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Project Page Link: https://review.jove.com/files_upload.php?src=21461373

Title: Reproducible 3D Glioblastoma Migration Assay with Magnetic Nanoparticle Mediated Spheroid Localization Under Hypoxic Conditions.

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**
- 3. Filming location:** Will the filming need to take place in multiple locations? **Yes**
If **Yes**, how far apart are the locations? The adjacent building, a few metres.
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in promotional materials. **No**

Current Protocol Length

Number of Steps: 13

Number of Shots: 44

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Brona Murphy:** We focus on visually capturing glioblastoma tumour migration, allowing the anti-invasive properties of therapies to be reliably and reproducibly examined.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Brona Murphy:** The incredible heterogeneity of glioblastoma is difficult to model. This protocol captures individual glioblastoma tumour invasiveness in a reproducible manner.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Brona Murphy:** This protocol enables the scalable high-throughput analysis of glioblastoma tumour-specific migration changes in response to conditions/therapies.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Brona Murphy:** Optimizing iron oxide concentration ensures minimal use and controlled spheroid positioning, greatly enhancing assay consistency, accuracy, and overall performance.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Brona Murphy:** Future studies can develop more representative brain ECM matrices to better mimic glioblastoma migration, improving translational relevance in in-vitro systems.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research was approved by the appropriate Institutional Review Board (IRB) or equivalent ethics committees at Erasmus MC, Rotterdam, The Netherlands, and The Hospital for Sick Children, Toronto, Canada

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Author's NOTES:

During filming on Friday, we made a few minor adjustments to the order of the script, and some steps were combined into a single shot where they naturally flowed together. The videographer clearly noted all changes during filming, so they should be easy for the editors to identify once the footage is ready for review.

Videographer's NOTES:

1. we sometimes did 2 or 3 shots in one go because they were part of the same manipulation. We made comments live when information was needed.
2. The same applies to sequence numbers when the space was too tight to fit the white board with the number in the frame.

2. Gliomasphere Dissociation and Matrigel Plate Preparation

Demonstrator: Niamh Scully

- 2.1. To begin, obtain the gliomaspheres in a culture flask and keep all the reagents ready under sterile conditions [1]. Using a 10-milliliter serological pipette and pipette aid, collect the gliomaspheres from the flask [2]. Transfer the gliomaspheres into a 15-milliliter conical tube [3], centrifuge at 300 *g* for 3 minutes [4], and discard the supernatant without disturbing the cell pellet [5].

- 2.1.1. WIDE: Shot of the culture and the required reagents in a sterile environment.

***Videographer:** Please obtain CU shot as well for this shot.*

- 2.1.2. Talent aspirating gliomaspheres from the culture flask with a 10-mL serological pipette and pipette aid.
 - 2.1.3. Talent transferring the gliomaspheres into a 15-mL conical tube.
 - 2.1.4. Talent placing the conical tube into the centrifuge.

- 2.1.5. Talent discarding the supernatant while leaving the cell pellet undisturbed.
- 2.2. Resuspend the pellet in 1 milliliter Accutase at room temperature [1]. Use a P1000 (P-1000) pipette to agitate the solution every 2 to 3 minutes [2] and incubate for a total of 10 to 15 minutes [3].
- 2.2.1. Talent resuspending the cell pellet in cell detachment enzyme medium.
- 2.2.2. Shot of the talent pipetting the suspension with a P1000 pipette.
- 2.2.3. Shot of the suspension being left aside between the agitations.
- 2.3. After incubation, add an equal volume of gliomasphere media to the cell suspension to deactivate the Accutase [1]. Centrifuge the single cell suspension at 300 g for 3 minutes at 37 degrees Celsius [2]. Then, resuspend the cell pellet in 3 to 5 milliliters of complete gliomasphere medium, depending on the pellet size [3]. Note the exact volume used for subsequent cell counting [4].
- 2.3.1. Talent adding gliomasphere media to the cell suspension in a tube.
- 2.3.2. Talent placing the tube into the centrifuge.
- 2.3.3. Talent gently resuspending the cell pellet in 3 to 5 mL complete gliomasphere medium and noting the volume.
- 2.3.4. Talent noting the exact volume used.
- 2.4. Take a 10-microliter aliquot of the single-cell suspension [1] and gently mix it with 10 microliters of Trypan Blue in a 1.5 milliliter microcentrifuge tube [2]. Load 10 microliters of this mixture into an automated cell counter [3].
- 2.4.1. Talent taking a 10 µl aliquot of the single-cell suspension.
- 2.4.2. Talent mixing the aliquot with 10 µl of Trypan Blue in a 1.5 milliliter microcentrifuge tube.
- 2.4.3. Talent loading the stained cell mixture into an automated cell counter.
- 2.5. Prepare a mastermix of the glioma cells in the gliosphere medium containing 1 microgram per milliliter propidium iodide and 0.05 microliters iron oxide nanoparticles [1]. Seed 500 single glioma cells per well from this mastermix into an ultra-low attachment 96-well spheroid microplate [2]. Incubate the plate for 48 hours at 37 degrees Celsius, 5 percent carbon dioxide and 21 percent oxygen in a humidified cell culture incubator to allow gliomasphere formation [3].
- 2.5.1. Talent preparing the glioma cell mastermix in gliosphere medium.
- 2.5.2. Talent seeding 500 single glioma cells per well into an ultra-low attachment 96-well spheroid microplate.
- 2.5.3. Talent placing the plate into the humidified cell culture incubator.
- Videographer's NOTE:** About shot 2.5.3 and 2.7.3 :they are the same (use the

same footage) and we didn't film it twice because the room with the incubator was busy.

- 2.6. To prepare the Matrigel, dilute the Matrigel extracellular matrix stock 1 to 10 in ice-cold complete neurosphere medium, after 48h of gliomasphere formation [1]. Perform a second 1 to 10 dilution to obtain the Matrigel working solution [2]. Keep the diluted Matrigel on ice [3].
 - 2.6.1. Talent diluting the Matrigel extracellular matrix stock with ice-cold complete neurosphere medium.
 - 2.6.2. Talent performing a second dilution of the Matrigel solution.
 - 2.6.3. Talent keeping the diluted Matrigel on ice.
- 2.7. Pre-chill the imaging plate and store it on ice [1]. Dispense 50 microliters of the Matrigel working solution into each well of a 96-well clear flat-bottom plate and microClear-bottom plate [2]. Incubate the plate at 37 degrees Celsius for at least 1 hour [3]. Then, remove 30 microliters from each well, leaving 20 microliters of polymerized Matrigel coating the bottom of each well [4].
 - 2.7.1. Talent placing the imaging plate on ice.
 - 2.7.2. Talent dispensing the 1:100 Matrigel working solution into the wells of the plate.
 - 2.7.3. Talent placing the plate into the incubator.
 - 2.7.4. Talent removing 30 microliters from each well while leaving 20 microliters of polymerized Matrigel at the bottom.
- 2.8. Spray the magnetic plate with 70% ethanol and wipe dry, under sterile conditions [1]. Place the Matrigel-coated microplate onto a magnetic 96-well plate drive [2].
 - 2.8.1. Talent spraying the magnetic plate with 70% ethanol and wiping it dry under sterile conditions.
 - 2.8.2. Talent placing the Matrigel-coated microplate onto the magnetic 96-well plate drive.
- 2.9. Pipette 80 microliters of medium containing the single gliomasphere from each well [1]. Gently dispense it onto the Matrigel-coated plate [2]. Treat the spheroids according to the experimental design [3]. Now, transfer the plate, with the magnetic drive, to the imaging system for live imaging [4].
 - 2.9.1. Talent aspirating 80 microliters of medium containing the single gliomasphere from a well.
 - 2.9.2. Talent gently dispensing the gliomasphere-containing medium onto the Matrigel surface.

- 2.9.3. Representative shot of the talent treating spheroids according to the experimental design.
- 2.9.4. Talent carrying the plate on the magnetic drive to the imaging system.

3. Live-Cell Imaging Setup and Image Acquisition

Demonstrators: Niamh Scully and Brenton Cavanagh

- 3.1. Configure a programmable inverted live-cell microscope [1] to acquire live-cell images [2]. Set the camera to 2 by 2 binning [3].

- 3.1.1. TEXT ON A PLAIN BACKGROUND:

Microscope settings:

- Plan-Apochromat 5× / 0.35 NA objective
- 0.5× tube lens
- Axiocam 506 camera, binning set to 2
- ZEN 3.1 Blue Edition software
- Enable automatic pixel-to-micrometer calibration in software

- 3.1.2. Talent acquiring live cell images.

- 3.1.3. SCREEN: 3_1_3_binning.mp4 0:02- 0:27

Video editor: Highlight binning set to '2 x 2' in the left panel in the first few seconds and then in info, image dimensions, highlight the scaling per pixel 3.645 μm x 3.645 μm .

- 3.2. Gently load the multi-well plate without the magnetic drive into the microscope [1]. Adjust the microscope stage acceleration and speed to 10% [2]. If applicable, select the plate material in the software so the optics are adjusted to match [3].

3.2.2, Talent gently loading the multiwell plate into the microscope without the magnetic drive. **Videographer's NOTE:** the talent told me about shot 3.2.2 to happen before 3.1.2, Vo is adjusted accordingly

3.2.1, SCREEN: 3_2_1_platespeed.mp4 0:02- 0:27 *Video editor: Highlight the stage acceleration and speed settings being adjusted to 10% in the right panel.*

3.2.3. SCREEN: 3_2_3_platematerials.mp4 0:55-1:15 *Video editor: Highlight the plate material being selected in the left panel.*

- 3.3. Adjust the magnification to encompass the entire invasive field of view [1]. Bring a gliomasphere into focus [3] and select the 5x objective and 0.5x tube lens to yield 2.5x magnification [2]. Check both the phase gradient contrast, transmitted light, and fluorescence channels [4]. Adjust the exposure time and light intensity to avoid

overexposure [5].

- 3.3.1. SCREEN: 3_3_1-6--find-and-set-exp.mp4 0:10 - 0:23
- 3.3.2. SCREEN: 3_3_1-6--find-and-set-exp.mp4 00:20-00:23
- 3.3.3. SCREEN: 3_3_1-6--find-and-set-exp.mp4 0:24 - 0:34
- 3.3.4. SCREEN: 3_3_1-6--find-and-set-exp.mp4 00:59-01:01 and 00:25-00:28
- 3.3.5. SCREEN: 3_3_1-6--find-and-set-exp.mp4 00:38-00:45 and 01:02-01:04

3.4. Assign a position that includes the x, y, and z coordinates [1]. Use hardware autofocusing to ensure consistent imaging of the same focal plane [2].

- 3.4.1. 3_4_1-add-positions.mp4 0:03-0:58 *Video Editor: Please speed up the footage to match the VO.*
- 3.4.2. 3_4_2-autofocusing.mp4 0:01-0:15

Results

4. Results

- 4.1. Gliomaspheres derived from newly diagnosed and recurrent patient tumors exhibited dual phenotypes and random cell-to-cell associations [1].
 - 4.1.1. LAB MEDIA: Figure 1A. *Video editor: Show the 2 microscope images and mark the different-looking cell shapes and the mixed cell arrangement.*
- 4.2. Transmitted light images of gliomaspheres cultured for 48 hours showed a dark, speckled appearance in gliomaspheres containing iron oxide nanoparticles [1].
 - 4.2.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the bottom panel and mark the dark speckled sphere.*
- 4.3. Gliomaspheres cultured without nanoparticles showed random localization [1], whereas those cultured with nanoparticles showed consistent central localization [2].
 - 4.3.1. LAB MEDIA: Figure 1D. *Video editor: Highlight the top images and show that the sphere appears in different positions.*
 - 4.3.2. LAB MEDIA: Figure 1D. *Video editor: Highlight the bottom images and show that the sphere is centered in each view.*
- 4.4. Representative transmitted light images showed invasion dynamics at 0 hours and 72 hours in vehicle treated gliomaspheres [1] and Drug X treated gliomaspheres [2].
 - 4.4.1. LAB MEDIA: Figure 5A. *Video editor: Highlight the line with circular dots marked 'vehicle' from 0h and 72h.*
 - 4.4.2. LAB MEDIA: Figure 5A. *Video editor: Highlight the line with square dots marked 'Drug X' from 0h and 72h.*
- 4.5. Quantification of the invasive area at 72 hours showed a significantly decreased invasion in Drug X treated gliomaspheres compared to vehicle, represented as total area covered at the time of fixation [1].
 - 4.5.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the bars labeled Vehicle and Drug X under "Area Covered 72h" and show the difference in height.*

1. Gliomasphere
Pronunciation link: No confirmed link found
IPA: /,gli:.oo'spi:/
Phonetic Spelling: glee·oh·sfeer
2. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /di,sou.si'eɪ.fən/
Phonetic Spelling: dih·soh·see·ay·shuhn
3. Matrigel
Pronunciation link: <https://www.howtopronounce.com/matrigel>
IPA: /'meɪ.trɪ,dʒel/
Phonetic Spelling: may·trih·jel
4. Serological
Pronunciation link: <https://www.merriam-webster.com/medical/serological>
IPA: /,sir.ə'lɑ:.dʒɪ.kəl/
Phonetic Spelling: seer·uh·laa·jih·kuhl
5. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sen.trə,fju:dʒ/
Phonetic Spelling: sen·truh·fyooj
6. Supernatant
Pronunciation link: <https://www.merriam-webster.com/medical/supernatant>
IPA: /'su:.pə,nei.tənt/
Phonetic Spelling: soo·pur·nay·tuht
7. Accutase
Pronunciation link: <https://www.howtopronounce.com/accutase>
IPA: /'æk.jə,teɪs/
Phonetic Spelling: ak·yuh·tays
8. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪn.kjə,bet/
Phonetic Spelling: in·kyuh·bayt
9. Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>
IPA: /'æl.ɪ,kwɑ:t/
Phonetic Spelling: al·ih·kwaat
10. Trypan Blue
Pronunciation link: <https://www.howtopronounce.com/trypan-blue>
IPA: /'traɪ.pæn blu:/
Phonetic Spelling: try·pan·bloo
11. Microcentrifuge
Pronunciation link: <https://www.howtopronounce.com/microcentrifuge>
IPA: /,maɪ.kroo'sen.trə,fju:dʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj

12. Propidium
 Pronunciation link: <https://www.howtopronounce.com/propidium>
 IPA: /proʊˈpɪd.i.əm/
 Phonetic Spelling: proh·pid·ee·uhm
13. Iodide
 Pronunciation link: <https://www.merriam-webster.com/dictionary/iodide>
 IPA: /ˈaɪ.əˌdaɪd/
 Phonetic Spelling: eye·uh·dyde
14. Nanoparticles
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nanoparticle>
 IPA: /ˌnæn.oʊˈpɑːr.tɪ.kəlz/
 Phonetic Spelling: nan·oh·par·tih·kuhlz
15. Spheroid
 Pronunciation link: <https://www.merriam-webster.com/dictionary/spheroid>
 IPA: /ˈsfɪr.ɔɪd/
 Phonetic Spelling: sfeer·oyd
16. Humidified
 Pronunciation link: <https://www.merriam-webster.com/dictionary/humidified>
 IPA: /hjuːˈmɪd.əˌfaɪd/
 Phonetic Spelling: hyoo·mih·duh·fyd
17. Matrigel
 Pronunciation link: <https://www.howtopronounce.com/matrigel>
 IPA: /ˈmeɪ.trɪˌdʒel/
 Phonetic Spelling: may·trih·jel
18. Extracellular
 Pronunciation link: <https://www.merriam-webster.com/medical/extracellular>
 IPA: /ˌɛk.strəˈsel.jə.lə/
 Phonetic Spelling: ek·struh·sel·yuh·lur
19. Neurosphere
 Pronunciation link: No confirmed link found
 IPA: /ˈnɔr.oʊˌsfɪr/
 Phonetic Spelling: nyur·oh·sfeer
20. Polymerized
 Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerize>
 IPA: /pəˈlɪm.əˌraɪzd/
 Phonetic Spelling: puh·lim·uh·ryzed
21. Ethanol
 Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
 IPA: /ˈeθ.əˌnɔːl/
 Phonetic Spelling: eth·uh·naal
22. Programmable
 Pronunciation link: <https://www.merriam-webster.com/dictionary/programmable>
 IPA: /ˈproʊ.græm.əˌbəl/
 Phonetic Spelling: proh·gram·uh·buhl

23. Inverted
 Pronunciation link: <https://www.merriam-webster.com/dictionary/inverted>
 IPA: /ɪnˈvɜː.təd/
 Phonetic Spelling: in·vur·tuhd
24. Apochromat
 Pronunciation link: <https://www.howtopronounce.com/apochromat>
 IPA: /ˌæp.ooˈkroʊ.mæt/
 Phonetic Spelling: ap·oh·kroh·mat
25. Objective
 Pronunciation link: <https://www.merriam-webster.com/dictionary/objective>
 IPA: /əbˈdʒek.tɪv/
 Phonetic Spelling: uhb·jek·tiv
26. AxioCam
 Pronunciation link: No confirmed link found
 IPA: /ˈæk.si.ooˌkæm/
 Phonetic Spelling: ak·see·oh·kam
27. Micrometer
 Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
 IPA: /maɪˈkraɪ.mə.tər/
 Phonetic Spelling: my·kraa·muh·tur
28. Magnification
 Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>
 IPA: /ˌmæɡ.nə.fəˈkeɪ.ʃən/
 Phonetic Spelling: mag·nuh·fuh·kay·shuhn
29. Fluorescence
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
 IPA: /flɔːˈres.əns/
 Phonetic Spelling: flaw·res·uhns
30. Overexposure
 Pronunciation link: <https://www.merriam-webster.com/dictionary/overexposure>
 IPA: /ˌoo.və.ɪkˈspoʊ.ʒər/
 Phonetic Spelling: oh·vur·ik·spoh·zher
31. Autofocusing
 Pronunciation link: <https://www.howtopronounce.com/autofocus>
 IPA: /ˌɔː.tuʊˈfoʊ.kə.sɪŋ/
 Phonetic Spelling: aw·toh·foh·kuh·sing
32. Phenotypes
 Pronunciation link: <https://www.merriam-webster.com/dictionary/phenotype>
 IPA: /ˈfiː.nəˌtaɪps/
 Phonetic Spelling: fee·nuh·types
33. Localization
 Pronunciation link: <https://www.merriam-webster.com/dictionary/localization>
 IPA: /ˌloʊ.kə.ləˈzeɪ.ʃən/
 Phonetic Spelling: loh·kuh·luh·zay·shuhn

34. Quantification

Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>

IPA: /ˌkwɑːn.tə.fəˈkeɪ.ʃən/

Phonetic Spelling: kwaan·tuh·fuh·kay·shuhn

35. Invasive

Pronunciation link: <https://www.merriam-webster.com/dictionary/invasive>

IPA: /ɪnˈveɪ.sɪv/

Phonetic Spelling: in·vay·siv