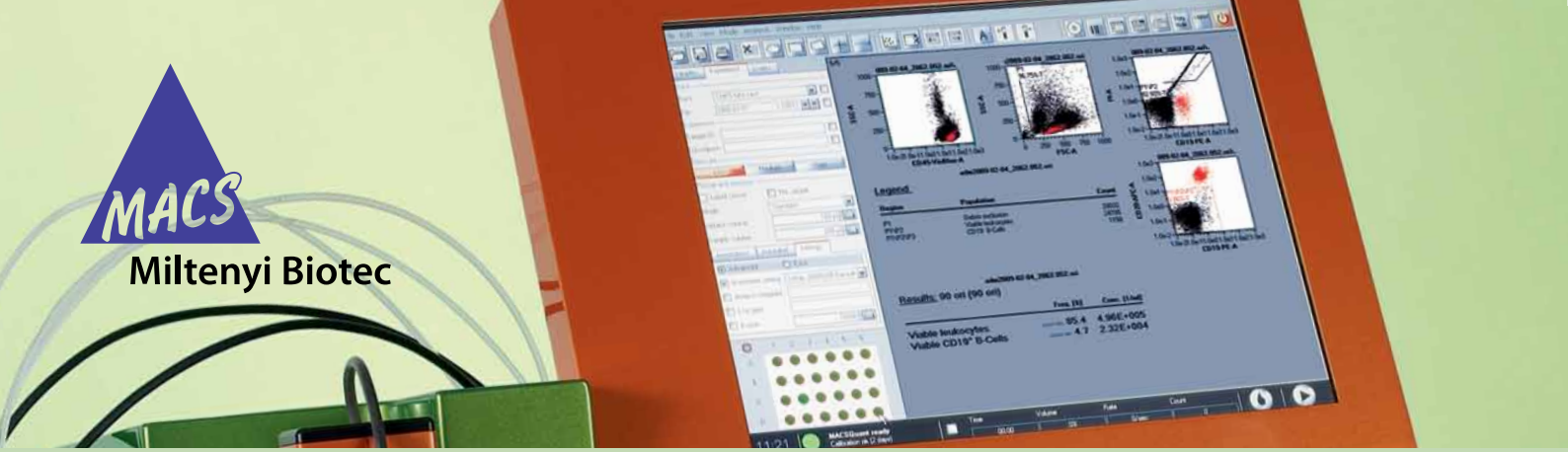

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Univariate cell cycle analysis using the MACSQuant® Analyzer

Cell cycle analysis

Background

Cell proliferation is characterized by four distinct phases: G1 phase (Gap1), S phase (DNA synthesis), G2 phase (Gap2) and M phase (mitosis). Non-proliferative or "resting cells" are referred to as being in G0 phase (Gap0). Over the last few years flow cytometry has been increasingly used to analyze cell cycle. This is achieved by performing either univariate analysis of deoxyribonucleic acid (DNA) content or multivariate analysis of DNA content and proteins associated with cell cycle. This application note discusses univariate cell cycle analysis.

Univariate analysis of cellular DNA

The fluorescent dyes propidium iodide (PI) or 4', 6'-diamidino-2-phenylindole (DAPI) bind to DNA stoichiometrically; that is, there is a direct correlation between the quantity of cellular DNA and the amount of DNA-bound probe[1]. Flow cytometry measures the fluorescence intensity of individual cells at a rate of several thousand cells per second. Using this approach a frequency distribution or histogram of DNA fluorescence can be displayed to show the G1/G0, S and G2/M cell cycle phases.[2, 3]

Materials and methods

Reagents and solutions

- Sample buffer (store at 4 °C): 1 g Glucose
  1 L Phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}; filter through a 0.22 µm filter
- Fixative: 70% ethanol, ice-cold
- Staining solution: Propidium iodide (50 µg/mL), RNase A (100 Kunitz units/mL) in sample buffer
  Prepare solution directly before use

Note: PI (order number: 130-093-233) is supplied at a concentration of 100 µg/mL.

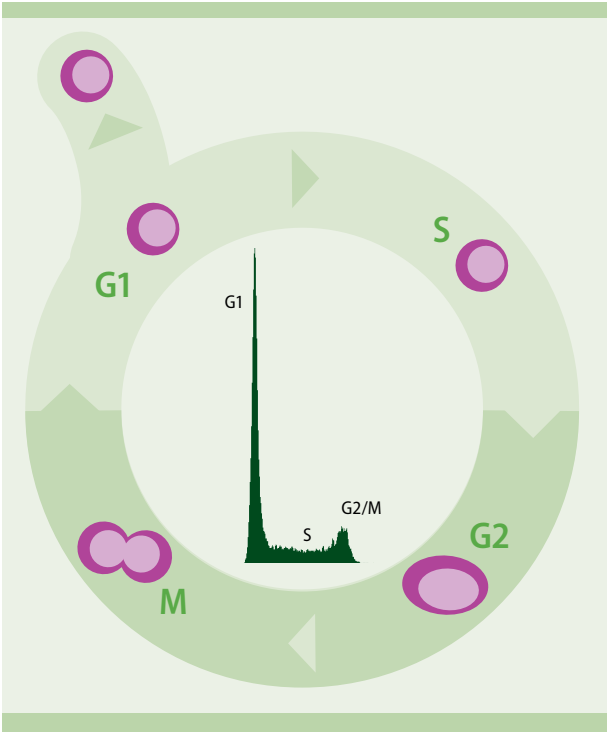

Figure 1: Cell cycle analysis by flow cytometry. G1/G0-phase cells are diploid (2N) and express half the DNA content of tetraploid G2/M phase cells (4N). S phase cells contain varying amounts of DNA between the G1 and G2 states. Average changes in DNA quantity are directly reflected by variations in fluorescence intensity as shown by the accompanying histogram.

Materials

- Monoclonal cell line (e.g. Jurkat cells)
- 15 mL conical tubes
- Centrifuge
- MACSQuant® Analyzer

Cell preparation

1. Determine cell number by performing an absolute cell count using the MACSQuant® Analyzer.
2. Centrifuge the harvested cell suspension at 300×g for 10 min at 4 °C. Aspirate supernatant completely.

Note: The centrifugation conditions can be modified in accordance with the cell type.

3. Resuspend the cell pellet in 1 mL sample buffer and wash the cells twice by adding 10–12 mL sample buffer for up to 1×10^7 cells.
4. Adjust the cell concentration to $1–3\times10^6$ cells/mL in sample buffer. Pipet 1 mL aliquots of the cell suspension into tubes for centrifugation.
5. Centrifuge the cells at 300×g for 10 min at 4 °C.
6. Aspirate supernatant without disturbing the pellet, e.g., leave approximately 0.1 mL/10^6 cells.
7. Vigorously vortex the pellet in the remaining buffer for approximately 10 s. Continue to vortex the cells and slowly add 1 mL of ice-cold 70% ethanol drop-by-drop to the pellet.
8. Cap the tubes and allow samples to fix in ethanol over-night (>18h) at 4 °C for maximum resolution of cellular DNA.

Staining

9. Prepare the staining solution (ingredients given above). **Caution:** Propidium iodide is a potential carcinogen. Use extreme care when handling. Wear nitrile gloves.
10. Briefly vortex the sample tube from step 8 and add 10 mL sample buffer.
11. Centrifuge at a higher speed than used previously (500×g for 10 min; 4 °C) and remove the tube with care. Aspirate the supernatant without disturbing the pellet.

Tip: Check to see that there is a visible pellet. If no pellet is visible, recentrifuge at a higher speed until one is seen. After ethanol fixation, cells require higher *g*-forces to form a pellet.

12. Gently vortex the tube to resuspend cells in residual buffer. Add 1 mL staining solution to each tube and vortex carefully. Incubate for 30–40 min at room temperature.

Tip: Gentle rocking will help to accelerate the staining process and ensure degradation of double-stranded RNA by RNase A.

13. Analyze the samples within 24 hours using the MACSQuant Analyzer.

Cell analysis using the MACSQuant® Analyzer

14. Open a new analysis window.
15. In the **Channels** tab, click **Advanced**. Activate the checkbox **Height**. Optional: Check **Width**.
16. Define the Experiment settings.

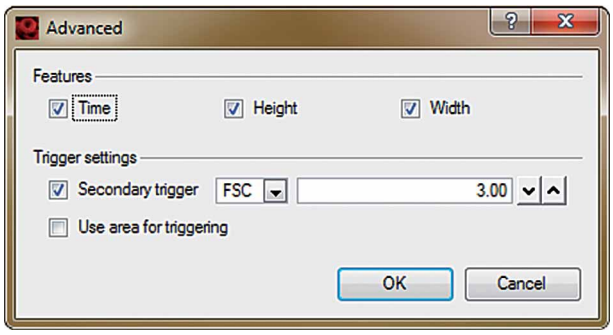

Figure 2: The parameter **Height** must be activated for cell cycle analysis. Optional: **Width** can also be activated.

17. Define the Instrument settings:
    - Set compensation to **None** (no compensation is required).
    - Set trigger to **FSC**. Ensure that the fluorescence channel **B3** is set to measure using linear acquisition.
    - Start acquisition.
    - Adjust the voltage-gains so that the peak modal value of cells in G1-phase is around 250 (see figure 5).

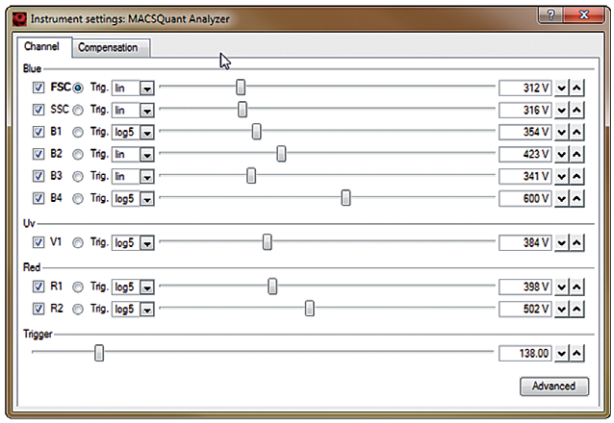

Figure 3: Example instrument settings for cell cycle analysis

Note: For optimal results it may be necessary to adjust the FSC and SSC voltage gain settings manually. It is recommended to acquire at least 10,000 events per sample.

Gating strategy

18. Draw a gating strategy as shown in figure 4 below.
    a. Draw region **P1** to exclude debris.
    b. Draw region **P1/Singlets** to exclude doublets from the analysis.

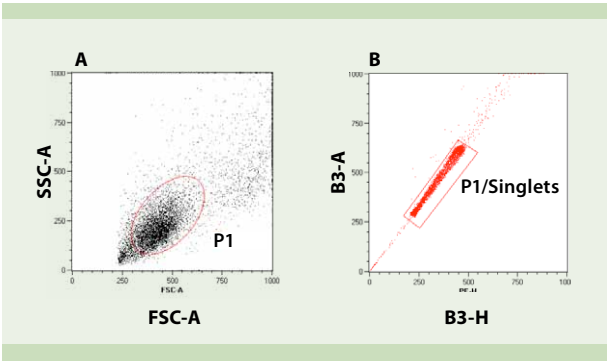

Figure 4: Cell cycle analysis of Jurkat cells A: Region P1 was drawn to exclude debris from the analysis. B: Doublet discrimination in a density plot of B3-Area vs. B3-Height.

What about doublets?

Doublets form when cells in close proximity are identified by the flow cytometer as a single event, i.e., cell coincidence or clumping during data acquisition. Therefore, a doublet may be falsely identified as a single tetraploid cell in G2/M-phase. Doublets can be discriminated by using two parameter measurements plotting pulsed-height (FL-H) against pulsed-area (FL-A).

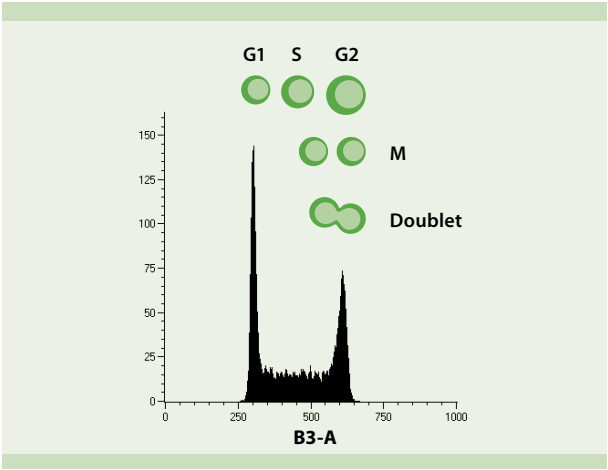

Figure 5: Diagram shows correlation between the stage of cell cycle and fluorescence intensity. Note that doublets could be misinterpreted as cells in G2- or M-phase.

Results

From a sample of Jurkat cells it was shown that 37.42%, 24.61% and 37.00% of the gated population (%-#) were in G0/G1-, G2/M- and S-cell cycle phases, respectively.

Region	%-#	Count	B3-A Mean	B3-A CV	B3-A Median
G0/G1	37.42	1849	304.54	3.10	302.95
G2/M	24.61	1216	604.95	2.37	606.30
S-phase	37.00	1828	453.74	15.22	452.75

Table 1: Statistical table summarizes results of the cell cycle analysis.

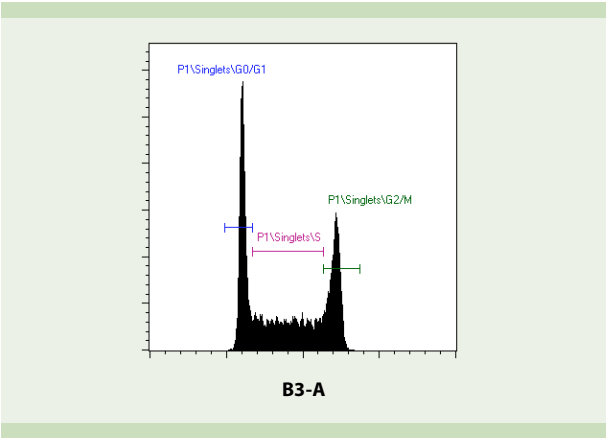

Figure 6: Cell cycle results displayed as a histogram. Intervals (⟼) were used to calculate statistics for table 1.

Conclusions

Cell cycle analysis can be accomplished with ease using the MACSQuant Analyzer in combination with MACSQuantify™ Software. For more information about additional cell analysis applications with the MACSQuant Analyzer, visit **www.macsquant.com**.

References

1. Darzynkiewicz, Z.; Huang, X., Analysis of cellular DNA content by flow cytometry. Curr Protoc Immunol 2004, Chapter 5, Unit 5 7.
2. Cunningham, R. E., Overview of flow cytometry and fluorescent probes for cytometry. Methods Mol Biol 1994, 34, 219–24.
3. Larsen, J. K.; Munch-Petersen, B.; Christiansen, J.; Jorgensen, K., Flow cytometric discrimination of mitotic cells: resolution of M, as well as G1, S, and G2 phase nuclei with mithramycin, propidium iodide, and ethidium bromide after fixation with formaldehyde. Cytometry 1986, 7, (1), 54–63.
4. Current Protocols in Cytometry Unit 5.7: Use of Flow cytometry for DNA Analysis. John Wiley & Sons, Inc., 1991.

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