Journal of Visualized Experiments

Live-3D-Cell Immunocytochemistry Assay of pediatric Diffuse Midline Glioma

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63091R1
Full Title:	Live-3D-Cell Immunocytochemistry Assay of pediatric Diffuse Midline Glioma
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Additional Information:	
Question	**Response**
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TITLE:

Live-3D-Cell Immunocytochemistry Assays of Pediatric Diffuse Midline Glioma

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SUMMARY:

This study presents a protocol of live-3D-cell immunocytochemistry applied to a pediatric diffuse midline glioma cell line, useful to study in real-time the expression of proteins on the plasma membrane during dynamic processes like 3D cell invasion and migration.

ABSTRACT:

Cell migration and invasion are specific hallmarks of Diffuse Midline Glioma (DMG) H3K27M-mutant tumors. We have already modeled these features using three-dimensional (3D) cell-based invasion and migration assays. In this study, we have optimized these 3D assays for live-cell immunocytochemistry. An antibody Labeling Reagent was used to detect in real-time the expression of the adhesion molecule CD44, on the plasma membrane of migrating and invading cells of a DMG H3K27M primary patient-derived cell line. CD44 is associated with cancer stem cell phenotype and tumor cell migration and invasion and is involved in the direct interactions with the central nervous system (CNS) extracellular matrix. Neurospheres (NS) from the DMG H3K27M cell line were embedded into the basal membrane matrix (BMM) or placed onto a thin coating layer of BMM, in the presence of an anti-CD44 antibody in conjunction with the antibody labeling reagent (ALR). The live-3D-cell immunocytochemistry image analysis was performed on a live-cell analysis instrument to quantitatively measure the overall CD44 expression, specifically on the migrating and invading cells. The method also allows visualizing in real-time the intermittent expression of CD44 on the plasma membrane of migrating and invading cells. Moreover, the assay also provided new insights into the potential role of CD44 in the mesenchymal to amoeboid transition in DMG H3K27M cells.

INTRODUCTION:

The ability of tumor cells to evade and disseminate through the surrounding tissue is a hallmark of cancer[1]. In particular, tumor cell motility is a characteristic feature of malignant tumors, whether it is a metastatic tumor type such as breast[2] or colorectal cancer[3] or a locally invasive type such as diffuse glioma[4,5].

Imaging has a central role in the investigation of many aspects of tumor cell phenotypes; however, live-cell imaging is definitely to be preferred when studying dynamic cellular processes such as migration and invasion, when changes in morphology and cell-cell interaction[6,7] occur and can be more easily examined over time. For live-cell imaging, different optical microscopy systems can be used, from phase contrast to confocal fluorescent microscopes, and image acquisition performed over a short or long period of time on an inverted microscope equipped with a chamber for temperature and CO_2 control, or in high-content image analysis systems which have built-in chambers, or alternatively in image systems that can sit in the incubator without the need to disturb the cells during the whole duration of the experiment. The choice of the system used is often dictated by a number of factors such as resolution needed, length of the overall acquisition time and time intervals, vessel type used and throughput of the assay (single chamber or multi-well plate), the sensitivity of the cells used (precious and/or rare cells) and phototoxicity of the cells if fluorophores are present.

With regard to fluorescent imaging in live mode, this can be achieved by transducing cells for the expression of fluorescent proteins either for stable expression or as an inducible system[8], by transient cell transfection, or by using cell dyes which are now available for live-cell labeling[7], for live-cell tracking as well as for labeling subcellular organelles[9].

A useful approach has been recently developed for live-cell immunocytochemistry, where an antibody recognizing a surface marker of choice can be bound to a labeling reagent, and upon addition to the culture media, cells expressing the specific marker can be readily imaged in real-time by live-cell imaging. The visualization and quantification of marker expression using such a system can be easily achieved when cells are grown in two-dimensional (2D) culture conditions[10].

In this study, we optimized protocols for live-3D-cell immunocytochemistry invasion and migration of pediatric diffuse midline glioma (DMG) patient-derived cells[11,12]. DMG are highly aggressive brain tumors affecting children, for the vast majority associated with the driver mutation K27M in histone H3 variants. DMG arise in the brain stem and the midline regions of the central nervous system (CNS) and are characterized by a highly infiltrative nature. This invasive capacity has been shown to be at least in part mediated by the intratumor heterogeneity and the cancer-stem-like features of DMG cells[7].

To exemplify our assays, an antibody labeling reagent (ALR) was used in combination with an antibody for CD44. CD44 is a transmembrane glycoprotein and adhesion molecule expressed on stem-cell and other cell types, associated with cancer stem cell phenotype and tumor cell migration and invasion[13]. The protocols include the sample preparation, the image acquisition in

brightfield and fluorescent mode, and the analysis on a live-cell analysis instrument that allowed to quantitatively measure in real-time the overall CD44 expression on the DMG cell membrane during 3D invasion and migration. The assays also allowed the possibility to visualize the intermittent fluorescent signal of CD44 on individual cells while migrating and invading. Interestingly an effect of the anti-CD44 antibody was also observed, which potentially acting as a blocking antibody, also seemed to reduce cell migration and invasion as well as to induce a switch of the invasion pattern from a collective mesenchymal-like to a more single-cell amoeboid-like phenotype.

PROTOCOL:

This protocol follows the guidelines of the institutions' human research ethics committees.

NOTE: This study was performed using Incucyte S3 and/or SX5 Live-Cell Analysis Instrument (referenced as live cell analysis instrument).

1. Generation of reproducibly sized tumor spheroids

NOTE: The protocol (section 1) described by Vinci et al. 2015[7,12], was used as reported below, with some modifications:

1.1. Collect the DMG H3K27M-mutant neurospheres (NS) and centrifuge at 170 x *g* for 10 min at room temperature (RT).

1.2. Incubate the NS with 500 µL of the accutase solution for 3 min at 37 °C to break them up.

1.3. Neutralize the accutase solution with tumor stem cell (TSM) medium[7] and centrifuge the cell suspension at 355 x *g* for 5 min at RT.

1.4. Resuspend the cell pellet in 1 mL of TSM medium and then count the cells using a cell counting chamber.

1.5. Dilute the cell suspension to obtain 2.5–5 x 10^3cells/mL and seed 100 µL/well into ultra-low attachment (ULA) 96-well round-bottom plates (see **Table of Materials**). Use a proper cell density to obtain individual NS of ~300 µm diameter, 4 days after cell seeding (250–500 cells/well for highly aggressive glioma cells).

1.6. Visually confirm the NS formation by using an inverted microscope 4 days after cell seeding

2. Preparation of the ALR/antibody complex and setup for the invasion assay

NOTE: For the antibody labeling procedure, the antibody labeling dyes protocol[10] for live-cell Immunocytochemistry is used with some modifications, as reported below. For the invasion assay, the protocol previously described by Vinci et al. 2015[12] is followed.

2.1. Consider the number of wells (e.g., 60 wells) to analyze and calculate the volume needed for each reagent. Also include the wells for the negative control (samples with ALR but without antibody).

2.2. Add 100 µL of sterile water to the ALR to rehydrate the reagent (final concentration = 0.5 mg/mL). Pipette to mix the solution.

NOTE: The reagent is light-sensitive; therefore, keep in the dark. Aliquot the leftover reagent and store at -80 °C (avoid freezing and thawing).

2.3. Mix the antibody with the ALR in the TSM medium (or appropriate cell growth media for the cell line of choice) in a round bottom multi-well plate or in an amber tube and protect from light.

2.4. Prepare enough quantity of the medium to dispense 25 µL/well at 3x final assay concentration. Incubate at RT for 15 min.

NOTE: A 1:3 molar ratio of antibody to ALR is recommended, with a final (1x) concentration of the test antibody <1.5 µg/mL. For the experiments in this protocol an anti-human CD44 mouse antibody is used (starting concentration 86 µg/mL) at a final concentration of 0.1 µg/mL (3x concentration = 0.3 µg/mL).Add the reagents in the following order: i) antibody; ii) ALR; iii) TSM medium. Mix by pipetting.

2.5. Dilute the background suppressor reagent (BSR) in TSM medium (or appropriate cell growth media for cell line of choice) at 1.5 mM (3x) to obtain at the end of the assay a final concentration of 0.5 mM.

2.6. Perform the invasion assay directly in the ULA 96-well round-bottom plate where cells were initially seeded. Check the NS visually using an inverted microscope before starting.

2.7. Gently and slowly remove 75 µL/well of the medium, avoiding touching the bottom of the well where the NS sits. Check the presence of the NS visually.

2.8. Gently add 25 µL of the BSR to each well.

2.9. Gently add 25 µL of the ALR/antibody complex to each well. Wait 2 or 3 min to let the ALR/antibody complex mix with the medium.

2.10. Check visually using an inverted microscope to ensure that each NS is centrally located at the bottom of the well. Avoid the formation of bubbles. If any bubble is present, remove it by using a needle.

2.11. Place the plate on ice and wait 5 min to let the bottom of the plate become cold.

2.12. With a pre-cooled p200 tip, dispense 75 µL/well of basal membrane matrix (BMM), placing the pipette tip on the internal wall of the well and avoiding touching the bottom of the well. Avoid the formation of bubbles and remove with a sterile needle the existing ones.

Note: Make sure to have thawed the BMM at 4 °C from the night before.

2.13. Leave the plate on ice for 5 min to let the BMM mix with the medium. Check visually using an inverted microscope the presence of the NS and that they are centrally located in the well. If not, centrifuge the plate at 4 °C at 180 x *g* for 5 min.

2.14. Transfer the plate in the live-cell analysis instrument (**Table of Materials**) placed within the incubator at 37 °C, 5% CO_2, 95% humidity.

3. Preparation of the ALR/antibody complex and setup for the migration assay

NOTE: For the antibody labeling procedure, the Labeling Dyes protocol[10] for Live-Cell Immunocytochemistry is used.

3.1. Consider the number of wells to analyze and calculate the volume needed for each reagent. Include also the wells needed for the negative controls. Check the NS visually using an inverted microscope before starting.

3.2. Use flat-bottom 96-well plates. Perform the coating procedure as described by Vinci et al., 2013[14]. For this study, the BMM is used as a thin coating.

3.3. Once the coating is ready, remove the excess of BMM coating with a p200 tip placing the tip in the edge of the well and avoiding touching the bottom. If working with multiple wells, use a multichannel pipette.

3.4. Cut a p200 tip, take 50 µL of the cell medium + NS from each selected well, and transfer it to a coated flat bottom well. Check the presence and the position of the NS in each well visually.

NOTE: Each NS must be centrally located in the well. Avoid leaning on the tip on the edge of the well during the transfer but drop the medium centrally in the well without touching the bottom. For highly migratory cells, consider a higher number of replicates than the standard three replicates. This is because when NS sits too close to the edge of the well, the migrating cells may cover a smaller area of the well.

3.5. Rehydrate the ALR as described above (steps 2.2–2.3).

NOTE: Reagent is light sensitive. See above for good handling procedures.

3.6. Mix the antibody with ALR in the appropriate complete cell growth media in a round bottom multi-well plate or in an amber tube and protect from light. Prepare enough quantity to dispense 50 µL/well at 3x final assay concentration. Incubate at RT for 15 min.

Note: Add the reagents in the order as indicated above (step 2.4).

3.7. Follow the same procedure as reported in step 2.5.

3.8. Gently add 50 µL of the BSR to each well.

3.9. Gently add 50 µL of the ALR/antibody to each well. Wait 2 or 3 min to let the reagents mix and check visually using an inverted microscope to ensure that most of the replicate NS are centrally located in the well.

3.10. Avoid the formation of bubbles and remove any existing ones by using a needle. Gently transfer the plate in the live-cell analysis instrument placed within the incubator at 37 °C, 5% CO_2, 95% humidity.

4. Live-cell analysis instrument setting for image acquisition

4.1. Scan the plates using the live-cell analysis instrument (for specifications, see **Table of Materials**) with scanning intervals starting from time point zero (t0) of the invasion and migration assays set up, respectively, after step 2.14. and 3.10. up to 96 h.

NOTE: Ensure to be able to dispose of the live-cell analysis instrument immediately after the starting of the invasion and migration assay. Depending on the tumor type, cells can start to invade or migrate from the NS already within 1 h from the assay setup.

4.2. On the live-cell analysis instrument software, select the option **Schedule to Acquire.** Click on the **+** tab and select the option **Scan on Schedule**.

4.3. On the software window **Create or Restore Vessel**, click on the option **New.**

4.4. Select the specific application in the live-cell analysis instrument for the invasion and migration acquisition. Select **Spheroid** scan type, 4x objective, **Phase+Brightfield** and **Green** image channels for the invasion assay. Select **Dilution Cloning** scan type, 4x objective and **Phase** and **Green** for the migration assay.

4.5. Select the plate type and define the wells to be scanned by highlighting them on the plate map.

4.6. Set up the scanning frequency (for the experiments in this protocol scanning frequency was 15 min for invasion and 30 min for migration).

4.7. Click on **Add to Schedule** and start the scan.

5. Live-cell analysis instrument setting for image analysis

5.1. Select the tab **Create New Analysis Definition**.

5.2. Select **Spheroid Invasion** or **Basic Analyzer** application for invasion and migration, respectively, on the tab.

5.3. Select the invasion and migration appropriate channels (for Invasion: **Phase+Brightfield – Green**; for Migration: **Phase – Green**) in the image channel.

5.4. Select few representative images from 3–4 wells for previewing and refining the analysis setting.

5.5. For the invasion assay, in the **Analysis Definition** tab, adjust the application settings in the **Brightfield** and **Green** channels with the following setting to generate a precise segmentation between the Whole Spheroid and Invading Cells (see **Figure 5**; blue mask):
Brightfield Segmentation: Whole Spheroid sensitivity = 50; Invading Cell sensitivity = 100; Clean Up = default.
Whole Spheroid Filters: set all parameters as default.
Invading Cells Filters: set all parameters as default.
Green Segmentation: Radius = 900.

5.6. For migration assay, adjust the application settings in the **Phase** and **Green** channels to generate a precise segmentation between the Confluence and Green Cells (see **Figure 5**, yellow and pink masks) with the following setting:
Phase: set all parameters as default.
Green Segmentation: Radius = 300; Threshold = 1000
Cleanup: Hole Fill = 400; Filters = default.
Whole Well: set all parameters as default.

5.7. Check that the analysis settings are correct for the NS by clicking randomly on several wells. The segmentation must outline the spheroid. If not, adjust the setting accordingly.

5.8. Select the wells and time points to analyze.

5.9. Save the **Analysis Definition** and click on **Finish**.

REPRESENTATIVE RESULTS:

Live-3D-Cell Immunocytochemistry protocol for invasion and migration is summarized in a straightforward and reproducible workflow in **Figure 1**. By seeding the DMG cells in ULA 96-well round-bottom plates, reproducible sized NS are obtained and used in the steps displayed. When the NS have reached the ideal size of ~300 µm (approximately 4 days post-seeding) the invasion[12] and migration[14] assays are initiated. The addition of the ALR/antibody complex together with the background suppressor in the medium of the individual NS, allows following the specific marker expression on the cell membrane, in live imaging and over time. The surface marker expression during the cell invasion and migration is easily monitored at intervals starting from t = 0 up to 96 h using the live-cell analysis instrument. This imaging system allows a fully automated image analysis.

A primary patient-derived cell line, QCTB-R059, was used to exemplify the invasion and migration proprieties of pediatric DMG tumor dissemination. QCTB-R059 was originally indicated as a pediatric thalamic glioblastoma (GBM) cell line[15]. Later on, it has been indicated as H3-K27M thalamic glioma cell line[16] or diffuse midline glioma (DMG) H3-K27M cell line[11], following the 2016 World Health Organization classification of brain tumors with the introduction of DMG H3F3A K27M-mutant as a new entity[17].

CD44, an adhesion molecule known to be involved in cell migration and invasion, was investigated. CD44 is expressed by QCTB-R059 cells as demonstrated by confocal images of immunofluorescent (IF) staining on 3D cell migration onto (**Figure 2**), and invasion into (**Figure 3**) BMM.

Taking into consideration that 3D invasion and migration are both non-static processes, we thought to investigate the expression of CD44 over time when cells are in movement. To do this we employed the live-cell immunocytochemistry assay and adapted the protocol for 3D assays. By using the ALR in complex with an anti-CD44 antibody, we are able to follow in real-time the expression of CD44 when the protein is expressed on the cell membrane while the cells evade the neurospheres and spread onto and into the BMM.

The live-3D-cell immunocytochemistry allows visualizing CD44 expression (**Supplementary Video 1** and **Supplementary Video 2)**. The representative frames of the time-lapse, for both migration and invasion (**Figure 4A**,**B**), show more in detail the intermittent expression of CD44 on the cell membrane. In particular, it is possible to see the green fluorescent signal to be on (green circle) and then off (black circle) on the same cell observed over time, suggesting that the expression of CD44 is on and off while cells are migrating and invading.

The migration and invasion processes are followed over 96 h, and as shown in **Figure 5**, QCTB-R059 cells show a high level of CD44, demonstrating that overall the expression observed with the live-cell immunocytochemistry is in line with the expression of CD44 obtained by IF shown on confocal images in **Figure 2** and **Figure 3**. Interestingly though, we also noticed that when the anti-CD44 antibody is used on live cells, it affects cell morphology, inducing a transition from mesenchymal-like to amoeboid-like invasion. It induces a reduction of the invasive and migratory capacity of these cells (**Supplementary File 1**). We cannot exclude, though, that the reduction in

cell migration and invasion observed is also in part due to an inhibition of cell proliferation.

The automated image analysis performed on the live-cell analysis instrument shows the quantification of CD44 expression and its increase over time, measured by the overall green fluorescent signal associated with the ALR (**Figure 5B**,**C**) for both migration and invasion. The quantifications are achieved as exemplified in **Figure 5B and Figure 5C**, with the automated image analysis set to segment all the area covered by the CD44 green-migrated cells (**Figure 4B**) and all the spread area covered by the CD44 green-invaded cells but excluding the neurosphere core (**Figure 5C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic workflow of Live-3D-Cell Immunocytochemistry Assays. The workflow shows the steps involved in the 3D invasion and migration live imaging methods, including representative images of pediatric primary DMG patient-derived cells (QCTB-R059) after invasion into (top panel; t = 96 h) and migration onto (bottom panel; t = 96 h) BMM. Bars = 800 µm.

Figure 2: CD44 expression in 3D tumor cell migration. Representative immunofluorescent confocal images of CD44 expression in primary DMG patient-derived cells (QCTB-R059) upon migration onto BMM. Timepoint = 96 h (red:CD44; blue: nuclei). Scale bars: 500 µm (upper panel) and 200 µm (lower panel).

Figure 3: CD44 expression in 3D tumor cell invasion. Representative immunofluorescent confocal images of CD44 expression in primary DMG patient-derived cells (QCTB-R059) upon invasion into BMM. Time point = 96 h (red:CD44; blue: nuclei). Scale bars: 250 µm (upper panel) and 100 µm (lower panel).

Figure 4: CD44 expression over time. Selected frames of QCTB-R059 migration (A) and invasion (B) time-lapse. Images were obtained on the live-cell imaging instrument. Green circle indicates the expression of CD44, black circle indicates no CD44 expression on the cell membrane of the same cell observed over time. Scale bars: 200 µm (**A**) and 100 µm (**B**).

Figure 5: Live-3D-Cell Immunocytochemistry Assays for CD44: migration and invasion. (**A**) Representative brightfield, fluorescent (ALR with anti-CD44 antibody), and merge images of QCTB-R059 cell immunocytochemistry migration, and invasion (96 h) are shown. Scale bars: 400 µm. (**B**) Quantification of CD44 overall expression relatively to migration (**B**) and invasion (**C**), determined by ALR-anti-CD44 image analysis on the live-cell imaging instrument. The curves show the Green Mean Intensity of CD44 expression over time. Values are mean ± SD. The two small figures in the plots display the segmentation applied for the analysis of the migration (**B**) where all area was considered and the invasion (**C**) for which the NS core part was excluded.

Supplementary Figure 1: Effect of anti-CD44 antibody on cell morphology and degree of cell motility. Representative images of QCTB-R059 invasion and migration assay show the effect of anti-CD44 antibody used for the live-3D-cell immunocytochemistry. Cells display a reduced invasion and migration capacity as well as the transition from a more mesenchymal-like to

amoeboid-like invasion pattern between the negative control (without anti-CD44 antibody) and CD44 (plus anti-CD44 antibody). Lower panel shows higher power magnification displaying a more clear view on the morphological appearance of the cells in the absence and the presence of the anti-CD44 antibody (white arrows). Scale bars: 800 µm upper panel and 100 µm lower panel.

Supplementary Video 1: Time-lapse video of QCTB-R059 3D cell migration on BMM in the presence of anti-CD44 antibody. Fluorescent green signal, representing the expression of CD44 on the cell membrane, is visualized over time by the conjugation of the anti-CD44 antibody with ALR.

Supplementary Video 2: Time-lapse video of QCTB-R059 3D cell invasion in BMM in the presence of anti-CD44 antibody. Fluorescent green signal, representing the expression of CD44 on the cell membrane, is visualized over time by the conjugation of the anti-CD44 antibody with ALR.

DISCUSSION:

The live-3D-cell immunocytochemistry we have adopted here for pediatric DMG invasion and migration could be easily adapted also for other highly invasive tumor cell types, including breast and colon cancer cell lines.

Different from previously performed live-2D-cell immunocytochemistry assays[10], when working in 3D, it is suggested to pay attention to some critical steps. In particular, for the invasion assay we describe, it is advised to add the ALR/antibody mix directly to the medium with NS in each well, prior to the addition of the BMM, and not in the BMM or on top of the BMM once gelled. This is to allow a good mix of the reagents with the medium and ensure more direct access of the reagents to the cell surface. Moreover, to ensure a better quality of the imaging, although the protocol includes the use of the BSR, we advise using phenol red-free medium and BMM.

Another point to consider for the live-cell immunocytochemistry is that any antibody binding an extracellular membrane protein on live cells may affect the protein function by altering its conformation or by occupying the binding site of a ligand or of a protein on an adjacent cell, therefore acting as a "blocking agent"[18,19]. While this approach may be useful as a therapeutic strategy[19], it may not be the primary goal of any experimental setup. Therefore, prior to performing a large set of experiments, different antibodies binding distinct epitopes of the same protein should be tested to also verify any potential "blocking" effect. In this study, we used a specific antibody to follow in real-time the expression of CD44 on the cell membrane of a highly aggressive pediatric DMG cell line in 3D invasion and migration. The protocol used allowed us to quantitatively measure the expression of CD44 over time on cells invading and migrating. Interestingly, in the presence of the anti-CD44 antibody, we also noted a reduction in cell motility in comparison to the cells with the ALR but in the absence of the antibody. We cannot exclude though also an inhibitory effect on cell proliferation. The acquisition of a different invasion pattern with a switch from mesenchymal-like to amoeboid-like cell morphology[20] was also observed in the presence of anti-CD44 antibodies. These unexpected results suggest that blocking CD44 may contribute to mesenchymal to amoeboid transition in pediatric DMG.

With regard to the limitations of this protocol, taking into consideration the resolution of the CCD camera of the Incucyte Live-Cell Analysis Instrument and its limited z-stack capability, the setup we present for the live-3D-cell immunocytochemistry assays may be used as a preliminary approach, on a large scale multi-well format, before moving on to a more in-depth analysis using either more powerful fluorescent imaging systems (e.g., confocal microscopes and high-content imaging system with a confocal modality such as the Operetta CLS or the Opera Phoneix) or a more refined approach for studying in real-time the expression of a surface protein via a reporter assay[21].

A broader application of the live-3d-cell immunocytochemistry presented here as a monoculture, could be a 3D co-culture assay established to image and analyze in real-time direct cell-cell interactions. In this case, two different ALR could be used with different fluorophores to bind proteins specifically expressed on the cell membrane on different cell types (e.g., tumor cell and immune cells). In this case, direct cell-cell contact may be analyzed with live imaging by the co-localization of the two different ALR/antibody complexes.

ACKNOWLEDGMENTS:

We would like to thank Dr. Silvia Soddu and Dr. Giulia Federici (Unit of Cellular Networks and Molecular Therapeutic Targets, IRCCS-Regina Elena National Cancer Institute, Rome, Italy) for access to the IncuCyte S3 Live Cell Imaging System in the preliminary set up of the imaging protocol. Moreover, we thank Bernadett Kolozsvari for the technical advice. The study was supported by the Children with Cancer UK grant (16-234) and The Italian Ministry of Health Ricerca Corrente. M Vinci is a Children with Cancer UK Fellow. R Ferretti is a recipient of Fondazione Veronesi Fellowship (2018 and 2019). The authors acknowledge Fondazione Heal for their support and the Children's Hospital Foundation for funding the Queensland Children's Tumor Bank.

DISCLOSURES:

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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Figure 1

Click here to access/download;Figure;figure 1.png

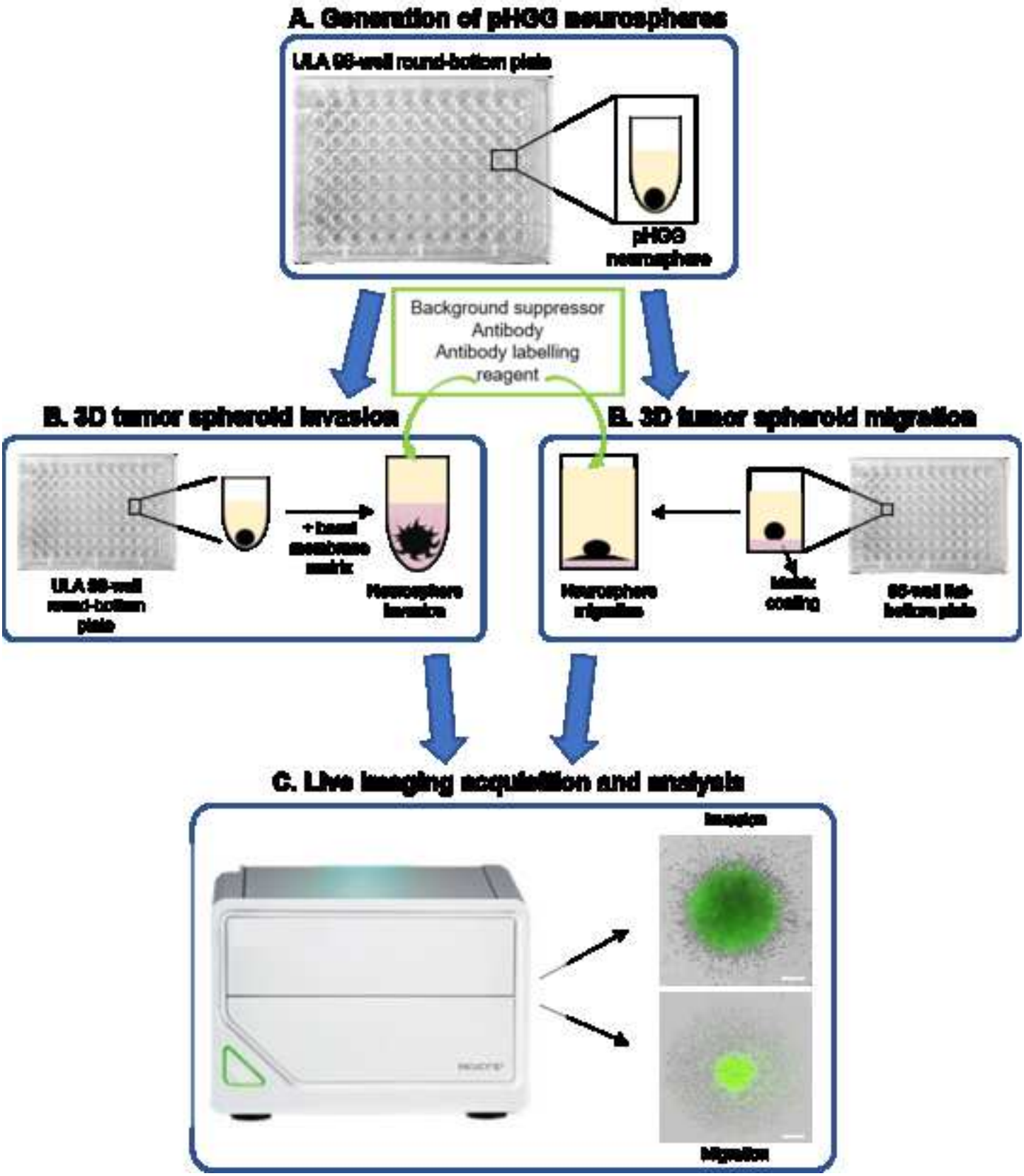

Figure 2

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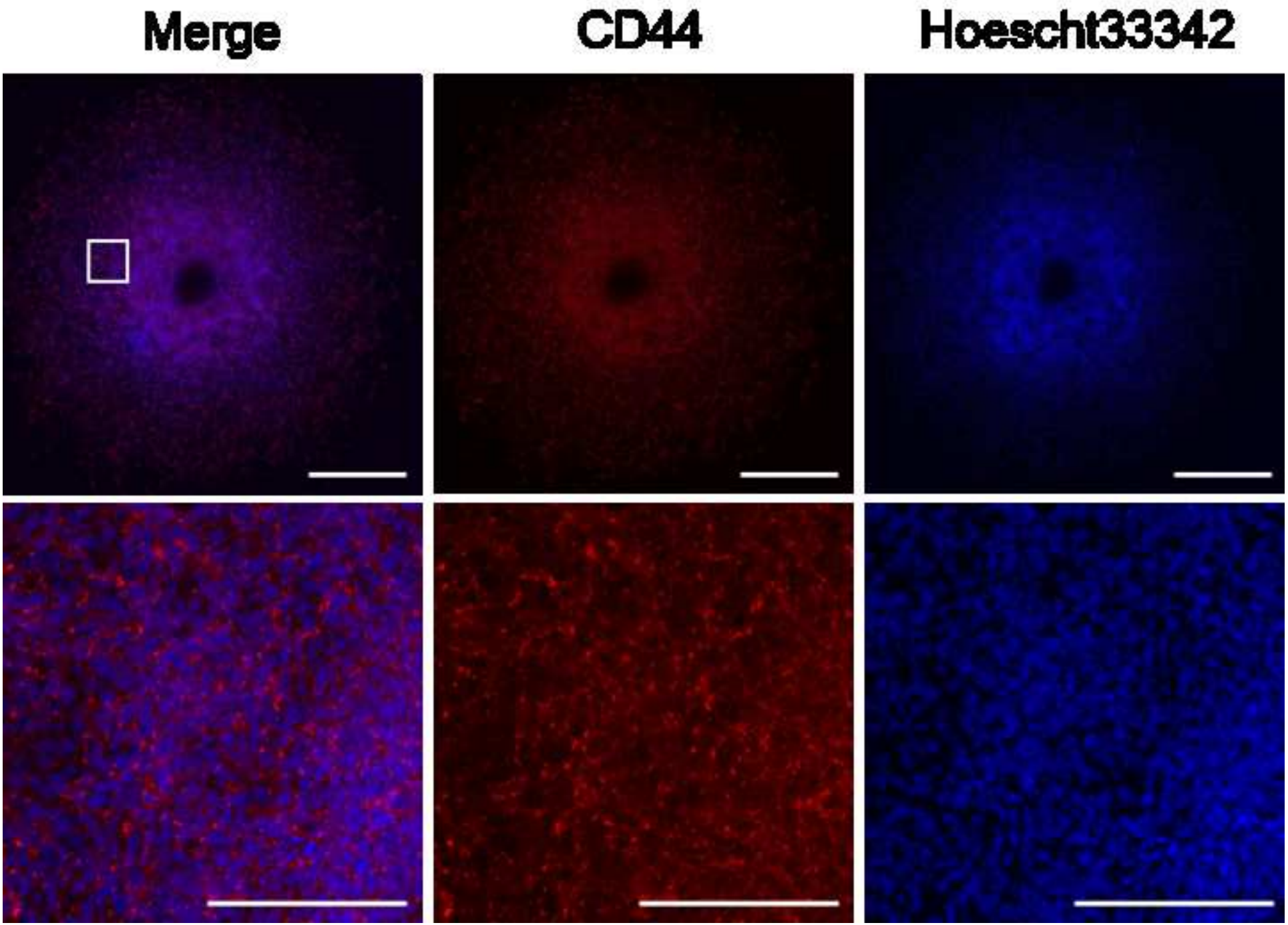

Figure 3

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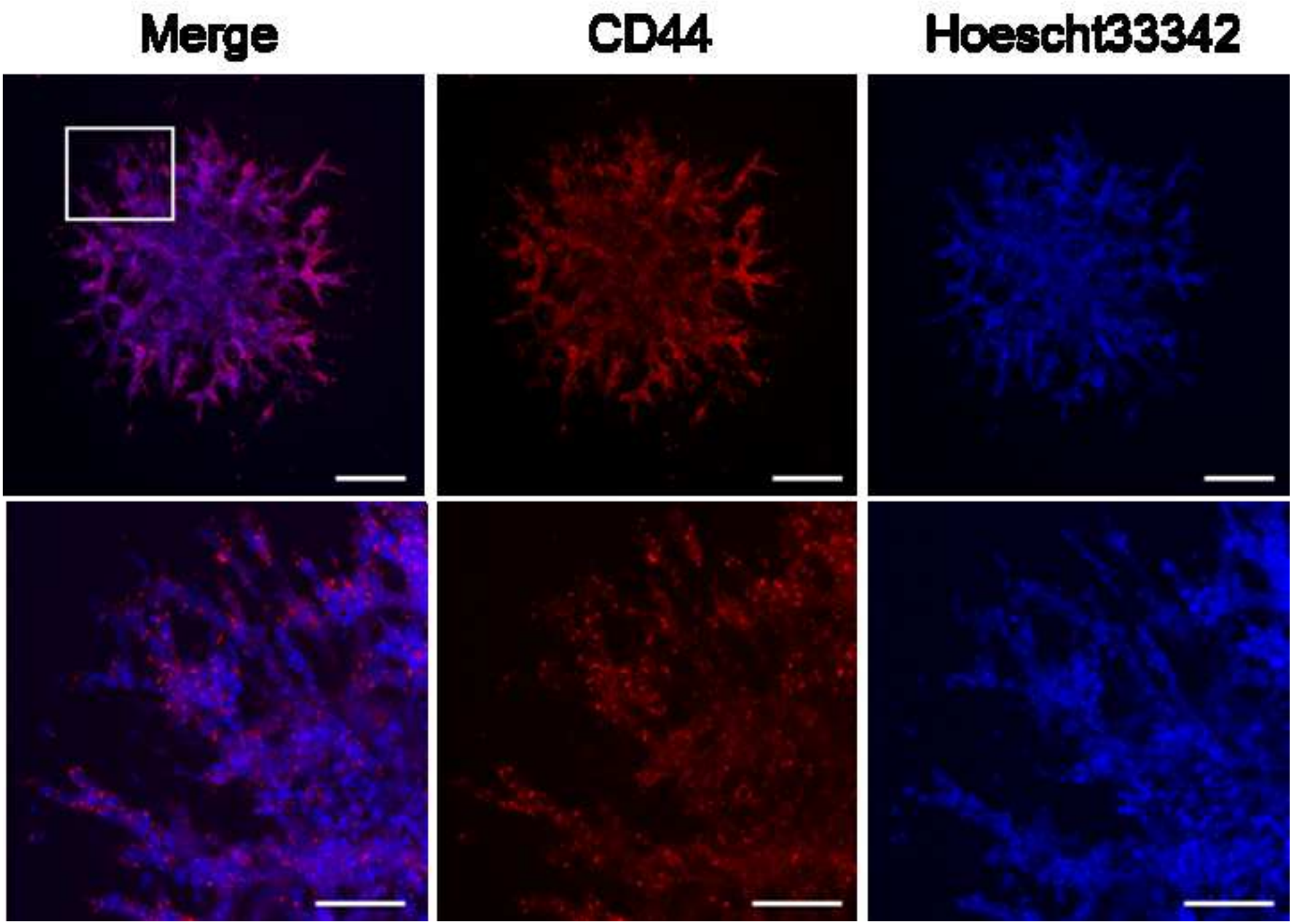

Figure 4

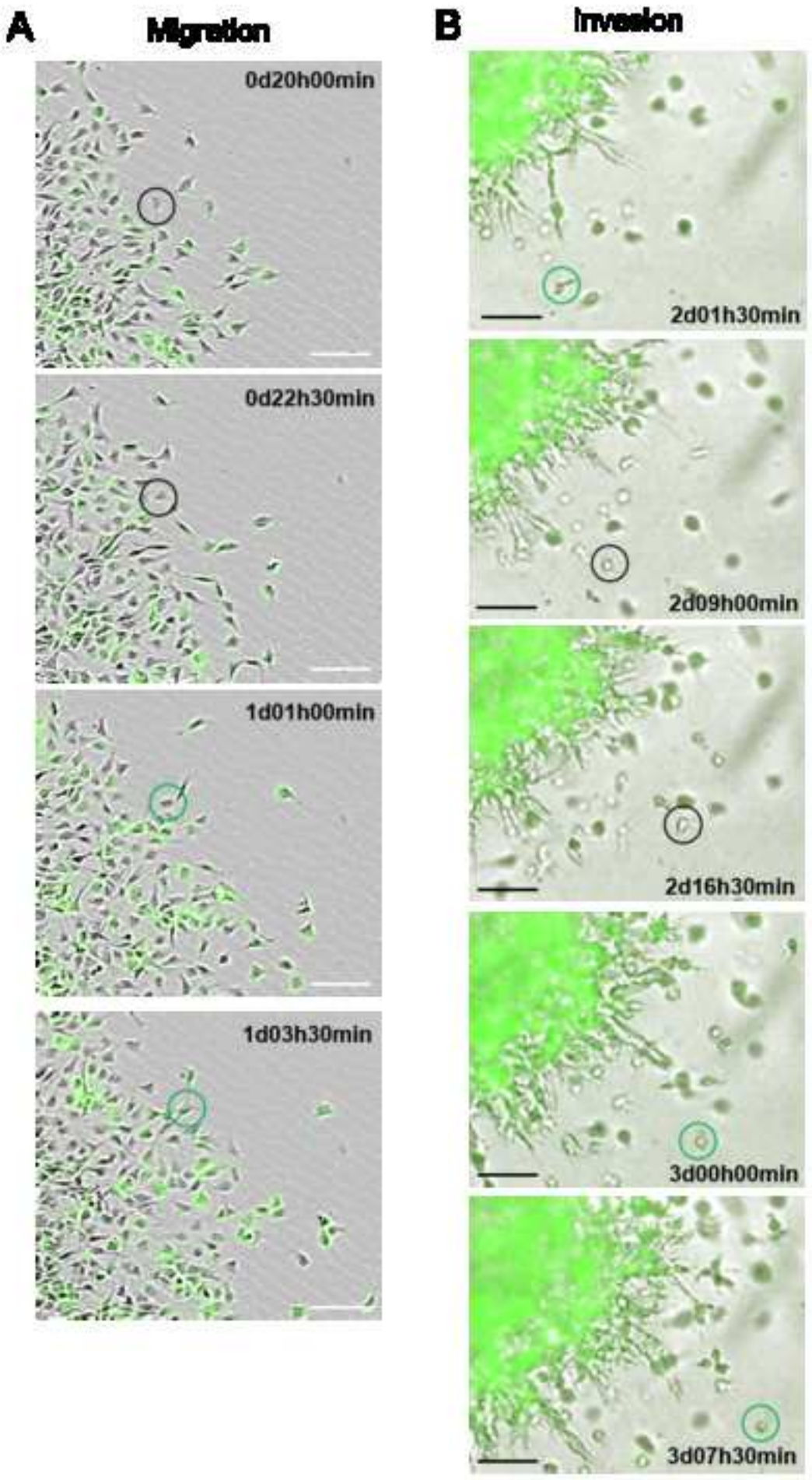

Figure 5

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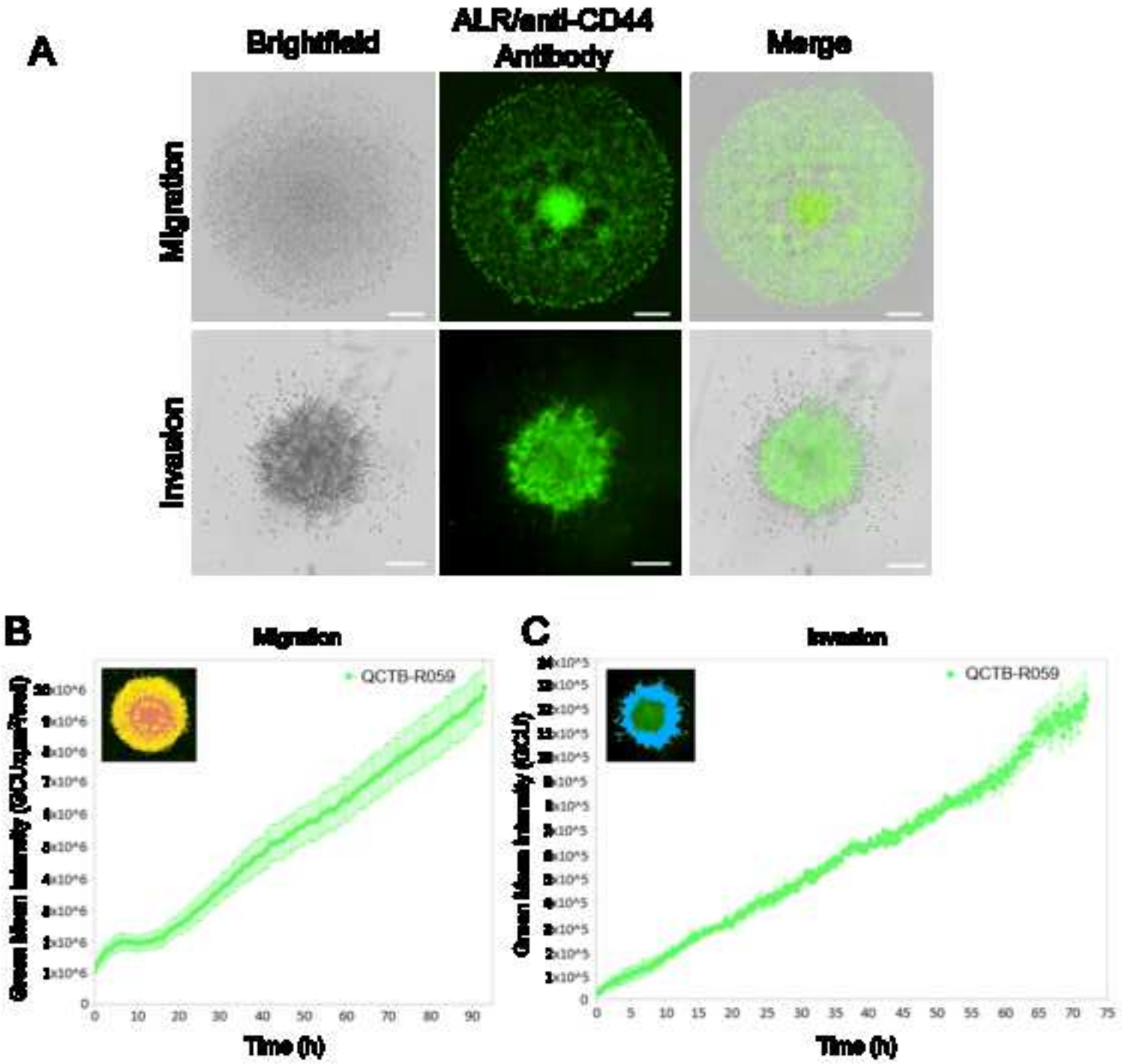
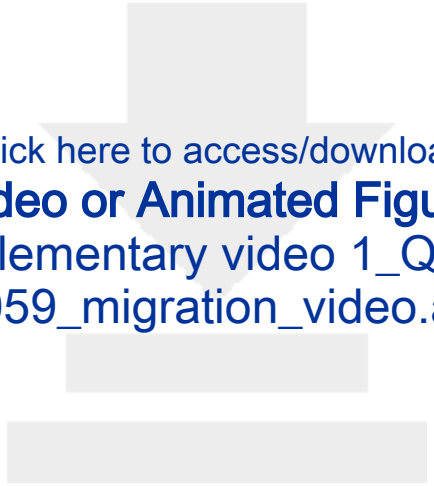


Supplementary Video 1



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Video or Animated Figure

Supplementary video 1_QCTB-R059_migration_video.avi

Supplementary Video 2

Click here to access/download

Video or Animated Figure

Supplementary video 2_QCTB-R059_invasion video.avi

Rebuttal letter for the JoVE63091 "Live-3D-Cell Immunocytochemistry Assay o f pediatric Diffuse Midline Glioma"

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues

We have proof read the manuscript.

2. Please provide an institutional email address for each author.

Please find below the e-mail address of the authors:

giulia.pericoli@opbg.net

ferrettiroberta@hotmail.it

andy.moore@health.qld.gov.au

maria.vinci@opbg.net

3. Please revise the following lines to avoid previously published work: 111-112,113-115,116-118,175-178,217-219. Please refer to the ithenticate report attached.

We have reviewed the lines and modified the text.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have reviewed this and removed and modified where appropriate.

5. Please define all abbreviations before use. For example, CNS, IF, etc.

We have now reviewed all the abbreviations and modified accordingly.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: IncuCyte FabFluor-488 Antibody Labeling Reagent, Opti-Green, FabFluor, Matrigel.

We have modified as required. In the new Table of Material, all the reagents are now named with a common non-commercial name which has been given for protocol purposes.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your

protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have modified the text as requested.

8. Line 96: Please specify the cells used in this study. What was the medium used? Specify the culture conditions. How was the cell density measured? How were the cells counted?

For the study we have used the QCTB-R059 primary patient-derived DMG cell line. We have provided the information about the medium and culture conditions (line 100) and references have also been provided. The cells were counted with a burker chamaber as indicated in the revised version.

9. Line 99: How was the neurosphere formation confirmed. Microscopically? What type of microscope was used?

We have provided the information required.

10. Line 108: How much volume is required for one well. How much was used in this experiment? What were the number of wells for the given protocol? Please specify.

We have provided additional information as required (line 106) and line (line 117).

11. Line 132: What is the selected size range? If the microscope step is to be filmed, please provide microscope settings.

The selected NS size range has been already specified at point 1.5 In any case this step is not necessary to film.

12. Line 149: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We have addressed this as requested now.

13. Line 200: Please specify the scanning frequency used.

This also has been addressed in the revised version.

14. Line 201: Were the measurements taken at Room temperature? If yes, please specify.

This has been specified in the text as required (point 2.10 and 3.8 of the protocol). The analysis to determine the measurements can actually be either performed in real time or later when the acquisitions have been completed. In any case, the measurements refer at 37°C, as the Incucyte Live-Cell Analysis Instrument featured in the protocol sit within the incubator, therefore cells are inside the incubator also during image acquisition time.

15. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be

visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The steps of the protocol that needs to be captured in the video have been highlighted as requested.

16. Line 296: Please correct the label to Figure 5

Ok this has been done

17. Line 298: Please revise “hs” to “h”.

Ok this has been done.

18. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We believe we have covered those points in the discussion really putting attention on the method aspects and its broader applications. We have added one citation in a paragraph where we refer to modifications and critical steps (line 346). We hope this is ok now.

19. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

As requested the references have been checked and the “&-sign” has been removed.

20. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have removed the figures from main manuscript as requested. Figures are now provided separately as indicated.

21. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

The table has been modified as requested.

22. Figure 1: Please replace the commercial terms with generic terms in the figure (e.g., revise "Matrigel" as "basal membrane matrix"). Remove the term Sartorius from 3. Please use upper case letters to label the figure instead of numbers.
23. Figure 2/3/4/5/ supplementary figure 1: Please include scale bars in all the images of the panel.

Scale bar has now being added to the all figures.

24. Figure 5: Please revise the unit in the Y-axis to "h" instead of "hours".

On Figure 5 hours is on the X-axis. The unit has been modified as requested.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Pericoli et al perform live-3D-cell immunocytochemistry for CD44 in one H2K27M diffuse midline glioma cell line, QCTB-R059 using matrigel for migration and invastion assays.

Major Concerns:
1. QCTB-R059 is mentioned as both DMG H3K27M cell line (line 29, others) and pediatric glioblastoma spheroids (line 277), which is confusing. This cell line has been previously described as primary pediatric thalamic GBM cells in an abstract for a AACR in 2015, which makes sense for both descriptions. I would provide a brief description of the cell line and cite previous references.

We thanks the reviewer for the comment. Due to the past and still on-going revised versions of the WHO classification of brain tumors, a different nomenclature has been used in the past few years. QCTB-R059 was originally indicated as a pediatric thalamic glioblastoma (GBM) cell line (Taylor K.R. et al., Nature Genetics 2014) while later on it has been indicated as a H3-K27M thalamic glioma cell line (Mount et al., Nature Medicine 2018) or diffuse midline glioma (DMG) H3-K27M cell line (Ferretti et al., BioTechniques 2021) given the new nomenclature applied to the midline glioma affected by the histone *H3F3A* K27M mutation. More recently a new nomenclature (*Diffuse midline glioma, H3 K27-altered*) has been introduced (Louis D. et al., Neuro-Oncology 2021), therefore we may see in future this same cell line renamed. A brief description and the references have now been added as suggested by the reviewer.

2. The authors claim that CD44 expression is turned on and off during migration and invasion, although Fig 4A and 4B are not very convincing. Can the authors quantify the amount of green signal?

Thank you for the comment. “The green signal” has been quantified over time for both invasion and migration as shown on Figure 5. The data refer to the bulk fluorescent intensity and it cannot be provided as single cell data with the system we feature in this article. In fact, the Incucyte Lice-Cell Analysis Instrument applications “ Spheroid invasion” and “Basic Analyzer”” used to analyse the 3D invasion and migration respectively, do not allow the quantification at the single cell level. This could potentially be done with alternative systems. We apologoize if it was not clear or confusing. We have clarified this in the text.

Minor Concerns:
Several minor spelling errors. There is a space between "o" and "f" in the title. Both um and µm are used. I think you use commas for decimal points in Italy, but should be period.

Thanks for the comments and suggestion. We have addressed them in the revised version.

Reviewer #2:
Manuscript Summary:
The paper is generally well written and easy to follow. A few grammatical errors were identified and should be scanned for by the copy editors prior to publication to improve the readability of the paper. The paper describes a method that incorporates plating pediatric brain cancer cells in or on top of Matrigel and then using an antibody/fluorophore conjugate to visualize protein expression during time lapse imaging. The method is well described and figure 1 provides an excellent overview of the protocol. The authors state that this method can be used to quantitatively assess protein expression in real time, although they data shown does not provide evidence of this. They also state their technique provides novel insight into the role of CD44 in DMG cells, but again the data supporting this statement is weak.

Major Concerns:
* The green fluorescence is difficult to see on cells that are away from the sphere. I recommend the settings for the phase image be darkened to enable to green to be more evident.

Thanks for the suggestion. We have improved the contrast as the reviewer suggests and we provide now a revised version of Figure 4, Figure 5 and Supplementary Figure 1. .
* The inset indicated at the top of figure 3 does not appear to be the image in the bottom row based on the pattern of red fluorescence. In order to support the statement that CD44 expression is not evenly distributed across the cell surface (or "accumulates in spots") a much higher resolution image where individual cells can be easily identified needs to be shown. Furthermore, while this may be evident in Figure 3, it is not obvious in Figure 2 (again - recommend a higher magnification be shown in Fig 2 if this statement is to be included).

We thank the reviewer for the comment based on which we have carefully reviewed the images in Figure 3 and have placed the insert in the right spot on the

upper panel image (10x) that now correspond to the image captured in the higher power images (25x) shown below. With regard to the CD44 localization on the cell membrane in spots, we believe that our observation is in line with previous reports that have described the localization of CD44 on tumour cell surface protrusions (*Martín-Villaris E. et al., "Podoplanin Associates with CD44 to Promote Directional Cell Migration" Molecular Biology of the Cell, 2010)* and in relation to the translocation from non-raft into raft domains on the cell membrane (*Sun F. et al., "Molecular mechanism for bidirectional regulation of CD44 for lipid raft affiliation by palmitoylations and PIP2", PLOS Computational Biology 2020*). In our images the "spots" observed may be more evident on the invasion (Figure 3) compared to the migration (Figure 2). Though, these aspects are not the focus of our method manuscript, therefore based on the reviewer suggestion we have removed this statement in the revised version.

* Supplementary videos should have a time stamp shown and each should be associated with a proper legend/description. I can not see the intermittent expression of CD44 in the videos. If this truly occurs please annotate the videos to make this obvious to the reader. If this statement is to be included as a scientific conclusion, then the number (or proportion) of cells where this is observed should be quantified.

Thank you for the comment and suggestion. The figure legends have now been provided for both supplementary videos and the time stamp has been added on the selected frames from both invasion and migration videos featured in Figure 4 and on the Supplementary videos 1 and 2. The circled cells displayed on the images in Figure 4 are intended to make more clear and evident the "intermittent" expression of CD44on the cell membrane. The circle on the individual cells featured in Figure 4 have been coloured in green and black to indicate fluorescent signal on and off respectively. The figure legend and the text have been modified to clarify this. We did not claim though that we can ***quantify*** the intermittent expression on the single cells, but only visualize this. The fluorescent signal associated to CD44 expression is measured and quantified overall not at single cell level. We apologize if it was confusing and following the reviewers comment, we have rephrased the text in the representative results. The single cell analysis of these assays although not possible with the Incucyte, it may be possible with other systems using the same images acquired by the Incucyte (*e.g.* ImageJ).

* Figure 5 - the values on both axes in C and D are not legible - please increase the size of the font. It is not clear what these graphs are showing, is it mean fluorescence intensity per field of view or per cell. In other words is it simply indicating that the tumor cells are increasing in number, rather than being a measure of migration or invasion?

Thank you for the suggestion. We have now increased the font of the values on the axes. As specified above, the analysis is relative to the overall fluorescent intensity associated with CD44 expression on invading or migrating cells but not on a single cell level. . Following the reviewer's comment we have modified the text in the representative results section relative to Figure 5 to clarify the points raised. We hope this is clearer now.

* The magnification of the images in Supplementary file 1 are too low to verify the statement that the cells transition from "mesenchymal-like to ameboid-like" phenotypes. The individual cells at the periphery of the spheres appear similar I the images shown. The paper cited by Pankova et al shows cells at a much higher magnification. The authors should show the cells at an equivalent magnification to support the statement or revise the discussion.

As said above the image system featured in our method is the Incucyte Live-Cell Analysis Instrument. Higher resolution images could be acquired for example in z-stack mode with a confocal microscope. As requested by the reviewer we provide higher power magnification. Despite the potential limitation compared to other imaging systems, we believe it is clearer now the different morphological appearance of the invading cells in the absence of the antibody (negative control), looking like invading in a more collective pattern with elongated morphology, compared to the condition in the presence of the anti-CD44 antibody, where in contrast, cells look generally invading as single cells and with a more rounded morphology.

* The discussion states that "we could quantitatively measure the expression of CD44 over time", however my interpretation of the graphs in figure 5 are showing increased green fluorescence across the entire sphere/well. This isn't necessarily an indicator of CD44 expression, but could simply be an indicator of increasing cell number. If this statement is to be retained, the authors need to show that CD44 expression does increase on a per cell level.

As mentioned above the system featured in the method does not allow a single cell analysis. For the migration assay, the CD44 fluorescent intensity on the cell membrane is measured over time for the cells in the entire well, while for the invasion the analysis applied was restricted to the invaded cells excluding the core of the NS as displayed on the schematic image shown on the graph. We cannot exclude though that together with cell motility there is also a cell proliferation component. Following the reviewer's comment we have clarified this in the discussion section.

* The discussion also states that CD44 antibody resulted in an inhibition in cell motility, however the data shown is insufficient to support that statement. While the spheres appear smaller, this is not necessarily an indication of reduced motility. An alternative could be that the antibody impedes proliferation. If the authors want to say that antibody binding to CD44 reduces motility, some quantitative data specifically comparing the motility of anti-CD44 treated cells, with control (untreated) cells should be shown. Alternatively the authors could add a nuclear dye such as NucLight red and count cell number - if the data shows that cellular replication is equal but sphere size is reduced, then it would support the statement that motility is impaired.

We agree with the reviwer that thre may be an additional effect of the anti-CD44 antibody on cell proliferation beside cell motility. We have now stated this into the discussion. As this is a method paper we believe this point could be left as a more open question for future more scientific based studies.

Minor Concerns:

* Figures appear to be misnumbered?

We have corrected this in the revised version.

* There are two figure legends labeled figure 4

Thank you, we have corrected this in the revised version.