

High throughput 3D tumor spheroid assays for target validation and drug evaluation

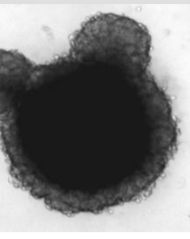
Abstract #4277

M. Vinci¹, S. Gowan¹, F. Boxall¹, L. Patterson¹, C. Lomas¹, W. Court¹, M. Mendiola², S. A. Eccles¹.

¹Cancer Research UK, Cancer Therapeutics Unit, The Institute of Cancer Research, Sutton, UK

²Laboratory of Molecular Pathology and Oncology, Research Unit, Hospital Universitario La Paz, Madrid, Spain

maria.vinci@icr.ac.uk

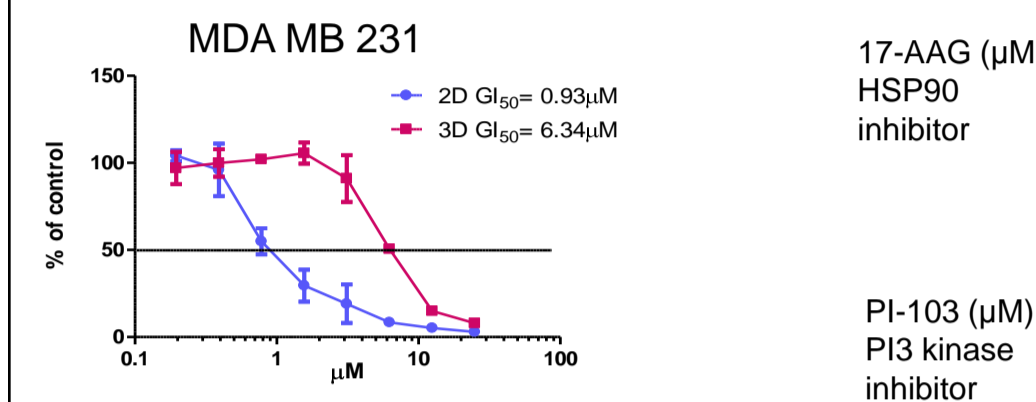


INTRODUCTION:

Standard 2D monolayer cultures used for target validation and drug screening studies do not adequately address the complexity of *in vivo* tumor pathophysiology. Although 3D tumor spheroids better represent a tumor microenvironment (with gradients of proliferation, oxygenation and drug access with evidence of altered signalling pathway activation) they have not been routinely used in high-throughput (HTS) mode.

The aim of the present study was to develop a suite of *in vitro* 3D spheroid-based assays to rapidly and accurately quantify key aspects of the malignant phenotype: growth, motility, invasion and angiogenesis

1. 2D versus 3D cell viability assay



Cell line	2D	3D
U87MG	0.32 0.1	1.3 1.0
KNS42	7.75 2.1	>25
MDA MB 231	0.75 0.1	6.2 0.5

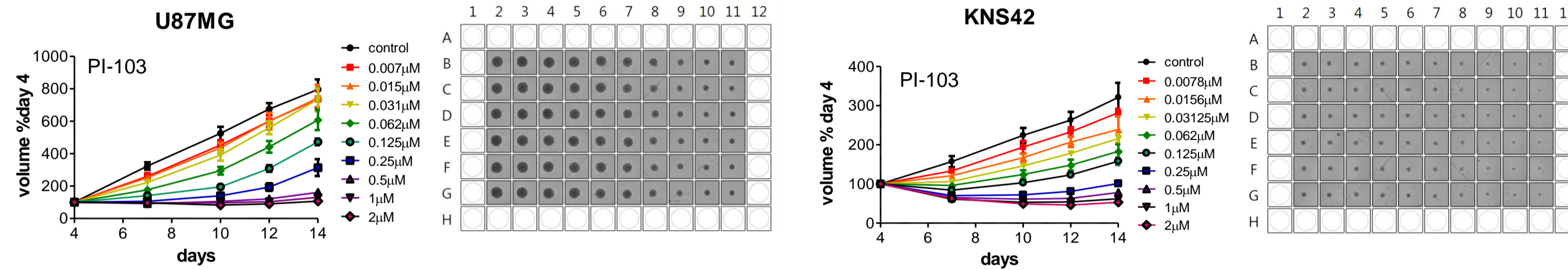
17-AAG (µM)
HSP90 inhibitor

PI-103 (µM)
PI3 kinase inhibitor

Cell line	2D	3D
U87MG	0.77 0.39	0.35 0.13
KNS42	1.2	0.66 0.3
MDA MB 231	2.39 0.69	>100

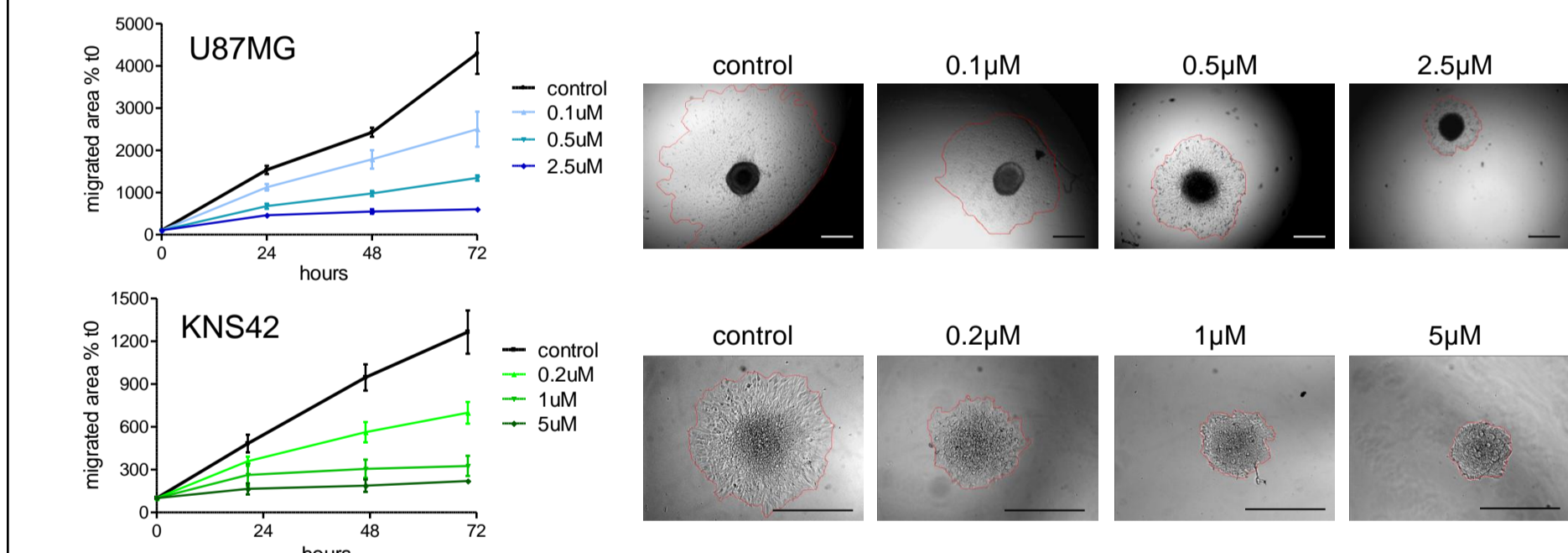
MDA MB 231 (breast carcinoma), U87MG and KNS42 (adult and paediatric GBM respectively) were grown in 2D and in 3D. 4 days later, both cultures were treated with 17-AAG (HSP90 inhibitor) and PI-103 (PI3 kinase inhibitor). 72 hrs later, a CellTiterGlo luminescence assay for cell viability was performed to determine GI_{50} values. In most cases 3D was more resistant than in 2D. Interestingly U87MG and KNS42 are more sensitive to PI-103 in 3D than in 2D.

2. Growth kinetics: PI-103 inhibits tumor spheroid (TS) growth in a dose-dependent manner



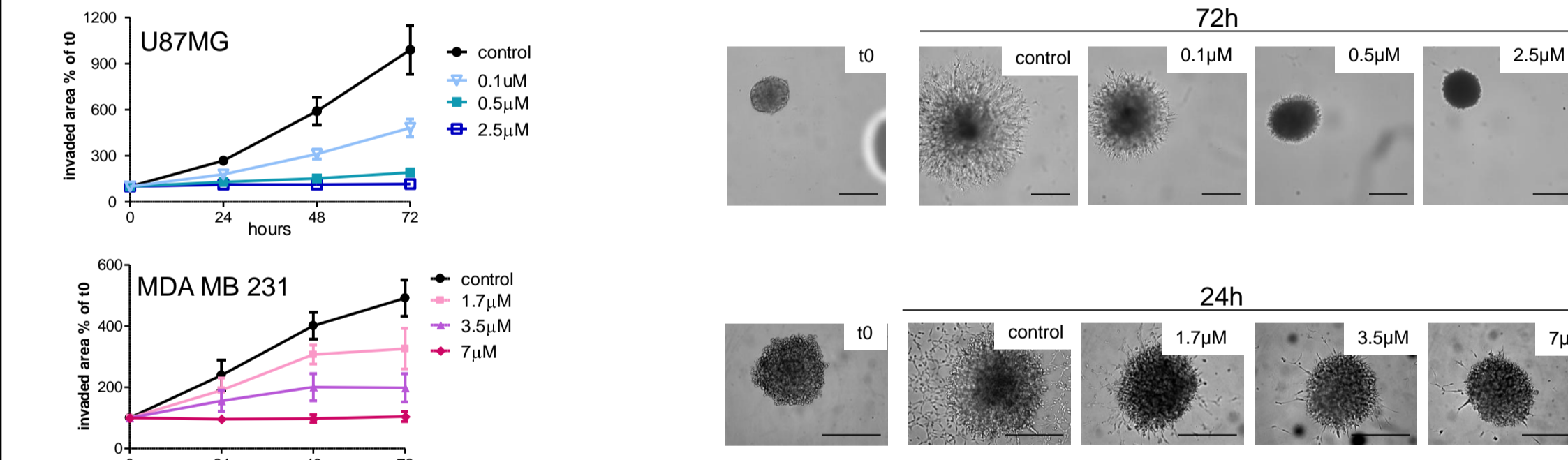
U87MG and KNS42 tumor spheroids were generated accordingly to our standardised method. At day 4 post initiation, TS were treated with the PI3K inhibitor PI-103 over a range of doses. Plates were imaged and analysed using the Celigo™ cytometer. Graphs show a clear dose dependent inhibition also shown on the print screen of the plates. Note the excellent reproducibility of the spheroids on each plate across the 6 replicates and/or conditions (intra-plate CV between 5.53% to 7.28%. The inter-plate CV value was 17.87%.

3. Tumor dissemination on matrix protein: 17-AAG inhibits U87MG and KNS42 haptotaxis



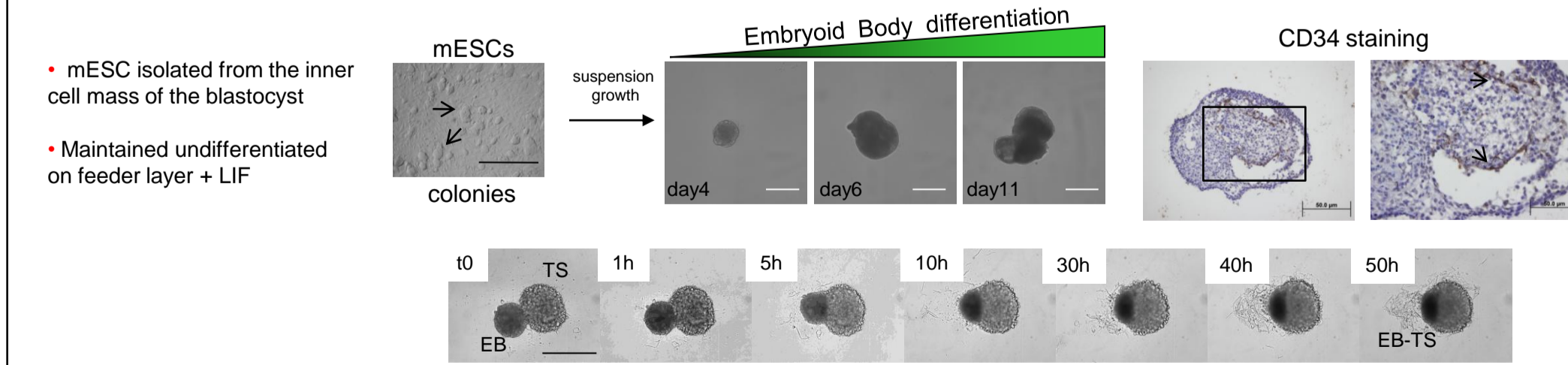
Haptotaxis (motility mediated by cell-substrate interactions) of U87MG and KNS42 tumorspheroids on gelatin is inhibited by 17-AAG at sub GI_{50} values in a concentration-dependent manner. Images were captured at intervals using an inverted microscope and analysed with ImagePro Plus software. Representative images are shown relative to the 72h time point. Scale bar : 500µm.

4. Tumor spheroid invasion into Matrigel™: 17-AAG dose-dependent inhibition of U87MG glioma and MDA MB 231 breast carcinoma



U87MG and MDA MB 231 tumor spheroids were embedded in matrigel and treated with 17-AAG. Graphs and images show inhibition of invasion at sub GI_{50} values. Images were captured at intervals using an inverted microscope or the Celigo™ cytometer and analysed with ImagePro Plus software. Representative images are shown relative to the 72h time point for U87MG and 24h time point for MDA MB 231. Scale bar: 500µm

5. EB-tumor spheroid co-culture for invasion and tumorangiogenesis



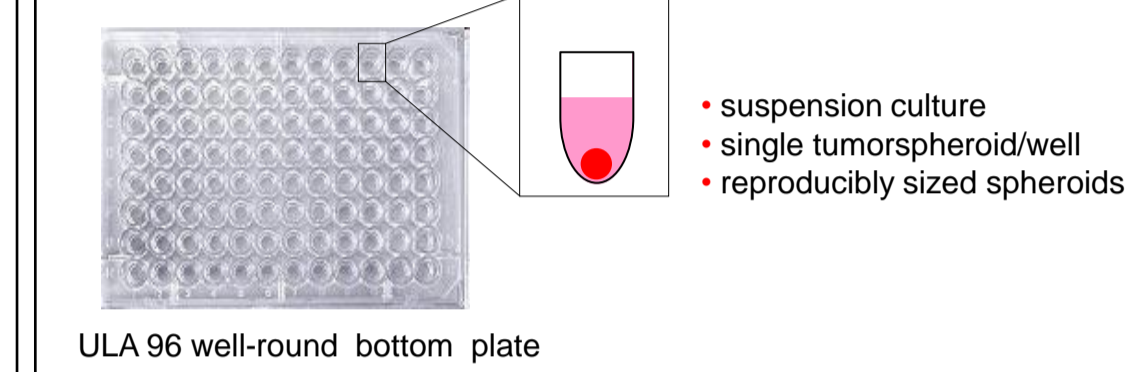
Mouse embryonic stem cells (mESCs) were differentiated into embryoid bodies (EBs) by growing in suspension culture in the ULA 96-well round bottom plates (upper panel). EBs represent a complex tissue including vascular elements as confirmed by the IHC showing CD34 staining of a 12day old EB. Time-lapse imaging of the TS-EB co-culture (lower panel) allows dynamic measurements of the relatively rapid attachment and coalescence of the two tissues (representing tissue invasion and angiogenesis). Such cultures allow the effects of drugs on both angiogenesis and spheroid growth/tissue invasion to be simultaneously assessed.

METHODS:

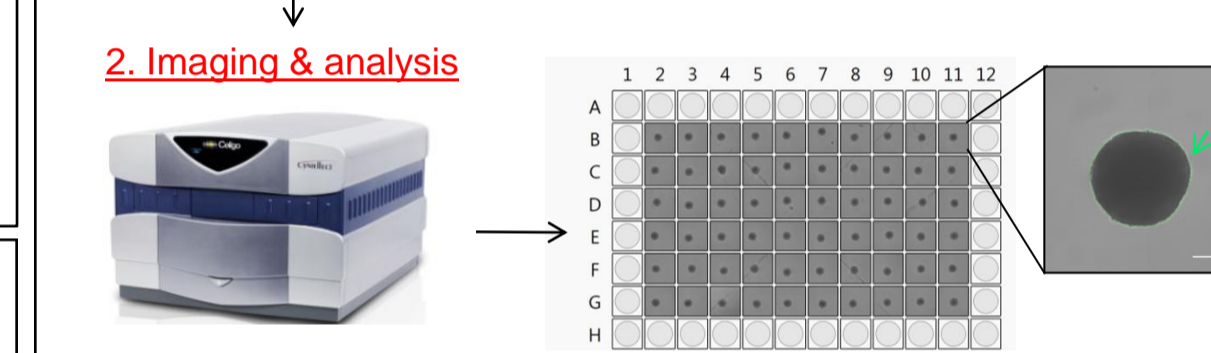
A standardised method was established for the generation of tumor spheroids in suspension culture using ultra-low attachment (ULA) 96-well round bottom plates. Spheroids were characterized in terms of growth kinetics, haptotaxis on extracellular matrix (ECM) protein, invasion into Matrigel™ and co-culture with embryoid bodies (EB) to model tissue invasion/angiogenesis. For growth kinetic assays, quantitative data were obtained using the Celigo™ cytometer that allows fully automated image analysis. For functional assays, quantitative data were obtained using the Celigo™ or microscopy. Cell viability was determined by the CellTiter Glo assay for direct comparison of 2D and 3D cultures. All the assay established are in 96 well format. Highly malignant human adult (U87MG) and paediatric (KNS42) gliomas and breast carcinoma (MDA MB 231) spheroids were treated with inhibitors of HSP90 (17-AAG) and PI3K (PI-103) to exemplify the power of the techniques.

High-throughput standardised method for tumor spheroids

1. Generation

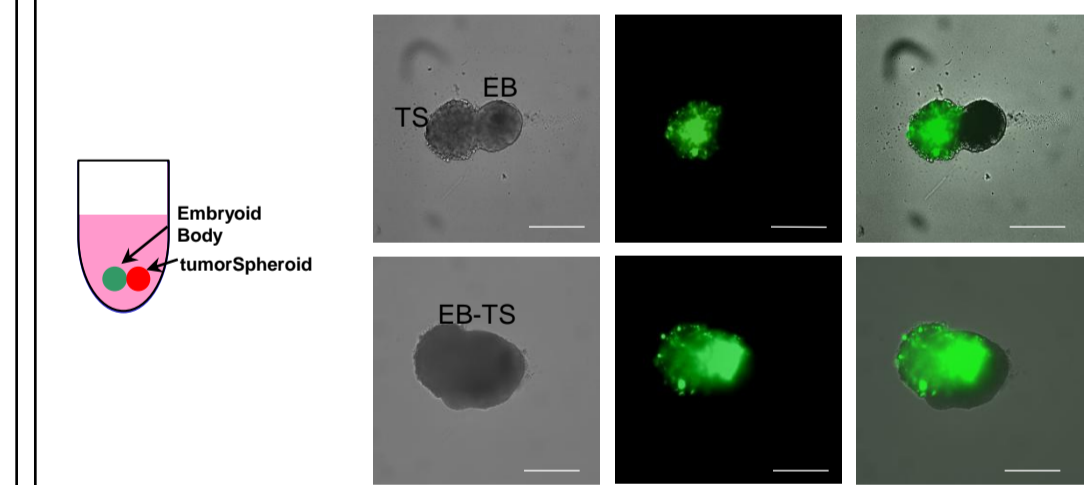


2. Imaging & analysis



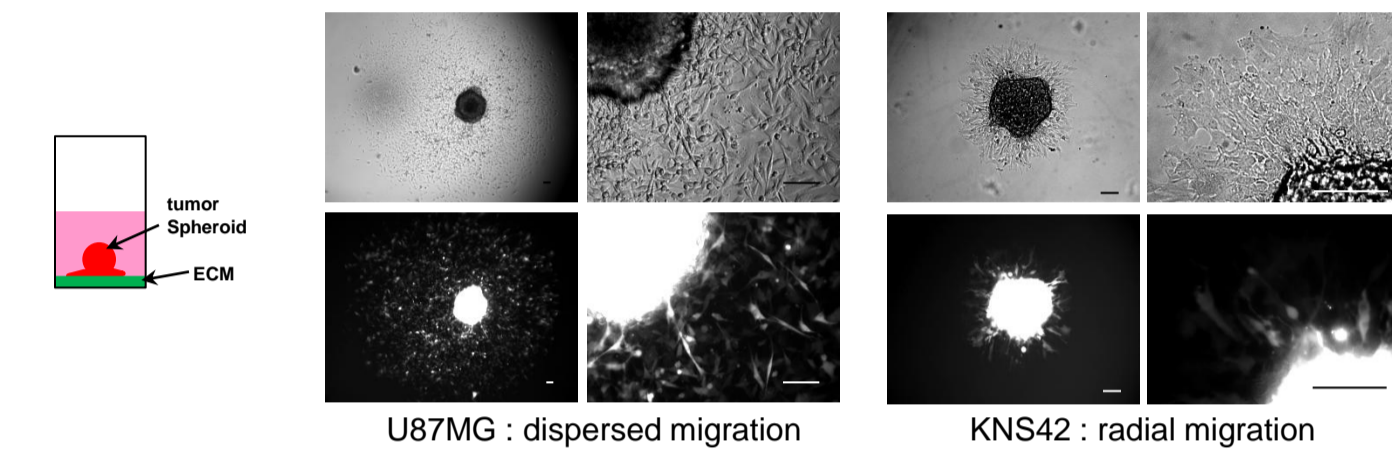
The method allows us to obtain a single tumor spheroid/well, highly reproducible in size. Imaging and analysis are fully automated using the Celigo™ cytometer.

TS-EB co-culture for invasion/angiogenesis



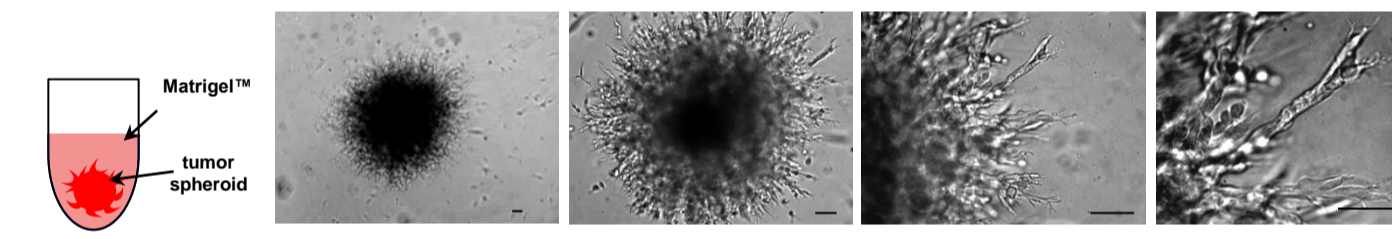
GFP transduced U87MG spheroids as well as Embryoid bodies (EB) were generated in ULA 96-well round bottom plates. The co-culture is initiated by transferring a single tumor spheroid (TS) and a single EB into the same well. Representative images (obtained using the Celigo™) show the invasion of the TS into the EB allowing us to identify the two bodies. Scale bar: 500µm. Cells can be differentially labelled for greater discrimination, and could be adapted for other tissues or for human EBs.

Tumor dissemination on ECM



tumor spheroids were generated using GFP-transduced U87MG and KNS42 cells and transferred onto a 96-well plate pre-coated with matrix proteins (e.g. gelatin). Note the differential migration pattern of the glioma cell lines. Representative images are shown (48h time point). Scale bar: 100µm.

Tumor spheroid invasion into Matrigel™



U87MG tumor spheroids were embedded into matrigel in ULA 96-well round bottom plates. Representative images (48h time point) show details of the invadopodia into the matrix demonstrating true 3 dimensional invasion. Scale bar: 100µm.

Summary

- We have pioneered a suite of 3D assays to address potential molecular therapeutic targets in tumorgrowth, migration, invasion and angiogenesis
- We have established a novel, simple, standardised high-throughput 96-well plate assay for growth of tumorspheroids and embryoid bodies
- The cultures allow detailed analyses under conditions that better recapitulate the *in vivo* microenvironment
- The systems provide real-time, dynamic, quantitative analyses rather than endpoint assays
- Spheroids can be readily be harvested for PD analyses and gene expression profiling
- Further sophistication is introduced by the EB-spheroid co-cultures, representing a complex tissue environment, enabling mutual invasion/angiogenic potential to be measured

We have developed a unique repertoire of novel 3D microplate assays reflecting key aspects of malignant cell behaviour and able to deliver rapid and reproducible results. We provide evidence that our methods potentially enhance target selection and the triaging of drug candidates prior to *in vivo* studies.

References
Balconi G, Spagnuolo R and Dejana E. Development of endothelial cell lines from embryonic stem cells: A tool for studying genetically manipulated endothelial cells in vitro. *Arterioscler. Thromb. Vasc Biol.* 2000 ; 20: 1443-51. Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: an in vitro model of mouse embryogenesis. *Exp Physiol.* 2000 Nov;85(6):645-51. Eccles S. Parallels in invasion and angiogenesis provide pivotal points for therapeutic intervention in cancer. *Int. J. Dev. Biol.* 48: 583-598 2004. Friedrich J, et al. Spheroid-based drug screen: considerations and practical approach. *Nat Protoc.* 2009. 4(3): 309. Wartenberg M, Donmez F, Ling FC, Acker H, Hescheler J, Sauer H. Confrontation cultures of embryonic stem cells with multicellular tumor spheroids to study tumor-induced angiogenesis. *FASEB J.* 2001 Apr;15(6):995-1005. Offer FA, Bigalke I, Schiefer J, Wirtz HC, Klosterhalfen B, Feichtinger H, Kirkpatrick CJ. Interaction of human malignant melanoma tumor spheroids with endothelium and reconstituted basement membrane: modulation by RGDS. *Int J Cancer.* 1993 May 28;54(3):506-12.

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