAVIS, MD William Beaumont Hospital, Royal Oak, Michigan LOUIS KAMENTSKY, PhD CompuCyte, Boston, Massachusetts MICHAEL KEENEY, ART, FIMLS London Health Sciences Center, London, Ontario, Canada KATHARINE MUIRHEAD, PhD SciGro, Inc., Malvern, Pennsylvania CARLETON STEWART, PhD Roswell Park Cancer Institute, Buffalo, New York

*Dartmouth Medical School/Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire ^Course Directors INFORMATION DATES/TIME: The course begins at 10:00 a.m., Sunday, July 18th, and concludes at noon, Friday, July 23rd. PLACE: Lectures and laboratories will be held at the Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire, USA. AUDIENCE: The Course is directed at individuals with some experience with flow or image cytometry. Register early because the Course is limited to approximately 45 participants. ACCOMMODATIONS: Rooms ($75 per night, single; $37.50 per night, shared double ACCREDITATION: The Dartmouth-Hitchcock Medical Center designates this continuing education activity for up to 40 hours in Category 1 credit towards the AMA Physician's Recognition Award. Each physician should claim only those hours of credit that he or she actually spent in the educational activity. TRAVEL: Dartmouth-Hitchcock Medical Center is served by the Lebanon, NH airport (10 REGISTRATION FEE: $1,075 (increasing to $1,195 after April 1). The fee includes course materials, refreshment breaks, 5 lunches, and 5 dinners (including reception dinner and lobster bake). REFUNDS: A $25 fee will be retained on all cancellations made before June 1. On June 1 or thereafter, half the fee will be retained. No refunds will be given after June 30, 1999 on tuition or accommodation. FURTHER INFORMATION: For course content information, contact by e-mail ClinCourse@Dartmouth.edu; by FAX to Alice Givan at 603-650-6130. For registration information or if you have any special needs, contact the Center for Continu ing Education in the Health SciencesHere are more details of the 12th Annual Clinical Course on Flow and Image Cytometry, to be held again this summer at Dartmouth Medical School ---- July 18th through July 23rd. If you are an ISAC member, brochure with application form will be mailed to you automatically within the next month. If not, you can request one from the Center for Continuing Education in the Health Sciences, Dartmouth-Hitchcock Medical Center, One Medical Center Drive, For those of you with flow-related web pages, please feel free to link the Course web site to listings of courses on your own page, thanks! Alice Givan Registration will begin at 8:30 a. m. on Sunday morning, July 18th. Following registration, we will start, at 10:00 a.m., a series of lectures on general principles of flow and image cytometry, including discussion of fluorochromes, compensa tion, and data analysis. This all-day session will be followed by a reception and dinner at the Hanover Inn and a keynote address by Dr.Louis Kamentsky. The schedule for the rest of the week will include morning and afternoon lectures on advanced topics, each followed by 3-4 hour blocks of hands-on laboratory and computer work. Participants will rotate through each of the seven practical modules. Evenings will provide time for question periods and panel discussions. [includes breakfast] - plus 8% tax) have been reserved for Course participants at the Marriott Residence Inn near the Medical Center campus. These rooms can be booked and paid for via the registration form. All rooms have private baths. The shared doubles are in limited supply. If you select the shared option, we will assign a roommate to you if you do not indicate a roommate preference on the registration form. minutes driving time), Manches ter, NH airport, (1.3 hrs.), Burlington, VT airport (2 hrs.), and Lo-

gan airport in Boston, MA (2.5 hrs.). Ground transportation serves most of these airports. Complete travel information will be sent upon registration. Although nearby parking is available, cars will not be required for transportation during the week of the course. , Dartmouth-Hitchcock Medical Center, One Medical Center Drive, Lebanon, New Hampshire 03756-0001 - Telephone (603) 650-1525.

EPITHELIALE ZELLEN IM LYMPHKNOTEN NACHWEISEN

Chapman (gvchapman@bigpond.com)
Hi everyone A surgical colleague wants to know if there is a method to detect minor numbers of epithelial cells in a lymph node by flow. He is particularly interested in nodes where there is no apparent sign of epithelial cells observable by microscopy. Any suggestions or methods would be appreciated.

Michael Ormerod (Michael_Ormerod@compuserve.com)
Many years ago, we detected small numbers of epithelial cells (breast carcinoma cells) in sections of lymph nodes using an immunohistochemical stain for epithelial membrane antigen (EMA) (The use of antisera to epithelial membrane antigen in detecting micrometastases in histological sections. J.P. Sloane, M.G. Ormerod, S.F. Imrie and R.C. Coombes. Brit. J. Cancer, 42, 392-398, 1980). You could use the same approach in flow. Having made a single cell suspension from the node, I would label the cells with EMA (rat monoclonal antibody from Harlan Sera-Lab Ltd.) and CD45. Alternatively, you could label with CD45, permeablise and label with monoclonals to cytokeratin. I would prefer the first approach as you do not need to fix or permeabilise the cells before labelling.

Snider Denis (sniderd@fhs.csu.McMaster.CA)
Saverio, We currently are sorting epithelial carcinoma cells from blood, post surgical using the Ber-EP4 mAb (common epithelial surface antigen). This is a Dako product (Code F 0860). Provided your cell isolation and preparation techniques do not destroy the surface antigen then you might expect accurately detect as little 0.1% epithelial cells, in a cell suspension from the node. We get that kind of detection level, when epithelial cells are mixed in a blood cell suspension.

Saverio Alberti (alberti@cmns.mnegri.it)
PCR for any epithelial-specific mRNA will do. People are doing that for breast carcinoma (Her-2/neu).

John Lawry (j.lawry@sheffield.ac.uk)
Dear Graeme, You will need to dissociate the lymph node, or at least a part of it. This should be fairly easy to do using scalpels and gently pressing the tissue through a steel mesh (60-100 gauge) using the plunger from a syringe. Or use the Medicons tissue dissociator. Or add some dilute collagenase (Type 4 @ 0.2mg/ml) and dissociate at 37oC for about 15 minutes. Cells will then need to be fixed, either 70% cold methanol; or 1% paraformaldehyde (5 mins), wash, 70% methanol 30mins+. Then stain cells using FITC conjugated EMA-type antibodies, or cytokeratin (CAM5.2, CK8,18,19) or using two stage staining with a second stage FITC conjugated antibody. This will enable epithelial cells to be identified by flow cytometry. If you wish, also add 50ug/ml propidium iodide, stain overnight and then do dual analysis with the FITC Ab (FL1) and DNA (FL2 or 3) to measure DNA ploidy and cell cycle of the positive cells. Best wishes, John.

tmtcyip (tmtcyip@ibm.net)
Timothy Yip wrote: Dear Graeme, We have tried using monoclonal antibodies to cytokeratin (I think is MNF-116 from Dako) to fish out a small no. of epithelial cells from a population of lymphoid cells by anti-mouse antibody conjugated magnetic bead. But, you have to fix the cells (with ethanol or

acetone) before doing it as the mAb does not penetrate to reach its target if not fixed. It will be even nice if you can add on a CD45 mAb together with cytokeratin antibody in two color sorting in flow. That gives a better separation between epithelial &lymphoid cells.

KI-67 MIT DEM FLOW

Kathy Altig (kaltig@swmedctr.com)
We are planning to perform Ki-67 on paraffin preserved tissue by flow. This will replace the ploidy analysis, as it seems to be out of favor. I am trying to find the best or most practical method and antibody. Suggestions? Thank you, SW Washington Med. Ctr.

Karen Semiao (karen.semiao@dakousa.com)
Dear Kathy, You may want to try DAKO Ki-67 (Code No. F0053) FITC conjugated antibody for paraffin-embedded samples. The antibody is directed against a nuclear antigen and requires permeabilization first (DAKO IntraStain). Our package insert describes the procedure in detail. Please feel free to call me with any questions or visit our website at www.dakousa.com Respectfully, Karen Semiao Product Manager Flow Cytometry DAKO Corporation

drs. ing. M.P.G. Leers (mathie.leers@wxs.nl)
Dear Kathy, When you use the Ki-S5 antibody from Boehringer Mannheim, then you can quantify the Ki67-positive cells in cell suspensions prepared from paraffin embedded tissues after trypsin digestion step. Ki-S5 is an antibody also directed against a formalin resistant epitope of Ki67.

STABILE ZELLINIE, DIE CD32 UND CD16 EXPRIMIERT

Jeffrey Stinson (jeffreystinson@mindspring.com)
Greetings to all. I am new to this listing and flow cytometry. I am a researcher at a small start-up biotech company, Sunol Molecular Corp. in Miramar, Florida. I want to do some experimentas to investigate the interaction of a humanized antibody(IgG1 and 3 / Kappa)w with human Fc Gamma receptors on the cell surface. I was wondering if anyone out there could recommend a stable cell line expressing high levels of human CD16 and/or CD32 on the surface. I tried search in the ATCC on line but without success. I'd really appreciate any help or advise, thanks.

Rafael Nunez (rafaeln@vetvir.unizh.ch)
Dear Jeffrey, just try U937, is very handy and has a lot of such receptors, cheers Rafael > Greetings to all.

Mike Clark (mrc7@cam.ac.uk)
I've just spotted this in the list and I think that U937 may not be suitable for your purposes. One problem with U937 is that like many human cell lines it expresses multiple FcR at the same time and so it is difficult to get clean results. Certainly U937 does express CD32 and it can be induced to express CD16 with gamma interferon treatment. However it also expresses the high affinity CD64 receptor. I have faced this problem myself several times when trying to characterise recombinant and also monoclonal antibodies such as our CAMPATH-1H (CD52) antibody. In the end I have resorted to using functional assays such as ADCC or phagocytosis with several different human donors as well as comparing these results with those obtained for binding to transfectants expressing single human FcR at a time. Using these approaches it has also been possible to identify differences with regard to allelic differences in FcR and Ig allotype.

H.-G. Kreysch (kreysch@merck.de)
Jeffrey, you may use U937 cells from ATCC as FcR positive cell line. The variant we use in our lab originally was obtained from ATCC

and was found to be contaminated with another line some years later(!?). After FACS sorting we got the cell line (U937-KR) which is CD64+ and CD32+ but CD16-.

WAS TUN, WENN DIE HELPERZELLEN CD4- NEGATIV WERDEN? KANN MAN SIE DURCH CD3+/CD8- DEFINIEREN?

Kathleen Schell (kschell@facstaff.wisc.edu)

Dear Flowers, We have an investigator who is studying cytokine production in response to mitogenic stimulus. We observe a down regulation of the CD4 molecule (in surface staining) by 24 hours that renders this population unidentifiable. They are looking at intracellular cytokines, and surface molecules are stained first, cells are fixed, permeabilzed, and then stained for cytokines. Has anyone seen this? We have repeatedly seen a decrease in the fluorescence of CD3 after PHA stimulation, but never to the point where the population can no longer be seen--this has been reported in the literature. Is there another way to identify the CD4 population using this kind of staining protocol? Cells not stimulated have good surface expression of CD4. Thanks in advance.

Calman Prussin (CPRUSSIN@atlas.niaid.nih.gov)

When you note "mitogenic stimulus" do you mean PMA/ionomycin? PMA down regulates surface CD4 expression at concentrations from 1 to 20 ng/ml and as early as 4 hours. By 24 hours there is little if any CD4 to be detected. There are several "tricks" to this problem, none of which is completely satisfying. 1. Stain intracellular CD4. My experience is that after PMA activation, intracellular CD4 stains far better than extracellular CD4. My guess is that CD4 is modulated off the cell surface. Try staining both CD4 and cytokines simultaneously. 2. 4-6 hours activation. Why are you activating for 24 h? This is not an ELISA. Cytokine generally peaks at 6-18h. The CD4 staining will get worse as time goes on. 3. Try staining for CD3+, CD8- cells. Yeah, I don't like it much either, but it works and has been used in a number of papers. 4. Titer your CD4 mAb. In my experience, CD4 staining is harder than the cytokine staining! Some CD4 clones work better than others in these situations. Does PHA give anyone much signal in regards to intracellular cytokines?

Mario Roederer (roederer@stanford.edu)

Kathy, CD4 is downregulated in a matter of minutes through nearly all stimulations (PKC-dependent phosphorylation of a cytoplasmic amino acid signals the molecule to be endocytosed). We have overcome this problem by simply pre-labelling cells with antibodies to CD4. When the CD4 molecule is internalized, so is the antibody and its fluorochrome. The major problem is the acidification and proteolysis that occurs during endocytosis, which kills many fluorochromes (FITC, PE, Cy5PE, APC, PerCP...). HOWEVER, if you include monensin from the beginning (monensin also inhibits Golgi export, and is a very cheap alternative to Brefeldin A), then the acidification and proteolysis does not occur--thereby rendering CD4 cells fully fluorescent! Note we use this protocol for 6 hour stimulations, not 24. I don't know if it will work for 24 hours... However, the alternative is to include the anti-CD4 antibody with the intracellular stains--after all, the CD4 is internalized, and should be labeled by IC staining. This presumes that it hasn't been fully degraded (and not resynthesized) during the 24 hour period, but it's definitely worth a try. Note that you MUST re-titre your anti-CD4 antibody if you want to use it for intracellular staining--the titre will be different than that for surface staining. Finally, we haven't yet published this information, but I've been presenting it at meetings for a couple of years. We are putting two papers out in the next few months that will have it; in the meantime, you can just reference personal communication if you wish. Mr

Keith Bahjat (kbahjat@ufl.edu)
Kathy, Dr. O'Gorman in Chicago has a nice technique for accomplishing this. He stains with CD3 and CD8, then gates the CD3+, CD8- events and assumes these to be the cells which would express CD4 (has they not been overstimulated). I believe he described this in a paper concerning a CD40L assay he developed. Keith Bahjat Graduate Assistant University of Florida

Maryalice Stetler-Stevenson (stetler@box-s.nih.gov)
I can't help but interject one caution to generally applying this. Gamma delta T-cells can be CD4-CD8- and can be a significant population in Asian patients and setting of mycobacterial infection. In addition, there are disease processes where there are high numbers of alpha beta T-cells that are CD4-CD8-- eg ALPS. Sooooo- this is a useful approach to detect recently CD4 positive T-cells in specific situations but is not generally applicable.

"Don't use CD3+CD8- (to define CD3+CD4+)"

Mario Roederer [mailto: roederer@stanford.edu]
Don't Use CD3+CD8-!!!
Having the advantage of 10-color FACS to explore all of the different T cell subsets simultaneously, I can assert definitively that this method can lead to significant artefact. CD3+CD8- T cells are comprised of at least two distinct subsets: CD4+ and CD4- T cells. In healthy adults, the ratio of these two subsets is around 6:1. In HIV-infected adults, especially advanced stages, this ratio can be much lower, for example 1:1. Therefore, the CD3+CD8- gate can be substantially contaminated with "double-negative" (DN) T cells. This is a significant problem, because DN T cells have a very different functional profile from CD4 T cells (and somewhat different from CD8 T cells). A large fraction of these cells make g-IFN, few make IL2, and none make IL4. Therefore, a CD3+CD8- gate, taken to be CD4 T cells, will significant OVER-estimate the gIFN production by CD4 T cells, and somewhat underestimate the IL2 or IL4 production. (Sorry, we haven't done other cytokines as yet). Again, in healthy adults, this is not such a major problem, although it can change the apparent CD4 gIFN production by 25-50% too high. In adults with low CD4 counts, however, the gIFN production by CD3+CD8- T cells is due principally to DN T cells, NOT to CD4 T cells. (Note that DN T cells are not the only contaminants to the CD3+CD8- subset. Depending on the CD8 antibody being used, you may also end up including the CD8-dull T cells. These cells are not that frequent in healthy adults, but, in many situations such as BMT, chemotherapy, and HIV (among others) this population can become quite prevalent. These cells also have a very high proportion of gIFN producers, and very low IL2 and nonexistent IL4 production.)
mr

Maurice R.G. O'Gorman (mogorman@nwu.edu)
Hello Kathy I am guilty of originally responding directly to you. After reading the latest round of "emails" i feel compelled to chime in on the list. As i had indicated in my original response to you, the CD3+CD8- gating strategy includes the activated CD4+ cells as well as CD3+ double negative cells. alternatives to selectively gate on the CD4 positive T cells, including mario's suggestions sound good but are a little tricky and will have to be validated for each specific system. the CD3+CD8- gating strategy will obviously not work in every possible situation (especially if you are studying in vitro activation of CD4 T cells in ALPS patients), but it certainly provides a very quick, easy and practical solution to capture the recently positive CD4 T cells (and the CD3+4/8 double negatives). i think everyone would agree that this gate does contain the CD4+ T cells. at the very least the ne-

gative gating method provides a very easy beginning to see if the substance you are looking for is in the cells in this gate, which in most instances (other than those alluded to earlier) will be predominantly CD4 T cells. if you get what you expect you will have to prove that the result is not do to contaminating 4/8 double negative T cells. if you don't get anything you have not wasted any time developing alternative CD4 gating strategies. i would be curious to know if CD4 binding potentiates/inhibits signalling, has this been investigated? or is it assumed that prelabelling with CD4 prior to stimulation has no effect on signalling? alternatively, if you choose cytoplasmic CD4 staining is the signal bright enough to cleanly separate the CD4+ from the CD4- T cells? either one of these methods may work, but i am not convinced that they have been validated to the extent that would preclude at least trying what is a much easier but admittedly not perfect solution. personally i would try the easiest method first and would not discount any suggestions. good luck

REAGIEREN T-ZELLEN NACH ANTI-CD4 BINDUNG ANDERS?


I would be curious to know if CD4 binding potentiates/inhibits signalling, has this been investigated? or is it assumed that prelabelling with CD4 prior to stimulation has no effect on signalling?


Mario Roederer (roederer@stanford.edu)

We actually looked into this rather carefully a while back, when we studied calcium mobilization (Roederer et al., Cytometry 21, 187-96; 1995). We found that staining with CD4 did not affect calcium mobilization at all. (Note that staining is done at room temperature). This does not mean that CD4 ligation does not induce any signal under these conditions, but in the absence of an affect on Ca mobilization, one of the primary T cell signals, I would hazard that it probably does not. Note that the antibody concentration used in these experiments is relatively low. (i.e., to induce Ca-mobilization, indeed to induce any T cell signal in a short assay period using anti-CD3, you have to use concentrations of 5-10 ug/ml of antibody; whereas if you measure proliferation, a multi-day assay, then concentrations several orders of magnitude lower are sufficient). BTW*(=by the way)*, all of this is moot if you are using PMA/Ionomycin, which basically triggers every signalling pathway that CD4 ligation could possibly hit anyway. Certainly the effect of anti-CD4 antibodies on the ICC staining should be considerably less than the effect of anti-CD3 that is used in the same stain, since CD3 ligation is a primary signal to the T cells--a much more powerful signal than CD4 and probably would mask the effect of CD4 if there were one. By the way, has anyone demonstrated that the CD3 staining is itself not affecting signalling (i.e., "validated" the CD3 stain)? >alternatively, if you choose cytoplasmic CD4 staining is the signal bright >enough to cleanly separate the CD4+ from the CD4- T cells? either one of >these methods may work, but i am not convinced that they have been >validated to the extent that would preclude at least trying what is a much >easier but admittedly not perfect solution. personally i would try the >easiest method first and would not discount any suggestions. The cytoplasmic CD4 stain does work as well as surface staining--and in fact is just as easy--you just mix the anti-CD4 antibody with the cytokine antibodies rather than prestaining. (You must use an antibody to an epitope that is not altered by the fixation used in prepping the cells--Picker has published this method and demonstrated that it works very well). Thus, I don't find the intracellular CD4 staining to be any more difficult than CD3+CD8-. (Our method, of pre-staining, does add some complexity, and I agree with you that if you are just looking around for an initial result, there's no need to use it. However, if you are performing this assay as part of a study where you are looking at a large number of

samples, then you might as well do it so as to avoid potential artefact, or at least to avoid having to go back to your positive samples to verify that the positivity is not due to contamination by double-negative T cells). Mr

WAS SIND PROPIDIUMIODID-POSITIVE ABER ANNEXIN-NEGATIVE ZELLEN?

S. Radioisotops Bellv. (SERV-RADBELL@bell.ub.es)
We have been analyzing apoptosis cell death in a human colon adenocarcinoma cell line, HT29. We have obtained very nice and clear results, but we usually detect a 3 to 5% annexin (-)/ PI (+) population. We can observe the same result with other cell lines and different stimuli, and I have been asked to explain what is happening there. Many articles don't make any difference between PI (+) cells, beeing annexin (+) or (-). Could someone suggest how to describe these cells ?

"Stray ye not too far from a fluorescence microscope"

ian titley (iant@icr.ac.uk)
I believe that HT29 is an adherent cell line, in a paper (van Engeland et al Cytometry 24:131-139 1996) a PI+ annexin- population is described and is attributed to scraping during harvesting and therefore the cells suffer mechanical damage. It appears that this population is only seen if cells are labelled with annexin before harvesting. If cells are labelled after harvesting then they appear as annexin+ PI+ and may be considered secondary necrotic false positive results. Trypsinisation and EDTA also appear to permiabilise cells to annexin. Ian Titley PhD Leukaemia Research Fund Centre at the Institute of Cancer Research 237 Fulham Road LONDON SW3 6JB UK Tel: +44 171 352 8133 ext5134 Fax: +44 171 352 3299 E-mail: iant@icr.ac.uk

Derek Davies (daviesd2@icrf.icnet.uk)
Hello Mireia, I don't think that you are the only one to have come across this problem which does seem to get largely ignored. I suspect that most people are interested in the early apoptotic population (Annexin+/PI-) and just lump together all the PI+ cells together into a "late apoptotic/necrotic" population. This is fine I think until you are called upon to define the Annexin-/PI+ population. At this stage you should bear in mind one of the Flow Commandments: "Stray ye not too far from a fluorescence microscope". You could look at your cell prep (or even sort the relevant populations onto a slide) and see what is what. Hazarding a guess, these cells could be very late dead cells that have lost a large part of their membranes.

Michael Ormerod (Michael_Ormerod@compuserve.com)
In my experience, PI +/ annexin - cells have probably died by necrosis. I have a data file (to be included in the second edition of my CD-ROM, when I finally complete it) of a PI/annexin stain of cultured human leucocytes. The granulocytes are dying by apoptosis and are PI positive/annexin negative, a population of lymphocytes are PI positve and annexin negativ. I have seen other examples. Although the dogma says that annexin will enter a necrotic cell and bind to PS on the internal face of the plasma membrane, I have not seen anyexperimental evidence (perhaps because, as yet, I have not looked very hard). The little evidence I have suggestes that, in some cells at least, this does not happen.

Eric Van Buren (aa9080@wayne.edu)
Mireia We have also seen free nuclei in the PI-positive/Annexin V-negative quadrant. Eric

Tom Frey (Tom_Frey@BDIS.Com)
Derek/Mireia/MIchael/Ian: Let me add my two cents to this thread. While I agree that there seem to be some broken cells that don't stain as brightly as you would expect with an-

nexin, I might suggest a second possiblity. Three background facts/assumptions: First, some models show a PI (or EB or 7-AAD) intermediate apoptotic population. Second, this could be due to a mechanism similar to that which causes apoptotic cells to be intermediate for fluorescein diacetate (as originally reported by M Ormerod I think). Third, I have reported (Cytometry 28:253) that in models I look at the surface staining with annexin follows the change in FDA staining. Given the above it would seem possible that a PI-intermediate, annexin-negative population could develop before the double positives. A time course or a careful look at the PI intensity might give you a hint about whether this is the case.

WERDEN UNSERE FLUORESZENZMARKER DURCH RAUMLICHT GESCHWÄCHT?

Keith Bahjat (kbahjat@ufl.edu)
This message brings up a point that I'd like to see addressed by the experts. In my hands, FITC (as well as PE, PerCP, APC, PI, 7-AAD, and ToPro-3) is very stable, and no precautions need to be taken while staining or running samples, as no degradation has ever been seen. Every clinical and research lab I've been in stains samples on the bench top and takes no special precautions to avoid any forms of light. But some researchers here do things like staining in the dark, encasing samples in aluminum foil, and using red filters on the room lights in the flow lab for fear of fluorochrome degradation. Based on my experience, I'm not sure why they fear the light to this extent. I've seen this happen using the fluorescent scope, but I think those are usually fluors like Cy2 and Cy3. Are some derivations of fluorescein more photolabile than others, or am I ruining my samples. thanks for any news and views.

Joseph Webster (J.Webster@centenary.usyd.edu.AU)
I am not claiming expert status, but I do have some observations from the operator's vantage point... We often see PerCP fade significantly over an hour or two in room light, the change was enough to ruin an experiment. I have seen tandem conjugates separate into mixtures of tandem plus separate fluorochromes. This separation was variable from tube to tube, and made compensation unworkable! I can't comment on the behaviour of PI in this context, though I don't remember such effects when I was reading lots of DNA samples years ago. I've not seen significant fading of FITC or PE, but I haven't looked very hard; I've always presumed they are less prone to fading. There are many variables in this can of worms, most of which I have no idea of. Most people here take the relatively easy precautions like keeping things covered (most of the time) and on ice unless room temperature (or higher) is specified. Joseph.

Richard Meister (meister.1@osu.edu)
I don't claim to be an expert on photobleaching, nor have I done any controlled studies on the topic, but here's my impression. Most of us who have taken photomicrographs of immunohistochem slides stained with FITC have probably experienced photobleaching of the green fluorescence; i.e., by the time you have focused and determined what field you want to shoot, the green fluorescence has diminished. In fact, mounting compounds are marketed to counteract photobleaching. However, I do all of my staining for flow work on the benchtop in a normally lit lab, with no apparent photobleaching. We do, however wrap our stained samples in Al foil if they are to be sitting around exposed to light (in a cold room, for instance) for any appreciable length of time. And we run the samples in a flow lab with dimmed lights. This is probably not necessary, but reduces eye strain from reflected glare off of the computer screen and makes my "customers" feel their samples are safe. I think the reason we see photobleaching in immunohistochem slides, but not so much in flow samples, has to do with the relative intensity of the illuminating light, and perhaps the wave length and cumu-

lative time of exposure. Compared to normal room light, the mercury arc lamp of an epifluorescence scope is really blasting away at the FITC molecules.

Steve G. Hilliard (steve@habanero.cb.uga.edu)
Keith, I always warn my clients about exposure to light, but I did directly test some FITC CD4/8 staining and saw no loss of signal. We ran the samples, put them in a brightly lit window for about 2 hrs, and ran them again, and there was no discernable effect. Funny, but I still caution them about exposure with PI. Just one data point....

Julie Auger (jauger@flowcity.bsd.uchicago.edu) Well, the overwhelming concensus to my query is that the photosensitivity of PerCP makes it difficult to work with on the sorters with high power lasers. I appreciate all the quick responses! I have received a number of great suggestions and will give it a go again. Thanks to all of you. Julie

EUROPEAN INTERNATIONAL CONFERENCE FUTURE TRENDS IN QUANTITATIVE CYTOLOGY FOR CLINICAL AND RESEARCH APPLICATIONS May 13-16, 1999, Hortobágy-Epona, Hungary

Janos Szollosi (szollo@jaguar.dote.hu)
Conference announcement: ISAC-SUPPORTED EUROPEAN INTERNATIONAL CONFERENCE FUTURE TRENDS IN QUANTITATIVE CYTOLOGY FOR CLINICAL AND RESEARCH APPLICATIONS May 13-16, 1999, Hortobágy-Epona, Hungary It will be the first International meeting of this type organised in Eastern Europe. The majority of participants are expected to be from Eastern and Central Europe, around 150 individuals. The conference will consist of short concise lectures covering three major topics: (I) IMAGE ANALYSIS AT MOLECULAR LEVEL (II) BIOTECHNOLOGY AT THE CELLULAR LEVEL (III) ADVANCES IN QUANTITATIVE FLOW CYTOMETRY The lecturers are chosen carefully to give a broad, yet contemporary, overview of analytical cytology on the eve of the 21st century. The focus of the presentations will be on new developments and how to meet the challenges and limitations of the rapidly evolving biotechnology. In addition to the plenary speakers, short oral presentations will be selected from submitted poster abstracts. Information about the conference can be obtained through E-mail: isac99@jaguar.dote.hu or from website: www.isac99.dote.hu Deadline for registration and sending abstract is: March 01. 1999. Contact person: Janos Szollosi Department of Biophysics and Cell Biology University Medical School of Debrecen, 4012 Debrecen, Nagyerdei krt. 98, HUNGARY Phone/Fax: (36) (52) 412-623, E-mail: szollo@jaguar.dote.hu

CD4+/CD57+ T-LYMPHOZYTEN

Maryalice Stetler-Stevenson (stetler@box-s.nih.gov)
Here's a weird one: 43 yo white male. 30.4%HCT, 625 K/UL plateets, WBC- 4.27 K/UL with 44.3% polys, 12% lymphocytes, 26.5% monocytes, 14.8% eosinophils and 2.4% basophils. 96% of lymphocytes are T-cells. 92% of Lymphocytes are CD4+,CD3+ and 4 % CD8+,CD3+. This is the cute part- 45% are CD4+, CD57+, CD3+ T-cells. They are mostly CD7+ (15% CD7-,CD3+), all CD2+ and CD5+ (a slightly dim population noted). They are TCR alpha beta +, CD16-, CD38+, and HLADR- as well as negative for B-cell antigens. How often have you seen CD4+CD57+ T-cells?

woodbl (woodbl@u.washington.edu)
Hi Mary, Yes, we have seen populations with

this immunophenotype (CD4+, CD57+ T-cells) occasionally in reactive conditions. They reportedly can be expanded in patients with rheumatoid arthritis, some hematologic malignancies (including CLL and hairy cell leukemia), and following solid organ transplantation. They have a cytotoxic activity. We recently have seen one case of presumed LGL-leukemia with this immuunophenotype, as has been reported in the literature.

Sean P. McDonough (spm13@cornell.edu)
For those who are interested, a very detailed analysis of NK associated immunophenotypes can be found in Leukemia and Lymphoma Vol 2:111-126, 1990 by Stephen J Richards and Colin Stephen Scott.

FALL MIT REKURRIERENDEN INFEKTEN

Brent Dorsett (brentd@nyct.net)
We have a 31 year old female who presented with recurrent, serious bacterial infections. She turns out to be pan hypoglobulinemic with IgG 51; IgA 8.6; IgM 7. WBC is 15,000 with 15% lymphs. In flow she had CD3 36%; CD4 24%; CD8 12%; CD5 40%; CD10 0%; CD19 57%; CD20 61%; CD22 58%; FMC7 23%; there was expression of CD23 and CD25 on about half of the B cells (CD20). There was some very weak expression of kappa and lambda on a few of these cells. Right now that's all I know. >From those of you with experience with immunodeficiency of the non-acquired type, is this consistent with Combined Variable Immunodeficiency. In general, since I have seen so few of these types of cases, I would be interested in any comments. Thanks,
Please excuse my bad habit of pressing the send button before I proofread. Actually she was 71 years old and I meant to say Common Variable not Combined Variable. Thanks again, Brent

Anna Porwit-MacDonald (Anja.Porwit@mb.ks.se)
Hi, CD25 is not normally expressed on B cells in PB. Have you checked CD103 and CD 11c? Even if there is no lymphocytosis the B/T cell ratio in the lymphocyte region is not normal and you see a B cell population that has no clear Ig pattern. How was lymphocyte morphology? (I'm thinking of hairy cell or NHL with villous lymphocytes that can have only a small population in PB!).

DETEKTION VON PARVOVIREN

John Parker (jsp7@cornell.edu)
Hi Flowers, I am a relative neophyte to flow and I am having some problems developing an assay to detect parvovirus (canine parvovirus infected cells). I have a purified mouse mab against the capsid protein which I have directly conjugated to Cy2. When I use this ab to stain infected cells on slides, I see a very clean separation of infected from non-infected cells, almost all the infected cell staining is in the nucleus and non-infected cells have no or negligible background. I am using mv1Lu cells, a mink lung cell line. However, when I trypsinize the cells from dishes and fix with 3% paraformaldehyde, then permeabilize with 0.1% triton in PBSA , stain and do flow. What I see is one broad peak which is shifted to the right on FL1 when compared to a narrow peak of mock infected cells treated the same way and stained with the same antibody. My interpretation of this is that I am not fixing the antigen in the cells and that it subsequently leaks and coats the surface of non-infected cells, hence the broad peak shifted to the right. My questions are: Is my interpretation reasonable, any other ideas? Any ideas on how I can solve this problem or pin-point what is happening? Thanks in advance for your help John

Mark A. KuKuruga (kukuru@umich.edu)
John John,First, a question . . . how does your PFA-Triton treatment compare with the protocol you use when looking at slides? Since you get good differential labeling in the latter,

I would take clues from that protocol to develop your flow technique. This doesn't always work, however . . . you might have to dramatically alter your fix/perm technique to get this to work in flow. Now, assuming you've developed a protocol for revealing the antigen that 1) retains the antigen, and 2) preserves its antigenicity . . . then perhaps your problem lies in the need to clear non-targeting excess antibody. This may simply require additional washing, or may require that you supplement your wash buffer with a little Triton to assist in the clearing. Beyond that, you could look into 1) eliminating background due to excessive PFA (causes increased autofluorescence), or 2) look into enhancing the signal by modifying the emission filter. Keep in mind . . . a fluorescence scope will rarely have filter sets comparable to those in a flow, unless you specifically set it up that way. So, your flow cytometer may not be selecting the proper emission wavelength, thus eliminating some of the specific signal. MAK.

plett@auhs.edu
Hi John This is a guess. Have you looked at a FL1 vs. FL2 plot? It could be that that procedure creates a high autofluorescence, or non-specific fluorescence, or your cells just have a high autofluorescence. The autofluorescent 'banana' on the FL1/FL2 plot could then make your two populations become one broad peak when looking at just one parameter. the fact that your infected cells are a broad peak may mean you really have two populations under that peak. Also, put some of those permeabilized cells fro flow on a slide and see what you see.

"Was spricht gegen eine CLL?"

B-ALL MIT CD5-EXPRESSION?

Black, Emily (EBlack@providence.org)
I'm looking to see if anyone is familiar with either the frequency of a T-cell marker CD 5 on a B-cell ALL or the significance of the cytogenetics result of a translocated 3,22 in this unusual case. The patient has no nodal involvement- no lymphoma, but the blood and marrow are full of young-looking blasts that mark with very mature Bcell markers-negative for CD 10, CD43, TDT and 34, and positive for CD 19,20,21,22,23,24,FMC-7, LAMBDA monoclonal, and CD 5. Is this CD 5 positive an unusual occurance that is worthy of publishing or further study?

Nebe, Thomas C. (thomas.nebe@ikc.ma.uni-heidelberg.de)
What argues against a regular B-CLL beside negativity of CD43? ALl other markers match with a leukemic B-NHL. Prolymphocytic leukemia PLL is a viable alternative if CD5 is dim. Please consult a local hemopathologist for morphology (PLL with nucleoli and more blast morphology) and experience.

Jonni S. Moore (moorej@mail.MED.UPENN.EDU)
Hi Emily, the phenotype you describe seems perfectly consistent with CLL--what is the clinical indication of ALL. The cytogenetic abnormality is not diagnostic of ALL either.. Jonni Jonni S. Moore, Ph.D. Director of Clinical and Research Flow Cytometry University of Pennsylvania School of Medicine 203 John Morgan Bldg. Philadelphia, PA 19104-6082 Phone: 215-898-6853

darber@smtplink.Coh.ORG
In our experience, we found CD5 expressed on only one of 52 precursor B and none of our B-ALLs. Could this be a case of blastic mantle cell lymphoma? Dan Arber

Glenda, M, Davison (gmd@samiot.uct.ac.za)
Dear Flowers, I must agree with Dan Arbor. In my experience the immunophenotyping is typical of Mantle cell lymphoma. We have however experienced a B-lineage ALL with

expression of CD5. This particular case had a typical common ALL phenotype with both CD19 and CD10 being positive. No other T-cell antigens were expressed. So although unusual, it is possible. Please let us know the outcome of this case. I would be very interested to hear your final decision. Happy New year to everyone, Glenda Davison Haematology Dept. Groote Schuur Hospital. Cape Town South Africa

Abby Kelliher (allena@helix.mgh.harvard.edu) Could this possibly be a CLL (Chronic Lymphocytic Leukemia)?

CD23+/CD8+ T-NHL?

Hier war die Frage nicht so interessant, die Antwort enthält aber nützliche Informationen:

Nebe, Thomas C. (thomas.nebe@ikc.ma.uni-heidelberg.de)

Dear Collegue Illingworth, I haven´t seen a CD23+CD8+ T-NHL so far, neither in our cases nor in NHL classification schemes, talks or literature. The subset analysis is not typical for a leukemic lymphoma. CD4/8 is like kappa lambda for polyclonality. Obviously the clinical information is insufficient and completely against CLL. The request for CLL is strange in the light of the current blood picture. May be some days ago there was a significant reactive CD8 lymphocytosis driven by a viral infection that now suppresses myelopoiesis in the bone marrow. It would have been desirable to prove this by CD57 and CD38 on CD8 or by incresed HLA-DR on monocytes (and morphology). As only 15% of lymphs are involved it will be hard to identify these in the blood smear but I recommend you should always try to. First of all you may want to contact the physician who is in charge of that patient to obtain further clinical information (fever, lymph nodes, time course etc.). I found these contacts were mostly positive and resulted in a positive feed back to the flow lab. Strange coexpressions occur and might be either artificial (eg. like anti-mouse Igs as discussed in the past here on the server or in cytometry) or related to autoimmmune, atopic (FceRI on monocytes etc.) or infectious diseases (eg. CD4 epitope on CD8 cells in HIV). Regards Thomas Nebe Universitätsklinikum Mannheim D-68135 Mannheim, GERMANY thomas.nebe@ikc.ma.uni-heidelberg.de

WAS IST BEI DER ANALYSE VON THROMBOS ZU BEACHTEN ?

Douglas S. Smoot (dsmoot@med.navy.mil)

Today I was asked about running platelets on my flow cytometer. I've never run them, so are there any special tricks that I should be aware of? I have a Coulter Elite, if that makes a difference. Thank you.

biocytex@biocytex.com

Hi Doug, Do not hesitate a minute. It's fun to play with platelets! We even do surface glycoproteins quantitation on them. Easy but : - prefer PRP for your 1st time, then go to whole blood (quicker); - prefer citrated blood and store at room temperature rather than in the cold ; - prefer no-wash IF protocols (we use standardized whole-blood, no-wash indirect IF protocols every day) - prefer log FS x log SS rather than linear for dual scatter plot (easier to find platelet cloud); - be sure to clean your sample tubing after other type of samples (Clenz or equivalent); - have a 0.22µm sheath filter on-line; - .... so many tricks .... Contact us if you need more infos. Don't worry , be happy ! Philippe Poncelet BioCytex

Derek Schulze, CRL (flow@post.queensu.ca)

We use a Coulter Elite for platlet activation studies on a regular basis without much difficulty. We use a pan-platelet marker to help pull them out of debris, but I have gated on light scatter with them in the past. You shouldn't have much difficulty (famous last words)

CD95 AUF LYMPHOZYTEN BEI JUGENDLICHEN

Antony Bakke (bakkea@ohsu.edu)
A clinician here is trying to diagnose an interesting patient. A boy originally diagnosed with lymphoma at 10 due to large lymph nodes and treated. He was later diagnosed with Kikuchi syndrome (necrotizing lymphadenitis) and had arthralgias and autoantibodies. His identical twin is healthy but with similar serology. At first we thought this might be an ALPS (autoimmune lymphoproliferative syndrome) patient. However, CD95 staining was positive with two different Mabs, but on only 15% of the lymphocytes. This is low compared to adults and a few newborns that I have tested. Does anyone have data on expected CD95 expression in teenagers? Do ALPS patients have variable expression? The reports I have seen indicate very low or no CD95 expression. Are there other syndromes that might fit? Thank you for any ideas, refs or data. Tony Bakke Tony Bakke, PhD Director, Clinical Immunology and Flow Cytometry Lab Dept of Pathology Oregon Health Sciences University Portland, OR

"Es macht Spaß mit Thrombos zu spielen"

biocytex@biocytex.com
Dear All, I remember seeing such data in (at least) the following papers : - Miyawaki,T et al., J. Immunol., 149, 3753 (1992) - McCloskey,TW et al. , Clin. Immunol. Immunopathol. , 87, 33 (1998). In this paper the median percentage of Fas+ CD4 lymphocytes was reported as 12%, which matches the 15% indicated by Tony Bakke. In our own experience, although we analyzed many healthy adult samples for quantitation of Fas expression on both Fas positive lymphocyte subsets (we call them Fas low and Fas high), we only tested two teen-ager samples in our series. The results were similar to adult values in terms of Fas expression levels and the percentages were : 70% / 30% Fas low / Fas high for an 8 y. o. boy, 35% / 65% Fas low / Fas high for an 11 y.o. boy , a result which looks more like those on adults >30 years old. Our cut-off between Fas low and Fas high is defined at 3,000 Fas antibody molecules bound per CD3+ (or CD4+) cell. We would be very interested in working a collaborative clinical study on this concept of Fas low and Fas high quantitative measurements to assess the "immune age" in a given population. Hope that helps. Season greetings. Philippe Poncelet, PhD Director, R&D

Tom Mc Closkey (thomasm@nshs.edu) Based on our work and other published data, 15% positive is reasonable for that age. J Immunol, 149: 3753-3758, 1992 Cell Immunol, 163: 303-308, 1995 Clin Immunol Immunupathol, 84: 46-55, 1997 Clin Immunol Immunopathol, 87: 33-41, 1998

IST MEIN FACSSCAN IN ORDNUNG? SOLL MAN IHN SELBER JUSTIEREN?

Jose A. Stoute (stoutej@net2000ke.com)
Dear all, I would like to get some advice on whether my FACscan needs further fine tuning. I recently called the BD engineer to come and recheck the alignment and sensitivity of our machine. He says there is no problem with it but I am not satisfied and would like to get a second opinion. Our main application is flowcytometry of red blood cells and I see two problems: 1. When we acquire using Log amplification for FSC and SSC we see a lot of background and have to increase the threshold on FSC to about 300. I understand that some background using these settings is normal to some extent but there was a time when my machine had no background at all using the same settings. This background is not due to impurities in our buffers because it is present even after filtering through 0.22 um filters. Also, it became worse after the engineer adjusted the machine. 2. The other problem is that the PMT gain that I have to use to have unstained cells in the first decade of FL1 seems

to be too high, around 700. We found the same when we used the FACscalibur across the hallway. The engineer said that he had chekked everything. I would appreciate any suggestions as to how to deal with the above problems.

Ann Atzberger (Ann.Atzberger@EMBL-Heidelberg.de)
Hallo Jose, some tips that might help 1. Try aligning it yourself: if you look at the optical configuration for the FSC;there's a lens mounted on a square holder, the top part of the holder is round with a hole in it. If you put a screwdriver (or anything that will fit) in the hole you can adjust it by moving it carefully, at the same time watch the FSC,SSC and FL signals. You need to run a sample and deactivate the laser shutter. Don't try unscrewing anything though, it can be difficult getting things back the way they were. If your machine still has a warrantee, don't do this! 2. Your flow cuvette needs cleaning, the company has a special cleaner for this. If the machine has been in use for several years it should be done. Makes a huge difference. 3.Most operators are inclined to put the threshold on SSC when working with LOG amplification. 4.Unstained red cells may well have such a high PMT setting. Being non-nucleated they might have less autofluorescence. I'm assuming we are talking about peripheral blood cells, non-chicken, non-fish, non-fixed. 5. Is your laser working properly. How old is it? good luck Ann

Peter Lopez (Peterl@Cytomation.com)
Hello Jose, I have to disagree with the suggestion of aligning the FACScan yourself. Moving the steering plate will change lots more than light scatter noise- it will also have an effect on fluorescence channels. I would suggest that you ask your service engineer to align the light scatter channels using log amplification, and minimize the noise that way. I believe light scatter is typically aligned by the service engineer using linear amplification. The only adjustment needed would be to the forward scatter obscuration bar. The fact that you're seeing similar PMT voltage requirements for your unstained material on the FACScalibur means that it is probably true- the intensity is low.

"Schrauben sie aber nichts auf"

SIND PI-SCHWACHE (SUB-G1) ZELLEN APOPTOTISCH?

Keith Bahjat (kbahjat@ufl.edu)
In 1995, a paper was published in J Imm Methods (Douglas, R.S. et al:188(1995) 219-228) where the authors stained cultured cells undergoing apoptosis with PI, then divided the sub G1 area into regions and sorted these events. Once sorted, these events (maybe cells, maybe not) had DNA extracted and run on an agarose gel which was then stained with Ethidium bromide, to demonstrate the laddering effect which had been demonstrated many times in apoptotic populations. Their finding was that the largest sub G1 population, which conaitned a majority of the sub G1 events, had NO DNA! Though this was not the main thrust of their paper (they were trying to provide a reproducible method for surface staining with DNA labeling), it is certainly relevant that many labs are measuring increases in debris (which apparently stains somewhat with PI) rather than fragmented nuclei. This is particularly disturbing considering that today, three and a half years after publication of this paper, I still read MANY publications that simply draw a region encompassing all sub G1 events and call these apoptotic cells. Most of this is debris, and last time I checked, no one had published that increased debris correlated with increased cell death (though it may!). It seems that this may be valid if you have a strong software modeling program to eliminate the debris (or use the technique in the paper of FL2-A vs. FL2-W, though my plots never look as clean as the ones they produced), but most papers just pull up a histogram in WinMDI and draw markers for Go/G1, S, G2/M, and sub G0/G1. Please

show me why I am wrong in thinking these methods are invalid, or explain why these papers are accepted for publication. Thanks for anyone and everyone's insight.

Telford, William Ph.D. (TelfordW@hss.edu)
Hello Keith... You are quite correct in your assessment - you should NEVER assume everything below the G1 peak in a DNA histogram is apoptotic without additional evidence. When our lab (Telford, King and Fraker, 191) and others (i.e. Nicoletti et al., 1991) published our early papers on this technique, murine thymocytes were the most common model for immune cell apoptosis. Mouse thymocytes give a very unambiguous apoptotic region at approximately 50% the fluorescent intensity of the G1 region - they also give a very "clean" form of cell death when killed with glucocorticoid or radiation treatment, with minimal debris generation and membrane blebbing. Ethanol fixation and PI staining worked very well in this system. The situation is much more complicated, of course, when dealing with any type of activated or constantly cycling cell, such as a mitogen-activated lymphocyte or a tumor line, or with actual patient samples. There ends up being a wide range of sub-G1 events in most apoptotic cell systems, and it is often difficult to tell what is debris and what is apoptosis. An almost complete absence of DNA is not necessarily an indication of cellular debris - we have identified two distinct apoptotic subpopulations in mouse T cells activated via mitogen, one with a subdiploid population similar to that seen with thymocytes, and one with extreme DNA loss. The second population is not debris - we can sort it, and find apoptotic cells with traditional cytoplasmic morphology but almost complete loss of DNA. We believe that this second type of cell death occurs in the cycling splenocyte fraction and is the result of a form of DNA fragmentation different from that seen in G0/G1 cells (hence the extreme loss of DNA upon ethanol fixation). It turns out that this type of cell death is functionally different from the first type as well - it is Fas-dependent, while the first type is Fas-independent (it can occur in lpr/lpr mice) is is regulated by CD30 (Telford et al., Cellular Immunology 182, 125 . We distinguish both of these types of cell death from debris by looking at PI fluorescence and forward scatter simultaneously - the cell with extreme DNA loss had a scatter measurment similar to the first apoptotic population, despite their dim DNA signal. Debris fell below both apoptotic populations. I guess the take-home message is that sub-G1 DNA content measurement can be a useful assay for apoptosis WHEN USED WITH CAUTION. It is great for some cellular systems (such as thymocytes), but can be of limited value in other systems where debris and apoptotic cells are difficult to distinguish. It is a good first assay to try, since it is easy and cheap. If you see multiple sub-G1 regions, you should probably sort them and check the morphology - this worked for us, and allowed us to distinguish two functionally distict forms of cell death. Use forward scatter along with DNA content to differentiate debris from apoptotic cells. If the sub-G1 pattern is too messy or ambiguous, then you should probably try another assay (annexin, TUNEL, DNA dye exclusion from unfixed cells, Phi-PhiLux, etc.). Regardless of how good the data looks, you should NEVER rely on one cell death assay anyway - always confirm your cell death data with at least one other assay, preferably one that looks at another manifestation of cell death than the one your primary assay detects. Good luck! Bill Telford Fannie E. Ripple Foundation Flow Cytometry Core Facility Hospital for Special Surgery

"Warum werden diese Papers zur Publikation angenommen?"

Donnenberg, Albert (donnenbergad@MSX.UPMC.EDU)
Keith- We still use the sub G1/G0 fraction as a measure of apoptosis. We have used granulocytes (cultured overnight) as an internal positive control (Res Immunol 146:11-21

1995.). They form a nice hypodiploid population. Anything dimmer than this population is debris and probably contains no DNA. Similarly, rbcs stained with PI can give you an idea of the fluorescent intensity of DNAless cells. It is not zero.

Tom Mc Closkey (thomasm@nshs.edu)
I agree that the literature includes many examples of use of this assay which are questionable. I strongly recommend to people that they should use fluorescence microscopy as a major part of establishing this assay. It accomplishes two goals: 1] veirifies that apoptosis is actually taking place 2] allows a count of the % of apoptotic cells as detected by nuclear morpholocgy to match up with the flow results I think it is also a good idea to simultaneously use another method, ie TUNEL to further verify the results. A negative and positive control should be used such as Jurkat cells in the presence/absence of anti Fas. Given careful setup of this assay and a standardized analysis scheme, this method provides an easy and inexpensive way to quantitate apoptosis. However, there are clearly opportunities to obtain inaccurate resul,ts by counting debris instead of appoptotic cells.

"...das ist Nonsense"
"No it is not..."

Michael G. Ormerod (Michael_Ormerod@compuserve.com) PI will stain anything containing DNA including necrotic cells and cell debris with DNA still in it. The 'sub-G1' peak from apoptotic cells may well be contaminated by other things. The statement that one sees occasionally in the literature that the region below G1 in the DNA histogram is the 'apoptotic region' is nonsense. Michael Ormerod

Donnenberg, Albert (donnenbergad@MSX.UPMC.EDU)
No its not quite nonsense, although it does have its limitations. In a well characterized system the hypo-diploid region of a PI profile can be a very valuable and simple way to quantify apoptosis. Things to watch: One apoptotic cell gives rise to many apoptotic bodies. Comparisons between treatment groups are valid, but arriving at "percent apoptotic" is more difficult. Identifying cells of interest with surface markers and running an internal positive control are helpful. We have used granulocytes cultured overnight to define the brightness of hypo-diploid apoptotic cells (i.e. how bright an event has to be in order to be considered an apoptotic cell and not space dust). Apoptotic Grans usually make a nice tight peak to the left of the G1 peak.

Tom Mc Closkey (thomasm@nshs.edu) In our experience, cells which can be morphologically described as necrotic show up in the G0 peak [early] or move far to the left on the DNA content histogram [late] such that they are offscale. They can be excluded by triggering on DNA so very low fluorescence events are excluded and light scatter can also help to eliminate them from the analysis. Certainly, though, everything that is left of G0 is not apaoptotic cells and you need some method to differentitate intact apoptotic cells from debris that fluoresces. We use 25% of the fluorescence of G0 cells as our cutoff. You should see a distinct peak of apoptotic cells with slightly reduced DNA content. The best way to veirify yuoor results is to count the cells under the scope and match up the numbers. Good luck, Tom

WAS SIND DIE CD8 SCHWÄCHEREN, CD3+ ZELLEN

Dalia and Natasha (d.khalil@mailbox.uq.edu.au)
Greetings! Our research group has noticed that in few patient's PBMC, there has been two populations of CD3+ CD8+ cells. One is of a high fluorescence and the other intermediate. We regularly observe the high fluorescent CD3+8+ population, but only a few have a definate second, intermediate fluo-

rescent CD3+8+ population. Has anyone also observed this second population, and do you know what type of cells these are?

biocytex@biocytex.com
Hi Dalia, A lymphocyte histogram always contains 2 populations of CD8+ cells.The CD8 high (bright) are exclusively T cells (CD3+) and the CD8 intermediate include both NK (CD3neg., generally representing the majority) and T cells (CD3+, TCR gamma-delta+). The CD8high express both alpha and beta chains of CD8 as heterodimers and bind high levels of most CD8 MAbs (120-160,000 molecules/ cell, see Bikoué et al. , Cytometry (1996),26:137-147). The CD8 interm. express only one chain as homodimers and bind lesser amounts of CD8 MAbs. This make them appear dimmer. We do see them in normal samples when doing CD8/CD3 dual staining. However the proportion of CD8 inter. T PBL is generally so low in healthy people that they often may not be taken into account.

smonard@adarc.org
We notice CD3+ CD8dimmer cells in samples that are very old, like three days. I suspect its a downregulation of surface receptors during their death throws. You could leave your blood lying around for a few days then stain again and see the difference. Rather a dull explanation I'm afraid. Happy Holidays Simon Monard

Mario Roederer (Roederer@Beadle.stanford.edu)
We have studied these cells extensively, and have even published a paper about them! It appeared in Blood, in November 1997, by K. Watanabe et al. Note that the following discussion applies only to human CD8 T cells; the murine system is a little different. First, CD8-dim CD3+ cells express just as much CD8 as do the CD8-bright cells! The reason they are "dim" is because they express CD8 alpha-alpha homodimers as opposed to CD8 alpha-beta heterodimers normally expressed on the "bright" CD8 T cells. Nearly all antibodies have a lower affinity for the homodimer than for the heterodimer. Since the CD8 antibodies are titred against the "bright" population, they are packaged at concentrations that do not saturate cells with homodimer CD8. Therefore, these cells appear "dim" because binding has not reach equilibrium. Incidentally, this is why some people see the dim cells, and others don't. If you use low concentrations of CD8, (say, at a point where "bright" cells are falling off in fluorescence), then the homodimer form doesn't bind enough antibody to resolve. If you use high concentrations, about 10-fold above saturating, then the "CD8-dull" cells are all of a sudden just as bright as "CD8-bright" cells! Obviously, the ability to detect these cells using CD8 antibodies alone will also depend on the precise CD8 clone being used. The only way to know the form of CD8 expressed is to counterstain with an antibody that reacts specifically to the beta chain (it's available from a couple of mfr's). The CD8+ CD8-beta negative cells are the homodimer cells. By the way, there is no antibody that specifically recognizes the CD8-alpha chain with the same affinity when the chain is expressed as a homodimer or as a heterodimer with CD8-beta. In our paper, we demonstrated that the "dim CD8" T cells can be either CD4+ or CD4-. The CD4- are about 50% gamma-delta T cells; the rest and all of the CD4+ are alpha-beta. These cells often express high levels of CD57 and perforin. The express good amounts of gamma-interferon when stimulated... There is a reasonable amount of evidence that these cells are extrathymically-derived. These cells are often elevated in individuals who have undergone significant immune remodelling, i.e., those treated with chemotherapy, BMT, or who have advanced HIV disease. We have even found antigen-specific responses in this population, but don't yet understand the immunoprotection that the cells may afford to the individual.

smonard@adarc.org
The cells with slightly higher CD3 intensity are probably gamma/delta T-cells, some are CD8 positive and some are not. Simon Monard

SHEATH PRESSURE DES XL

Philip Marder (marder@iquest.net)
I'm trying to determine the operating sheath pressure of our Coulter XL cytometer. I'm unable to find this data in the manuals and their technical help on the telephone was not informative. Anybody out there have a clue on this? Thanks, Phil Marder

Jean-Pierre Godet (godetj@wanadoo.fr)
Hi, Philip! The sheath pressure of Coulter XL is 4 PSI, sample pressures are 3.72 (low), 3.92 (medium) and 4.12 PSI (high).

RAUMTEMPERATUR VERÄNDERT SORTIERERGEBNISSE

Andy Oberyszyn wrote: Hi all you FLOWers! This subject was briefly discussed in the past and I know that all of you who sort can vouch for this, however, does anybody have any "Hard evidence" for room temperature affecting sorting? Our buildings heating/cooling system is not reliable (+/- 15F on any given day) and we've been asked to "justify" having an A/C unit put in. Any info would greatly be appreciated! Thanx in advance!

Michael Ormerod (Michael_Ormerod@compuserve.com)
The viscosity of water is temperature dependent. Any change in viscosity will alter the speed of the jet and hence the distance between the nozzle of the flow chamber and the point at which a droplet is formed (break-off point). For this reason, it is advisable to fit air-conditioning in a sorting laboratory to ensure that a constant temperature is maintained.

almei_g (almei_g@med.unr.edu)
Dear Andy I had that problem before. After 4 h of having the sorter on (because the room would start to warm up) I could not get stable sortings anymore, and that especially happened during the summer. Since we installed an A/C unit in the room, we never had this kind of trouble again, and have experienced a noticeable difference in the quality of sorting. Graca Almeida-Porada, M.D, Ph.D. University of Nevada VA Medical Center

Houston, Jim (Jim.Houston@stjude.org)
From having a sorter in various environments, I would suggest putting a fan on the power supplies if the room temperature exceeds 75 degrees or warms up gradually by the end of the day. My Vantage is in an extremely controlled room. I still run fans on the power supplies. It will shift after about 2 hours without these fans.

WELCHEN CD34ER SOLL MAN VERWENDEN?

lwk (hkleewk@netvigator.com)
Dear Flowers, Recently, we have tried to incoporate the CD34 in the acute leukaemia panel. As you known, cellular expression of CD34 is quite complicated. Some of the Flowers have used the clone:581 in stem cell estimation. But I am so puzzle whether this clone is too specific in leukaemia immunophenotyping. Some of the Flowers have suggested me to use a cocktail of CD34 from the Coulter Immunotech clone. I will be grateful if someone can give me some practical experience in using CD34 for immunophenotyping but not just stem cell estimation. Thank you. Regards, W.K.LEE Dept. of Path. United Christian Hospital, Hong Kong

D. Robert Sutherland
Dear Dr. Lee, Choose a CD34 antibody that detects a class III epitope, i.e., 581 (Coulter Immunotech) or 8G12 (HPCA2, BDIS). If possible, use PE conjugates, although both also work well as FITC conjugates. These reagents all work interchangeably in the ISHAGE Guidelines for CD34+ cell enumeration. 581PE:Cy5 also works well but beware of other reagents conjugated with PE:Cy5 as they may stain CD64 (Fc gamma III receptors) via Cy5. This is a problem for at least one class III antibody that we have tested in

which the PE appears to have been overconjugated with Cy5. If in doubt, test the chosen clone/conjugate against one of the validated antibody conjugates listed above. Regarding the 'pool' of PE antibodies sold by Coulter Immunotech: This pool contains two class I antibodies (Immu 409 and Immu 133) and a class II antibody (QBEnd10). While QBEnd10 PE on its own works interchangably in the ISHAGE method and has a decent signal-to-noise ratio, overall it is not quite as bright as the class III antibodies cited above. Class I antibodies, since they are dependent for their binding on the presence of certain sugars on the mucin-like CD34 molecule, are thought by many to be incapable of detecting all CD34+ cells in all clinical samples, and because of this, are not recommended for enumeration or leukemia immunophenotyping purposes. Additionally, several of the class I antibodies cannot be conjugated with negatively charged fluorochromes such as FITC because they bind to negatively charged sialic acid residues on the CD34 antigen, further reducing their utility. Even as PE conjugates, they tend to exhibit poor signal-to-noise ratios compared to the class III counterparts. Furthermore, we and others have mapped the class II epitope to the same region of the CD34 molecule as the clustered class I epitopes at the far N-terminus of the antigen. Thus, FITC conjugates of the class II reagents also fail to detect CD34+ cells efficiently, further reducing the flexibility and utility of the class II antibodies in the above applications. From these observations, it is apparent that even as non-conjugates, Immu 133, Immu 409 and QBEnd10 in the 'pool' are competing for closely apposed epitopes on the CD34 molecule. PE conjugation greatly compounds this problem because PE itself is a larger molecule than the antibody and indeed, several studies have demonstrated that class I and class II reagents interfere with the binding of each other. On top of all this, in the 'pool' you have three antibodies which between them contain IgG1 and IgG2 heavy chains, and both kappa and lambda light chains. The net effect is that you have three antibodies, (neither of which alone is as good as a single class III reagent) competing for their binding sites (as opposed to the hoped for 'additive binding'), while at the same time one has to account for the non-specific binding of three different antibodies of different heavy and light chain types (all of which may be additive!!). There have been claims that the 'pool' sees CD34+ cells that class III and class II reagents individually do not detect. These observations have not been confirmed and may merely be an example of additive non-specific binding. I hope this make sense. Good luck, Rob Sutherland, Associate Professor of Medicine, Oncology Research, Toronto Hospital, Toronto (AKA the Frozen North)

WIE DEFINIERT MAN NAIVE UND MEMORY-ZELLEN (KORREKT)?

Susan Schmitt (SSchmitt@specialtylabs.com)

Any recommendations for the best marker combination to identify CD4 helper naïve and memory cells?

Mario Roederer (Roederer@Beadle.stanford.edu)

Susan, Identifying naive T cells in the human is nontrivial and you must take care to do it properly! In any case, you must use at least 3 colors to do so: CD4 or CD8, and a pair of antibodies including a CD45 isoform (as noted below). There are several ways to uniquely identify T cell subsets in the human. Currently, the best are the combined use of CD45RA with one of CD11a, CD62L, or CD27. Naive T cells are CD45RA+ and CD62L+, CD27+, and CD11a-dull. You may use CD45RO in place of CD45RA, noting that its expression is inverse to CD45RA; thus, naive T cells are CD45RO- and CD62L+, CD27+, CD11a dull. Don't make the mistake of using CD45RO and CD45RA in the same stain; you get no additional information from using either one alone (see notes below for more info). Also note that CD45 isoform expression is very different on CD4 and CD8 T cells! Therefore, you CANNOT use isotype gates for identifying naive T cells, and you CANNOT use the same

gate set for CD4+ cells as for CD8+ cells. In particular, the CD8+ memory cells that are "CD45RA-" actually express a good amount of CD45RA! You will need some experience with staining healthy adults to see where to set the gates. CD62L is probably the best antigen to use in combination with CD45RA. It is bright; the CD62L+ cells are easy to distinguish from CD62L- cells. The main problem with CD62L is that it falls off cells during freeze/thaw protocols, and cannot be reliably used on frozen blood samples. CD27 is a good choice as well, although far fewer functional studies have been done with CD27+CD45RA+ cells to really prove that these are pure naive T cells. CD11a is the most robust identifier of naive vs. memory cells in the CD45RA+ subset. However, it is the most difficult to use, because you must set a gate that distinguishes bright from dull cells, without that much separation. Again, a bit of experience looking at healthy adult samples will teach you quickly where to set this gate. CD45RA and/or CD45RO staining is insufficient without an additional marker (CD62L, CD27, or CD11a). There is a distinct set of CD45RA+CD45RO- memory T cells. In the CD8 compartment, this subset averages around 10-30% of total CD8; in CD4 it averages 5% of total CD4 (in healthy adults). In adults with acute or chronic disease such as HIV, these percentages go way up. In advanced HIV disease, CD4 T cells can be more than 50% CD45RA+, but nearly all are memory T cells! In unpublished studies using 10-color flow cytometry, I have correlated the exact expression of all of these markers (together with functional studies to verify memory/naive as well as possible). I do find that about 1% (CD4) or 5% (CD8) of the "naive" T cells, when identified using CD45RA with CD62L OR with CD27 are actually activated memory T cells! If you use CD45RA, CD62L, and CD27 altogether (a 4-color combination with CD4 or CD8), then this percentage drops by an order of magnitude. Thus, a three color combination of CD4/8 with CD45RA and CD62L or CD11a identifies naive T cells to about 95-99% purity. CD11a was much better, achieving nearly 100% purity. mr

"CD62L ist wahrscheinlich der beste Marker den man mit CD45RA kombinieren kann"

biocytex@biocytex.com

Dear All , In response to Susan's request, Mario Roederer recently wrote : < Identifying naive T cells in the human is non trivial and you must take care to do it properly... Thus a three-color combination of CD4/CD8 with CD45RA and CD62L (or better CD11a) identifies naive T cells to about 95-98% purity.> We suggest that quantitative measurement of Fas (CD95) cell surface expression may also be a valuable approach for differentiating naive and memory T cells. Indeed, when developping a new quantitative FCM method for measuring the expression of Fas (CD95) on whole blood T (or T4/T8 subsets) lymphocytes, we observed the following : - Healthy adults display two distinct sub-populations of T lymphocytes with different levels of Fas. We call them Fas low and Fas high rather than Fas (-) and Fas (+) because quantitative FCM shows us Fas low cells are clearly distinct from negative control staining. In fact they span on the fluorescent scale from background up to 3,000 Fas sites/cell . Fas high cells span from 3,000 to 20,000 Fas sites/ cell with a mean level of 10,500 +/- 1,500 in our series. The value 3,000 is the place of the valley between both peaks in samples where both Fas low and Fas high are equally represented (~50% of each). This quantitative approach thus provides an objective and standardized way of placing a gate between dull and bright cells. - When counter-staining was made with CD45RA-PE , Fas low cells appeared 45RA high and vice-versa. This is in accordance with many papers in the literature which have already qualified Fas high (Fas+) T lymphocytes as activated/memory T cells and subsequently positionned naive T cells among Fas low (Fas- in the literature) cells. In particular, Miyawaki accordance with many papers in the literature which have already

qualified Fas high (Fas+) T lymphocytes as activated/memory T cells and subsequently positionned - In pathological situations where nearly all T cells are memory T cells, i.e. , advanced HIV disease and immune reconstitution after bone marrow transplantation (BMT), we often observed single peak distributions restricted to Fas high T lymphocytes. In many cases <10% Fas low cells were present. After BMT in adults, recovery of normal percentages of Fas low T cells were observed only after one year or more. Thus, we suggest that the flow cytometric quantitative evaluation of Fas (CD95) expression on T cells subsets might be a powerful and standardized approach to recognize and count naive T lymphocytes in whole blood samples. We have already generated some data with an outside clinical site which could be available fairly soon to support this point of view. We arealso initiating some works with CD95 during liver transplant. We consider it an important topic and we would be glad to hearing comments from the list. Philippe PONCELET Director R&D BioCytex Dear All , In response to Susan's request, Mario Roederer recently wrote : < Identifying naive T cells in the human is non trivial and you must take care to do it properly... Thus a three-color combination of CD4/ CD8 with CD45RA and CD62L (or better CD11a) identifies naive T cells to about 95-98% purity.> We suggest that quantitative measurement of Fas (CD95) cell surface expression may also be a valuable approach for differentiating naive and memory T cells. Indeed, when developping a new quantitative FCM method for measuring the expression of Fas (CD95) on whole blood T (or T4/T8 subsets) lymphocytes, we observed the following : - Healthy adults display two distinct sub-populations of T lymphocytes with different levels of Fas. We call them Fas low and Fas high rather than Fas (-) and Fas (+) because quantitative FCM shows us Fas low cells are clearly distinct from negative control staining. In fact they span on the fluorescent scale from background up to 3,000 Fas sites/cell . Fas high cells span from 3,000 to 20,000 Fas sites/cell with a mean level of 10,500 +/- 1,500 in our series. The value 3,000 is the place of the valley between both peaks in samples where both Fas low and Fas high are equally represented (~50% of each). This quantitative approach thus provides an objective and standardized way of placing a gate between dull and bright cells. - When counterstaining was made with CD45RA-PE , Fas low cells appeared 45RA high and vice-versa. This is in accordance with many papers in the literature which have already qualified Fas high (Fas+) T lymphocytes as activated/ memory T cells and subsequently positionned naive T cells among Fas low (Fas- in the literature) cells. In particular, Miyawaki T. et al. (J. Immunol., 149, 3753 (1992)) showed that newborns have almost no Fas high (Fas+) T cells and that the percent of Fas high cells increases with age. - In pathological situations where nearly all T cells are memory T cells, i. e. , advanced HIV disease and immune reconstitution after bone marrow transplantation (BMT), we often observed single peak distributions restricted to Fas high T lymphocyafter bone marrow transplantation (BMT), we often observed single peak distributions restricted to Fas high T lymphocytes. In many cases <10% Fas low cells were prese Thus, we suggest that the flow cytometric quantitative evaluation of Fas (CD95) expression on T cells subsets might be a powerful and standardized approach to recognize and count naive T lymphocytes in whole blood samples. We have already generated some data with an outside clinical site which could be available fairly soon to support this point of view. We arealso initiating some works with CD95 during liver transplant. We consider it an important topic and we would be glad to hearing comments from the list. Philippe PONCELET Director R&D BioCytex

EXPRIMIEREN GAMME-DELTA-ZELLEN CD2?

Maryalice Stetler-Stevenson (stetler@box-s.nih.gov)

Not all gamma delta T-cells express CD2. Up to 22% of the gamma delta T-cells were CD2 negative in a series we studied of healthy normal donors who had greater than

5% gamma delta T-cells in their peripheral blood. There are some ethnic differences.

HISTOGRAMM-DATEN NACH EXCEL EXPORITEREN

David McFarland (David.McFarland@mcmail.vanderbilt.edu) OK. I know that you can export statistics from CellQuest to Excel, but I have a client that is interested in plotting data from histograms(a single parameter) in coordinate system (x,y) where x is the channel number and y is the number of events in that channel. I looked through the archives and didn't see anything about this. Can anybody help me out? Thanks.

Mario Roederer (Roederer@Beadle.stanford.edu) FlowJo (available from www.treestar.com/flowjo/) has the capability of exporting either raw FCS data or the histogram data for analysis in other programs like Excel. For Histograms, all you have to do is "Copy" while viewing the histogram, and "Paste" into Excel spreadsheet. BTW, FlowJo can also export kinetics data in the same way. Mr

Hi David, You can use the "Export Histogram Data" feature of FCS Assistant, you can download the current version from one of my websites <http://facsmac.med.cam.ac.uk/fcsa.html> where there is an on-line manual. or <http://www.angelfire.com/biz2/rayh/)> (currently being built, stored as a .zip archive) or from Geoff Osborne's <http://jcsmr.anu.edu.au/facslab/facs.html>. (stored as a Binhex archive) It exports a column of frequency data in tab-delimited ascii for each parameter in an FCS file. You'll have to gate prior to saving, and it doesn't include the channel number, but that can be added by Excel. Ray

ANNEXIN ASSAY

Laboratorium Medisch Spectrum Twente (labmst@euronet.nl) I'm using MNC to study the induction of apoptosis in subsets of lymphocytes (CD4, CD8, CD19). Who can tell me more about the combination of Annexin V and 7-AAD as a viability-marker? Questions: - Is anyone using a similar assay?; - Which concentration of 7-AAD is the best to use?; - How long is the incubation-time of 7-AAD?; - How are the results of using 7-AAD above Propidiumiodide in combination with Annexin V? I'm looking forward hearing from you! Renate Weghorst MST-hospital Enschede The Netherlands

Nebe, Thomas C. (thomas.nebe@ikc.ma.uni-heidelberg.de)
Dear Collegues, as pointed out 15 min on ice (a must) has also been the result of our validation assays. We see no difference between PI and 7-AAD for labeling of apoptotic cells. The stock should be 1 mg/ml i.e. a 1:100 or 1:500 dilution. 2µg/ml was the optimum in our hands but depends on final voulumes and cell density. Find your own optimum. Be aware of the solvent for your dye. We prefer DMF over DMSO and it should be diluted out more than 1:500 as stated above. Be aware that the phophatidyl serine flip flop is rather fast (seconds) and any harm to your cells like azide does it (pipet contamination from azide containing monoclonals, sheath fluid...). Once well established it might be one of the most valuable clinical FCM tests. We obtained good clinical results for acute leukemia, NHL, HIV and autoimmune diseases either by in-vivo or in-vitro treatment (not yet published). Of course you need a standardized in-vitro run out time (4 or 20 hrs). Thomas Dr.med. C. Thomas Nebe Universitätsklinikum Mannheim Institut für Klinische Chemie Theodor-Kutzer-Ufer 1-3 D-68167 Mannheim Tel. +49 621 383-3485 FAX +49 621 383-3819 thomas.nebe@ikc.ma.uni-heidelberg.de PSE 75-1445

Keith Bahjat (kbahjat@ufl.edu)
We use 7-AAD with our Annexin V assays. We use this at 1 ug per ml of cell suspension. We add the 7-AAD when we add Annexin V FITC and let it incubate for 15 minutes. 7-AAD is made as a 0.1 ug per ul stock, then 10 ul are added for every ml of cell suspensi-

on (i.e. we add 5 ul to our 500 ul total final volume of binding buffer for Annexin V assays) We have found (as was reported in a Cytometry article for which I don't have the reference) that 7-AAD tends to be less "leaky" than PI. i.e. all cells that stain PI positive will not stain 7-AAD positive. It seems to require a larger pore for entry into a cell. Additionally, we have found that keeping the cells cold while staining (as suggested by Immunotech) significantly improves results. Good luck. Keith Bahjat kbahjat@ufl.edu

DETEKTION VON EBV-ERKRANKUNG

Christian Awaraji (cawaraji@rri.on.ca) What we have done is actually detect EBV transformation by detecting the LMP expression (Dako) using a BD lysing solution for permeation (15 min). Good luck, Christian On Tue, 13 Oct 1998, Frederic Preffer wrote:

Tom Just (tom.just@DAKO.DK) Hi Frederic, Several groups have detected EBV infected cells by flow using in situ hybridization. Two relevant references that I know of are: Crouch, J. et al., Epstein-Barr virus suspension cell assay using in situ hybridization and flow cytometry. Cytometry 29, 50-57, 1997. Just, T. et al., Flow cytometric detection of EBV using peptide nucleic acid probes. J. Vir. Methods 73, 163-174, 1998. If you need further information you are wellcome to contact me. Best wishes Tom Just

PÄDIATRISCHE NORMALWERTE FÜR DIE IMMUNOPHÄNOTYPISIERUNG

Nebe, Thomas (thomas.nebe@ikc.ma.uni-heidelberg.de)
The best I know is from the van Dongen group at Rotterdam: Comans-Bitter WM et al (1997) Immunophenotyping of blood lymphocytes in childhood The Journal of Pediatrics, 130, 388-393 Dr.med. C. Thomas Nebe Universitätsklinikum Mannheim Institut für Klinische Chemie Theodor-Kutzer-Ufer 1-3 D-68167 Mannheim Tel. +49 621 383-3485 FAX +49 621 383-3819 thomas.nebe@ikc.ma.uni-heidelberg.de PSE 75-1445
Try this... Cytometry, Sep 15 1994, 18(3) p129-139 Quality control in flow cytometry for diagnostic pathology: II. A conspectus of reference ranges for lymphocyte immunophenotyping Dominique Cossali

AML M7 VERSUS PLATELET ADHERENCE TO BLASTS

Cynthia Aller (caller@execpc.com)
We diagnose many acute leukemias per year. Often we have encountered acute myelogenous leukemias that stain positively with our monoclonal antibodies to CD41 and CD61, which indicates to us that this patient may have a diagnosis of AML-M7. It is known that platelets adhere to blast cells and the standard recourse is to visually inspect the cells on a fluorescent microscope for the adherence of the platelets to the blast cells. This is often difficult, however, when the blast population is a minor population within, say, bone marrow. I am looking for a method to show that the binding of anti-CD41 and anti-CD61 to the blast cells is not platelet adherence but true cellular staining by flow cytometry. I have performed a literature search and have found two references to this phenomenon, including Blood, 79(9):2399-2403, 1992, and Blood86(10):3771-3782 (1995). These papers noted the false-positive platelet adherence to myeloid blasts and the second paper included a method using 5mM EDTA to eliminate the binding of the platelets to the blasts. We have tried this method without success. What have other members of the flow cytometry community done with this problem? Perhaps there is another method for preventing or eliminating the binding of the platelets to the blasts? I would appreciate any input that I might receive with regards to this problem.
Walter Sharp (denby@compuserve.com)

Dear All, I'd like to get a concensus opinion from the clinical "flowers" since I'm having a "difference of opinion" with a flow lab here. Which CD do you use for detecting M7's - CD41, 42b or 61? My experience of CD42b is that it is the least reliable of the three and can be negative where 41 &/or 61 are positive. The lab here (not where I am!) uses 42b on it's own and I'm a bit concerned that they're likely to miss some. Wal Sharp

Maryalice Stetler-Stevenson (stetler@box-s.nih.gov)

We use 41+61 in FITC and 42b in PE with 45PerCP. We get alot of secondary leukemias and are always thinking of the possibility of megakaryocytic lineage. Maryalice

Nebe, Thomas (thomas.nebe@ikc.ma.uni-heidelberg.de)

Dear collegue, we use CD41 and CD61 for M7 including intracellular staining with fix&perm from the company An der Grub. There was one case of discrepany between us and the German reference lab at Berlin where they found CD61 only in the cytoplasm. We have skipped CD42 because of redundancy. In addition fluorescence microscopy is required to ensure that the positive signal is not derived from platelets sticking to the blast cells. Furthermore a characteristic feature is the membrane blebbing of the peripheral blasts (may be seen sometimes in other leukemias). Bone marrow often shows punctio sicca because of myelofibrosis. In the marrow smear the blebbing sometimes reminds to platelets. Children with Down´s syndrome are at high risk. M7 is close to M0 in terms of immaturity. It´s often preceeded by MDS. So it´s not only a question of CD42. Kind regards Thomas Nebe Dr.med. C. Thomas Nebe Universitätsklinikum Mannheim Institut für Klinische Chemie Theodor-Kutzer-Ufer 1-3 D-68167 Mannheim Tel. +49 621 383-3485 FAX +49 621 383-3819 thomas.nebe@ikc.ma.uni-heidelberg.de

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