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# Time-resolved fluorescence imaging and analysis of cancer cell invasion in the 3D spheroid model

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**TITLE:**

# Time-Resolved Fluorescence Imaging and Analysis of Cancer Cell Invasion in the 3D Spheroid Model

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**KEYWORDS:**

cancer spheroid, invasion, fluorescent labeling, longitudinal imaging, time-lapse microscopy, microfabrication

**SUMMARY:**

Presented here is a protocol for the fabrication of a spheroid imaging device. This device enables dynamic or longitudinal fluorescence imaging of cancer cell spheroids. The protocol also offers a simple image processing procedure for the analysis of cancer cell invasion.

**ABSTRACT:**

The invasion of cancer cells from the primary tumor into the adjacent healthy tissues is an early step in metastasis. Invasive cancer cells pose a major clinical challenge because no efficient method exist for their elimination once their dissemination is underway. A better understanding of the mechanisms regulating cancer cell invasion may lead to the development of novel potent therapies. Due to their physiological resemblance to tumors, spheroids embedded in collagen I have been extensively utilized by researchers to study the mechanisms governing cancer cell invasion into the extracellular matrix (ECM). However, this assay is limited by (1) a lack of control over the embedding of spheroids into the ECM; (2) high cost of collagen I and glass bottom dishes, (3) unreliable immunofluorescent labeling, due to the inefficient penetration of antibodies and fluorescent dyes and (4) time-consuming image processing and quantification of the data. To address these challenges, we optimized the three-dimensional (3D) spheroid protocol to image fluorescently labeled cancer cells embedded in collagen I, either using time-lapse videos or longitudinal imaging, and analyze cancer cell invasion. First, we describe the fabrication of a spheroid imaging device (SID) to embed spheroids reliably and in a minimal collagen I volume, reducing the assay cost. Next, we delineate the steps for robust fluorescence labeling of live and fixed spheroids. Finally, we offer an easy-to-use Fiji macro for image

processing and data quantification. Altogether, this simple methodology provides a reliable and affordable platform to monitor cancer cell invasion in collagen I. Furthermore, this protocol can be easily modified to fit the users' needs.

**INTRODUCTION:**

During the cancer progression, cancer cells can acquire a motile and invasive phenotype, enabling them to escape the tumor mass and invade into the surrounding tissues[1]. Eventually, these invasive cancer cells can reach and grow inside secondary organs, a process called cancer metastasis[1]. Metastasis causes more than 90% of cancer-related deaths[2]. One reason for this is that, while localized tumors are clinically manageable, no efficient methods exist for the elimination of invasive cancer cells once metastatic spreading has occurred. Therefore, the emergence of invasive cancer cells and the transition from a localized to an invasive disease is posing a major clinical challenge. Determining how cancer cells initiate and sustain an invasive behavior may lead to the development of novel potent therapies.

The 3D spheroid model is an ideal platform to investigate the motile behavior of cancer cells under controlled, yet physiologically relevant conditions[3]. Indeed, in this assay, spheroids of cancer cells are embedded inside extracellular matrix (ECM), for example collagen I, which mimics a simplified tumor. Then, imaging is used to visualize the invasion of cancer cells from the spheroid into the collagen matrix. However, multiple challenges limit this procedure.

The first challenge occurs at the embedding step, where the liquid collagen matrix can spread across the dish surface, causing the spheroid to touch the bottom of the dish. Consequently, cells from the spheroid spread on the two-dimensional (2D) surface, breaking the three-dimensional (3D) spheroid morphology. Increasing the volume of collagen is an efficient, but costly solution. To prevent cells from spreading on the 2D surface, while maintaining a minimal volume of collagen, we developed a spheroid imaging device (SID) by bounding a 1 mm-thick, 3-hole polydimethylsiloxane (PDMS) insert onto a glass bottom dish.

The second challenge of the spheroid assay is the labeling of cancer cells in spheroids, which is limited by the poor penetration of antibodies and fluorescent dyes, an effect that increases with the spheroid size. While the ideal solution for labeling cells is the establishment of cell lines stably expressing fluorescent protein(s), this option is mostly restricted to immortalized cell lines and is limited by the availability of fluorescent protein chimeras. Here, we describe an optimized protocol for immunofluorescence staining of fixed spheroids, as well as the efficient use of a cytoplasmic dye to label cells immediately before embedding the spheroid.

The third challenge of the spheroid assay is the lack of simple Fiji macros for semi-automated quantification of cell invasion over time. To address this challenge, we describe a simple methodology to analyze the spheroid area over time. We illustrate the advantages of this protocol using the 4T1 and 67NR cell lines as examples.

**PROTOCOL:**

**1. Fabrication of a Spheroid Imaging Device (SID) to optimize spheroid embedding (Duration 1 day)**

1.1. Create the spacer using a 3D printer (**Figure 1A** and **Supplementary File 1**).

1.2. Weigh out a 10:1 (wt/wt) ratio of base polymer:crosslinker in a plastic cup [e.g., 20 g of ethylbenzene base polymer and 2 g of silicone resin crosslinker to create polydimethylsiloxane (PDMS)].

1.3. Thoroughly mix the PDMS solution in the plastic cup using a disposable pipette.

1.4. Place the plastic cup into a vacuum chamber to remove the air bubbles from the mixture. Quickly release the vacuum pressure to remove the small amount of air trapped at the surface of the mixture and dissipate the remaining air bubbles.

1.5. Incubate the 3D printed spacer at 100 °C for 5 min to increase its flexibility.

1.6. Clean the two glass plates that will be used to construct the PDMS mold thoroughly by wiping them with 100% isopropanol. If the glass plates were previously used to cast PDMS, be sure to remove any remaining old PDMS by gently scraping the glass plates with a razor blade and wiping them with 100% isopropanol.

1.7. Construct the mold by placing the 3D printed spacer flush in-between the two clean glass plates.

1.8. Seal the mold using large binder clips on the outside edges of the glass plates. Place two binder clips on the bottom edge and one on the top corner (**Figure 1B**).

1.9. Inspect the top part of the mold to ensure that the spacer is flush with the glass plates. This guarantees that no deformations will be present and that an even sheet of PDMS will be created.

NOTE: If the resulting sheet is not of uniform thickness, the clips need to be adjusted slightly.

1.10. Cut the tip of a disposable pipette (~2 cm from the tip) and add the PDMS mixture at a slow and constant rate to the top left corner of the mold. Pour the mixture slowly to prevent the creation of large air pockets.

1.11. Place the mold into a vacuum chamber to remove air bubbles that formed during the pour.

1.12. Cure the PDMS by incubating the mold at 100 °C for 1 h.

1.13. Retrieve the mold from the incubator and allow it to cool to touch.

1.14. Remove the binder clips and glass plates from the spacer containing the cured PDMS.

1.15. Use a razor blade to cut through the seal that was created on all four sides of the mold between the spacer and the glass plate. With all four sides cut into, begin pulling apart the mold to reveal the PDMS sheet in the spacer.

1.16. Carefully peel the new PDMS sheet off of the spacer using tweezers.

1.17. On a cutting mat, punch out 17.5 mm diameter PDMS disks from the sheet, and then punch three evenly distributed 5.5 mm diameter holes, using different size biopsy punches. Here these 3-hole PDMS disks are referred as "inserts" (**Figure 1C**).

NOTE: Nonuniform cuts made into the PDMS, or a faulty bind via plasma treatment, may result in the future leaks.

1.18. Clean each insert by gently removing any dust particles using tape.

1.19. Stick the inserts onto a piece of double-sided tape and wrap the tape around the lid of a 10 cm Petri dish.

1.20. Place the lid of the Petri dish in the plasma machine, along with the open 35 mm glass bottom dishes.

1.21. Activate the surface of the inserts and of the glass via plasma treatment for 1 min at 300 mTorr. A hand-held plasma wand can be used.

1.22. Using tweezers, quickly attach the upside, i.e., treated side, of one insert (**Figure 1D**) to the glass part of a glass bottom dish (**Figure 1E**). Repeat for all dishes.

1.23. Use pointer finger and thumb to apply an even pressure while rotating the glass bottom dish. This will ensure stable securement of the insert to the glass bottom dish.

1.24. Incubate the SIDs (**Figure 1F**) at 60 °C for 20 min to strengthen the adhesion between glass and PDMS.

1.25. Perform a second round of plasma treatment on the SIDs, using the same settings as in step 1.21 or with the hand-held wand. This will render the free PDMS surface adhere to the poly-L-lysine in the coating solution (see 1.26).

1.26. Freshly prepare the following solutions.

1.26.1. Coating solution: 1x PBS containing 0.01% (vol/vol) poly-L-Lysine [e.g., add 10 µL of 0.1% (vol/vol) poly-L-Lysine to 90 µL of 1x PBS].

1.26.2. Crosslinking solution: Distilled water containing 1x (vol/vol) glutaraldehyde [e.g., add 10 µL of 10x glutaraldehyde to 90 µL of distilled water].

1.26.3. Storage solution: 1x PBS containing 10x (vol/vol) Penicillin-Streptomycin [e.g., add 1 mL of 100x Penicillin-Streptomycin to 9 mL of 1x PBS].

1.27. Add 35 µL of coating solution per each hole and incubate for 1 h at room temperature.

1.28. Aspirate the poly-L-Lysine and rinse the entire SID 3 times with distilled water.

1.29. Add 35 µL of crosslinking solution per each hole incubate for 30 min at room temperature.

1.30. Aspirate the crosslinking solution and rinse the entire SID 3 times with distilled water.

1.31. Add 70% ethanol into each SID and place under ultraviolet (UV) light for 30 min.

1.32. Under the hood, aspirate ethanol and rinse the SID 3 times with distilled water.

1.33. Add 2.5 mL of storage solution per each SID.

NOTE: At this stage, the SIDs can be stored at 4 °C for a week. It was observed that the binding strength between PDMS and glass decreases over time. Beyond a week, users are recommended to test the SIDs for potential leakage before use. To do so, aspirate the storage solution and add 35 µL of 1x PBS into each hole. After use, PDMS inserts can be peeled off the glass bottom dishes. To achieve optimal cleaning of the glass bottom dishes, several washes with isopropanol and hydrochloric acid should be used to remove PDMS residues.

## 2. Spheroid formation and embedding into collagen (Duration 4 days)

NOTE: For live imaging of spheroids, longitudinally or in time-lapse videos, use a cell line expressing a cytoplasmic and/or nuclear fluorescent protein. If such a cell line is available, follow the steps described in this section. Alternatively, in the section 3, a protocol is proposed to label cancer cells in spheroids using a cytoplasmic dye.

2.1. Form spheroids of 4T1 and/or 67NR cells using the hanging drop technique, as previously described[4–7], with 3,000 cells/40 µL droplet and a 3-day incubation time. Add the bovine atelocollagen I solution last and maintain all solutions on ice, at all time.

2.2. Identify the correctly formed spheroids using a bright-field microscope.

2.3. Fill a 15 mL conical tube with 8 mL of pre-warmed complete medium.

2.4. Collect spheroids with a P1000 pipette and transfer to the 15 mL conical tube. Wet the pipette tip by pipetting some complete medium in and out to prevent spheroids from sticking to the inside walls of the pipette tip and limit spheroid loss.

2.5. Allow spheroids to sink to the bottom of the tube and carefully wash the spheroids by exchanging the medium. Repeat twice.

2.6. Prepare a 5 mg/mL collagen I solution according to the manufacturer's recommendations (alternate gelation procedure, see exemplary calculation below). Replace the distilled water with complete medium. When calculating the volume of medium to be used, take into account that spheroids will be added in 20 µL of complete medium [e.g., for one SID, 3 x 30 + (3 x 30) x 20 % = 108 µL of 5 mg/mL collagen I is needed (prepare 20 % extra to account for pipetting loss when handling viscous fluids); 22 µL of complete medium + 10.8 µL of 10x PBS + 1.2 µL of 1 M NaOH + 54 µL of 10 mg/mL collagen I stock].

2.7. Collect all spheroids in 20 µL of medium, using a P200 pipette, and add them to the collagen I solution prepared in step 2.6.

2.8. Slowly mix the solution by pipetting up and down to avoid heterogeneity in the collagen I concentration, limiting bubble formation. Keep the solution on ice.

2.9. Remove the storage solution from the SID(s) and wash 3 times with 3 mL of 1x PBS. After the final wash, leave the SID(s) dry so not to dilute the collagen I solution.

2.10. Start a timer and dispense 30 µL of the collagen I solution containing one spheroid into one of the three holes of the SID. Visually ensure that a single spheroid is contained in the 30 µL.

2.11. Repeat step 2.10 twice more to fill all the 3 holes of a SID.

2.12. Use a 10 µL pipette tip to re-center the spheroid if it is located close to the PDMS border. If two or three spheroids end up dispensed in one of the holes, same pipette tip can be used to separate the spheroids from each other. Stop the timer.

NOTE: Frequently inverting of the SID upside-down and upside-up, throughout the period of the collagen I polymerization and solidification, ensures that the spheroid is positioned at the vertical center of the ECM layer, and prevents invasion of cells in 2D. The frequency of inverting (flipping) should be maximized. As the flipping frequency is controlled by the spheroid "dispensing time" measured in 2.10-2.12, the dispensing time should be minimized. In our lab, dispensing time is 2 min on average.

2.13. To vertically center the spheroid in the collagen layer, flip the SID upside-down and incubate at 37 °C for dispensing time.

2.14. Flip the SID upside-up and incubate at 37 °C for dispensing time.

NOTE: The length of time that the spheroids spend in the upside-down orientation should be equal to the time spent in the upside-up orientation.

2.15. Repeat steps 2.13 and 2.14 for 30 min, until the collagen I polymerizes.

2.16. Add 2.5 mL of complete medium/SID and if needed, acquire an image of the spheroids for the initial timepoint.

2.17. Repeat steps 2.10-2.16 if multiple SIDs are used.

## 3. Fluorescence labeling of spheroids

3.1. Live imaging (Duration 6-7 days)

NOTE: If a cell line expressing a cytoplasmic and/or nuclear fluorescent protein is available, follow the steps described in the section 2. Alternatively, for cytoplasmic labeling, the following protocol is proposed.

3.1.1. Follow steps 2.1-2.5 of the protocol described in the section 2.

3.1.2. Dilute the cytoplasmic dye to 25 µM in 200 µL of serum-free medium.

NOTE: While a red cytoplasmic dye is used in this experiment, any other available color should be suitable. Cancer cells were labeled inside spheroids using nuclear dyes diluted to 20 µM in 200 µL of serum-free medium (see **Table of Materials**).

3.1.3. After the final wash, resuspend spheroids in the cytoplasmic or nuclear dye solution and incubate at room temperature for 20 min, protected from light.

NOTE: With this approach, labeling of the cells in the spheroid center will not be efficient. To achieve labeling of all cells, incubation can be extended overnight, by placing the dish on a rocker. Alternatives are described in Discussion.

3.1.4. Wash spheroids 3 times in complete medium.

3.1.5. Proceed to steps 2.6-2.17 of the protocol described in the section 2.

3.1.6. Image spheroids via time-lapse imaging every 10 min for 24-72 h (**Figure 2**), or via longitudinal imaging, daily, for up to 7 days (**Figure 3**). Use a laser scanning confocal microscope, 10x air objective (0.4 numerical aperture and 3.1 mm working distance), 1024 x 1024 pixels, exposure time 8 µs/pixel, pinhole 90 µm, 6 x 15 µm z-steps and 3 fields of view-each containing one spheroid.

NOTE: For time-lapse imaging, use minimal laser power and equip the microscope with an environmental chamber with temperature, humidity and gas control. Culture the embedded spheroids at 37 °C for > 8 h or overnight prior to imaging to minimize the time on the microscope.

3.2. Immunofluorescence staining of spheroids (Duration 2 days)

NOTE: The procedure described here is adapted and optimized from previously published protocols[8,9]. This method can be used after the protocol outlined in the sections 2 and 3.1.

3.2.1. Freshly prepare the following solutions.

3.2.1.1. Fixing solution: 1x PBS containing 4% PFA (vol/vol) [e.g., add 5 mL of 16% (vol/vol) PFA to 15 mL of 1x PBS].

3.2.1.2. Fixing and permeabilizing solution: 1x PBS containing 4% PFA (vol/vol) and 0.5% Triton X-100 (vol/vol) [e.g., add 50 µL of Triton X-100 to 10 mL of fixing solution].

3.2.1.2. Blocking solution: 1x PBS containing 1% FBS (vol/vol) and 1% BSA (wt/vol) [e.g., dissolve 100 mg of BSA in 10 mL of 1x PBS, then add 100 µL of FBS].

3.2.1.3. Washing solution: 1x PBS containing 0.05% Tween 20 (vol/vol) [e.g., add 25 µL of Tween 20 to 50 mL of 1x PBS].

3.2.2. Remove the culture medium from the SID(s) and wash once with warm 1x PBS.

3.2.3. Add 2 mL of fixing and permeabilizing solution per SID and incubate at room temperature for 5 min.

3.2.4. Remove the fixing and permeabilizing solution and add 2 mL of fixing solution per SID and incubate at room temperature for 20 min.

3.2.5. Wash 3 times with the washing solution.

3.2.6. Add 2 mL of blocking solution per SID and incubate at 4 °C for 24 h with mild shaking.

NOTE: At this step, samples can be incubated at 4 °C, over the weekend. Please note that a longer blocking time might interfere with the immunofluorescence labeling procedure.

3.2.7. Dilute primary antibodies in blocking solution.

3.2.8. Add 150 µL of blocking solution with primary antibodies/well in a 48-well plate.

3.2.9. Using fine tweezers, carefully detach the plug of collagen I containing a spheroid and transfer into a well. Repeat if multiple spheroids are labeled. Wipe any liquid that might remain on the tweezers when working with different antibodies, to prevent contamination.

3.2.10. Incubate overnight at 4 °C with mild shaking.

3.2.11. Add 300 µL of washing solution to empty and full wells.

3.2.12. Carefully transfer the plug of collagen I containing a spheroid into a "wash" well.

3.2.13. Wash 3 times with washing solution for 2 h, at room temperature and with mild shaking.

NOTE: Transferring the plugs of collagen I containing a spheroid, instead of aspirating the solutions, can help reducing sample loss and sample damage.

3.2.14. Dilute secondary antibodies, 4',6-diamidino-2-phenylindole (DAPI) and/or phalloidin in blocking solution.

3.2.15. Add 150 µL of blocking solution with secondary antibodies, DAPI and/or phalloidin per well.

3.2.16. Using tweezers, carefully transfer the collagen plug containing the spheroids into the well. Repeat if multiple spheroids are labeled.

3.2.17. Incubate at room temperature for 1 h with mild shaking.

3.2.18. Repeat steps 3.2.11 and 3.2.12.

3.2.19. Wash 3 times with washing solution for 30 min, at room temperature with mild shaking.

3.2.20. Using a razor blade, cut a 3-hole PDMS insert into three parts so that each part contains a hole.

3.2.21. Place two pieces of PDMS onto a microscope slide.

3.2.22. Add a drop of mounting solution using a P200 tip with the end cut off. Prevent bubble formation.

3.2.23. Carefully transfer a collagen I plug into each hole.

NOTE: If the spheroid was positioned at the top of the collagen plug, invert the collagen plug so that the spheroid ends up closer to the cover glass.

3.2.24. Place a coverslip on top and seal using tape.

3.2.25. Let the sample dry at room temperature for 10 min, protected from light.

3.2.26. Store samples at 4 °C, protected from light until imaging.

## 4. Image processing to analyze cancer invasion over time

NOTE: The format required for this macro is a single-channel x,y,t image saved as a .tiff file.

4.1. If multiple z-slices were acquired (*i.e.* x,y,z,t image), open the image in Fiji and select **Image | Stacks | Z Project.** It is recommended to use the **Max Intensity** option. Alternatively, a single z-slice can be used to run the macro. Save the image as a .tiff file.

4.2. Create a separate "Processing" folder on the Desktop.

4.3. Select **File | Save As | Image Sequence**. Use the TIFF format, update the digits number according to the number of frames, check the box to use slice label as file name and select **Ok**. Select the "Processing" folder created in step 2.

4.4. Open one image from the "Processing" folder. It should be an x,y image, corresponding to a single timepoint.

4.5. Select **Image | Adjust | Auto Threshold**. In the dropdown menu select the method **Try all** and check the box for white objects on black background.

4.6. A montage image appears showing the result for each automated thresholding method.

4.7. Identify the best automated thresholding method, for example **RenyIEntropy**.

4.8. To confirm the choice for the automated thresholding method, open any other image from the "Processing" folder and test the chosen thresholding method using **Image | Adjust | Auto Threshold.**

4.9. Close all images.

4.10. Download the SpheroidAreaTime macro (**Supplementary File 2**).

4.11. Open the Fiji macro by drag-and-drop.

4.12. In line 58, update the automated thresholding method as needed.

4.13. In line 62, update the size range according to the cell dimensions.

4.14. Select **Run**.

4.15. Select the “Processing” folder and type in “Processing” as the Parent folder, then select **Ok**.

4.16. Once the run is over, save the table “Summary”. The first column indicates the name of the image; the third column indicates the spheroid area; the sixth, seventh and eight columns specify the parameters for an ellipse fitted onto the spheroid.

4.17. The “Processing” folder now contains a processed image for each time point, with the extension _SpheroidArea.

NOTE: If the spheroid center is dim, fill it with white using the selection tools, for all timepoints, and then proceed to step 3. Similarly, the space around the spheroid can be filled with black to “clean” the image. If the image is clean, comment the line 61 to speed up the run.

**REPRESENTATIVE RESULTS:**

Due to its biocompatibility, PDMS is widely used for microfabrication of confining wells, stamps and molds, which revolutionized micropatterning and microfluidic devices. In the method described here, it is used to create SIDs, customizable wells that optimize spheroid embedding and imaging procedure. **Figure 1** illustrates the major components used in the fabrication of the SIDs. To cast the PDMS mold, a 1-mm thick spacer is 3D printed (**Figure 1A**,**B**), placed between the two glass plates, sealed with large clips. Pouring and baking PDMS in the space between the plates forms an 1-mm thick sheet of PDMS. The SID schematic (**Figure 1C**) indicates the dimensions of the optimal device, however slight variation occurs in the hole distances, due to manual punching of holes using biopsy punches (**Figure 1D**). **Figures 1D,F** indicate clean circular cuts with evenly spaced holes within the PDMS insert.

Using the SIDs facilitates efficient embedding, and hence recording of the invasion of cancer cells inside collagen I using time-lapse (**Figure 2**, **Video 1**) or longitudinal (**Figure 3**) imaging. Despite using the same inversion frequencies for all the SIDs during the collagen I polymerization, spheroid positions inside the collagen plug will slightly vary. Therefore, it is important to use an objective with a long working distance (>1 mm). Otherwise, focusing and imaging may be difficult for spheroids positioned close to the top of the collagen plug. In contrast, spheroids positioned close to the bottom of the collagen plug, will have cells which move to the glass surface and migrate on the glass, instead of invading into the collagen I matrix. Videos of such spheroids need to be discarded. The thickness of the collagen plug, here approximately 800 µm, is controlled by the volume dispensed in each hole of the SID and adjusted to invasion distances spheroids exhibit in this protocol. Thickness of the collagen plug can be lowered by dispensing a lower volume of collagen I in each hole of the SID, when using smaller or less invasive spheroids.

For proper analysis of the invasion using the Fiji macro, it is critical for cancer cells to be properly labeled over the course of the imaging session. As noted in the step 4.17., the image processing step allows for image correction if the labeling is sub-optimal. While we illustrate longitudinal imaging over the course of 6 days (**Figure 3**), which requires the stable expression of cytoplasmic

and/or nuclear fluorescent protein, similar longitudinal imaging could be performed over a shorter time using labeling with dyes.

Following live imaging, we present some results from the immunolabeling procedure for the epithelial cadherin (E-cadherin), cortactin and F-actin (**Figure 4**). In these examples, we used the 4T1 and 67NR cell lines. **Figure 5** shows step-by-step illustration of the image processing procedure using the Fiji macro to measure the area of the spheroid over time.

**FIGURES AND TABLE LEGENDS:**

**Figure 1: Fabrication of the SIDs.** (**A**) Top view schematic representation of the spacer. (**B**) 3D printed spacer. (**C**) Top view schematic representation of the SID. A 3-hole PDMS inserts (**D**) is bound to a glass bottom dish (**E**), creating the final SID (**F**). Dimensions are in millimeters.

**Figure 2: Live imaging of spheroids.** Representative 20 h and 40 h timepoints from Video 1, maximum projection of a 4T1 spheroid. 4T1 cells were labeled using a cytoplasmic dye. Scale bar, 100 µm.

**Figure 3: Longitudinal imaging of spheroids.** Representative micrographs (maximum projection) of a mixed 4T1/67NR spheroid imaged daily. 4T1 and 67NR cells stably express the cytoplasmic mScarlet and green fluorescent protein (GFP), respectively. Scale bar, 100 µm.

**Figure 4: Immunofluorescence imaging of spheroids.** Representative micrographs (maximum projection) of a 4T1 spheroid fixed 2 days after embedding in collagen I. E-cadherin (cyan, A), cortactin (yellow, B) and F-actin (magenta, A and B) were labeled. Scale bar, 100 µm.

**Figure 5: Image processing analysis.** Step-by-step illustration of the image processing procedure using the Fiji macro (**A**) to measure the area of the spheroid over time (**B**).

**Video 1: Representative video of a 4T1 spheroid imaged every 10 min for 46 h and 10 min. 4T1 cells were labeled using a cytoplasmic dye.** Scale bar, 100 µm.

**Supplemental file 1: 3D model of the spacer (STL file)**.

**Supplemental file 2: SpheroidAreaTime macro.**

**DISCUSSION**:

The 3D printed spacer was designed to create 1-mm thick sheets of PDMS that can then be used to easily create various shapes of PDMS, as required by the experimental applications. Due to the simplicity of its fabrication and the freedom to alter the design, this method of PDMS casting was chosen for the initial design of the SID. If high volume of SIDs is required, production can be made more efficient by creating a 3D-printed mold, which already contains PDMS disks with three equally spaced holes, and reducing the process to one step. This would eliminate the need to punch out each disk, along with the subsequent three holes, and decrease the overall preparation time.

While the 3-hole punch is developed for use with 35 mm glass bottom dishes, other sizes are available which allow for more holes and hence, more spheroids to be imaged in parallel. In addition, custom-size cover glass is also commercially available, which, in combination with 3D printed holders, can allow for high-throughput spheroid assays. With such an approach, limiting factor is the speed of data acquisition- for example, in our multicolor time-lapse confocal imaging, acquiring 3D stack of a single spheroid requires approximately 2.5 minutes. Therefore, acquiring 3D stacks for 3 spheroids in the SID requires approximately 8 minutes. As a result, to maintain our preferred frequency of imaging at 10 minutes per stack, we cannot increase the number of holes in the SIDs.

To record and quantify the invasion of living cancer cells in the 3D spheroid model, bright-field imaging can be used[5]. However, fluorescence microscopy is preferred, as it provides increased contrast, and ease and precision in the image processing. If the generation of a cell line expressing a cytoplasmic and/or nuclear fluorescent protein is not possible, we propose the use of the cytoplasmic dyes. As the retention time of cytoplasmic dyes inside cells is three days in our imaging conditions, cells should be labeled following the 3-day period in hanging drop, and immediately before the embedding. Labeling of cells post-embedding may non-specifically label the collagen, and reduce cell labeling. Imaging for longer than 3 days post-embedding requires the use of a different spheroid seeding protocol[10] or cell lines stably expressing fluorescent protein(s).

We successfully formed and imaged spheroids containing anywhere from 60 to 5,000 cells/drop. Small spheroids are ideal for recording invasion over multiple days, as their entire invasion area can easily fit into a single field-of-view of higher magnification (20x-30x) objectives. In addition, they can easily be labeled throughout with cytoplasmic or nuclear dyes. Finally, due to the reduced scattering, each cell in the spheroid can be visualized and segmented. However, small spheroids are barely visible with naked eyes and may require additional labeling with tissue markers. In contrast, larger spheroids are easier to handle, but more susceptible to sinking to the bottom of the dish, due to their weight. Moreover, cells in the spheroid center are not always labeled when using dyes, or visible using confocal microscopy, which has penetration depth of approximately 100 micrometers. To visualize all cancer cells throughout the large spheroids using time-lapse imaging, multiphoton microscopy can be used, offering an extra advantage of collagen fibers visualization by second harmonic generation (SHG) without the need for labeling. Also, imaging can be done with light-sheet microscopy[8,11,12] , providing reduced image acquisition time and hence allowing for high-throughput spheroid assays, but also requiring more data storage and advanced image processing. If time-lapse videos are not required, our labeling procedure for fixed 3D spheroids can be further combined with optical clearing[8,10]. In addition, cryosectioning of the embedded spheroids can eliminate issues with penetration of dye or antibody during labeling, as well as penetration of light during imaging. In our hands, however, successful cryosectioning was limited to early stages of invasion and non-invasive cells, due to the technical challenges in preservation of long and fragile invasive strands.

Our protocol is also compatible with the use of nuclear dyes, to label cancer cells inside the spheroids, enabling single cell tracking of time-lapse data. The Fiji plugin TrackMate[13] can be used

to automate cell tracking and extract motility parameters of single cells, such as velocity, instantaneous speed and persistence.

**ACKNOWLEDGMENTS**:
We would like to thank members of Temple Bioengineering for valuable discussions. We thank David Ambrose at the flow cytometry core (Lewis Katz School of Medicine) for his assistance with cell sorting and Tony Boehm from the IDEAS Hub (College of Engineering, Temple University) for help with the 3D printing. We also thank our funding resources: American Cancer Society Research Scholar Grant 134415-RSG-20-034-01-CSM, Conquer Cancer Now / Young Investigator Award, National Institutes of Health, R00 CA172360 and R01 CA230777, all to BG.

**DISCLOSURES**:
The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Fig1.psd

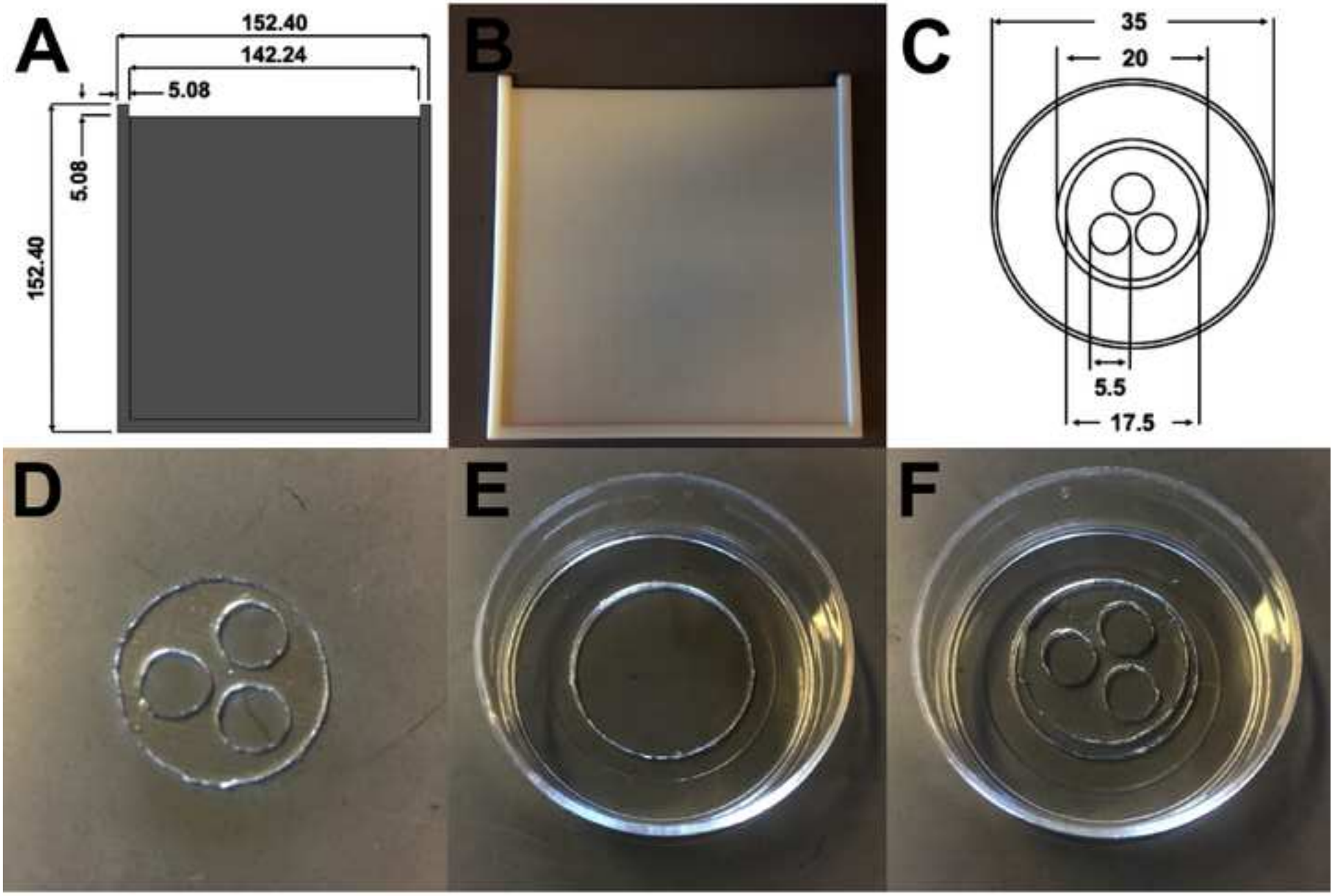


Figure 2

Click here to access/download;Figure;Fig2.psd

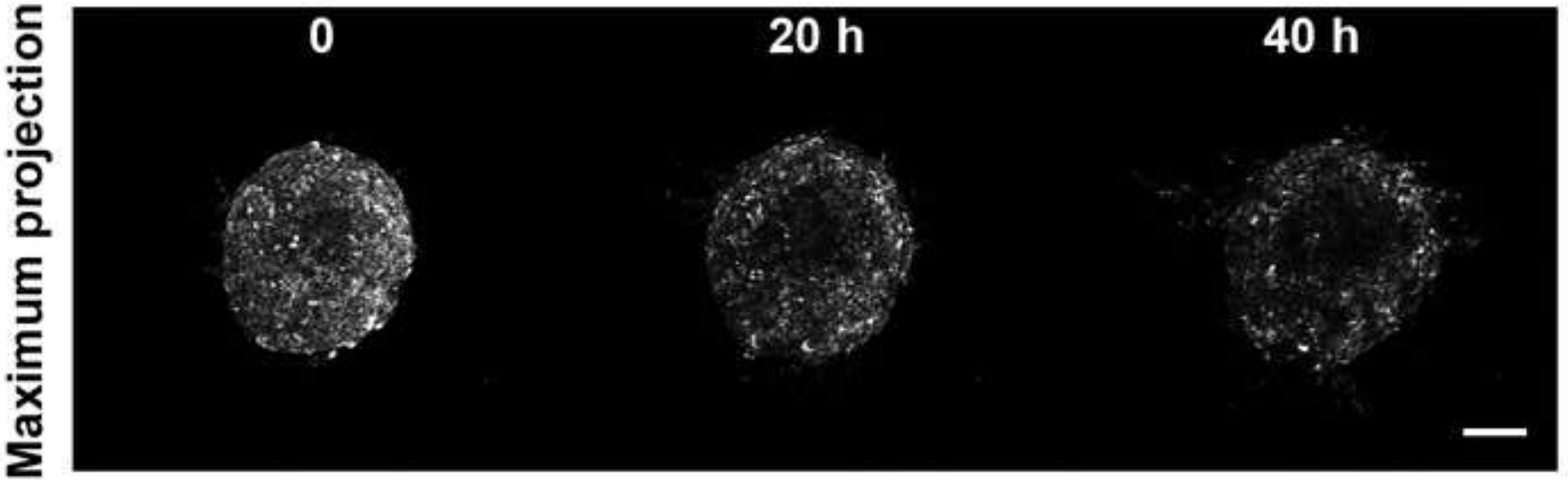


Figure 3

Click here to access/download;Figure;Fig3.psd

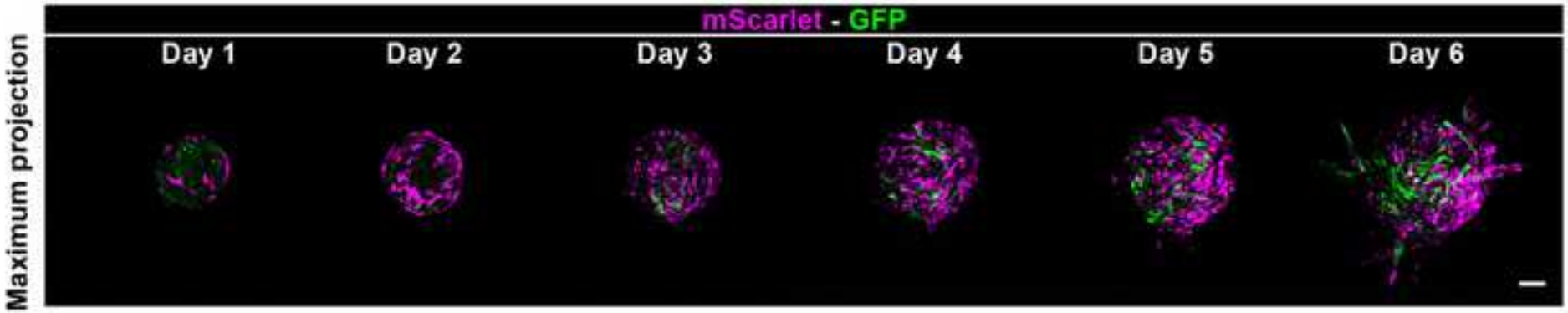


Figure 4



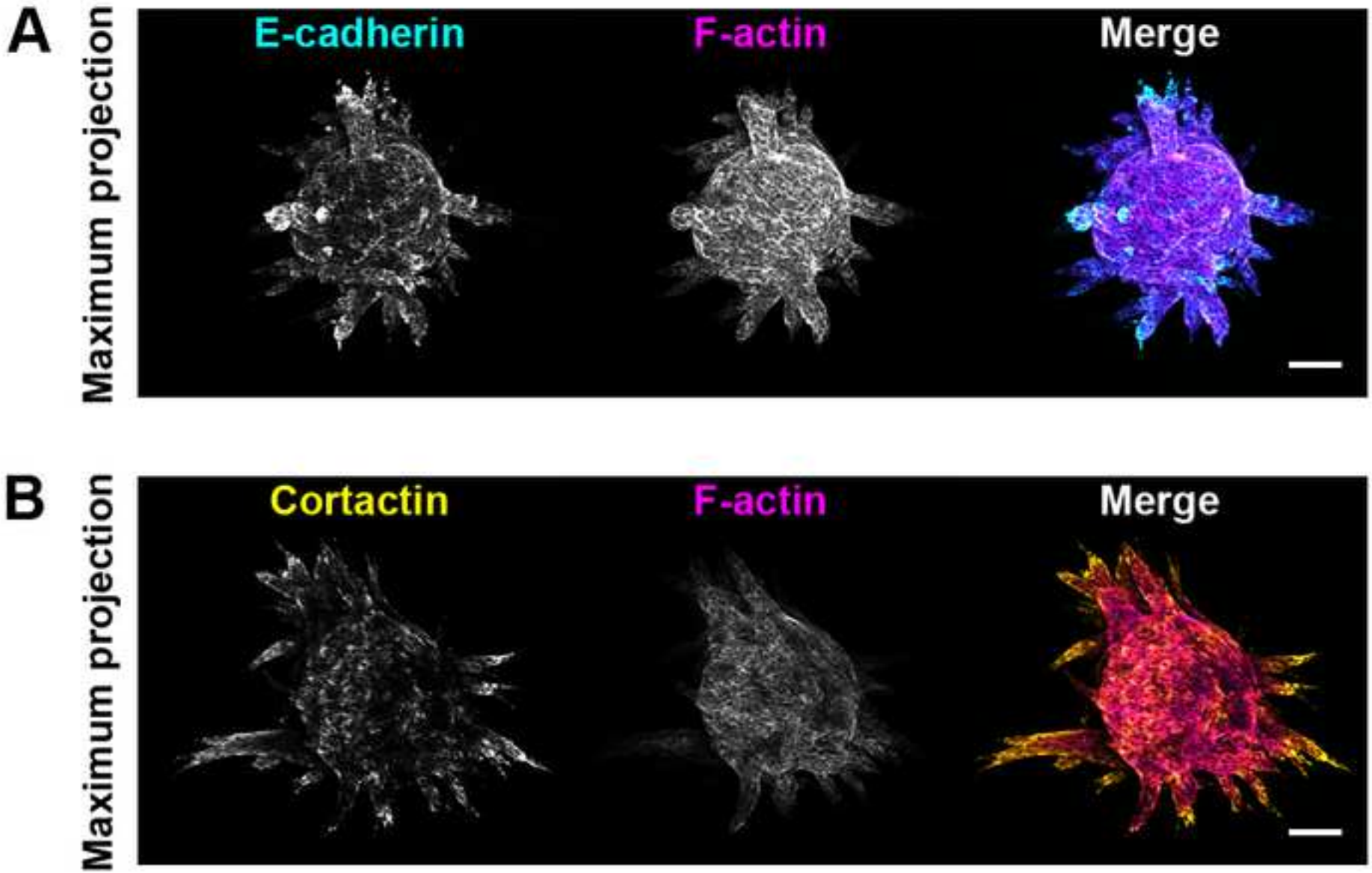
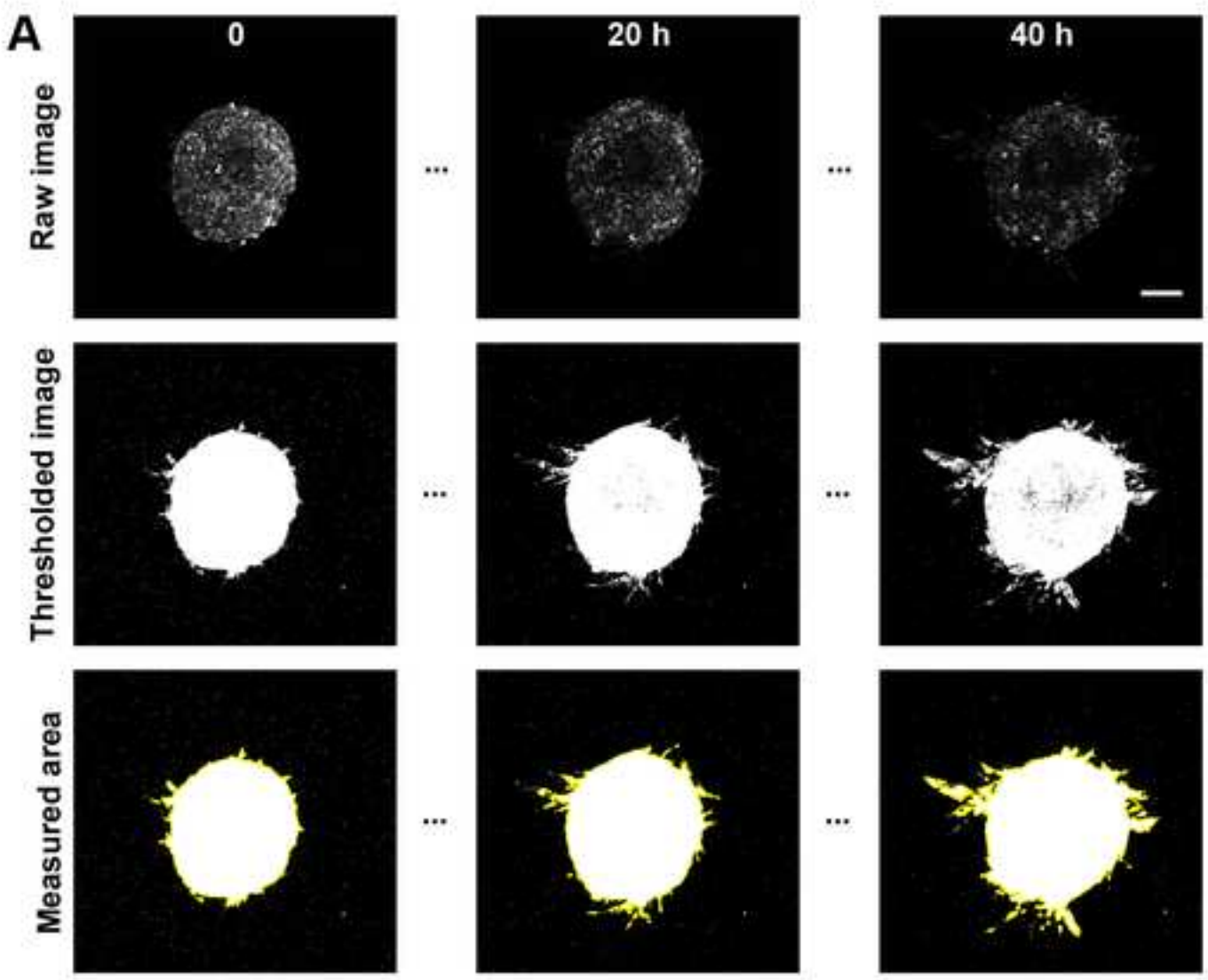


Figure 5






B

Summary

| Slice | Count | Total Area | Average Size | %Area | Major | Minor | Angle |
|---|---|---|---|---|---|---|---|
| MAX_Image0001-C10000.tif | 3 | 83887.923244 | 27962.641081 | 43.167171 | 125.044162 | 107.468478 | 130.933146 |
| MAX_Image0001-C10001.tif | 3 | 83741.374470 | 27913.791490 | 39.377497 | 122.416593 | 108.326508 | 122.791878 |
| MAX_Image0001-C10002.tif | 4 | 83692.010572 | 20923.002643 | 41.978412 | 96.385802 | 81.415383 | 95.712791 |
| MAX_Image0001-C10003.tif | 4 | 83883.288319 | 20970.822080 | 35.272330 | 95.330245 | 82.140674 | 83.764870 |
| MAX_Image0001-C10004.tif | 3 | 83883.290131 | 27961.096710 | 39.485187 | 125.206112 | 107.320814 | 97.797813 |
| MAX_Image0001-C10005.tif | 3 | 83944.991951 | 27981.663984 | 40.129933 | 125.616881 | 107.385955 | 105.397349 |
| MAX_Image0001-C10006.tif | 4 | 83939.563163 | 20982.391791 | 37.312183 | 96.617282 | 81.552030 | 76.064457 |

Video 1

Click here to access/download

**Video or Animated Figure**

Video 1.avi

| Name of Material | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 1 N NaOH | Honeywell Fluka | 60-014-44 | |
| 10X Dulbecco's phosphate-buffered saline (PBS) | Gibco | SH30028.LS | |
| 16% paraformaldehyde (PFA) | Alfa Aesar | 43368-9M | |
| 1X Dulbecco's phosphate-buffered saline (PBS) | Gibco | 20012027 | |
| 4',6-diamidino-2-phenylindole (DAPI) | Invitrogen | D1306 | |
| 48-well plate | Falcon | T1048 | |
| Alexa Fluor 647 phalloidin | Life Technologies | A20006 | |
| Anti cortactin antibody | Abcam | ab33333 | 1 to 200 dilution |
| Anti E-cadherin antibody | Invitrogen | 13-1900 | 1 to 100 dilution |
| Bovine atelocollagen I solution (Nutragen) | Advanced Biomatrix | 501050ML | |
| Bovine serum albumin (BSA) | Sigma Aldrich | A4503-50G | |
| CellTracker Red CMTPX Dye | Invitrogen | C34552 | |
| Conical tubes | Falcon | 352095 | |
| Coverslips | FisherBrand | 12-548-5E | |
| Disposable container | Staples | | Plastic cups |
| Disposable transfer pipette | Thermo Scientific | 202 | |
| DMEM | Fisher Scientific | 11965118 | |
| Double-faced tape | Scotch | | |
| Ethanol | Sigma Aldrich | E7023-500ML | |
| Fetal bovine serum (FBS) | Bio-Techne | S11550 | |
| Fluoromount-G | eBioscience | 00-4958-02 | |
| Glutaraldehyde | Sigma Aldrich | G5882-100mL | |
| Hoescht nuclear stain | Thermo Fischer | 62249 | |
| Isopropanol | Thermo Fischer | S25371A | |
| MatTek dish (glass bottom dish) | MatTek Corporation | P35G-1.5-14-C | |
| Methyl cellulose | Sigma Aldrich | M6385-100G | |

| | | | |
|---|---|---|---|
| MilliQ water | | | |
| Penicilin/streptomycin solution | Thermo Fischer | 15140122 | |
| Petri dish | Corning | 353003 | |
| Pipet tips | Fisherbrand | 02-707 | |
| Pipets | Gilson | F167300 | |
| Poly-L-Lysine | Sigma | P8920 | |
| Primary antibodies, user specific | | | |
| Rat Tail Collagen I | Corning | 47747-218 | |
| Razor Blade | Personna | 74-0001 | |
| Secondary antibodies, user specific | | | |
| Slides | Globe Scientific | 1354W-72 | |
| Sylgard 184 Silicone | Dow Corning | 4019862 | |
| Tape | Scotch | | |
| Triton X100 | Sigma Aldrich | 10789704001 | |
| Tween 20 | Sigma Aldrich | 655204-100ML | |

| Name of Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 100 C and 60 C oven | Fisherbrand | | |
| 10X objective | Olympus | UPLXAPO10X | |
| 3D printer | Stratasys | Object350 CNX2 | |
| Aspirator | | | Use either a building vaccum line or a vacuum pump |
| Binder clips | Staples | 10669 | |
| Biopsy punch (set) | Cole-Parmer Instrument Co. | 06298-90 | |
| Cutting mat | W.A.Pormtan LTD. | CM912GBN | |
| Environmental chamber | Live Cell Instrument | | |
| Fine tweezers | Miltrex | MH18-949 | |
| Glass plates | ChemGlass | CG-1904-17 | |
| Horizontal shaker | Scilogex | 834111019999 | |
| Laser scanning confocal microscope | Olympus | FV1200 | |
| Plasma cleaner | Plasma Etch | PE-25 | Air plasma treatment was used |
| Refridgerator with internal power outlet | Thermo Scientific | TSG12RPGA | |
| Scale | Accuris | | |
| Spacer | | | The 1 mm-thick spacer needs to be 3D printed |
| Timer | FisherBrand | 5004 | |
| UV lamp | | | UV lamp of the cell culture hood can be used |
| VeroWhite | Stratasys | OBJ-03258 | 3D printing material |

**Editorial comments:**

**Changes to be made by the Author(s):**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

>>>We carefully proofread the manuscript.

**2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points**

>>>The manuscript was formatted according to the guidelines.

**3. Please provide at least 6 keywords or phrases.**

>>> "Microfabrication" was added to the list.

**4. Please ensure all abbreviations are defined during the first time use.**

>>>The authors ensured that all abbreviations are defined during the first time use.

**5. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"**

>>>We rephrased the short abstract to 40 words: "Presented here is a protocol for the fabrication of a spheroid imaging device. This device enables dynamic or longitudinal fluorescence imaging of cancer cell spheroids. The protocol also offers a simple image processing procedure for analysis of cancer cell invasion."

**6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets.**

>>>The references in the manuscript were formatted according to the guidelines.

**7. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: MatTek, Nutragen, etc.**

>>>We removed all commercial language from the manuscript and used generic terms instead. All commercial products are referenced in the table of materials.

**8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes. We also cannot have non-numbered steps in the protocol section.**

>>> The manuscript was formatted according to the guidelines.

**9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions**

**should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."**
**10. The Protocol should contain only action items that direct the reader to do something**
**11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.**
**12. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.**
**13. Please ensure you answer the "how" question, i.e., how is the step performed?**
>>>We ensured that the protocol is in the imperative tense with only 2-3 actions per step and that the "how" question was answered for every step of the protocols in the manuscript.

**14. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.**
>>>The protocol is below the 10-page limit. The 3-page limit for filmable content was highlighted in yellow.

**15. Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.**
>>>We expanded the Representative Results section including sub-optimal experiments.

**16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."**
>>>All figures in the manuscript are original.

**17. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.**
>>>Figures legends were updated to include a title and short description.

**18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:**
**a) Critical steps within the protocol**
**b) Any modifications and troubleshooting of the technique**
**c) Any limitations of the technique**
**d) The significance with respect to existing methods**

**e) Any future applications of the technique**
>>> We updated the Discussion to cover the listed points.

**19. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.**
>>>The references in the manuscript were formatted according to the guidelines.

**20. Please sort the materials table in alphabetical order.**
>>>The materials table was modified.

_____

**Reviewers' comments:**

**Reviewer #1:**

**Manuscript Summary:**

**this manuscript by Perrin et al. describes a protocol to generate and visualise 3D cancer cell spheroids, with fixed and live cell imaging. Despite this being a well-used technique, a simple and detailed protocol for labelling and imaging is missing. Therefore, this will represent a valuable resource for researchers interested in cancer cell invasion in 3D systems. The methods are clearly described and easy to follow**

**Major Concerns:**

**none**

**Minor Concerns:**

**It would be beneficial to provide more details about the imaging part (for instance providing acquisition settings for some of the systems that the group used before), highlighting whether different colour cell trackers make any difference (why do they recommend the red one?). Moreover, the cell tracker red is not listed in the material list**

>>>We thank the reviewer for pointing out the lack of details regarding the imaging part. A note containing the detailed imaging parameters was added (see protocol step 3.1.6. for the updates as well as the table of materials). The red cell tracker was added to the list of materials. We did not try using different color for the cell tracker. However, we expect any other available color to be suitable (see protocol step 3.1.2. for the updates).

**Reviewer #2:**

**Manuscript Summary:**

**The manuscript describes a method for time-lapse spheroid imaging embedded in a collagen matrix, using fluorescence confocal microscopy. Some sections of the manuscripts are more or less novel and interesting. The first section on making the PDMS mould for holding the spheroids is of potential interest for the community. The spheroid formation and labelling is much more classical and has previously been reported extensively. The image analysis section is very limited given the data collected, the users will be able to do much more than measuring the spheroid area. One do not need every 10 min time lapse with a complex experimental set-up for such a crude and basic image analysis analysis.**

**Major Concerns:**

**1. As being submitted to Jove, I expected a video showing how to make the PDMS mould (SID) and eventually how to place the spheroids inside the Collagen I and inside the SID. I don't think anyone could reproduce it it from the text provided. It seems very fiddly and complicated and having a video showing the protocol and steps would be helpful. I single picture of the finished product (Figure 1) is definitively not enough. I thought that the supporting video was part of**

**Jove requirements, and that I missed the video, but could not find it anywhere and was very disappointed. A video is needed to illustrate all steps to make the SID.**
>>>We thank the reviewer for addressing the lack of supporting video. According to Jove Author Guidelines, the video production may begin upon manuscript acceptance, and the video will be available to the community together with the revised manuscript. We also thank the reviewer for pointing out the lack of information in Figure 1. We added 2 panels in Figure 1 to better illustrate the fabrication of the SIDs.

**2. The image analysis guide provided is too superficial. As mentioned above, if one do time-lapse microscopy every 10 min at single cell level, they can extract much more information than the spheroid area (this could be obtained by 1 image/day, no time-lapse). The authors should make a strong emphasis on using a nuclear label for time-lapse experiments and cell tracking, which enable single cell tracking (no overlap of nuclei). This is not even discussed in the discussion and must be included. With the video they show, they cannot easily track single cells, but with stable lines labelled with a Histone H2B-fluorescent protein, they could then track single cell velocity, direction, displacement etc by using Trackmate-Fiji. This would need to be included, as otherwise the complex method of sample preparation is not really useful.**
>>>We thank the reviewer for pointing out the superficial imaging processing part. We expanded this section and added the potential use of a nuclear dye to track individual cancer cells and extract motility parameters (see the note following the step 3.1.2. and the last paragraph of the Discussion for the updates).

**Minor Concerns:**
**There are so many details missing everywhere, which makes it impossible to reproduce the technique proposed. It is hard to list them all. Some examples are:**

**STEP 1**
**1. provide the exact model/shape/dimensions for the spacer to be 3D printed. 1mm is not enough**
>>>We thank the reviewer for addressing the missing dimensions of the 3D printed spacer. The dimensions have been added to the protocol as well as a picture of the spacer in figure 1. In addition, we are providing the 3D drawing of the spacer as the Supplemental file 1.

**2. What is the base polymer-crosslinker? Where do you purchase it? which volume do you prepare?**
>>>We thank the reviewer for pointing out the missing details concerning the base polymer/crosslinker in the protocol. The active chemicals and measured weights have been added to the point 1.2 of the protocol, while the commercial information regarding the product used has been made available on the materials list.

**3. "Remove any PDMS residues with a razor blade and wipe clean with 100% isopropanol." where are these PDMS residue coming from? Are you not taking new dishes? The razor blade would scratch the glass, could you explain (and show with a video), what you are doing?**

>>>We thank the reviewer for addressing the language used in this step of the protocol. This step (1.6) of the protocol has been updated to describe the origin of the PDMS residues. In addition, during the video production, we will make sure to clearly illustrate this step.

**4. point 6-14 are not clear and should be shown. Also Provide detailed pictures to show the steps.**

>>>We thank the reviewer for addressing the steps written in points 1.6-1.14. The steps have been reviewed and edited accordingly to create a smoother protocol for the reader. In addition, pictures were added to Figure 1 to illustrate these steps.

**STEP 2**

**5. "7. Collect all spheroids in 20 µL of medium, using a P200 pipette, and add to the collagen 1 solution." Volume of Collagen solution?**

>>>We thank the reviewer for pointing out the confusion. We added an exemplary calculation for the preparation of the 5 mg/mL collagen I solution (see steps 2.6 and 2.7).

**6. "10. Start a timer and dispense 30 µL of the collagen 1 solution containing one spheroid into each hole" How do you control how many spheroids you pipet? You mention to collect all spheroids and suddenly you are supposed to be able to pipet only 1 of them?**

>>>We thank the reviewer for pointing out the confusion. The experimenter visually ensures that only one spheroid is pipetted with the 30 µl. If multiple spheroids are accidentally collected and dispensed in a hole, a 10 µL pipette tip is used to separate the spheroids from each other (see added step 2.12).

**7. "Repeat twice to fill one SID and stop the timer." So you end up with 3 spheroids in each hole??? This does not make sense at hole, why repeating it twice?**

**8. What do you mean by flipping frequency? This comes out of the blue with no introduction**

**9. Step 11-12 seem very fiddly**

>>>We thank the reviewer for pointing out the confusions. The steps have been reviewed and edited accordingly to create a smoother protocol for the reader. In addition, during the video production, we will make sure to clearly illustrate this step.

**10. How do you control the thickness of the collagen layer? Does it affect imaging?**

>>>We thank the reviewer for pointing out these aspects. The effect of collagen thickness on the imaging are discussed in the representative results part, in the second paragraph.

**STEP 3**

**11. No indication of type of objective, do you need a specific working distance?**

>>>We thank the reviewer for pointing out the lack of details regarding the type of objective. The information was added to the protocol, as a Note following point 3.1.6, and included in the Materials list.

**STEP 4**

**12. As mentioned above, step 4 is far too superficial. A much deeper image analysis is required for such imaging conditions and set-up.**

>>>We thank the reviewer for pointing out the superficial imaging processing part. We expanded this section and added the potential use of a nuclear dye to track individual cancer cells and extract motility parameters (see note following step 3.1.2. and the last paragraph of the Discussion).

**Figure legends**

**13. Indicate which objectives were used. For the movie, how long was the time-lapse?**

>>>We thank the reviewer for pointing out the lack of information and adjusted the legends accordingly.

**Reviewer #3:**

**Manuscript Summary:**

**the authors present a clear and useful protocol for the easy controlled embedding of cancer spheroid in a minimal amount of collagen for cell /migration invasion assays. Moreover, the authors provide staining strategies for both live and fixed specimens. Finally, a straightforward ImageJ image processing pipeline for the calculation of the spheroid's projected area is described. This contribution is a valid aid for researchers starting to perform assays based on cellular spheroids.**

**Major Concerns:**

**I have no major concerns.**

**Minor Concerns:**

*** "The spacer can be 3D printed using the length and width of the glass plates": which type of filament/resin is suggested? What type of 3D-printer? - this information is key in order to properly reproduce the protocol. Any biocompatibility/cytotoxicity issues? The authors should briefly comment on this point.**

>>>We thank the reviewer for addressing the lack of details in regard to the 3D printed spacer. The information was added to the protocol and the Materials list. PDMS is widely used for its biocompatibility; we now pointed that out in the first sentence of the results section.

*** The authors should provide in the main text a figure showing the CAD-drawing of the spacer, so that the reader can grasp at a glance the general idea without needing to open the source file with a specialized CAD-software.**

>>>We thank the reviewer for pointing out the need for a figure displaying the 3D printed spacer. Figure 1 has been updated accordingly to present a general idea of the spacer and the mold being used so readers do not need to open a specialized software.

*** "Clean the two glass plates": which cleaning procedure, which solvent is used?**
>>>We thank the reviewer for addressing the absence of the used solvent in the protocol. The protocol has been updated to include the 100% isopropanol cleaning step that was previously absent.

*** "Perform a second round of plasma treatment on the SIDs": is there any alternative to plasma etching? - Not every group has this device available, therefore a suggestion for an alternative etching method (e.g. chemical) would be helpful.**
>>>We thank the reviewer for pointing out the need for an alternative etching method. We are pointing out that a hand-held plasma wand, which is more affordable, can be used.

*** "After the final wash, resuspend spheroids in the cell tracker solution and incubate at room temperature for 20 min, protected from light.": by using this procedure, not all the cell in the inner layer of the spheroid will be stained. Could the authors elaborate on this point?**
>>>We thank the reviewer for pointing out the need to elaborate on the labeling of cancer cells inside the spheroid. In the Note following 3.1.3, as well as in the Discussion, we describe the possible modifications to the approach, including extended incubation, creation of smaller spheroids, and cryosectioning, to ensure complete labeling and visibility of all the cells throughout the spheroid.

Supplemental File 1-SolidWorks spacer file for 3D printing

Click here to access/download

**Supplemental Coding Files**

JoVE_3D_Spacer (2).SLDPRT

Spheroid_Macro