

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. This can manifest itself in several ways. For instance, RBC lytic agents whilst removing blood samples of erythrocytes can strip off antigens from other cells or cause unwanted lysis of nucleated reticulocytes. Alternatively, the use of whole peripheral blood necessitates the use of additional antibodies to exclude the RBC from analysis. Neither approach is suitable where samples are derived from adherent cell cultures and the contaminant is debris resulting from mechanical removal of cells from the surface of a culture flask. 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 a novel DNA/RNA binding dye CyTRAK Orange in flow cytometric analysis is described here.

Description of the dye

CyTRAK Orange is a fluorescent dye that will stain the nucleus and cytoplasm in both live and fixed cells (figure 2). Detailed excitation and emission spectra are shown in figure 1 (Exλ_{max} 510nm / Emλ_{max} 610 nm). The mode of binding is consistent with the anthraquinone family, typically DNA minor groove binders with A-T/A-T specificity. They preferentially bind dsDNA and then dsRNA.

Fig.1

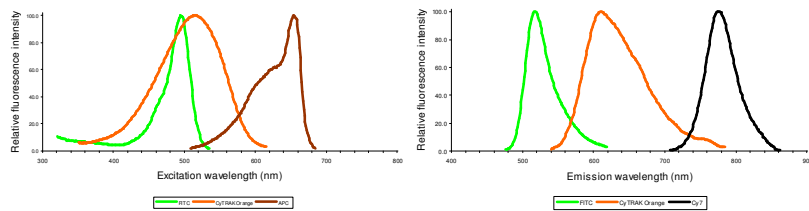
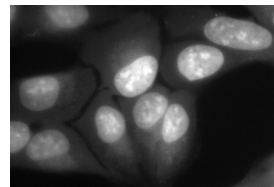


Fig.2



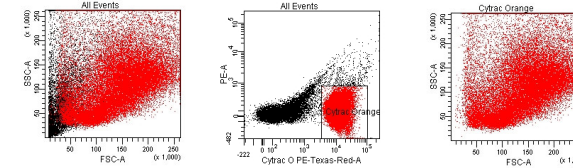
Materials & Experimental Procedures

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 counting in a haemocytometer. For CEC detection the antibody EpCAM was used. To assist in the enumeration of CEC TruCount beads were added to the Whole Blood.

3. The use of CyTRAK Orange in distinguishing mouse bone marrow from debris in a multicolour experiment

Fig.5

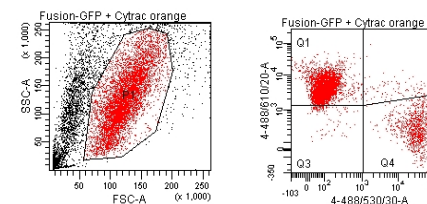


Population	#Events	%Parent	PE-A Mean	Cytrac O P. Mean
All Events	50,000	###	152	9,271
Cytrac Orange	37,533	75.1	106	11,638

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 8 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.

4. The use of CyTRAK Orange with mouse embryonic stem (ES) cells

Fig.6

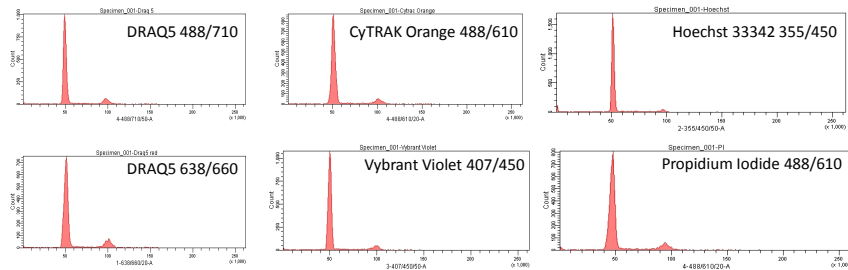


CyTRAK Orange was used to stain human fibroblasts (Q1), which were then fused with mouse ES cells (GFP-positive, Q4). The result can be seen above showing a dual population of GFP-positive CyTRAK Orange-positive i.e. the fused cell population (Q2).

Results

1. Comparison of nuclear staining properties of CyTRAK Orange with other DNA dyes

Fig.3



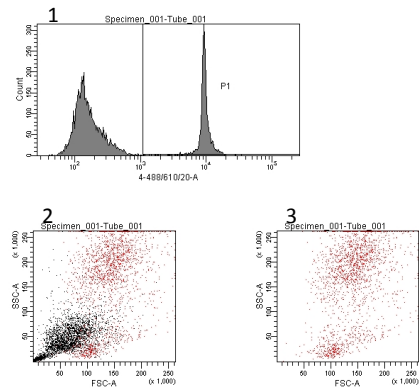
Cells used: fixed rat lymphocytes.

From left to right: DRAQ5 Ex/Em 488/710nm, CyTRAK Orange 488/610, Hoechst 33342 355/450, DRAQ5 638/660, DyeCycle Violet 407/450, Propidium iodide 488/610

Note: further experiments (not shown here) determined that CyTRAK Orange was not suited as a live cell DNA content dye due to the degree of RNA binding and corroborated by directly exchanging CyTRAK Orange for Propidium iodide in a Fix/Perm and RNase methodology.

2. The use of CyTRAK Orange to avoid need for RBC lysis reagents

Fig.4



A whole blood sample incubated with CyTRAK Orange to enable DNA gating to avoid the use of red cell lysing reagents.

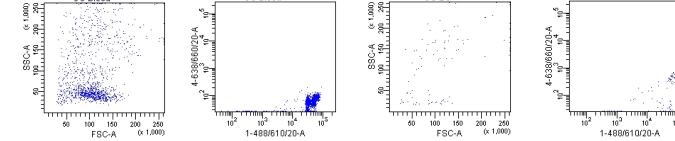
Plot 1 - Red cell and nucleated cell differential staining

Plot 2 - Scatter plot without red cell exclusion

Plot 3 - Scatter plot with red cell exclusion by gating on P1(CyTRAK Orange)

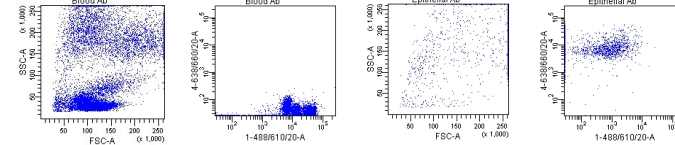
5. The use of CyTRAK Orange as a threshold parameter to aid the detection of circulating epithelial cells

Fig.7



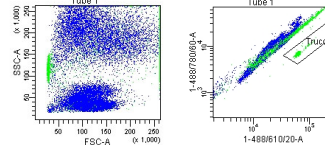
CyTRAK Orange stains both leukocytes and epithelial cells. CyTRAK Orange is not excited by the 638 nm laser allowing its use with APC, APC conjugates and PE-Cy7 and analogues.

Fig.8



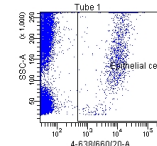
The EpCAM (APC conj.) antibody does not stain leucocytes, only epithelial cells.

Fig.9



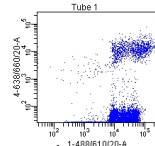
The sample of blood is thresholded on CyTRAK Orange so as to visualise APC-negative leucocytes, APC-positive epithelial cells and providing a fluorescent thresholding parameter for Trucount beads enabling absolute values to be determined for the quantitation of epithelial cells within peripheral blood samples doped with varying concentrations of epithelial cells

Fig.10a



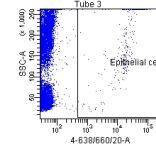
589 cells /ul

Fig.10b



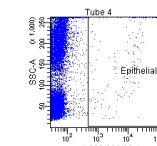
408 cells/ul

Fig.10c



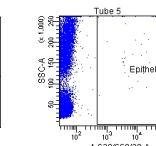
310 cells/ul

Fig.10d



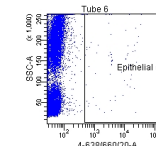
300 cells/ul

Fig.10e



260 cells/ul

Fig.10f



250 cells/ul

The data above present the basis for a method to detect epithelial cells within a peripheral blood sample. The sample has been spiked with epithelial cells to validate a procedure for the detection of low numbers of circulating epithelial cells. Since Trucount tubes were used threshold was set on a fluorescent parameter ensuring the counting of all Trucount beads, and consequently the accurate determination of the absolute numbers of epithelial cells circulating in the sample samples. The numbers of cells detected was in good agreement with the numbers of cells seeded in.

Conclusions

CyTRAK Orange has enabled us to improve our flow cytometric techniques allowing us to:

- report more accurate phenotype percentages by using the dye as a DNA enabled gate excluding any debris from analysis, this is also an alternative to CD45 gating if cells of interest are CD45 negative.
- avoid lysis of a whole blood sample when this is thought to be detrimental to the antigens under investigation