16.07.2018

Bing Wu, Ph.D.

Review Editor JoVE,

Dear Sir,

We wish to thank the Editor and the Reviewers for helping us to improve the quality and clarity of our manuscript. Reviewer comments are addressed below point-by-point as they appear in the review, and relevant changes have been incorporated into our revised manuscript. Answers to Reviewer comments appear after each comment in *italics*.

**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.

*The manuscript has been thoroughly proofread.*

2. Figure 4: Please include a space between the number and unit of the scale bar (i.e., 500 µm).

*This has been corrected, see new Figure 6.*

3. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

*The Long Abstract has been revised; to more clearly state the goal of the protocol, see specifically, lines 78-80.*

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

*The Introduction has been rephrased to include a clear statement of the goal, see lines 136-138.*

5. Please define all abbreviations before use (DDW, RT, SD, etc.).

*Abbreviations have all been defined at first appearance in the manuscript.*

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

*Spaces have been added between numbers and units throughout the manuscript.*

7. 2.1: Please add more details to this step. This step does not have enough detail to replicate as currently written. For instance, what are the trypsinization reactions condition? What medium and what volume is used to wash?

*Details have been added to this step, see lines 192-198.*

8. There is a 2.75 page limit for filmable content. Please highlight 2.75 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

*The text for the video has been highlighted.*

9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

*The highlighted steps mentioned above in comment 8 form a logical flow from one text to the next.*

10. As we are a methods journal, please revise the Discussion to 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

*The Discussion section has been revised to include the above points.* *a) Critical steps within the protocol: embedding the spheroid in collagen, see lines 527-532. b) Any modifications and troubleshooting of the technique: re-seeding of cells when the occupancy is too low, see lines 532-534. c) Any limitations of the technique: the size and geometry of the HMCA described in the manuscript are designed to hold relatively small cell clusters (up to 150 µm). For analyzing cell migration from larger cell structures, designing a new array will be necessary, see lines 534-537. d) The significance with respect to existing methods: see lines 498-525 and 539-545. e) Any future applications of the technique: see lines 566-568.*

11. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

*References have been changed to meet the Reviewer’s specifications - journal names are no longer abbreviated and volume and issue numbers are included.*

12. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

*Changes to Table of Equipment and Materials have been made as per Editor’s above request.*

**Reviewers' comments:**

**Reviewer #1:**

Summary of the key results:

Orit et al. report a micro-chamber array composed of agarose, collagen I, which can also include hyaluronan to form cancer spheroids to study invasion. Improved in vitro models to understand invasion are needed, as some in vivo models do not reproduce the physiology of human tumours. Using a PDMS stamp pyramid-shaped micro-wells are made in agarose that support spheroid formation in hydrogels prepared on collagen-based gels. Spheroid area and cell invasion are measured using confocal microscopy over several days. The methods describe quantification and analysis of these readouts using one ovarian and one breast cancer cell line with Matlab.

**Revisions:**

1. Line 63: Lower case hydrogel

*This word has been removed from the short abstract, instead the acronym was added, see line* 63.

2. Line 65: Remove space for extracellular

*Space has been removed, see line 65.*

3. Line 86: Define HMCA

*HMCA has been defined in the title, see lines 1-2.*

4. Line 90: Define HTS

*HTS has been removed from the long abstract and defined at first appearance, on lines 125-126.*

5. In the abstract, describe the cell types used and the use of Fisetin

*Cell types and the use of Fisetin have been described in the revised long abstract as per Reviewer’s request, see lines 85-89.*

6. Line 95: Reference needed

*Reference has been added and subsequent references re-numbered, see line 96 (reference 1).*

7. Line 127: Reference needed

*Reference has been added and subsequent references re-numbered, see line 129 (reference 14).*

8. Protocol 1: Describe the dimensions of the pyramid microwells and the stamp need to produce the desired device

*The dimensions of MCs and PDMS stamp (same dimensions but reverse structure) have been described in the revised manuscript, see lines 158-161. In addition, a new figure has been added, presenting a cross section of an MC with its dimensions, see new Figure 1.*

9. Protocol 1.1: How is LMA sterilized?

*LMA powder is dissolved in sterile PBS in a sterile bottle, while stirring and heating to 80 °C, making it semi-sterile. Thereafter, when LMA gelation has been achieved in the wells, the whole device is sterilized by UV light in a dedicated instrument. This step has been added to the protocol as section 1.5, see lines 182-183. The UV instrument has been added to the Table of Equipment and Materials, see line 14.*

10. Protocol 3.2: How is sterility ensured using an ice bucket in the biosafety cabinet? Can something better be used?

*The ice bucket is thoroughly cleaned with 70% ethanol and placed inside a biological hood for at least 10-20 minutes – enough time to be sterilized by the air flow.*

11. What difference is there between 48 and 72 h spheroids?

*Usually, 72-hour spheroids are larger than 48-hour spheroids. Incubation time is calibrated and determined according to the type off cell line and the size of spheroids used in the experiment. This parameter is kept constant throughout all the experiments of the same kind. For MCF7 cells, 48-hour spheroids were used, whereas for HeLa cells, 72-hour spheroids were used for invasion assay. This has been described on lines 206-207 in Protocol 2.4.*

12. Line 222: After collagen mixture add (ECM gel) so it is clear what is being referred.

*Protocol 6.3, (ECM mixture) has been added on line 260. This definition (ECM mixture) is more correct than (ECM gel) which was suggested by the Reviewer, due to the fact that the ECM (at this stage of the Protocol) has not yet become a gel.*

13. Line 227: Switch pour to pipette

*This has been changed as per Reviewer’s suggestion; see line 265, Protocol 6.4.*

14. Line 228: Is the plate sealed with parafilm when cooled at 4°C?

*The plate is only covered with its lid without parafilm sealing. This has now been detailed on line 266, Protocol 6.4.*

15. Line 239: Switch possibility to option

*This has been switched as per Reviewer’s request; see line 276, Protocol 7.2.*

16. Line 256: Is spheroid center of mass accurate considering the geometry of the wells are pyramids? Perhaps consider geometric center as you are not accounting for the density of cells in the spheroid

*The geometry of the micro well does not influence the shape of the spheroids. The cells are unable to attach to the hydrogel, thereby attaching to each other and forming a 3D floating object. The shape of the object is independent; see new Figure 5A&C, in which the images demonstrate properly the structure of the MC (truncated upside down square-shaped pyramid) and the independent structure of the spheroid (spherical shape). The center of mass at each time point is calculated by averaging the X and Y coordinate values of all pixels in the ROI which defines the spheroid.*

17. Line 297: Report the correct number of significant digits based on detection accuracy

*Based on detection accuracy of 10 measurements for one sample and calculation of standard error, the measurement error starts at the fifth digit after the decimal point for sectional area (mm2) and at the third digit after the decimal point for sphericity (a.u.). This has been corrected in the revised manuscript, see lines 340-343.*

18. Line 304: Switch pour to pipette

*This has been switched as per Reviewer’s request, see line 358.*

19. Line 318: Report the correct number of significant digits and units

*The correct significant digits for the slopes are at the sixth digit after the decimal point based on error evaluation by the least squares regression method. Units of the slopes were added as well. This has been corrected in the revised manuscript, see lines 370-373.*

20. Line 320: Describe what Fisetin targets

*Fisetin activity and targets are now described and referenced; see lines 374-378 and reference 20.*

21. Line 363: Switch X4 to 4X

*This has been changed throughout the revised manuscript as per Reviewer’s request, see lines: 274, 401, 427, 437, 444, 462 and 472.*

22. Line 366: Switch 3 day old to day 3

*This has been changed* to 3-day *throughout the revised manuscript, see lines: 323, 337, 344, 348, 355, 431, 443, 449-450, 459, 469 and 477.*

23. Line 384: How are the linear regressions adjusted for each curve? What does this do? *Could you mean calculated?*

*Linear regressions are adjusted using the automatic linear trendline option in excel software, this option uses the TREND function. TREND function fits a straight line (using the least squares method) to the arrays of known\_y's and known\_x's and returns new numbers in linear trend, matching the known data points. We calculated the slope of the linear trendline for each one of the spheroids and then executed the t-test on the slopes to determine if the two samples (collagen and collagen+HA) are significantly different. The trendline presented in the new Figure 5 has the mean slope of all the slopes in each sample.*

24. Line 398: Define the acronym DETA

*This has been defined at first appearance, see line 387.*

24. Line 406: Switch X10 to 10X

*This has been changed as per Reviewer’s request, see lines: 233, 239, 274, 400, 452 and 478.*

25. Figure 4B: Spheroids are missing from some micro wells how often does this occur?

*In some of the experiments, while adding the ECM mixture to the spheroid array (or during other manipulations such as medium change), some of the spheroids become dislocated from their MCs. This is the reason for the slow and gentle medium removal and ECM addition while leaning the tip on the hydrogel array edge (see Protocol steps 6.2 and 6.3.). Dislocation of 10-30% of the spheroids occurs in most experiments. However, in invasion protocol, which includes covering the spheroids with ECM, time 0 of the experiment is determined right after spheroids are embedded in ECM, hence missing spheroids are not included in the experiment. From that point on, spheroids will rarely dislocate.*

26. Figure 4D: x- and y-axis are difficult to read

*The figure has been replaced with a clearer one, as per Reviewer’s request (see new Figure 6D).*

27. Figure 5B: Some red areas of invading cells are not in micro wells are these artifacts of the software detection.

*This is not an artifact. This happens in collective migration when the entire object is moving inside the ECM and changes its position relative to the MC.*

**Major revisions:**

1. Long abstract has too much of a focus on introduction information and limited details about how this model can answer questions about understanding and developing drugs to prevent metastasis.

*The long abstract has been revised accordingly, see lines 69-91.*

2. This method does not address the limitations reported for in vivo breast or ovarian metastasis models.

*We reported that in-vivo models for metastatic disease are expensive, have environments unlike the human microenvironment including ECM composition and non-tumor cells, and lack the ability to separate and control parameters. Indeed, the current in-vitro model is less expensive than most in-vivo animal models. Moreover, we have the ability with this method, to control parameters such as:*

*1. Spheroid size and composition, determined by the numbers and kinds of cells loaded into the array,*

*2. ECM composition, by choosing ECM components adapted to human levels and to the type of cancer as well.*

*3. Microenvironment cellular composition, by co-culturing tumor spheroids with cancer associated fibroblasts or immune cells in order to measure their influence on tumor invasion.*

*However, in this work we did not exemplify all model options but rather focused on the principles.*

3. The model is not described as a high-throughput system for performing drug screening in the method described. 6-well systems are not scalable to large arrays of drugs (100-1000's).

*We have changed the text to read 'medium throughput', see lines 85 and 149-150. It is correct that a 6-well plate does not enable a large array of drugs. However, the sample size for each drug tested is very large (450 spheroids), which makes the measurement more accurate and lowers the number of repeats. This has been explained in the revised manuscript on lines 539-543. In reality, each macro well of a 6-well plate contains 4 times the number of spheroids than a 96-well plate.*

4. Please detail why or cite the importance of inter-spheroid interaction for studying metastasis in drug screening.

*The HMCA technology facilitates formation, treatment and continuous monitoring of individual multicellular structures derived from a distinct small number of cells confined to micro-chambers. Survival and function of individual cells/small cell clusters are enabled due to the fact that the cells/spheroids share the same space and medium and inter-spheroid interaction via soluble factors is possible. This explanation has been added to Discussion, see lines 522-525.*

5. Protocol 5.3: The stiffness of hydrogels with collagen I and with hyaluronan are not defined in the method these should be reported.

*Stiffness information for collagen and collagen with HA has been added to Discussion and referenced accordingly, see lines 557-560 and references 29-33.*

6. Describe advantages of this model to other spheroid systems which also enable detection of cell viability?

*This model enables detection of cell viability as well. A new Protocol section (step 3) and a new figure to exemplify this ability in the protocol have been added in the revised manuscript; see lines 209-226 in Protocol and lines 346-353 in Representative results. Also see new Figure 4 and Figure Legend lines 442-447. Detection of cell viability is conducted here after spheroid formation; in addition, it could also be performed after ECM addition and at the end of the invasion process as well. Optionally, other dyes detecting cell viability could be used; for details see lines 223-226.*

7. The coefficient of variation described for these spheroids is quite high. Please discuss this in the context of other spheroid models relying on cell aggregation and how this variation exists between different micro-wells in other wells.

*The CV described here (52.80%) is higher, compared to some other spheroid models. Castillo et al. (doi: 10.1038/srep28375) compares 3 different methods for spheroid formation and report a CV (sectional area) of 6-23%. The methods that Castillo et al. describe (hanging drops and low attachment 96 microplate) attain cell distribution by pipetting cells directly to each well/drop. Sato et al. create embryoid bodies from pluripotent stem cells (doi.org/10.1038/srep31063) and report a CV (diameter of object) of 19-30%. Sato et al. attain cell distribution by loading cell suspension on top of the MC array and cells distribute by gravity into the MCs. Because spheroid size and homogeneity depend on initial number of cells per spheroid, the variation in cell distribution after cell loading derived from different methods should be considered. Therefore, comparing our CV to Sato et al. (same loading system) in the same parameter (diameter) will be more relevant. Considering spheroid diameter, we achieve a CV of 19-30% with average 24.52%, very similar to Sato et al. However, others (Lee et al. (DOI: 10.1063/1.3687409) who use loading by gravity as well), report lower CV values (sectional area) of 5.5-8.9%.*

*Our solution is to analyze spheroids at single-element resolution, which is more accurate than averaging the whole population, and reduces CV to 15.48% for sectional area and 7.45% for spheroid diameter. In some experiments, spheroids are analyzed by initial cell loading number. The CV between different wells for cell loading (24.37%) is similar to the one reported for hanging drops and low attachment plate (pipette cells directly to each well). The reply was briefly clarified in the revised manuscript as well; see lines 326-328.*

8. While the authors describe quantitative methods to characterize cell invasion they do not discuss methods to characterize cell viability.

*See our reply to comment #6. This work focused on invasion assay. Although viability screens using fluorescent probes are feasible in the HMCA plate, they were not thoroughly explored in this work, and thus, not discussed in detail in our manuscript.*

9. Include a 3D reconstruction image of the spheroids so the geometry can be confirmed.

*Confocal microscopy was not used in this work, but rather wide field microscopy. Geometry was previously checked by image acquisition at several focal planes and by histologic sections. See Supplementary Figure 1 in Reference #16 (doi.org/10.18632/oncotarget.21610). Other histological section data have been submitted but not yet published.*

10. Graph the number of disengaged cells from more than five spheroids considering there are 450 micro chambers as reported in line 357.

*A histogram which shows the distribution of cumulative value of disengaged cells has been added; see new Figure 8E and description on lines 416-419 in Results. The Figure Legend has been changed as well on lines 485-486.*

11. There are many unsubstantiated claims in the discussion:  
Homogeneous spheroids

*The text has been rephrased: "spheroid populations in which approximately 50% of the spheroids are comparable in size"; see line 518 and our reply to comment #7 as well.*

Reliable analysis

*The reliability of HMCA plate results depends on their compatibility with parameters and outcomes derived from other methods or methodologies. Such agreement was previously demonstrated for breast cancer spheroids (reference #16,) and in the current study for HeLa spheroid invasion capacity in the presence of MMW-HA.*

Practical to screen thousands of drugs

*This sentence relates to other methods available in the market and has been omitted from the revised manuscript in Discussion. With the HMCA technology, we can screen "thousands of spheroids per plate"; see lines 539-5*40.

Please include references: DOI: 10.3389/fonc.2017.00293

*This reference (#8) has been added to the Introduction and subsequent references have been re-numbered; see line 121.*

**Reviewer #2:**

Manuscript Summary:  
Orit et al reported a cell culture system for analyzing the cancer cell invasion. The authors prepared microwell array structures using agarose hydrogel, seeded cells and formed spheroids, and finally embedded the spheroids in collagen gel. Quantitative analysis of cell migration was performed by analyzing the spheroid shape. The presented concept is interesting, but there are several points that need intense improvement and/or further clarification before publication in this journal.

Major Concerns:

(1) First of all, the authors did not properly explain the advantages of the current approach compared to previously reported methods. For example, microchannel-based invasion assays (Chung, Lab Chip. 2009, 269) and spheroid-based invasion assays (Evensen, PLoS One, 2013, e82811; Yamamoto, Plos One, 2014, e103502) have already been reported. More recently, hydrogel fiber-based assay was reported (Sugimoto, Lab Chip 2018, 1378). All these examples used hydrogels for analyzing 3D migration/invasion assay of cancer cells, but the authors did not cite these important literatures, and that is a critical weakness of this paper.

*Advantages of the current approach over other previously reported hydrogel-based methods have been included in the Introduction and Discussion; see lines 115-126, 498-525, citing above references; see lines: 123 reference #9, 124 reference #12, 504 reference #24 and 506 reference #25, as per Reviewer’s suggestion.*

(2) The authors did not distinguish the proliferation and migration abilities of the cancer cells, so it was unclear what the authors actually analyzed by using the presented system.

*The most commonly used assay to quantitate invading cells is the Boyden chamber which uses ECM coated membrane. Cells that move through the ECM from one side of the membrane to the other are defined as invading cells. In the current work, we measure cells which leave the spheroid body and move through the surrounding ECM. This is considered an invasion process – otherwise the cells would continue to proliferate in/atop the spheroid body (as demonstrated in control experiments with spheroids lacking an ECM cover). Moreover, the possibility to stain cells with markers for proliferation and for invasion as well, in order to distinguish between the states was not in the scope of this work (although possible in the HMCA device). However, in kinetic BF images, cells were defined, which after drifting apart from the spheroid body became round and sometimes split into two round cells, thereafter changing again to regular/long shape and continuing their movement. There are works which report that invading cells switch between the states* *(DOI: 10.1158/0008-5472).*

(3) It was unclear why the authors used two types of cancer cells for different experiments. The authors should clarify why there was a need to choose different cell types for different experiments.

*Two kinds of cancer cells were used in order to exemplify the two kinds of invasion and their analysis. MCF7 cells commit collective invasion whereas HeLa cells commit single-cell invasion.*

(4) The experimental procedures were not properly described. For example, the authors did not describe the concept of data processing, but just explained the software guides, and this is not appropriate. The software used for statistical analysis was not shown. It was unclear how many cells/colonies the authors observed for obtaining the data.

*The concept of data processing was described as a Note in the Protocol section (right after step 7.5, see lines 284-297). Excel software was used for result processing and statistical analysis and this information has been added to the revised manuscript; see lines 306-307 and to the Table of Equipment and Materials, see line 22. Each figure legend indicates the number of spheroids that were observed and analyzed (n).*

(5) The authors did not describe the reproducibility of the experimental results. Some of the descriptions in Results were not incomprehensible. For example, it is difficult to claim that the aggregates were "typically spherical".

*All experiments were carried out at least twice, and each condition was duplicated in all experiments. Results have been re-checked and incomprehensible parts have been revised. The description "typically spherical" has been changed to 'more spherical' for accuracy; see line 338.*

Minor Concerns:

(6) Abbreviations should be defined for the first time when they appear. Refs. 11 and 12 are identical. The authors should check entire manuscript once again if it is easily comprehensible.

*Abbreviations have been defined at first use throughout the revised manuscript as per Reviewer’s suggestion, and duplicate reference removed.*

We thank you for your consideration.

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