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## Protocol Article

# Fabrication, characterization, and implementation of engineered hydrogels for controlling breast cancer cell phenotype and dormancy

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## A B S T R A C T

A better understanding of how microenvironmental factors regulate cancer dormancy is needed for development of new therapeutic strategies to control metastatic recurrence and disease progression. Modeling cancer dormancy using engineered, *in vitro* platforms is necessary for investigation under well-defined and well-controlled microenvironments. We present methods and protocols to fabricate, characterize, and implement engineered hydrogels with well-defined biochemical and physical properties for control over breast cancer cell phenotype in three-dimensional (3D) culture. Changes in hydrogel adhesivity, crosslink density, and degradability induce a range of phenotypic behaviors in breast cancer cells including: (1) high growth, (2) moderate growth, (3) single cell, restricted survival dormancy, and (4) balanced dormancy. We describe a method of classifying hydrogel formulations that support each of these phenotypic states. We also describe a method to phenotypically switch cancer cells from single cell dormancy to high growth by dynamically modulating ligand density, thereby recapitulating reactivation and metastatic recurrence.

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## A R T I C L E I N F O

**Protocol name:** Methods to characterize hydrogel properties, Quantification of cellular metrics, Phenotypic classification of cancer cell states

**Keywords:** Biomaterials, Tissue engineering, Hydrogel, PEG, Cancer, Dormancy, Metastasis, Relapse, Microenvironment

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## Specification Table

Subject Area:	Engineering
More specific subject area:	Biomaterials, Tissue Engineering, Cancer
Protocol name:	Methods to characterize hydrogel properties, quantification of cellular metrics, and phenotypic classification of cancer cell states
Reagents/Tools:	Included in each section of the protocol
Experimental Design:	MDA-MB-231 metastatic breast cancer cells are cultured within PEG-based hydrogels with varying adhesivity and crosslink density. Encapsulated cells are assessed for viability, early apoptosis, proliferation, metabolic activity, and 3D morphology over 15 days. Quantified metrics are used to classify hydrogel formulations into groups supporting various cancer cell states. Matrix adhesivity is also dynamically modulated in long-term culture to switch encapsulated cancer cells from dormancy to metastatic relapse.
Trial Registration:	N/A
Ethics:	N/A

## Value of the Protocol

- The influence of matrix adhesivity and crosslink density on phenotypic cancer cell states is demonstrated.
- Systematic classification of hydrogel formulations based on phenotypic cellular metrics can be applied to study a wide range of 3D cancer cell behavior ranging across spontaneous apoptosis, single cell dormancy, balanced dormancy and metastatic growth.
- Dynamic increase in matrix adhesivity facilitates reactivation of dormant tumor cells into proliferative state and facilitates future investigation of metastatic relapse.

## Method details

### Introduction

A major challenge underlying treatment of metastatic cancer is the presence of dormant tumor cells in various organs; at the primary site and in secondary sites post dissemination [1,2]. These dormant cell populations are difficult to detect and treat owing to their low proliferation, quiescent nature, and high degree of resistance against standard chemotherapeutics. Investigating the molecular mechanisms underlying tumor dormancy is necessary to develop new therapeutic strategies to eliminate dormant populations in a targeted, specific manner. Unfortunately, *in vivo* models, owing to their physiological complexity and poor control of experimental conditions, are not optimal for investigating the dormancy phenomenon [3]. Hence, *in vitro* platforms with well-defined, user-controlled properties are useful for systematic investigation of microenvironmental factors that regulate dormancy in a reproducible, higher-throughput fashion.

The microenvironment of the primary tumor as well as secondary metastatic sites is an important regulator of the phenotypic plasticity exhibited by tumor cells and influences the switch between tumor dormancy and metastatic reactivation [4,5]. In particular, specific physical (stiffness, pore size) and biochemical (adhesivity, degradability) properties of the extracellular matrix (ECM) have been posited to influence the phenotypic states of cancer cells and thereby induce dormancy [3,6]. Hence systematic tuning of these properties in engineered synthetic matrices and adopting a materials-directed approach in controlling cancer cell states may provide an avenue for investigating tumor dormancy *in vitro*.

Toward this goal, we developed a set of sixteen, poly(ethylene glycol) (PEG)-based hydrogel formulations with well-defined physical and biochemical properties that can be used for encapsulation and three-dimensional (3D) culture of cancer cells for extended time periods (~75 days) that induce various phenotypic states, including tumor dormancy, via tuning of the hydrogel properties [7,8]. We present methods to fabricate these hydrogels, characterize their properties

(ligand density, elasticity, swelling, degradability, diffusivity, and pore size), evaluate cellular metrics (viability, proliferation, early apoptosis, metabolic activity, cell/cluster morphology) corresponding to various phenotypic states (high growth, moderate growth, single cell restricted survival dormancy, and balanced dormancy), classify hydrogel formulations based on the phenotype induced, and reactivate single, dormant tumor cells into a highly proliferative state.

### Hydrogel stock solution preparation

This protocol describes the methods for preparation of stock solutions that constitute the PEG-based hydrogel precursor.

#### Materials/equipment

- Phosphate-buffered saline (PBS, sterile)
- PEG-PQ-PEG macromer (PEG-PQ, 3.4 kDa) (For synthesis protocol, see Refs. [7,9])
- PEG-RGDS macromer (3.4 kDa) (For synthesis protocol, see Refs. [7,10,11])
- Lithium acyl phosphinate (LAP) (For synthesis protocol, see Refs. [11,12])
- N-vinyl pyrrolidinone (NVP, Sigma-Aldrich)

1 Prepare PEG-PQ stock in PBS (20 % w/v) (200 mg/mL or 10 mg/50  $\mu$ L).

2 Prepare PEG-RGDS stock in PBS (20 mM) (68 mg/mL or 6.8 mg/100  $\mu$ L).

3 Prepare LAP stock in PBS (30 mg/mL or 3 mg/100  $\mu$ L).

**Note:** LAP powder may need to be sonicated for 15–20 min and vortexed thoroughly for complete dissolution.

**Note:** Protect all solutions containing LAP from light using aluminum foil to prevent unwanted photopolymerization and store at 4 °C for subsequent use.

4 Prepare NVP stock in PBS (10  $\mu$ L/mL or 10  $\mu$ L + 990  $\mu$ L PBS).

5 Prepare final precursor solution with PEG-PQ = 5 % w/v, PEG-RGDS = 0, 1, 5, or 10 mM, LAP = 3 mg/mL and NVP = 0, 0.5, 1, or 2  $\mu$ L/mL and adjust with PBS (Table 1). Mix all components thoroughly to ensure uniform mixing.

### Cell preparation

This protocol describes the method of preparation of cancer cell suspension used subsequently for hydrogel encapsulation.

#### Materials/equipment

- MDA-MB-231 breast cancer cells cultured in tissue culture flasks coated with human fibronectin (10  $\mu$ g/mL, Sigma-Aldrich)
- Cell culture media (Dulbecco's Modified Eagle Medium (DMEM), Thermo Fisher + 10 % (v/v) fetal bovine serum (FBS), Thermo Fisher + 1 % (v/v) penicillin-streptomycin, Lonza)
- Serum-free media (DMEM + 1 % (v/v) penicillin-streptomycin)

**Table 1**

Composition of hydrogel formulations used for cell encapsulation.

Constant components	Variable components	
	[PEG-RGDS] (mM)	[NVP] ( $\mu$ L/mL)/(mM)
PEG-PQ: 5% w/v; LAP: 3 mg/mL	0	0.0/0.0
	1	0.5/4.7
	5	1.0/9.4
	10	2.0/18.7

- Trypsin (0.25 %, Lonza)
- Phosphate-buffered saline (PBS, sterile)

- 1 Replace cell culture media with serum-free media in tissue culture flask 48 h prior to cell encapsulation for synchronizing the cell cycle of MDA-MB-231 cells.
- 2 On the day of encapsulation, aspirate media from flask and rinse cells with PBS.
- 3 Trypsinize cells from flask and centrifuge cells.
- 4 Aspirate media to obtain cell pellet, resuspend cell pellet in 1 mL media.
- 5 Count cells in hemocytometer.

**Note:** Cell encapsulation can be performed in the range of  $1\text{--}50 \times 10^6$  cells/mL,  $10 \times 10^6$  cells/mL is recommended. At this density, for 100  $\mu\text{L}$  of polymer precursor,  $1 \times 10^6$  cells are needed.

### *Cell encapsulation in hydrogels*

This protocol describes the method for encapsulation of cancer cells within PEG-based hydrogels with well-defined composition via UV-initiated photocrosslinking and subsequent 3D culture.

#### *Materials/equipment*

- Cancer cell suspension in media prepared earlier
- Cell culture media (Dulbecco's Modified Eagle Medium (DMEM), Thermo Fisher + 10 % (v/v) fetal bovine serum (FBS), Thermo Fisher + 1 % (v/v) penicillin-streptomycin, Lonza)
- Hydrogel precursor solution prepared earlier
- Parafilm (sterilized with ethanol)
- Tweezers (sterilize with ethanol)
- Glass slide
- Well plate
- UV light source (Blak-Ray flood UV lamp, wavelength: 365 nm, intensity: 10 mW/cm<sup>2</sup>)

- 1 Pipette an equivalent volume of cell suspension containing  $1 \times 10^6$  cells and transfer into a clear microcentrifuge tube.
- 2 Centrifuge the cell suspension to obtain cell pellet.
- 3 Warm up the UV light lamp for 15 min prior to crosslinking. On a glass slide, stretch a strip of Parafilm for use as a hydrophobic surface.
- 4 From the cell pellet, carefully aspirate supernatant media and resuspend cell pellet in the polymer precursor solution.

**Note:** Try to remove all the media as remaining media can dilute the polymer precursor solution and inhibit crosslinking. Minimize the time that cells are in direct contact with the liquid polymer precursor, usually 4–5 min is long enough. Mix the cell pellet quickly and uniformly in the precursor solution.

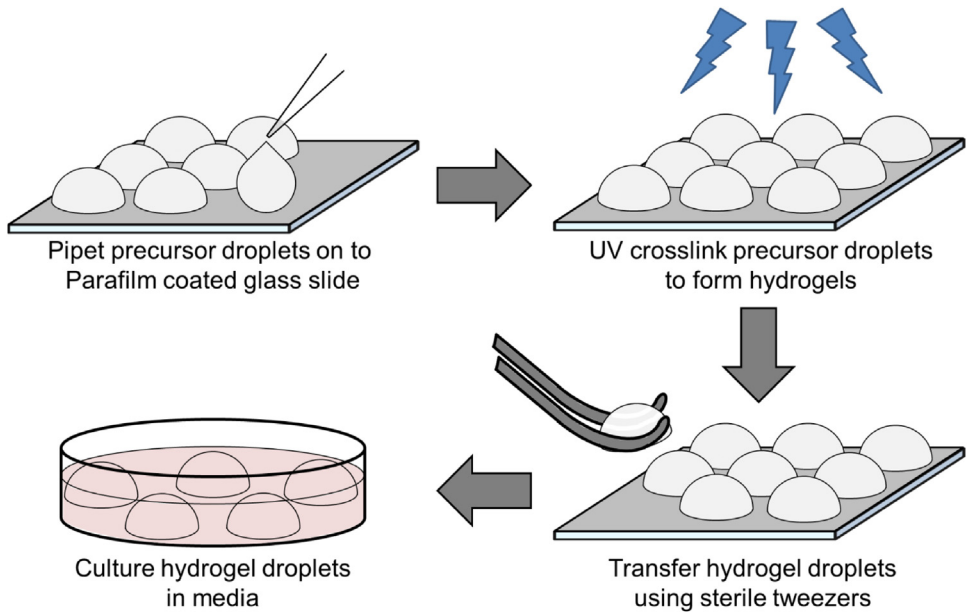
- 5 Pipette 3  $\mu\text{L}$  droplets of cell-laden precursor solution onto the hydrophobic Parafilm surface (~15–20 droplets) (See Fig. 1 Ref. [7]) and crosslink under the UV lamp (Blak-Ray flood UV lamp, wavelength: 365 nm, intensity: 10 mW/cm<sup>2</sup>, height of UV lamp from Parafilm surface: 8 in.) for 1 min (Fig. 1).

**Note:** Pipetting of hydrogel droplets must be completed as rapidly as possible to prevent cells from settling to the bottom of the droplets.

**Note:** Due to the fast polymerization kinetics of the LAP photoinitiator, the crosslinking time can be reduced to 30 s; however, 1 min crosslinking time is suggested to ensure maximum PEG-RGDS conjugation to the PEG-PQ matrix.

- 6 Fill 1 well of a 6-well plate with cell culture media. Using sterile tweezers, carefully pick up each hydrogel droplet and transfer to the media in the well plate.
- 7 Maintain cell-laden hydrogels in culture changing media every 4 days or as necessary.

**Note:** Cell-laden hydrogels can be maintained for at least 15 days in culture for quantification of cellular metrics.



**Fig. 1.** Schematic of hydrogel fabrication. A glass slide is covered with Parafilm and hydrogel precursor solution (containing cells) is pipetted as 3  $\mu\text{L}$  droplets on the hydrophobic surface. After 1 min UV exposure, crosslinked hydrogels are transferred with sterile tweezers into well plates containing media.

### Characterization of PEG-RGDS incorporation

This protocol describes a method to quantify the concentration of PEG-RGDS that is incorporated into the hydrogel during the photocrosslinking process. Particularly, the increase in incorporation efficiency of PEG-RGDS in the presence of the NVP co-monomer is demonstrated. PEG-RGDS is mixed with ratiometric concentration of fluorescently labeled PEG-RGDS-Alexa Fluor<sup>®</sup> 488 and the fluorescence images of the hydrogel precursor prior to crosslinking, immediately after crosslinking (to account for photobleaching), and after overnight rinsing (to remove unconjugated moieties) is acquired. Fluorescence intensities are quantified via FIJI to obtain the final incorporation efficiency.

#### Materials/equipment

- PEG-PQ macromer
- PEG-RGDS macromer
- PEG-RGDS-Alexa Fluor<sup>®</sup> 488 (PEG-RGDS-488) (For synthesis protocol, see Ref. [7])
- Lithium acyl phosphinate (LAP)
- N-vinyl pyrrolidinone (NVP)
- PBS
- Polydimethyl siloxane (PDMS) (SYLGARD<sup>™</sup> 184 Silicone Elastomer Kit, Dow)
- Spacers/shims (thickness: 500  $\mu\text{m}$ )
- Biopsy punch (diameter: 3 mm)
- Glass slides
- Well plate
- Glass coverslips
- UV lamp
- Zeiss AxioObserver Z1 inverted fluorescent microscope equipped with Zeiss AxioCam MRm camera

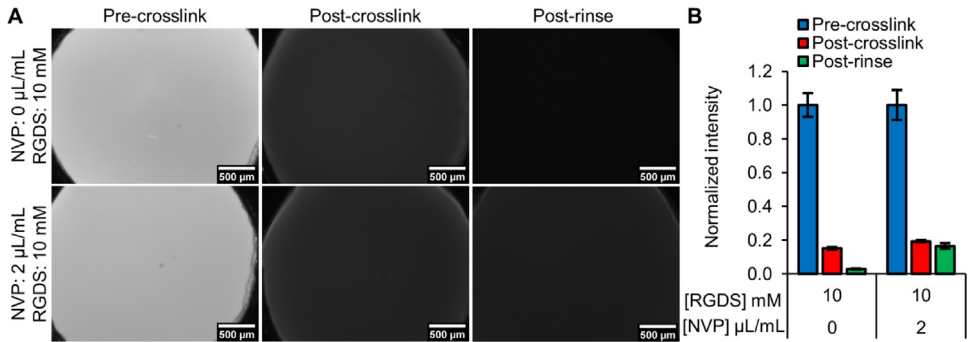
- 1 Mix PDMS base elastomer and curing agent in the ratio of 10:1 on a weighing scale and degasify using a vacuum chamber.
- 2 Cure a thin (500  $\mu\text{m}$ ) sheet of PDMS at 70 °C between two clean glass slides fitted with 500  $\mu\text{m}$  thick shims as spacers.
- 3 Punch 3 mm diameter holes in the PDMS sheet using the biopsy punch to make PDMS molds.
- 4 Prepare PEG-PQ, PEG-RGDS, NVP and LAP stock in PBS as described in the previous section.
- 5 Prepare PEG-RGDS-488 stock in PBS (10 mM) (68 mg/mL or 6.8 mg/100  $\mu\text{L}$ ).
- 6 For preparation of the final solution, the total RGDS concentration (PEG-RGDS + PEG-RGDS-488) should be kept constant at 1, 5, or 10 mM. The final concentration of PEG-RGDS-488 should be constant at 0.5 mM; adjust the PEG-RGDS concentration accordingly to 0.5, 4.5, or 9.5 mM.
- 7 Prepare final precursor with PEG-PQ = 5 % w/v, PEG-RGDS = 1, 5, or 10 mM, LAP = 3 mg/mL and NVP = 0, 0.5, 1, or 2  $\mu\text{L}/\text{mL}$  and adjust with PBS. Mix all components thoroughly.
- 8 Fix PDMS molds on glass a coverslip and pipet 3  $\mu\text{L}$  of precursor solution into the molds.
- 9 On the microscope and camera, pre-set exposure time (100 ms) and excitation intensity (20 mW/ $\text{cm}^2$ ) settings. Set the coverslip with precursor solution on the microscope stage and image precursor solution using a GFP filter cube (excitation: 450–490 nm, emission: 500–550 nm).  
**Note:** Keep intensity and exposure settings constant throughout the entire experiment.  
**Note:** Acquire images 200  $\mu\text{m}$  from the bottom of the coverslip to ensure imaging at the same z-location in all samples.  
**Note:** Acquisition settings will vary between microscopes. Adjust the excitation intensity and acquisition time as needed.
- 10 Remove the coverslip from the stage and photocrosslink the polymer precursor solution under the UV lamp for 1 min.
- 11 Without removing the PDMS mold, re-image the crosslinked hydrogel again using the same acquisition settings.  
**Note:** Comparing fluorescence intensities of the images from the un-crosslinked precursor solution and immediately post-crosslinking will provide the ability to quantify the amount of photobleaching and to correct for it.
- 12 Remove the PDMS mold, transfer the hydrogels to well plates with PBS buffer and incubate overnight at room temperature to remove un-crosslinked moieties.
- 13 Transfer the hydrogels to coverslips, add a few drops of PBS, and re-image using the same microscope settings.  
**Note:** Comparing the fluorescence intensities of images immediately post-crosslinking and after overnight rinsing will give the relative percentage of RGDS conjugated in the final hydrogels.
- 14 Analyze images using Fiji software (NIH, Version 1.52h) to quantify fluorescence intensities (Fig. 2). Quantify background fluorescence of a representative non-gel area and representative gel area of the image. Subtract background intensity from the gel fluorescence intensity to obtain true fluorescence intensity. Normalize intensity values based on the values obtained prior to crosslinking.  
**Note:** Use the following method and Fiji functions to analyze fluorescence intensity:  
Analyze > Set Measurements > (Check box for Mean Gray Value)  
Analyze > Measure

**The** following assumptions are made to quantify the conjugation efficiency using this approach:

- 1 PEG-RGDS and PEG-RGDS-488 have the same incorporation efficiency (no competitive behavior).
- 2 The molecular weights of PEG-RGDS and PEG-RGDS-488 are similar (based on molar ratios used during synthesis).
- 3 Overnight rinsing ensures complete removal of unconjugated moieties.

The following equations are used to quantify the concentration of conjugated PEG-RGDS in the hydrogels:

$$\text{Relative bleaching (B)} = \frac{I_1}{I_0} \quad (1)$$



**Fig. 2.** Quantifying PEG-RGDS incorporation into hydrogels. (A) Representative fluorescent images of the hydrogel precursor solution before crosslinking (left column), post-crosslinking (middle column), and post-rinsing (right column) for two different hydrogel formulations (0 NVP and 10 mM PEG-RGDS (top row); 2 μL/mL NVP and 10 mM PEG-RGDS (bottom row)). SB = 500 μm. (B) Measured fluorescence intensities of the hydrogels normalized to the pre-crosslink values demonstrate a decrease in intensity due to photobleaching and rinsing of unconjugated moieties. Eqs. (1)–(3) are used to quantify the incorporation efficiency and incorporated PEG-RGDS concentration from the measured intensities.

where  $I_0$  is the fluorescence intensity prior to crosslinking and  $I_1$  is the fluorescence intensity immediately after crosslinking which accounts for bleaching.

$$\text{Relative conjugation (C)} = \frac{I_2}{I_1} \quad (2)$$

where  $I_2$  is the fluorescence intensity after rinsing and removal of unconjugated moieties.

$$\text{Conjugated PEG – RGDS concentration ([RGDS])} = C \times R \quad (3)$$

where  $R$  is the initial PEG-RGDS concentration in the prepolymer solution ( $R = 1, 5$  or  $10$  mM PEG-RGDS).

### Characterization of hydrogel elasticity

This protocol describes a method to quantify the compressive modulus of hydrogels, in particular, the changes in compressive modulus resulting from increased NVP co-monomer concentration (increased crosslink density) in PEG-PQ hydrogels. Of note in this assay is the fact that, incorporation of PEG-RGDS was not deemed necessary in the hydrogel formulation as the presence of cell-adhesive ligands has been demonstrated to have negligible effect on the bulk modulus of the hydrogels [13].

### Materials/equipment

- MDA-MB-231 breast cancer cells
- PEG-PQ macromer
- PEG-RGDS macromer
- Lithium acyl phosphinate (LAP)
- N-vinyl pyrrolidinone (NVP)
- PBS
- Tweezers
- Vernier calipers
- Glass slides
- Well plate
- PDMS molds (diameter: 3 mm, thickness: 1 mm)
- UV lamp
- Universal Testing System 3340 Series (Instron)

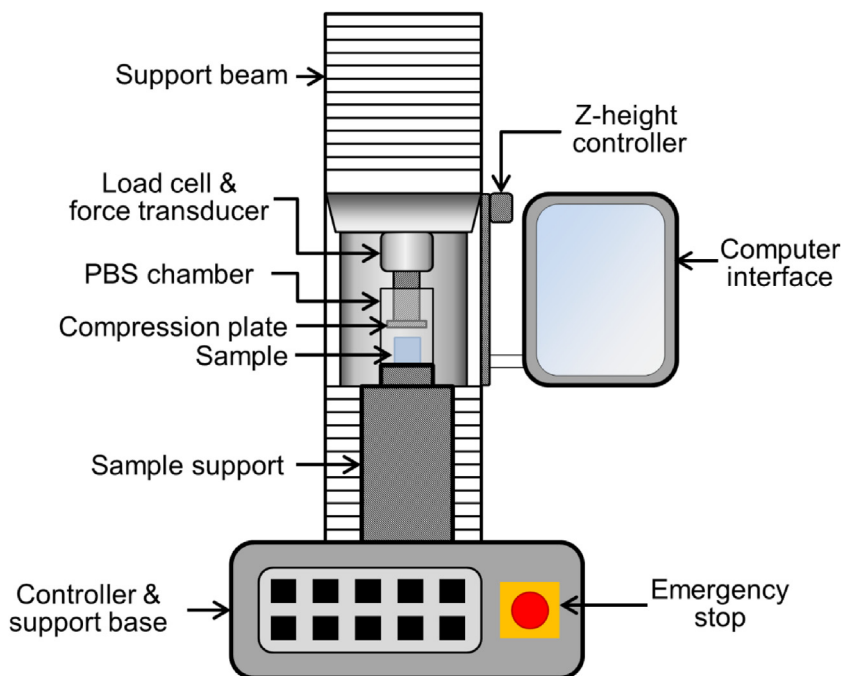
- 1 Prepare PDMS containing 3 mm diameter holes and a thickness of 1 mm as per previous instructions.
- 2 Prepare the precursor solution with PEG-PQ = 5 % w/v, PEG-RGDS = 1 mM, LAP = 3 mg/mL and NVP = 0, 0.5, 1, or 2  $\mu\text{L}/\text{mL}$  in PBS.
 

**Note:** PEG-RGDS is necessary only when encapsulating cells. When making acellular gels, PEG-RGDS can be omitted if desired.
- 3 Pipet 15  $\mu\text{L}$  of precursor solution or precursor with MDA-MB-231 ( $10^6$  cells/mL) into PDMS molds affixed on a glass slide.
- 4 Photocrosslink under the UV lamp for 1 min.
- 5 Transfer acellular hydrogels to a well-plate with PBS and incubate overnight to achieve equilibrium swelling. If encapsulating cells, transfer hydrogels to a well plate with cell culture media and incubate for 1–15 days as desired.
 

**Note:** Acellular hydrogels can be tested on day 1 post-crosslinking. Cell-laden hydrogels can be tested either on day 1 to characterize the initial elasticity or at some later time point (day 15 for example) to characterize elasticity after cell-mediated hydrogel degradation.
- 6 On the Instron system, set up the unconfined compression system with platens and a liquid bath chamber filled with warm PBS (Fig. 3).
 

**Note:** Use a 10 N load cell with a compression rate of 2  $\mu\text{m}/\text{s}$  for 100 s with an initial load of 0.02 N to ensure uniform contact between the hydrogel and compression platens.
- 7 Measure the hydrogel thickness and diameter prior to loading and hydrogel diameter at the end of the compression cycle using calipers.
- 8 Load hydrogel samples between parallel plates and proceed with compression testing to obtain force vs. displacement measurements.
 

**Note:** Avoid cracking or fracturing of the hydrogel under compression. If necessary, reduce the compression rate or maximum compression value.



**Fig. 3.** Schematic of mechanical testing system. An Instron compression testing system is used to measure compressive moduli of hydrogels. The hydrogel sample is placed in between parallel plates surrounded by a PBS chamber and undergoes unconfined compression as controlled by the load cell and z-height controller.



9 Obtain stress vs. strain values and calculate the slope of a linear fit (within the first 20 % of the strain compression) to determine the compressive modulus (Fig. 4).

**Note:** The Instron setup generates force (N) vs. displacement (mm) data that is used for calculating stress and strain for each compression step. Calculate area of compression from the hydrogel diameter. The area should increase with linear increase in diameter during compression. Divide force values with area values to obtain stress values for each compression step.

$$\text{Stress (Pa)} = \frac{\text{Force (N)}}{\text{Area (m}^2\text{)}} \quad (4)$$

Divide the displacement values by initial hydrogel thickness for each compression step to obtain strain values.

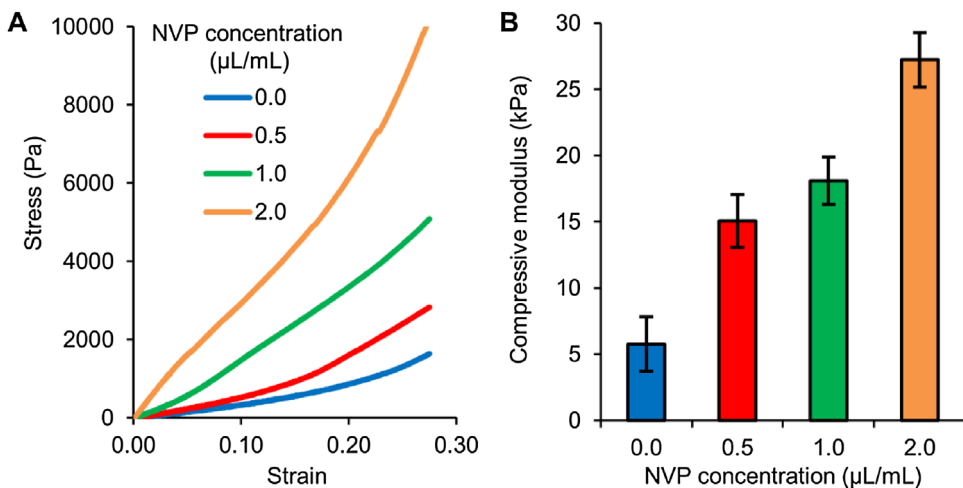
$$\text{Strain} = \frac{\text{Displacement (mm)}}{\text{Hydrogel thickness (mm)}} \quad (5)$$

### Characterization of hydrogel swelling

This protocol describes a method to quantify the swelling ratio of hydrogels, especially, the reduction in swelling ratio with increased NVP concentration (increased crosslink density).

### Materials/equipment

- PEG-PQ macromer
- Lithium acyl phosphinate (LAP)
- N-vinyl pyrrolidinone (NVP)
- PBS
- Tweezers
- Glass slides
- Well plate
- PDMS molds (diameter: 3 mm, thickness: 1 mm)
- UV lamp
- Precision weighing balance (Mettler-Toledo)



**Fig. 4.** Hydrogel elasticity measurements. (A) Representative stress-strain curves and (B) compressive modulus of PEG-PQ hydrogels with varying concentrations of NVP.

- 1 Prepare the polymer precursor solution with PEG-PQ = 5 % w/v, LAP = 3 mg/mL, and NVP = 0, 0.5, 1, or 2  $\mu\text{L}/\text{mL}$  in PBS.
- 2 Pipet 15  $\mu\text{L}$  of precursor solution into PDMS molds affixed on a glass slide and photocrosslink under UV lamp for 1 min.
- 3 Transfer hydrogels to a well-plate with PBS and incubate overnight to achieve equilibrium swelling.
- 4 Weigh swollen hydrogels on a carefully tared balance.
 

**Note:** Wick off excess PBS from hydrogels using a Kimwipe prior to weighing.
- 5 Allow the hydrogels to dry in ambient air for 6 h.
- 6 Weigh the dried hydrogels.
- 7 Calculate the swelling ratio using the equation below (Fig. 5):

$$\text{Swelling ratio} = \frac{\text{Swollen Weight} - \text{Dry Weight}}{\text{Dry Weight}} \quad (6)$$

#### Characterization of hydrogel degradation

This protocol describes a method to quantify changes in the hydrogel degradability, especially, due to incorporation of the non-degradable NVP co-monomer in the hydrogel. Methacryloxyethyl thiocarbonyl rhodamine B (containing free acrylate end groups) is conjugated to the PEG-PQ hydrogel matrix at low concentrations, thereby fluorescently labeling the hydrogels. The reduction in fluorescence intensity of hydrogels under the action of collagenase IV is monitored over time to quantify the rate of degradation of hydrogels.

In particular, this protocol helps quantify the inherent degradability of hydrogel matrices under the action of a digesting enzyme and not the ability of cells to degrade the hydrogel matrix. Encapsulated cells may have varying MMP profiles and secretion rates depending on matrix conditions, which would make it difficult to account for cell-induced matrix degradation.

#### Materials/equipment

- PEG-PQ macromer
- Lithium acyl phosphinate (LAP)
- N-vinyl pyrrolidinone (NVP)
- PBS

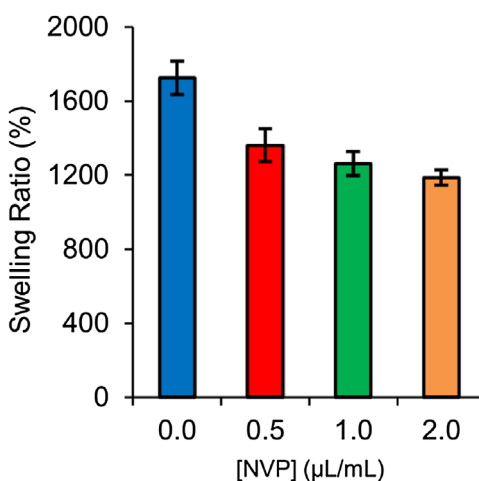


Fig. 5. Hydrogel swelling ratio. Variation in the swelling ratio of PEG-PQ hydrogels with varying concentrations of NVP.

- Deionized (DI) water
- Collagenase IV (Worthington)
- Methacryloxyethyl thiocarbonyl rhodamine B (PolyFluor<sup>®</sup> 570, Polysciences)
- PDMS molds (diameter: 3 mm, thickness: 500  $\mu\text{m}$ )
- 0.22  $\mu\text{m}$  syringe filter (polyethersulfone, Millex-GP)
- UV lamp
- Zeiss AxioObserver Z1 inverted fluorescent microscope equipped with Zeiss AxioCam MRm - camera and/or heated incubation stage
- 37 °C Incubator
- Water bath

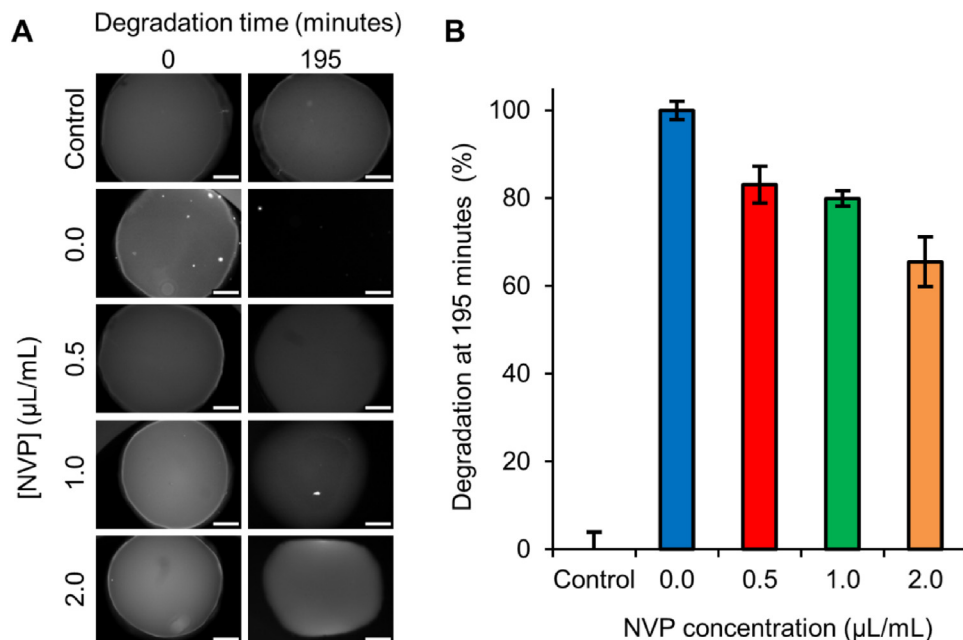
- 1 Prepare a stock solution of rhodamine B in PBS. Dissolve 2 mg of powder in 1 mL of DI water with vigorous vortexing and/or sonication. Filter the solution through a 0.22  $\mu\text{m}$  filter to remove undissolved particulates. Mix the resulting solution with PBS in a ratio of PBS:rhodamine B = 80:20 to obtain the working solution.  
**Note:** Dissolution of the rhodamine B in DI water takes  $\sim$ 30 min. Complete dissolution may not be achieved. Double filtration may be necessary to remove particulates.
- 2 Prepare the polymer precursor solution with PEG-PQ = 5 % w/v, LAP = 3 mg/mL and NVP = 0, 0.5, 1, or 2  $\mu\text{L}/\text{mL}$  in the above buffer.
- 3 Pipet 3  $\mu\text{L}$  of precursor into PDMS molds affixed on glass coverslips and photocrosslink under UV lamp for 1 min.
- 4 Allow the hydrogels to swell overnight in PBS at room temperature.
- 5 Prepare collagenase IV solution in PBS to a final concentration of 100  $\mu\text{g}/\text{mL}$  and warm to 37 °C in a water bath.
- 6 Transfer the swollen hydrogels to 96 well plates with PBS (1 hydrogel/well) and incubate at 37 °C for 30 min.
- 7 Image hydrogels using rhodamine filter cube (excitation: 538–562 nm, emission: 570–640 nm) with an excitation intensity of 27 mW/cm<sup>2</sup> and acquisition time of 100 ms.  
**Note:** Acquisition settings will vary between microscopes. Adjust the excitation intensity and acquisition time as needed.
- 8 Replace the PBS buffer with warm collagenase solution.
- 9 Acquire fluorescence images every 15 min.  
**Note:** Make sure that collagenase buffer stays warm; either use a heated incubation stage or return to 37 °C incubator between imaging cycles.  
**Note:** Include a control group: PEG-PQ hydrogel with only PBS buffer (no collagenase) to serve as reference to account for any photobleaching that may occur during image acquisition.
- 10 Analyze the images using FIJI software to quantify fluorescence intensities (Fig. 6).

### *Characterization of hydrogel mesh size*

This protocol describes a method to quantify changes in the hydrogel mesh size, especially, due to incorporation of additional NVP (leading to additional crosslink density and reduced porosity) in the hydrogel matrix.

### *Materials/equipment*

- PEG-PQ macromer
- Lithium acyl phosphinate (LAP)
- N-vinyl pyrrolidinone (NVP)
- PBS
- Deionized (DI) water
- Glass slides
- 0.22  $\mu\text{m}$  syringe filter (polyethersulfone, Millex-GP)
- 96-well plates



**Fig. 6.** Hydrogel degradation. (A) Representative fluorescent images of PEG-PQ hydrogels labeled with methacryloxyethyl thiocarbonyl rhodamine B and photopolymerized with varying NVP concentrations exposed to collagenase IV over time. SB = 500  $\mu\text{m}$ . Control refers to a PEG-PQ hydrogel in PBS buffer without any collagenase IV. (B) Corresponding percent degradation of hydrogels with varying NVP concentration as measured at 195 min post exposure to collagenase IV.

- PDMS molds (diameter: 3 mm, thickness: 500  $\mu\text{m}$ )
- Fluorescein isothiocyanate (FITC)-dextran (Molecular weight: 150 kDa, Sigma-Aldrich)
- Plate reader (Biotek Synergy)
- UV lamp

- 1 Prepare the polymer precursor solution with PEG-PQ = 5 % w/v, LAP = 3 mg/mL and NVP = 0, 0.5, 1, or 2  $\mu\text{L/mL}$  in PBS.
- 2 Pipet 3  $\mu\text{L}$  of precursor solution into PDMS molds affixed on a glass slide and photocrosslink under the UV lamp for 1 min.
- 3 Allow the hydrogels to swell overnight in PBS at room temperature.
- 4 Dissolve FITC-dextran in DI water at 1 mg/mL. Filter the solution through a 0.22  $\mu\text{m}$  filter to remove particulates.
- 5 Remove PBS and add FITC-dextran to the hydrogels; incubate at 4  $^{\circ}\text{C}$  for 48 h.
- 6 Post-incubation, transfer the dextran-swollen hydrogels into a 96-well plate and blot off excess dextran with a Kimwipe.
- 7 Add 200  $\mu\text{L}$  of DI water to each well and collect 50  $\mu\text{L}$  of solution from the well every 15 min and pipet it into a new 96-well plate.
- 8 Analyze the fluorescence intensity of the collected solutions in the new well plate (Excitation: 490 nm, Emission: 525 nm) using a plate reader.
- 9 Add 50  $\mu\text{L}$  of DI water to each hydrogel sample after each collection time point to compensate for the collected solution. Vortex well plate momentarily to homogeneously distribute diffused dextran within each well plate.
- 10 Continue collection for  $\sim$ 4 h or until no significant change in fluorescence intensity is observed.
- 11 Tabulate the quantified fluorescence intensities and subtract background fluorescence intensity measured at the 0 min time point to obtain corrected average intensities for each time point.

12 For each hydrogel condition, calculate the total intensity value obtained through all time points. This is designated as  $M_\infty$  (representative of the cumulative mass of released FITC-dextran). Also for each time point, calculate the cumulative intensity released till that time point. This is designated as  $M_t$  (representative of the mass of released FITC-dextran till time  $t$ ).

13 For each time point, calculate  $M_t/M_\infty$  and  $[\log \text{normal}(1 - M_t/M_\infty)]$ . Plot  $[\log \text{normal}(1 - M_t/M_\infty)]$  vs. time and estimate the slope from the linear fit.

**Note:** With reference to Eq. (7), the slope of the linear fit corresponds to  $\left[-\frac{4\delta^2}{D\pi^2}\right]$  and the intercept corresponds to  $\left[-\frac{4\delta^2}{D\pi^2}\ln\frac{\pi^2}{8}\right]$

14 Calculate the value of  $D$ , the effective diffusion coefficient, from the slope.

15 Calculate the mesh size (average pore diameter) of the hydrogel matrix using Eq. (6) and the value of  $D$  obtained above.

**Note:** The Solver function in MS Excel can be used to solve Eq. (8).

The cumulative mass of FITC-dextran released is analyzed from the measured fluorescence intensities and used to calculate the diffusion coefficient of FITC-dextran in the hydrogel according to the equation below [14]:

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2} \exp\left[\frac{-D\pi^2 t}{4\delta^2}\right] \quad (7)$$

where  $M_t$  is the mass of released FITC-dextran at time  $t$ ,  $M_\infty$  is the cumulative mass of FITC-dextran,  $M_t/M_\infty$  is the fractional mass of FITC-dextran released,  $D$  is the effective diffusion coefficient, and  $2\delta$  is the hydrogel thickness (500  $\mu\text{m}$ ).

The theoretical mesh size of the hydrogels is estimated using the hindered solute diffusion in solvent-filled-pores model based on the following equation (Fig. 7):

$$\frac{D}{D_0} = (1 - \lambda^2) \left(1 - 2.1044\lambda + 2.089\lambda^3 - 0.948\lambda^5\right) \quad (8)$$

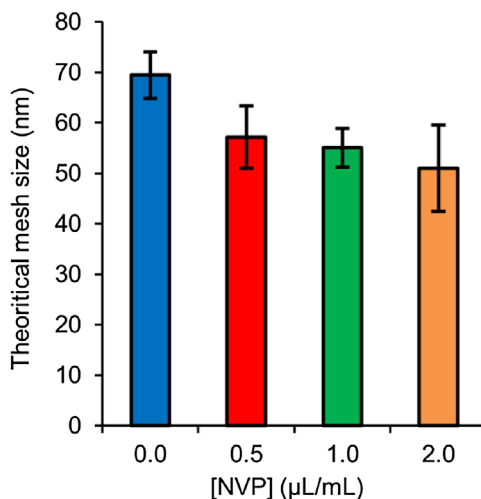
where  $D$  is the effective diffusion coefficient of 150 kDa FITC-dextran in hydrogels,  $D_0$  is the diffusion coefficient of 150 kDa FITC-dextran calculated from the Stokes–Einstein's equation ( $2.09 \times 10^{-7} \text{ cm}^2/\text{s}$ ), and  $\lambda$  is a characteristic ratio of the FITC-dextran hydrodynamic diameter to the average pore diameter of the hydrogel matrix. The Stokes' radii and hydrodynamic diameter of 150 kDa dextran was estimated to be 8.5 nm and 17 nm respectively, as provided by the manufacturer.

### Quantification of cell viability

This protocol describes an assay to quantify cell viability and viable cell density within PEG-based hydrogels post 3D encapsulation using a standard cell viability kit and image analysis. Changes in these metrics over time form the basis for subsequent classification of cancer cell phenotype within engineered hydrogel formulations.

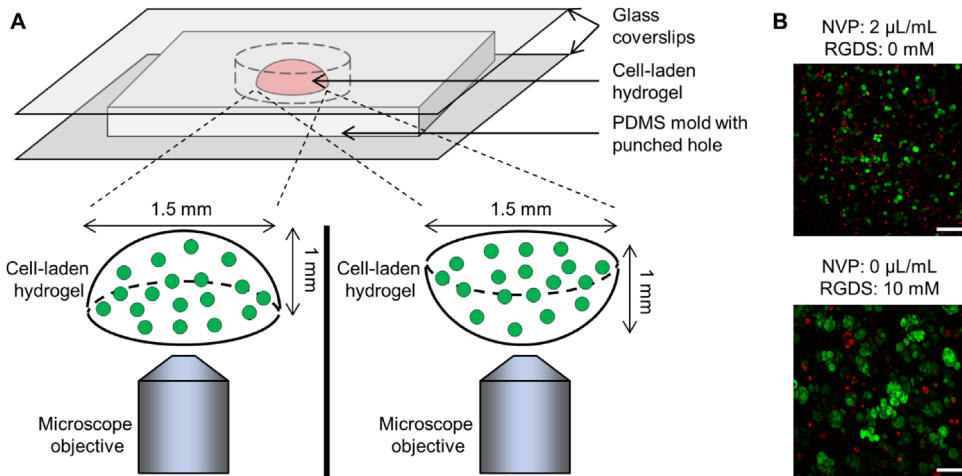
### Materials/equipment

- Cell-encapsulated hydrogels
- Warm PBS (sterile)
- Live/dead cell viability kit (Invitrogen) (calcein AM + ethidium homodimer)
- Tweezers (sterilized with ethanol)
- Glass coverslips
- 24-well plate
- PDMS molds (diameter: 3 mm, thickness: 500  $\mu\text{m}$ )
- Confocal microscope (Zeiss LSM 710/880) or
- Structured illumination microscope (Zeiss AxioObserver Z1 inverted microscope with Zeiss Apotome imaging system and Hamamatsu ORCA-Flash 4.0LT camera)



**Fig. 7.** Hydrogel mesh size. Variation in the theoretical mesh size of PEG-PQ hydrogels with varying concentrations of NVP.

- 1 Add 1 mL of warm PBS into 1 well of a 24-well plate. Using sterile tweezers, transfer each cell-laden hydrogel from the culture media to the warm PBS. Incubate for 5 min at room temperature to remove media.
- 2 Replace with fresh warm PBS again and incubate for additional 5–10 min.  
**Note:** As media inside the hydrogel diffuses into the surrounding PBS, hydrogels will change color from pink to whitish.
- 3 Prepare the staining solution in warm PBS according to manufacturer's recommended concentration.
- 4 Remove the PBS and add the staining solution to the hydrogels. Incubate in dark for 20–30 min at room temperature.
- 5 Remove the staining solution and add fresh PBS to remove excess staining solution. Incubate for 10 min at room temperature.
- 6 Remove PBS and replace with fresh PBS.
- 7 Prepare imaging setup (Fig. 8A):
  - a Clean 2 glass coverslips and PDMS molds with ethanol. Attach the mold to one coverslip and squeeze out trapped air bubbles using tweezers.
  - b Add a drop of PBS into each of the punched holes in the PDMS molds.
  - c Using tweezers, transfer each hydrogel sample into a punched PDMS mold.
  - d Add a 2nd coverslip on top of the PDMS mold to make a sandwich-like setup.  
**Note:** Make sure all holes are completely filled with PBS and there are no air bubbles trapped between the coverslips adjoining the hydrogels as fluorescence signal can get distorted during imaging.
- 8 Transfer the hydrogel-PDMS mold setup onto the microscope stage.
- 9 In acquisition settings, choose GFP (excitation: 450–490 nm, emission: 500, 550 nm) and rhodamine (excitation: 538–562 nm, emission: 570–640 nm) channels. Optimize exposure time and excitation intensity based on fluorescence intensity of stained samples.
- 10 Acquire z-stacks (z-height: ~150 μm, step size: 3 μm) (Fig. 8B).  
**Note:** Complete imaging within 1–2 h of staining. Stained samples can be stored for 2–3 h in PBS at room temperature.  
**Note:** The hydrogel-PDMS mold setup can be flipped to acquire z-stacks from the opposite side of the hydrogel and to acquire images from a larger volume if desired (Fig. 8A).
- 11 Analyze the z-stacks using FIJI or other image analysis software to quantify the number of live cells, dead cells, and cell density per unit volume. Calculate the percent viability and viable cell density.



**Fig. 8.** Quantification of cell viability. (A) Schematic of the setup of the cell-laden hydrogel between a punched PDMS mold affixed between two glass coverslips. The setup can be flipped to acquire z-stacks from either side of the hydrogel. (B) Representative maximum intensity z-projections from 3D image stacks of MDA-MB-231 cells labeled with calcein AM (green, live cells) and ethidium homodimer (red, dead cells) on day 15 post-encapsulation in two hydrogel formulations. SB = 100  $\mu\text{m}$ .

**Note:** Background noise and contrast enhancement may be necessary to distinguish individual cells.

**Note:** Viable cells in dense clusters may not be distinguishable from each other. To compensate, Hoechst stain (10  $\mu\text{L/mL}$  in PBS) can be included in the staining solution to label the nuclei separately and imaged using the DAPI channel (excitation: 330–370 nm, emission: 430–480 nm).

**Note:** For image analysis, counting can be manual or automated. The manual method takes more time but can be more accurate. The automated method is faster if the images are sharp and cells are individually distinguishable in each plane. The FIJI code for the automated method is provided below:

```
run("Z Project . . .", "projection=[Max Intensity]");
run("Subtract Background . . .", "rolling = 50");
run("Enhance Contrast", "saturated = 0.35");
run("Threshold . . .");
run("Make Binary");
run("Open");
run("Fill Holes");
run("Watershed");
run("Analyze Particles . . .");
```

### Quantification of early apoptosis

This protocol describes an assay to quantify early apoptosis of cells cultured within PEG-based hydrogels via fluorescent immunolabeling and quantification of Annexin V. This assay is necessary to demonstrate that encapsulated cells that undergo death, do so via spontaneous apoptotic process, and not due to nutrient limitations, hypoxia, or necrosis.

### Materials/equipment

- Cell-encapsulated hydrogels
- Warm PBS (sterile)
- CF568 Annexin V (Biotium)

- Hoechst 33342
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
- Sodium chloride (NaCl)
- Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ )
- Calcium chloride ( $\text{CaCl}_2$ )
- DI water
- Tweezers (sterilized with ethanol)
- Glass coverslips
- 24-well plate
- PDMS molds (diameter: 3 mm, thickness: 500  $\mu\text{m}$ )
- Confocal microscope or structured illumination microscope (as above)

1 Prepare HEPES-buffered saline (HBS). To prepare 100 mL of 1x HBS, dissolve 876.6 mg NaCl + 28.392 mg  $\text{Na}_2\text{HPO}_4$  + 1191.5 mg HEPES in 90 mL DI water. Stir well to dissolve. Adjust to pH 7.0. Bring to 100 mL volume with DI water.

2 Prepare Annexin V binding buffer (HBS +2.5 mM  $\text{CaCl}_2$ ): To 100 mL of 1X HBS add 27.745 mg  $\text{CaCl}_2$ , stir well.

3 Prepare staining solution: Add CF568 Annexin V to 1X binding buffer at 5  $\mu\text{L}/\text{mL}$  (prepare a  $\sim 500$   $\mu\text{L}$  volume for each well of a 24-well plate that holds 10–15 hydrogel droplets).

4 Stain cells with the staining solution at 37 °C for 20–30 min, protected from light.

**Note:** Staining for longer than 30 min is not recommended as cells might start apoptosing if kept in buffer for too long.

5 Wash the hydrogels with 1X binding buffer 2 times, 5 min. each.

6 Add Hoechst stain (10  $\mu\text{L}/\text{mL}$  in 1X binding buffer) from the stock solution (10 mg/mL) for nuclear counterstaining. Incubate for 10 min.

7 Load hydrogels between glass coverslips with punched PDMS molds and fill with 1X binding buffer (as described in the previous section).

**Note:** Make sure all holes are completely filled with PBS and there are no air bubbles trapped.

8 Acquire fluorescent z-stacks of hydrogels within 1 h of staining on a confocal or structured illumination microscope. For CF568 Annexin-V, use the rhodamine channel (excitation: 538–562 nm, emission: 570, 640 nm). For Hoechst, use the DAPI channel (excitation: 330–370 nm, emission: 430–480 nm). Optimize exposure time and excitation intensity based on fluorescence intensity of labeled samples (z-height:  $\sim 150$   $\mu\text{m}$ , step size: 3  $\mu\text{m}$ ) (Fig. 9).

**Note:** Positive Annexin V staining should follow an approximate circular appearance (around the cell membrane).

**Note:** The hydrogel-PDMS mold setup can be flipped to acquire z-stacks from the opposite side of the hydrogel and to acquire a larger volume as described earlier.

**Note:** For a positive control, incubate cell-laden hydrogels with 1  $\mu\text{g}/\text{mL}$  Staurosporine. From the stock solution at 1 mg/mL, dilute 1:1000 in culture media and add to cell-laden hydrogels for 1 h at 37 °C and 5 %  $\text{CO}_2$ . Remove the media and proceed with above steps.

9 Analyze z-stacks using FIJI or other image analysis software to quantify the percentage of Annexin V positive cells.

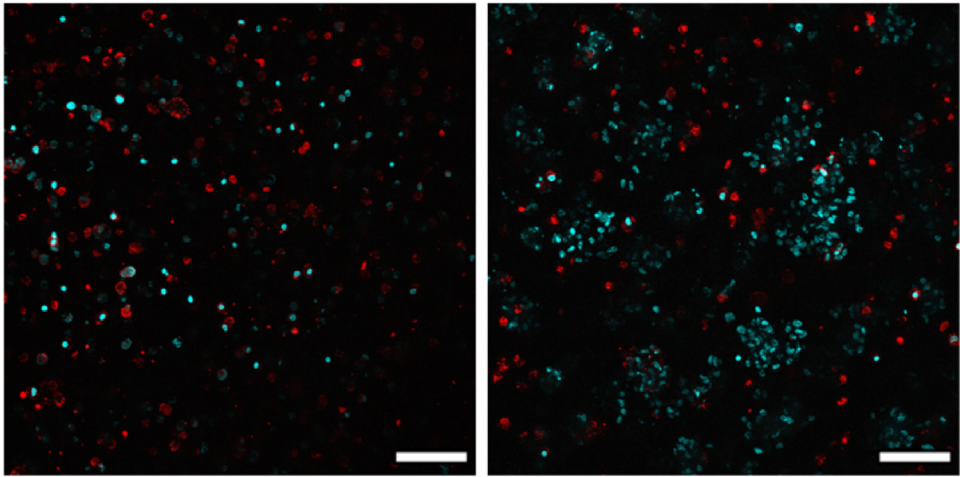
**Note:** When using FIJI, the manual counting method is preferred to obtain accurate cell counts. Load the z-stack in FIJI and use the following code to improve the z-stack for manual quantification:

```
run("Subtract Background . . .", "rolling = 50");
```

```
run("Enhance Contrast", "saturated = 0.35");
```

Using the multi-point tool, click on individual cells and mark them as they are counted. Keep the marks on the counted cells visible throughout the process. Progress through each plane of the z-stack and ensure same cells are not counted more than once. Note the total number of cells counted at the end.



NVP: 2  $\mu$ L/mL, RGDS: 0 mMNVP: 0  $\mu$ L/mL, RGDS: 10 mM

**Fig. 9.** Quantification of early apoptosis. Representative maximum intensity zprojections from 3D image stacks of MDA-MB-231 cells stained with CF568 Annexin V (red, early apoptotic cells) and Hoechst 33342 (cyan, nuclei) on day 15 post-encapsulation in two hydrogel formulations. SB = 100  $\mu$ m.

### Quantification of cell proliferation

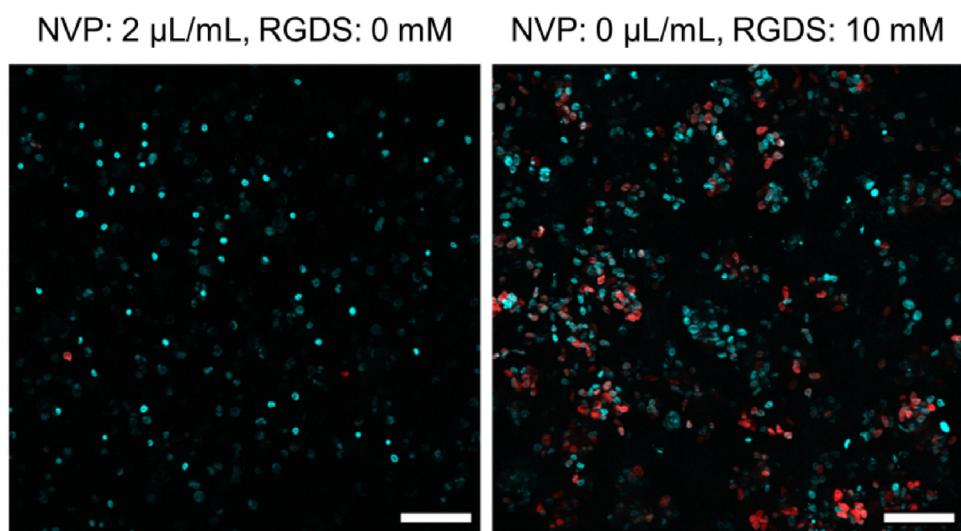
This protocol describes an assay to quantify cell proliferation within PEG-based hydrogels via fluorescent labeling of EdU incorporated in the nuclei of dividing cells. The results of this assay provide another metric for quantification of cell phenotypes.

### Materials/equipment

- Cell-encapsulated hydrogels
- Warm PBS (sterile)
- Click-iT Plus<sup>®</sup> EdU Imaging kit (Invitrogen)
- Tweezers (sterilize with ethanol)
- Glass coverslips
- PDMS molds (500  $\mu$ m thickness) with punched holes (3 mm diameter)
- DI water
- 4 % paraformaldehyde
- PBS-T (PBS + 1 %w/v bovine serum albumin + 0.2 %v/v Triton-X)
- Blocking buffer (PBS + 3 %v/v fetal bovine serum)
- Confocal microscope or structured illumination microscope (as above)

- 1 Prepare stock solutions of 5-ethynyl-2'-deoxyuridine (EdU), 1X reaction buffer, and 10X buffer additive according to manufacturer's protocol. Store as recommended.
- 2 Labeling with EdU: Prepare a working solution of 10  $\mu$ M EdU in cell culture media from the stock solution.
- 3 Using tweezers, transfer cell-laden hydrogels into a separate 24-well plate. Incubate with EdU working solution at 37  $^{\circ}$ C for 24 h.  
**Note:** The incubation time can be varied depending on the experimental condition and cell doubling time.
- 4 After 24 h, remove media and rinse hydrogels twice with warm PBS for 10 min each rinse to completely remove media.
- 5 Add 4 % paraformaldehyde to hydrogels and incubate at room temperature for 30 min.

- 6 Remove fixative and rinse twice with PBS (5 min each).  
**Note:** Fixed samples can be stored at 4 °C for a few days.
- 7 Add PBS-T to hydrogels and incubate at room temperature for 30 min.
- 8 Remove PBS-T, add blocking buffer to hydrogels, and incubate for 30 min; else samples can be stored for a few days at this step.
- 9 Prepare Click-It reaction cocktail as below:
  - a For a total volume of 1 mL of reaction cocktail, dilute 10X buffer additive in DI water to 1X (10  $\mu$ L 10X stock +90  $\mu$ L of DI water).  
**Note:** Prepare 1X buffer additive fresh each time within 1 h of assay, do not store.
  - b To 1X reaction buffer (86  $\mu$ L 10X stock +774  $\mu$ L DI water), add copper sulfate solution: 40  $\mu$ L, Alexa Fluor<sup>®</sup> 568 picolyl azide: 1  $\mu$ L, 1X buffer additive and mix well.  
**Note:** The choice of fluorophore for labeling incorporated EdU can be chosen by the user.
- 10 Remove blocking buffer from hydrogels and add this reaction cocktail to the hydrogels. Incubate at room temperature for 2 h, protected from light.
- 11 Remove the cocktail from the hydrogels and rinse twice with PBS-T (5 min each).
- 12 Prepare Hoechst working solution (10  $\mu$ L/mL in blocking buffer) and add to the hydrogels. Incubate at room temperature for 15 min.
- 13 Rinse with PBS twice to remove excess Hoechst.
- 14 Load the hydrogels with PBS under glass coverslips and punched PDMS molds (as described earlier).
- 15 Acquire fluorescent z-stacks of hydrogels on a confocal or structured illumination microscope. For Alexa Fluor<sup>®</sup> 568, use the rhodamine channel (excitation: 538–562 nm, emission: 570–640 nm). For Hoechst, use the DAPI channel (excitation: 330–370 nm, emission: 430–480 nm). Optimize the acquisition time and excitation intensity based on the fluorescence intensity of labeled samples (z-height:  $\sim$ 150  $\mu$ m, step size: 3  $\mu$ m) (Fig. 10).  
**Note:** The hydrogel-PDMS mold setup can be flipped to acquire z-stacks from the opposite side of the hydrogel and to acquire a larger volume image as describe earlier.
- 16 Analyze z-stacks using Fiji or image analysis software to quantify their percentage of EdU positive cells.



**Fig. 10.** Quantification of proliferation. Representative maximum intensity zprojections from 3D image stacks of MDA-MB-231 cells fluorescently labeled for EdU (red, proliferative cells) and Hoechst 33342 (cyan, nuclei) on day 15 post-encapsulation in two hydrogel formulations. SB = 100  $\mu$ m.

**Note:** Manual quantification of cell proliferation is preferred as described above in the early apoptosis assay. After background subtraction and contrast enhancement of the z-stack, use the multi-point tool to mark each cell plane-by-plane taking care not to mark each cell twice. At the end of the last plane, note the total number of marked cells to obtain the cell number.

### *Quantification of metabolic activity*

This protocol describes an assay to quantify metabolic activity of cells cultured within PEG-based hydrogels using an Alamar Blue assay. Though this assay does not differentiate between distinct modes of metabolism (glycolysis vs. oxidative phosphorylation), it serves as an important metric for phenotypic classification.

#### *Materials/equipment*

- Cell-encapsulated hydrogels
- Warm PBS (sterile)
- Phenol red-free media (Phenol red-free DMEM + 10 % (v/v) FBS + 1 % (v/v) penicillin-streptomycin)
- Alamar Blue reagent (Thermo-Fisher)
- Tweezers (sterilize with ethanol)
- 96-well plates
- Plate reader (Biotek Synergy)

- 1 Post-encapsulation, culture the cells in phenol-red free media throughout the entire assay.
- 2 Prepare the working solution. Mix the stock bottle thoroughly prior to use. Add 10  $\mu\text{L}$  of the 10X stock to 100  $\mu\text{L}$  of phenol-red-free media (for each hydrogel droplet). Scale up as necessary. Warm the working solution in a water bath.
- 3 With tweezers, transfer the hydrogels into a 96-well plate (one hydrogel/well).
- 4 Rinse twice with PBS (5 min each).
- 5 Add the working solution to the hydrogels (100  $\mu\text{L}$ /well) and incubate for 4 h at 37 °C.  
**Note:** At the end of the incubation period, there should be a color change from bluish to reddish/pinkish. Incubation time can be varied based on the metabolic activity of the cells and the sensitivity of the plate reader. Incubation time can be increased to 8 h if the cells have very low metabolism.
- 6 Transfer the hydrogels to a new 96-well plate with phenol-red-free media using tweezers. In the same well plate, include 2 controls: (1) phenol-red-free media without hydrogels or Alamar blue reagent and (2) hydrogels in phenol-red-free media without Alamar blue reagent.
- 7 Use a plate reader to measure the fluorescence intensity (Excitation: 550 nm, Emission: 600 nm). Subtract readings from unstained hydrogels to remove background noise.
- 8 Display results either as absolute values or normalized to day 0 control values.  
**Note:** The normalized metabolic activity can be divided by the viable cell density for each hydrogel formulation and each time point to obtain a cellular metabolic activity (activity on a per cell basis), which can then be compared across groups.

### *Quantification of single cells and cell cluster properties*

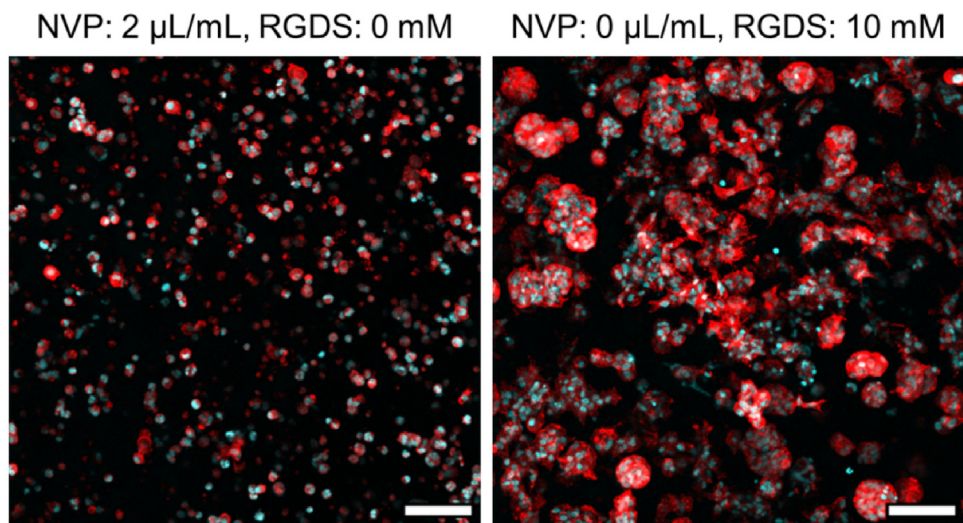
This protocol describes an assay and image analysis techniques to quantify metrics related to 3D morphology of encapsulated cells and cell clusters. This quantification provides direct visual evidence of cluster formation from single encapsulated cells over time, invasiveness of single cells and resulting clusters, and relative density of cells/clusters within the 3D hydrogel matrix.

#### *Materials/equipment*

- Cell-encapsulated hydrogels
- Warm PBS (sterile)

- Hoechst 33342
- Alexa Fluor<sup>®</sup> 568 Phalloidin (Invitrogen)
- Tweezers (sterilize with ethanol)
- Glass coverslips
- PDMS molds (500  $\mu\text{m}$  thickness) with punched holes (3 mm diameter)
- 4 % paraformaldehyde
- PBS-T (PBS + 1 %w/v bovine serum albumin + 0.2 %v/v Triton-X)
- Blocking buffer (PBS + 3 % (v/v) FBS)
- Confocal microscope or structured illumination microscope (as above)

- 1 With tweezers, transfer the hydrogels from the culture media into a 24-well plate.
- 2 Rinse twice with PBS (10 min each) to completely remove the media.
- 3 Add 4 % paraformaldehyde to hydrogels and incubate at room temperature for 30 min.
- 4 Remove fixative and rinse twice with PBS (5 min each).  
**Note:** Fixed samples can be stored at 4 °C for a few days.
- 5 Add PBS-T to hydrogels and incubate at room temperature for 30 min.
- 6 Remove PBS-T, add blocking buffer to hydrogels, and incubate for 30 min.
- 7 Prepare Alexa Fluor<sup>®</sup> 568 Phalloidin in blocking buffer (25  $\mu\text{L}/\text{mL}$ ).
- 8 Remove blocking buffer from hydrogels and add Phalloidin stain. Incubate for 30 min at room temperature.
- 9 Remove Phalloidin stain and add Hoechst working solution (10  $\mu\text{L}/\text{mL}$  in blocking buffer). Incubate for 10 min at room temperature.
- 10 Rinse twice with PBS (10 min each).
- 11 Load hydrogels with PBS under glass coverslips and punched PDMS molds (as described earlier).
- 12 Acquire fluorescent z-stacks of hydrogels on confocal microscope. For Alexa Fluor<sup>®</sup> 568, use the rhodamine channel (excitation: 538–562 nm, emission: 570, 640 nm). For Hoechst, use the DAPI channel (excitation: 330–370 nm, emission: 430–480 nm). Optimize image acquisition settings based on fluorescence intensity of labeled samples (z-height:  $\sim 150$   $\mu\text{m}$ , step size: 3  $\mu\text{m}$ ) (Fig. 11).  
**Note:** The hydrogel-PDMS mold setup can be flipped to acquire z-stacks from the both sides of the hydrogel and to capture a larger volume as described earlier.



**Fig. 11.** Quantification of cell/cluster morphology. Representative maximum intensity zprojections from 3D image stacks of MDA-MB-231 cells labeled with AF568 Phalloidin (red, F-Actin) and Hoechst 33342 (cyan, nuclei) on day 15 post-encapsulation in two hydrogel formulations. Scale bar = 100  $\mu\text{m}$ .

- 13 Analyze z-stacks using Fiji or other image analysis software by manually outlining individual cells and/or cell clusters to quantify the following:
- a percentage of the cell population residing as individual cells
  - b percentage of the cell population residing in cell clusters
  - c percentage of the cell population residing as rounded individual cells
  - d percentage of the cell population residing as invasive individual cells
  - e percentage of the cell population residing as non-invasive cell clusters
  - f percentage of the cell population residing as invasive cell clusters
  - g single cell density
  - h cluster density

**Note:** A roundness value less than 0.80 indicates invasive cell/cell cluster.

**Note:** For quantifying cell clusters, set measurements in Fiji (Analyze > Set Measurements > Check boxes) for the following parameters: area, Feret diameter, aspect ratio, circularity, and roundness.

**Note:** A minimum of three cells in contact with each other was considered as a cluster.

**Note:** Manual outlining in Fiji can be conducted using either the 'Polygon selection' tool or the 'Freehand selection' tool.

#### *Classification of cell states and phenotypic dormancy metrics*

This method describes the analytical steps to classify hydrogel formulations, based on quantified cellular metrics, into groups that support specific cancer states: (1) high growth, (2) moderate growth, (3) single cell, restricted survival dormancy, and (4) balanced dormancy. A multitude of metrics can be used to quantify cell phenotype but this approach relies on temporal changes in live cell density, dead cell density, and metabolic activity as the basis for classification.

#### *Materials/equipment*

- None

- 1 Assemble quantified cellular metrics (viability, viable cell density, early apoptosis, proliferation, metabolic activity, cell/cluster morphology) collected through 15 days of culture for each of the 16 hydrogel formulations.
- 2 Normalize the viable cell density and metabolic activity with respect to day 0 for each hydrogel formulation. Plot the data as a scatter plot (See Fig. 6A in Ref. [7]).  
**Note:** Three distinct clusters will appear. Groups with high values ( $>1$ ) of normalized viable cell density and metabolic activity are labeled as a 'high growth state'. Groups with low values ( $<1$ ) of both metrics are labeled as a 'single cell, restricted survival dormancy state'. Groups with intermediate values ( $\sim 1$ ) are clustered as 'Cluster A'.
- 3 From the quantified viability and viable cell density values, calculate the viable (live) cell densities and dead cell densities for days 5, 10, and 15 post encapsulation. Add these values to obtain the 'new live cell density' and the 'new dead cell density' measured through days 5–15. Calculate the standard deviations corresponding to these two metrics and obtain the average of these standard deviations.
- 4 For hydrogel formulations in Cluster A, plot the 'new live cell density' vs. 'new dead cell density' as a scatter plot. Plot the two average standard deviations as straight lines representing the error band of these metrics.  
**Note:** Hydrogel formulations which fall above the error band (those with higher new live cell density and lower new dead cell density) are clustered and labeled as a 'moderate growth state'. Hydrogel formulations which fall within the error band (those with balanced new live and dead cell densities) are clustered and labeled as a 'balanced survival dormancy state (Cluster B)'.

### *Reactivation of dormant tumor cells*

This protocol describes a method to reactivate single dormant cancer cells toward an invasive, proliferative state that is reminiscent of metastatic recurrence. This phenotypic switch is induced via a dynamic increase in ligand density in long-term culture, which is simulative of dormant single cells experiencing increased matrix adhesivity with deposition of cell-permissive extracellular matrix components in the native dormant niche.

#### *Materials/equipment*

- MDA-MB-231 breast cancer cells
- PEG-PQ macromer
- PEG-RGDS macromer
- Lithium acyl phosphinate (LAP)
- Warm PBS (sterile)
- Parafilm (sterilized with ethanol)
- Tweezer (sterilize with ethanol)
- Glass slide
- Well plate
- UV lamp

- 1 Prepare PEG-PQ stock in PBS (20 % w/v) (200 mg/mL or 10 mg/50  $\mu$ L).
- 2 Prepare PEG-RGDS stock in PBS (20 mM) (68 mg/mL or 6.8 mg/100  $\mu$ L).
- 3 Prepare LAP stock in PBS (30 mg/mL or 3 mg/100  $\mu$ L).
- 4 Prepare final polymer precursor solution with PEG-PQ = 5 % w/v and LAP = 3 mg/mL and adjust with PBS. Mix all components thoroughly.
- 5 Encapsulate cells in this hydrogel formulation at 10 million cells/mL as described earlier and maintain in culture for 40 days. Replace media every 4 days.
- 6 Conduct quantification of cell viability, viable cell density, and proliferation on 0, 15, and 30 days post encapsulation as per above protocols.
- 7 On day 40, prepare an RGDS precursor solution with a final concentration of PEG-RGDS = 10 mM and LAP = 3 mg/mL in PBS. Mix thoroughly.
- 8 Using tweezers, transfer cell-laden hydrogels into a microcentrifuge tube filled with PBS. Rinse twice in PBS for 10 min each to completely remove media.
- 9 Remove PBS and add the PEG-RGDS/LAP precursor solution to the hydrogels. Incubate at 37 °C for 1 h to allow diffusion of PEG-RGDS into the hydrogel.
- 10 Warm up the UV lamp. Transfer the hydrogels from a microcentrifuge tube to a Parafilm covered glass slide. Photocrosslink for 1 min.
- 11 Transfer the PEG-RGDS crosslinked hydrogels into a well plate with culture media and maintain in culture for an additional 35 days.
- 12 Conduct quantification of cell viability, viable cell density, and proliferation on 5, 15, and 35 days post PEG-RGDS coupling (See Fig. 7 in Ref. [7]).

#### *Conclusions*

We presented protocols and methods pertaining to fabrication of PEG-based hydrogels with well-defined and well-controlled physical and biochemical properties and 3D culture of MDA-MB-231 cells for the investigation of tumor dormancy and metastatic recurrence. We also described methods of quantifying cellular metrics of 3D cultured cells and implementing these metrics to phenotypically classify hydrogel formulations into groups that support specific cancer cell states. The systematic phenotypic classification of hydrogel formulations provides a roadmap toward designing engineered matrices for regulating cell fates for various applications, including tumor dormancy and metastasis. We also described the method for dynamically increasing ligand density in cell-laden hydrogels (via incorporation of PEG-RGDS) to induce reactivation of single dormant tumor cells into invasive



proliferative populations, reminiscent of metastatic recurrence. These methods will potentially be useful for mechanistic investigations of tumor dormancy, not only for MDA-MB-231 cells, but also other breast cancer cell lines and other cancer types. In the future, this engineered *in vitro* platform could potentially aid in discovery and development of dormancy-associated molecular targets and drugs toward those targets.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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