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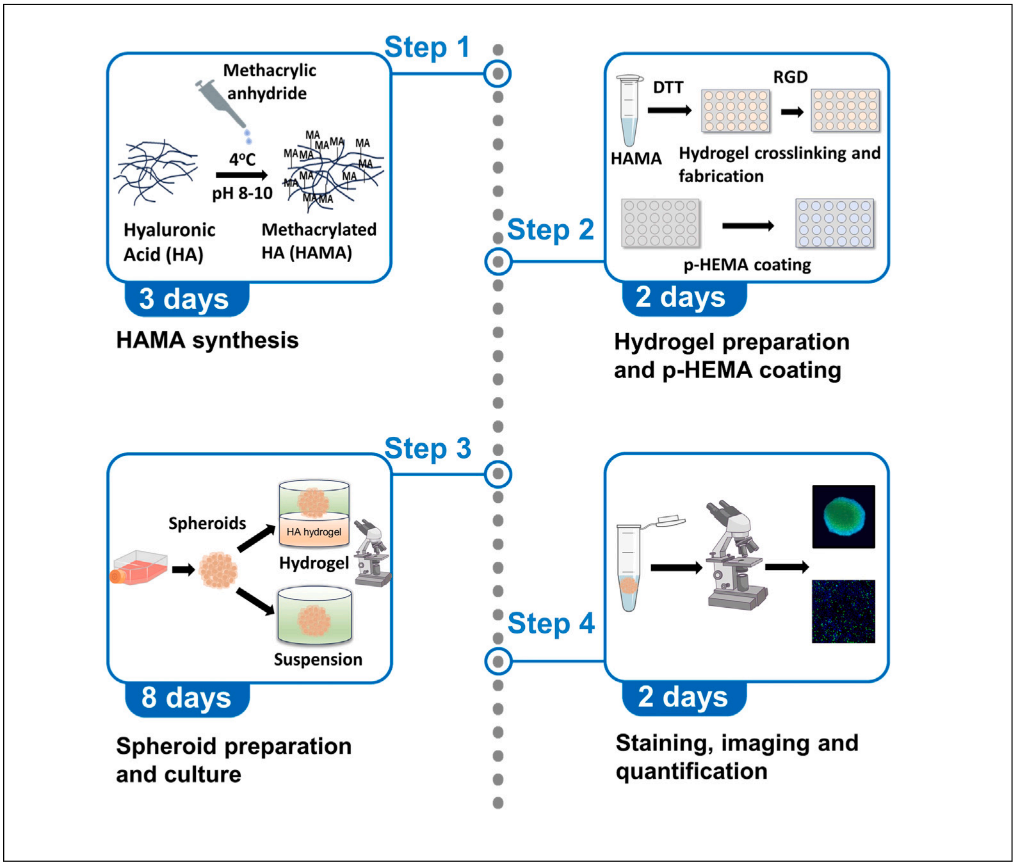

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Highlights

Steps for preparation of hyaluronic acid hydrogels

Generation of BMBC spheroids in hydrogel and suspension

Spheroid characterization via cross-sectional area, cell numbers, and EdU staining

Here, we present a protocol to generate dormant brain metastatic breast cancer (BMBC) spheroids utilizing hyaluronic acid (HA) hydrogels. We describe the steps for construction of spheroids from human BMBC cell lines MDA-MB-231Br and BT474Br3, HA hydrogel preparation, and spheroid plating on HA hydrogels and in suspension culture. We then detail the impact of HA hydrogel on the dormant phenotype of spheroids by measuring spheroid cross-sectional area, cell numbers, and EdU staining.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol for generating dormant human brain metastatic breast cancer spheroids *in vitro*

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SUMMARY

Here, we present a protocol to generate dormant brain metastatic breast cancer (BMBC) spheroids utilizing hyaluronic acid (HA) hydrogels. We describe the steps for construction of spheroids from human BMBC cell lines MDA-MB-231Br and BT474Br3, HA hydrogel preparation, and spheroid plating on HA hydrogels and in suspension culture. We then detail the impact of HA hydrogel on the dormant phenotype of spheroids by measuring spheroid cross-sectional area, cell numbers, and EdU staining.

For complete details on the use and execution of this protocol, please refer to Kondapaneni et al.[1]

BEFORE YOU BEGIN

Protocol overview

In this protocol, we describe detailed steps to generate dormant brain metastatic breast cancer (BMBC) spheroids by employing a hyaluronic acid (HA) hydrogel-based culture platform. Here, we utilized two different BMBC cell lines: MDA-MB-231Br and BT474Br3. By following the below mentioned protocol, we were able to develop a biomimetic *in vitro* culture platform that generates dormant BMBC spheroids, which can be further used to study the impact of microenvironment derived cues on the regulation of the dormant state. Additionally, these dormant spheroids could be utilized for drug screening in order to develop an effective treatment strategy for metastatic tumors, since the dormant cancer cells are known to evade current treatment strategies.[2]

Synthesis of hyaluronic acid methacrylate (HAMA)

Timing: 2–3 days

This section provides the detailed protocol to prepare hyaluronic acid methacrylate utilizing sodium hyaluronate, methacrylic anhydride and 5 M NaOH solution.

1. Dissolve 250 mg of sodium hyaluronate in 24.75 mL of deionized water to obtain a 1 wt % solution in a 50 mL glass beaker.
   a. Place the solution in a 4°C refrigerator and stir it at 500 rpm for 12–16 h.
   b. Place a syringe pump and a 5 mL syringe containing 5 M NaOH, while placing the sodium hyaluronate solution in the 4°C refrigerator.

Note: All the methacrylic anhydride additions should be carried out in 4°C refrigerator. Once you reach methacrylation of ~85%, rest of the steps (extraction) can be carried out at 25°C.

2. After 12–16 h, manually adjust the pH of the solution between 8 to 10 and add 100 μL of methacrylic anhydride.
3. Place the solution at 4°C and start infusing NaOH through syringe pump at 3.5–3.75 μL/min and stir the solution at 700 rpm.
   a. After 45 min of methacrylic anhydride addition check the pH of the solution and make the second methacrylic anhydride addition (100 μL).
   b. Continue adding 100 μL methacrylic anhydride every 45 min by carefully monitoring the pH of the reaction mixture.

⚠ CRITICAL: At any time of the reaction, pH of the reaction mixture should be maintained between 8 and 10. If the pH of the reaction mixture raises above 10, try to adjust the pH by altering the flowrate of NaOH solution to 3.25 μL/min.

4. After the 14^{th} addition, collect 1 mL of the reaction mixture and add 5 mL of ice-cold acetone and centrifuge for 3 min at 1000 rcf to extract HAMA.
   a. Later, dissolve the extracted HAMA in 600–1000 μL of deuterium oxide (D_2O) and perform nuclear magnetic resonance (NMR) analysis to obtain the degree of methacrylation.
   b. Upon attaining the required degree of methacrylation, extract the HAMA from the reaction mixture by adding 5-fold excess of ice-cold acetone (125 mL of acetone) to the reaction mixture and stirring at 500 rpm for 20 min.
   c. Later, dry the extracted HAMA in a fume hood for 2 h, flash freeze in liquid nitrogen, and lyophilize for 12–16 h in a freeze dryer.

⚠ CRITICAL: If the degree of methacrylation is less than 85%, add a couple of methacrylic anhydride additions and test the degree of methacrylation. Usually, 14−16 additions of methacrylic anhydride are sufficient to achieve a degree of methacrylation ~85%.

Note: In case all the required methacrylic anhydride additions were not done in a single day, stop infusing NaOH and stir the reaction mixture at 500 rpm for 12 hours at 4°C . Next day, adjust the pH (8–10) manually by adding 5 M NaOH solution dropwise and start the remaining methacrylic anhydride additions by infusing 5 M NaOH.

Cell culture

Timing: 5–14 days

This section provides information on the cell types utilized and their culture procedure.

5. Prepare respective cell culture media at least 24 h ahead before starting cell culture.
   a. Add 9 mL of respective cell culture media in a T75 flask and add 1 million of cells into the flask.
   b. Later, place the flask in a 37°C and 5% CO_2 incubator and culture the cells as adherent cultures by changing the cell growth media for every two days until the cells are confluent.
   c. Once the cells are confluent (usually 5–7 days for MDA-MB-231Br and 12–16 days for BT474Br3 cells), remove the cell media and wash the cells with 9 mL of PBS.
   d. After aspirating PBS from flask, add 3 mL of 0.25% trypsin, and incubate for 3 min at 37°C.
   e. Next, add 9 mL of cell media and collect the cell suspension into a 15 mL falcon tube and centrifuge it at 1000g for 3 min.
   f. After centrifugation, remove the media solution by pipetting and use the cell pellet for spheroid preparation.

⚠ CRITICAL: Thaw cell stocks (MDA-MB-231Br or BT474Br3) in a water bath (1 vial containing 1 million cells are sufficient to perform the experiments. For both the cell lines, cells with passage number below 30 were utilized in this study).

Note: MDA-MB-231Br media composition: 89% DMEM high glucose + 10% FBS + 1% Penicillin streptomycin. BT474Br3 media composition: 89% DMEM/F12 (1:1) + 10% FBS + 1% Penicillin streptomycin.

Note: Once the cells are adhered after seeding cells from a stock, change the media to remove dimethylsulfoxide (DMSO).

Preparation of polyhydroxyethyl methacrylate (p-HEMA) solution

Timing: 12–18 h

This section describes the steps involved in preparing p-HEMA solution.

6. Weigh 500 mg of p-HEMA and dissolve it in 25 mL of 95% Ethanol in a 50 mL glass beaker and cover it with a parafilm tape.
   a. Stir the mixture (500 rpm) at 65°C for 3 h and later switch off the heater and stir it for 12–16 h at 20°C–25°C (this process needs to be done in a fume hood). This gives a 20 mg/mL solution.
   b. Next day, collect the p-HEMA solution in a 50 mL tube and store it at 4°C. If stored properly, you can use it up to 3 months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich	D6429
DMEM/F12 (1:1)	Gibco	11320-033
Fetal bovine serum (FBS)	VWR Life Sciences	97068-085
Penicillin-Streptomycin	Gibco	15140-122
Trypsin	Gibco	25200-072
Poly (2-hydroxyethyl methacrylate)	Sigma-Aldrich	P3932-25G
Growth factor reduced (GFR) Matrigel	Corning	354230
Phosphate-buffered saline (PBS)	Gibco	14190-144
Accutase	Gibco	A11105-01
Sodium hyaluronate (66–90 kDa)	Lifecore Biomedical	HA60k-5
Methacrylic anhydride	Sigma-Aldrich	276685
Dithiothreitol (DTT)	Sigma-Aldrich	D9163-5G
Integrin binding peptide (RGD)	AnaSpec	
4% Paraformaldehyde	Thermo Fisher Scientific	J19943-k2
Triton X-100	Alfa Aesar	A16046
Bovine serum albumin (BSA)	Sigma-Aldrich	A9647-50G
0.4% Trypan blue stain	Invitrogen	T10282
Deuterium oxide	Thermo Scientific	426931000
95% Ethanol	Pharmco	111000190
Critical commercial assays		
Click-iT EdU Cell Proliferation Kit	Thermo Fisher Scientific	C10337

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
MDA-MB-231Br	Generously provided by Dr. Lonnie Shea, University of Michigan	Narkhede et al.[3]
BT474Br3	Generously provided by Dr. Dihua Yu, University of Texas MD Anderson Cancer Center	Saldana et al.[4]
Software and algorithms		
ImageJ	https://imagej.net/ij/download.html	N/A
cellSens	https://www.olympus-lifescience.com/en/software/cellsens/	N/A
Other		
Conical bottom plate	Thermo Fisher Scientific	249952
96-well flat bottom plate	Corning	351172
Cell counting chamber slides	Invitrogen	C10283
Biological safety cabinet	Thermo Fisher Scientific	1337
Centrifuge	Eppendorf	5424 R
CO_2 Incubator	Panasonic	MCO-170AICUVL-PA
Confocal inverted microscope	Olympus	IX83P2ZF

STEP-BY-STEP METHOD DETAILS

Non-adhesive coating of conical bottom 96-well plate

Timing: 12–18 h

This section explains the steps involved in generating non-adhesive cell culture surfaces.

1. Sanitize the conical 96-well plate by spraying 70% ethanol and place the plate in the laminar hood. Also, bring p-HEMA solution into the hood.
   a. Depending on the total number of spheroids (irrespective of cell density) required for the experiment, coat twice as many wells with 35 μL of p-HEMA solution.

   Note: For example, if 20 spheroids are required to set up an experiment, we recommend coating 40 wells with p-HEMA. Make sure the entire surface of the well is coated with p-HEMA solution.

   b. After coating the required number of wells, leave the plate in the laminar hood for 12–16 h for drying with open lid (this allows ethanol to evaporate leaving a non-adhesive coating on the well).
   c. You can use the plate right away next day or you can store the plate in the laminar hood for a week by wrapping it in a paraffin film.

Cell spheroid preparation

Timing: 1–2 h

This section describes the steps involved in preparing cell spheroids.

2. In this study, we prepared spheroids (1 spheroid/well) using 10,000 cells.

   Note: Upon reaching 80% confluency level (around 5 to 6 million cells), passage the cells using 0.25% trypsin and count the cells.

   a. Depending on the cell count, prepare 1 million cells in 1 mL of respective cell media, this gives 1000 cells per 1 μL of media.

⚠ CRITICAL: To attain 10,000 cells in each well, 90 μL of cell media and 10 μL of 1 million cells/mL dilution was added to the p-HEMA coated conical bottom plate.

b. After adding 10,000 cells to each well in the p-HEMA coated plate. Spheroid formation can be initiated by centrifuging plates at 1000g for 10 min at 25°C.

Note: Before centrifuging the plate make sure that the weight of the p-HEMA coated plate and balance plate are same. Use weigh balance to measure weight.

Note: Irrespective of the cell density required to prepare spheroids, volume added to each well in a p-HEMA coated 96 well conical bottom plate is always maintained constant at 100 μL.

c. After centrifuging the plate, add 2.5 μL of GFR Matrigel to each well at a concentration of 2.5% on v/v basis.

Note: Matrigel should be placed in an ice bath (4°C) at least 2 hours before to addition. To add Matrigel, use pipette tips stored at −20°C.

d. Later, incubate the plate at standard cell culture conditions for 12–16 h and the spheroids will be ready for culture within 24 h.
e. By following this protocol, we can prepare one spheroid per well even with lower cell densities (100 cells) without any issue.

Note: It is recommended to prepare twice the number of spheroids than required for the experiment.

Note: This procedure will only yield one spheroid per well, regardless of the cell density utilized.

HA hydrogel preparation

Timing: 1–2 h

This section details the steps involved in fabrication of HA hydrogels.

3. Weigh 5 mg of HAMA in an Eppendorf tube and add 85 μL of DMEM to dissolve HAMA. Next, vortex and centrifuge the tube, and place it in a 37°C water bath for 1 h to dissolve HAMA.
   a. Meanwhile, prepare 0.1 M dithiothreitol (DTT) solution by weighing 15.4 mg and dissolving it in 1 mL of DMEM.
   b. After 1 h, take out the Eppendorf tube and vortex it and check whether HAMA is dissolved, by visual inspection.
   c. If HAMA is dissolved completely, sterilize the tube with 70% ethanol before placing it in the laminar hood.

Note: If not place the tube in water bath for additional 20 minutes at 37°C.

4. Add 10 μL of 0.1 M DTT cross-linker solution to the tube and vortex.
   a. Centrifuge the tube in a mini centrifuge (at 3200 xg) for 20–30 s.
   b. Immediately transfer the 95 μL of HAMA and DTT solution to a well of a 96 well flat bottom plate.
   c. Incubate the plate for 12–16 h for gelation in a 37°C and 5% CO_2 incubator.

Note: This formulation results in a ~0.4 kPa hydrogel, as measured via compression testing on RSA-G2 solid analyzer (TA Instruments).[3,5]

⚠ CRITICAL: A maximum of 8 hydrogels can be prepared in a single Eppendorf tube by scaling up the above mentioned quantities. If multiple Eppendorf tubes are used to prepare gel precursor solution, add DTT solution to second tube when you have completely transferred gel solution of 1st tube into the wells of a 96 well flat bottom plate.

HA hydrogel functionalization:

Timing: 4 h

This section explains the steps for functionalizing the HA hydrogels with an integrin binding peptide for supporting cell adhesion.

5. Next day, wash the gels by adding 100 μL of DMEM for 30 min to remove any excess DTT present in the hydrogel.
   a. Meanwhile, prepare 1 mg/mL RGD solution from a 5 mg/mL stock (stored in −20°C) and store the 1 mg/mL solution at 4°C for an hour.

   Note: Always freshly prepare 1 mg/mL RGD solution.

   b. After 30 min, take out the DMEM and switch on the UV light of the laminar hood for 30 min. During this time, the lid of 96-well flat bottom plate should be removed.
   c. After 30 min, switch off the UV light and add 25 μL of prepared 1 mg/mL RGD solution to each hydrogel. Incubate the hydrogels in the presence of RGD solution for 3 h in the laminar hood.
   d. After 3 h, remove the RGD solution and wash the hydrogels with 100 μL of DMEM. Next, seed spheroids on top of the hydrogel.

Non-adhesive coating of flat bottom 96-well plate for suspension culture

Timing: 12–18 h

This section details the steps involved in preparing p-HEMA coated well plates for suspension culture.

6. Sterilize a flat bottom 96-well plate with 70% ethanol and place it in the laminar hood.
   a. Coat each well with 50 μL of p-HEMA solution (see preparation of polyhydroxyethyl methacrylate (p-HEMA) solution section).

   Note: Entire surface of the well must be coated with p-HEMA solution.

   b. After coating the required number of wells, leave the plate in the laminar hood for 12–16 h for drying with open lid.
   c. You can use the plate right away or you can store the plate in laminar hood by wrapping it with a paraffin film.

Transfer of spheroids on to HA hydrogel and suspension culture

Timing: 30–45 min

This section details the procedure of transferring spheroids from conical bottom plate to suspension culture and also on to HA hydrogels.

7. Take out the conical bottom plate containing spheroids from the incubator and bring it into the laminar hood after sanitizing the plate.

⚠ CRITICAL: Before transferring the spheroids, cut the tip of a 200 μL pipette using scissors to increase the orifice of pipette tip, which helps to transfer the whole spheroid along with 100 μL of media without any breakage.

a. By utilizing the cut pipette tips, take out the spheroid along with 100 μL of media from conical bottom plate and place it on top of a HA hydrogel by pointing the tip towards the center of the gel.

Note: For suspension culture, transfer the spheroids to the p-HEMA coated flat bottom plate.

b. After transferring the required number of spheroids along with 100 μL of media, place the plate in the incubator.

⚠ CRITICAL: Once the spheroids are transferred, check the spheroids under microscope to make sure all the spheroids are intact, and they did not break during transfer. If the spheroid looks good (with sphericity index > 0.8, as shown in Figures 2 and 4 and without any breakage), move on to the next gel. If not pipette it out immediately and transfer another spheroid.

c. To keep the media addition method consistent between HA hydrogel and suspension culture, we always supplement the existing media with 50 μL of respective cell media on day 2, 4 and 6.

Note: Utilize 10 μL pipette for adding media, and make sure the spheroids are not disturbed during media addition.

d. Capture images of spheroids on top of the HA hydrogel or in suspension culture using a confocal microscope. Images were taken on day 1, 3, 5 and 7.

Spheroid cross-sectional area measurements

⏲ Timing: 30–45 min

This section outlines the steps for quantifying cross-sectional area of cell spheroids.

8. Image J software was utilized to calculate the cross-sectional area of spheroids cultured on HA hydrogel and in suspension culture.
   a. First, set the measurements that need to be measured (area, shape descriptions).
   b. Next, set the scale depending on the magnification used to capture images.
   c. Once the scale and measurements are set, select the freehand selections tool, and draw the line along the periphery of the spheroid, then select analyze and click measure for the cross sectional area and shape descriptions of spheroids to be displayed.

Note: If any growth had been observed along the spheroid periphery include them.

⚠ CRITICAL: Multiple cell spheroids with different cells densities ranging from 2500 to 50,000 cells were prepared to test whether spheroid cross sectional area can be utilized to quantify cell spheroid growth. Cross-sectional area calculations depicted that with increase in cell numbers, the area of spheroids increased linearly indicating cross-sectional area calculations can be employed for assessing cell spheroid growth patterns (Figure 1).

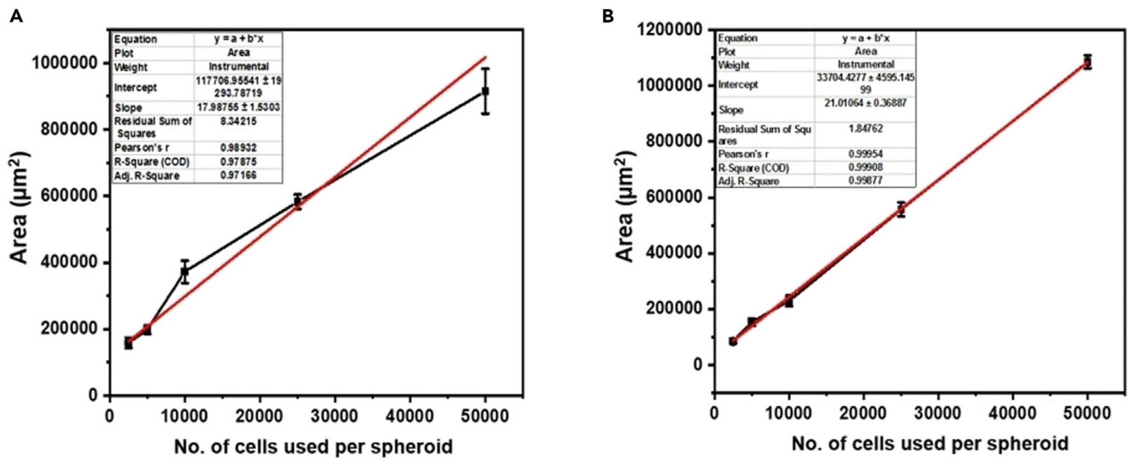

Figure 1. Cross-sectional area measurements can be employed to evaluate growth of cell spheroids *in vitro*
Cross sectional area measurements of (A) MDA-MB-231Br cell spheroids and (B) BT474Br3 cell spheroids as a function of number of cells used for making spheroids. N $\geq$ 6 replicates per condition. Data reported as mean $\pm$ S.E.M.

Note: Irrespective of the cell density utilized to prepare cell spheroids, the technique we utilized will only yield 1 cell spheroid per well.

Measuring total number of cells present in spheroids

Timing: 45–90 min

This section details the procedure for dissociating cell spheroids into single cells.

9. On day 7, collect all the cell spheroids cultured on HA hydrogel and in suspension culture individually into an Eppendorf tube and aspirate the cell media and wash the spheroid thrice with 400–600 μL of PBS.

   Note: Always centrifuge the Eppendorf tube before aspirating.

   a. After PBS wash, add 400 μL of Accutase to the spheroid and incubate the tube at 37°C for 5 min.
   b. After 5 min of incubation, manually re-suspend spheroids in the Accutase solution with a 200 μL pipette tip for 5 more minutes.

   Note: This would allow the spheroid to mechanically disintegrate. Later, incubate the spheroid for 5 minutes and repeat the same process for 40 minutes.

   c. After 40 min, centrifuge the Eppendorf tube in micro-centrifuge (at 330 *g*) for 5 min to allow single cells to settle at the bottom of the tube.
   d. Aspirate the accutase carefully and wash the cells twice with 400 μL of PBS and add 100 μL of PBS and re-suspend cells in the PBS.

   Note: After this step, cells can be used for staining of various biological markers.

   e. Take out 10 μL of PBS containing cells and add to a new Eppendorf tube and add 10 μL of 0.4% trypan blue stain. Mix both the solutions and add 10 μL of mixture to a cell counting chamber slide and insert the slide into countess II hemocytometer to measure the number of cells.

f. Divide the total number of cells value by the number of spheroids dissociated per condition to get the value of total number of cells present in each spheroid.

EdU staining for assessing proliferation vs. dormancy

Timing: 18–24 h

This section describes the steps for performing EdU staining on whole spheroid as well as at the single cell level.

10. On day 7, aspirate the media and add 90 μL of fresh media to each well. Then add 10 μL of 10 μM EdU containing media solution to each well. Incubate the plate for 12–16 h in an incubator.
    a. To prepare 10 μM EdU solution.
        i. Add 99 μL of cell media to an Eppendorf tube.
        ii. Add 1 μL of EdU stock solution. Mix the two liquids by vortexing.

    Note: EdU dilution should be done in a sterile environment.

    b. Next day, collect the cell spheroids into an Eppendorf tube and disintegrate the spheroids into single cells (as mentioned in measuring total number of cells present in spheroids section).

    Note: For staining whole cell spheroids directly, retrieve each spheroid and add them to a well of a 96 well plate. After transferring all the spheroids to respective wells, centrifuge the plate for 1 min at 1000g and aspirate the EdU containing media, and follow the similar procedure mentioned for single cells.

    c. Once the spheroids are dissociated into single cells, add the single cells in a 96 well plate and fix the cells for 20 min by adding 100 μL of 4% paraformaldehyde.

    Note: Depending on the number of spheroids dissociated, add single cell suspension to equivalent number of wells in a 96 well plate.

    d. After 20 min, remove the solution and wash with 100 μL of PBS. Before aspirating the solution from the plate, centrifuge the plate for 1 min at 1000g, to allow cells to settle at the bottom.
    e. Later, permeabilize cells by adding 100 μL of 0.25% triton X solution for 15 min at 20°C–25°C. After washing, block the cells for 30 min in the presence of 5% BSA solution. Before aspirating the solution from the plate, centrifuge the plate for 1 min at 1000 *g*, to allow cells to settle at the bottom.
    f. Next, add 50 μL of the reaction cocktail prepared as per the manufacturer's protocol (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fmp10338.pdf) and incubate the plate in dark at 20°C–25°C for 25–30 min.

    Note: Switch off lights while preparing the reaction mixture and do not expose the stock solutions to light.

    g. Later, aspirate the reaction mixture and wash the cells with PBS. Before aspirating the solution from the plate, centrifuge the plate for 1 min at 1000g, to allow cells to settle at the bottom.

h. Further, counter stain the cell nucleus with 100 μL of 14.3 μM DAPI solution for 5 min at 20°C–25°C in the dark. After 5 min, aspirate DAPI solution and wash cells twice with PBS. Cells will be ready for imaging. Before aspirating the solution from the plate, centrifuge the plate for 1 min at 1000g, to allow cells to settle at the bottom.
i. To prepare 14.3 μM DAPI solution:
   i. Add 1998 μL of PBS to an Eppendorf tube.
   ii. Add 2 μL of DAPI stock solution (14.3 mM). Mix the two liquids by vortexing.

Fluorescence intensity measurements

Timing: 45–90 min

This section details the steps for measuring the fluorescence intensity of cell spheroids.

11. Fluorescence intensity measurements were performed using cellSens Dimension software. Clear bottom 96 well plates can be used for imaging. Positioning the spheroid at the center of the well before capturing images is recommended.

Note: To capture the image of whole spheroid in one field of view, perform imaging at 4X magnification.

a. In the software, open the fluorescent EdU stained images in "count and measure" tab and create multiple rectangular regions of interest (ROI) of same dimension within the area of each spheroid.

Note: The dimension of the rectangular ROI should be kept constant for all spheroids.

b. To cover maximum area, randomly chose at least four ROIs within a spheroid.
c. Determine the mean color intensity by using the "measurements and ROI" functions available in the software. The mean color intensity was then plotted.

CRITICAL: Set the exposure time of confocal microscope to 100 milliseconds and EM gain to 5 in the acquisition tab of the cellSens dimension software for acquiring fluorescent images. Similarly, for acquiring bright-field images reduce the exposure time to 50 milliseconds with EM gain set to 5. Under the 'adjust display' tab the fixed scaling can be manually set between 2000 and 4000. It is important that fluorescent images of different spheroids are acquired under identical settings for comparing the fluorescent intensity of EdU.

EXPECTED OUTCOMES

By following the above-mentioned protocol, we were able to generate dormant BMBC spheroids (one spheroid/well) *in vitro*. In particular, we found that when the spheroids (prepared using 10 k cells) were cultured on top of HA hydrogel for a period of 7 days, no difference was noticed in cross-sectional area of spheroids from day 1 to day 7 (Figure 2). However, in suspension culture, cross-sectional area of spheroids increased significantly from day 1 to day 7 (Figure 2). In addition to cross-sectional area measurements, we have dissociated spheroids cultured on both HA hydrogel and in suspension culture into single cells on day 7 to verify whether the growth observed via area measurements translates into an increase in cell number per spheroid. We found that spheroids cultured in suspension culture contained significantly higher single cell populations compared to those in HA hydrogel (Figure 3). Next, we evaluated the percentage of proliferating cells through EdU incorporation method,[5,6] to quantify differences in growth patterns between dormant and proliferative states. Consistent with the previous finding, a significant increase in the percentage of

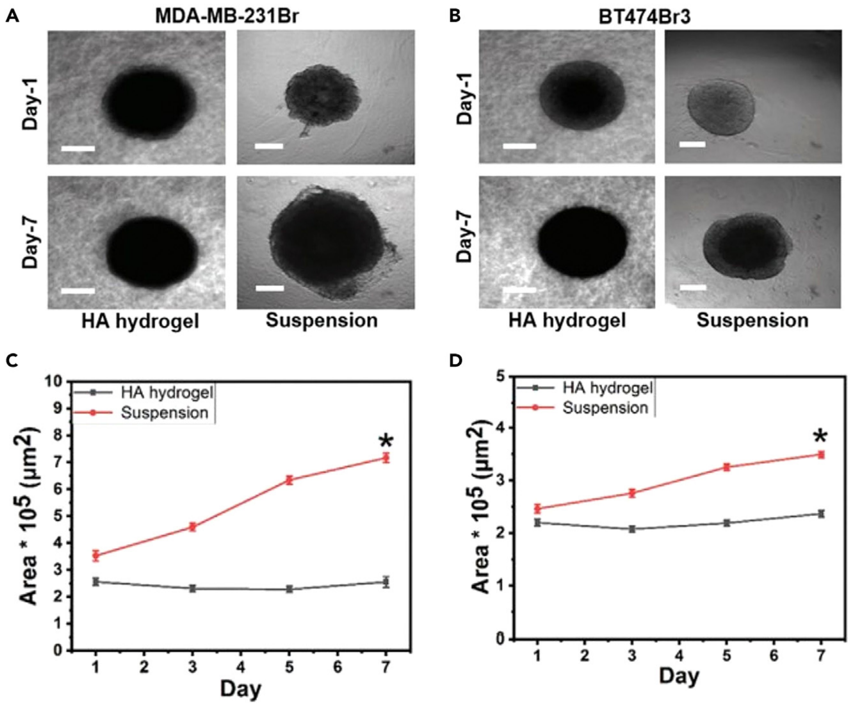

Figure 2. Hyaluronic acid hydrogels restricted growth in BMBC spheroids compared to suspension cultures over a period of 7 days

Bright field images of MDA-MB-231Br (A) and BT474Br3 (B) spheroids, Scale bars, 200 μm. Cross-sectional area of MDA-MB-231Br (C) and BT474Br3 (D) cell spheroids in HA hydrogel and in suspension culture. N ≥ 12 replicates for each condition. * indicates statistical significance ($p < 0.05$). Data reported as mean ± S.E.M. Figure taken from RV Kondapaneni et al.[1], and reprinted with permission from John Wiley and Sons.

proliferating cells was seen in suspension culture (proliferating spheroids) vs. the HA hydrogel (dormant spheroids) as quantified via mean intensity measurements and percentage of EdU positive cells (Figures 4 and 5). In prior work, dormant vs. proliferative states have been identified by a lower ratio of p-ERK to p-p38 levels and vice-versa.[6–9] In addition to the cross-sectional area measurements, EdU staining, levels of dormancy marker p-p38 and proliferative marker p-ERK were

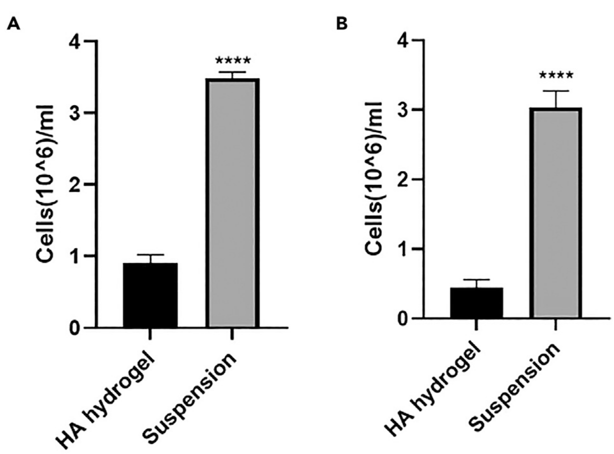

Figure 3. Quantification of total number of cells in spheroids

Total number of cells in (A) MDA-MB-231Br or (B) BT474Br3 spheroids cultured in suspension was significantly higher than in spheroids cultured on HA hydrogel at day 7. Data reported as mean ± S.D. N ≥ 10 spheroids per each condition, ****$p < 0.0001$.

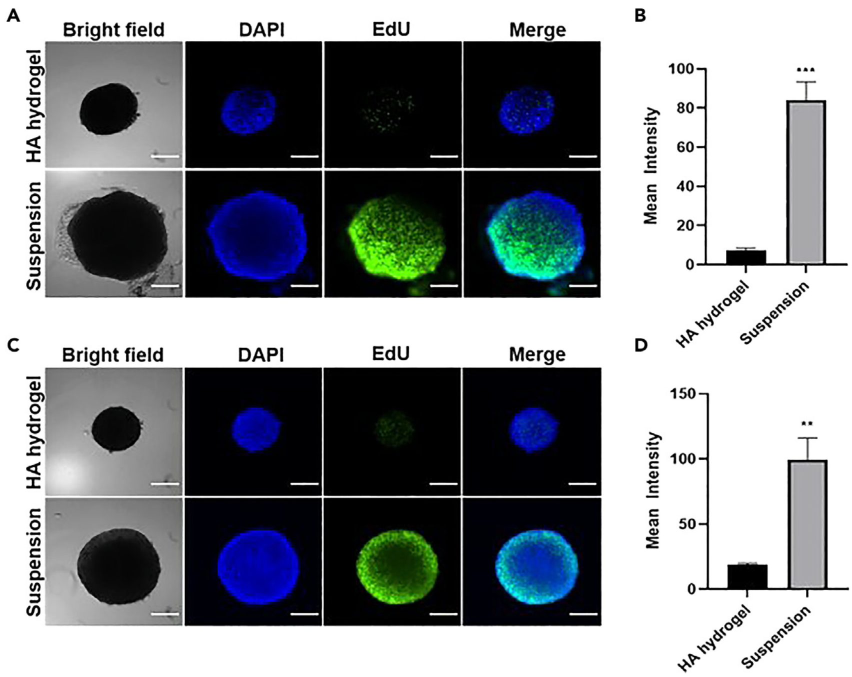

Figure 4. EdU positivity of whole spheroids

(A) Representative fluorescent images showing higher EdU positivity of MDA-MB-231Br spheroids cultured in suspension.

(B) Mean EdU color intensity of MDA-MB-231Br spheroids cultured in suspension was significantly higher than spheroids cultured on HA hydrogel at day 7 (***$p < 0.001$).

(C) Representative fluorescent images showing higher EdU positivity of BT474Br3 spheroids cultured in suspension.

(D) Mean EdU color intensity of BT474Br3 spheroids cultured in suspension was significantly higher than spheroids cultured on HA hydrogel at day 7 (**$p < 0.01$). Blue - DAPI (nucleus), green - EdU. Data reported as mean $\pm$ S.D. N $\geq$ 6 replicates per each condition. Scale bars, 200 μm.

analyzed. The percentage of p-p38 positive cells was significantly higher while the percentage of p-ERK positive cells was lower in spheroid cultures on HA hydrogel vs. suspension.[1] Additionally, HA hydrogel can also induce a dormant state in lower cell density spheroids.[5]

LIMITATIONS

Selective use of BMBC cell lines and brain extracellular matrix mimetic HA hydrogel in our protocol does not completely represent the complex *in vivo* tumor microenvironment. Therefore, the intricate interactions among the multitude of cellular and acellular components *in vivo* (as noted in mouse models of BMBC[10]) are only partially reflected in our approach. Possibly, the biomechanical cues engaged in inducing dormant phenotype of the spheroids in our *in vitro* culture system may only encompass a fraction of the interactions ongoing *in vivo*. However, our system provides a controllable environment to probe the impact of specific cues on cell phenotype. This protocol is primarily focused on developing dormant BMBC spheroids; however, it may be adapted for generation of dormant spheroids of other brain metastatic cancer cell lines, including patient derived cells in the future.

TROUBLESHOOTING

Problem 1

Irregular or non-uniform shape of spheroids.

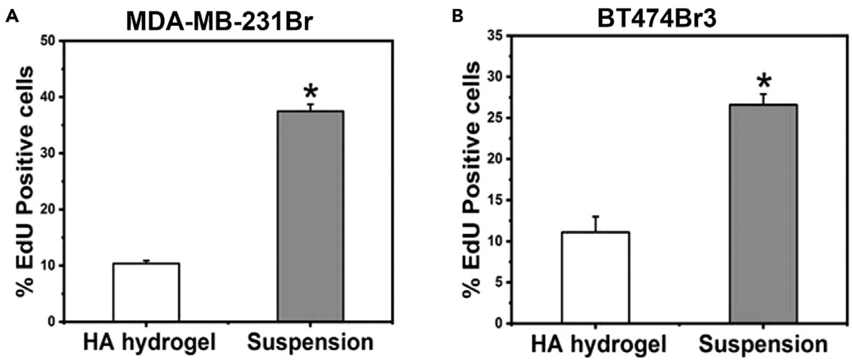

Figure 5. Percentage of EdU positive cells were higher in suspension cultures compared to HA hydrogels
Quantification of EdU positive cells present in MDA-MB-231Br (A) and BT474Br3 (B) cell spheroids. N $\geq$ 6 replicates for each condition. * indicates statistical significance ($p < 0.05$). Data reported as mean $\pm$ S.E.M. Figure taken from RV Kondapaneni et al.[1], and reprinted with permission from John Wiley and Sons.

Potential solution

Uneven coating of poly-HEMA solution in conical bottom plates that were used to prepare tumor spheroids can lead to formation of spheroids with variable shapes and sizes. Care must be taken to dispense an equal volume of coating solutions into each well. Additionally, it should also be ensured that the coating solution is allowed to dry off completely before seeding the cells.

Problem 2

Spheroids disintegrate during the transfer process.

Potential solution

Spheroids should be handled very carefully with minimal pipetting to avoid mechanical damage. Pipette tips with larger holes (made by cutting off the tip with scissors) will allow gentle and easy movement of spheroids into and out of the pipette tips. It also helps to set the pipette at lower settings to avoid larger suction force which could potentially damage the spheroids.

Problem 3

Hydrogel polymerizes quickly.

Potential solution

The crosslinking reaction starts immediately with addition of crosslinker, so it is advised to keep all the setup (including plates and pipettes) ready beforehand so that the hydrogel can be quickly transferred to respective wells before polymerization.

Problem 4

Incomplete spheroid disruption for downstream processes.

Potential solution

Manual disruption of spheroid into single cells can be achieved by incubating in Accutase or Trypsin EDTA at 37°C for 15–30 min with intermediate trituration with a pipette. Washing the spheroids with PBS before the addition of dissociating agents can help in removing the traces of expended media which could block the activity of dissociating agents. Incubating longer with relatively gentler trituration can help to disperse the spheroids without damaging the cells.

Problem 5

Loss of cells during the washing steps.

Potential solution

To avoid excessive loss of single cells during the washing steps, after each centrifugation, the supernatant should be aspirated out slowly from the top while touching sides of the wall without disturbing the cells at the bottom. Pipette can be set at lower settings to avoid abrupt forceful aspiration of the supernatant.

Problem 6

Attachment of cell spheroids in p-HEMA coated plates.

Potential solution

The p-HEMA coating should be uniform to avoid any uncoated spots in the well, to which the cancer cells can attach and proliferate. If this does not occur, cell spheroids will grow as adherent cultures instead of free-floating spheroid cultures.

RESOURCE AVAILABILITY

Lead contact

Requests for any additional information should be directed to the lead contact, Dr. Shreyas S. Rao (srao3@eng.ua.edu).

Technical contact

For any technical questions related to the protocol, please contact Dr. Raghu Vamsi Kondapaneni (kondapaneniraghuvamsi@gmail.com).

Materials availability

No new materials were either generated or utilized in this study.

Data and code availability

Not applicable.

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AUTHOR CONTRIBUTIONS

S.S.R. and R.V.K. conceived the methodology. R.V.K. and S.K.G. performed the experiments. R.V.K., S.K.G., and S.S.R. wrote the original draft. R.V.K., S.K.G., L.A.S., and S.S.R. reviewed and edited the final draft. S.S.R. and L.A.S. acquired resources. S.S.R. supervised the work and acquired the funding for this protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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