Cell cycle analysis of human brain tumour stem cells using the Coulter DNA Prep reagents kit.

APPLICATION NOTE

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IN THIS PAPER YOU WILL

- Learn how to prepare cells from brain tissue
- Learn how to stain tissue for cell cycle analysis by flow cytometry
- Gate single events for clarity in discriminating cell cycle phases using the CytoFLEX flow cytometer

Principal of the Technique

Cell cycle analysis for quantification of DNA content is one of the earliest and most utilized applications by flow cytometry. Mammalian, yeast, plant and bacterial DNA can be stained by a variety of dyes that bind in proportion to the amount of DNA present in the cells [Fig 1]. DAPI, Hoechst 33342, DRAQ5, 7-AminoactinomycinD (7AAD), Vibrant Green, Sybr Green and Propidium Iodide (PI) are among the dyes frequently used. Dr. Awtar Krishan from the Harvard Medical School presented the first protocol using PI for cell cycle analysis in 1975 [1]. The protocol consisted of an alcohol fixation step followed by RNase treatment as PI also binds to double stranded RNA, and finally staining with an optimized concentration of PI. The total length of this procedure was approximately two hours.

The Coulter DNA Prep reagent kit is a fast and easy way to stain for DNA content using PI. The kit consists of two ready to use buffers: the LPR reagent which permeabilizes the cells (and lyses red blood cells if using whole blood) and the DNA staining solution which already contains RNase. The total length of this procedure is approximately 15 minutes.

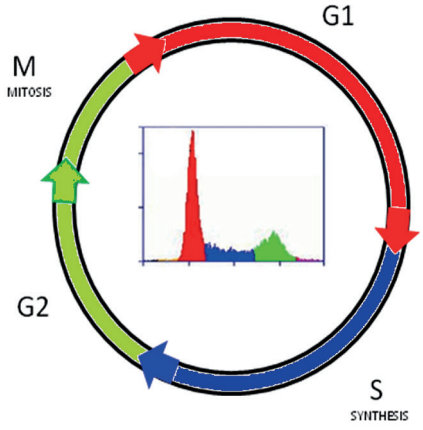

Figure 1:
DNA content increases as cell move from G1 to S to G2 phase of cell cycle. Cells in G2/M would be approximately twice as bright as cells in G1[1].

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Introduction

We previously identified a rare abnormal stem cell that drives the formation of brain tumours [2] and termed it brain tumour initiating cell (BTIC). Further characterization of BTICs has important implications in our understanding and treatment of brain tumours. Current diagnostic, genetic and therapeutic approaches focus on the bulk tumour rather than targeting the rare cancer stem cell, and this may explain the poor response to present treatment. BTICs that escape therapy may also metastasize to other organs.

Our serum-free culture conditions enrich for BTICs, allowing us to study the effects of genetic manipulation (i.e. up or downregulation of genes) and screen potential drug compounds on this usually rare population. Ultimately, our research may generate selective therapies that specifically target the BTIC, thereby improving the prognosis and survival of adults and children with brain tumours.

The cell cycle is a highly regulated series of events which leads to cell division and duplication. Monitoring cell cycle behavior *in vitro* may help our search for more targeted therapies.

In this study, we used the Coulter DNA prep reagents kit to assess the cell cycle of a metastatic brain tumour (BT478) and a recurrent medulloblastoma (MB) cell line (D458) in normal and BTIC-enriching culture conditions. Furthermore, we added two drug compounds to the MB cell line for 48 hours and observed the effect on the cell cycle.

Cell Culture

D458, a cell line derived from a recurrent human MB, is normally cultured in DMEM high glucose media supplemented with 20% FBS. Prior to analysis, the culture conditions were changed to BTIC enriching Neural Stem Cell (NSC) complete media for several days. The metastatic brain tumour BT478 was also cultured in NSC complete for several days before analysis.

Media preparation

NSC basal media Stock solution: 480 mL DMEM:F12, 5 mL N2 supplement, 5 mL HEPES 1M, 3.0 g Glucose, 1 mL N-acetylcysteine and 10 mL NSF-1

NSC complete media (made fresh prior to use): NSC basal media, 10 ng/mL EGF, 10 ng/mL bFGF, 5 ng/mL LIF and 10 µL/mL antibiotic-antimycotic

Drug treatment

1X10^6 cells MB BTICs in NSC complete media were treated with either DMSO (control), compound X or compound Y for 48h.

Sample preparation for flow

All tumour-spheres were enzymatically dissociated using liberase, cell counts adjusted to 1-3x10^6/mL and filtered through a 70 µm filter before staining with the Coulter DNA Prep reagents kit.

Sphere Dissociation[3]

Reagents required:

Liberase TM Research grade
Sterile PBS
Trypan blue
DNAse

Procedure:

1. Evaluate spheres under microscope: if there are many, consider splitting the culture into multiple tubes for dissociation
2. Transfer culture to 15 mL conical (Falcon or Corning) tube(s)
3. Add 2-3 mL sterile PBS to rinse plate and recover more spheres if necessary
4. Centrifuge at 1200 rpm for 5 minutes, remove supernatant and resuspend in 1- 2 mL sterile PBS
5. Add 10 µL liberase
6. Incubate in 37^{0}C water bath for 5 minutes. Remove and visually evaluate spheres, if multiple clumps seen, pipette up and down with a 1000 µL pipette tip.
7. If clumps are still visible after an additional 5 minutes, add another 10 µL of liberase and return to water bath for 2-3 minutes
8. Centrifuge at 1200 rpm for 5 minutes
9. Remove supernatant and resuspend in 1-2 mL sterile PBS
10. Add 2 µL DNAse to 1 mL cells in PBS and incubate in 37^{0}C water bath for 5 minutes.
11. Add 5 mL sterile PBS and centrifuge at 1200 rpm for 5 minutes
12. Remove supernatant and resuspend pellet
13. Transfer 10 µL of cell suspension into 10 µL trypan blue dye in an eppendorf, mix well
14. Transfer 10 µL to counting slide and perform cell count on Countess (or a ViCell)
15. Adjust cell count in appropriate buffer or media if necessary for specific purpose, i.e. 3 million/mL for cell cycle analysis

DNA Prep Reagent Kit

Materials/equipment required

Coulter DNA Prep Reagents Kit
Single cell suspensions @ 1-3x10^6/mL
CytoFLEX

Staining Protocol:

1. Transfer 100 µL of cell suspension into a 12x75 mm polypropylene tube
2. Add 100 µL of LPR to 100 µL single cell suspension and vortex for 10 seconds
3. Add 2 mL of DNA Prep Stain and vortex for 10 seconds
4. Leave at room temperature for 15 minutes, analyze

List of reagents

Reagent	Company	Cat#
DNA Prep Reagent Kit	Beckman Coulter	6607055
Dulbecco's PBS	Gibco	14190-144
Liberase TM Research Grade	Roche	05 401 119 001
NH_4Cl RBC Lysis Buffer	Stem Cell Technologies	07850
DMEM high glucose	Invitrogen	11965-118
Serum	Seradigm	1400-500
DMEM:F12	Invitrogen	11320-082
DMEM high glucose	Invitrogen	11965-092
Hyclone FBS	Thermo Scientific	SH30396.03
N-2 supplement	Invitrogen	17502-408
1M HEPES	Wisent	330-050
Glucose	Invitrogen	15023-021
N-acetylcysteine	Sigma	A9165
NSF-1	Lonza	CC-4323
EGF Recombinant Human Protein	Invitrogen	PHG0311
FGF Basic Human recombinant	Invitrogen	PHG0261
Leukemia inhibitory Factor	Cedarlane	LIF1010
Ultra pure EDTA 0.5M	Gibco	15575
DNAse vial (D2)	Worthington biochemical	LK003170
Flow-Check™ Pro Fluorospheres	Beckman Coulter	A69183
Antibiotic-antimycotic	Wisent	450-115-EL

Results

For flow cytometric analysis, a dual-region gating strategy was utilized: an initial region on FS and SS was used to eliminate debris (Fig 2A) and a second region on FL3 area (PI-A) versus FL3 height (PI-H) to eliminate doublets (Fig 2B); both regions are applied for DNA content histograms.

Cell cycle analysis was performed using the Dean-Jett-Fox algorithm of FlowJo 10 software (Fig 3A). The percentage of cells in each phase of the cell cycle is thus calculated for the metastatic brain tumour sample (Fig 3B). The cell cycle of the recurrent MB cell line D458 under normal DMEM-FBS culture conditions (Fig 4A) was compared to BTIC enriching serum-free (NSC) media conditions (Fig 4B). Treatment of D458 with compound X (Fig 4C) did not appear to affect cell cycle at 48 hours, but cell cycle arrest in the G2/M phase was observed following treatment with compound Y (Fig 4D).

Neurosphere cultures are often difficult to grow and challenging to analyse by flow cytometry due to their tendency to aggregate. The DNA prep reagents kit provides us with an easy and efficient method to evaluate cell cycle and is a valuable tool in our quest to a better understanding of BTICs.

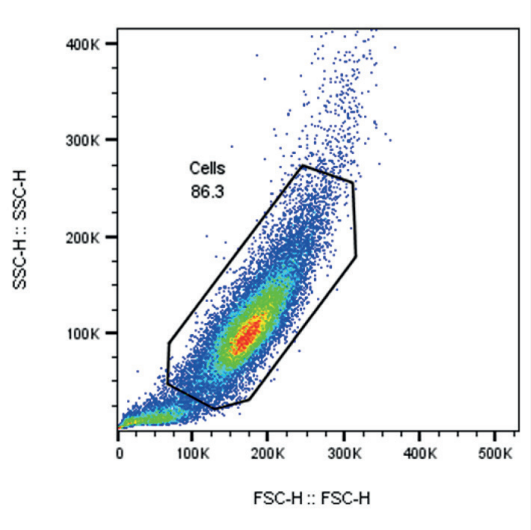
References

1. Krishan A. The Journal of Cell Biology 1975;66:188-193
2. Singh SK, et al. Nature 2004;432(7015):396-401
3. Venugopal C, et al. Journal of Visual Experimentation 2012;25:(67).

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the authors cannot guarantee a similar appearance with the use of other flow cytometers.

A



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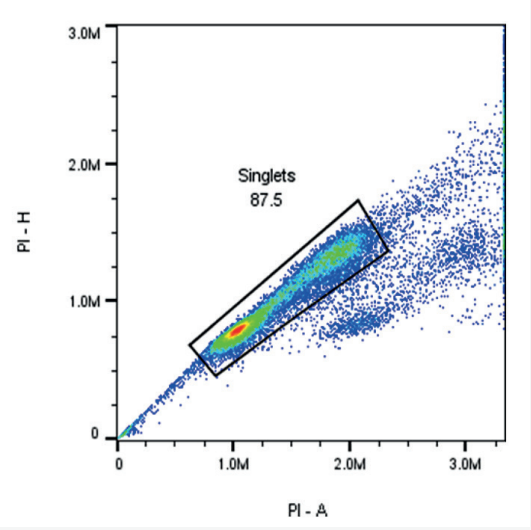
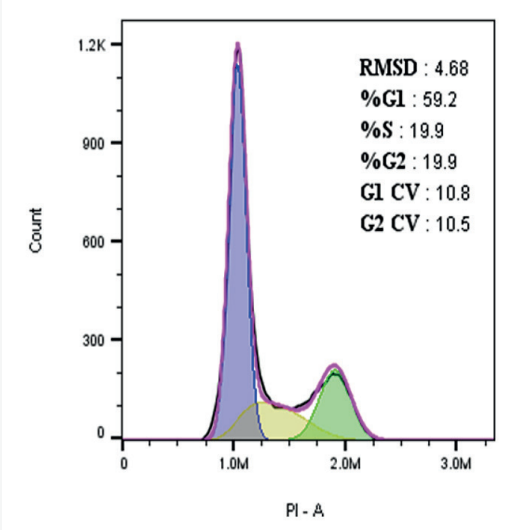


Figure 2. Flow Cytometry gating strategy:
An initial region (Cells) was set on FS and SS to eliminate debris (A).
A second region (Singlets) was set on PI-A vs PI-H to eliminate doublets (B).
Cells resulting from these two regions were further analyzed for cell cycle distribution.

A



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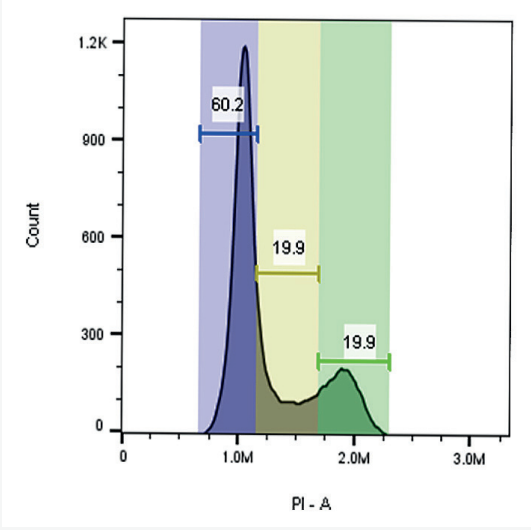

Figure 3. Cell Cycle analysis:
Cell cycle analysis of each sample was performed using FlowJo 10. The Dean-Jett-Fox algorithm was used to calculate the percentage of cells in each phase of the cell cycle (A).
The example above represents the cell cycle distribution of BT478 , a metastatic brain tumour grown in NSC complete medium. (B)
G0-G1 phase (blue), S phase (light brown and G2-M phase green).

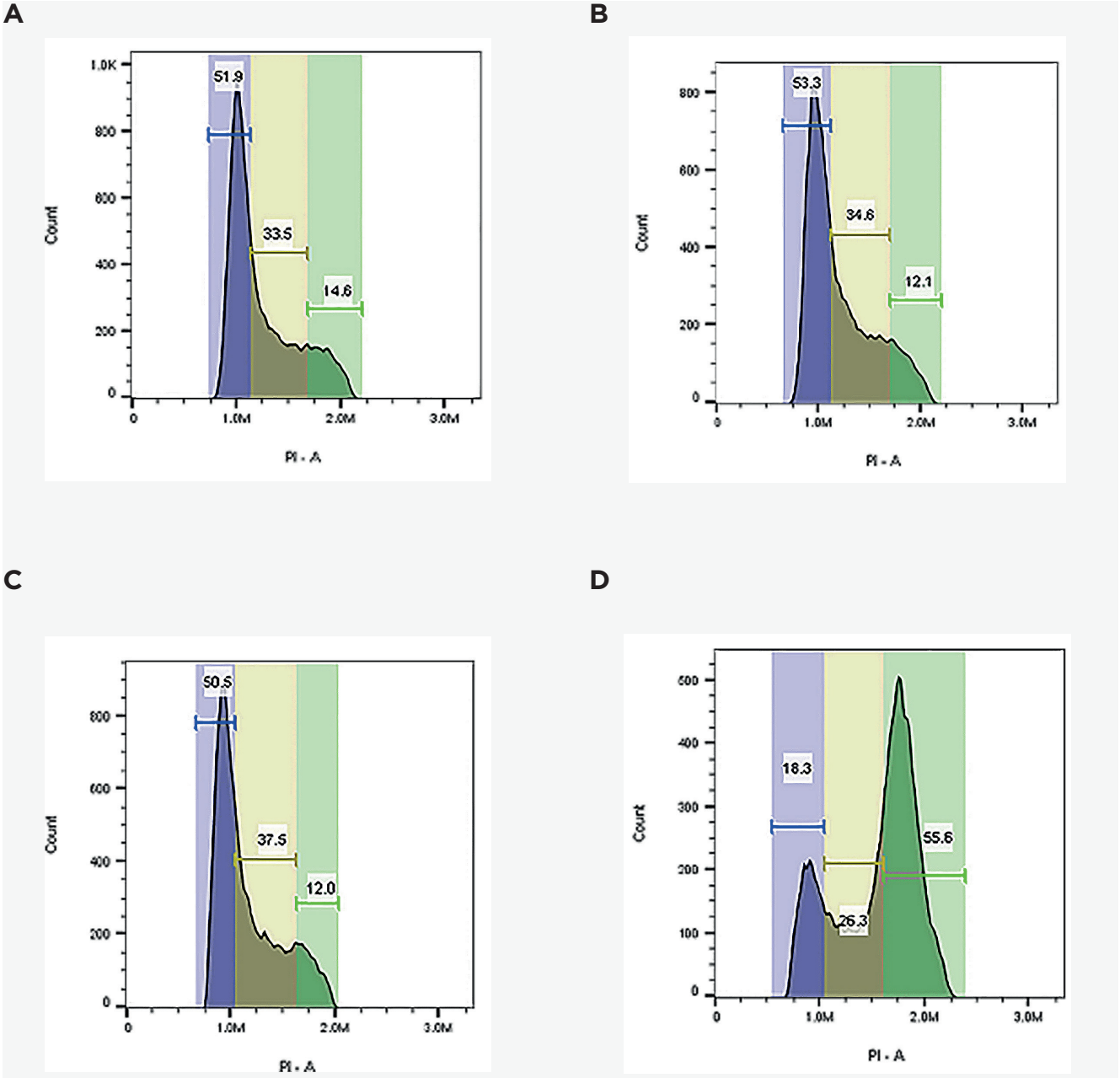

Figure 4. Cell cycle analysis of D458, a recurrent meduloblastoma cell line.
(A) D458 in normal media conditions.
(B) D458 in BTIC enrichment condition (NSC medium).
(C) D458 treated with compound X for 48h.
(D) D458 treated with compound Y for 48h.