Optimisation of viability assay for DIPG patient-derived cell lines

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Background:

Evaluation of the efficacy of M4K compounds in DIPG patient-derived cell lines is essential before any promising compounds can be further tested in mouse xenograft models. This approach can aid in narrowing down clinical compound candidates and reduce the time, resources and animal sacrifice needed downstream.

A robust and efficient readout for the changes in the viability of the DIPG cells needs to be established before it can be used to evaluate the M4K compounds. In addition, the amount of cells to be seeded at the beginning of the experiment has to be optimised to avoid overcrowding and starvation of the cells after extended culture times. Overcrowding and starvation will lead to increased cell death and prevent accurate estimation of the potency of M4K compounds (EC50).

Experimental design:

The wells of 96-well plate with transparent bottom will be coated with laminin to synchronise the mode of growth (adherent or non-adherent) among the different patient-derived lines. Additionally, this will promote even distribution of cells and allow most cells to be in focus when images are taken.

An increasing cell number (125 to 20000) will be seeded into each well. The amount of cells after 7 days of growth in 100µl of medium will be measured based on the area occupied and the total amount of ATP present in each well. In addition, propidium iodide which stains dead cells will be added at the beginning the experiment. At seeding number that are not saturating, the amount of cells after 7 days should remain proportional to the initial seeding number.

Figure 1. The timeline of this experiment.

Detailed protocol:

Medium composition

Tumour Stem Medium (TSM) Base

50% Neurobasal-A Medium (1X) (Thermofisher 10888022)

50% D-MEM/F-12 (1X) (Thermofisher 11320074)

10mM HEPES Buffer (Thermofisher 15630056)

1mM Sodium Pyruvate MEM (Thermofisher 11360039)

0.1mM MEM Non-Essential Amino Acids Solution (Thermofisher 11140035)

1X GlutaMAX-I Supplement (Thermofisher 35050038)

1X Antibiotic-Antimycotic (Thermofisher 15240062)

Additional components for TSM complete

1X B-27 Supplement Minus Vitamin A (Thermofisher 12587010)

20ng/ml Recombinant Human EGF (Peprotech AF-100-15)

20ng/ml Recombinant Human FGF-basic (Peprotech AF-100-18B)

10ng/ml H-PDGF-AA (Peprotech 100-13A)

10ng/ml H-PDGF-BB (Peprotech 100-14B)

2μg/ml Heparin (Sigma H3149-10KU)

Cell lines to be used

HSJD-GBM-02	Glioblastoma
SU-DIPG-IV	DIPG mutant ALK2
SU-DIPG-06	DIPG WT ALK2
HSJD-DIPG-07	DIPG mutant ALK2
HSJD-DIPG-08	DIPG WT ALK2
HSJD-DIPG-11	DIPG WT ALK2
SU-DIPG-XXI	DIPG mutant ALK2

Plate layout

		Set 1 for Cell TiterGlo																					
Dilution				HSJD-GBM-02			SU-DIPG-IV			SU-DIPG-06			HSJD-DIPG-07					HSJD-DIPG-11			SU-DIPG-XXI		
TSM-complete		Cells/well		1	2	3	4	5	6	7	8	9	10	11	12			1	2	3	4	5	6
0ul	1500ul	20000	A														A						
750ul	750ul A	10000	B														B						
750ul	750ul B	5000	C														C						
750ul	750ul C	2500	D														D						
1125ul	750ul D	1000	E														E						
750ul	750ul E	500	F														F						
750ul	750ul F	250	G														G						
750ul	750ul G	125	H														H						
		Set 2 for Propidium Iodide/Hoerchst/Calcein-AM staining																					
				HSJD-GBM-02			SU-DIPG-IV			SU-DIPG-06			HSJD-DIPG-07					HSJD-DIPG-11			SU-DIPG-XXI		
				1	2	3	4	5	6	7	8	9	10	11	12			1	2	3	4	5	6
		20000	A														A						
		10000	B														B						
		5000	C														C						
		2500	D														D						
		1000	E														E						
		500	F														F						
		250	G														G						
		125	H														H						

Before starting (Day 1)

1) Prepare 100ml TSM complete and store at 4 degree Celsius.

2) Pre-coat wells of clear-bottom black 96-well plate (Greiner 655090) with 50µl of laminin diluted 1:100 in DPBS (final concentration of 0.01mg/ml) overnight at 37 degree Celsius.

3) Prepare 5mM (1000X) Propidium Iodide stock solution for dead cell staining by reconstituting lyophilised powder in appropriate volume of sterile water

Preparation of single cell suspension (Day 2)

For suspension DIPG cells (all except HSJD-DIPG-008 and SU-DIPG-IV)

1) Collect all cells in a 50ml tube.

2) Pellet cells at 300xG for 5 minutes.

3) Save supernatant in a separate tube.

4) Resuspend cell pellet in 1ml of TrypLE express and incubate at 37 degree Celsius for 5 minutes.

5) Add supernatant to the resuspended cells and pipette up and down 5 times to break up clumps.

6) Pellet cells at 300xG for 5 minutes.

7) Discard supernatant and dislodge cell pellet by tapping.

8) Resuspend cells in 10ml TSM-complete.

9) Pass cell suspension through cell strainer to remove clumps.

10) Count cells and adjust to desired concentration.

For adherent DIPG cells (HSJD-DIPG-008 and SU-DIPG-IV)

1) Transfer medium and dislodged cells to a 50ml tube.

2) Incubate flask with TrypLE express at 37 degree Celsius for 5 minutes (1ml for T25 flask and 3ml for T75 flask).

3) Add saved medium and dislodged cells back to the flask and collect everything in a 50ml tube.

4) Pipette up and down 5 times to break up clumps.

5) Pellet cells at 300xG for 5 minutes.

6) Discard supernatant and dislodge cell pellet by tapping.

7) Resuspend cells in TSM-complete.

8) Pass cell suspension through cell strainer to remove cell clumps.

9) Count cells and adjust to desired concentration.

Seeding cells into 96-well plate

1) Add 1µl of 5uM Propidium Iodide per ml of single cell suspension.

2) Prepare serial dilutions of different cell lines.

3) Wash wells once with 100µl DPBS right before adding cells.

4) Incubate plates in a lunchbox with extra humidification to minimise evaporation in 37 degree Celsius incubator with 5% carbon dioxide for 7 days.

Image-based quantification (Day 9 – after 7 days in culture)

1) Acquire images of all of the wells using Celigo imaging cytometer.

2) Imaging channels:

Confluency: brightfield

Propidium Iodide (dead cells): λ_{ex} ~530nm, λ_{em} ~620nm

End point CellTiter Glo 3D ATP measurement (Day 9 – after 7 days in culture)

1) Thaw CellTIter Glo reagent in 4 degree Celsius fridge overnight and aliquot into 10ml fractions. Extra tubes were frozen again at -20 degree Celsius. 20ml is required for this experiment.

2) Add 100µl of room temperature CellTiter-Glo reagent to each well and shake at 200rpm for 5 minutes.

4) Incubate in the dark at room temperature for further 25 minutes without shaking.

5) Measure total luminescent signal using Clariostar plate reader.

Results:

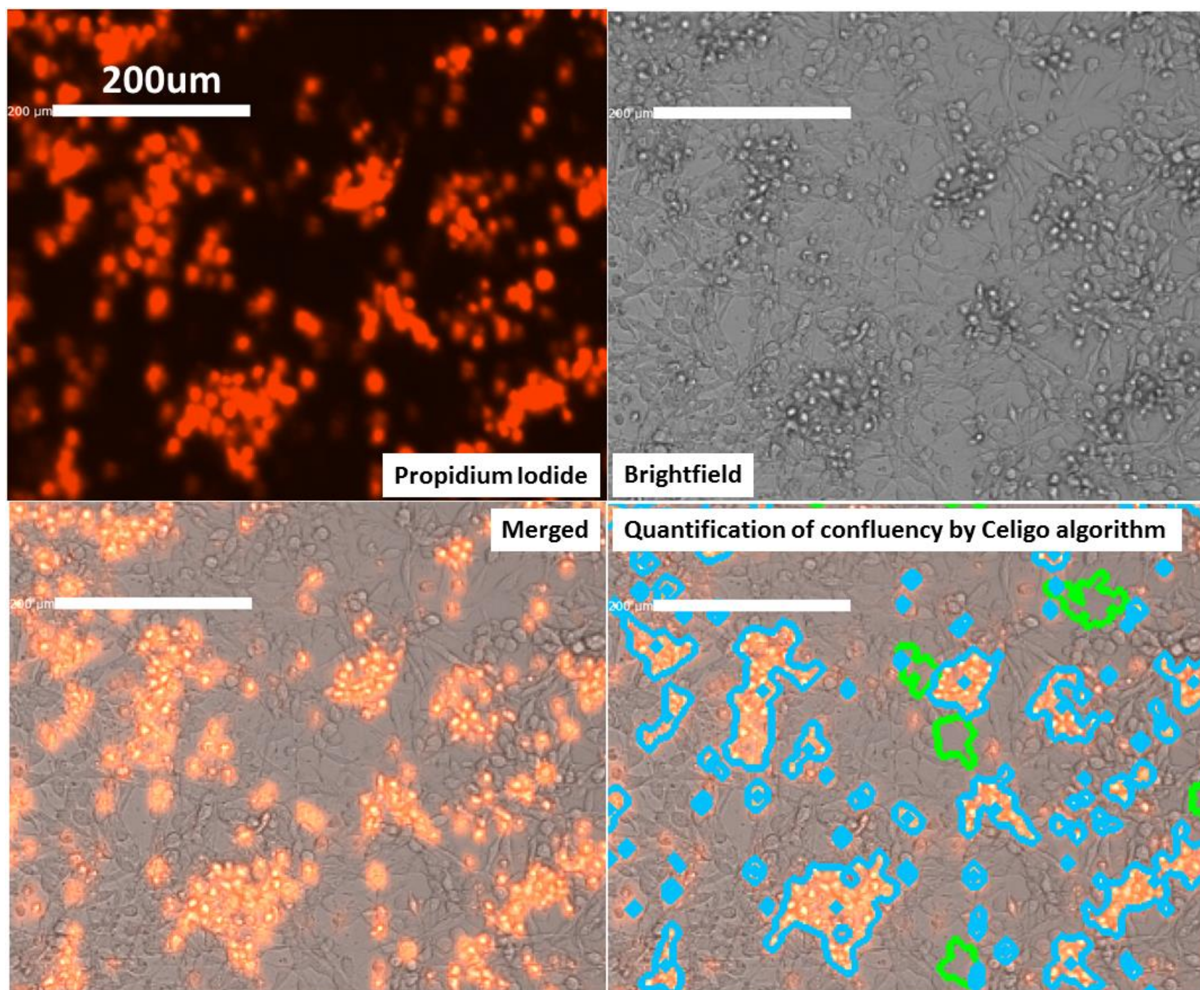

Figure 2. Demonstration of how Celigo algorithm quantifies the area occupied by total cells and area occupied by propidium iodide signal. Images of HSJD-DIPG-007 cells after 7 days in culture were used.

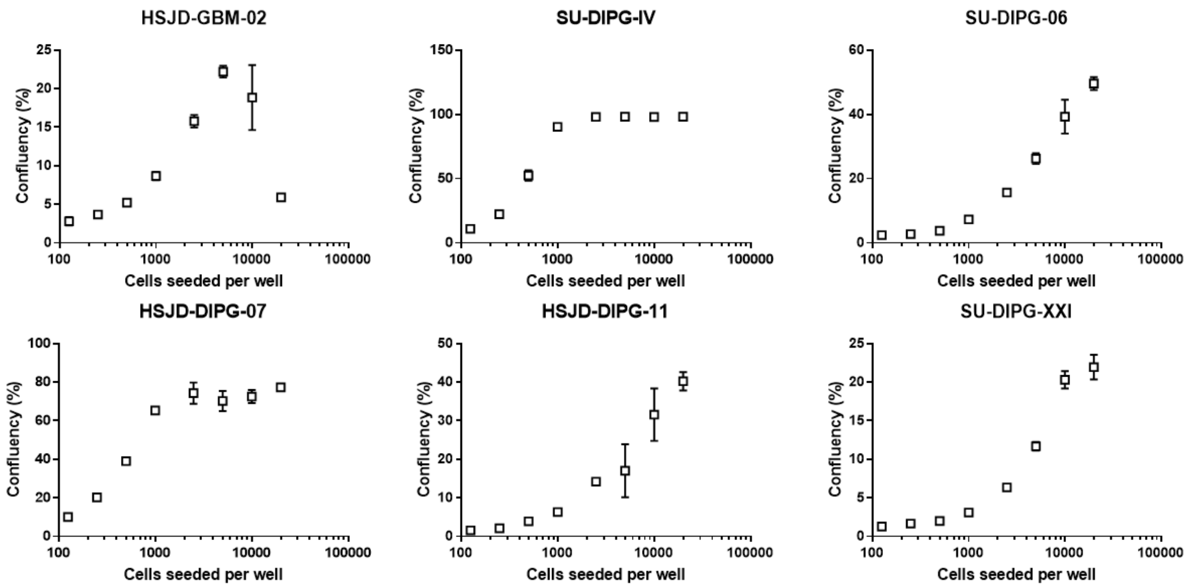

Figure 3. Area of the well occupied by each patient-derived cell lines after 7 days in culture expressed in percentage of total imaged area (confluency %).

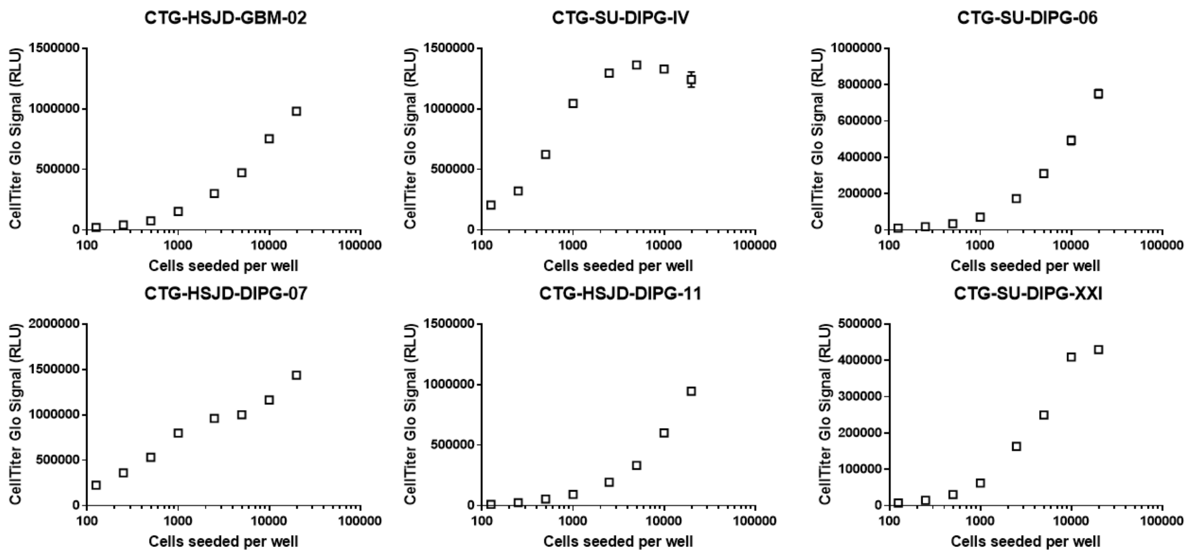

Figure 4. Luminescence signal detected after treating the wells with CellTiter Glo 3D reagent. ATP content is proportional to the amount of luminescence signal and is an indicator of cell viability.

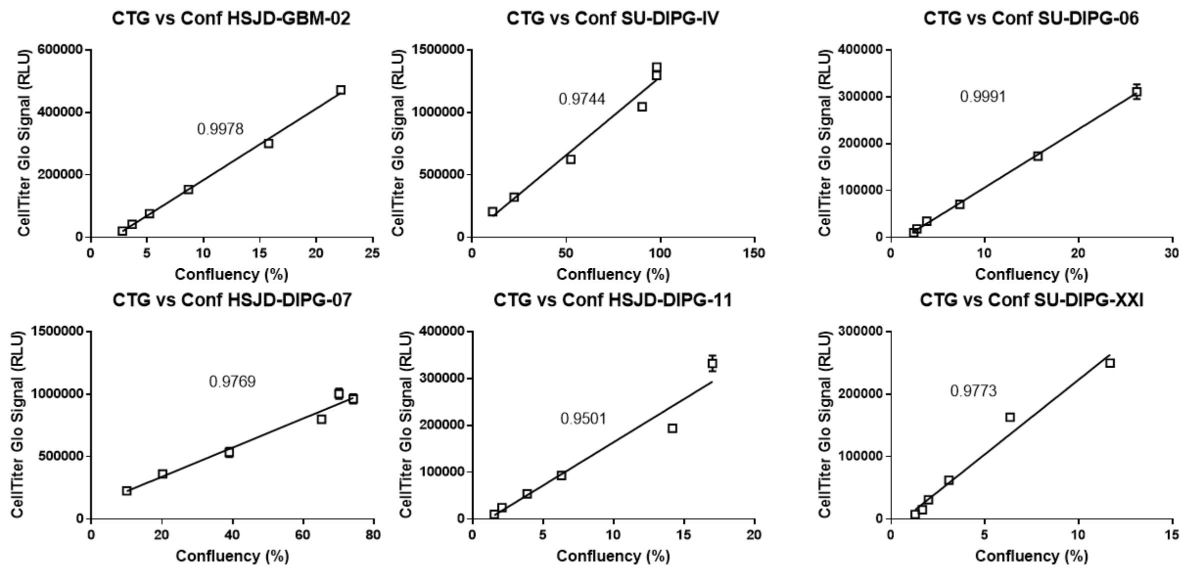

Figure 5. The viability of the cells (CellTiter Glo signal) correlates almost perfectly with the area occupied by the cells (confluency) below 5000 cells seeded per well. R squared values of the two measurements are indicated next to the fitted curves. A perfect correlation will have an R squared value of 1.

Cell viability (CellTiter Glo signal A.U.)

Cell/well	Factor	GBM-02	Factor	DIPG-IV	Factor	DIPG-06	Factor	DIPG-07	Factor	DIPG-11	Factor	DIPG-XXI	Factor
20000	2	979988.5	1.3	1241316	0.9	749846.9	1.5	1435798	1.2	944414.7	1.6	429256.6	1.0
10000	2	753214.6	1.6	1328580	1.0	493017.7	1.6	1161801	1.2	601180.8	1.8	409022.2	1.6
5000	2	471582.6	1.6	1362398	1.1	310512.9	1.8	1001663	1.0	331935.2	1.7	249426.4	1.5
2500	2.5	300399.9	2.0	1294832	1.2	173001.1	2.5	961103.4	1.2	193401.3	2.1	162920.9	2.6
1000	2	153102.8	2.0	1044247	1.7	70393.87	2.0	798351.8	1.5	92963.17	1.7	62183	2.0
500	2	75804.87	1.8	623139.8	1.9	34412.93	1.9	532817.6	1.5	53926.43	2.2	30807.03	2.1
250	2	41980.3	2.1	321443.7	1.6	18238.97	1.8	361058.8	1.6	24456.7	2.4	14993.33	2.1
125		20382.37		205726.3		10021.93		225546.1		10271.1		7275	

Cell death (Mean propidium iodide signal A.U.)

Cell/well	Factor	GBM-02	DIPG-IV	DIPG-06	DIPG-07	DIPG-11	DIPG-XXI
20000	2	19.51246	46.28425	23.84945	44.58629	21.35611	32.09126
10000	2	9.908865	27.31845	17.95764	25.32902	12.89795	14.50378
5000	2	3.709662	17.21245	14.7126	13.46435	6.863876	7.669858
2500	2.5	0.781443	10.58454	7.422064	5.097167	2.359125	2.298878
1000	2	ND	3.210848	2.822382	2.638094	0.122234	0.334665
500	2	ND	0.477858	0.143969	0.917128	ND	ND
250	2	0.060071	1.454555	1.389297	1.103822	ND	0.330363
125		ND	ND	ND	0.147469	ND	ND

Selected for future assays

Figure 6. Cell seeding numbers chosen for future experiments.

Findings:

1) Celigo and its algorithm is able to quantify the area occupied by the cells accurately.

2) SU-DIPG-IV and HSJD-DIPG-007 proliferate quickly. Seeding too many of these cells at the beginning of the experiment will saturate both confluency and CellTiter Glo readouts.

3) CellTiter Glo signal and confluency are good indicators of the actual quantity of cells in the well. Below 5000 cells seeded per well, both sets of measurements correlated almost perfectly with each other.

4) Cell death (as indicated by propidium iodide signal in **Figure 6**) in relation to total cell at endpoint increases when too many cells were seeded at the beginning of the experiment.

5) Initial cell seeding numbers for each patient-derived cell lines that do not lead to overcrowding at the end of the experiment can be chosen based on the linearity of CellTiter Glo signal and the intensity of propidium iodide.