Identification and Characterization of Brain Tumor Initiating Cells (BTICs) using the Beckman Coulter CytoFLEX

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- How to prepare single cell suspension from brain tumor
- Staining and appropriate flow cytometry controls for immunophenotyping brain tumor cells
- Gating strategy to identify brain tumor initiating cells

Background

Glioblastoma (GBM), the most common primary brain tumor in adults, is one of the most aggressive human cancers, feared for its near uniformly fatal prognosis. Surgery, conventional chemotherapy and radiation have proven ineffective in eradicating this tumor, leading to inevitable tumor re-growth and patient relapse, and a median survival of <15 months.

A rare subset of brain tumor initiating cells with stem cell properties, termed BTICs, are known to resist current chemo and radiation therapy regimens. Current diagnostic, genetic and therapeutic approaches to brain tumors focus on every cell in the tumor, rather than the rare cancer stem cell. Higher fractions of BTICs escaping therapy may explain why some patient tumors, initially responsive to chemo-radiotherapy, aggressively relapse.

In our lab, we have selected four markers to compare expression patterns of BTICs from naïve and in vitro chemo- and radiation- treated GBM samples: CD133, a transmembrane glycoprotein which identifies a stem cell like population having the ability to self-renew, differentiate and recapitulate the original tumor when injected into the brains of immune-compromised mice [1]; CD15, a surface marker which also identifies a stem cell like population capable of recapitulating the original tumor in xenografts [2]; Bmi1, a polycomb group protein and master regulator of NSCs, implicated in brain tumor progression and maintenance and finally, Sox2, a neuro-developmental transcription factor thought to induce a BTIC state and sustain tumor propagation [3].

Evaluating the presence and co-expression patterns of CD15-CD133-Bmi1-Sox2 in tumors that are treatment-naïve and those that have evolved over the course of treatment will enable us to expand our understanding of the cellular diversity observed in brain tumors and thereby develop targeted therapies toward unique clones that persist over the course of treatment. Instruments such as the Beckman Coulter CytoFLEX will prove invaluable in completing this proposal as we aim to improve the survival and prognosis of children and adults with brain tumors.

In this study, we have established a protocol for these four markers in a primary naïve human GBM and a normal human neural stem cell samples, using the Beckman Coulter CytoFLEX.

Protocol

Upon arrival in the lab, all brain samples are mechanically disaggregated with scissors or scalpels, followed by enzymatic digestion with Liberase Blendzyme. The cells are placed into serum-free neural stem cell media and left undisturbed for several days with media top-ups as required, after which media is changed as necessary. Neural stem cells will soon form neurospheres which are ultimately dissociated with Liberasee and filtered before staining for flow cytometry analysis. The addition of DNAse is helpful with samples that tend to clump. Final concentration is adjusted to $1x10^6$/mL and surface staining is performed on 100 µL aliquots in 12x75 mm tubes.

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Detailed protocol can be found in reference number 4.
Full list of reagents can be found in Table 1.

Media

NSC basal media Stock solution (500 mL)	
DMEM:F12	480 mL
N2 supplement	5 mL
1M HEPES	5 mL
Glucose	3.0 g
N-acetylcysteine	1 mL
NSF-1	10 mL

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

Culture of normal and brain tumor tissues

Reagents

Sterile PBS
Liberase
Red Blood Cells (RBC) lysis buffer
NSC complete media

- Add 200 µL thawed Liberase to 15 mL of PBS, place into 37°C water bath.
- Place RBC lysis buffer in 37°C water bath.
- In sterile biological safety cabinet, wash specimen in container with PBS to remove excess red blood cells.
- Transfer specimen to a sterile petri dish.
- Using fine scissors or scalpels and forceps, disaggregate tissue to slurry consistency.
- Collect sample with 10 mL regular pipette or forceps and transfer fragments into pre-warmed PBS/Liberase.
- Place on incubator-shaker and set to 37°C, for 15 minutes at speed 30 RPM.
- Filter through cell strainer (70 µm).
- Spin cells down at 1200 RPM for 5 minutes.
- Remove supernatant, evaluate cell pellet (size & presence of RBC).
- Resuspend pellet in 1 mL PBS.
- Add an appropriate amount of RBC lysis buffer (4-12 mL based on RBC).
- Incubate at room temperature for 5 minutes.
- Spin cells down at 1200 RPM for 5 minutes; evaluate cell pellet.
- Wash once with 5-10 mL of sterile PBS (based on pellet size).
- Resuspend in 10 mL NSC complete medium and transfer to 10 cm ultra-low plate.
- For the first week of culture, do not change the media: top-up only with 1-2 mL if cells need more media.
- Continue to observe culture and change media as necessary.

Sphere Dissociation

Reagents required

Liberase
Sterile PBS
Trypan blue
DNAse

Procedure

- Evaluate spheres under microscope: if there are many, consider splitting the culture into multiple tubes for dissociation.
- Transfer culture to 15 mL conical (Falcon or Corning) tube(s).
- Add 2-3 mL sterile PBS to rinse plate and recover more spheres if necessary.
- Centrifuge at 1200 RPM for 5 minutes.
- Remove supernatant and resuspend in 1-2 mL sterile PBS.
- Add 10 µL Liberase.
- Incubate in 37°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. Return to water bath for an additional 5 minutes.
- If aggregates are still visible, add 10 µL more Liberase and return to water bath for 2-3 minutes.
- Centrifuge at 1200 rpm for 5 minutes.
- Remove supernatant and resuspend in 1-2 mL sterile PBS.
- Add 12 µL DNAse to 1 mL cells.
- Incubate in 37°C water bath for 5 minutes.
- Add 5 mL sterile PBS.
- Centrifuge 1200 RPM for 5 minutes.
- Remove supernatant and add 1 mL PBS.
- If passaging, resuspend in media and transfer to culture dish.
- If performing cell count, resuspend in sterile PBS to obtain desired turbidity.
- Transfer 10 µL of cell suspension into 10 µL trypan blue dye in an Eppendorf, mix well.
- Transfer 10 µL to counting slide and perform cell count on Countess.
- Adjust cell counting appropriate buffer or media if necessary for specific purpose, i.e. $1x10^6$ cells/mL for flow cytometry assays.

Staining protocol for flow cytometry analysis

Reagents required

Fixable Live/Dead Infra Red stain
BD Cytofix/Cytoperm fixation permeabilization kit
PBS+EDTA (PBS + 2 mM EDTA)
Flow antibodies

Laser	405 nm					488 nm					638 nm		
Fluor	KrO	V450	V610	V660	V780	FITC	PE	ECD	PC5	PC7	APC	APC AF700	APC AF750
Marker		Anti-Sox2				Bmi1	CD15				CD133/2		L/D
Clone											293-C3		Near-IR

Surface followed by internal staining

- Add 1 µL Live/Dead IR stain per $1x10^6$ cells/mL .
- Incubate on ice for 20 minutes.
- Meanwhile, add external antibodies/isotype controls to labeled flow tubes.
- Wash cells once with equal volume of PBS-EDTA (centrifuge at 1100 RPM for 3 minutes) decant and blot.
- Resuspend in sufficient PBS-EDTA to have 120 µL per test tube required (i.e. ~$1.2x10^5$ cells).
- Transfer 120 µL of cell suspension into labeled flow tubes with antibodies/isotype controls.
- Incubate 15 minutes at room temperature (in dark).
- Add 1 mL PBS-EDTA to each flow tube, centrifuge at 1100 RPM for 3 minutes, decant and blot and resuspend well.
- Add 250 µL fix-perm solution to each tube, incubate 20 minutes on ice.
- Wash once with 1 mL perm/wash buffer.
- Resuspend in 100 µL perm/wash buffer and add appropriate amounts of intracellular antibody or isotype control.
- Incubate 30-45 minutes on ice.
- Wash once in 1-2 mL perm/wash buffer.
- Resuspend in 250 µL PBS+EDTA.

Equipment required

Centrifuge
Pipettes (1000, 200, 20, 2 µL) and sterile tips
MoFlo XDP
CytoFLEX

Table 1. List of reagents

Reagent	Company	Cat#
CD133/2 (293-C3) APC	Miltenyi Biotec	130-090-854
IgG2b APC	Miltenyi Biotec	130-092-217
CD15 PE	Beckman Coulter	IM1954U
IgG2a PE	Beckman Coulter	A09141
Bmi1 FITC	R&D systems	IC33341F
IgG2a FITC	R&D systems	IC003F
Mouse anti Sox2 V450	BD Biosciences	561610
MsIgG1 V450	BD Biosciences	560373
Dulbecco's PBS	Gibco	14190-144
BD Cytofix/Cytoperm	BD Biosciences	554714
Liberase TM Research Grade	Roche	05 401 119 001
Live/dead Fixable near-IR	Invitrogen	L10119
NH4Cl RBC Lysis Buffer	Stem Cell Technologies	07850
DMEM:F12	Invitrogen	11320-082
N2 supplement	Invitrogen	17502-408
1M HEPES	Wisent	330-050
Glucose	Invitrogen	15023-021
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
BD CompBeads particle set	BD Biosciences	552843
Antibiotic-antimycotic	Wisent	450-115-EL

Results

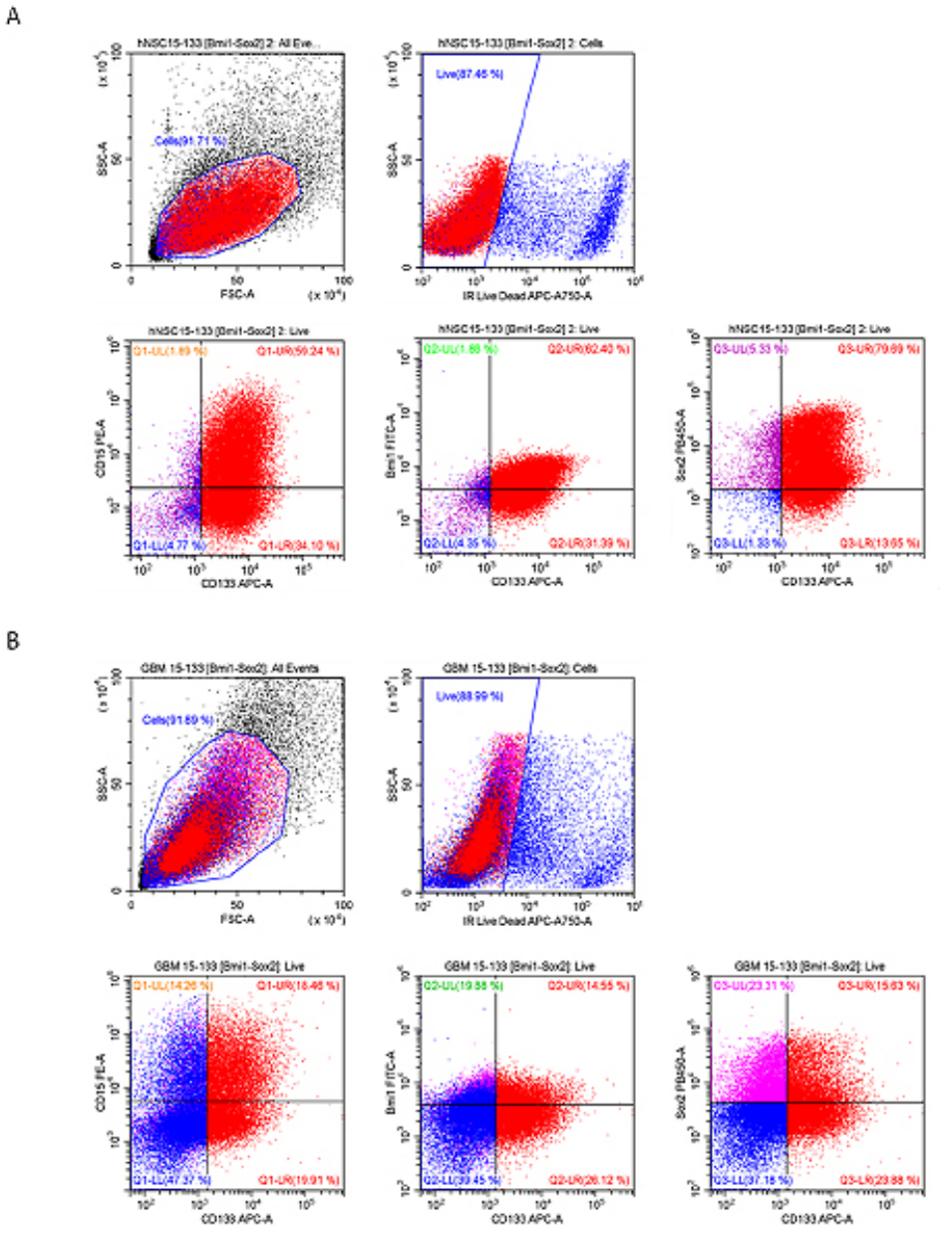

Figure 1 - Expression patterns of CD15, CD133, Bmi1 and Sox2 on hNSCs
(A) and primary human GBM
(B): initial gate is applied to Forward and Side Scatter to eliminate debris and doublets; dead cells are eliminated based on IR Live/Dead viability dye; all dot plots are gated on both cells and live.
Compensations values are calculated using BD Compbeads. Final gating is adjusted using isotype controls.

References

1. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD and Dirks PB. Nature 2004;432:396-401.
2. Son MJ, Woolard K, Nam DH, Lee J, Fine HA. SSEA-1 is an enrichment marker for tumor-inititating cells in human glioblastoma. Cell stem cell 2009;4:440-452
3. Suva ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD, Riggi N, Chi AS, Cahill DP, Nahed BV, Curry WT, Martuza RL, Rivera MN, Rossetti N, Kasif S, Beik S, Kadri S, Tirosh I, Wortman I, Shalek AK, Rozenblatt-Rosen O, Regev A, Louis DN and Bernstein BE. Cell 2014;157:580-594.
4. Venugopal C, McFarlane NM, Nolte S, Manoranjan B, Singh SK. Journal of visualized experiments : JoVE 2012.

Authors Notes

Not all brain tumor samples can be successfully cultured in vitro. If we observe that a sample has not progressed within a few days, we try culturing on poly-ornithine and laminin-coated plates.

Enzymatically digested tissue and neurosphere cultures may tend to aggregate over a short period of time, which makes them unsuitable for flow cytometry. The addition of DNAse after neurosphere dissociation with Liberase appears to reduce aggregation in some cases and allows for cytometric analysis as well as sorting.

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 author cannot guarantee a similar appearance with the use of other flow Cytometers.

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