

Inexpensive CD4+ T lymphocyte counting by flow cytometry – relevance to developing countries

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Acknowledgements

This study was presented as a late breaker at the XIII International AIDS Conference, July 2000, Durban, South Africa (Abstract LbPp112). We would like to thank I Storie, L Whitby and K Goodfellow (Sheffield) and Mr. E. Mbena (Dar-es-Salaam) for their excellent technical assistance. This study in the U.K. was supported by the Fifth Framework Programme (FP5; Quality of Life) of the European Commission (grant No. QLRT-199-30436 to DB and GJ). The contribution from Tanzania and Sweden (by FM, EL and GB) was supported by a grant to the TANSWED HIV programme from the Swedish International Development Co-operation Agency (SIDA), Department of Research Co-operation (SAREC).

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Summary

Background: Recently, efforts have been made to make anti-retroviral therapy available to resource-poor countries. Several vaccines are also to enter large field trials in the near future. In contrast, CD4 counting remains mostly unavailable in developing countries due to its complexity and high costs. Thus, we introduced the concept of "primary CD4 gating".

Methods: CD4 cells were counted in a single tube of whole blood containing a single CD4 antibody (Ab). CD4+ lymphocytes were identified as cells with high CD4 fluorescence intensity and low side scatter. A volumetric "single-platform" cytometer, the Ortho Cytoron *Absolute*, that does not require beads for absolute counting as it operates with precise Hamilton syringes to deliver known volumes was used as the 'gold-standard'.

Results: All six CD4 Ab's conjugated to four different fluorochromes performed well in "primary CD4 gating". When compared to the full reagents panel on the Cytoron, the primary gating counted in average +2 cells/mm³ (n=552; Bland-Altman limits of agreement [LA] -23 to +27; R²=0.999). Manual lymphocyte gating was used to calculate CD4% values; these were 0.2% higher than values obtained by the conventional method (n=277; LA -3.9% to +4.3%; R²=0.980). Fixative *TransFix* did not alter CD4 counts, and allowed to show the precision of CD4 counts in Tanzania – as an example of QC to support local African technology.

Conclusions: **Primary CD4 gating is as accurate as the more complex panels. As only one Ab is needed, this represents at least 85% cost savings in reagent, and there is no need for expensive microbeads. Thus primary CD4 gating on volumetric flow cytometers represent an affordable test for CD4 counting in resource-poor settings.** This new technology is promoted by (i) the availability of CD4 Abs, (ii) the development of a new fixative *TransFix* for facilitate trouble-free sample transport (iii) as well as the use of cells stabilized for long periods to serve as standards during international QC procedures. **These studies define the specification for a new breed of inexpensive volumetric 'CD4-counters'.**

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Introduction: This study is part of an international collaboration to establish the concepts and practice² of affordable CD4 testing in developing countries (a '*minimalist*' approach in terms of costs) with maximum quality assurance utilizing stabilized cellular controls and standard reagents ('*maxi-quality*'). The principal collaborators in the full project are G. Janossy (London), W. Göhde (Münster), D. Barnett (Sheffield), B. Brando (Milano), G. Biberfeld (Stockholm), D. Glencross (Johannesburg) and F. Mandy (Ottawa).

Absolute CD4 counts can be performed in two different ways: (i) either by '**double platforms**' using a combination of flow cytometry (for CD4% values) plus haematology (for lymphocyte and WBC counts), or (ii) by '**single platform**' using dedicated flow cytometers alone with direct counting facility. During the last decade cytometry performed on single platform has become an exceptionally precise technology where inter-laboratory variations are much lower (13.7%) than those seen on double platforms (23.4%) or on the diverse haematological analysers^{1,2}.

Single platforms, accurate but still complex, can use two approaches. Some are '**volumetric**' (Cytoron, Ortho³, Galaxy, DAKO) and can determine volumes directly. Others are '**bead-based**' (FACS-Calibur; BD; Elite, Coulter) and need known numbers of microbeads added to the tubes to calculate absolute counts.

In order to introduce affordable CD4 tests into practice six issues need to be considered:

1. Advantages of **volumetric 'single-platform' cytometers**³ in terms of running costs.
2. Introduction of **primary CD4 gating**⁴ with the use of a single CD4 reagent (instead of 6-9 reagents as currently used in expensive CD4 assays).
3. The availability of monoclonal **CD4 antibodies**⁴ conjugated to fluorochromes detectable with inexpensive red diode lasers.
4. **Fixatives** for short term use (e.g. TransFix from NEQAS)⁵ for inexpensive trouble-free sample transport
5. **Standard cells**¹ fixed for long periods to facilitate participation in international Quality Control (e.g. by NEQAS)
6. The search for **simple volumetric flow cytometers**⁶ that handle well in tropical conditions is now on

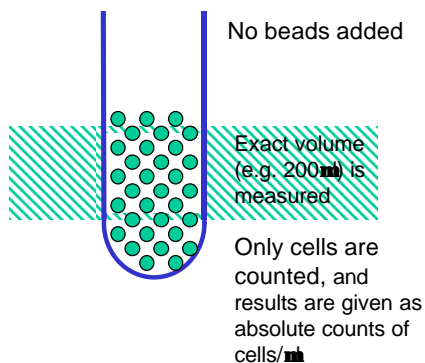
¹ Barnett et al. *Brit.J.Haem.* 106: 1059-62.1999. ² O'Gorman & Nicholson *Clin Diag Lab Immunol* 7: 333-9.2000. ³ Marcolino et al. *Cytometry* 22: 48-59. 1995

⁴ Janossy, Jari & Goehde *Brit.J.Haemat.* 111: in press Dec. 2000. ⁵ Jari et al. submitted for publication and this poster ⁶ Doornbos et al. *Cytometry* 15: 267-271. 1994

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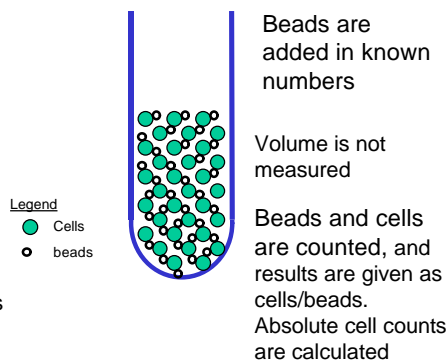
1. Advantages of volumetric 'single-platform' cytometers³ in terms of running costs

Volumetric (Ortho, Dako, Partec)



No hidden costs

Bead-based (BD, Coulter)



Beads cost \$5-16 (£3-10) per test. Beads are useful to 'calibrate' volumetric machines once a day but very wasteful when added to every sample tested (100-300/day in a busy lab!)

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2. The concept of primary CD4 gating

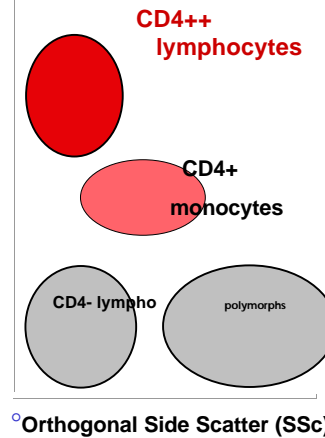
1 IF channel + 1 side scatter

CD4

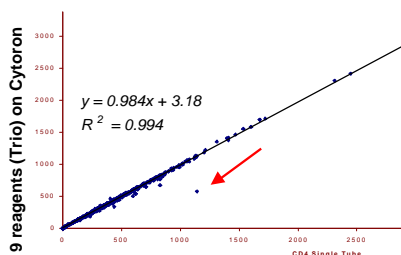
CD4 count and WBC+Ly counts

- Sherman et al J Imm Meth. 222: 209-217, 1999
 - comparison with double platform equipment
- this study: volumetric absolute count
 - comparison with single platform
 - CD4 alone
 - CD4 + CD8

As this is a volumetric analysis, absolute counts are provided. No expensive beads are required. **One single CD4 antibody replaces** a whole panel of reagents (6-9 antibodies, depending upon the manufacturers' and FDA recommendations). Can such a simple test be accurate? (see next)



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Inexpensive 'primary CD4 gating' in a single tube using a single CD4 antibody compared to the expensive CD4 test ('state of art' method using 9 reagents on Cytoron). The correlation between the results is extremely high ($r^2 = 0.994$). The counts are the same except in 1 out of 600 cases. This case (↖) shows weak CD4 staining and it is aberrant patient (T-ALL?).

'primary CD4 gating' in a single tube The same results have also been analysed by the more discriminative Bland-Altman test (Janossy, Jani and Goehde: *Br.J.Haematol.* 114: 1196. 2000). There was virtually no bias (-4 with confidence interval 95%: -2 to -6). The conclusion is therefore that in adults using a single CD4 antibody with 'primary CD4 gating' gives the same results as the expensive assays

3. The availability of monoclonal CD4 antibodies

conjugated to fluorochromes detectable with inexpensive red diode lasers

CD4 monoclonal antibodies are available in different forms conjugated to different fluorochromes such as FITC, PE, PE-Cy5, APC and Cy5. All 5 CD4 monoclonal antibodies, conjugated to different fluorochromes, were excellent for primary CD4 gating (CD4++, small side scatter for lymphocytes in Gate A) and effectively discriminated from monocytes (CD4+, intermediate side scatter in Gate B).

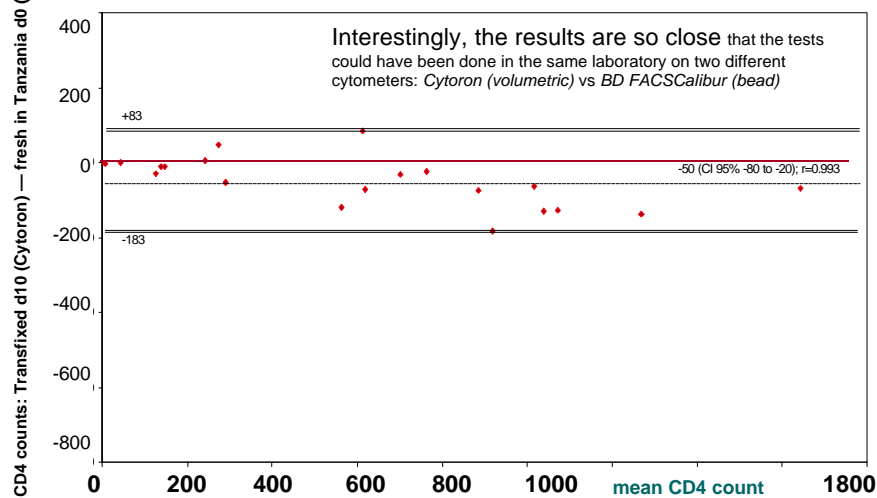
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4. Fixatives for short term use (TransFix from NEQAS) for inexpensive trouble-free sample transport

- Specimen transportation between clinical sites is increasing globally
- **Clinical trials** are increasingly requesting immunological & haematological profiles
- In developing countries HIV infected individuals (or cells !!!) may have to travel long distances to receive adequate CD4 counts - and the postal services can not guarantee acceptable temperatures during the varied duration of transport
- TransFix introduces two important factors during transport: **tolerance to timing & sample integrity**
- Several haematology and immunology guidelines have published specific time frames for time to completion of analysis. The time limits can now be extended without dangers of deterioration
- Decreased burden on the staff, budget and courier costs
- More rational planning of laboratory work

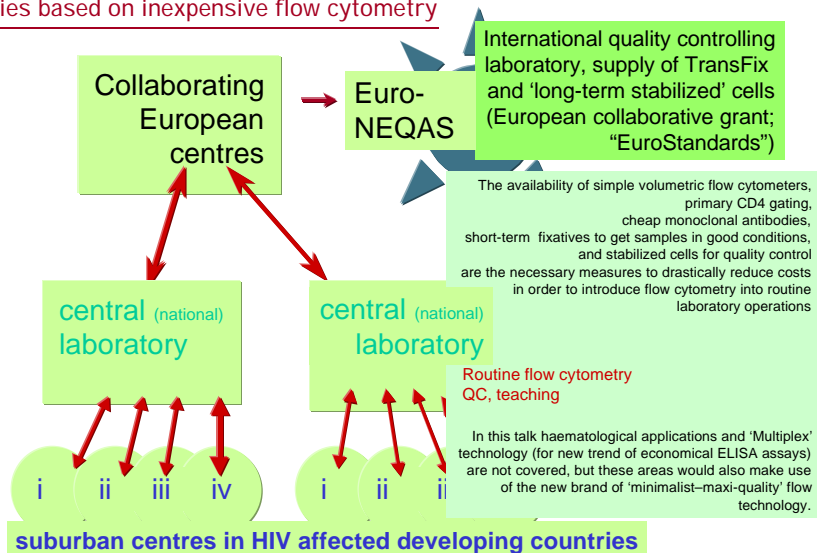
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Comparison of fresh Tanzanian samples with Transfixed samples tested in London (travelling for 10 days at room temp.)



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Overview of new collaborative possibilities for developing countries based on inexpensive flow cytometry



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Conclusions:

1. Volumetric flow cytometry ✓
 2. 'primary CD4 gating' = one CD4 reagent is enough ✓
 3. this CD4 Ab is available to run on cheap diode lasers ✓
 4. blood can be fixed (TransFix): plenty of time to send – even under tropical conditions ✓
 5. QC is available using stabilized samples ✓
- All works fine! ✓

If introduced, these measures start a new chapter in the development of clinical flow cytometry

Costs:

Normally:

\$13-25 per test:

\$5-16 bead : **not needed**

\$10 for 6-9 antibody:

replaced by 1 single CD4 Ab

Flow cytometer:

\$ 70,000 : that does not work in Africa (too complicated, needs stable electricity, expensive to run, etc.)

to be replaced by a small, inexpensive volumetric machine using a cheap red diode laser

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