Different methods to evaluate the effects of M4K compounds on DIPG patient-derived cell lines

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Background:

Aside from determining the optimal numbers of DIPG cells to be seeded for viability assay, it is also important to validate that measurements using CellTiter Glo are in agreement with other methods of determining cell viability.

Experimental design:

I chose to compare the EC50 values of M4K2009 on HSJD-DIPG-007 when measured using different methods. A simplified comparison of the different measurement methods is summarised in the following table.

Methods	Principle	Pros	Cons	Ex/Em
CellTiter Glo	Lyses all cells and quantifies the amount of ATP present in the well	• Fast • Sensitive • Robust	• High cost • Endpoint measurement only	luminescence
Brightfield confluency	Images of the wells are taken and area occupied by cells is determined by software.	• Cost • Can be performed any time	• High variability • Difficult to standardise analysis parameters between cell types	Brightfield
Propidium Iodide	Dye fluoresces strongly when bound to nucleic acid. Cannot diffuse through intact cell membrane.	• Low cost • Can be performed any time	• High variability • Difficult to standardise analysis parameters between cell types • Will not detect cells that have highly degraded DNA	535nm/ 617nm (red filter)
Calcein AM	Dye can diffuse into all cells but will only fluoresce after it is reduced by active reductases.	• Sensitive	• Slow • High cost • Endpoint measurement only	495nm/ 515nm (green filter)
Hoechst 33342	Dye can diffuse into all cells and fluoresces after binding to DNA. Number of cell nuclei in an image can be determined by software.	• Low cost	• Slow • Endpoint measurement only	361nm/ 497nm (blue filter)

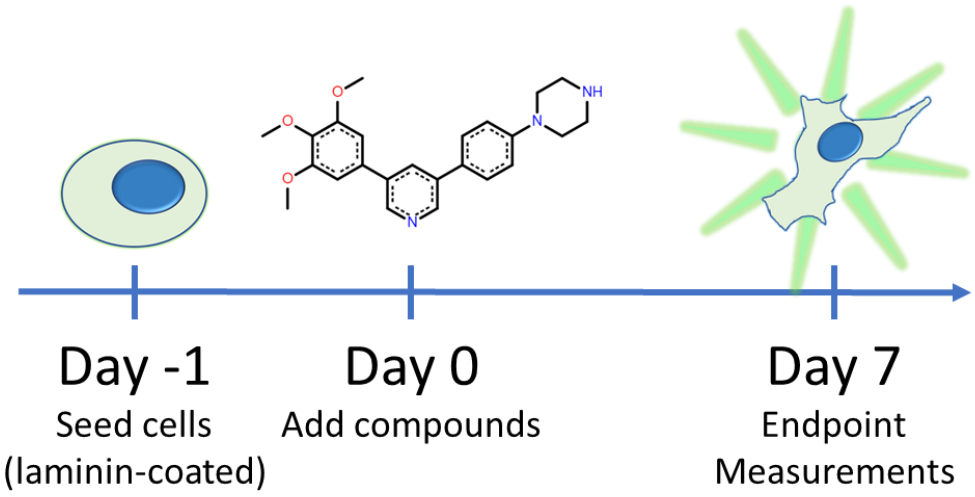


Figure 1. The timeline of this experiment.

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)

Stains

Calcein AM

4mM in DMSO

3uM final concentration (1333X)

[564061 BD Biosciences, 1mg dissolve in 250ul DMSO for 4mM stock] store at -20 degree Celsius

Hoerchst 33342

10mg/ml stock in water

0.005mg/ml final concentration (2000X)

[B2261-25MG, dissolve whole bottle in 2.5ml of sterile water] store at 4 degree Celsius

Propidium iodide

5mM stock in water

5uM final concentration (1000X)

[P4864-10ML Sigma-Aldrich] store at 4 degree Celsius

Cell lines to be used

HSJD-DIPG-07	DIPG mutant ALK2 (R206H)

Plate layout

Set 1 for Cell TiterGlo								
	HSJD-DIPG-07 (1000 cells + M4K2009 serial dilution)							
	10	11	12	Final [M4K2009]	(1000X)			
A				10uM	10mM	24ul parent+36ul DMSO		
B				5uM	5mM	30ul A+30ul DMSO		
C				2.5uM	2.5mM	30ul B+30ul DMSO		
D				1uM	1mM	30ul C+45ul DMSO		
E				0.5uM	0.5mM	30ul D+30ul DMSO		
F				0.25uM	0.25mM	30ul E+30ul DMSO		
G				0.1uM	0.1mM	20ul F+30ul DMSO		
H				DMSO	DMSO			
Set 2 for Propidium Iodide/Hoerchst/Calcein-AM staining								
	HSJD-DIPG-07 (1000 cells + M4K2009 serial dilution)							
	10	11	12	Final [M4K2009]	(1000X)			
A				10uM	10mM	24ul parent+36ul DMSO		
B				5uM	5mM	30ul A+30ul DMSO		
C				2.5uM	2.5mM	30ul B+30ul DMSO		
D				1uM	1mM	30ul C+45ul DMSO		
E				0.5uM	0.5mM	30ul D+30ul DMSO		
F				0.1uM	0.25mM	30ul E+30ul DMSO		
G				0.01uM	0.1mM	20ul F+30ul DMSO		
H				DMSO	DMSO			

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)

HSJD-DIPG-007 grows in suspension

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.

Seeding cells into 96-well plate

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

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

3) Seed 90ul of cell suspension per well.

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

Adding compounds to cells

1) Dilute compounds to 10X desired final concentration in TSM-complete supplemented with 5uM Propidium Iodide.

2) Add 10ul of 10X compounds per well.

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:

Brightfield: confluency

Red: Propidium Iodide (dead permeable cells)

Green: Calcein AM (reduced-form)

Blue: Hoechst 33342 (cell nuclei)

3) Master staining solution: 2X Calcein-AM, 2X Hoechst stain, 1X Propidium Iodide, dilute in DPBS

4) Mix well and dispense 100ul into each well slowly without flushing out cells

5) Incubate for 30 minutes at 37 degree Celsius

6) Image all wells and perform the appropriate software analysis

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. 2.5ml 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 20 minutes without shaking.

5) Measure total luminescent signal using Clariostar plate reader.

Results:

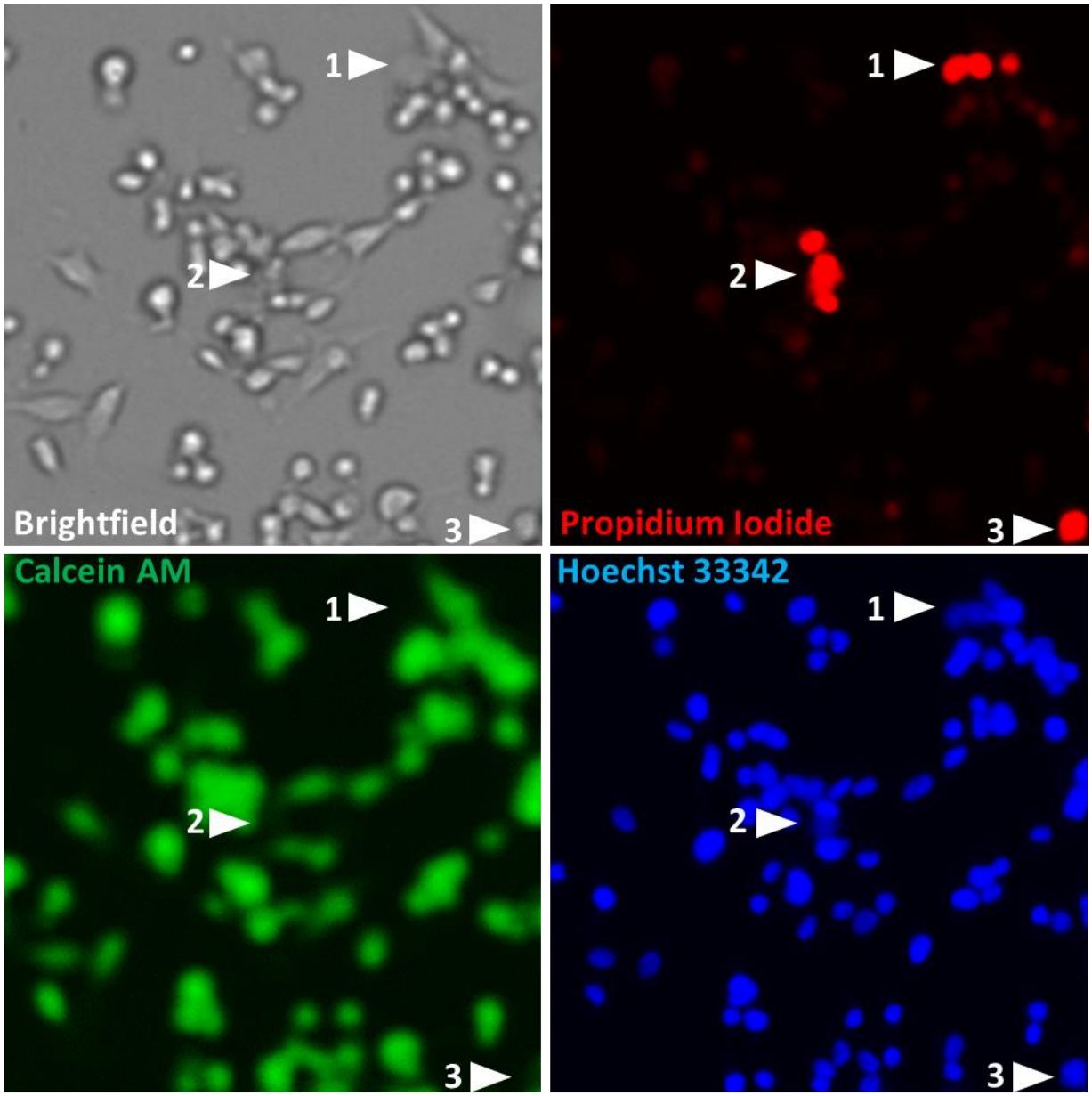
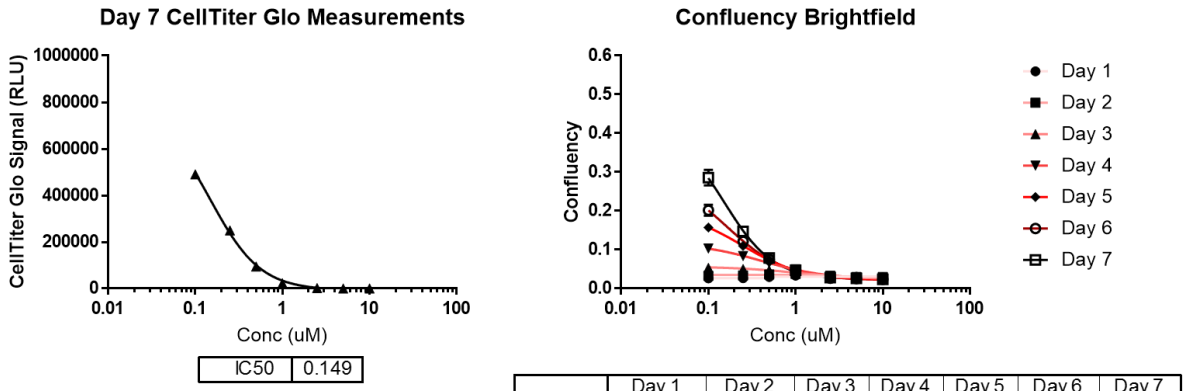
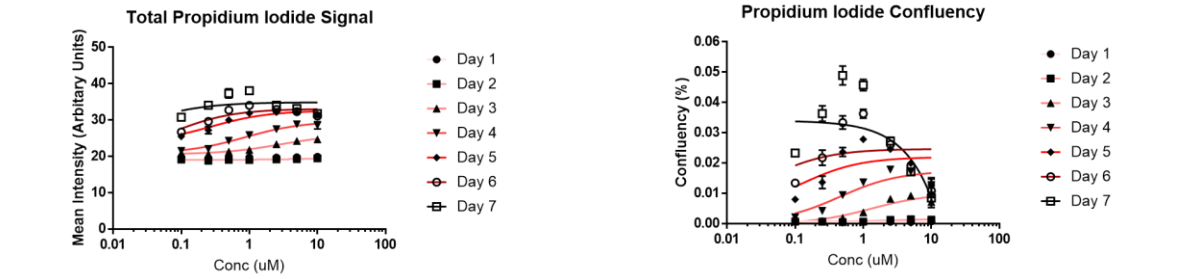


Figure 2. Demonstration of how different stains serve as the indicator of various cell aspects. Dead cells (marked as 1, 2 and 3) that are stained by Propidium Iodide (Red) are not stained by Calcein AM (Green). They are still stained by Hoechst 33342 (Blue), albeit at slight lower intensity due to DNA degradation. Depending on the form and extend of cell death, they might no longer be visible in brightfield (based on Differential Interference Contrast).



	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
IC50	~ 0.4765	~ 2.481	1.106	0.414	0.245	0.1648	0.1543

Figure 3. EC50 of M4K2009 determined based on the amount of ATP in each well (left) and total area occupied by cells (right). Brightfield images were acquired every 24 hours.



	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
IC50		~ 1.141e+016	2.887	0.8598	0.2669	0.08264	0.04467

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
IC50	0.08418	4.076	1.318	0.4549	0.1242	0.04813	~ 32550805991279

Figure 4. EC50 of M4K2009 determined based on the intensity of dead cell stain (left) and area occupied by the stain (right). Propidium iodide signal was imaged every 24 hours.

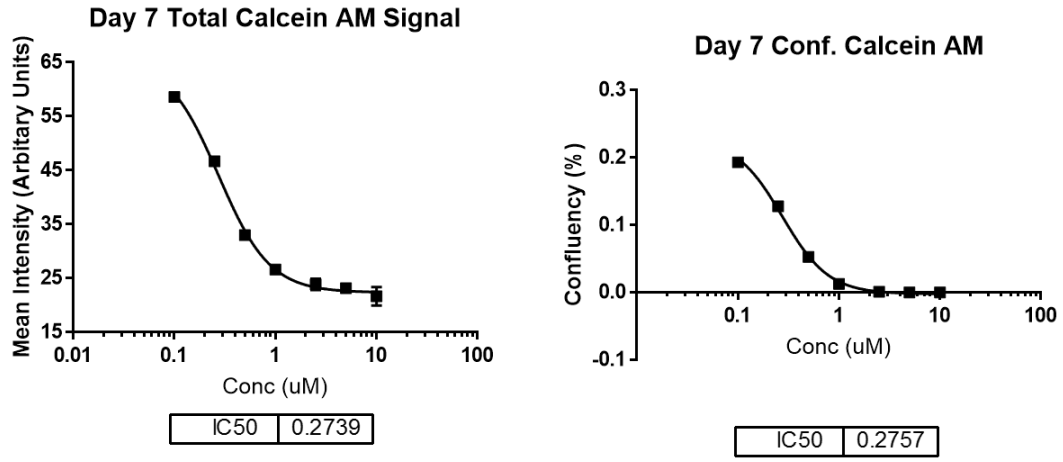


Figure 5. EC50 of M4K2009 determined based on the metabolic activity of the cells (reduction of Calcein AM stain). Both signal intensity (left) and area covered by the signal (right) were examined.

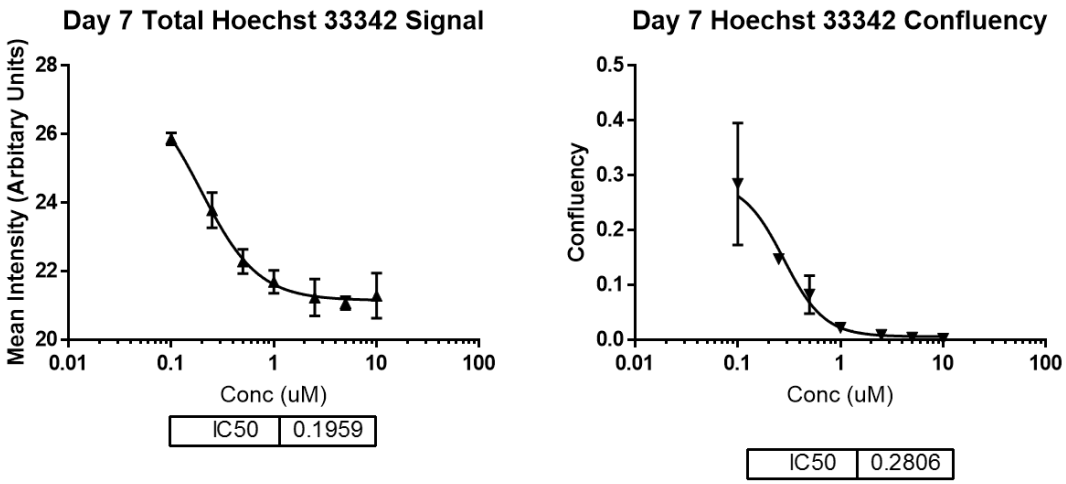


Figure 6. EC50 of M4K2009 determined based on the total number of cells (staining of all cell nuclei). Both signal intensity (left) and area covered by the signal (right) were examined.

Findings:

1) 7 days of compound treatment gave the best assay window for EC50 estimated based on the confluency of the cells.

2) CellTiter Glo is superior over other methods of quantification in term of robustness and number of steps involved.

3) Propidium iodide staining of dead cells is not suitable for EC50 estimation because most dead cells likely became too degraded to be stained after 7 days of compound treatment.

4) EC50 estimation based on Calcein AM staining of metabolically active cells was robust but not as sensitive as CellTiter Glo or cell confluency.

5) EC50 estimation based on the amount of nuclei stained by Hoechst 33342 is relatively variable.

In a nut shell:

In future experiments, the EC50 of M4K compounds on the viability of DIPG patient-derived cell lines will be estimated based on CellTiter Glo signal and cell confluency.