

Cell Cycle Tutorial

Contents

Experimental Requirements	2
DNA Dyes	2
Protocols.....	3
PI Parameter & Analysis Setup.....	4
PI Voltage Adjustments	6
7-AAD Parameter Setup	6
To-Pro3 Parameter Setup	6
DAPI & Hoechst 33342 Parameter & Analysis Setup	7
DAPI & Hoechst 33342 Voltage Adjustments	8
Cell Cycle Analysis Software	8

Experimental Requirements

1. Cells in suspension
2. 70% ice-cold ethanol
3. PBS
4. RNase
5. 37°C incubator
6. DNA dye of choice *e.g.* PI, DAPI, 7-AAD, Hoechst 33342
7. Flow Cytometer, BD LSR II or BD FACSCanto II

DNA Dyes

There are numerous fluorescent DNA dyes used for multiple purposes not all are suitable for flow cytometric cell cycle analysis. Only fluorescent DNA dyes that bind to DNA in a linear manner can be used for flow cytometric analysis of the cell cycle. Thus as DNA content of a cell increases the fluorescent signal from the DNA dye increases proportionally in a linear manner.

Commonly used DNA dyes used for flow cytometric cell cycle analysis include propidium iodide (PI), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), 7-amino-actinomycin D (7-AAD), ToPro-3 and Hoechst 33342. There are numerous other dyes such as Sytox16 a green fluorescent DNA dye as well as other live cell cycle dyes, such as DRAQ5, and the new range of dyes from Invitrogen.

The common dyes, PI ex 350 & 488nm em 619nm, 7-AAD ex 488nm em 650nm, ToPro-3 ex 633nm em 660nm and DAPI ex 350nm em 461nm are for use with ethanol fixed cells only. Hoechst 33342 ex 350 nm em 461nm is the commonly used DNA dye for live cell cycle analysis.

PI is normally used at 50 µg/ml, 7-AAD at 25 µg/ml, ToPro-3 10nM, DAPI at 1µg/ml. Hoechst 33342 titrated for each cell type and has to be incubated with live cells at 37°C for varying times for each cell type. A reasonable starting point for most cells is 10 µg/ml for 45 minutes at 37°C.

Fixed Cell Staining Protocols for PI, 7-AAD, ToPro-3 and DAPI

1. Harvest cells washing in PBS.
2. Fix pelleted cells in ice-cold 70% ethanol by adding with a Pasteur pipette on a vortex. Leave cells at 4°C from 30 mins to a week.
3. Pellet cells at approximately 2,000 rpm for 5 mins. Wash twice in PBS.
4. Pellet cells, wash twice in PBS.
5. Add 50 µl RNase (100 µg/ml Sigma) and incubate at RT or 37°C for 15 mins.
6. Add 200 µl of PI (50 µg/ml Sigma P4170); or 7-AAD (25 µg/ml Sigma); or ToPro-3 (10 nM Invitrogen); or DAPI (1 µg/ml Sigma).
7. Analyse by flow cytometry collecting 25,000 events per sample.

Live Cell Cycle Analysis with Hoechst 33342

1. Harvest cells washing in PBS.
2. Add 10 µg/ml Hoechst 33342 to cells incubate at 37°C for 45 minutes
3. Transfer cells to Falcon 5 ml tubes
4. Add 5 µg/ml PI for viability
5. Analyse by flow cytometry collecting 25,000 events per sample.

PI Parameter and Analysis Setup

PI can be detected in the 575/26 nm, 610/20nm and 670/14nm channels on the BD LSRII and the 575/26nm channel on the BD FACSCanto II.

The parameter setup for cell cycle analysis is special setup compared to immunophenotyping. Aggregates of G₁ cells have the same DNA content as G₂m cells, to distinguish these the instrument can separate these by measuring the time it takes for the two types of cells to pass through the laser beam by a fluorescent parameter width measurement. Aggregates of cells take longer to pass through the laser of the flow cytometer and thus appear higher up on the fluorescent width scale, see diagram below.

PE 575/26nm Channel Cell Cycle Analysis

Fig 1a

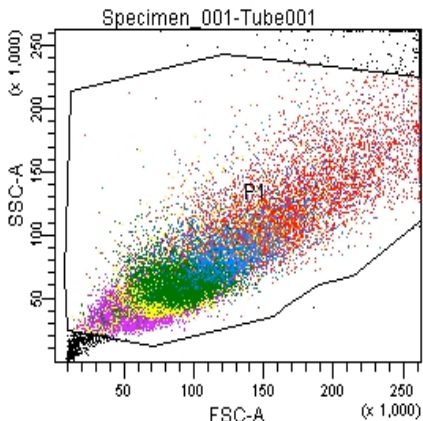
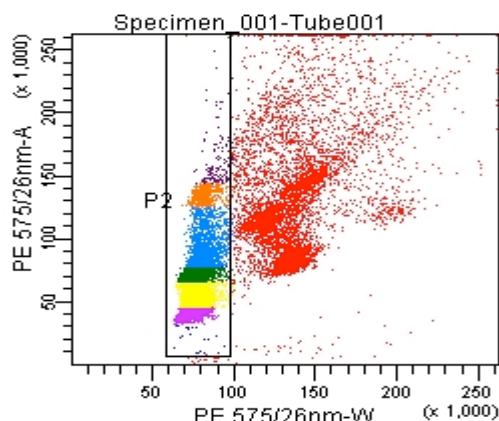
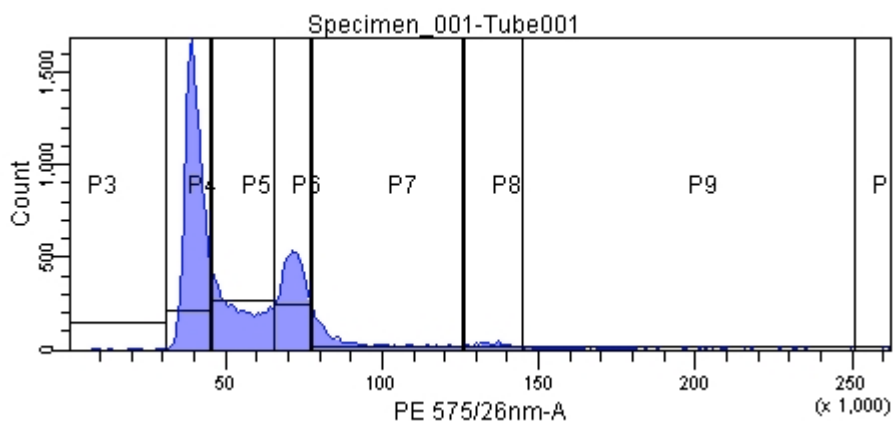


Fig 1b



Initial cell population gating is placed on FSC v SSC (cell size v granularity) (Fig1a). This cell population gate is then placed on PE 575/26nm-W(idth) v PE 575/26nm-A(rea) plot (Fig1b). Doublets appear to the right of single cell analysis gate P2. Single cell gate P2 is then displayed as a histogram using PE 575/26nm-A parameter (Fig2)

Fig2



Standard cell cycle analysis of the cell cycle profile is shown in Fig2 with gates P4, P5 and P6 gating on G₁, S phase and G₂m with 46%, 22% and 21% respectively.

PE-Texas Red 610/10nm Channel Cell Cycle Analysis

Fig 3a

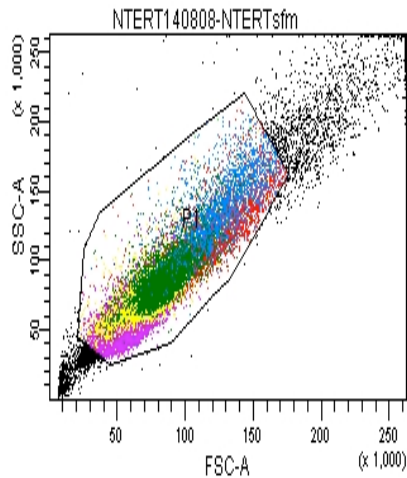


Fig 3b

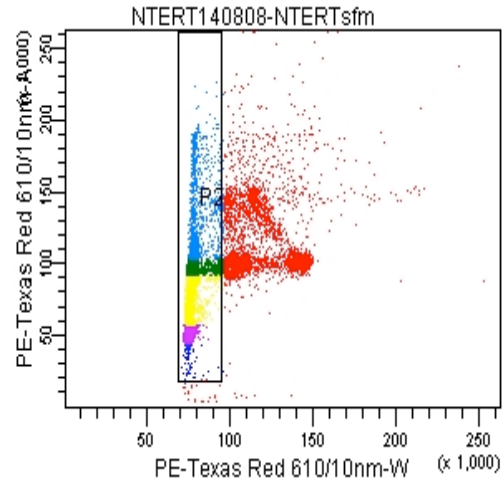
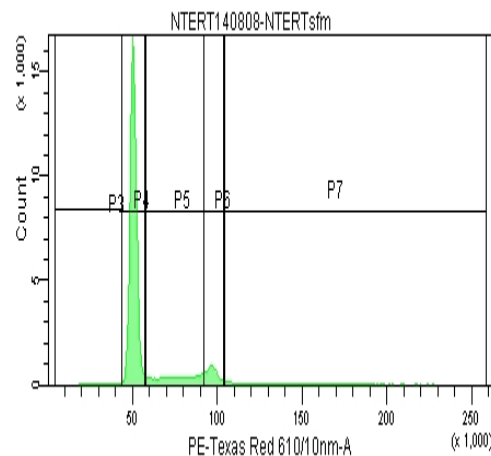


Figure 3 a and b shows the parameter display when using the PE-Texas-Red Channel for detection of PI.

Fig 4

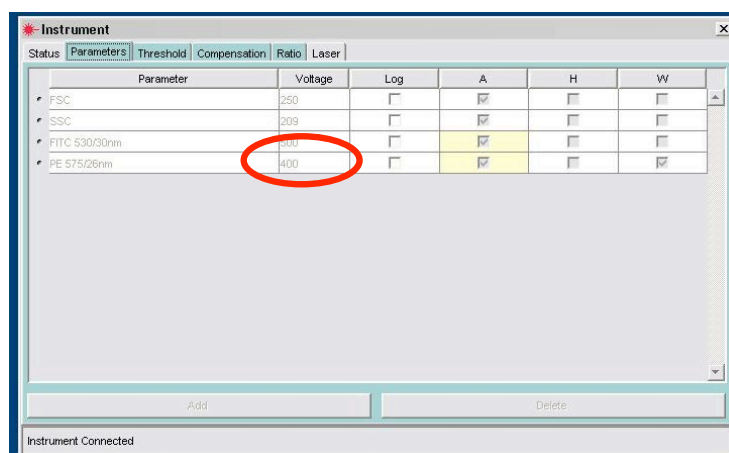


Standard cell cycle analysis of the cell cycle profile is shown in Fig2 with gates P4, P5 and P6 gating on G₁, S phase and G₂m with 78%, 13% and 8% respectively.

PI Voltage Adjustments

For clinical use of flow cytometric cell cycle analysis cells should be counted and the volume of PI solution adjusted so all samples have the same number of cells per volume. For easy analysis of research samples (not clinical) the voltage (see highlighted area in figure 5) on the PE 575/26nm-A or PE-Texas-Red 610/20-A can be altered from one sample to the next to place the G₁ in the same position for each sample. This allows the researcher to not move the gates when analysing the data making the data more reproducible, see Figure 5 below.

Fig 5



7-AAD Parameter Setup

The parameter setup when using 7-AAD is as described above but using the PE-Cy5 670/20 nm channel on the BD LSR II or the 650LP nm channel on the BD FACSCanto II.

To-Pro3 Parameter Setup

The parameter setup when using To-Pro-3 is as described above but using the APC 660/20 nm channel on the BD LSR II and the BD FACSCanto II.

DAPI & Hoechst 33342 Parameter & Analysis Setup

DAPI and Hoechst 33342 have similar excitation and emission profiles so the parameter setup is using the UV 440/40nm channel on the BD LSRII. This procedure cannot be carried out well on the violet 440/40nm channel on the BD LSRII or the BD FACSCanto II .

The analysis setup for DAPI is shown below in Figure 6 using the Area and Width parameters on the UV 440/40nm channel on the BD LSRII.

Fig 6a

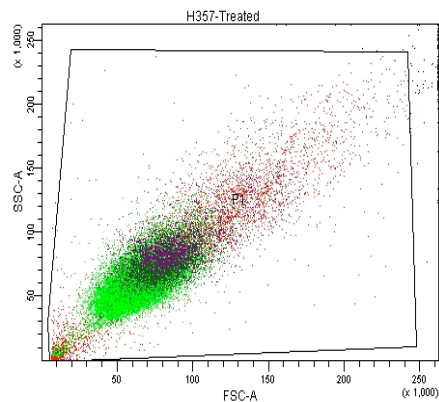
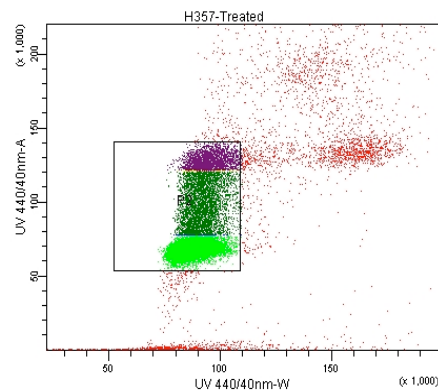
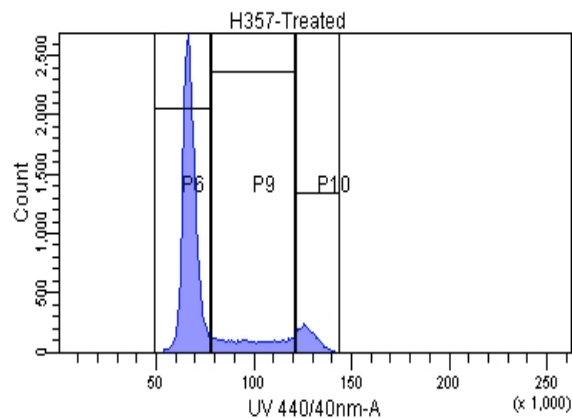


Fig 6b



The final analysis of the cell cycle profile is shown in Fig7 with gates P4, P5 and P6 gating on G₁, S phase and G₂m with 73%, 7% and 14% respectively.

Fig 7



DAPI & Hoechst 33342 Voltage Adjustments

For easy analysis of research samples the voltage on the UV 440/40nm-A can be altered from one sample to the next to place the G_1 in the same position for each sample. This allows the researcher to not move the gates when analysing the data making the data more reproducible.

Cell Cycle Analysis Software

Cell Cycle analysis of research samples can be adequately done by the straight linear gating as illustrated above. If the research requires more stringent analysis the Flow Cytometry Core Facility has deconvolution software that allows the investigator to calculate the area under the curve for G_1 , S phase and G_2 m. The tree Star inc software FlowJo has numerous special analysis platforms purpose made for flow cytometric analysis. The Cell Cycle Platform employs two mathematical models the Dean/Jett/Fox analysis and the Watson Pragmatic, see example below in figure 8a and b.

Fig 8a

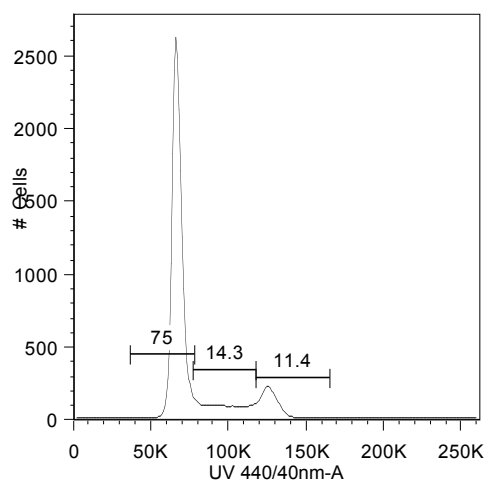


Fig 8b

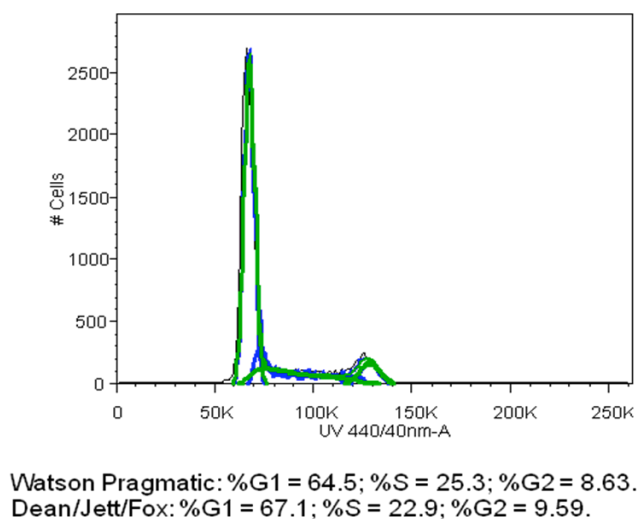


Figure 8a shows cell cycle data analysed in the standard manner. Figure 8b shows analysis by FlowJo models Watson Pragmatic and Dean/Jett/Fox algorithms.

Please note that no software models can analyse Sub G_1 data for estimations of apoptosis.