Pharm Res (2018) 35: 155
https://doi.org/10.1007/s11095-018-2431-4

RESEARCH PAPER

Three-Dimensional Printing of Cell Exclusion Spacers (CES) for Use in Motility Assays

Christen J. Boyer[1] • David H. Ballard[2] • Jungmi W. Yun[1] • Adam Y. Xiao[1] • Jeffery A. Weisman[3] • Mansoureh Barzegar[1] • Jonathan Steven Alexander[1]

Received: 31 March 2018 / Accepted: 15 May 2018 / Published online: 4 June 2018

ABSTRACT

Purpose Cell migration/invasion assays are widely used in commercial drug discovery screening. 3D printing enables the creation of diverse geometric restrictive barrier designs for use in cell motility studies, permitting on-demand assays. Here, the utility of 3D printed cell exclusion spacers (CES) was validated as a cell motility assay.

Methods A novel CES fit was fabricated using 3D printing and customized to the size and contour of 12 cell culture plates including 6 well plates of basal human brain vascular endothelial (D3) cell migration cells compared with 6 well plates with D3 cells challenged with 1uM cytochalasin D (Cyto-D), an F-actin anti-motility drug. Control and Cyto-D treated cells were monitored over 3 days under optical microscopy.

Results Day 3 cell migration distance for untreated D3 cells was 1515.943μm ± 10.346μm compared to 356.909μm ± 38.562μm for the Cyt-D treated D3 cells ($p < 0.0001$). By day 3, untreated D3 cells reached confluency and completely filled the original voided spacer regions, while the Cyt-D treated D3 cells remained significantly less motile.

Conclusions Cell migration distances were significantly reduced by Cyto-D, supporting the use of 3D printing for cell exclusion assays. 3D printed CES have great potential for studying cell motility, migration/invasion, and complex multi-cell interactions.

KEY WORDS 3D printing · invasion assays · migration assays · motility assays · personalized medicine · three-dimensional printing

ABBREVIATIONS

CES	Cell exclusion spacers
Cyto-D	Cytochalasin D
D3 cells	Human brain vascular endothelial cells
FDA	Food and drug administration
Migration/ invasion	Migration and invasion
PBS-EDTA	Phosphate-buffered saline/ ethylenediaminetetraacetic acid

INTRODUCTION

Migration and invasion (migration/invasion) assays are common methods used to study cell motility, development, inflammation, wound healing and invasion of malignant cells in cancer (1,2). In migration/invasion assays, several approaches including restrictive barrier (cloning wells), inserts or specialized equipment (scratch tools) are used to create defects in monolayers which can be used to monitor the influence of drug treatments on the rate and completeness of cells 'in-filling which may provide information about therapeutic effectiveness of different agents. Some authors have described 'ad hoc' solutions for producing cell patterning, such as using parafilm inserts (3). Such solutions are inexpensive but not highly reproducible, and more cost-effective durable inserts which can restrict cell adhesion to create cell patterns for migration/invasion assays would be a useful tool. 3D printing is one

Guest Editors: Tony Zhou and Tonglei Li

✉ Jonathan Steven Alexander
jalexa@lsuhsc.edu

[1] Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana, USA

[2] Mallinckrodt Institute of Radiology, Washington University School of Medicine, St Louis, Missouri, USA

[3] Department of Anesthesiology, Washington University School of Medicine, St Louis, Missouri, USA

potential low cost solution that could be used to accomplish this goal. 3D printing is projected to be a disruptive manufacturing technology, which may enable labs around the world to fabricate custom assays in house and on-demand. 3D printing technologies also have ability to easily incorporate many bioactive test substances including antibiotics, chemotherapeutics, and hormones either as a pre-print additive or a post-print binder (4–10). These compositional and surface modifications allow highly complex models to be created which can even incorporate bioprinting (11,12).

This study describes a novel 3D printed restrictive insert spacer approach which is an alternative to the 'scratch' wound assay. This assay does not create a surface defect which is often a result of the scratch, with variable removal of underlying matrix. Additionally, this study demonstrated the feasibility of this approach in a migration assay using the actin microfilament toxin Cytochalasin-D (Cyto-D) (1uM) as a canonical motility suppressing test agent (13), and with gelatin-coated migration surfaces.

METHODS

3D Printed Insert Fabrication

Migration inserts and stabilization clamps were created on TinkerCAD (AutoDesk) and designed to fit various tissue culture plates (see Fig. 1). Outer clamps were added to prevent spacer movement and allow for a constant interface connection between the 3D print and the polystyrene plates (see Fig. 2). PLA filament (e-Sun) was used to 3D print inserts (supports on) using a consumer grade Lulzbot Taz6 3D printer (Lulzbot). Supports were carefully removed and the inserts were soaked in 70% ethanol for 4 h and allowed to air dry in a sterile tissue culture hood.

Cell Culture Migration Setup

Human brain vascular endothelial cells (D3) (passage 15) were grown to confluency in T-25 polystyrene flasks with EndoGro-MV culture media (MilliporeSigma). Cells were washed with phosphate-buffered saline/ethylenediaminetetraacetic acid (PBS-EDTA), trypsinized, collected through centrifugation, and re-suspended in EndoGro-MV media. 3D printed inserts were placed in 12 well plates and 2 mL of cell-suspended media was pipetted in each well (1 mL on each side of the spacer) and circular motion was applied to ensure cells were evenly dispersed in the wells before attaching. During pipetting of cells, a set of tweezers was used to apply downward force on the inserts to enable contact with plates during pipetting. Plate covers were added and the 3D printed stabilization clamps were applied to each side of the plate. D3 cells were incubated until confluency was reached, and then the 3D printed spacer inserts were removed and fresh media was replaced. For 12 well plates, 6 wells were replenished with fresh EndoGro-MV media and 6 wells with EndoGro-MV media containing 1μM Cyto-D. Cell motility was monitored for 3 days under optical microscopy and migration distances were analyzed with ImageJ software.

Underlying Extracellular Matrix Comparison

To study the effects of CES and scratch assays on ECM coatings, 12-well tissue culture plates were coated with biotinylated porcine skin gelatin solution ($NaHCO_3$ buffer, 1:40 dilution) and incubated overnight (16 h) at 4 degrees Celsius.

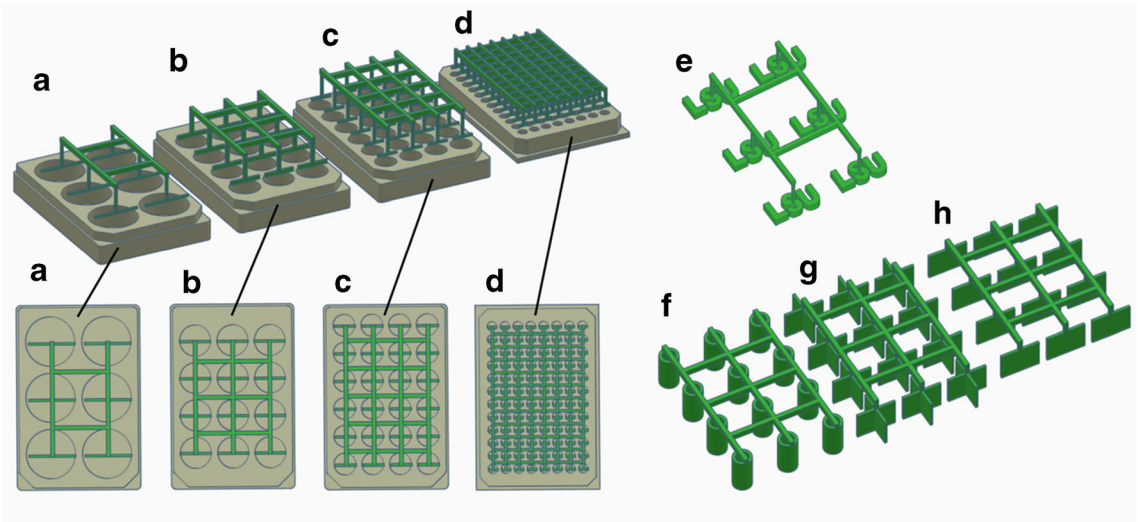

Fig. 1 Insert spacer designs for different tissue culture plates. Images of (A) 6 well single spacers, (B) 12 well single spacers, (C) 24 well single spacers, (D) 96 well single spacers, (E) 6 well spacers with LSU geometry, (F) 12 well circular spacers for multi-cell interactions, (G) 12 well spacers with quadrant geometry for multi-cell interactions, and (H) 12 well spacers with tall single spacers for multi-cell interactions.

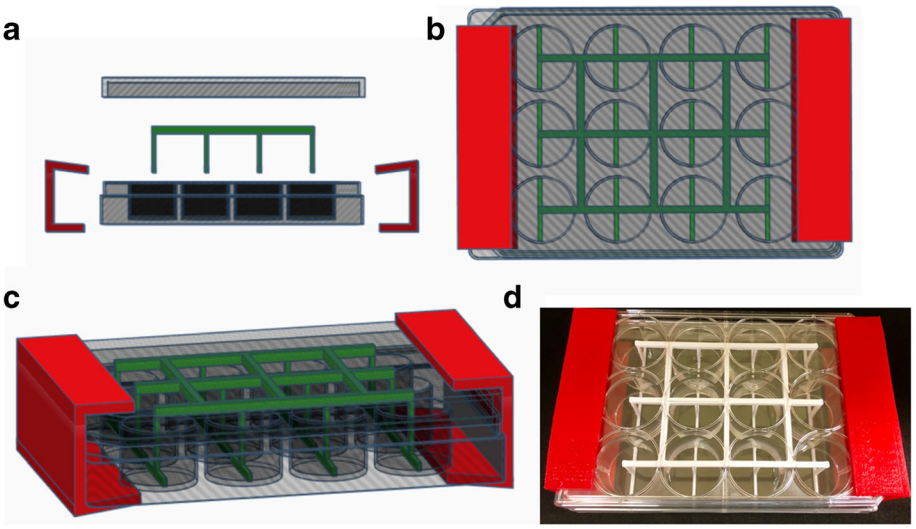

Fig. 2 Images of a 12 well single spacer insert with external stabilizer clamps. Fig. 2 shows (**a**) side view in open configuration, (**b**) top view in closed configuration, (**c**) side view in closed configuration, and (**d**) top view of 3D printed model in closed configuration.

Gelatin solutions were removed and plates were washed with 1X phosphate buffered solution (PBS) twice. The 3D printed CES migration procedure was repeated on gelatin-coated surfaces using the previously tested 3D printed CES by ethanol re-sterilization. Additional gelatin-coated plates were setup without the 3D printed CES for scratch assay comparisons. Once D3 cells were confluent, the 3D printed spacers were removed and other plates were exposed to scratch assays. For plates that did not contain the CES, wells were scratched with either a p-200 plastic pipette tip or a metal blade. Media was removed and plates were then exposed to fluorescein isothiocyanate (FITC)-avidin (1:50 dilution in media) for two hours. Wells were washed twice with 1X PBS to remove non-bound FITC-avidin and fresh media replaced. Gelatin coated surfaces exposed to scratching and CES were monitored through fluorescent microscopy.

RESULTS

The utility of 3D printed spacers as a cell motility assay was confirmed in a set of experiments designed to monitor cell migration. The Lulzbot Taz6 was capable of printing nine individual 12-well inserts at a time. Due to the heated bed feature on the printer, the bottoms of the spacers displayed smooth and flat uniform bottoms that allowed for a tight interface with the polystyrene plates during cell culture. The stabilization clamps were created to prevent movement of the spacers and results showed that the spacers maintained continuous interface with the polystyrene plates. Upon removal of the 3D printed spacers, empty zones were left over from the spacer position and demonstrated tight contact with the polystyrene plates (day 0) (see Fig. 3). Some areas of the exclusion zones did display dead and live cells, which may have entered during the placement of stabilization clamps. Overall, cell confluency was clearly visible with distinct linear barriers left over from the spacers regions at day 0 of migration (see Fig. 3).

Cell migration distance on day 0 was set to 0 µm for both treated and untreated D3 cells. At day 1, (see Figs. 4 and 6) untreated D3 cells began to migrate and towards the empty spacer regions. D3 cells treated with Cyto-D at day 1 also appeared to migrate, but at a slower rate (see Figs. 4 and 6). Day 1 cell migration distance for untreated D3 cells was 439.137µm ± 98.461µm ($n = 6$) and for the Cyto-D treated D3 cells was 182.797µm ± 43.070µm (n = 6). By day 3, untreated D3 cells reached confluency and completely filled the original voided spacer regions, while the Cyto-D treated D3 cells remained significantly less motile (see Figs. 5 and 6). Day 3 cell migration distance for untreated D3 cells was 1515.943µm ± 10.346µm (n = 6) and for the Cyto-D treated D3 cells was 356.909µm ± 38.562µm (n = 6). For treated *vs.* untreated D3 cells at day 1, there were significant differences (Day 1, $P = 0.0002$). For treated *vs.* untreated D3 cells at day 3, there were significant differences (Day 3, $P < 0.0001$). Overall, results supported the use of consumer grade 3D printed spacers for use in motility assays with bioactive agents.

Wells coated with gelatin ECM were exposed to 3D printed CES and traditional scratch assays. For metal scratched wells, the gelatin ECM coating and the plastic surfaces were damaged (see Fig. 7a and white arrow). The damage to the plastic also altered the refraction of light through the plates. For plastic (pipette tip) scratched wells, the ECM surface was altered and appeared to have a more rough texture patterned based on the scratch direction (see Fig. 7b and white arrow). For 3D printed CES, the gelatin ECM appeared unmodified and undamaged (see Fig. 7c). No damage to the plastic plates were observed for the 3D printed CES and the plastic (pipette tip) scratching.

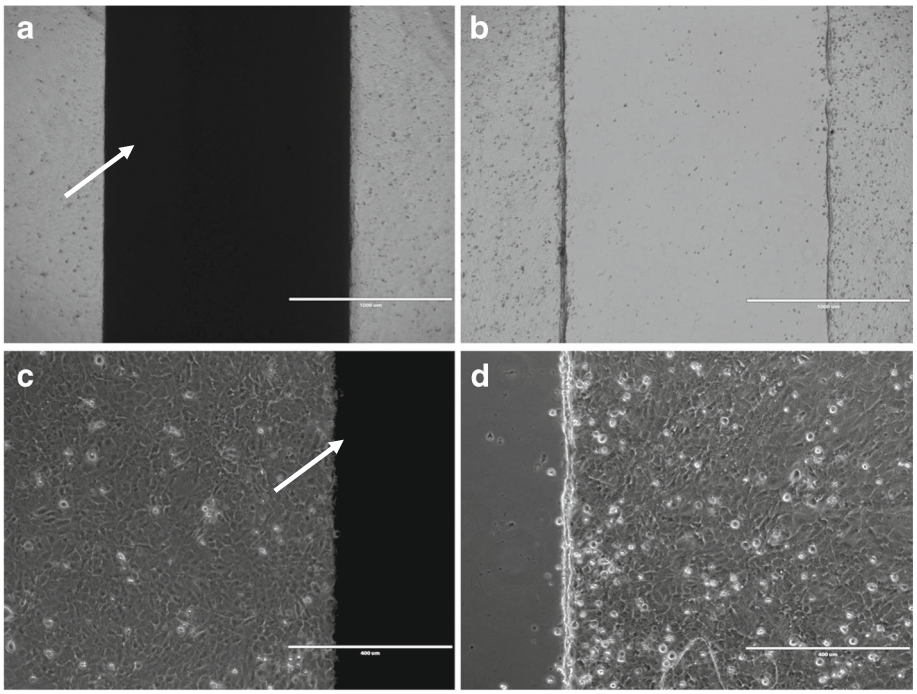

Fig. 3 Confluent D3 cells in apparatus at day 0 before (left) and after spacer removal (right). Fig. 3 shows low magnification (**a**) and higher magnification (**c**) micrographic images of D3 cells surrounding the 3D printed spacer (white arrows denote the spacer). Images (**b**) and (**d**) show D3 cells at low and high magnification respectively on day 0 immediately after removal of the 3D printed spacer. Scale bar for (**a**–**b**) = 1000 μm, and for (**c**–**d**) = 400 μm.

DISCUSSION

The present work describes the development of a novel 3D printed insert spacer design for use in cell migration and invasion assays. These customizable insert spacers can be printed on demand, customized to the size, width, and shape of same cell, dual cell, or multi cell migration/invasion assays by a low-cost desktop 3D printer. 3D printing is not limited to CES designs for motility assays, as other migration assays can easily be printed. For example, 3D printed PLA transwell shapes can be combined with micron-pore meshes through dipping the transwells in chloroform or chlorinated solvents, and then applying to meshes and allowing it to dry. Additionally, 3D printed guides with slits for consistent scratch assays and patterned scratch assays may also be created for a variety of tissue culture plates. In the present work, 3D printed spacers separated the D3 cells through exclusion. This study showed significantly lower motility in D3 cells treated with Cyto-D, an

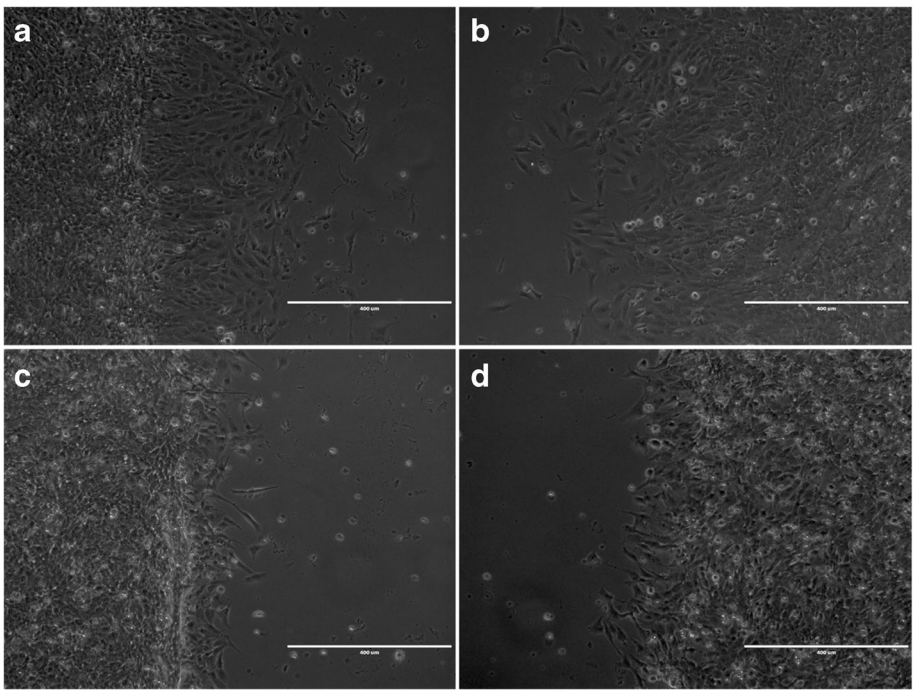

Fig. 4 D3 cells on day 1 after spacer removal with and without motility suppression (Cyto-D, 1uM). Fig. 4 (**a**) and (**b**) show migration patterns of untreated D3 cells contrasted with cells treated with Cyto-D (**c**) and (**d**). Scale bar for (**a**–**d**) = 400 μm.

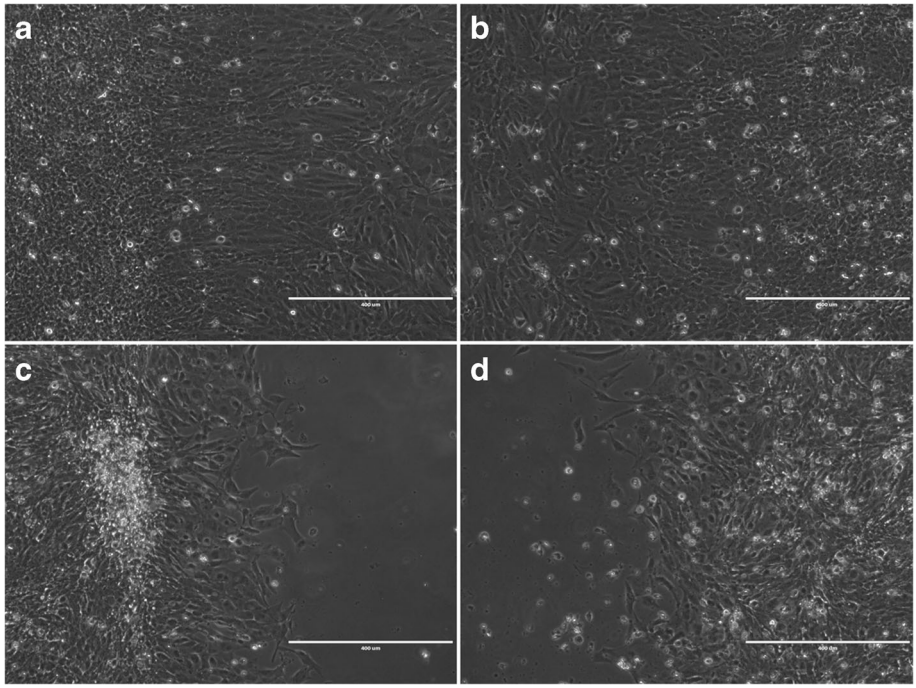

Fig. 5 D3 cells on day 3 after spacer removal. Fig. 5 (**a**) and (**b**) show untreated D3 cells reaching confluency. By comparison, Fig. 5 images (**c**) and (**d**) show marked inhibition of migration in D3 cells treated with Cyto-D. Scale bar for (**a**–**d**) = 400 μm.

inhibitor of actin polymerization (13). This high and low motility model demonstrated using our 3D printed insert spacer system, serves as a proof-of-concept model for creating 3D printed insert spacers for experiments requiring migration/invasion assays.

As an additive manufacturing techniques 3D printing techniques e.g. fused deposition modeling, can incorporate drugs, hormones and other compounds into 3D printed constructs. It is suggested drug-loaded CES prints would remain in the plates throughout the study. Similar to bacterial zones of inhibition, it is expected that highest drug absorption rate would be nearest to the 3D printed CES and would allow for cellular interface studies with medicated 3D printed devices.

Several *in vitro* studies have now incorporated antibiotics, hormones, and iodine into medical devices and implants such as catheters, meshes, and stents (4–8). Previously (9) reported *in vitro* studies show that 3D printed vascular stent constructs could be iodized, which made these constructs highly resistant to supporting bacterial growth in culture assays. Tappa *et al.* (10) incorporated estrogen and progesterone into 3D printed intrauterine devices which demonstrated the utility of such devices as contraceptives and drug-delivery devices. Such incorporation of drugs and hormones into 3D printed insert spacers represents a novel approach to further 'customize' migration/invasion assays using the advantage of 3D printing technologies to study migration and repulsion effects of materials introduced into the inserts used in such assays.

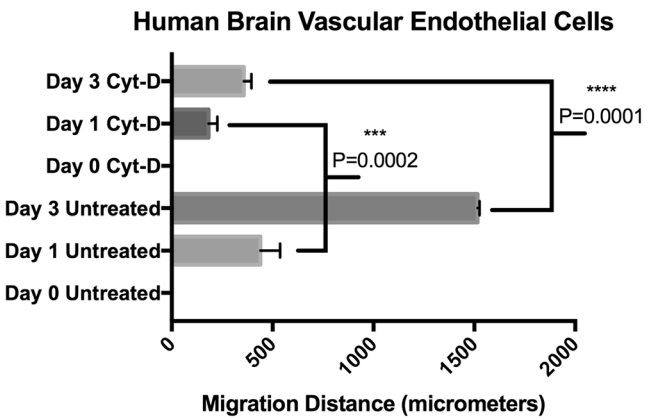

Fig. 6 Graphical comparison of distances of cell migration into spacer area following removal of the 3D printed mask in untreated and Cyto-D (1uM) treated D3 cells at days 1–3 ($n = 6$).

Several 3D printing approaches for tissue-like constructs and scaffolds for tissue growth have been described. Kane *et al.* (11) printed simple tissues such as human-shaped ears, which have been successfully grafted onto dorsal skin in mice as a proof-of-concept. One promising methodological advance was described by Laronda *et al.* (12), who developed 3D printed bioprosthetic ovaries, implanted them into mice following post-oophorectomy, showed that this *de novo* synthetic ovary-like construct with follicles restored fertility (12). Similar to incorporating drugs or compounds into the structure of 3D printed constructs, bioprinting of tissue and scaffolding for tissue could potentially incorporate living cells/tissues into the 3D printed insert spacers described in the present study for novel customizable migration/invasion assays.

3D printing still needs to overcome several regulatory and financial challenges before it can be commercialized for wide use in clinical practice and personalized medicine (14). In the United States, Food and Drug Administration (FDA) regulatory barriers exist for each material and application and 'ad

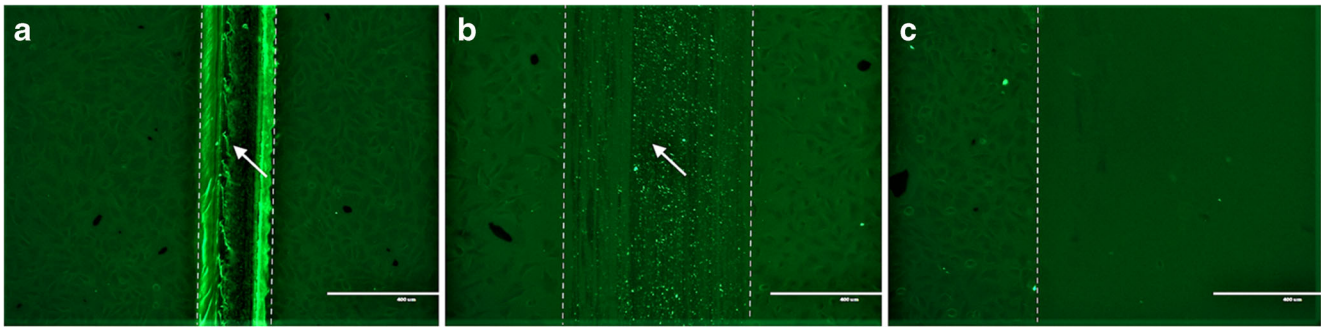

Fig. 7 Confluent D3 cells after scratching and spacer removal on gelatin matrixes. Fig.7 shows D3 cells on gelatin coated surfaces with (**a**) metal scratching, (**b**) plastic pipette tip scratching, and (**c**) 3D printed CES after removal. The white arrows point to areas of gelatin matrix damage. The white dotted lines show scratching (**a**–**b**) and CES (**c**) perimeters. Scale bar for (**a**–**c**) = 400 μm.

hoc' and '*de novo*' procedural devices, such as described in the present study would have to follow traditional approval pathways, demonstrating product efficacy prior to introduction for personalized medicine technologies (7,15,16). A full review of examples of FDA approve 3D printed devices is beyond the scope of this manuscript, but excellent summaries are available (15–17). Moreover, the FDA has a narrative published on their perspective of 3D printed constructs and the approval process (14).

There are limitations to the present approach, such as the diversity of consumer-grade 3D printers on the market and the different printing resolutions associated with each type. Also, the ability to design custom structures requires computer aided design skill sets. All 3D printers have different resolutions and capabilities. Resin 3D printers can have high resolution printing ability near 50 µm while fused deposition modeling (FDM) resolution can range from 100 to 1000 µm. The exclusion zone size will be limited to the printer type and resolution. For 3D printers with heated beds, another limitation and important feature is that the first layer is more flattened and wider than the rest of the print. For this study, the CES bar shape was designed with 1 mm bars, and the first layer resulted in 1.5 mm bars and zones. Overall, the exclusion zones will be limited to the printer type and resolution.

Although control and 1uM Cyto-D treated D3 cells showed significant differences, this study did not compare the described 3D printed insert spacers to commercially available migrations assays. Rather, this approach was intended to demonstrate the feasibility of applying 3D printed insert spacers in migration assays as a 'proof-of-concept' study which is inexpensive, rapidly adjusted and 'druggable' in that test agents can also be added to, or coated onto these inserts. This study did compare the underlying ECM coatings for scratch assays and the 3D printed CES. Both metal and plastic scratching altered the underlying ECM. Metal scratching damaged the ECM and the plastic plate, and it is suggested that metal scratching is not an ideal approach. Plastic scratching (pipette tip) produced roughened gelatin surfaces and it is not known if the altered surface effects the overall migration rate and pattern. It is possible that the cell migration is either enhanced or delayed due to the ECM surface roughness created through scratching.

Future studies to address these ideas will compare 'ad hoc' 3D printed insert spacers with commercial products for comparison. This study suggests that consumer grade 3D printing is a valuable option and tool for the research community, especially to those that have limited funding. As more and more laboratories around the world obtain affordable consumer grade 3D printers, techniques like this can be easily and cheaply integrated into experiments. Additionally, 3D printing allows for more rapid on-demand structures and modifications that can be designed to fit most cell culture plates, essentially democratizing manufacturing. Another advantageous feature is the ability to use a variety of materials for 3D fabrication.

At writing, current cell migration kits on the market range in price $380–$510 for 24 assays, and these price entries may be a limiting factor for many labs around the world. The 3D printed 12-well insert with stabilization clamps in this study cost $1.19 to fabricate (PLA material cost). In comparison for 24 assays, the 3D printed CES system costs around $2.38 (material cost). The 3D printed CES system developed is 159–214 times more cost effective than some of the current cell migration systems on the market. In a review of cost per assay: leading systems on the market cost $15.83 to $21.25, and the 3D printed CES insert cost $0.09. To make the 3D CES price even cheaper, the 3D printed CES could be designed in such a way that minimal supports would be needed during the printing process, thereby dramatically reducing the overall material costs. Additionally, the 3D printed CES can be reused and sterilized through gamma irradiation or soaking in ethanol. For labs that already possess 3D printers, this may be a viable and cost-effective option for studying cell migration. In conclusion, this study demonstrates the feasibility of 3D printed cell exclusion spacer inserts in migration/invasion assays. These 3D printed spacers are suitable for motility agent testing, complex cell migration and invasion assays, and multi-cell interaction studies.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors would like to thank Louisiana State University Health Sciences Center Shreveport for supporting this research. The authors have no conflicts of interest to disclose.

REFERENCES

1. Justus CR, Leffler N, Ruiz-Echevarria M, Yang LV. In vitro cell migration and invasion assays. J Vis Exp. 2014;(88) http://www.jove.com/video/51046/in-vitro-cell-migration-and-invasion-assays. Accessed March 22, 2018.
2. Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, Hengstschläger M, *et al.* In vitro cell migration and invasion assays. Mutat Res Rev Mutat Res. 2013;752(1):10–24.
3. Javaherian S, O'Donnell KA, McGuigan AP. A Fast and accessible methodology for micro-patterning cells on standard culture substrates using Parafilm™ inserts. Neves NM, editor. PLoS One. 2011;6(6):e20909.
4. Weisman JA, Nicholson JC, Tappa K, Jammalamadaka U, Wilson CG, Mills DK. Antibiotic and chemotherapeutic enhanced three-dimensional printer filaments and constructs for biomedical applications. Int J Nanomedicine. 2015;10:357–70.
5. Ballard DH, Weisman JA, Jammalamadaka U, Tappa K, Alexander JS, Griffen FD. Three-dimensional printing of bioactive hernia meshes: in vitro proof of principle. Surgery. 2017;161(6):1479–81.
6. Weisman JA, Ballard DH, Jammalamadaka U, *et al.* 3D printed antibiotic and chemotherapeutic eluting catheters for potential use in interventional radiology: in vitro proof of concept study. Acad Radiol. 2018; [Ahead of print]; https://doi.org/10.1016/j.acra.2018.03.022.
7. Ballard DH, Trace AP, Ali S, Hodgdon T, Zygmont ME, DeBenedectis CM, *et al.* Clinical applications of 3D printing: primer for radiologists. Acad Radiol. 2018;25(1):52–65.
8. Hodgdon T, Danrad R, Patel MJ, Smith SE, Richardson ML, Ballard DH, *et al.* Logistics of three-dimensional printing: primer for radiologists. Acad Radiol. 2018;25(1):40–51.
9. Boyer CJ, Ballard DH, Weisman JA, Hurst S, McGee DJ, Mills DK, *et al.* Three-dimensional printing antimicrobial and radiopaque constructs. 3D Print Addit Manuf. 2018;5(1):29–35.
10. Tappa K, Jammalamadaka U, Ballard DH, Bruno T, Israel MR, Vemula H, *et al.* Medication eluting devices for the field of OBGYN (MEDOBGYN): 3D printed biodegradable hormone eluting constructs, a proof of concept study. PLoS One. 2017;12(8):e0182929.
11. Kang H-W, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. Nat Biotechnol. 2016;34(3):312–9.
12. Laronda MM, Rutz AL, Xiao S, Whelan KA, Duncan FE, Roth EW, *et al.* A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. Nat Commun. 2017;8:15261.
13. Casella JF, Flanagan MD, Lin S. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. Nature. 1981;293(5830):302–5.
14. Di Prima M, Coburn J, Hwang D, *et al.* Additively manufactured medical products–the FDA perspective. 3D Print Med. 2016;2:1.
15. Christensen A, Rybicki FJ. Maintaining safety and efficacy for 3D printing in medicine. 3D Printing in Medicine. 2017;3 https://doi.org/10.1186/s41205-016-0009-5.
16. Leng S, McGee K, Morris J, Alexander A, Kuhlmann J, Vrieze T, *et al.* Anatomic modeling using 3D printing: quality assurance and optimization. 3D Printing in Medicine. 2017;3 https://doi.org/10.1186/s41205-017-0014-3.
17. Morrison RJ, Kashlan KN, Flanangan CL, Wright JK, Green GE, Hollister SJ, *et al.* Regulatory considerations in the design and manufacturing of implantable 3d-printed medical devices. Clin Transl Sci. 2015;8(5):594–600.