Protocol
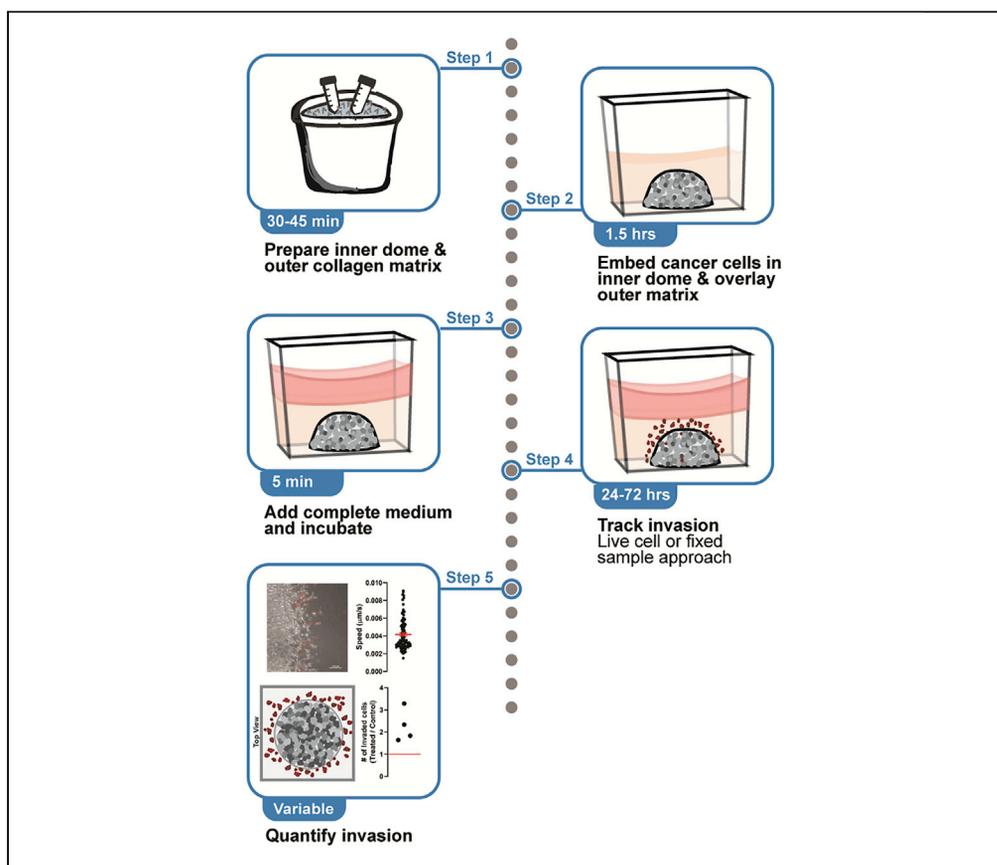
Optimized *in vitro* three-dimensional invasion assay for quantifying a wide range of cancer cell invasive behavior

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Highlights

Optimized 3D invasion assay to examine cells with a wide range of invasive activity

Customized microenvironment with collagen and chemoattractant

Applications for fixed and live-cell imaging

Quantification of global and individual differences in invasive behavior

We describe a three-dimensional (3D) *in vitro* assay for quantifying cancer cell invasion into a 3D microenvironment with defined biochemical and biophysical properties. Researchers can quantify invasion dynamics (e.g., cell motility and directionality) and examine morphological changes during invasion, using live-cell and confocal imaging techniques. Together, these advantages over existing *in vitro* invasion assays, such as transwell-based assays, provide researchers with a valuable tool to gain insight into the mechanisms regulating cancer cell invasion.

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

Protocol

Optimized *in vitro* three-dimensional invasion assay for quantifying a wide range of cancer cell invasive behavior

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SUMMARY

**We describe a three-dimensional (3D) *in vitro* assay for quantifying cancer cell invasion into a 3D microenvironment with defined biochemical and biophysical properties. Researchers can quantify invasion dynamics (e.g., cell motility and directionality) and examine morphological changes during invasion, using live-cell and confocal imaging techniques. Together, these advantages over existing *in vitro* invasion assays, such as transwell-based assays, provide researchers with a valuable tool to gain insight into the mechanisms regulating cancer cell invasion.
For complete details on the use and execution of this protocol, please refer to Padilla-Rodriguez et al. (2018) and Watson et al. (2021).**

BEFORE YOU BEGIN

The study of invasion using *in vivo* experimentation has significantly advanced our understanding of cancer cell dissemination and metastasis. However, *in vivo* approaches present several challenges, including time required for tumor development and the inability to manipulate aspects of the tumor microenvironment. Transwell-based *in vitro* invasion assays, such as the Boyden chamber assay, were developed to overcome these challenges. However, the experimental setup of these assays requires cells to break through a porous synthetic membrane with a significant thickness, relative to the dimension of a cell, thus restricting the invasion of several cancer cell types with low invasive potential. Here, we present a 3D *in vitro* invasion assay with a definable microenvironment, into which cells can readily invade, thus allowing the quantification of cancer cell invasion with a wide range of invasive potential. Furthermore, researchers can quantify overall invasiveness by counting the number of invaded cells; invasion dynamics, such as cell speed and direction; and examine dynamic morphological changes during invasion, using live-cell and confocal imaging techniques to capture changes in the actin cytoskeleton. Together, this assay provides researchers with the ability to better understand the mechanisms regulating migration and invasion in cancer cells.

The protocol below describes steps to prepare a glass bottom device for 3D invasion. We have optimized the assay for the breast cancer cell lines, SUM159, MCF7, and MDA-MB-231; the prostate cancer cell lines, DU145 and PC3LN4; and the macrophages, RAW 264.7 cells.

Silanization of coverglass-bottom chamber slide

⏱ Timing: 3.5 h

This section describes steps to prepare the 8-well chamber slide for setting up the assay. We used coverglass bottom chambers with non-removable wells. This process is performed to functionalize the glass by silanization (Aratyn-Schaus et al., 2010), which enables the 3D matrix to be cross-linked to the glass and prevents it from lifting off over the duration of the assay. These volumes are for a single 8-well chambered coverglass slide with non-removable wells, user should scale volume up for larger wells in multi-well chamber slides. The solutions in this section must be prepared right before each use.

1. Prepare a working area in the fume hood to fit an orbital shaker.
2. Using a glass serological pipette, prepare 4 mL of a 2% silane solution in 100% isopropanol in a glass beaker.

   Note: Glass serological pipettes are preferred over plastic pipettes due to the corrosive property of silane.

3. Pipette 500 μL of 2% silane solution into each well of the chamber slide.
4. Incubate silane solution in chamber slide with gentle shaking on orbital shaker for 10 min at 20°C–22°C.
5. Decant or aspirate the silane solution into an appropriate waste container and then rinse chamber slide about 8 times with deionized (DI) water. For each rinse submerge chamber under water, then invert and tap on paper towel.
6. After final rinse, do a longer wash for 10 min with DI water with gentle shaking.
7. After washing, decant DI water and place chamber slide on lab bench to allow complete drying (∼1 h).

   Note: To expedite drying, chamber slides could be placed in an oven set to 37°C–40°C until completely dry (∼30 min).

8. Prepare a 1% final glutaraldehyde solution in 1× PBS. Pipette 500 μL of 1% glutaraldehyde solution into each well of the dry chamber slide.
9. Incubate glutaraldehyde solution with chamber slide on the orbital shaker set to low speed (≤60 RPM) for 30 min at 20°C–22°C.
10. Decant or aspirate 1% glutaraldehyde solution and rinse chamber slide with DI water as described in 5.
11. After final rinse, wash for 10 min as described in 6.
12. After wash, decant DI water and place chamber slide on lab bench for drying.
13. Once dry, chamber slides are ready to use and can be stored at 20°C–22°C for up to one week.

   Note: Once silanized, the chamber slide should be used within one week. If the assay is not performed within the week, the chamber slide can be re-silanized. Be cautious repeated silanization will result in thinning of the glass making it fragile.

   ⚠ CRITICAL: (3-Aminopropyl)trimethoxysilane (silane) and Glutaraldehyde are corrosive. Perform all steps in the fume hood with proper personal protective equipment (PPE).

UV sterilization of chamber slide

⏱ Timing: 10 min

14. Place uncovered chamber slide and lid under a UV light with wavelength 100–280 nm for 10 min.
15. Once sterilized, replace lid and treat as sterile.
16. Place chamber slide in incubator set at 37°C to pre-warm.

⚠ CRITICAL: UV sterilization requires proper PPE. To minimize exposure to UV light, perform sterilization in a biosafety cabinet equipped with a UV light with the protective sash completely closed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isopropanol molecular biology grade	Thermo Fisher Scientific	BP26184
(3-Aminopropyl)-trimethoxysilane	Sigma-Aldrich	Cat # 281778
Glutaraldehyde Aqueous 25%, EM Grade	Electron Microscopy Sciences	Cat # 16220
DMEM (Dulbecco's Modified Eagle's Medium)	Corning	Cat # 10-013-CV
Collagen Type I, rat tail	Sigma-Aldrich	Cat # 08-115PB
Ethylene glycol-bis (succinic acid N-hydroxysuccinimide ester) (PEG-di(NHS))	MilliporeSigma	Cat # E3257
Insulin Growth Factor (human)	Sigma-Aldrich	Cat # I3769
Sodium Hydroxide	Thermo Fisher Scientific	Cat # S318-100
Matrigel Matrix Growth Factor Reduced (GFR) Basement Membrane Matrix	Corning	Cat # 354230
0.25% Trypsin, 2.21 mM EDTA, 1× sodium bicarbonate	Corning	Cat # 25-053-CI
Dulbecco's Phosphate Buffered Saline	Corning	Cat # 21-030-CV
16% Paraformaldehyde (Formaldehyde) Aqueous Solution, EM Grade	Electron Microscopy Sciences	Cat # 15710
Hoechst 33342	Thermo Fisher Scientific	Cat # H1399
DAPI	Thermo Fisher Scientific	Cat # D1306
Critical commercial assays		
Nunc Lab-Tek II Chambered Coverglass	Thermo Fisher Scientific	Cat # 155411
Steriflip-GP Sterile Centrifuge Tube Top Filter Unit	MilliporeSigma	Cat # SCGP00525
Bright-Line Hemacytometer	MilliporeSigma	Cat # Z359629
Fisherbrand Paper pH strips; range 0–14	Thermo Fisher Scientific	Cat # 13-640-508
Fisherbrand Paper pH strips; range 6–8	Thermo Fisher Scientific	Cat # 13-640-510
Experimental models: Cell lines		
Human: MCF7 (Breast)	Joan Brugge, Harvard	N/A
Human: SUM159 (Breast)	Joan Brugge, Harvard	N/A
Human: MDA-MB-231 (Breast)	Joan Brugge, Harvard	N/A
Human: DU145 (Prostate)	ATCC	Cat # HTB-81
Human: PC3LN4 (Prostate)	Andrew Kraft, University of Arizona	N/A
Mouse: RAW 264.7 (Macrophage)	ATCC	Cat # TIB-71
Software and algorithms		
Nikon NIS Elements	Nikon	NIS Elements Ar (Advanced Research)
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/

MATERIALS AND EQUIPMENT

The inner dome serves as the initial cell reservoir and the outer matrix constitutes the surrounding 3D space into which the cells invade (Figure 1A). This assay allows for customization by modifying the outer matrix in either a biochemical or biophysical manner. The outer matrix can be modified to include a chemoattractant such as a growth factor (GF) or cytokine (Figure 1B and Table 1) or be modified to change the stiffness via PEG-di(NHS) concentration or density via collagen concentration (Figure 1C and Tables 2 and 3, respectively).

Note: The Ethylene glycol-bis (succinic acid N-hydroxysuccinimide ester) (PEG-di(NHS)) is used for crosslinking the matrix. By using a higher concentration of PEG-di(NHS) we increase the stiffness of the matrix without increasing the density.
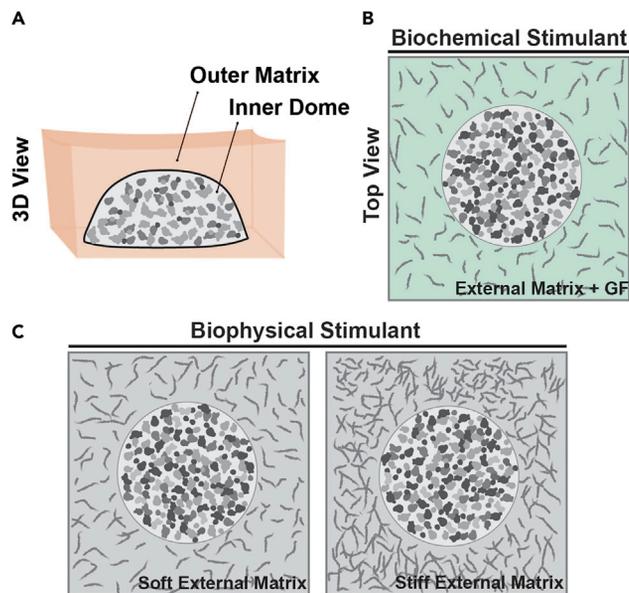
Figure 1. Illustration of 3D invasion assay
(A) 3D rendering of the invasion assay with the inner dome in the center with embedded cells and the outer matrix encompassing the dome.
(B) Illustration of a top view of a 3D in vitro invasion assay with a biochemical stimulant such as growth factors (GFs) or cytokines.
(C) Illustration of a top view of a 3D in vitro invasion assay with a biophysical stimulant, such matrix stiffness and/or matrix density.

⚠ CRITICAL: When preparing the matrix, keep collagen stock on ice.

Note: The prostate cell lines, DU145 and PC3LN4, and the macrophage cell line, RAW 264.7, have not been optimized with the collagen inner dome matrix.

Different cell lines required specific optimization of the protocol, mostly due to differences in invasive activity. For example, MCF7 cells, which are luminal ER+ breast cancer cells and considered to have a relatively low invasive activity, required a longer incubation time of 72 h to observe a significant number of invading cells (i.e., enough invading cells needed for quantitative analysis). More aggressive cell lines, such as SUM159, required a shorter time of 16 h to exhibit a substantial invasive phenotype.

Another factor that required cell-specific optimization was the number of cells embedded in the inner dome. For instance, more aggressive cell lines required a lower number of cells embedded in the inner dome. This was particularly relevant when examining cells with mesenchymal properties, such

Table 1. Experimental setup for invasion assay with chemoattractant

Reagent	Dome Final conc.	Outer matrix Final conc.
No-additive Media	n/a	n/a
Collagen Type I, rat tail	1 mg/mL	1 mg/mL
IGF (10 μM)	n/a	20 nM
PEG-di (NHS) (50 mg/mL)	62.5 μg/mL	625 μg/mL
NaOH (0.1 M)	pH 7.0	pH 7.0
Total Volume	200 μL	2 mL

Table 2. Experimental setup for invasion assay with outer matrices of different stiffness

Reagent	Dome Final conc.	Outer matrix (soft) Final conc.	Outer matrix (stiff) Final conc.
No-additive Media	n/a	n/a	n/a
Collagen Type I, rat tail	1 mg/mL	1 mg/mL	1 mg/mL
PEG-di (NHS) (50 mg/mL)	62.5 μg/mL	6.25 μg/mL	625 μg/mL
NaOH (0.1 M)	pH 7.0	pH 7.0	pH 7.0
Total Volume	200 μL	2 mL	2 mL

as SUM159 and MDA-MB-231 cells, which are highly contractile (i.e., exert strong forces on the surrounding matrix); a high number of these embedded cells resulted in the global contraction of the inner dome leading to its dissociation from the outer matrix.

In addition, some cell types required adjustment of the stiffness of the outer matrix. In the initial experiments using the prostate cell lines, DU145 and PC3LN4, a PEG concentration of 625 μg/mL in the outer matrix (which is what was used for the breast cancer cell lines) suppressed their invasive behavior. Decreasing the PEG concentration 10-fold to 62.5 μg/mL allowed their invasion into the outer matrix.

Finally, specific adjustments in the final complete media composition might be needed for some cell types. For instance, in our initial experiments, the macrophage RAW264.7 cell line showed little to no invasion. However, implementing the use of lipopolysaccharide (LPS), a known attractant for macrophages, in the media resulted in invasion and proved to be an appropriate positive control.

STEP-BY-STEP METHOD DETAILS
Preparation of inner dome and outer collagen matrix

Ⓘ Timing: 30–45 min

This step is for the preparation of both the inner dome and outer matrix with the biochemical stimulant setup (Figure 2A, Table 1). Each solution is prepared in excess.

1. For the 8-well chamber slide, a total volume of 200 μL will be prepped for the inner dome and a total volume of 2 mL will be prepped for outer matrix. See Tables 1, 2, and 3 for the starting and final concentrations and example volumes needed for inner dome and outer matrix solutions. All collagen matrices should be prepared on ice.

    Note: Initial concentrations of collagen varies based on the extraction batch from the vendor; be mindful to adjust calculations accordingly.

    Note: Matrigel may also be used for the inner dome if it is appropriate for the experimental design.

Table 3. Experimental setup for invasion assay with outer matrices of different density

Reagent	Dome Final conc.	Outer matrix (low density) Final conc.	Outer matrix (high density) Final conc.
No-additive Media	n/a	n/a	n/a
Collagen Type I, rat tail	1 mg/mL	1 mg/mL	3 mg/mL
PEG-di (NHS) (50 mg/mL)	62.5 μg/mL	62.5 μg/mL	62.5 μg/mL
NaOH (0.1 M)	pH 7.0	pH 7.0	pH 7.0
Total Volume	200 μL	2 mL	2 mL
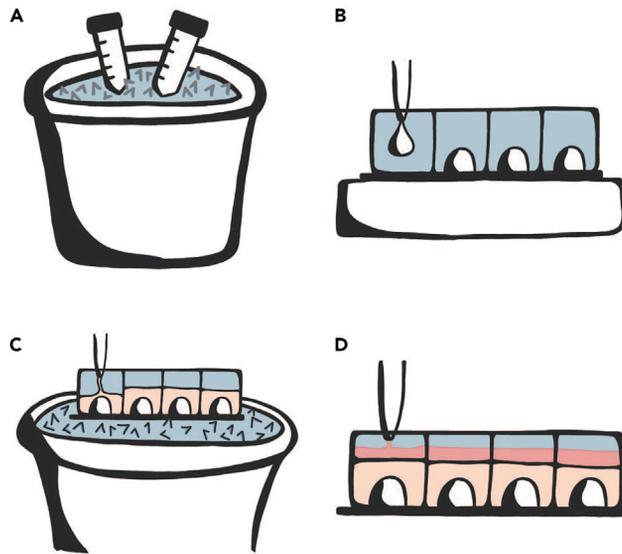
Figure 2. Visual walkthrough of the 3D *in vitro* invasion assay
(A) Preparation of the inner dome and outer matrix solutions in separate tubes on ice.
(B) Plating of inner dome with embedded cancer cells. After plating, chamber slide is incubated for 20 min in a cell culture incubator set to 37°C.
(C) Chamber slide is placed on ice while pipetting outer matrix solution. After pipetting, keep chamber slide on ice for 3 min. Incubate chamber slide for 45 min in a cell culture incubator set to 37°C.
(D) Collagen matrices are polymerized, and invasion assay is created. Add growth media to each chamber well.

> *Note:* The collagen concentration may be varied for the outer matrix to modify the matrix density (See Table 3).

2. Pre-chill 1.5 mL tubes and 0.1 M NaOH on ice.
3. No-additive media is used as a diluent for collagen. Determine the volume of no-additive media needed to achieve a final collagen concentration of 1 mg/mL and a total volume to 200 μL and 2 mL for the inner dome and outer matrix, respectively. Mix the no-additive media with the corresponding collagen volumes in prechilled conical tubes.
4. Mix by slowly pipetting up and down, avoiding the introduction of bubbles.
5. Add PEG to conical tubes for a final concentration of 0.625 mg/mL.

> *Note:* PEG is a homobifunctional cross-linking reagent that mainly couples to primary amine containing molecules when buffered at pH 7.5. It is soluble in DMSO and can be kept at 20°C–22°C to thaw during collagen preparation. The PEG concentration may be varied for the outer matrix to modify the matrix stiffness (See Table 2).

6. Add the chemoattractant, such as IGF1, to the outer matrix only, for a final concentration of 20 nM.

> *Note:* We have optimized IGF1 as effective chemoattractant for MCF7 cells, which express insulin-growth factor receptor (IGFR); other growth factors could be used based on receptor expression.

7. Use pH strips to adjust the pH of collagen solutions to approximately 7. For accuracy, start with pH strips ranging 0–14 then for finer tuning use strips ranging from pH 6–8.

> *Note:* The phenol in phenol-containing media, such as DMEM, could be used as a pH indicator for the 'coarse' titration step. Around pH 7, the media will turn salmon-pink in color from a bright yellow corresponding the initial acidic pH.

Table 4. Cell line specific optimization

Cell line	Cell number	Incubation time	Inner dome matrix	Outer matrix PEG concentration
MCF7	100,000 (Col.) 100,000 (Mat.)	48–72 h	Collagen or Matrigel	6.25–625 µg/mL
SUM159	30,000 (Col.) 75,000 (Mat.)	24–36 h	Collagen or Matrigel	6.25–625 µg/mL
MDA-MB-231	30,000	24–36 h	Collagen	6.25–625 µg/mL
DU145	100,000	24–48 h	Matrigel	6.25–62.5 µg/mL
PC3LN4	100,000	24–48 h	Matrigel	62.5 µg/mL
RAW 264.7	200,000	48–72 h	Matrigel	62.5 µg/mL

8. Degas the collagen solutions 10–15 min on ice. A 50 mL Steriflip-GP sterile centrifuge tube filter unit could be used to create the needed vacuum. Microfuge tubes are placed with the lid open in the 50 mL conical. Once done, close the lid to the tubes and keep on ice.

   Note: Degassing of both the inner dome and outer matrix for at least 10 min is important for reducing bubble formation. Without degassing, microscopic bubbles could expand during polymerization and interfere with the assay.

   Note: Rat tail collagen is optimal for this assay because it has faster gelation time than bovine collagen, which prevents the cells from settling to the bottom of the inner dome during polymerization. Rat tail collagen also polymerizes at 37°C where bovine collagen begins to polymerize at 20°C–22°C, which could lead to a variance in polymerization between chamber wells and experiments. Finally, rat tail collagen has been shown to be more *in vivo*-like with irregular fibrils (Muller-Rath et al., 2007).

   ⚠ CRITICAL: All steps involving collagen preparation must be done on ice, as the collagen solution can begin to polymerize at ambient temperatures. Furthermore, solutions used in collagen preparation must be prechilled on ice. Micropipettes do not need to be chilled.

Embedding cancer cells in inner dome and overlaying outer matrix

⏱ Timing: 1.5 h

This section describes the process of embedding cells in the inner dome and onto the chamber slide and overlaying it with the outer matrix (Figures 2B–2D). The following calculations are for plating two experimental conditions for MCF7 cells in four chamber wells each. Cells can be cultured in the invasion assay for up to 3–4 days, after which point, the outer matrix may begin to lift off the glass bottom of the chamber. The ideal number of cells to embed in the inner dome will depend on the cell line used, refer to Table 4 for recommendations for optimized cell lines. Too many cells will deplete growth media quickly and may cause the inner dome to contract, resulting in separation from the outer matrix. Too few cells will compromise the quality and accuracy of the quantification of invaded cells due to a small sample size.

9. Trypsinize and collect cancer cells per routine protocols.
10. Calculate the total number of cells per condition to be collected. For example, MCF7 cells need 100,000 cells per inner dome, plus 50,000 for excess to account for pipetting errors.
11. Pellet 450,000 total cells needed for each condition in a 1.5 mL tube by centrifugation at 200 × *g* for 2 min. Carefully aspirate as much supernatant as possible without disturbing the pellet.
    a. Be sure to aspirate most of the media to minimize diluting the collagen during embedding.

    Note: When aspirating the media in step 10, remove as much media as possible without disturbing the cell pellet. Excess media will dilute the final concentrations of matrix in inner dome
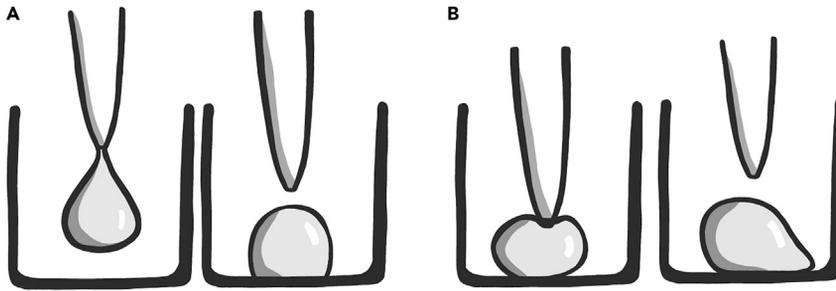
Figure 3. Illustration of correct plating method for the inner dome in the chamber well
(A) Inner dome with embedded cells should be plated by letting the mixture drip onto the bottom of the well by gravity.
(B) Incorrect plating of the inner dome. Users should avoid ejecting the collagen matrix forcibly onto the coverglass-bottom the chamber well as this will result in a non-symmetrical shape.

components leading to poor collagen polymerization. Note that the high cell density in itself also alters the biophysical properties of the matrix in the inner dome.

12. Resuspend each cell pellet with 45 μL of inner dome solution while keeping the tube on ice. Ensure thorough mixing by carefully pipetting until homogeneous with care not to introduce bubbles.
13. Once mixed, plate collagen-embedded cells by pipetting 10 μL of dome solution containing cells in the following manner and as shown in Figure 3A.
14. Fully eject the contents of the pipette to form a hanging droplet and allow it to drip and contact the coverglass in the middle of the chamber well.
    a. Avoid touching the glass bottom with the pipette tip during this process, which might lead to uneven dispensing resulting in an asymmetrical inner dome.

    Note: The shape of the inner dome is critical. We have observed that cell invasion is altered when the inner dome is non-symmetrical (for instance tear shaped), which is consistent with previous research showing that invasion is altered by the geometrical shape of tumors (Nelson and Bissell, 2006). Incorrect plating of inner dome droplet shown in Figure 3B.

15. Repeat for all chamber wells.
16. Carefully place chamber slide in tissue culture incubator set at 37°C and incubate for 20 min.
17. Remove chamber slide from incubator and let it cool on ice for about 5 min.
18. Once cooled, slowly pipette 125 μL of the outer matrix in diagonally opposed corners of the chamber, to a total volume of 250 μL, then use a 20 μL pipette tip to carefully spread the outer matrix around the chamber by dragging the 20 μL pipette tip against all four chamber walls to evenly distribute the outer matrix around the inner dome.
19. Once the outer matrix has been added, keep chamber slide on ice for additional 2 min. Remove chamber slide from ice and allow to reach 20°C–22°C for about 5 min.

    Note: After overlaying the inner dome with the outer matrix on ice, the gradual transition to 37°C will affect the structure of the collagen fibers. Leaving for a longer time at 20°C–22°C before putting it in the incubator will create longer and thicker fibers.

20. Transfer chamber slide to tissue culture incubator set at 37°C for 45 min.
21. Pipette 350 μL of the full growth media of the investigated cells, into chamber wells.
22. Media changes should be performed daily, aspirating media using a micropipette, not vacuum suction, to avoid disturbing the outer matrix.
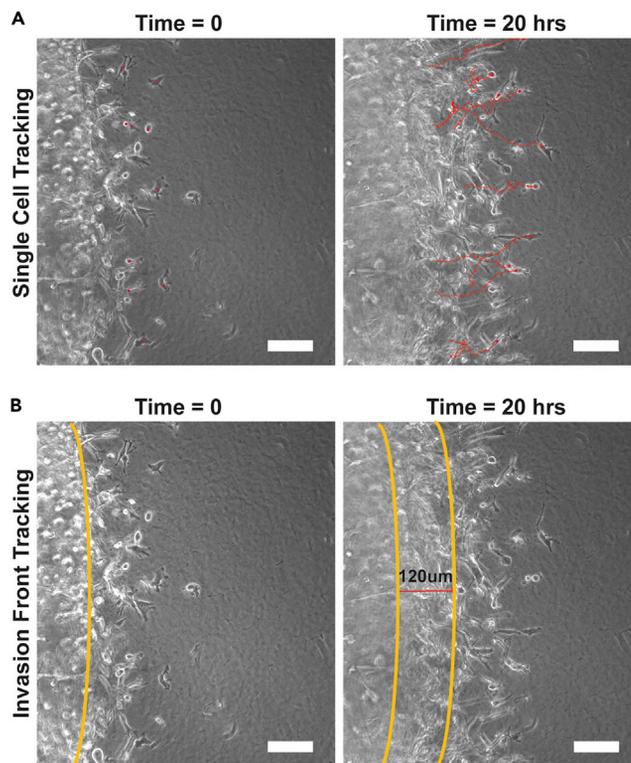
Figure 4. Examples of single cell tracking and invasion front measurement with SUM159 cells
(A) Single cell tracking can be done to generate data for cell speed, trajectory, and path length.
(B) Invasion front analysis can be done to measure the distance the dome wall invades outward. Scale bar is 100 μm.

    a. For researchers performing drug treatments, drug compounds can be added to the growth media when plating.

Live-cell imaging and analysis

   ⏱ Timing: Setup 0.5 h, Run-time: 12–20 h

This step describes a method for live-cell imaging and analysis of invading cells into the outer matrix (Figure 4 & Methods video S1). Objectives with long working distance are recommended for imaging to capture invasion into the outer matrix deeper into the 3D setup. A microscope equipped with an environmental chamber and automated stage is used for live-cell imaging of this assay. The small volume of an 8-well chamber slide format is susceptible to evaporation; therefore, users should carefully monitor media levels and ensure proper humidification in the chamber.

   Note: If live-cell imaging does not apply to the cell type because invasion requires 24–72 h, the user may skip to step 36, "Sample fixation".

23. Define the range and step size of the z-series to capture the height of the inner dome, based on the objective in use.
    a. For example, with the 20× objective, a z-series range of 40–50 μm is typically sufficient to capture the inner dome and a z-step size of 1.5 μm has been used previously (Padilla-Rodriguez et al., 2018) to image invading cells.
24. Determine a time course acquisition appropriate to capture the migration of cancer cells. For SUM159 cells an acquisition of 1 frame/5 min for 20 h captures the invasion from the inner dome to the outer matrix.

25. Using an image processing software, such as NIS Elements or ImageJ, manually track the invading cells to analyze their speed and directionality (Figure 4A) or measure the shift of the invasion front from the initial to final time point (Figure 4B).

Optional: Remove the chamber slide lid and fill the chamber well with serum containing media so that it contacts the 22 × 50 mm glass coverslip, allowing capillary pressure of the media to secure the coverslip to the chamber slide. This reduces evaporation of the growth media during long-term imaging. Additionally, this minimizes the meniscus of the media by reducing the concavity, thereby preventing the optical distortions that occur during brightfield imaging through an air-water interface in small volumes (Horn and Zantl, 2006). It is important to note that this method cannot be used with drug treatments because it will cause the drug containing media to infuse into the neighboring well.

Optional: Stain cells with Hoechst 33342 8–12 h to allow for binary tracking instead of manual.

Note: Acquisition parameters including exposure time, range of the z-series, z-step size, and the image acquisition interval must all be carefully considered if using fluorescent light. Fluorescent light is toxic to living cells and over exposure will result in phototoxicity. An example of live-cell time-lapse imaging is provided (Methods video S1).

See Methods video S1 for time-lapse of cell tracking analysis for SUM159 cells in the in vitro invasion assay.

Live-cell quantification using NIS elements

⏱ Timing: Variable

This section describes a method to track invading cells into the outer matrix using Nikon's NIS Element software. This section examines SUM159 cells over a 20 h image acquisition.

26. Open file to be analyzed and split multipoints so each point can be examined independently.
27. Crop image using the import/export option and selecting Crop ND Document.
    a. Under "Time dimensions" type '5' into the "Step" and select "Crop".
    b. This will crop the file into 25 min increments. This can be adjusted based on user needs.
28. Open the Tracking module through Analysis Controls.
29. Under Tracking module select "Define new ROI".
30. Using the circle function, create an ROI on the cells you want to track.
    a. Select cells that will stay in the field of view throughout the video.
31. Can modify ROI by right clicking to select "ROI Properties".
32. Manually track the ROI to the center of each cell for each frame.
33. The data will be collected under the Tracking module.
34. Export all data to excel.
35. Repeat for each multipoint.

Sample fixation

⏱ Timing: 30 min

This section describes a method to fix the chamber slide for creating a large-stitched image with a z-stack of the entire chamber well.

36. Prewarm sterile 1× PBS in 37°C water bath.
37. Prepare 4 mL of 4% paraformaldehyde (PFA) in 1× PBS and warm to 37°C in water bath.

38. Remove chamber slide from cell culture incubator and place on a heating block set to 37°C.
39. Carefully aspirate growth media from chamber wells using a micropipette and wash each chamber well with 400 µL of warm 1× PBS.
40. Aspirate PBS and pipette 400 µL of warm 4% PFA into each chamber well. Fix at 37°C for 10 min.
41. Prepare 4 mL of a 1 µg/mL solution of DAPI in sterile 1× PBS and keep solution in the dark at 20°C–22°C.
42. Aspirate PFA solution and wash with 1× PBS.
43. Aspirate PBS and pipette 400 µL of DAPI solution into each well. Incubate 8–12 h at 4°C, keeping the chamber slide in the dark. To prevent evaporation, secure parafilm around the chamber slide.
44. The following day, aspirate DAPI solution from chamber wells and add 400 µL of 20°C–22°C 1× PBS. Can be kept in 1× PBS until imaged.

Note: Staining of the actin cytoskeleton using fluorescently labeled Phalloidin may also be performed in parallel with DAPI.

CAUTION: Paraformaldehyde is toxic, corrosive, and carcinogenic. All steps involving PFA should be performed in a fume hood with proper PPE. Discard PFA waste in a manner consistent with regulations.

Fixed invasion assay imaging and quantification

⊙ Timing: Variable

This step describes the methodology for imaging chamber wells that have been fixed and stained with DAPI and performing image analysis.

45. A large-stitched image is required to capture the inner dome and the surrounding outer matrix within 500 µm of the inner dome. Importantly, the z-dimension range is determined visually by moving through z at the intersection of the inner dome and outer matrix until no more cells invading into the outer matrix are visible.
    a. This will vary depending on the height of the inner dome and can range from 40–80 µm.
46. Acquisition of both brightfield (DIC) and fluorescence (DAPI) will be necessary for the analysis as described below.
47. To process the image stack, first generate a maximum intensity projection image using the z from the large-composite z-series of DAPI. Using the maximum projection image, generate a binary mask by thresholding the DAPI signal.
    a. Adjust the thresholding histogram of the DAPI channel to delineate individual nuclei of cells that have invaded into the outer matrix (Figure 5A).
48. Create a binary ROI of the entire initial inner dome using the DIC channel (Figure 5B). The boundary of the inner dome can be distinguished in the DIC channel by identifying the boundary where cells transition from a cluster to invasive.
    a. The chamber well can also be imaged as mentioned above after initial plating to use as a reference.
49. Using the binary operation tools, subtract the ROI of the cells within the dome from the DAPI binary mask of the invaded cells in the outer matrix (Figure 5C).
50. Additional manual refinement of the DAPI binary mask may be necessary to segment single cells.
51. Quantify the number of invaded cells by using an object count module in image processing software, such as NIS Elements or ImageJ.

Note: To minimize XYZ drift during acquisition, acquire the images in this order: 1) acquire lambda, 2) acquire z-series, and 3) acquire large image, thereby ensuring proper alignment of brightfield and fluorescent channels across the entire large-composite z-series.
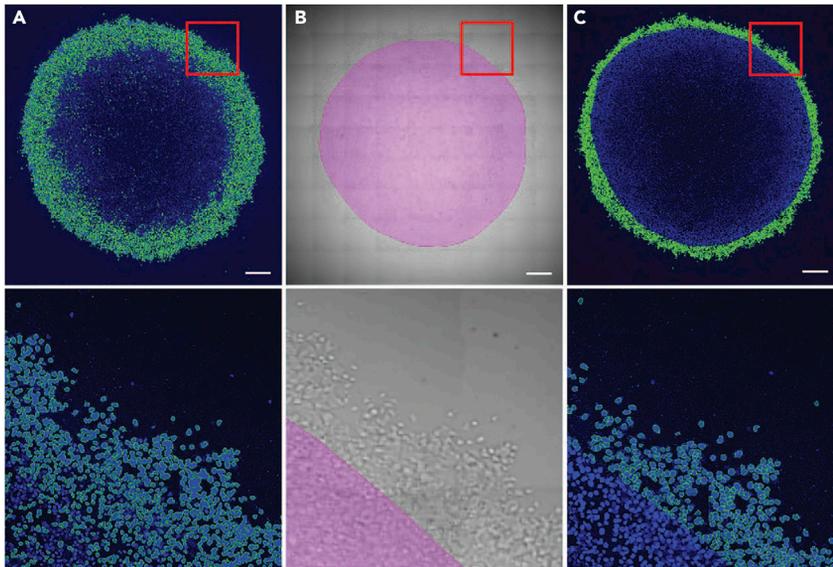
Figure 5. Image analysis and quantification workflow for MCF7 cells

(A) Using a maximum intensity projection in z of DAPI generate a binary mask by thresholding the DAPI signal and adjust thresholding histogram of the DAPI channel to delineate individual nuclei of cells that have invaded the outer matrix. Bottom panel shows zoomed in area.

(B) Using the brightfield channel, create a mask around the inner dome of embedded cells. Bottom panel shows zoomed in area.

(C) Subtract the inner dome mask from the DAPI mask to create a layer identifying all cells that have invaded into the outer matrix. Object count can be used to quantify number of invaded cells. Scale bar is 500 μm.

Fixed-cell analysis using NIS elements

> ⏱ Timing: Variable

This section describes a method to quantify invading cells in the outer matrix using Nikon NIS Element software (20 h file).

52. Open large-stitched image of whole chamber well.
53. Create Maximum Intensity Projection in Z using ND Processing.
54. Threshold using the DAPI channel to create a binary layer (Figure 5A).
    a. Use the Binary Toolbar to adjust the binary layer to cleanly identify the cells invading into the outer matrix.
    b. Save the binary layer.
55. Create a "Non-Invaded" binary layer by using the Binary Toolbar to draw a circle around the edge of the inner dome (Figure 5B). Save the "Non-Invaded" binary layer.
56. Create an "Invaded" binary layer by using the Binary Operations to subtract the "Non-invaded" binary layer from the DAPI binary layer (Figure 5C).
57. Adjust the "Invaded" binary layer using the Binary Toolbar to make sure that the segmentation includes single cells. This is especially relevant for cells that invade as clusters.
58. Use the "Object Count" feature to count the number of invaded cells.

EXPECTED OUTCOMES

The described protocol has several advantages over existing *in vitro* invasion assays. First, it is exceedingly customizable, allowing the user to modify the biochemical and biophysical properties of the matrix to best suit the requirements of the experiment, and to quantify changes in invasion under specific microenvironmental conditions. Second, the coverglass-bottom chamber format is

compatible with multiple imaging modalities, including live-cell microscopy and confocal imaging of fixed samples. Importantly, while transwell-based assays rely on fixed, endpoint data after a relatively short assay duration, the open-ended experimental design of the presented assay permits researchers to examine invasion for up to 3 days and, using live-cell imaging, to quantify invasion dynamics. These advantages allow users to gain insight into cellular behavior during invasion. Lastly, the image analysis and invasion quantification presented here can be performed using a variety of imaging software, increasing the accessibility of this assay to many potential users. Together, the experimental advantages over existing *in vitro* and *in vivo* methodologies position the 3D *in vitro* invasion assay as a highly amenable platform capable of addressing diverse experimental questions.

Successful execution of this assay will result in quantifiable invasion of cells into the outer matrix. This assay allows a fold change comparison of invasion across multiple conditions. This assay has been used to study the effect of outer matrix stiffness on invasion in MCF7 cells (Puleo et al., 2019). It has also been used to demonstrate the effects of matrix conditioning on invasion in SUM159 cells (Watson et al., 2021). An example of quantification of live-cell invasion through cell tracking is shown in Figure 4 and quantification of the number of invaded cells in an entire chamber well using fixed image analysis is shown in Figure 5.

LIMITATIONS

Since this protocol is highly customizable, it requires several important optimization steps to fit the experimental parameters under investigation; therefore, it is not an "off-the-shelf" invasion assay, such as the Boyden chamber assay. Variables that will require optimization include the number of cells embedded in the inner dome, the overall incubation time, and the imaging parameters. Although acquisition of the 3D invasion assay can be done on various microscopes with multiple objectives, and utilize many software modules for analysis, some optimization may be necessary. Due to the structure of the outer matrix, tracking cells may be difficult if the cells move outside the acquired z range. Creating the large-stitched image for each chamber well may be time consuming depending on the total number of wells, the scan area, and the efficiency of the software for stitching. The quantification may also be time consuming due to the accuracy of the binary masking, which will dictate how much manual object segmentation may need to be done by hand. Or in some cases, if the software does not have the ability to mask on intensity, the segmentation and counting may be entirely manual.

TROUBLESHOOTING

Problem 1

The inner dome or outer matrix does not polymerize.

Steps 16–17 (inner dome) and 19–20 (outer matrix) from section "embedding cancer cells in inner dome and overlaying outer matrix".

Potential solution

In addition to temperature, two variables influence the polymerization of the collagen – pH and PEG concentration. Ensure the pH is around 7.0 by using more granular pH indicators and fresh 0.1 M NaOH solution. Importantly, slightly acidic environments create shorter fibers, and basic longer fibers. In addition, double check the final concentration of PEG; PEG can alter gelation at higher concentrations, in part by altering the pH.

Problem 2

There are bubbles (and/or microscopic bubbles) forming at the interface between the inner dome and outer matrix.

Steps 17–18 from section "embedding cancer cells in inner dome and overlaying outer matrix".

Potential solution

In addition to possible bubbles in the gels (which are remedied by longer degassing time $\geq$ 10 min), there are bubbles that form at the interface between the two matrices. We have noticed that the difference in temperature leading to air expansion is the major cause of this problem. To mitigate this issue, extend the time for chilling the inner dome at 20°C–22°C, then on ice, after removing it from the incubator to overlay it with the outer matrix.

Problem 3

Invasion is not observed after 72 h.

Potential solution

Each cell line behaves differently in response to 3D collagen matrix and not all cell lines possess invasive potential. Nonetheless, to boost invasion, increase the concentration of the chemoattractant in the outer matrix or use a different reagent/growth factor. On the other hand, the outer matrix may be too stiff, reducing PEG concentration to decrease the amount of crosslinking may help. For example, as described above, the prostate cell lines exhibited invasion at 10-fold lower PEG concentration, compared that concentration used for breast cancer cells.

Problem 4

The inner dome is contracting and tearing away from the outer matrix. This will be evident the next day after plating the experiment.

Potential solution

This issue is seen with mesenchymal like cells that are highly contractile, such as SUM159 and MDA-MB-231; These cells "pull in" the collagen of the inner dome resulting in separation of the inner dome and outer matrix. To mitigate this problem, reduce the number of cells embedded in the inner dome incrementally by 25%–30%.

Problem 5

Drifting in the z and xy dimensions during live-cell imaging.

Potential solution

During acquisition of the z-stacks for live-cell imaging of invasion, a drift might occur especially in the z dimension, leading to a major erroneous change in the translocation of the cells. If this drift occurred in the early time points and then stabilized, it is most probably caused by a change in temperature in the culture; this is especially common when the cells are transported from the tissue culture facility to the microscope (which is usually in a separate room). To solve this issue, wait about 15 min before starting to set up the time lapse acquisition protocol. On the other hand, if the drift was not avoidable (due to problems in the microscope hardware or software), a drift correction should be applied before proceeding to image analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ghassan Mouneimne, gmouneimne@arizona.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any datasets/code.

AUTHOR CONTRIBUTIONS

S.M.H. optimized assay conditions, created image analysis figures and examples of assay, and wrote and revised the manuscript. M.P.R. provided initial protocol and created illustrations used in manuscript. A.C. optimized conditions for prostate and macrophage cell lines. J.A.S. contributed to the execution of experiments, image analysis, and editing the manuscript. All authors reviewed the manuscript. S.S.P. contributed to editing the manuscript. N.A.W. contributed to editing the manuscript. G.M. was responsible for conceptualization of methods, securing funding, and editing the manuscript.

DECLARATION OF INTERESTS

S.M.H. is an employee of Roche Diagnostics. G.M. has equity in MeCo Diagnostics LLC. M.P.R., A.C., J.A.S., S.S.P., and N.A.W. have no declarations of interests.

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