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Enhancing Scaffold-Free Spheroid Models: 3D Cell Bioprinting Method for Metastatic HSC3- Oral Squamous Carcinoma Cell Line

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Abstract

3D *in vitro* systems offer advantages over the shortcomings of bi-dimensional models by simulating the morphological and functional features of *in vivo*-like environments, such as cell-cell and cell-extracellular matrix interactions, as well as the co-culture of different cell types. Nevertheless, these systems present technical challenges that limit their potential in cancer research requiring cell line- and culture-dependent standardization. This protocol details the use of a magnetic 3D bioprinting method and other associated techniques (cytotoxicity assay and histological analysis) using oral squamous cell carcinoma cells, HSC3, which offer advantages compared to existing widely used approaches. This protocol is particularly timely, as it validates magnetic bioprinting as a method for the rapid deployment of 3D cultures as a tool for compound screening and development of heterotypic cultures such as co-culture of oral squamous cell carcinoma cells with cancer-associated fibroblasts (HSC3/CAFs).

Keywords: Oral cancer; Spheroids; 3D cell culture; Protocols; Magnetic bioprinting.

Introduction

Preclinical cell-based *in vitro* assays have significantly advanced our understanding of tumor biology and aided the development of new drugs with antitumor potential [1]. *In vitro* models are performed to assess the potential risks and adverse effects associated with compounds or drugs of interest in order to minimize the complexity, expense and ethical issues incumbent upon research involving animal models [2–4]. Due to low cost, simplicity and reproducibility, most of these assays are commonly performed using two-dimensional (2D) monolayers of immortalized human cancer-derived cell lines, which does not accurately mimic cell organization, nor provide appropriate conditions to observe interactions within a tumor microenvironment *in vivo* [4,5]. Thus, the advent of three-dimensional (3D) approaches capable of reproducing primary tumor characteristics represents an important alternative.

3D cell culture systems overcome the limitations of bi-dimensional models by allowing for the reproduction of morphological and functional features in a simulated tumor microenvironment. As these models reproduce interactions between cells and non-cellular components by enabling the production of extracellular matrix (ECM) and the co-culturing of tumoral and non-tumoral cell types, the modeling of heterotypic interactions, a significant challenge when using 2D systems, becomes feasible [4–6]. “3D culturing” has been widely used to describe some 3D structures [3,6]. Several methods are available for the development of spheroid cultures, a commonly used type of 3D model. Spheroid cultures can be broadly categorized according to the presence or absence of scaffolds; scaffold-based models incorporate protein gels [7] or synthetic and semi-synthetic hydrogel materials that provide cell support and resemble the ECM [8,9], while scaffold-free models comprise non-adherent and suspension culture techniques that depend on additional methods to facilitate aggregation and spheroid formation [10–12]. Since the materials used in scaffold-based models can interfere with many cellular processes, potentially misrepresenting what occurs in the *in vivo* tumor microenvironment [13], scaffold-free techniques have been widely adopted, with the most recent being magnetic-based 3D technology [14].

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The magnetic spheroid technology commercialized by Greiner Bio-One induces spheroid formation via cellular magnetization. Cells are initially incubated overnight in a bi-dimensional apparatus with biocompatible NanoShuttle™ magnetic nanoparticles (50 nm) composed of iron oxide, gold and poly-L-lysine [13,15,16]. The adsorption of these nanoparticles, or beads, on the cell surface, establishes electrostatic interactions with the membrane, resulting in the magnetization of cells [17]. A magnetic plate is placed below the magnetized cells, which drives cellular aggregation and spheroid formation. Following incubation, there are three types of magnetic-based models available: levitation, bioprinting, or ring formation [6]. Similar methods have previously been employed to simulate such tissues and tumor microenvironments in glioblastoma [18], breast cancer [13,19], lung [20], kidney [21], and pancreatic carcinomas [22] that show *in vivo*-like protein expression and ECM [23].

It is essential to highlight that while 3D models offer many advantages, issues related to the morphology of the formed structures, as well as reproducibility of size, shape, and integrity, pose significant challenges [23]. Furthermore, in scaffold-free spheroid models, the ECM formation is influenced by the specific cell line used. As a result, there are often noticeable differences based on the techniques used to form these spheroids [24–26]. These shortcomings associated with 3D cell culturing have driven the development of specific protocols being published for different cell lines. Moreover, authors often make a range of modifications to optimize standardized protocols according to their research goals [6].

Oral squamous cell carcinoma (OSCC) represents the most frequent oral malignancy, corresponding to 80-90% of tumors in the oral cavity [27]. There is a rising incidence in young adults with an increased aggressiveness compared to older patients. In addition, metastatic OSCCs have a poor prognosis, with a median overall survival of less than one year [28]. Thus, there is a growing imperative to study biological behavior (e.g., cell proliferation, invasion, metastasis), drug resistance [29] and developing new drugs [30] using *in vitro* models that mimic OSCC [31,32].

This protocol was previously validated by our team to create heterotypic spheroids to provide a reliable evaluation of Cell-in-Cell (CIC) structures in OSCC [33]. According to this method, the present study proposes an optimized magnetic 3D bioprinting protocol to shape spheroids using metastatic HSC3 cells derived from a human tongue OSCC. The optimized method described here can support the use of heterotypic cultures and can be used to investigate tumor behavior and compounds or drugs screening, representing a relevant tool for the performance of translational oncology in Oral squamous cell carcinoma.

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Materials

Biological Materials

In this study, we used Human oral squamous carcinoma cell line (HSC3, JCRB Cell Bank, JCRB0623) up to the 8th passage and OSCC-derived cancer-associated fibroblasts (CAF1; R.D.C-Unicamp, IRB 4.706.681) up to the 10th passage. IMPORTANT: It is essential to ensure the authenticity of the cells of interest (i.e., using short tandem repeat analysis) and that they are free of mycoplasma.

Reagents

Table 1
Products used are listed including catalog number, provider and storage condition, when applicable.

Reagents	Catalog number	Provider	Storage condition
DMEM high glucose	12800-058	Gibco™,Life Technologies	4°C
DMEM/F-12	11320033	Gibco™,Life Technologies	4°C
DMEM/F-12, Glutamax™	10565018	Gibco™,Life Technologies	4°C
Fetal Bovine Serum (FBS)	210520K	Gibco™,Life Technologies	- 20°C
Newborn Calf Serum	26010066	Gibco™,Life Technologies	- 20°C
Hydrocortisone (0.8%)	50-23-7	Sigma-Aldrich™	4°C
Penicillin-Streptomycin (100 U/mL)	15070-063	Gibco™,Life Technologies	4°C
GLI Inhibitor GANT61	G9048-5MG	Sigma-Aldrich™	- 20°C

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Cisplatin	15400054	Gibco™,Life Technologies	- 20°C
Phosphate buffered saline, pH7.4	10010-023	Gibco™,Life Technologies	4°C
Trypsin-EDTA (0.5%)	15400054	Gibco™,Life Technologies	4°C
NanoShuttle™	657846	Greiner Bio-One	4°C
Trypan Blue Solution (0.4%)	15250061	Gibco™,Life Technologies	Room Temperature
Cell Titer-Glo™ Cell Viability Assay 3D	G9683	Promega	- 20°C
Paraformaldehyde, 4%	J61899	Gibco™,Life Technologies	4°C
Trypan Blue Solution (0.4%)	15250061	Gibco™,Life Technologies	Room Temperature

NOTE: The reagents are available for purchase from distributors and manufacturers other than those listed here. With regards to histological processing, reagents such as xylol, alcohol and hematoxylin and eosin stain were not purchased by the team but were rather provided by the local institution's histotechnology department. Therefore, any compounds of choice can be used. For our protocol we used Cisplatin and GANT61, in stock of 5 mg/mL prepared in dimethyl sulfoxide (0.5% DMSO). The compounds were diluted in culture medium before each experiment. NanoShuttle™ magnetic nanoparticles can be stored at 4°C for 1 year. Do not freeze nanoparticles. For CellTiter-Glo™ 3D Assay, prepare the reagent according to the CellTiter-Glo™ 3D Cell Viability Assay manufacturers. Thaw the reagent at 4°C overnight and equilibrate to room temperature prior to use.

Equipment

Table2
When applicable, equipment used is listed including catalog number, provider, and storage condition.

Equipment	**Catalog**	**Provider**
Incubator (37°C, 5% CO_2)	Galaxy 170R	New Brunskwick
Laminar flow cabinet	211971030822	Nuaire Laboratory Equipment
Refrigerator (4°C)	926292866	Electrolux

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Freezer (-20°C)	926292866	Electrolux
Cell culture flasks, 75 cm^2	658175	Greiner Bio-One
Pipette controller	20190658	Ionpipet
Pipette tip, 100–1000 µL	KK25430	Gilson
Pipette tip, 20–200 µL	TA76433	Gilson
Pipette tip, 2-20 µL	HA56369	Gilson
Hemocytometer (Neubauer chamber) or cell counter	Z359629	Bright-Line, Cambridge Instruments
Magnetic drive consisting of an array of 96 neodymium magnets (96-Well BiO Assay™ Kit)	655846	Greiner Bio-One
Magnetic drive consisting of an array of 24 neodymium magnets (24-Well Bio-Assembler™)	662840	Greiner Bio-One
MagPen™ (optional)	657850	Greiner Bio-One
96 Well Plate, With Lid, Ultra-Low Attachment Surface	3474	Costar®, Corning, Promega
24 Well Plate, With Lid, Ultra-Low Attachment Surface	662970	Costar®, Greiner Bio-One
Opaque 96-well plate	30396	SPL
Multi-Mode Microplate Reader	26 170-1022	Filter Max F3, Molecular Devices
Inverted microscope	AMEX1200	EVOS XL, ThermoScientific
Microscopic slides	K5-7105	Olen, MYLABOR

Note: Equipment can be purchased from distributors and manufacturers other than those listed. The authors affirm no commercial relations nor conflicts of interest.

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Software

1. ImageJ (NIH, Bethesda, Maryland, USA)
2. GraphPad Prism (Dotmatics, Boston, Massachusetts, USA)
3. SoftMax Pro 6 Software (Molecular Devices, version 6.2.1)

Note: Software can be obtained from manufacturers other than those listed here. The authors affirm no commercial relations nor conflicts of interest.

Procedure

Homotypic Spheroid formation assays

Timing: 40 minutes, incubation time-4 hours.

Homotypic spheroid formation assay using HSC3 cells and 96-well plates - standardization of ECM production, spheroid size, and nanoparticle quantity. Magnetic spheroids can be used for either the development of homotypic or heterotypic cultures or to mimic healthy or diseased tissue. This protocol employed HSC3 cells, plated on repellent 96-well plates, evaluated at three different concentrations ($2.5x10^3$, $5x10^3$, $7.5x10^3$) and using 4 hours as a standard bioprinting time. Nonetheless, this same protocol is applicable to different cell types and densities, and can be used with different plates (e.g., 24-well plates). **Note:** Do not autoclave the magnetic drive beforehand, as demagnetization will occur under exposure to high temperatures. Cleaning with 70% ethanol (vol/vol) is sufficient. Perform all cell culturing steps under sterile conditions.

1. Obtaining the number of cells suitable for the assay.
    a. Seed HSC3 cells (JCRB Cell Bank, JCRB0623) in a T75 flask (filter cap) and culture until 70–80% confluence is achieved in an incubator under an atmosphere of 37°C, 5% CO_2.
    b. Detach the cancer cell line cultured in standard tissue culture flasks by incubating with 1 mL of 0.5% trypsin-EDTA (10X) solution for 5 minutes.
        i. Neutralize cells with complete DMEM (containing FBS 10%), centrifuge for 5 minutes at 300 x g, and resuspend the cell pellet in 1mL of complete medium.
    c. Count the number of cancer cells in suspension using a hemocytometer (*see* NOTE 1). Trypan blue exclusion can be used to identify live cells.
        i. Calculate the number of cells and quantity of medium that will be needed to perform the assay of interest (*see* NOTE 2). Calculate the number of cells considering more wells than needed (e.g., when plating 12 wells, calculate enough cells for 13.5 or 14 wells). During centrifugation and homogenization, volumes can vary due to bubble formation or during pipetting.
        ii. Next, calculate the volume of magnetic nanoparticles required for the assay of interest at a ratio of 0.4 µL to every $1x10^4$ cells (*see* NOTE 3). Determine the final volume of plating suspension culture (*see* NOTE 4).

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2. Magnetizing the cells
    a. Centrifuge three times for better magnetic bead incorporation (400 x *g* for 5 minutes), resuspending carefully in between centrifugation steps. **Optional:** In some protocols, magnetization is performed by overnight incubation in a 2D culture flask [17], followed by cell dissociation (trypsin), resuspension, transfer to an ultra-low attachment surface, and a levitation step involving the use of a specific magnetic drive (*see* NOTE 5).
    b. Place magnetized cells in 100 µL of standard DMEM culture medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.8% hydrocortisone medium in each well of a non-adherent 96-well cell culture plate and maintain as necessary. After plating, the bioprinting magnetic drive ("Concentration Drive", Greiner Bio-One) must be kept under the low-adherence plates containing the spheroids in an incubator under an atmosphere of 37 °C, 5% CO_2. Spheroid bioprinting can be performed at differing time durations; standardization of bioprinting time for each cell type used is recommended. According to our group experience with HSC3 cell line characterization, and the data obtained, we established a time of 4 hours for bioprinting. Spheroids can be monitored via an inverted microscope. Upon the conclusion of bioprinting, the magnetic drive can be removed.
    c. After 15 minutes, 3D structures will begin to form. The bioprinting time duration established for the HSC3 cell line was 4 hours using a "Concentration Drive" magnetic plate (Greiner Bio-One). Figure 1 illustrates all steps in the process of creating homotypic magnetic spheroid cultures using HSC3 cells and 96-well plates.

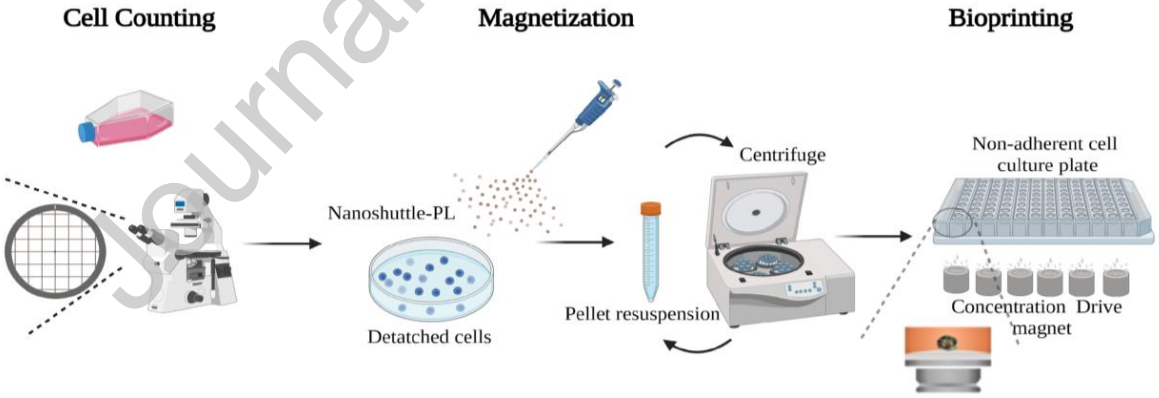

Figure 1. Overview of the steps required to create spheroid monocultures using 96-well plates. Created with BioRender.com

    d. After carefully removing the "Concentration Drive" magnetic drive, return the plate to an incubator under an atmosphere of 37°C, 5% CO_2. Maintain the spheroid culture for as long as desired for the selected assay(s) (*see* NOTE 6). Culture medium should be changed every 2 days. Carefully remove the existing medium using a 200

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µL pipette, then replace the same volume with fresh medium and return the plate to the incubator. Place magnetic drive (Holding Drive) under plate when changing medium to avoid spheroid aspiration.

Heterotypic spheroid formation assay

Timing: 60 - 70 minutes, incubation time-24 hours.

Magnetic spheroids can be used in monocultures or co-cultures (heterotypic) to mimic healthy or diseased tissue. This protocol employed HSC3 cells co-cultured with CAFs on repellent 24-well plates, with two cell ratios (2:1 and 1:1 HSC3/CAF) and two cell densities ($3x10^5$ and $1x10^5$ cells/well) evaluated. Nonetheless, this protocol applies to different cell types and densities, as well as different plates (e.g., 96-well plates) (*see* NOTE 7). Do not autoclave the magnetic drive beforehand as demagnetization will occur under exposure to high temperatures. Cleaning with 70% ethanol (vol/vol) is sufficient. Perform all cell culturing steps under sterile conditions.

3. Obtaining the number of cells suitable for the assay.
    a. Seed the cells lines of interest and culture until 70–80% confluence is achieved in an incubator under an atmosphere of 37°C, 5% CO_2.
    b. Detach the cell lines cultured in standard tissue culture flasks by incubating with 1 mL and 2 mL of 0.5% trypsin-EDTA (10X) solution for HSC3 cells and CAFs, respectively. Concentration and volume of dissociation agents may differ according to the chosen cell type(s). Avoid the use of exceedingly high concentrations of dissociation agents, as these may cause harm to cells.
        i. Neutralize cells with complete DMEM medium (supplemented with 10% FBS for HSC3 cells and 10% newborn calf serum for CAFs). Collect each cell type separately, then centrifuge, and resuspend the resulting cell pellets in 1mL of respective complete medium.
    c. Count the number of cells in suspension using a hemocytometer (*see* NOTE 1). Trypan blue exclusion can be used to identify live cells.
        i. Calculate the number of cells and quantity of medium that will be needed to perform the assay of interest (*see* NOTE 2). Cell density calculation must be adjusted for heterotypic spheroids. The total number of cells predicted for each well will consist of subsets of each cell population (HSC3 and CAF cells) in accordance with the desired ratios. Calculate the number of cells considering more wells than needed (e.g., when plating 12 wells, calculate enough cells for 13.5 or 14 wells). During centrifugation and homogenization, volumes can vary due to bubble formation or during pipetting.
        ii. Next, calculate the volume of magnetic nanoparticles required for the assay of interest at a ratio of 0.4 µL for every $1x10^4$ cells (*see* CRITICAL 3). Determine the final volume of the plating suspension culture (*see* CRITICAL 4).

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4. Magnetizing the cells
    a. Centrifuge three times for better magnetic bead incorporation (400 x g for 5 minutes), resuspending carefully in between centrifugation steps. The medium selected for heterotypic spheroid culturing should also be used for plating. In this protocol, the selected medium was DMEM F-12 Glutamax™ (Gibco®, Life Technologies), supplemented with 10% FBS, 0.8% hydrocortisone and 100 U/mL penicillin-streptomycin. Remember that the final volume per well must consider both the volume of each cell type as well as the other plating components. **Optional:** In some protocols, magnetization is performed by overnight incubation in a 2D culture flask [17], followed by cell dissociation (trypsin), resuspension, transfer to an ultra-low attachment surface, and a levitation step involving the use of a specific magnetic drive (*see* NOTE 5).
    b. Place magnetized cells in 1000 µL of DMEM F-12 Glutamax™, supplemented with 10% FBS, 0.8% hydrocortisone and 100 U/mL penicillin-streptomycin in each well of a non-adherent 24-well cell culture plate and maintain as necessary. After plating, the bioprinting magnetic drive ("Concentration Drive", Greiner Bio-One) must be kept under the low-adherence plates containing the spheroids, which need to be kept on the magnet in an incubator under an atmosphere of 37°C, 5% CO_2.
    c. Spheroid bioprinting can be performed at differing time durations; standardization of bioprinting time for each cell type used is recommended. Spheroids can be monitored via an inverted microscope. Upon the conclusion of bioprinting, the magnetic drive can be removed. After 15 minutes, 3D structures will begin to form. The bioprinting time duration established for co-cultured HSC3 and CAF cells was 24 hours using a "Concentration Drive" magnetic plate (Greiner Bio-One). Figure 2 illustrates all steps in the process of creating heterotypic magnetic spheroid cultures using HSC3 cells and CAFs with 24-well plates.

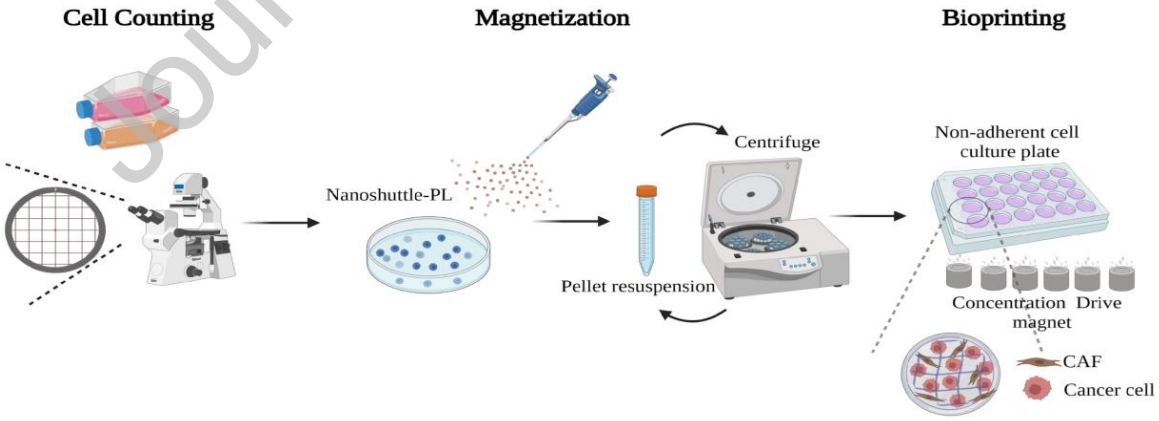

Figure 2. Schematic overview of the protocol used to generate heterotypic magnetic spheroids. The step-by-step process of creating heterotypic cultures was performed with HSC3 and CAF cells using 24-well plates. Created with BioRender.com

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d. After carefully removing the "Concentration Drive" magnetic drive, return the plate to an incubator under an atmosphere of 37 ºC, 5% CO_2. Maintain the spheroid culture for as long as desired for the selected assay(s) (*see* CRITICAL 6). **Note:** Culture medium should be changed every 2 days. Carefully remove the existing medium using a 1000 µL pipette, then replace the same volume with fresh medium and return the plate to the incubator. Place magnetic drive (Holding Drive) under plate when changing medium to avoid spheroid aspiration.

Spheroid diameter assay

Timing: 30 minutes (following spheroid formation).

To carry out diameter measurements, homotypic spheroids were obtained by performing the spheroid standardization steps in section 1, while heterotypic spheroids were obtained using the spheroid standardization steps in section 3. Importantly, this assay can be performed with spheroids of different cell compositions, densities, and using different repellent plates (e.g., 24-well plates).

5. Evaluation of diameter.
   a. Follow all steps in sections 1 and 3 to obtain homotypic or heterotypic spheroids, respectively, according to the desired assay.
   b. Carefully place the repellent plate containing the spheroids (without a magnetic drive) onto the inverted microscope for imaging.
   c. Photograph spheroids on an inverted microscope (under 10X and 20X objectives). Ensure the appropriate positioning of each spheroid to capture its full extent and borders. From the obtained images, the spheroid diameter can be measured using millimeter ruler and Image J software (*see* NOTE 10). Spheroid size can be evaluated unidimensionally by measuring the largest diameter. **Optional:** Alternatively, other measurements can be made using the same software program. Ensure that images of all spheroids are captured using a single microscope with an established scale, which is crucial to obtain accurate spheroid measurements.

Spheroid viability assay

Timing: 40 minutes (following spheroid formation).

To carry out the viability assay, homotypic spheroids were obtained following spheroid standardization steps (section 1) using repellent 96-well plates (Ultra-Low Attachment Surface) at a concentration of $5x10^3$ cells/well, and then transferred to an opaque-walled multiwell plate. Nonetheless, this assay can be performed with spheroids of different cell compositions, densities, and using different repellent plates (e.g., 24-well plates).

6. Cellular Viability Assay using Cell Titer-Glo® 3D.

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a. Follow all steps in sections 1 and 3 to obtain homotypic or heterotypic spheroids, respectively, according to the desired assay(s).
b. Follow all steps in section 5 "spheroid diameter assay" (*see* NOTE 10 *and* 11).
c. Carefully transfer each spheroid individually, together with its culture medium, from a repellent plate to an opaque 96-well plate using a magnetic tool (MagPen™ 657850) or a 1000 µL pipette tip. To preserve spheroid integrity, 1000 µL pipette tips must be previously cut to increase tip diameter to avoid damaging spheroids during aspiration. During transfer, confirm spheroid aspiration in the pipette tip and change tips regularly (*see* Troubleshooting section).
d. Add CellTiter-Glo® 3D Reagent at a ratio of 1:1 considering the plated volume of each well (e.g., on a 96-well plate, add 100 µL of CellTiter-Glo® 3D Reagent to 100 µL of medium containing cells) (*see* NOTE 12). Use the CellTiter-Glo® 3D reagent at room temperature, protected from light at all times.
    i. To induce cell lysis, gently agitate the plate for 5 minutes. Agitation is essential for effective ATP extraction from spheroids.
    ii. For stabilization, place the plate aside at room temperature for 25 minutes.
e. After obtaining luminescence readings, data analysis can be performed using GraphPad Prism Software. Spheroid viability can be evaluated at different time points. Here, we evaluated at 0, 12, 24, 36, 48, 60, 72, and 84 hours, with time 0 being the moment when the magnetic drive was removed. Figure 3 summarizes the protocol used for magnetic spheroid viability assessments.

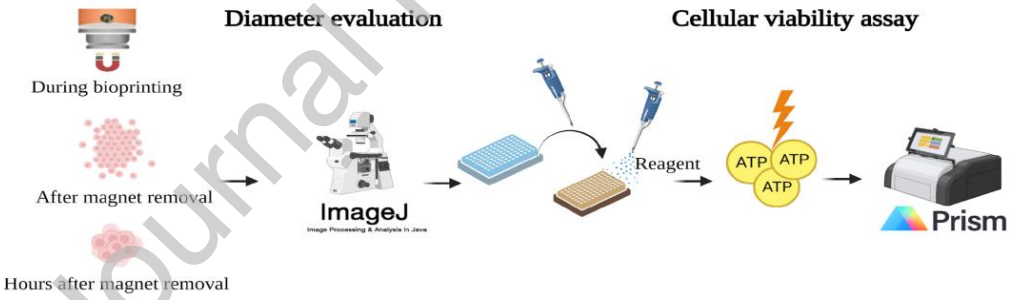

Figure 3. Overview of the steps used to assess magnetic spheroid viability. Created with BioRender.com

Compound screening

Timing: 50 minutes (following spheroid formation).

To perform compound screening, homotypic spheroids were obtained using repellent 96-well plates (Ultra-Low Attachment Surface) at a concentration of $5x10^3$ cells/well (established during the spheroid standardization process—Section 1), with the spheroids subsequently transferred to an opaque-walled multiwell plate. Importantly, this assay can be performed with spheroids of different

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cell compositions, densities and using different repellent plates (e.g., 24-well plates). The steps described in this assay are similar to those in Section 6 “Spheroid viability assay”.

7. Compound screening using CellTiter-Glo® 3D.
    a. Follow steps a-c from section 6 “Spheroid viability assay”.
    b. Add the compounds of interest to each well and then incubate each plate for 24 hours. As spheroid viability can be evaluated at different times, it is therefore recommended to standardize the time of analysis for each compound of interest.
    c. After compound incubation, photograph the spheroids using an inverted microscope (10X and 20X objectives). Spheroid diameter can be measured from the obtained images using Image J software (*see* NOTES 10 *and* 11). Spheroid size (contraction) can be used as a proxy for cell viability, which constitutes a simplified metric to evaluate toxicity [23].
    d. Carefully transfer the spheroids to a repellent opaque 96-well plate using a pen (MagPen™ 657850) or a pipette with an angled (cut) tip (*see* Troubleshooting table).
    e. Repeat the same viability assay protocol described in steps d-e of section 6 “Spheroid viability assay”.
    f. Obtain luminescence readings. Values can be analyzed using GraphPad Prism Software (version 5.0). Spheroid viability can be evaluated at different time points (0, 24, 48 and 72 hours), in which Time 0 represents the moment of magnet removal. Figure 4 summarizes the protocol to assess magnetic spheroid viability following exposure to compounds of interest.

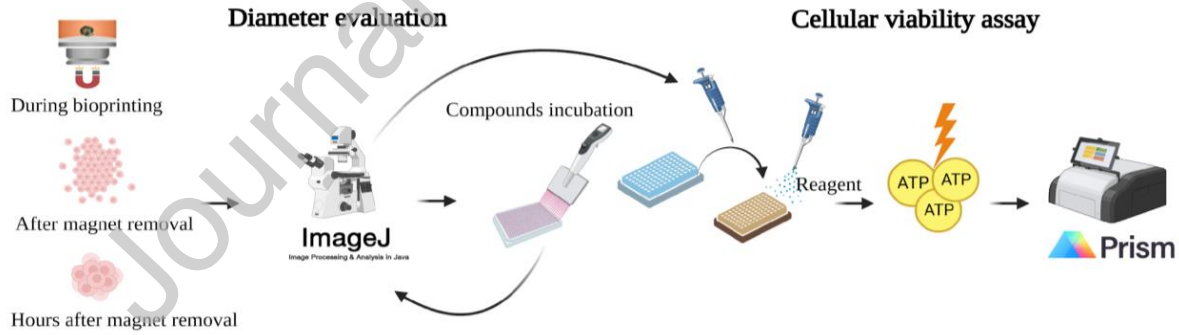

Figure 4. Overview of spheroid cell viability assessment after exposure to compounds of interest. Created with BioRender.com

Histological analysis of spheroids

Timing: At least 24 hours (after spheroid formation) due to overnight fixation.

For histological analysis, homotypic and heterotypic spheroids were obtained using 24-well plates. Each spheroid was processed into paraffin blocks and sectioned for slide assembly.

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8. Compound screening using CellTiter-Glo® 3D.
    a. Follow all steps in sections 1 and 3 to obtain homotypic or heterotypic spheroids, respectively, according to the desired assay.
    b. In a laminar flow cabinet, place the plate containing spheroids above the magnetic drive to keep the spheroids in place during media removal. Use 1000 µL pipettes to remove media from wells.
    c. Carefully wash wells with 300 µL of PBS 1X. When pipetting the washing solution, carefully avoid dispensing it near the spheroids by placing the pipette tip close to the edge of each well. To remove PBS 1X, tilt the spheroid plate with the magnetic drive below gently to one side. To avoid accidental aspiration of spheroids during this step, do not remove the magnetic drive from under the culture plate.
    d. Remove the magnetic drive from under the culture plate. Dispense 500-1000 µL of fixing solution (paraformaldehyde, 4% in PBS 1X) into each well. To detach the spheroids from the plate surface, gently dispense the fixing solution using a pipette close to each spheroid. Repeat until all spheroids are seen floating in the fixing solution.
    e. Using 1000 µL tips, collect spheroids from all wells. In order to preserve spheroid integrity, 1000 µL pipette tips must be previously cut at the end to increase tip diameter, allowing for safe spheroid aspiration. Place each spheroid in an individually labeled eppendorf tube and store in a fixing solution overnight at 4ºC.
    f. The following day, before collecting the spheroids, ensure that cassettes and film papers are available and labeled. At room temperature, collect the spheroids from the eppendorf tubes using previously cut 1000 µL pipette tips. Avoid collecting excessive amounts of fixing solutions. Gently transfer each spheroid from its tube onto a piece of film paper.
    g. Fold the labeled pieces of film paper containing spheroids to avoid sample loss during processing. Place each folded sample into an individual cassette.
    h. Place all cassettes in a flask and briefly wash with running water. Remove the water from the flask containing the cassettes and fill it with 70% alcohol. Some laboratories may have specific handling requirements for histological processing of spheroids. In this protocol, spheroids are placed in 70% alcohol for processing. Please confirm the specific requirements of your lab or institution.
    i. Process the spheroids into paraffin blocks. Paraffin-embedded spheroids can be sectioned using a micrometer at a thickness of 0.4 µm, mounted onto slides, and then stained with Harris hematoxylin and eosin (HE) or other phenotypical staining techniques to perform immunohistochemistry or immunofluorescence to verify epithelial cell phenotype and cell-cell interactions. Figure 5 summarizes the histological processing protocol and analysis of paraffin-embedded spheroids.

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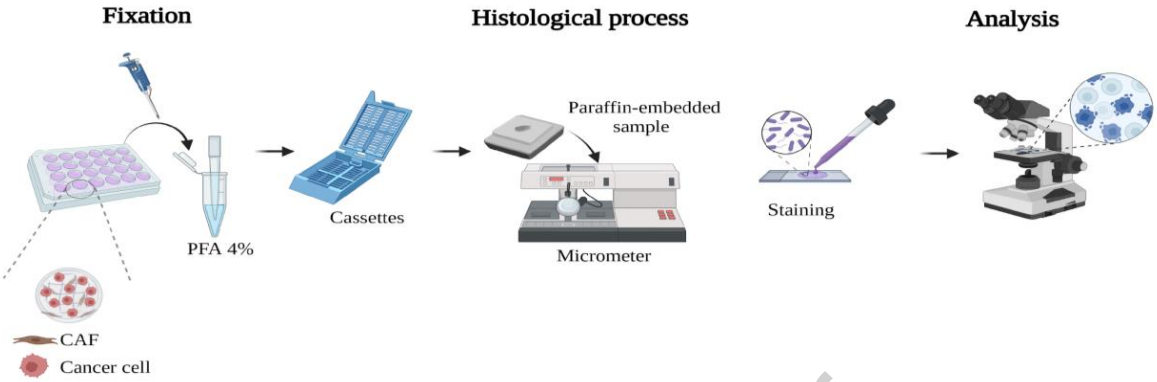

Figure 5. Schematic overview of histological processing and analysis of paraffin-embedded spheroids obtained through magnetic culture protocol. Created with BioRender.com

CRITICAL:

NOTE 1. Determine the number of cells available for the assay. After centrifugation, resuspend the cell pellet with 1 mL of the medium. Two eppendorf tubes need to be prepared: add 90 µL of the medium to the first and 80 µL of medium plus 10 µL of Trypan Blue Solution to the second tube. Add 10 µL of the medium containing cells in the first tube and homogenize. Take 10 µL of the homogenized cell suspension medium from the first tube and add to the second tube containing the Trypan Blue Solution and homogenize. Position the Hemocytometer (Neubauer chamber), add 10 µL of the second tube suspension under the coverslip over the chamber, and determine the number of viable cells. After counting, analyze if the total amount of cells available is enough for the desired assay.

NOTE 2. Determine the number of spheroids needed for the assay and consider the number of wells needed. Calculate the total amount of cells needed for the assay (e.g., 10 spheroids with 5,000 cells each = you will need 50,000 cells in total). The size of the spheroid can vary based on the number of cells plated (e.g., one spheroid with 5,000 cells will be smaller than one with 20,000 cells) and time of culture (e.g., in early stages, the spheroids tend to show a larger diameter because the cells are still in the process of aggregation, whereas in later stages of culture, the spheroids normally show smaller diameters; the spheroid contraction is a marker of viability [16]. It is important to note that exceedingly large spheroids (plated with a higher number of cells) induce cell death and necrosis in their center due to poor oxygenation (which is not recommended) [34].

NOTE 3. Calculate the total number of beads for the assay. The manufacturer recommends 1µL of beads for every 10,000 cells but suggests that optimization of this proportion can be made. We reduced this proportion to 0.4 µL of beads for every 10,000 cells. In order to standardize the ideal number of cells and the bioprinting time, cells were plated in different concentrations: $2.5x10^3$, $5x10^{3,}$ and $7.5x10^3$ cells/well.

NOTE 4. Determine the final volume needed for the assay. Each well has a working volume of 75-200 µL. The volume required to form spheroids is 100 µL per well. To calculate the final volume required

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for the assay, the number of spheroids needs to be determined first; based on this information, it is possible to calculate how many cells, beads, and medium are going to be needed for the assay (e.g., 10 spheroids of 5,000 cells each = you will need 50,000 cells in total; using 100 µL per well and 10 wells = a final volume of 1000 µL; 50,000 cells require 2 µL of beads to be magnetized; after counting, if the determined volume containing the 50,000 cells was 20 µL, then the final volume would be: 2 µL of beads + 20 µL of cells + 978 µL of the medium, totalizing 1000 µL of final volume to be distributed, etc.). The following formula summarizes how to calculate the final volume needed for the assay.

$$FV = CV + BV + MV - (CV + BV)$$

FV: Final volume
CV: Cells volume
BV: Beads volume
MV: Medium volume

NOTE 5. The ECM is a complex network composed of an array of macromolecules with structures and properties that vary in a cell-specific manner [27]. One of the most important contributions of 3D bioprinting is that it has been tailored to favor ECM formation and deposition for the establishment of *in vitro* models. The levitation step aims to induce ECM formation, as was shown previously [17]. However, "*de novo*" ECM assembly of the scaffold-free spheroid is generated in a cell line-dependent manner, resulting in significant variations in deposited ECM, in accordance with the spheroid methodologies variation [24–26]. We found that the HSC3 cells deposit a large amount of ECM, which interferes with the levitation step, requiring its exclusion in the standardization of this specific protocol.

NOTE 6. The plate containing the spheroids can be removed from the magnetic drive and cultured long-term without the use of magnetic force.

NOTE 7. During standardization, our team concluded that four hours of incubation with the magnetic drive was enough time to achieve spheroid formation. However, using the magnetic drive for longer periods does not result in spheroid alterations [3]. Therefore, for heterotypic culture, this protocol established 24 hours as an appropriate incubation time.

NOTE 8. During the standardization of the heterotypic spheroid culture, different ratios may be used for different cell types. Meanwhile, different cell lines may display different growth and proliferation rates. Therefore, in order to achieve the desired cell density and 70-80% confluence of all cell types simultaneously, flasks of different sizes may be used (e.g., T175 or T225 flasks). For example, HSC3 cells are smaller and replicate faster than CAFs. Meanwhile, CAFs are larger primary cells, meaning passage increases are not recommended. Therefore, HSC3 cells were cultured in smaller flasks, dissociated, and subcultured, while CAFs were cultured in a larger flask and slowly reached the desired confluence for plating.

NOTE 9. For heterotypic spheroids, an adjustment is required during cell count and volume determination. The total number of cells predicted for each well will be composed of both cell populations (in this case, HSC3 and CAF cells). Therefore, to determine this value, use the desired

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ratio of cells for each population and the total number of cells per well-established to determine the amount and volume of each type of cell used. For example, if the total number of cells is $3x10^5$ cells/well and the desired ratio is 2:1 of HSC3 and CAFs, respectively, then you will need $2x10^5$ HSC3 cells and $1x10^5$ CAFs per well. This should be taken into consideration when defining the volume of suspended cells during plating. In our standardization, we evaluated two cell densities ($3x10^5$ and $1x10^5$ cells/well) as well as two ratios (2:1 and 1:1 HSC3/CAFs). After our standardization, heterotypic spheroids composed of $1x10^5$ cells/well at a ratio of 1:1 HSC3/CAFs presented the best results.

NOTE 10. Note that spheroid viability is inversely proportional to its diameter. Drug-treated spheroids present larger diameters than non-treated ones. According to Tseng et al.[23], spheroid size (contraction) can be used as a simple metric for toxicity.

NOTE 11. Note that spheroid diameter must be evaluated before the Cell Titer-Glo® 3D protocol. Therefore, we need to photograph the spheroids before the assay. Since Cell Titer-Glo® 3D protocol needs to be performed in an opaque plate, performing the assay before diameter evaluation would interfere with spheroid visualization and imaging, limiting measurement obtainment.

NOTE 12. As an experimental control, plate two wells containing the total volume of supplemented medium only (200 µL) and two wells containing medium and CellTiter-Glo® 3D Reagent (100 µL of each, totaling 200 µL).

Troubleshooting

Table 3

Troubleshooting table content potential problems during protocol performing. Each problem is indicated with the occurring step.

Step	Problem	Possible reason	Solution
Plating	During the centrifugation steps, homogenization of the mixture of beads and cells was not possible, compromising the plating process.	High number of cells during plating may have resulted in the rapid formation of extracellular matrix.	Reduce the number of cells per plating and optimize bead concentration.
Microscope evaluation	During imaging, when placing the culture plate under the microscope, spheroids often migrated to the periphery of the wells, making it difficult to visualize	Natural spheroid migration towards the contact border of the wells.	Before photographing, position the magnet under the plate to center

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	them under the microscope.		spheroids in the wells.
Spheroid handling	Spheroids can be transferred using a specific pen (MagPen™ 657850) or a pipette tip. However, some spheroids can be retained or disintegrated during aspiration.	The pipette tip has a narrow opening, not allowing spheroid passage.	Cut the pipette tip to avoid loss of spheroids during transfer when necessary.
Spheroid formation	We tried the approach of growing small spheroids and bonding them together with the largest diameter magnet. This resulted in misshapen spheroids, making standardization unfeasible.	Formation of extracellular matrix in each small spheroid, not allowing their disassembly and reaggregation.	Avoid using this approach.
Spheroid formation	There is no homogenous solution after performing the levitation step followed by the bioprinting step.	High formation of extracellular matrix.	Perform only one technique (levitation or bioprinting) and standardize which one best suit your cell type.
Viability Assay	During the transfer to an opaque-walled multiwell plate, possible bubbles may appear, leading to unreliable results provided by the luminometer.	Fast release of the spheroids and their contents during pipetting	Gently pour the spheroid and its contents slowly into the wall of the well. If any bubbles persist, sterile needles can be used.

Data Analysis

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To determine the EC_{50} (effective concentration of 50%) of Cisplatin and GANT61 in the spheroids, the relative luminescence values (RLU) obtained from the luminometer were calibrated in relation to DMSO values. The following operation was performed in an Excel table: Compound RLU ÷ DMSO RLU (average). These results were compiled in the GraphPad Prism software version 6.03, and the EC_{50} values were acquired through non-linear regression from three independent experiments carried out in triplicate. For the other tests, GraphPad Prism version 5.0 was also used, and the difference between groups was evaluated by the ANOVA test (analysis of variance) followed by the Student-Newman-Keuls test ($p \leq 0.05$). The statistical analysis of this study considered the "p" value corresponding to alpha (α) less than or equal to 5% as the significance level.

Anticipated Results

To illustrate results typically obtained using our magnetic culture protocol, the next sections describe homotypic HSC3 spheroids and heterotypic HSC3/CAF spheroids obtained following the standardization steps detailed in each section of the protocol, as well as cytotoxicity assay and histological analysis.

Images obtained from homotypic spheroid formation.

The results below demonstrate the optimization of the 3D magnetic spheroid protocol for the formation of homotypic HSC3 spheroids. At a cell density concentration of $2.5x10^3$ cells/well using 96-well plates, although spheroid formation was observed at 4 hours of bioprinting, this cell density was insufficient to maintain spheroid integrity 24 hours after bioprinting. By contrast, at a cell density of $5x10^3$ cells/well (96-well plates), spheroid integrity was maintained, and this concentration was then used for all further experimentation involving 96-well plates. Regarding bioprinting incubation time, our results indicate that spheroids formed using a 4-hour bioprinting time-maintained integrity and consistency 24 hours later. Therefore, a bioprinting time of 4 hours is considered a sufficient incubation time, i.e., removing the magnet after 4 hours of bioprinting can be considered an initial timepoint (time 0) after which functional experimentation can be conducted.

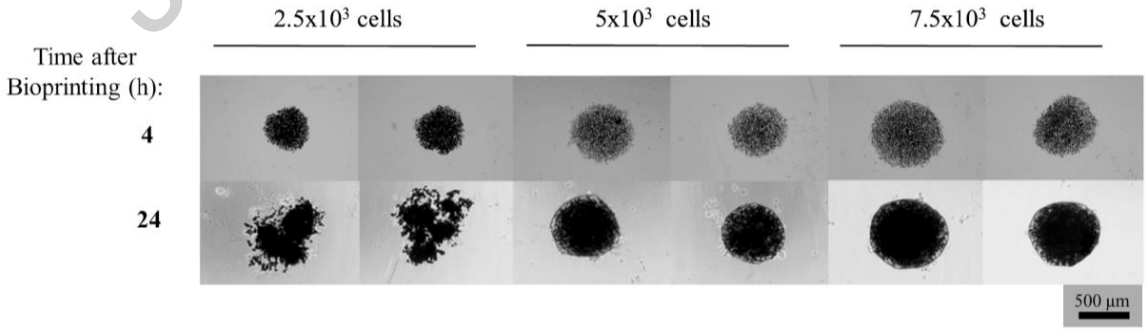

Figure 6. Examples of homotypic spheroids obtained using the 3D magnetic spheroid protocol. Inset images illustrate spheroids formed using different cell concentrations ($2.5x10^3$, $5x10^3$, $7.5x10^3$) at a 4

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hours bioprinting time, as well as 24 hours following spheroid formation. Images were obtained via an inverted microscope (EVOS XL, Thermo Scientific) using a 20X objective. Scale bar: 500 µm.

Images obtained from homotypic and heterotypic spheroid formation.

The following results demonstrate how the 3D magnetic spheroid protocol can be optimized for the formation of heterotypic HSC3/CAF spheroids. In Figure 7, images of heterotypic spheroids appear larger than homotypic spheroids, with both groups displaying integrity, reproducibility, and consistency at different cell densities ($3x10^5$ and $1x10^5$, using 24-well plates) and ratios (2:1 and 1:1) at 24 and 48 hours after bioprinting.

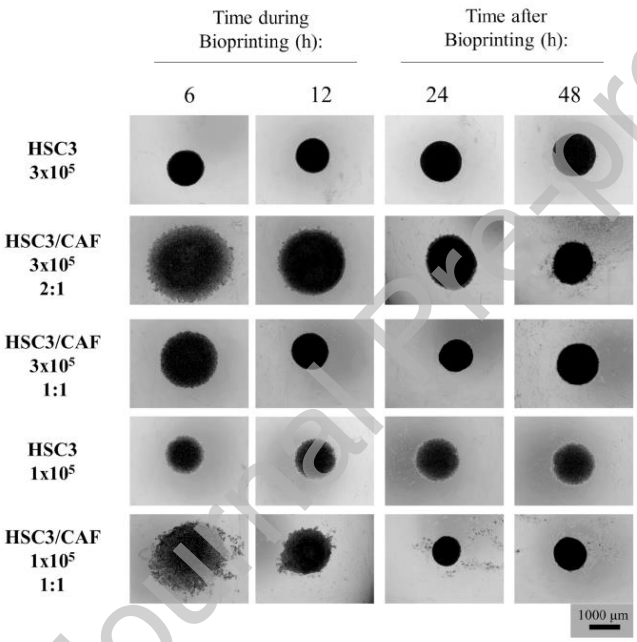

Figure 7. Images of homotypic and heterotypic spheroid formation assays involving different cell concentrations ($3x10^5$ and $1x10^5$) and cell ratios (2:1 and 1:1). Representative images of spheroid formation at 6, 12, and 24 hours of bioprinting. After a 24 hours bioprinting time, the magnetic drive was removed, and spheroid integrity was assessed 24 and 48 hours later. Images obtained via an inverted microscope (EVOS XL, Thermo Scientific) using a 4X objective. Scale bar: 1000 µm.

Viability evaluation of homotypic spheroids.

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In accordance with the previous standardization steps, untreated spheroids were cultured at a concentration of $5x10^3$ using a bioprinting time of 4 hours. Following magnetic incubation, spheroids were evaluated at different time points (0, 12, 24, 36, 48, 60, 72, and 84 hours) as shown in Figure 8. Given that the extracellular matrix is not yet fully formed at the initial timepoint (Time 0), the resulting images reveal a larger spheroid diameter (Figure 8). At 12 hours after bioprinting, spheroids appear well-formed (Figure 8), leading to increased cell viability (Figure 9C) that varies at later time points (from 12 to 84 hours). Regarding spheroid diameter (Figure 9B), no variations were observed after time 0.

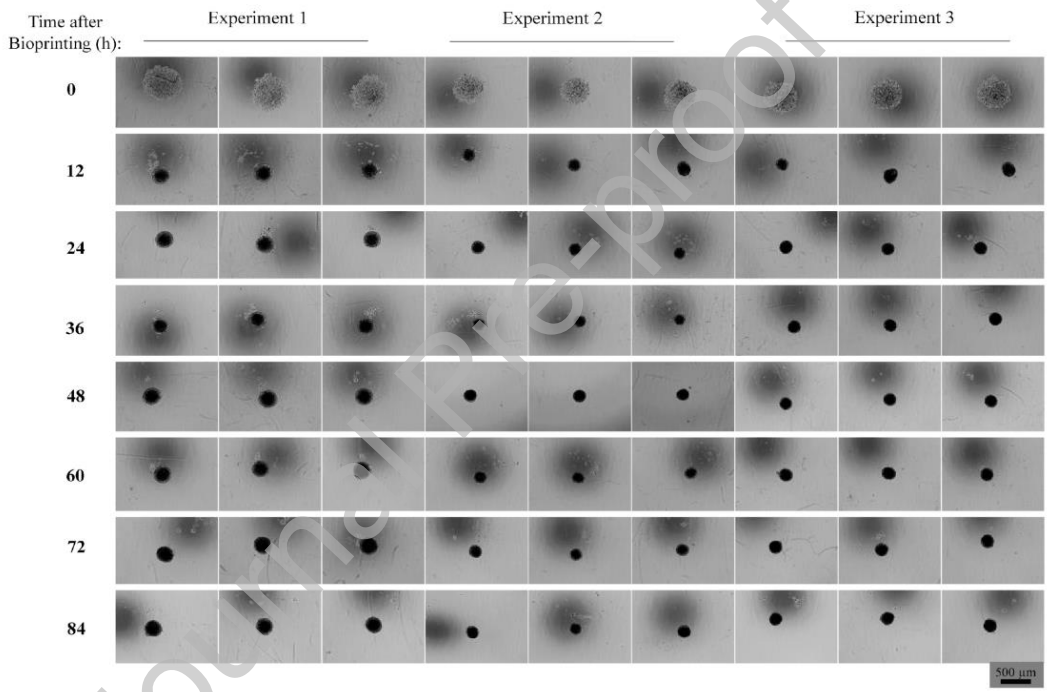

Figure 8. Representative images from viability assays. Spheroid formation and diameter are evaluated at different time points (0, 12, 24, 36, 48, 60, 72, and 84 hours after bioprinting). Spheroid images were obtained from three independent experiments performed in triplicate using an inverted microscope (EVOS XL, Thermo Scientific) with a 10X objective. Scale bar: 500 µm.

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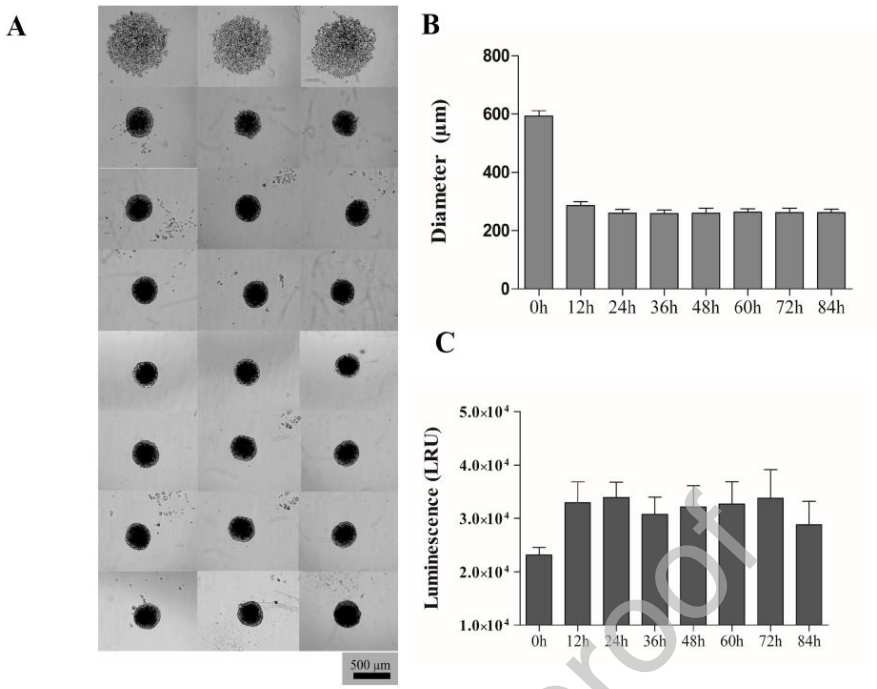

Figure 9. Spheroid diameter evaluation and luminescence assessment of homotypic spheroids. **a)** Representative images of spheroids at different time points (0, 12, 24, 36, 48, 60, 72, and 84 hours after bioprinting) using a more powerful objective (20X). Spheroid images were obtained using an inverted microscope (EVOS XL, ThermoScientific). **b)** Measurements obtained using a calibrated millimeter ruler and ImageJ version 1.8 (NIH, Bethesda, Maryland, USA). Values converted to micrometers (µm) correspond to three independent experiments performed in triplicate. **c)** Spheroid viability was evaluated using CellTiter-Glo® 3D at different time points (0, 12, 24, 36, 48, 60, 72, and 84 hours after bioprinting). Data are displayed as mean ± SEM corresponding to three independent experiments performed in triplicate. Scale bar: 500 µm.

Compound screening

In order to demonstrate the feasibility and reproducibility of bioprinted spheroids for drug screening, homotypic spheroids were cultured at a previously standardized cell concentration ($5x10^3$) using a 4-hour bioprinting time, then treated with different concentrations of the cisplatin (331.5, 165.7, 82.8, 41.4, 20.7, 10.3 and 5.1 µM) and GANT61 (100, 75, 60, 50, 40, 30, 15, 5 and 1 µM). Cisplatin, a well-known chemotherapy agent used in clinical practice, was used as a positive internal control. The concentrations are chosen from dose-dependent curves on a 2D model (data not shown). After cisplatin and GANT61 exposition, spheroids were monitored at 0 and 24 hours after bioprinting. Representative images indicate a larger spheroid diameter, approximately 600 µm, at the time of bioprinting conclusion (Time 0), with contraction evident at later time points following chemotherapeutic exposure. Notably, after 24 hours, spheroid contraction appeared to vary in accordance with different concentrations of GANT61 and cisplatin (data not shown) added after bioprinting; the diameter was reduced to 200 µm, as observed in Figure 10.

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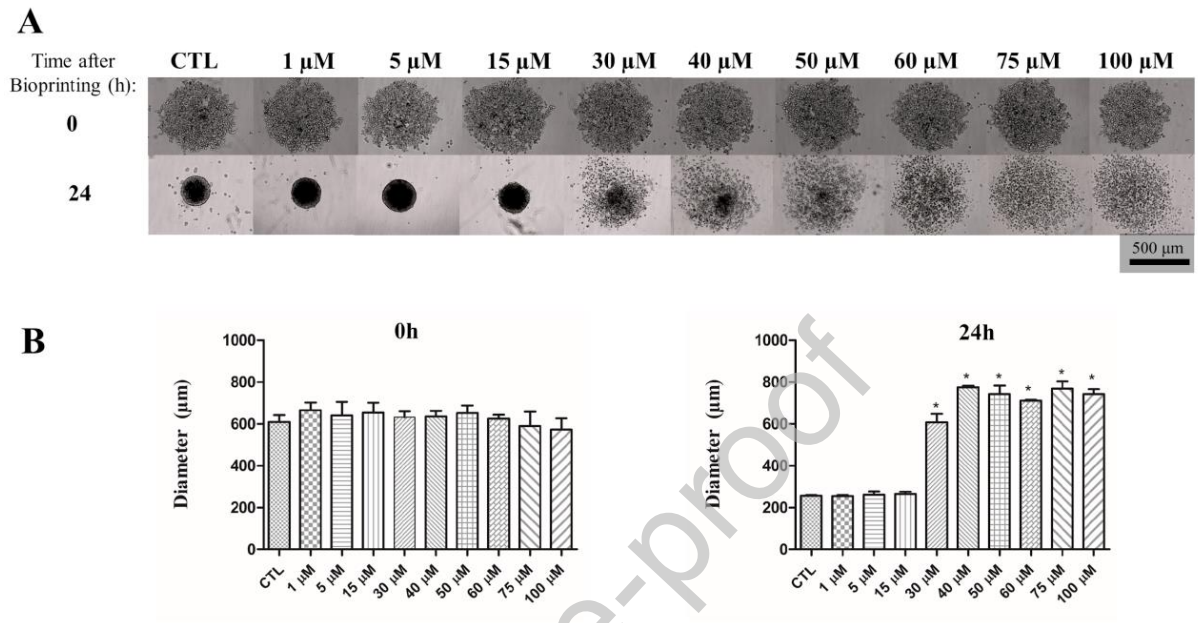

Figure 10. Spheroid profile following chemotherapy treatment. **a)** Representative images of spheroids at 0 and 24 hours after bioprinting following treatment with chemotherapeutic GANT61. Spheroid images were obtained using an inverted microscope (EVOS XL, Thermo Scientific) with a 20X objective. Scale bar: 500 µm. **b)** Spheroid diameter evaluation of homotypic spheroids after chemotherapy treatment. Measurements were obtained using a calibrated millimeter ruler and ImageJ version 1.8 (NIH, Bethesda, Maryland, USA). Values converted to micrometers (µm) correspond to three independent experiments performed in triplicate. CTL: negative control. (*) $p \leq 0.05$ compared to CTL by ANOVA (analysis of variance) followed by Student Newman-Keuls test.

To evaluate how the 3D model proposed would affect cell response to chemotherapy, 3D cultures were treated for 24 hours with either cisplatin or GANT61 followed by a cell viability assay. Therefore, the effective concentration (EC_{50}) of GANT61 was also compared on the HSC3 cells in 2D- and 3D- cell cultures as shown in Figure 11. The HSC-3 cell lines show an increase in the EC_{50} values of cisplatin and GANT61 in the 3D model compared to the 2D, suggesting 3D-bioprinting as a tumoral resistance model.

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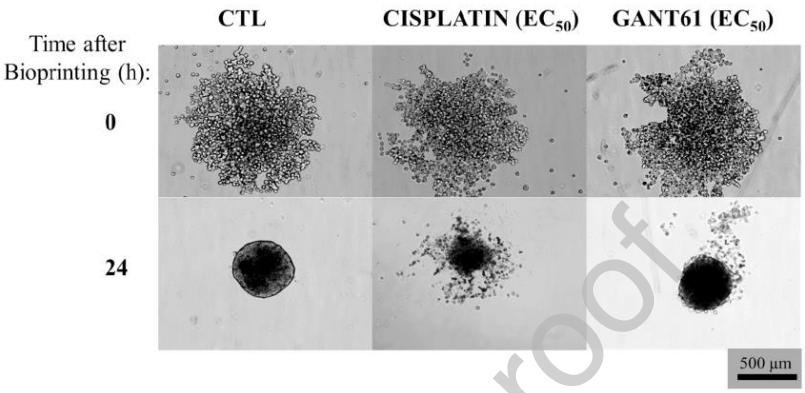
A



B

	CISPLATIN EC_{50} (µM)	GANT61 EC_{50} (µM)
HSC-3 2D model	15.4 (5-16.7)	10.8 (9-13.2)
HSC-3 3D model	30.3 (16-58)	19.8 (18.4-21.4)

Figure 11. Effect of chemotherapeutics 3D models. **a)** Representative images of spheroids at 0 and 24 hours after bioprinting following treatment with chemotherapeutics, cisplatin and GANT61. Spheroid images were obtained using an inverted microscope (EVOS XL, Thermo Scientific) with a 20X objective. Scale bar: 500 µm. CTL: negative control. **b)** 2D- and 3D-cell culture viability were evaluated using CellTiter-Glo® 3D after chemotherapeutics treatment at 24 hours. The data of EC_{50} values correspond to µM by non-linear regression analysis and are displayed as mean ± SEM (95% confidence interval) corresponding to three independent experiments performed in triplicate.

Histological analysis

Different approaches, such as histological analysis, can be utilized to analyze homotypic and heterotypic magnetic spheroids. Methods are conducted similarly in both 2D and 3D culture systems. For this protocol involving a cell density of $3x10^5$ cells using 24-well plates, histomorphological analysis was performed on both homotypic HSC3 (control spheroids) and

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heterotypic HSC3/CAF spheroids (1:1 ratio) (Figure 12). Through the cellular composition of heterotypic spheroids, it is possible to perceive characteristics compatible with each cell type. Toward this end, Siquara and collaborators [33] have described typical aspects of the HSC3 cell line and fibroblast cells in heterotypic spheroids, such as pleomorphic and polyhedral cells and fusiform and elongated cells, respectively. In addition, images of the HSC3 cell line and CAF in conventional 2D culture indicate the different morphology between these cell cultures from spheroids (N).

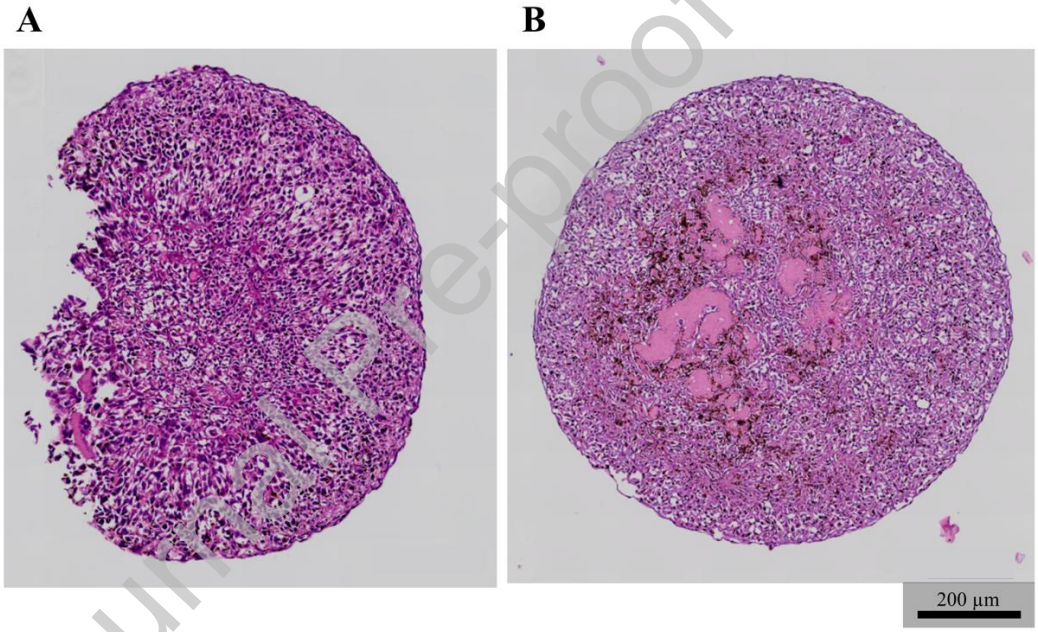

Figure 12. Histological analysis of 3D heterotypic culture. Representative images of spheroids at 24 hours after bioprinting. Spheroid zone formation can be seen in homotypic (a) and heterotypic (b) spheroids. H/E stains. Scale bar: 200 µm.

Perspectives and Limitations

The use of 3D models requires standardization of protocols, investment in analysis tools and protocols, as well as the purchase of consumables for 3D culture tests. In this study, HSC3 cells and combinations of HSC3/CAF cells were successfully bioprinted into spheroids, homotypic and heterotypic, with adequate reproducibility of size, shape, and integrity, offering a simple assay for

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the determination of general functional assays as cytotoxicity in 3D environment and drug resistance. Nevertheless, the need to understand and characterize the selected cell type remains a major challenge. The HSC3 cell line is a common model for studying metastatic squamous cell carcinoma, presenting a matrix-rich phenotype and replicating the aggressive behavior of OSCC [35]. On the other hand, the property of HSC3 cells of promoting high extracellular matrix deposition after bioprinting shows the need for a redefinition of parameters, such as the adequacy of the number of cells and beads, the need to attach small aggregates during the formation of spheroids, and the need for steps and time adjustment during the levitation and bioprinting protocol. Regarding heterotypic spheroids, we do not aim to identify and locate cancer cells and fibroblasts differentially. So, further assays such as immunophenotyping would be of great importance to identify and characterize cellular morphology and expression.

Therefore, these results emphasize that there are still challenges and obstacles that must be addressed, as demonstrated here, highlighting the need for the development of specific protocols for different types of cell lines to elevate the 3D bioprinting protocol for OSCC as a relevant tool in translational oncology.

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Author contributions

Methodology, investigation, software, validation, formal analysis, writing – original draft and writing – review and editing, T.B.S.d.A., R.L.R.N. and L.d.O.S.d.R.; conceptualization, investigation and methodology, I.N.B.; supervision and resources, R.D.C., D.W.L., B.S.d.F.S.; R.B.D.; validation, data curation, formal analysis, writing – original draft and writing – review and editing and supervision, V.A.O.S.; methodology, resources, validation, data curation, supervision, project administration and funding acquisition, C.A.G.R.

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Declaration of Competing Interests

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The authors declare no conflict of interest.