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Characterization of the effects of migrastatic inhibitors on 3D tumor spheroid invasion by high-resolution confocal microscopy

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TITLE:

Characterization of the Effects of Migrastatic Inhibitors on 3D Tumor Spheroid Invasion by High-Resolution Confocal Microscopy

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KEYWORDS:

cancer cell migration, glioma, spheroid, 3D invasion assay, inhibitor, confocal microscopy

SUMMARY:

The effects of migrastatic inhibitors on glioma cancer cell migration in three-dimensional (3D) invasion assays using a histone deacetylase (HDAC) inhibitor are characterized by high-resolution confocal microscopy.

ABSTRACT:

Drug discovery and development in cancer research is increasingly being based on drug screens in a 3D format. Novel inhibitors targeting the migratory and invasive potential of cancer cells, and consequently the metastatic spread of disease, are being discovered and considered as complementary treatments in highly invasive cancers such as gliomas. Thus, generating data enabling the detailed analyses of cells in a 3D environment following the addition of a drug is required. The methodology described here, combining spheroid invasion assays with high-resolution image capture and data analysis by confocal laser scanning microscopy (CLSM), enabled detailed characterization of the effects of the potential anti-migratory inhibitor MI-192 on glioma cells. Spheroids were generated from cell lines for invasion assays in low adherent 96-well plates and then prepared for CLSM analysis. The described workflow was preferred over other commonly used spheroid-generating techniques due to both ease and reproducibility. This, combined with the enhanced image resolution attained by confocal microscopy compared to conventional wide-field approaches, allowed the identification and analysis of distinct morphological changes in migratory cells in a 3D environment following treatment with the migrastatic drug MI-192.

INTRODUCTION:

Three dimensional spheroid technologies for preclinical drug discovery and the development of potential cancer drugs are increasingly being favored over conventional drug screens; thus, there is more development of migrastatic – migration and invasion preventing – drugs. The rationale behind these developments in cancer treatment are clear: 3D spheroid assays represent a more

realistic approach for screening potential anti-cancer drugs as they mimic 3D tumor architecture more faithfully than cell monolayer cultures, recapitulate drug-tumor interactions (kinetics) more accurately, and allow the characterization of drug activity in a tumor-related setting. In addition, the rise of resistance to chemotoxic drugs in many cancer types and high death rates among cancer patients due to metastasis potentiated by the ability of cancer cells to migrate to distant tumor sites supports the inclusion of chemotherapeutic agents targeting the migratory potential of cancer cells as adjuvant treatment in future clinical cancer trials[1]. This is particularly the case in highly invasive cancers, such as high-grade glioblastomas (GBM). GBM management includes surgery, radiotherapy, and chemotherapy. However, even with combination treatment, most patients relapse within 1 year of initial diagnosis with a median survival of 11-15 months[2,3]. Huge advances in the field of 3D technology have been made over the last few years: rotative systems, microfabricated structures and 3D scaffolds, and other individual assays are being continually improved to allow routine testing on a large scale[4-7]. However, results obtained from these assays must be analyzed in a meaningful manner because data interpretation is often hindered by attempts to analyze 3D-generated data with 2D image analysis systems.

Despite being preferable in terms of image acquisition speed and reduced photo-toxicity, most wide-field systems remain limited by resolution[8]. Thus, apart from data read-outs relating to drug efficacy, detailed effects of drug action on 3D cellular structures of migrating cells are inevitably lost if imaged using a wide-field system. Conversely, confocal laser scanning microscopy (CLSM) captures high quality, optically sectioned images that can be reconstructed and rendered in 3D post-acquisition using computer software. Thus, CLSM is readily applicable to imaging complex 3D cellular structures, thereby enabling interrogation of the effects of anti-migrastatic inhibitors on 3D structures and in-depth analyses of cell migration mechanisms. This will undoubtedly guide future migrastatic drug development. Here, a combined workflow of spheroid generation, drug treatment, staining protocol, and characterization by high-resolution confocal microscopy is described.

PROTOCOL:

1. Generation of cell spheroids

Day 1

1.1 Prepare the standard culture medium as required by the cell line under investigation.

1.2 Carry out all tissue culture–associated steps in a tissue culture hood using sterile handling techniques.

1.3 Trypsinize and count cancer cells. Use 20 mL of cell suspension per plate. Keep the cell suspensions in clearly labeled sterile universal tubes.

1.4 Add a predetermined number of cells to each well. Both the initial number of cells and ultimate spheroid size required depends upon the proliferation rate of the cell line being

investigated.

NOTE: For established glioma cell lines such as U251 and KNS42[9,10], 5 x 10^3 cells/mL will produce a microscopically visible spheroid (200 or 800 µm) after 4 days of incubation.

1.5 Resuspend the cells in universal tubes by gentle inversion to avoid cell clumping. Pipette 200 µL of the cell suspension into each well of a 96-well plate. If all wells are not required, it is advisable to add 200 µL of 1x PBS to each empty well to avoid evaporation.

1.6 Incubate the cells in an incubator as normal at 37 °C.

NOTE: Cell lines such as glioma cancer cell lines will form spheroids within 24 h. Allow 3D cellular architecture to form by incubating the spheroid for 72 h.

Day 2

1.7 Check cells by bright-field microscopy after 24 h. Depending upon the cell line, cells may have formed a spheroid detectable in the bottom of the well.

NOTE: Established glioma cancer cell lines readily form spheroids within 24 h. Patient-derived glioma cancer cell lines may take up to 1-2 weeks.

2. Collagen invasion assay

Day 3

2.1 Place collagen, 5x culture medium, 1 M NaOH, and one 20 mL tube on ice.

2.2 Carefully and slowly add 10.4 mL of cold collagen into a chilled culture tube. Avoid bubbles. This quantity of collagen is enough for one 96-well plate. Upscaling is possible, but it is recommended that one 20 mL tube per plate is prepared at a time.

2.3 Gently add 1.52 mL of cold sterile 5x culture medium. Avoid bubbles.

2.4 Just before use, gently add 72 µL of cold sterile 1 M NaOH. Keep solution on ice.

2.5 Mix gently by pipetting. Avoid bubbles. Efficient mixing leads to a color change (from red to orange-red (pH 7.4) in the medium. Leave the mixture on ice until use.

2.6 Crucial step: Remove 190 µL of supernatant from the 96-well plate prepared on day 1. Be very careful not to disturb the spheroids that formed in the bottom of the well. Use the pipette at an angle towards the side, not the center, of the well.

2.7 Gently add 100 µL of the collagen mix to each well. To prevent any spheroid disturbance,

pipette the mix down the side of the well. Avoid bubbles. Keep any remaining collagen mix in the 20 mL tube at room temperature to assess polymerization.

2.8 Incubate plate in the incubator for at least 10 min to allow the collagen to polymerize. As a guideline, if the leftover collagen has set, becoming semisolid and sponge-like, the spheroids are ready to be treated with inhibitor.

2.9 Add the drugs or inhibitors at 2x concentration to the culture medium. Add the medium gently to each well (100 µL per well). Again, pipette the medium down the side of the well to avoid spheroid disturbance.

2.10 Observe and image each spheroid by bright-field microscopy at times T = 0 h, 24 h, 48 h, and 72 h to assess drug activity. Then return the plate to the incubator.

NOTE: Depending on the invasive behavior of the cell line, migration away from the original spheroid core may be observed from 24 h onwards.

3 Preparation of collagen embedded spheroids and migratory cells for confocal microscopy

3.1 Place the plate in a tissue culture hood and gently remove the supernatant (200 µL). Again, take care not to disturb the spheroid and avoid touching the collagen, as this may interfere with the collagen plug.

3.2 Replace the supernatant with 100 µL of 1x PBS. Repeat this wash step 3x.

3.3 Remove the final wash and replace with 4% formaldehyde in 1x PBS (100 µL per well).

CAUTION**:** Formaldehyde is a potential carcinogen. Handle with care in accordance with health and safety guidelines.

3.4 Place the 96-well plate on a lab bench, cover with foil, and leave for 24 h at room temperature.

3.5 Carefully remove the formaldehyde and replace with 1x PBS. Repeat this 1x PBS wash 3x.

3.6 Prepare 0.1% Triton X-100 in 1x PBS. Remove the 1x PBS wash and replace with 100 µL of the Triton X-100 solution. Incubate for 30 min at room temperature. In the meantime, prepare the blocking solution with 1x PBS and 0.05% skimmed milk powder and mix thoroughly.

3.7 Remove Triton X-100 and wash 3x with 1x PBS. Add 100 µL of blocking solution to each well and incubate for 15 min.

3.8 Dilute the required primary antibody in blocking buffer at the predetermined

concentration. Here, use anti-mouse IgG acetylated tubulin antibody (1:100).

3.9 Centrifuge the primary antibody-blocking buffer mix for 5 min at 15,682 x *g*. Carefully remove the blocking solution and add the supernatant (25−50 µL) to each well. Incubate in the dark at room temperature for 1 h.

3.10 Remove the antibody solution and wash 3x with 1x PBS (100 µL per well).

3.11 Dilute the secondary antibody in the blocking buffer at the recommended or predetermined concentration in addition to any additional fluorescent stains. Here, use 1:500 anti-mouse fluorophore-488 conjugated antibody, phalloidin-594 (1:500) for actin staining, and the DNA stain (DAPI).

3.12 Again, centrifuge the secondary antibody solution for 5 min at 13,000 rpm.

3.13 Remove the blocking solution from each well and add 25−50 µL of the secondary antibody/phalloidin/DAPI mix. Incubate in the dark for 1.5 h at room temperature.

3.14 Remove secondary antibody-dye solution and wash 3x with 1x PBS (100 µL per well).

3.15 Carefully lift individual collagen plugs by suction with a plastic pipette (200 µL) onto the center of a high-quality plain glass slide.

3.16 Add one drop of a suitable mountant to the collagen plug, ensuring the plug is completely covered. Avoid bubbles.

3.17 Apply coverslip of the optimal thickness for the microscope objective that will be used for imaging and allow to set overnight. Store the slides at room temperature in the dark.

4 Fluorescence microscopy

4.1 Capture fluorescent images using a suitable confocal microscope.

REPRESENTATIVE RESULTS:

Three-dimensional spheroid technology is advancing the understanding of drug-tumor interactions because it is more representative of the cancer-specific environment. The generation of spheroids can be achieved in several ways; low adherence 96-well plates were used in this protocol. After testing several products from different manufacturers, the plates used here were chosen because they consistently performed best in terms of successful spheroid production and uniformity. The replacement step, where the growth medium is replaced with the collagen matrix, is a critical point of the protocol; great care must be taken to remove most of the medium without disturbing the spheroid itself. Automated imaging for the characterization of drug-induced effects by wide-field microscopy may be considered to remove any handler bias, but currently commercially available instruments remain considerably more expensive than the

imaging approaches outlined here.

Wide-field epifluorescence microscopy allows examination of the effect of drug activity on cell migration and invasion. However, the resolution attained from wide-field microscopy is not good enough to allow detailed interpretation of results with regards to drug effect on cell morphology (**Figure 1**). Here, the preparation of glioma spheroids and migrating cells through easily reproducible staining protocols is described, followed by imaging using a confocal microscope. From the wide-field microscopy image analysis, it was evident that different morphological changes had occurred in the glioma cells following treatment with the MI-192 inhibitor, but clearly-defined details were lacking. Confocal microscopy confirmed the initial findings, and these higher resolution images allowed the assessment of the effect of MI-192 even further. Significant differences between untreated (control) spheroids, migrating cells (**Figure 2**), and treated spheroids and cells (**Figure 3**) became evident. Whereas the adult glioma cell line U251 appeared to migrate in 'spikes', radiating away from the original spheroid core with single cells detaching, the pediatric cell line KNS42 adopted a sheet-like migration pattern with few distinct cell spikes. Previously, different migration patterns among different cell lines (here the adult glioma cell line U251 and the pediatric cell line KNS42) were observed, potentially reflecting the cell type they arose from and site of tumor isolation. Crucially, an increase of acetylated tubulin with increasing inhibitor concentration (from 0.1−10 µM) was also uncovered, not only in the migrating cells, but also in the spheroid-associated cells. This was not evident in the initial wide-field microscopy acquired images. Further imaging would also allow the quantitative analysis of protein expression levels as a cellular response to treatment with migrastatic inhibitors.

In this study, it was demonstrated that the cells changed morphologically in response to treatment; cell rounding became apparent with increasing inhibitor concentrations and cell death at the highest inhibitor concentration (10 µM), with nuclear fragmentation evident in U251 cells and collapsed microtubules and nuclear fragmentation in KNS42. These findings are in keeping with previous observations that the anti-migratory activity of MI-192 on glioma cell lines is concentration dependent[13,14]. The overall workflow of the protocol is depicted in **Figure 4**.

FIGURE AND TABLE LEGENDS:

Figure 1. Spheroid cell invasion into collagen imaged by wide-field microscopy. Representative images of the cell lines U251 and KNS42 are shown after treatment with the inhibitor MI-192 at the anti-migratory concentration of 1 µM and at 24 h intervals. A control spheroid with no treatment is also shown. Potential anti- or pro-migratory effects are detected as highlighted (arrows). This is especially noticeable in KNS42 with seemingly no migration in either the control or treated spheroids. All images were taken at 4x. Scale bar = 1,000 µm. Scale bar in enlarged images for SF188 = 200 µm, KNS42 = 1,000 µm.

Figure 2. The effect of the migrastatic inhibitor MI-192 on glioma cell line U251 revealed by confocal microscopy. Fixed and stained glioma spheroids and migratory cells display distinct migratory phenotypes. Migratory cells close to the original spheroid edges are shown. U251 cell spheroids are characterized by spikes radiating away from the original spheroid with increasing cell rounding in cells apparent with increasing inhibitor concentration (arrow indicates cell spike).

Scale bar = 10 µm. Labels: red = actin, green = acetylated tubulin, blue = DAPI. For each image, a single representative optical section was captured with all settings with both pre- and post-image capture maintained for comparative purposes. All images were subsequently processed.

Figure 3. The effect of the migrastatic inhibitor MI-192 on the glioma cell line KNS42 revealed by confocal microscopy. KNS42 migration is characterized by sheetlike protrusions with single cell spikes (arrow indicates cell sheet). At the lowest inhibitor concentration this phenotype appears to be pronounced but is lost with increasing inhibitor concentration (scale bar = 10 µm); labels: red = actin, green = acetylated tubulin, blue = DAPI. For each image a single representative optical section was captured, with all settings with both pre- and post-image capture maintained for comparative purposes. All images were subsequently processed.

Figure 4. Summary of workflow. Incorporated in this workflow is the generation of spheroids, embedding in collagen, drug treatment, fixing, staining, and imaging by confocal microscopy.

DISCUSSION:

A novel way to create cancer cell spheroids for identification of migrastatic drug activity using high-resolution confocal microscopy is described. The use of low adherent plates over other techniques, such as hanging drops[15], has facilitated a means of generating reproducible and uniform spheroids for use in the collagen migration and invasion assays. The critical points in this protocol are the removal of growth medium from the 96-well plate prior to the cell spheroid embedding in a collagen matrix and the careful handling of the collagen plugs containing the spheroids thereafter. New technologies such as digital microfluidics[16] are now available and, although more expensive, could provide an alternative to the manual removal of media and fluids. The main limitation of the described protocol is that it is time-consuming when single spheroids are imaged and then analyzed manually by bright field microscopy in order to assess inhibitor drug activity. In addition, the reagents required for all steps of the process are expensive, and specialized equipment, namely a high-resolution confocal microscope, is also required. Optimal cell numbers required for seeding and the time taken to obtain a cell spheroid of the required size also must be predetermined prior to commencement of the collagen invasion assay.

The development of drugs targeting cancer cells, large-scale drug screening, and the characterization of drug activity for drug modification and improvement increasingly rely on 3D cell assays and technology. This protocol can be optimized and adapted for use with other experimental assays and numerous other cell types of importance in other disease systems. To date, the interpretation of data generated microscopically has been hampered by the application of wide-field microscopy, with images acquired offering limited resolution and plagued with inherent image blur resulting from light originating outside of the focal plane. The wide-field microscopy images shown here were acquired manually using an EVOS imaging system, a process that was time-consuming and could potentially introduce handler bias. New technologies, including automated workstations and analysis platforms[17,18], are now available but remain costly and are therefore still unavailable to many research laboratories. In addition, further software developments could aid in the analysis of high-resolution 3D rendered images to

accurately quantify phenotypic changes such as those noted here, and therefore the efficacy of inhibitors on cell migration. Furthermore, the application of super-resolution fluorescent imaging techniques that are increasingly becoming standard in many research laboratories will provide further insight into the migratory behavior and morphology of cells grown in 3D spheroid structures and treated with inhibitor drugs.

It was established that it is possible to fix and stain spheroids, following treatment with a migrastatic inhibitor, when embedded in collagen. This method was easy to perform, with all steps completed in 96-well plates, followed by mounting the collagen plugs containing the spheroids onto coverslips for imaging. In assessing the effect of inhibitor activity on cancer cell morphology, confocal microscopy was used to elucidate drug activity. Initial findings on the anti-migratory effect of inhibitor MI-192 from low-resolution images were confirmed by high-resolution confocal microscopy. This particular inhibitor targets histone deacetylase 3 (HDAC3). HDACs are enzymes involved in the epigenetic regulation of gene expression and have recently been of increasing interest as potential targets in cancer drug development. Previous experiences with MI-192 have shown that, at low concentrations, it regulates the acetylation of tubulin, leading to hyperacetylation and stabilization of microtubules. Stabilized microtubules are less dynamic, with a potential effect on migratory activities of cells. It was ascertained that the effect observed was present in both representative pediatric and adult glioma cell lines. A concentration-dependent increase in the tubulin acetylation status of both glioma cell lines was uncovered, a finding that has implications for the preselection of patients when considering complementary treatment with migrastatic drugs.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure

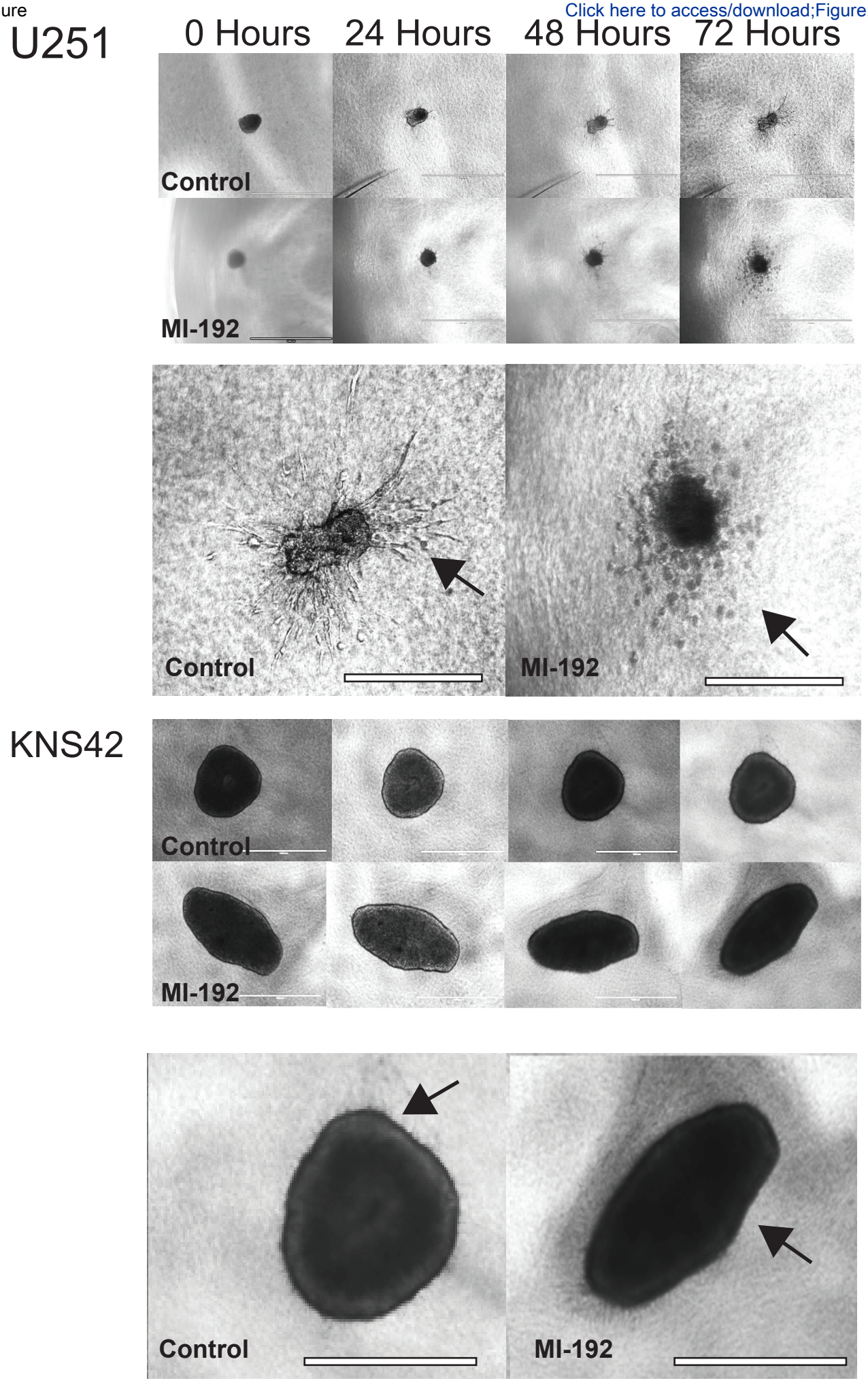

Fig 1.

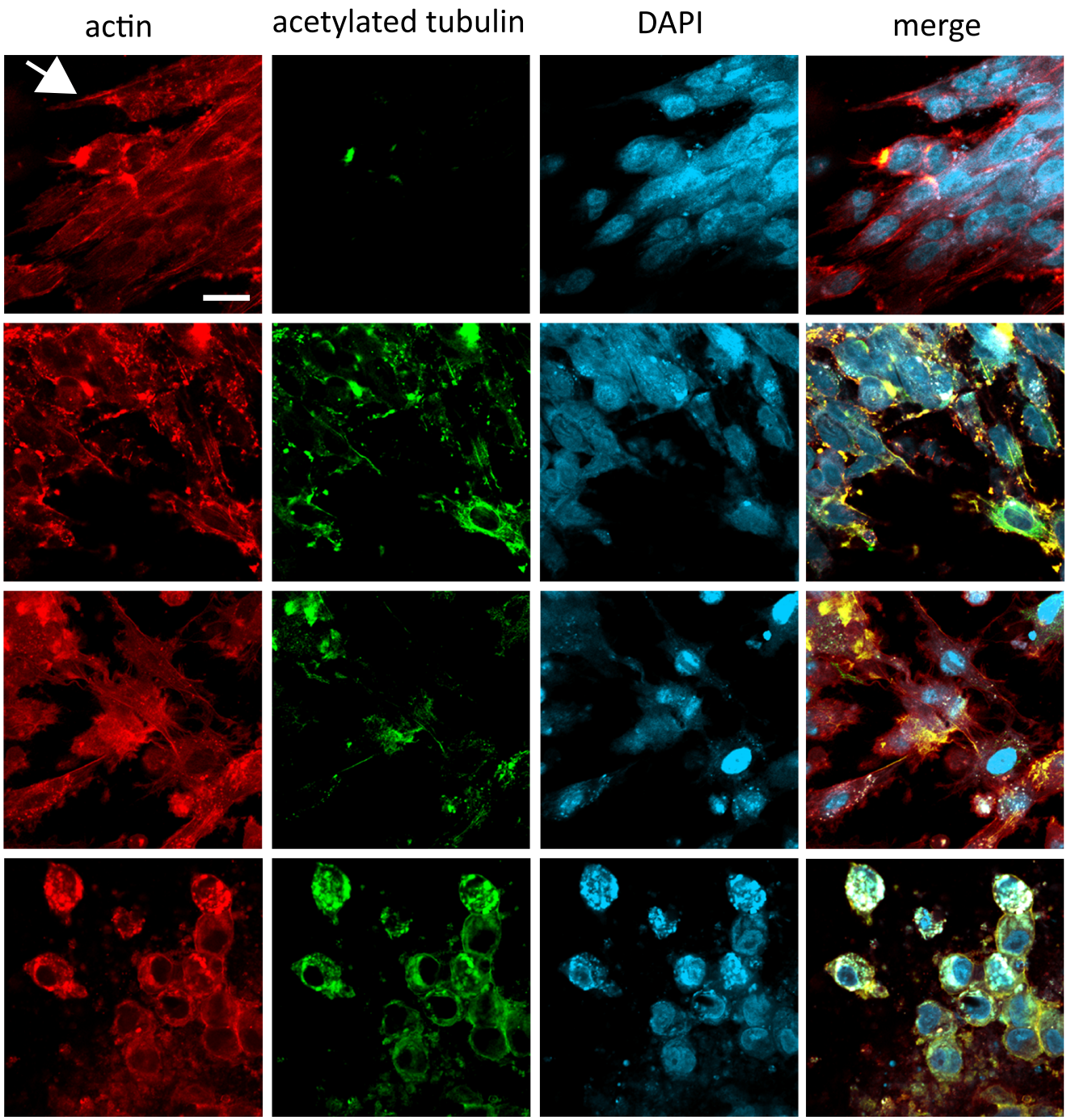

Fig. 2

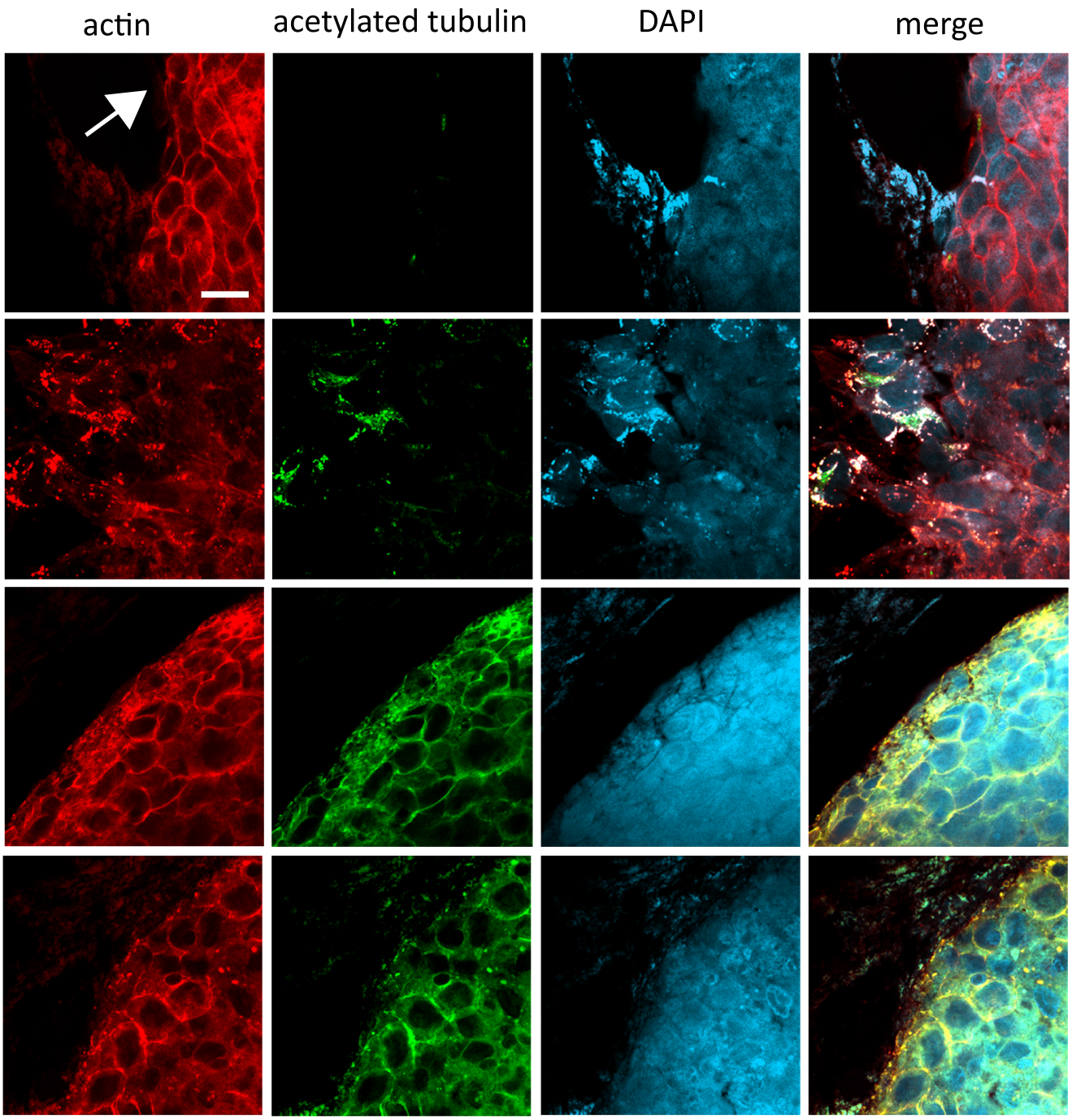

Fig. 3

Fig. 4

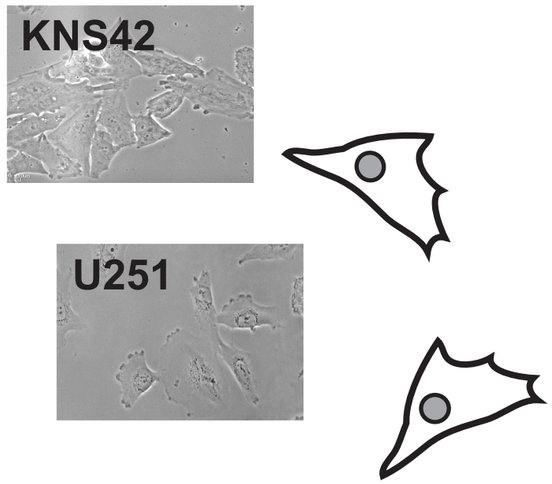

Table of Materials

Click here to access/download;Table of Materials;Copy of JoVE_Materials Harmer et al.xlsx

Name of Material/ Equipment	Company	Catalog Number
Collagen I, rat tail, 100 mg	Corning	354236
Coverslips	various	various
DMEM powder	Sigma	D5648
Foetal calf serum	Sigma	F7524-500ML
Glass slides	various	various
High glucose DMEM	Gibco	41965062
Inhibitor	Tocris	various
Mountant	various	various
NaOH (1 M)	various	various
Paraformaldehyde	various	various
Pastettes (graduated pipette, 3ml)	various	various
PBS, sterile for tissue culture	Sigma	D1408-500ML
Pen/strep (antibiotics)	Sigma	P4333
Primary antibody, secondary antibody	various	various
Sodium bicarbonate	Sigma	S5761

Sodium pyruvate	Sigma	P5280
Trypsin	Sigma	T4049
Ultra low attachment plates	Sigma/Nunc	CLS7007-24EA
Stripettes (serological pipettes, sterile, 5ml and 10 ml)	various e.g. Costar	CLS4488-50; CLS4487-50
various multichannel (50 - 250 uL) and single channel pipettes (10 uL, 50	various	various
Widefield microscopy	various	various
Zeiss LSM 880 CLSM equipped with a F	Zeiss	quote from manufacturer

Comments/Description

for glioma invasion assay; this is offered by many distributors/manufacturers and will need to be determined for both the type of assay inten

for microscopy imaging

needed at 5X concentration for collagen solution for glioma invasion assay; this may be purchased in powdered form, made up in double dist

needed for cell culture of glioma cell lines

for microscopy imaging

needed for cell culture of glioma cell lines

various - according to experimental design; inhibitors can be purchased from manufacturers such as Selleckchem and Tocris. These manufact

for microscopic imaging

NaOH can be either purchased at the required molarity or prepared from solid form. The prepared solution should be filter sterilized using a

for fixing spheroids and cells; make up at 4%, caution health hazard, ensure that health and safety regulations are adhered to for collagen sol

for invasion assay, solution
removal

needed for cell culture of glioma cell lines and washes for staining

needed for cell culture of glioma cell lines

there are many manufacturers for these reagents, for secondary labelled antibodies we recommend Alexa Fluor (Molecular Probes). Here we

needed for collagen solution for glioma invasion assay at 5X concentration

needed for collagen solution for glioma invasion assay at 5X concentration

for trypsinisation

for glioma invasion assay; a low adherent plate is required, with 96-well plates preferred to allow for large-scale screening of compounds und
for tissue culture and
collagen preparation
for cell and spheroid
handling
for observation of spheroid
generation and spheroid
Confocal images were
captured using a Zeiss

ded and cell lines used. For glioma cancer cell lines Collagen rat tail type 1 (e.g. Corning) is the preferred choice. Collagen should be stored at 4oC

tilled water and, depending upon final composition of the growth medium, completed with any additives required. The complete 5X solution sho

urers offer detailed description of inhibitor characteristics, links to associated references and suggestion of working concentrations. As with all in

syringe filter system. One M NaOH is corrosive and care should be taken during solution preparation.

ution for invasion assay

used for primary antibodies mouse anti-acetylated tubulin antibody (1/100, Abcam). For secondary antibodies we used 1/500 anti-mouse Alexa

ler investigation. There are several low adherence plates commercially available; it is advisable to test a variety of plates for optimum spheroid ge

C, in the dark, until required. It is not advisable to mix collagen from different batches as this may affect the consistency of the polymerized collag

uld be filtered through a syringe filter system (0.22 microns) before use.

hibitors, they may be potentially toxic and should be handled according to health and safety guidelines. Inhibitors are prepared as stock solutions

Fluor 488 conjugated antibody, Molecular Probes. For nuclear stain we used DAPI (many manufacturers) and the actin stain Phalloidin (many ma

eneration. In our experience Costar Ultra Low Cluster with lid, round bottom, works best for the generation of spheroids from glioma cancer cells

ɡen.

ɨ as recommended by the manufacturer. As an example we used the migrastatic inhibitor MI-192 to demonstrate the use of such inhibitors. We h

nufacturers) both used at recommended dilution of 1/500.

in terms of 100% spheroid formation and reproducibility. These plates were also successfully used for the generation of glioma spheroids from p

ıave tested a range of migrastatic inhibitors in this way with comparable results.

patient-derived material, bladder and ovarian cancer cells in our laboratory. In addition, stem or progenitor neurospheres can be used in these pla

ates to facilitate the generation of standardized neurosphere-spheroids

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Jove manuscript: TITLE: Characterisation of the effects of migrastatic inhibitors on 3D tumour spheroid invasion by high-resolution confocal microscopy

Dear editor,

We thank you for the comments from the reviewers and have now addressed the questions/suggestions raised for this manuscript.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Jane Harmer et al describes a methodology to study the effects of novel inhibitors on migration and invasion of glioblastomas in a three dimensional culture system using confocal laser scanning microscopy.

Major Concerns:
The authors should explain the advantages of spheroid culture method over the standard neurosphere assay method. What are the advantages of making spheroids (cell aggregation) over the neurospheres (ball of cells forming mainly by proliferation of a single cell (stem cells or progenitor cells)? There is a clear difference between these two and this should be addressed in their discussion or they can alternatively suggest the use of neurospheres in their invasion assay method.

Response: We agree that this technique can be easily used to generate spheroids from neurospheres for improved standardisation of spheroid size and ease to use these for invasion assays. We have actually used neurospheres from stem cell like glioma cells which also works very well in this assay. We focussed on the generation of spheroids from established cell lines as these are more accessible to most researchers. We will point out the use of neurospheres in this assay.

Minor Concerns:
The patterns of cell migration from the periphery of the spheroids are different in the cell lines used in this study. Is this phenomenon cell line dependent or the size of of spheroids plays a role here?

Response: The patterns of cell migration differ from cell line to cell line. We have observed distinct migration patterns for paediatric and adult glioma cell lines potentially reflecting their site of origin they were isolated from or the cell type they arose from. From our own experiences this is independent of spheroid size. We have commented on this in the manuscript now.

How thick is the collagen plug containing the spheroid when fixed? Doesn't this thickness cause any limitation for microscopy?

Response: The generated spheroids we used here are between 200 and 800 microns embedded in a semi-solid collagen matrix. When the plug is transferred to the glass slides the plug spreads out onto the slide without compromising the contained spheroid allowing the application of the cover slip and viewing by microscopy.

Reviewer #2:

Manuscript Summary:

This manuscript outlines the use of high-resolution confocal imaging to characterize the effects of a migrastatic inhibitor, MI-192, in glioma cell invasion from 3D spheroids into a surrounding matrix of collagen I. The method described appears to include the necessary steps and critical alerts for successful application by other researchers. However, the protocol would be improved by addressing a range of concerns.

General concerns:

The title states migrastatic inhibitors yet only examines the one inhibitor, MI-192, which is a bit misleading although the method, line 126, describes the use of inhibitors more generally.

Response: We appreciate that we should have pointed out that we used the inhibitor MI-192 as an example to investigate migrastatic inhibitors. We have tested a range of different inhibitors in this manner and achived comparable results. We will address this in the manuscript.

Terminology such as "stripettes" and "pastettes" should be avoided and items more fully described.Inclusion of catalog numbers in the text would be helpful.

Response: We have added the stripettes and pastettes used in the materials section.

Similarly, it would be helpful tp alphabetise contents in Table of Materials.

Response: We have done this in the Table of Materials.

The table is difficult to follow with catalog numbers and comments found on the next page.

Response: We will add the materials as the original spreadsheet.

It would be helpful to provide few more, albeit brief, details such as information on the type of inhibitor (e.g. HDAC inhibitor) and the relevance of acetylated tubulin or to provide the details earlier such as selection of adult versus paediatric glioma cell lines.

Response: We have addressed this in the text with details on HDAC inhibitors and the cell lines used.

Specific protocol description concerns:

In 1.1.1 Generation of cell spheroids, line 93, does (200; 800 microns) represent respective sizes of U251 and KNS42 spheroids?

Response: These are the sizes observed for both cell lines when plated out at the particular cell density (1000 cells/200 microliter)

In 1.1.2 Collagen invasion assay, is 5x incubation medium actually 5x culture medium? If so, please change the terminology.

Response: Corrected.

In 4 Preparation of embedded spheroids, although the work flow in figure 3 indicates that the spheroids are fixed at 72 h, the method does not explicitly state this.

Response: We have addressed this is section 3.10.

In 4.1, presumably the 4% formaldehyde is prepared in PBS?

Response: Yes, we have added 1X PBS in the text.

In 5.1 Confocal microscopy, what were the number and step size of optical slices captured by confocal microscopy? If a single optical section was captured, the protocol needs to state this.

Response: This information has been included in section 5.1

In Figure 1, a scale bar for the enlarged spheroid images is needed while the arrows don't particularly add to the identification of invasive cells.

Response: We have added the scale bars, the arrows are useful to highlight the relatively low resolution to identify individual migratory cells.

In contrast, arrows indicating invasive spikes of U251 cells in figure 2A and sheet-like invasion of KNS42 cells in 2B would be helpful. Similarly, if there are any detached U251 cells in 2A, they should be indicated.

Response: We have added arrows for clarification.

Has the research group attempted to quantify changes to cell morphology with migrastatic inhibitors? This would be a useful addition to the method.

Response: This is indeed a very good question which we are currently addressing for out next publication.