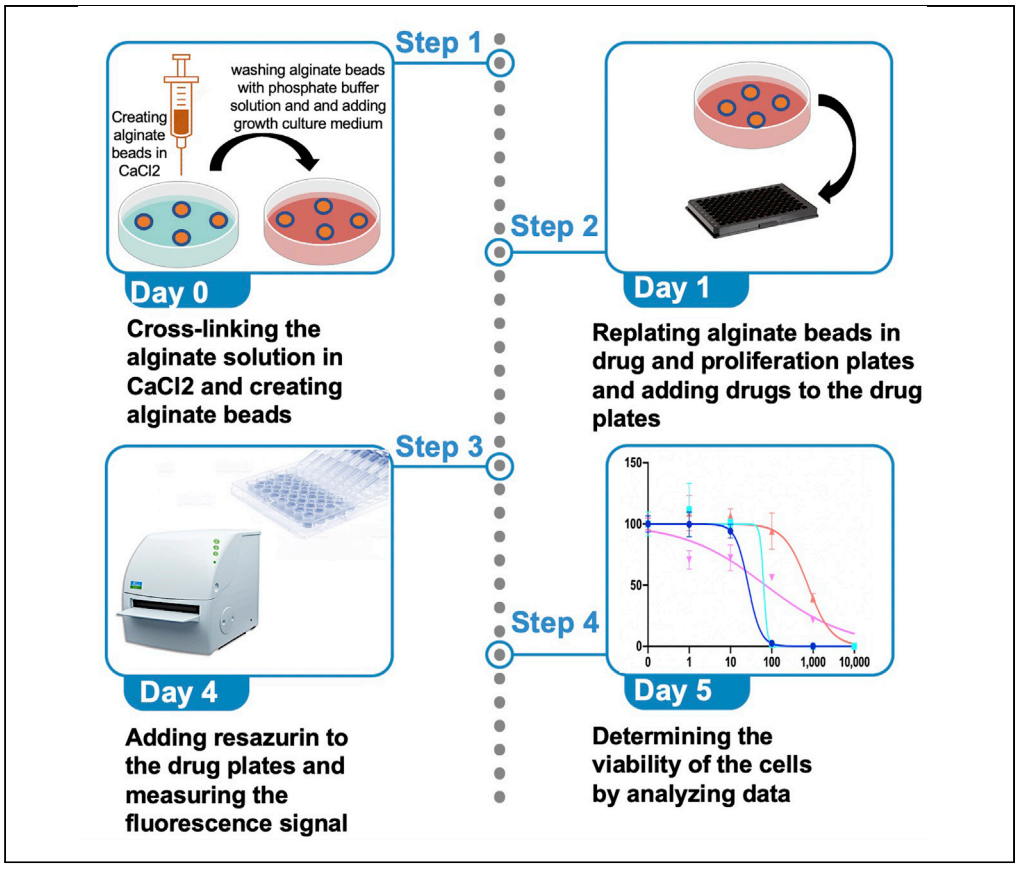
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Alginate-based 3D cancer cell culture for therapeutic response modeling



Two-dimensional (2D) culture of tumor cells fails to recapitulate some important aspects of cellular organization seen in *in vivo* experiments. In addition, cell cultures traditionally use non-physiological concentration of nutrients. Here, we describe a protocol for a facile three-dimensional (3D) culture format for cancer cells. This 3D platform helps overcome the 2D culture limitations. In addition, it allows for longitudinal modeling of responses to cancer therapeutics.

Farideh Davoudi, Samar Ghorbanpoor, Satoshi Yoda, ..., Aaron N. Hata, Henning Willers, Cyril H. Benes

fdavoudi@mgh.harvard.edu, fdavoudi@partners.org (F.D.) cyrilbenes@gmail.com (C.H.B.)

HIGHLIGHTS

A detailed protocol on hydrogel-based 3D culture of patient-derived tumor cell lines

No binding sites for cells in hydrogel polymers allowing for pure interaction of cells

Longitudinal 3D proliferation assays and drug-response assessments

Quick and easy recovery of 3D-cultured cells for downstream experiments

Davoudi et al., STAR Protocols *2*, 100391
June 18, 2021
https://doi.org/10.1016/j.xpro.2021.100391

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Alginate-based 3D cancer cell culture for therapeutic response modeling

Farideh Davoudi,[1,3,4,*] Samar Ghorbanpoor,[1] Satoshi Yoda,[1] Xiao Pan,[2] Giovanna Stein Crowther,[1] Xunqin Yin,[1] Ellen Murchie,[1] Aaron N. Hata,[1] Henning Willers,[2] and Cyril H. Benes[1,*]

[1]Massachusetts General Hospital, Center for Cancer Research, Harvard Medical School, 149 13th Street, Boston, MA 02129, USA

[2]Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114, USA

[3]Technical contact

[4]Lead contact

*Correspondence: fdavoudi@mgh.harvard.edu or fdavoudi@partners.org (F.D.), cyrilbenes@gmail.com (C.H.B.)
https://doi.org/10.1016/j.xpro.2021.100391

SUMMARY

Two-dimensional (2D) culture of tumor cells fails to recapitulate some important aspects of cellular organization seen in *in vivo* experiments. In addition, cell cultures traditionally use non-physiological concentration of nutrients. Here, we describe a protocol for a facile three-dimensional (3D) culture format for cancer cells. This 3D platform helps overcome the 2D culture limitations. In addition, it allows for longitudinal modeling of responses to cancer therapeutics.
For complete details on the use and execution of this protocol, please refer to Lhuissier et al. (2017), Lehmann et al. (2016), Liu et al. (2016), and Duval et al. (2011).

BEFORE YOU BEGIN

General preparations

1. Check the safety data sheet (SDS) for all the materials and use proper personal protective equipment (PPE).
2. Autoclave all the non-sterile equipment , i.e., spatula, pipettes, and pipette tips.
3. Inactivate complements in fetal bovine serum (FBS) and Dialyzed FBS by heating at 56°C for 30 min before adding into culture media.
4. Prepare the two different culture media, i.e., high-glucose & high-glutamine and low-glucose & low-glutamine culture media
5. Warm culture media, resazurin solution, and phosphate buffered saline (PBS) in a water bath at 37°C.

Note: Taking into account that 3D cell growth is generally slower than cell growth in 2D, having enough cells for 3D cultures is important for the proliferation test and seeding densities should be optimized. In addition, cells grow slower in low-glucose & low-glutamine culture media.

Note: All the steps are performed inside the biological safety cabinet under sterile conditions (BSL-2).

Preparing 1% w/v alginate powder in high-glucose & high-glutamine (HG) and low-glucose & low-glutamine (LG) culture media

Timing: 2 h

6. Pour 100 mL of pre-warmed (37°C) culture media into a vacuum filtration bottle containing a stir bar.

Note: Use one vacuum filtration bottle for HG and a separate one for LG culture media.

7. Weigh out 1 g of sodium alginate powder and add it slowly to the culture media.
8. Place the vacuum filtration bottle on a magnetic hot plate stirrer (35°C and 400 RPM) for 2 h.

Note: The sterile alginate solution can be kept at 4°C for up to a month.

Mixing cells with the alginate solution

Timing: 2 h

9. Use 2 mL of TrypLE Express enzyme to collect cells from a 100 mm TC-treated culture dish.
10. Neutralize TrypLE by adding 4 mL of culture media containing FBS after cells are detached from the dish (5–15 min depending on the cell line).
11. Centrifuge cells at 150 *g* for 5 min and count them.

Note: The calculation is based on 10 μL alginate solution per bead. For cells that grow fast with doubling time of 24–48h, start the proliferation test with 1,000 cells/bead. For cells with slower growth rate, start the proliferation test with 2,500 cells/bead.

Note: For the proliferation test, prepare at least three concentrations of cells, i.e., 1,000, 2,500 and 5,000 cells/bead for each of HG and LG culture media.

12. Based on your calculation, centrifuge cells again at 150 *g* for 5 min, aspirate culture media and add the desired volume of alginate solution (based on the previous calculation) to the cells and resuspend them very well with a 1,000 μL pipette.

Note: Ideally, at least 5 mL of cell suspension in alginate should be prepared.

Cross-linking the alginate solution and creating alginate beads

Timing: 1–2 h

13. Add around 25 mL of sterile $CaCl_2$ 100 mM solution to a 100 mm TC-treated culture dish.

Note: one culture dish is needed per each cell concentration and per each culture medium.

14. Take up the cell suspension in alginate solution in the syringe and eject air bubbles in the syringe.
15. Keep the needle tip around 3 cm above the CaCl2 solution surface, hovering the needle in a circular motion, dispense alginate beads from the syringe at one bead/second. This avoids clumping the alginate beads together.

Note: The needle used in this protocol to drop 10 μL alginate solution was a 21 G × 1–1/2" needle.

Alternatives: For cell suspension of more than 5 mL or creating alginate beads with smaller sizes (< 2 mm), a pump device and a size-adjustable nozzle may be used.

16. Allow the alginate beads to cross-link in the CaCl2 solution for 20 min.

17. Aspirate CaCl2 solution by tipping the plate at an angle. Avoid puncturing the alginate beads.
18. Wash the beads with 15 mL of culture media containing 1% Antibiotic-Antimycotic for 15 min.

Note: PBS or another buffer without CaCl2 should not be used for this step.

Note: Alginate beads that float on top of the surface should be discarded.

Re-plating alginate beads for proliferation and drug tests

Timing: 2–3 h

19. Re-plate each alginate bead in each individual well of a black clear-bottomed 96-well plate using the spatula.

Note: There will be two types of plates:
   a. Plates for the proliferation test measured by a cell viability assay. These plates are read on Day 1 and then over time for the duration of the experiment. Six to ten replicates are needed for the proliferation test.
   b. Plates for the drug-response test. Three replicates are needed for the drug-response test.

Note: Beads without cells, "empty beads" are needed for proliferation assays to allow subtracting assay background signal. These can be prepared the same way as cell containing beads by simply omitting the cells.

Note: Alginate beads are about 2 mm in diameter (Figure 1).

20. Add 150 µL of culture media to each well. Also, add PBS to the surrounding empty wells to minimize evaporation.

Note: Alginate beads can be maintained in culture for up to one month depending on the cell line. Culture media need to be changed after 48 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospho-ALK (Tyr1282/1283) (D39B2) Rabbit mAb	Cell Signaling Technology	Cat#9687
ALK (D5F3®) XP® Rabbit mAb	Cell Signaling Technology	Cat#3633
Phospho-Paxillin (Tyr118) Antibody	Cell Signaling Technology	Cat#2541
β-Actin (13E5) Rabbit mAb	Cell Signaling Technology	Cat#4970
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	Cat#7074
Chemicals, peptides, and recombinant proteins		
Alginic acid sodium salt	Sigma-Aldrich	Cat#180947
$CaCl_2$ 100 mM aqueous solution	Fisher Scientific	Cat#AAJ62905K2
Resazurin sodium salt	Sigma-Aldrich	Cat#R7017
Staurosporine	Selleckchem	Cat# S1421
Hoechst 33342, trihydrochloride, trihydrate - 10 mg/mL solution in water	Life Technologies	Cat#H3570
PBS (phosphate buffered saline), pH 7.4	Thermo Fisher Scientific	Cat#10010023
DMEM, high glucose (25 mM)	Thermo Fisher Scientific	Cat#11995065

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DMEM, low glucose (5 mM), no glutamine	Thermo Fisher Scientific	Cat#11054020
RPMI Medium 1640	Thermo Fisher Scientific	Cat#72400-047
Fetal bovine serum	Thermo Fisher Scientific	Cat#26140079
Dialyzed fetal bovine serum	Thermo Fisher Scientific	Cat#26400044
Glutamine	Thermo Fisher Scientific	Cat#2503008
Antibiotic-antimycotic	Thermo Fisher Scientific	Cat#15240062
UltraPure™ 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	Cat#15575020
TrypLE™ Express Enzyme (1×)	Thermo Fisher Scientific	Cat#12604039
Critical commercial assays		
LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells	Thermo Fisher Scientific	Cat#L3224
CellTiter-Glo® 3D Cell Viability Assay	Promega	Cat#G9681
Deposited data		
Analyzed data	This paper	N/A
Experimental models: Cell lines		
Patient-derived lung and breast tumor cell lines	MGH Center for Cancer Research	N/A
NCI-H3122	Sigma	Cat#95111734
Software and algorithms		
GraphPad Prism 8	GraphPad Software, Inc.	N/A
Microsoft Excel	Microsoft Corporation.	N/A
ImageXpress Micro XL High-Content Screening System	Molecular Devices	Series#XL138945
Other		
Stericup Quick Release-GP sterile vacuum filtration system	Millipore	Cat#S2GPU05RE
96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates	Corning	Cat#3904
Falcon® 6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate	Corning	Cat# 353046
100 mm TC-treated Culture Dish	Corning	Cat#430167
Falcon® 15 mL high-clarity polypropylene (PP) conical centrifuge tubes	Corning	Cat#352096
10 mL Stripette™ Serological Pipets, Polystyrene	Corning	Cat#4488
Syringe PP/PE without needle	Alridch	Cat#Z116874
BD PrecisionGlide™ 21 G × 1–1/2" Hypodermic Needles	Becton Dickinson	Cat#305167
Fisherbrand™ Stainless Steel Lab Spatula with PTFE Coating	Fisher Scientific	Cat#13-820-059
Stir bar	N/A	N/A
Magnetic hot plate stirrer	N/A	N/A
Orbital shaker	N/A	N/A
Multichannel pipette	N/A	N/A
Corning™ Costar™ Sterile Disposable Reagent Reservoirs	Fisher Scientific	Cat#07-200-130
D300e Digital Dispenser	Tecan	N/A
T8+ Dispensehead Cassettes	Tecan	30097370
EnVision Plate Reader	PerkinElmer	N/A

MATERIALS AND EQUIPMENT

High-glucose & high-glutamine culture media (HG)	
Reagent	Volume
DMEM, high glucose (25 mM)	450 mL
Fetal Bovine Serum	50 mL
Antibiotic-Antimycotic	5 mL
Store at 4°C.	

Low-glucose & low-glutamine culture media (LG)	
Reagent	Volume
DMEM, low glucose (5 mM), no glutamine	450 mL
Dialyzed Fetal Bovine Serum	50 mL
Glutamine	1115 µL
Antibiotic-Antimycotic	5 mL
Store at 4°C.	

Note: LG culture medium contains glucose and glutamine concentrations comparable to their human plasma levels.

STEP-BY-STEP METHOD DETAILS

Preparing resazurin sodium salt stock solution for resazurin cell viability assay: Day 1

Timing: 1:30 h

1. Weigh out 1 g of resazurin sodium salt.
2. Pour 400 mL of pre-warmed RPMI culture media into a vacuum filtration bottle containing a stir bar.

Note: RPMI is not supplemented with FBS or antibiotic-antimycotic.

3. Place the vacuum filtration bottle on a magnetic hot plate stirrer (35°C and 400 RPM).
4. Add the resazurin sodium salt into the culture media slowly.
5. Cover the bottle with aluminum foil to protect it from light.
6. Allow it to mix for 1 h on the stirrer.
7. After 1 h of filter the resazurin using another vacuum filtration bottle.

Note: Store the stock in 4°C for up to one month protected from light.

Resazurin read-out: Day 1 and at the end of each time-point for drug-response test

Timing: 4:30 h

8. Dilute 4 mL of pre-warmed resazurin stock with 6 mL of serum-free pre-warmed RPMI culture media.

Note: The dye is light-sensitive.

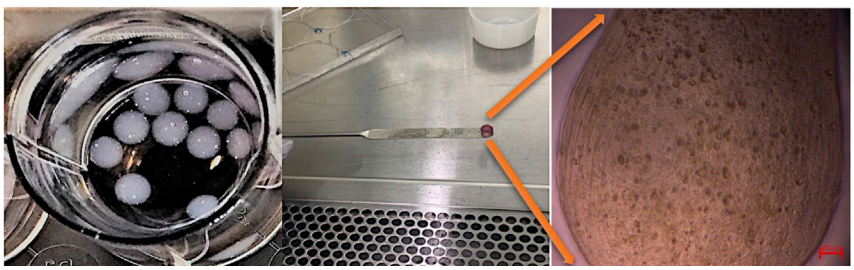


Figure 1. Alginate beads crosslinked with CaCl2
Scale bar represents 500 µm.

9. Add resazurin at 1:10 ratio (15 μL per well of 150 μL culture media) to each well of the 96-well plate containing alginate beads.
10. Protect the plates from light by wrapping them in aluminum foil.
11. Place the plates on an orbital shaker at low speed for 5 min.
12. Incubate plates for 4 h at 37°C.
13. Measure fluorescence: 555 nm excitation/585 nm emission.
14. After reading the plates, aspirate the resazurin with a multichannel pipette and wash with PBS twice for 5 min.
15. Aspirate PBS and replace it with fresh culture media.
16. Place the plates at 37°C if further proliferation measurement is to be taken.

Note: Resazurin-based assay allows to measure viability across several reads.

Note: The non-fluorescent resazurin dye is irreversibly reduced by viable, metabolically active cells to generate a strongly fluorescent product, resorufin, which can be detected by fluorescence microscopy

Note: Changing growth culture media, adding resazurin sodium salt, and washing steps are all performed using a multichannel pipette. The pipette tip needs to touch the bottom of the well at an angle of 45° to avoid the aspiration of alginate beads.

CellTiter-Glo (CTG) cell viability assay: Day 1 and at the end of each time-point for drug-response test

Timing: 3 h

17. Thaw and warm the CTG reagent.
18. Add CTG reagent at 1:3 ratio (50 μL per well of 150 μL culture media) to each well of the 96- well plate containing alginate beads by using a multichannel pipette.
19. Place the plates on a shaker at low speed for 30 min in 20°C.
20. Measure luminescence by a luminescence plate reader compatible with CTG-based assay.
21. Discard the plates after reading.

Note: CTG viability assay uses luciferase as the detection enzyme. Luciferase requires ATP in order to generate luminescent signal. Therefore, the signal is proportional to the amount of metabolically active cells.

Assessment of resazurin/CTG penetration in alginate beads

Timing: ~6 days

22. Re-plate the beads containing 3 different concentrations of cells, for example, 2,500, 5,000 and 10,000 cells as well as empty beads in each well of 96-well plates.
23. Add 200 μL of culture media.

Note: Use 6 replicates for each cell density including DMSO control.

24. Grow cells in 3D for 24 h and 72 h until the cells form larger colonies.
25. Administer staurosporine (1 μM) to cells in 3D alginate culture using a dispensing method of choice.

Note: Staurosporine is a cell-permeable alkaloid and a non-selective protein kinase inhibitor which induces apoptosis. Induction of apoptosis and activation of caspase-3 can lead to a

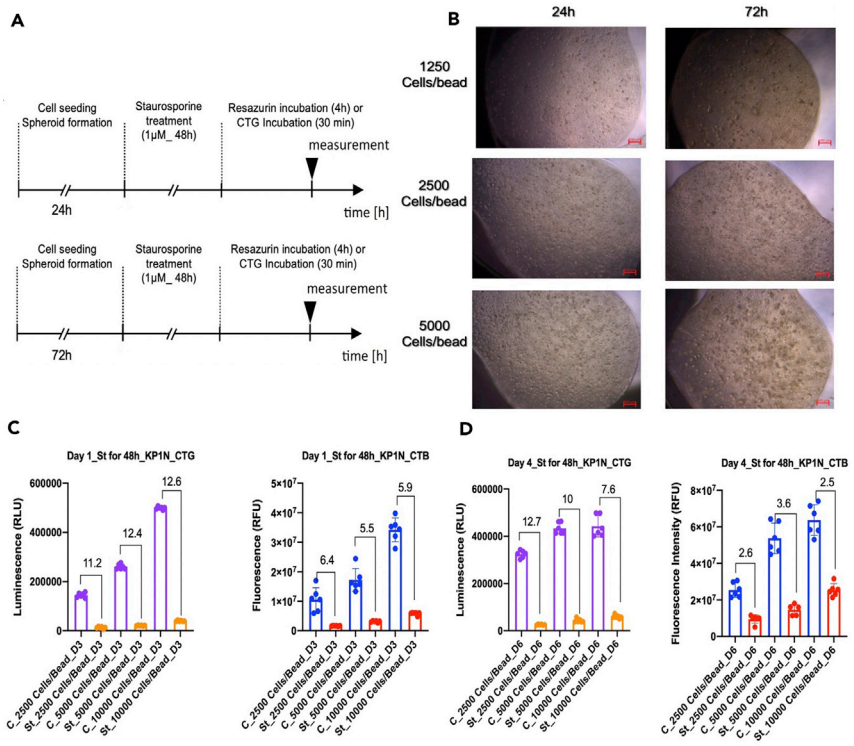


Figure 2. Expected viability measurement achieved in alginate cultures

(A) Experimental setup: KP1N cells were cultured in alginate at 3 different concentration of cells, 2500, 5000 and 10000 cells per bead for 24 h and 72 h. Then, 3D cultured cells treated with staurosporine (1 μM) or DMSO for 48 h, before resazurin and CTG assays were performed.

(B) Bright-field microscopy of KP1N cultured in 3D cultures for 24 h and 72 h before staurosporine treatment. Scale bars represent 500 μm.

(C) CTG luminescent. and resorufin fluorescence intensities of KP1N cells after growing for 24 h in 3D cultures and subsequent treatment with staurosporine (1 μM) or DMSO for 48 h (n = 6 for each condition) are shown. Data are presented as mean ± SD; fold changes in DMSO controls compared with staurosporine treatment are indicated. C, control; St, staurosporine; Cell-Titer-Blue (Resazurin).

(D) CTG luminescent and resorufin fluorescence intensities of KP1N cells after growing for 72 h in 3D cultures and subsequent treatment with staurosporine (1 μM) or DMSO for 48 h (n = 6 for each condition) are shown. Data are presented as mean ± SD; fold changes in DMSO controls compared with staurosporine treatment are indicated. C, control; St, staurosporine; CTB, Cell-Titer-Blue (Resazurin).

decrease in cell-cell contacts by cleavage of E-cadherin. We expect that the potent apoptosis inducer, staurosporine, results in loss of cell viability in loose cell colonies at Day 1 and a subsequent decrease in viability assays signals (Figure 2). In contrast, in the compact cell colonies of Day 4, we expect that cells display an increase in resorufin upon staurosporine treatment compared with the DMSO controls only if resazurin dye cannot penetrate into those colonies of cells.

26. After 48 h of treatment, assess the cell viability and proliferation capacity with the resazurin assay and compare it with the CTG assay (as explained above).
27. Normalize the results from resazurin and CTG assays to the reading for negative control (empty beads) and analyze them in GraphPad Prism software.

LIVE/DEAD cell viability assay: Once every week

Timing: 3 h

28. Thaw the assay dyes.
29. Dilute stock of 4 mM calcein-AM to 2 μM, stock of 2 mM ethidium homodimer-1 to 3 μM, and stock of 16.2 mM Hoechst to 33 μM in the culture media.
30. Aspirate the culture media from each well containing alginate beads by using a multichannel pipette and add 150 mL of the prepared solution to each well.
31. Incubate the cells in 37ºC for 2 h.
32. Image the plates with ImageXpress (or any other fluorescence microscope) using the following settings:
    a. Objective magnification: 10×
    b. Filter for Calcein-AM (FITC type set-up): excitation: 475/34 nm & emission: 536/40 nm, Ethidium Bromide dimer (Texas Red type set-up): excitation: 560/32 nm & emission: 624/40 nm, Hoechst 33342 (DAPI type set-up): excitation: 377/54 nm & emission: 447/60 nm
    c. Exposure: FITC: 50 ms, Texas Red: 100 ms, DAPI: 250 ms
    d. Range: 500 μM
    e. Step-Size: 50 μM

    Note: The settings have been provided as guidelines, they can be different depending on the specific instrument used.

33. Discard the plates after imaging.

    Optional: Hoechst stain is added to stain all nuclei.

    Note: Green-fluorescent calcein-AM indicates the presence intracellular esterase activity of live cells (Figure 3) and red-fluorescent ethidium homodimer-1 indicates the loss of plasma membrane integrity of dead cells.

Drug-response test: Day 1

Timing: 1 h

34. Prepare a stock concentration for each of the experimental drugs (typically 10 mM or 5 mM).
35. Add drugs to beads at final desired doses.
36. Place the plates on an orbital shaker at low speed for 5 min.
37. Incubate the plates at 37°C.

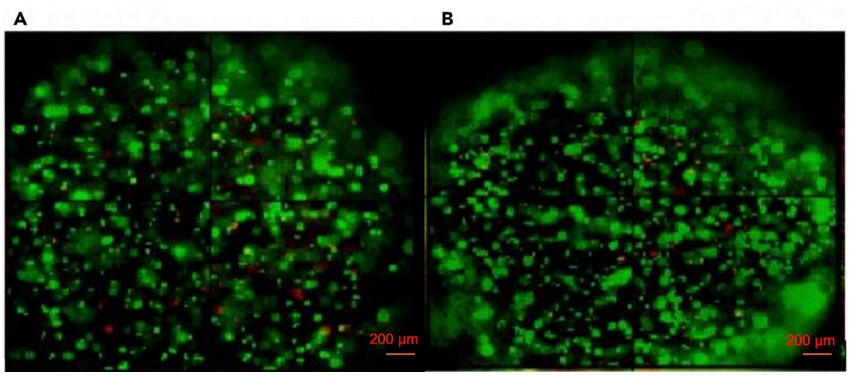


Figure 3. LIVE/DEAD viability assay of 3D-cultured cells
Calcein-AM indicates the intracellular esterase activity of live cells in an ER (+) and HER-2 (+) patient-derived breast cancer cell line embedded in one alginate bead at a concentration of A) 10,000 cells/bead, and B) 15,000 cells/bead on day 6 after bead making. Scale bars represent 200 μm.

38. Read the plates using the resazurin assay at the end of the time-point.

Note: To compare drug-response in 2D and 3D cultures, cells are also seeded in 96-well plates for 2D experiments. The end time-point for 2D experiments in this protocol is Day 4 when cells generally reach a proliferation rate of at least 4 times their Day 1 of seeding. To eliminate the effect of different proliferation rates in 2D vs 3D on the drug-response test, the treatment for 3D cultures was concluded at a time-point that cells reached a proliferation number (number of divisions) comparable to their 2D cultures on day 4. Given the slower growth rate of cells in 3D compared to 2D cultures, we needed to culture some cell lines in 3D at a higher density of cells and for a longer period of time. Culturing cells at higher densities also corrected the effect of LG culture media on the proliferation rate whenever cells could not grow at lower densities.

Culturing cells in alginate beads —> Starting treatment on day 1 while cells are embedded in alginate —> Measuring cells viability when their proliferation is comparable to 2D cultures on day 4

Radiation treatment: Day 1

39. Plate the beads in 96-well plates with 5–10 replicates for each condition.

Note: There will be one control plate and one plate per each radiation dosage.

40. Irradiate the plates, except for the control plate, on Day 1.
41. Read the control plate on Day 1 by using the resazurin assay.
42. Read all plates over time by using the resazurin assay.

Recovering cells from alginate beads for downstream experiments

Timing: 30 min

Note: For downstream experiments, beads are cultured in either 6-well plates or 100 mm tissue culture dishes. The culture media need to fully cover the beads in the plates.

43. Add 1:5 UltraPure™ 0.5M EDTA to the culture media containing beads and wait for 5 min in 20°C.
44. Aspirate the solution using a serological pipette and centrifuge it at 150 *g* for 5 min.
45. Aspirate the culture media and resuspend the cell pellet in PBS.
46. Centrifuge the cell suspension at 150 *g* for 5 min.
47. Aspirate PBS and use the cell pellet for downstream experiments.

Cell lysis for western blot assay

48. Label the tubes accordingly.
49. Add lysis buffer to the cell pellet recovered from alginate beads on ice for 20 min.
50. Centrifuge the lysate solution at 16,000 *g* for 15 min in 4°C.
51. Transfer the supernatant into a new tube and store at −80°C.

EXPECTED OUTCOMES

Several patient-derived breast, lung cancer and pancreatic ductal adenocarcinoma (PDAC) cell lines could grow in alginate beads using either HG or LG culture medium for more than one month. As expected, cells grew slower in LG culture media.

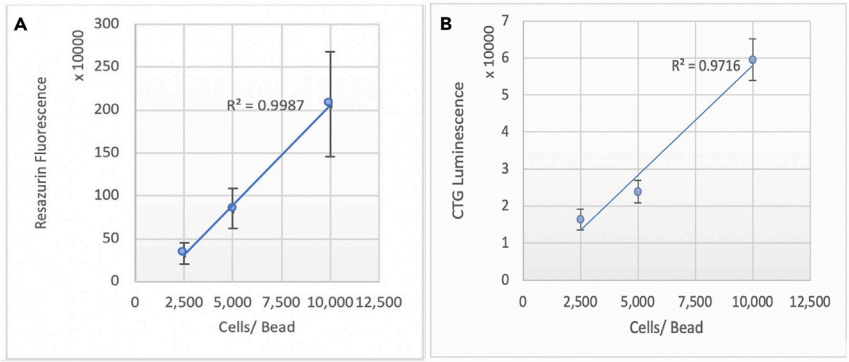


Figure 4. The linear correlation between embedded cell number and resazurin or CTG signal

(A) Resazurin fluorescence and the number of cells per each alginate bead in a KRAS-mutant patient-derived lung cancer cell line_ day 1 and B) Cell-TiterGlow (CTG) luminescence and the number of cells per each alginate bead in an ALK-rearranged patient-derived lung cancer cell line_ day 0. Data are presented as mean ± SD.

Multiple viability assays including CTG, resazurin and Live/Dead assays were compatible with this 3D system. Based on the staurosporine test, tight cell-cell interactions in 3D cultured cells did not hamper resazurin and CTG uptake and their subsequent reduction to either resorufin or luminescent signal (Figure 2D). We also observed that the measurement of cell viability performed using either resazurin or CTG assay yielded very similar results. Resazurin was mainly used in this protocol because it provides a non-cytotoxic assay and the stain could be easily washed and replaced with culture media. As a result, each alginate bead could be followed for cell proliferation over time. The number of the cells per each alginate bead was linearly correlated with both resazurin and CTG assays (Figure 4).

Based on visual observation and subsequent culture, there was little damage to the cells during recovery from alginate (Figure 5).

We also aimed to illustrate potential differences between the drug response in 2D vs 3D cultures and in the two different culture media. For this study, MGH 006-1, an anaplastic lymphoma kinase (ALK)-rearranged non-small cell lung cancer (NSCLC) patient-derived cell line with different response to the first generation of ALK inhibitors, crizotinib, in 2D cultures versus what was observed in the clinic and *in vivo* experiments was chosen.

The ALK rearranged NCI-H3122 cell line known to be highly sensitive to crizotinib *in vitro* (2D) and *in vivo* (sub-cutaneous xenograft in mouse) was chosen as the control cell line. This cell line displayed a

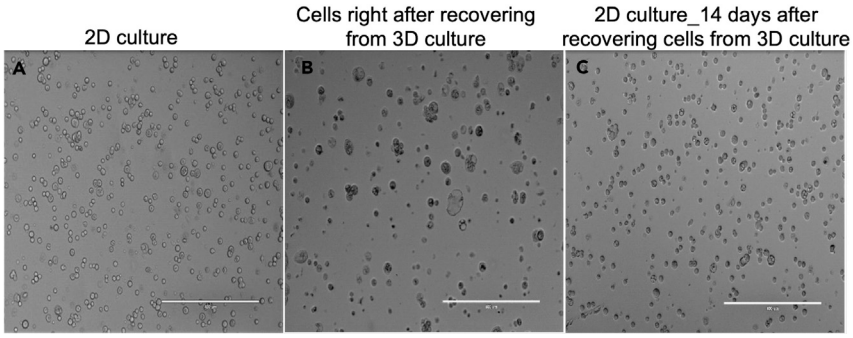


Figure 5. Morphology of 2D-cultured versus 3D-cultured cells

Morphology of an ER (+) and HER-2 (+) patient-derived breast cancer cell line in (A) 2D culture, (B) 3D culture_ Day 7, and (C) 2D culture after 14 days of recovering cells from 3D culture. Scale bars represent 400 μm.

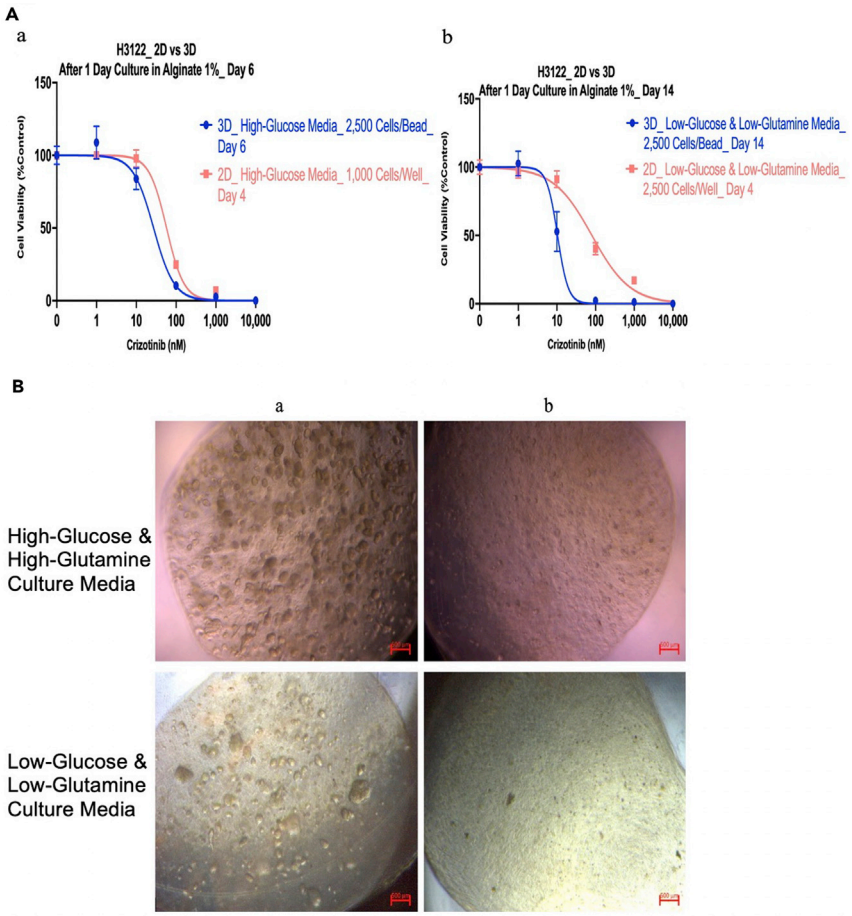


Figure 6. Crizotinib-sensitive H3122 cell-line

(A) Drug-response test in 2D vs 3D in a) in high-glucose & high-glutamine (HG) and b) in low-glucose & low-glutamine (LG) culture media and (B) Drug-response test in 3D in HG on day 6 and in LG culture media on day 14 in a) DMSO (control), and b) 100 nM crizotinib. Scale bars represent 500 μm.

proliferation rate in alginate beads comparable to its 2D cultures in HG and LG culture media after 6 and 14 days, respectively. The cellular density for 2D cultures were 1,000 cells and 2,500 cells per well of 96-well microplates for HG and LG culture media, respectively. The 3D cellular density was 2,500 cells/alginate bead for both culture media. As expected, this cell line was sensitive to crizotinib in both 2D and 3D cultures (Figures 6).

We found that contrary to 2D cultures, MGH 006-1 was sensitive to crizotinib in 3D cultures (Figure 7A). Similar sensitivity to crizotinib was observed in the patient from whom the cells were derived and also in *in vivo* experiments (Figure 7B). It was previously shown that the inhibition of ALK signaling in 2D cultures could result in an increase in SRC activity perhaps through a negative regulatory signal which normally coordinates ALK and SRC activities (Crystal et al., 2014). We further explored this by combination treatment with SRC inhibitor, dasatinib, and found this could overcome drug-resistance to crizotinib in 2D cultures of MGH 006-1 (Figure 7A; Table 1). No additional benefit over single agent crizotinib was observed in 3D.

Alginate beads allow for extensive time of culture without passage contrary to 2D cultures that need to be passaged once reaching confluence or before. To illustrate how extensive culture time before drug treatment can be achieved in the alginate cultures, we show an example of a treatment regimen following 14 days of culture in alginate:

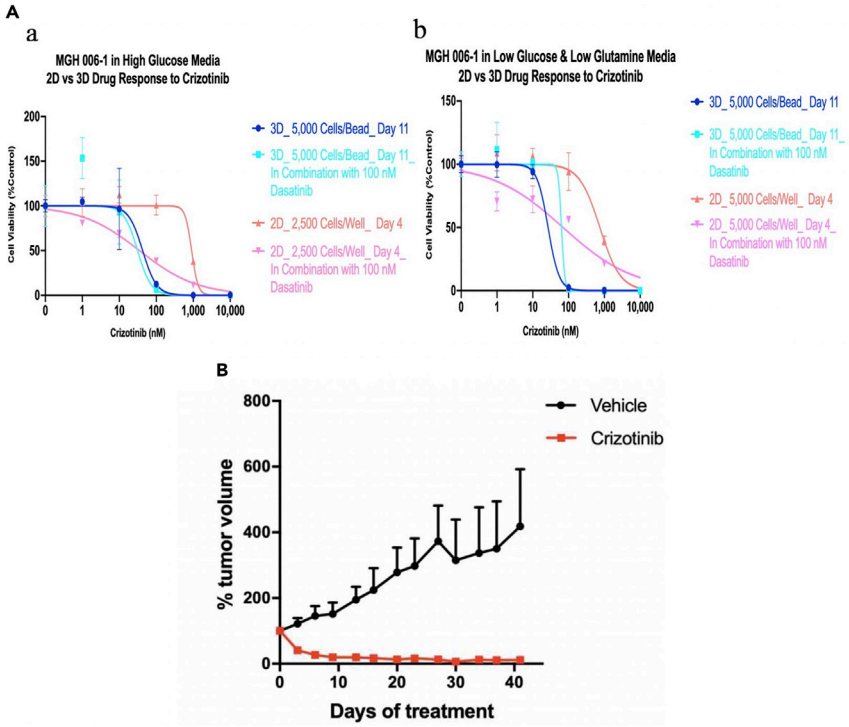


Figure 7. Drug-response test in the patient-derived cell line MGH 006-1 in 2D versus 3D cultures

MGH 006-1: the 2D crizotinib-resistant ALK-rearranged non-small cell lung cancer patient- derived cell-line showed sensitivity to crizotinib in 3D cultures similar to the *in vivo* experiments as well as the response in the patient (A) Drug-response test in 2D vs 3D in high-glucose & high-glutamine (HG) and in low-glucose & low-glutamine (LG) culture media, and (B) Subcutaneous xenografts showed sensitivity to crizotinib. Scale bars represent 500 μm.

Culturing cells in alginate beads —> Starting treatment on Day 14 while cells are embedded in alginate —> Measuring cells viability after 5 days

A similar response to what was observed in experiments without extensive pre-treatment culture was seen (Figure 8A). Consistent with a role of a SRC-FAK signaling modulating drug response in 2D (Crystal et al., 2014) but not in 3D, western blotting showed higher SRC activity (as indicated by paxillin phosphorylation status at tyrosin 118, which is known to be phosphorylated by SRC) in 2D versus 3D and inhibition by the SRC inhibitor, dasatinib in 2D cultures (Figure 8B).

Table 1. The SRC inhibitor Dasatinib overcomes drug resistance in 2D cultures

	2D high glucose media 75,000 cells/well day 4	2D low glucose & low glutamine media 150,000 cells/well day 4	3D high glucose media 150,000 cells/beads day 11	3D low glucose & low glutamine media 150,000 cells/beads day 11
Control	3.1	2.5	4.3	2.4
Crizotinib	1.7	2.1	1.1	0.9
Dasatinib	4.2	1.6	5.8	3.2
Crizotinib+ Dasatinib	0.9	0.3	1.0	1.0

MGH 006-1; the fold-change in the number of cells compared to the day of seeding/embedding in 2D versus 3D cultures in the presence of ALK and SRC inhibitors compared to DMSO control; to count the 3D-cultured cells, they were recovered from the alginate beads by using EDTA; Dasatinib overcomes drug-resistance to crizotinib in 2D cultures. No additional benefit over single agent crizotinib was observed in 3D cultures by adding dasatinib.

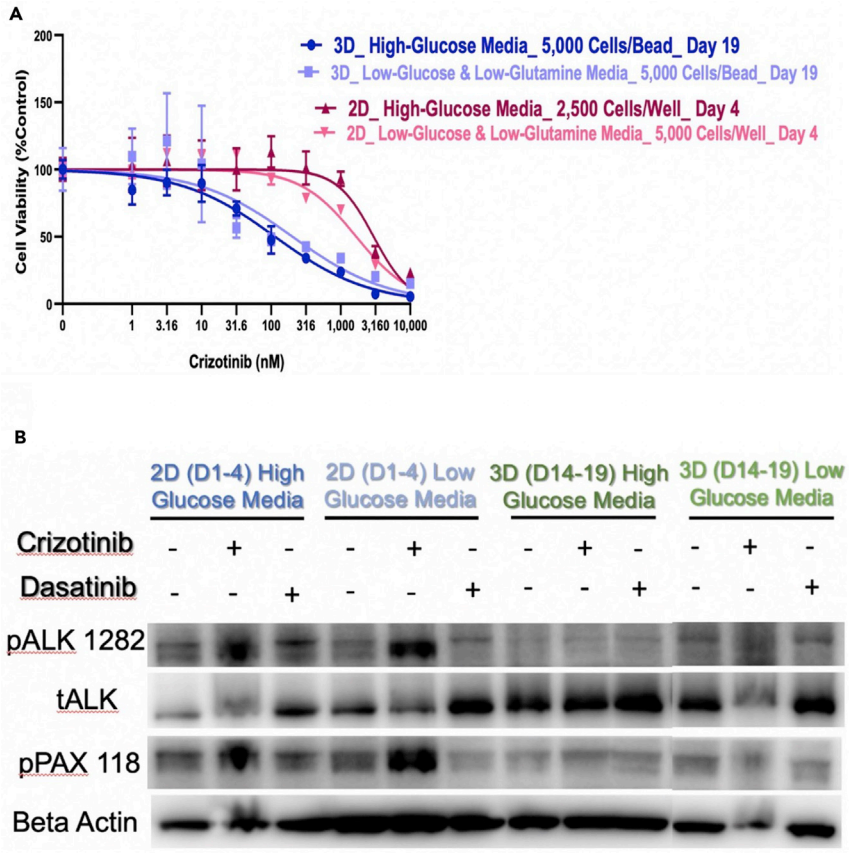


Figure 8. Pre-treatment 3D culture of MGH 006-1 for 14 days indicating similar response to crizotinib

MGH 006-1: the 2D-crizotinib resistant ALK-rearranged non-small cell lung cancer patient- derived cell-line showed sensitivity to crizotinib after 14 days of continuous culture in 3D (A) Drug-response test in 2D vs 3D in high- glucose & high-glutamine and in low-glucose & low-glutamine culture media, and (B) Increased SRC activity that as measured by phosphorylated paxillin was seen only in 2D cultures upon ALK inhibition. Data are presented as mean ± SD.

Throughout the experiments, we did not find any substantial difference in the drug-response test in LG versus HG culture media.

We further explored the drug-response test in both 2D and 3D cultures by using two other cancer cell lines; an additional ALK-rearranged patient-derived lung cancer cell line (MGH 049-1) and a KRAS- mutant PDAC (PDAC 7110) cell line. Similarly to the results obtained with MGH006-1 cell line, higher sensitivity to anti-tumor agents was seen in 3D compared to 2D cultures (Figure 9).

We also explored potential utility of the alginate based cultures to study cellular sensitivity to ionizing radiation. Previous data have shown that the KRAS-mutant NCI-H1703 cell line is more radioresistant compared to the isogenic wild-type cell line *in vitro* and *in vivo* cultures (Wang et al., 2017; Gurtner et al., 2020). This is in keeping with clinical observations that KRAS mutant tumors are relatively resistant to radiation therapy. Irradiation of alginate-based 3D cultures yielded the expected differential sensitivity to radiation with KRAS mutant cultures showing higher cell survival than KRAS wild-type cultures (Wang et al., 2017) (Figure 10).

LIMITATIONS

In this protocol, resazurin sodium salt, which is non-cytotoxic, was used for the proliferation assay to enable us to monitor the proliferation and metabolic activity of the encapsulated cells within a bead

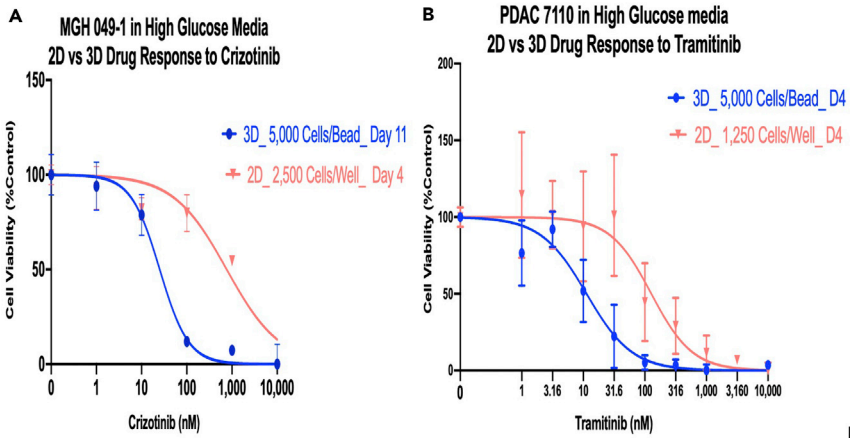


Figure 9. Drug-response test in 2D versus 3D cultures of two patient-derived cancer cell lines

(A) MGH 049-1 ALK-rearranged non-small cell lung cancer patient-derived cell-line and (B) PDAC 7110 KRAS-mutant pancreatic ductal adenocarcinoma cell lines demonstrate more sensitivity to anti-tumor molecular therapeutics in 3D cultures compared to 2D cultures. Data are presented as mean ± SD.

over time. However, one of the limitations of this assay is that at lower concentration of the cells the background signal is too high to detect viable cells. To overcome this limitation, several beads can be combined in a single measurement well or alternatively, a higher concentration of cells can be used at the time of bead fabrication.

TROUBLESHOOTING

Problem

Contamination of culture: The alginate sodium powder from Sigma is not sterile, which can be a source of contamination.

Potential solution

To overcome the risk of contamination the two culture media are supplemented with antibiotic- anti-mycotic. Also, alginate beads can be washed with culture medium containing antibiotic-antimycotic once for 10–15 min right after the crosslinking step.

Problem

Beads are not forming during the initial dispense of alginate into the calcium chloride solution.

Potential solution

To reduce the initial bead formation failure rate, use a vacuum filtration bottle containing a magnetic stir bar placed on a magnetic hot plate stirrer (35ºC and 200 RPM) instead of a 10 cm TC plate. We have also observed that priming the calcium solution with a 1/100 volume of alginate solution immediately before initiating bead formation can minimize the initial bead formation failure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Farideh Davoudi (fdavoudi@mgh.harvard.edu, fdavoudi@partners.org).

Materials availability

This study did not generate new unique reagents.

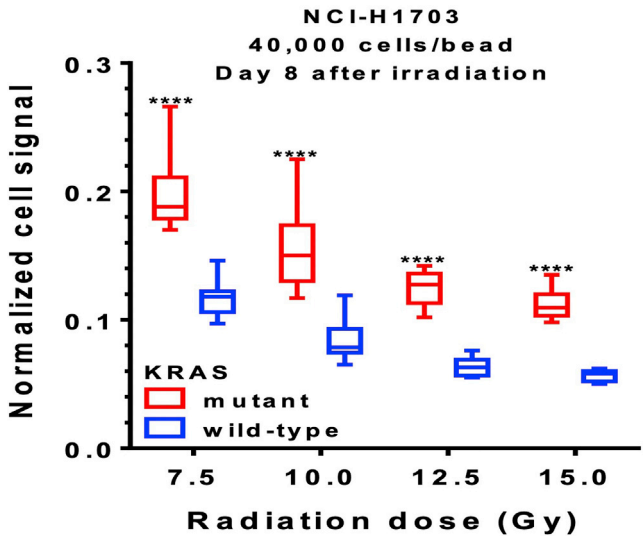


Figure 10. KRAS-mutant NCI-H1703 cell line is more radioresistant than the KRAS-wild-type isogenic line; 3D-cultured H1703 cells underwent irradiation on day 1 of culture

Data represent cells response to different doses of irradiation after 8 days normalized to control beads on day 1. Data are presented as median [IQR].

Data and code availability

This study did not generate/analyze datasets/code.

ACKNOWLEDGMENTS

This work was supported by NIH grant U01-CA220714 (H.W. and C.H.B.) and in part by a sponsored research agreement from Novartis (C.H.B.).

AUTHOR CONTRIBUTIONS

Conceptualization, F.D. and C.H.B.; investigation, F.D., C.H.B., H.W., A.N.H., S.G., S.Y., X.P., G.T.S., X.Y., and E.M.; writing – original draft, F.D. and S.G.; writing – review & editing, F.D. and C.H.B.; funding acquisition, C.H.B. and H.W.; supervision, F.D., C.H.B., and H.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Crystal, A.S., Shaw, A.T., Sequist, L.V., Friboulet, L., Niederst, M.J., Lockerman, E.L., Frias, R.L., Gainor, J.F., Amzallag, A., Greninger, P., et al. (2014). Patient-derived models of acquired resistance can identify effective drug combinations for cancer. Science *346*, 1480–1486.

Duval, E., Bigot, N., Hervieu, M., Kou, I., Leclercq, S., Galéra, Ph., Boumediene, K., and Baugé, C. (2011). Asporin expression is highly regulated in human chondrocytes. Mol. Med. *17*, 816–823.

Gurtner, K., Kryzmien, Z., Koi, L., Wang, M., Benes, C.H., Hering, S., Willers, H., Baumann, M., and Krause, M. (2020). Radioresistance of KRAS/TP53-mutated lung cancer can be overcome by radiation dose escalation or EGFR tyrosine kinase inhibition in vivo. Int. J. Cancer *147*, 472–477.

Lhuissier, E., Bazille, C., Aury-Landas, J., Girard, N., Pontin, J., Boittin, M., Boumediene, K., and Baug, C. (2017). Identification of an easy to use 3D culture model to investigate invasion and anticancer drug response in chondrosarcomas. BMC Cancer *17*, 490.

Lehmann, R., Gallert, C., Roddelkopf, T., Junginger, S., Wree, A., and Thurow, K. (2016). 3 dimensional cell cultures: a comparison between manually and automatically produced alginate beads. Cytotechnology *68*, 1049–1062.

Wang, M., Han, J., Marcar, L., Black, J., Liu, Q., Li, X., Nagulapalli, K., Sequist, L.V., Mak, R.H., Benes, C.H., et al. (2017). Radiation resistance in KRAS-mutated lung cancer is enabled by stem-like properties mediated by an osteopontin-EGFR pathway. Cancer Res. *77*, 2018–2028.

Liu, Y., Wang, Sh., Sun, D., Liu, Y., Liu, Y., Wang, Y., Liu, Ch., Wu, H., Lv, Y., Ren, Y., Guo, X., Sun, G., and Ma, X. (2016). Development of a biomimetic chondroitin sulfate-modified hydrogel to enhance the metastasis of tumor cells. Sci. Rep. *6*, 29858.