DNA/RNA Binding Anthraquinone Dyes in Flow Cytometric Sample Gating

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Introduction
Flow cytometry has become a mainstay of cellular analysis and sorting. However, the degree of sample preparation impacts significantly on the quality and reproducibility of analysis. For instance, RBC lytic agents remove blood samples of erythrocytes but can strip cells of antigens or cause unwanted lysis of late nucleated erythroblasts whilst the use of whole peripheral blood may require use of additional antibodies to exclude RBCs from analysis. Neither approach is suitable where samples are derived from adherent cell cultures and the contaminant is debris resulting from mechanical detachment of cells. The use of a fluorescent DNA binding dye can overcome these issues by labeling intact nucleated cells which can then be gated for discreet immunophenotypic analysis. The use of novel DNA/RNA binding anthraquinone dyes DRAQ5 and CyTRAK Orange in flow cytometric analysis is described here. In addition, a review of relevant methods from the published literature is provided to further exemplify the strategy being proposed in each case.

Description of the dyes
DRAQ5 and CyTRAK Orange are fluorescent dyes that stain the nucleus in live and fixed cells. Detailed excitation and emission spectra are shown below (DRAQ5: Exλ<sub>max</sub> 646nm / Emλ<sub>max</sub> 697 nm & CyTRAK Orange: Exλ<sub>max</sub> 510nm / Emλ<sub>max</sub> 610 nm). Usefully, DRAQ5 can also be excited by the 488nm laser line, which is not evident from the absorbance profile. The mode of binding for both is consistent with the anthraquinone family, typically being DNA minor groove binders with A-T/A-T specificity. They preferentially bind dsDNA and then dsRNA, to a greater degree by CyTRAK Orange.

Materials & Experimental Procedures
All samples were analyzed with an LSRII flow cytometer. For all experiments CyTRAK Orange, DRAQ5 and Vybrant Violet were used at a final concentration of 10 µM (2µl/ml). Hoechst 33342 and Propidium Iodide were used at a concentration of 10 µg/ml. Cells at an approximate concentration of 0.5 million/ml were stained for 30 minutes at 37°C. For circulating epithelial cell (CEC) detection experiments a colon carcinoma cell line (H630) was added at decreasing numbers after freezing. A whole blood sample was incubated with CyTRAK Orange to enable DNA gating to avoid the use of red cell lysis reagents. A whole blood sample was incubated with CyTRAK Orange to enable DNA gating to avoid the use of red cell lysis reagents.

Use of DRAQ5 for a rat bone marrow differential:

Use of DRAQ5 for a human bone marrow differential:

2. The use of anthraquinone dyes in distinguishing bone marrow cells from RBCs and debris in multicolour cell differential experiments

Above is an ungated scatter dot plot from a mouse bone marrow, events are then separated into CyTRAK Orange-negative (debris) and positive (DNA), the gating logic is then for CyTRAK Orange-positive events and any gating required so as to give consistent phenotype percentages. Of B Mouse bone marrow samples the percentage CyTRAK Orange-positive cells were: 75.1%, 81.8%, 74.0%, 75.7%, 72.9%, 69.3%, 78.2% and 67.8%. These variations in sample purity would give rise to inconsistent phenotype percentages proportional to the size and distribution of the gating and the varying degree of debris within the sample. Similarly, the panel to the right (in purple) shows a human bone marrow sample analysed by forward and side scatter, without (R) and with the aid of DRAQ5 (DNA) as a gate for intact nucleated cells, and specifically haematopoietic progenitor cells which would be indistinguishable from the blanket of debris and RBCs.

Results

1. Comparison of nuclear staining of DRAQ5 and CyTRAK Orange with other DNA dyes
A whole blood sample was incubated with CyTRAK Orange to enable DNA gating to avoid the use of red cell lysing reagents. As in the earlier BM examples, the anthraquinone DNA dye provides a live cell gate for nucleated cells in a mo lyse, no wash methodology.
3b. Use of anthraquinone dyes to avoid need for RBC lysis reagents in human blood
Recent examples from the published literature describing the use of DRAQ5 in a practical human whole blood differential by flow cytometry are described below.

- Pers. Comm. Dr Terence Hoy. At its simplest, a washed whole blood sample can be stained with antibodies against CD45 & CD4 (FITC) and against CD14 & CD8 (PE) and DRAQ5. Using this approach, gating on DRAQ5, it would be possible to get relative enumeration of neutrophils, lymphocytes (incl. T-cell subsets) and monocytes. With the addition of a RBC lysis step it may be possible to retain all SSC and FSC light scatter information. An alternative might be to use a sphering agent to narrow the distribution of non-nucleated cells.

- Beyond the CD45 Gate: Cytometrically Defining Multilineage Hematopoiesis Utilizing DRAQ5. Flye et al, GLUTICA 12, Milwaukee October 2003 Poster Abstract 7.
This described a similar strategy to go beyond gating of a CD45+ population. Using density gradient separated unfixed bone marrow aspirates a combination of CD45-FITC, CD71-PE and CD34-EDC with DRAQ5 was found to be suitable. Where necessary CD41 was included for megakaryocytes. By this method it was possible to ascertain nucleated cell populations in fresh material, DNA S-phase and the myeloid:erythroid ratio on a single laser cytometer with standard filter set up.

A subsequent method used FITC-CD3/CD16/CD20, PE-CD64/CD33 and PCs-CD45 and DRAQ5™ on a 488 nm laser cytometer (using Fl1-Fl4). This was proposed as a reference method for differential leukocyte counting and that, with FSC & SSC, would allow segregation of lymphocytes, monocytes, granulocytes, activated/ immature granulocytes, basophils, eosinophils, nucleated red blood cell precursors and potentially other nucleated (but CD45 negative) cells such as stem cells and blood parasites. Fluorescent beads could be added to provide absolute counts.

While, most recently, a refined one-tube flow cytometry methodology was directly compared to microscopy and cell counter. The use of DRAQ5 along with 4 groups of antibodies (CD36, CD203/CD138, CD45, CD16/CD56) on the 5 fluorescence channel Beckman Coulter FC500 permitted the classification of 10 classes of nucleated cells. The key components of the methodology were an optimised RBC lysis, no-wash antibody and nuclear dye labeling and FlowCount Beads for cell enumeration. Additionally, following dilution, the DRAQS-, CD36+ events were counted as platelets.

An alternative approach using the Laser Scanning Cytometer (LSC™) makes the essential clear distinction between leukocytes and erythrocytes. DRAQ5 was used as the trigger for leukocyte analysis by CD45-FITC & CD14-PE in a Neubauer counting chamber and further dilution permitted erythrocyte counting altogether from a 10ul blood sample.

4. Use of anthraquinone dyes to distinguish intact single cells from tissue digestes
Below is a digest of pancreas stained with a bodipy-labeled ligand. As pancreatic islet cells are particularly fragile care is taken not to over-process the sample. The resulting histogram shows a possible two overlapping populations heavily stained with a bodipy-tagged ligand (top left & centre). Without a further discriminator the sample is thus useless. Staining the sample with DRAQ5 allows simple and reliable gating of remaining intact single cells (top right) which can then be further analysed (lower plots).

5. Use of anthraquinone dyes to distinguish intact single cells from adherent tissue culture
Scraping adherent cells from the generates large amounts of debris. The culture with DRAQ5 allows simple and reliable gating of the intact cells as shown below in the resulting scatter plot and DNA content histogram, permitting gating of the intact ES cells.

6. Use of anthraquinone dyes as a threshold parameter aid detection of rare circulating epithelial cells

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7. Use of anthraquinone dyes to detect rare circulating endothelial cells in human whole blood

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Conclusions
Cell permeant DNA/RNA-binding anthraquinone fluorescent dyes can allow more accurate reporting of phenotype percentages by using the dye as a DNA enabled gate excluding any debris from analysis, and this is also an alternative to CD45 gating if cells of interest are CD45 negative. It avoids the lysis of a whole blood sample when this is thought to be detrimental to the antigens under investigation. Moreover, DRAQ5 permits DNA content analysis in live cells.

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