ARTICLE

A method for dissociation of oral cancer cell spheroids generated by magnetic 3D bioprinting

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Abstract
Spheroid 3D cultures yield a better representation of diverse cellular events in comparison with 2D cultures. However, the ability to investigate some specific phenotype/cell biology-related outcomes of experimental interventions by flow cytometry is somewhat limited in spheroid cultures, as it requires a single cell suspension with good preservation of membrane-bound proteins. In this study, we describe a method combining enzymatic and mechanical dissociation of large spheroids of five different oral squamous cell carcinoma (OSCC) cell lines (H314, SCC9, SCC25, UM-SCC1 and HSC3). Spheroids were generated using magnetic 3D bioprinting. Efficacy of dissociation and viability after dissociation were evaluated by flow cytometry. All spheroids were successfully dissociated into single cells with low cell aggregation using this method, except for spheroids of HSC3 cells. H314 and SCC9 spheroids yielded the highest number of events per microliter and therefore also the largest total number of events. H314 and SCC25 spheroids presented the better results for cell viability after dissociation. H314 spheroids also gave the largest total number of viable cell events. The dissociation method described can be useful for researchers interested in flow cytometry-based analysis of spheroid cultures.

Keywords Spheroids · Dissociation · OSCC · 3D bioprinting

Introduction

Tridimensional cultures have been increasingly used in the past decade (15,761 hits on PubMed since 2012 versus 5623 hits from 1968 to 2011) due to their higher capacity to reiterate various microenvironmental characteristics observed in vivo when compared with bidimensional cultures (Białkowska et al. 2020; Pinto et al. 2020). It is now widely acknowledged that 3D cultures are better representations of diverse cellular phenomena and thus have been used in multiple applications, from cell biology to pharmaceutical research over a wide range of areas: immune response, oncology, developmental biology, metabolomics, pathophysiology (Pinto et al. 2020; Longati et al. 2013; Lee and Kim 2021).

As a general concept, 3D cultures can be established using a variety of approaches, including spheroids, organoids, scaffold approaches, microfluidics/lab-on-chip techniques, 3D bioprinting, hydrogels, scaffold-based approaches, hanging drop, magnetic levitation, microcarrier beads or microfluidic devices. There are particular characteristics and advantages of using each approach that makes each method better suited for a given purpose, which have been reviewed elsewhere (Białkowska et al. 2020; Pinto et al. 2020).

Spheroid cultures are defined as tridimensional cellular aggregates assembled in an environment that does not allow cell adhesion to a substrate (Sant and Johnston 2017; Langhans 2018). These type of 3D cultures were initially described over 5 decades ago and were used as a means to better recapitulate tumor cell resistance to radiation therapy (Sutherland et al. 1971). Since then, various other cell types have been successfully used in spheroid cultures including hepatocytes, stem cells, neuronal cells, cardiomyocytes, multiple neoplastic cell lines, fibroblasts, endothelial cells, osteoblasts, chondrocytes and adipocytes.

Spheroid 3D cultures are characterized by a gradient of exposure to molecules in suspension in the culture medium, which decreases from outer layers towards its core. The core of the spheroid is also characterized by reduced oxygen tension. These characteristics recapitulate the conditions in the microenvironment of solid tumors. Thus, in spheroid cultures combination of lack of adhesion to a solid substrate, increased cell-to-cell and cell-to-matrix contacts via integrins and other cell adhesion molecules and the gradient of nutrient and oxygen tension ultimately shape the phenotype of a given cell very differently than what is observed in bidimensional cultures. Thus, even in single cell type spheroids there can be heterogeneous colonies of cells in distinct 'areas' of the spheroid, showing different levels of proliferation, viability and distinct phenotypes. However, the very characteristic of microenvironmental-shaped cell phenotype of spheroid 3D multicellular cultures that is a major advantage in the reiteration of in vivo conditions poses an important limitation in assessing of phenotype outcomes by flow cytometry, which requires dissociation of these 3D structures into single cell suspensions. Thus, an essential step to enable the use of powerful multiparameter flow cytometry assessments of phenotype and cell biology outcomes in 3D spheroid cultures is the effective dissociation and resuspension of cells. This represents an important methodological challenge, as it needs to be both efficient, for adequate resuspension of multicellular aggregates of considerable toughness, and gentle to avoid inducing phenotype-altering cellular stress, preserve cell viability, as well as the integrity of the cell membrane and the membrane-bound proteins.

There are a number of studies reporting data obtained after dissociation of 3D spheroid cultures (Grässer et al. 2018; Metzger et al. 2021; Velletri et al. 2022) including some studies addressing specifically the methods for dissociation (Grässer et al. 2018; Metzger et al. 2021), but none have investigated dissociation of large spheroids established by magnetic aggregation/levitation. In this study we report a method combining enzymatic and mechanic forces for the efficient and gentle dissociation of spheroids that is suitable for subsequent flow cytometry analysis.

Materials and methods

Cells and reagents

Five different OSCC cell lines (H314, SCC9, SCC25, UM-SCC1 and HSC3) were used in the experiments. All cell lines were grown bidimensionally in cell culture-treated polystyrene dishes (Corning #436107) in DMEM/F12 supplemented with 400 ng/mL hydrocortisone, 10% heat-inactivated FBS and 1% penicillin/streptomycin. Culture medium, supplements and Trypsin–EDTA were purchased from ThermoFisher Scientific. Tumor dissociation enzyme mix was purchased from Miltenyi Biotec (#130-095-929). Fluorescent viable cell stain was from BioGems (#62510-00).

Establishment of spheroid cultures

We used a bioprinting magnetic field method to form spheroids from OSSC cell lines, wherein cells are magnetized with biocompatible magnetic nanoparticles (Tseng et al. 2013; 2014, 2015; Castro-Chavez et al. 2013; Kim et al. 2013; Daquinag et al. 2013). Cells are quickly aggregated by mild magnetic forces and subsequently (i.e., after removal of the magnetic field) the established aggregates can mature and mimic characteristics of native tissues (Tseng et al. 2013; Daquinag et al. 2013; Souza et al. 2010).

After obtaining a single cell suspension of the cell lines in a 1000 µL volume of complete culture medium, cells were mixed by repeated gentle up-and-down pipetting (10 times) with magnetic nanoparticles (NanoShuttle-PL, Greiner Bio-One) at the ratio 1 µL of nanoparticles: 20,000 cells (the manufacturer does not inform the concentration of nanoparticles) After initial mixing, cells are centrifuged (400×*g*, RT, 5 min) and resuspended. This cycle of centrifuging/resuspension was repeated two additional times to increase contact between cells and magnetic nanoparticles. After the last centrifuging/resuspension cycle, cell suspension (1×10^5 cells/spheroid/well) was transferred to an ultra-low attachment (ULA) 96-well plate and placed in the CO_2 incubator exposed to the magnetic field by placing the spheroid drive magnet under the 96-well ULA plate. The spheroid drive was kept under the 96-well ULA plate for 3 h to promote initial cell aggregation and then removed. Spheroids were observed at 24, 48 and 72 h on brightfield microscopy. Spheroids were kept in culture for 72 h before dissociation to allow for establishment of a defined microenvironment, including production of extracellular matrix proteins and eventual changes associated with microenvironmental changes and cell-to-cell/cell-to-matrix interactions.

Dissociation method

Different enzyme-based approaches and enzymatic/mechanical combinations were extensively tested empirically and based on related literature reports (supplementary Table 1). The condition that has effectively dissociated the spheroids for flow cytometry is described.

Two spheroids from each cell line were transferred to 1.5 mL microfuge tubes using low retention 200 µL micropipettes with the tip cut off to increase the bore diameter and avoid shear fluid stress or inadvertent binding of the spheroid to the inner surface of the pipet tip during transferring. Microfuge tubes containing culture medium and the spheroids were centrifuged (400×*g*, RT, 5 min), the culture medium was removed, and the spheroids resuspended in the enzymatic mix. The incubation was done for 10 min at 37 °C in a thermomixer (Thermomixer® confort, Eppendorf) adjusted for 1400 RPM as the mechanical force component in the dissociation process (Table 1).

After completion of the dissociation procedure, cells were resuspended by gently pipetting up-and-down 10 times and the solution filtered through a 70 µm cell strainer to remove large aggregates. The microfuge tube was washed twice with 1000 µL of DMEM to collect all dissociated loose cells and also filtered into the same microfuge collection tube through the 70 µm cell strainer. The tubes were centrifuged (3000 rpm, RT, 5 min) the supernatant carefully discarded through pipetting and cells resuspended in PBS.

Efficacy of dissociation

Efficacy of dissociation was assessed by the number of events/µl, total number of events and by the presence of doublets assessed in FSC-Area × FSC-Height dot blots on a flow cytometer (Cytoflex, Beckman Counter).

Table 1 Dissociation conditions used in spheroid 3D cultures

Enzyme	Dilution	Mechanical and thermal stimulus	Time
Tumor dissociation enzyme mix	1100 µl DMEM, 50 µl Enzime H, 25 µl Enzime R and 12.5 µl Enzime A* (Tumor Dissociation Kit, human. Miltenyi Biotec, cat# 130-095-929)	Thermomixer at 1400 rpm and 37 °C	10 min enzymatic and mechanical dissociation

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Cell viability

The suspension from dissociated spheroids were stained with a fluorescent viability stain according to the manufacturer instructions (BioGems, #62510-00), ressupended in 500 µl of PBS, and data was acquired on the flow cytometer (Cytoflex, Bekman-Coulter).

Statistical analysis

Data was analyzed using GraphPad Prism 9 (GraphPad Software Ind., San Diego, CA, USA). Spheroid area comparisons were done using Two Way ANOVA followed by Tukey post-hoc analysis. All the other comparisons were done using Kruskal–Wallis One Way ANOVA followed by Dunn's multiple comparisons test. The significance level was set at 95% ($p < 0.05$) in all analyses.

Results

Spheroid culture formation

The bioprinting magnetic formation of spheroids generated large spheroids (100,000 cells used in the initial establishment of the 3D cultures) of five different OSCC cell lines in 24 h (Fig. 1). The spheroids presented a rounded morphology at all experimental periods (24, 48 and 72 h) with differences among the distinct cell lines in spheroid density, diameter/area and regularity (Fig. 1A). H314 spheroids were more 'loosely' aggregated. SCC25 cells generated more irregular spheroids. HSC3 cells formed the most regular and dense spheroids. UM-SCC1 generated the smallest spheroids; moreover, these spheroids were surrounded by more cellular debris. Regardless of the OSCC cell line used, the spheroid diameter continuously decreased (Fig. 1B), whereas the density continuously increased over time (Fig. 1A).

Efficacy of dissociation

The dissociation of spheroids should be gentle and generate a sufficient number of events for further downstream analyses by flow cytometry. The minimum amount of 10,000 events is considered the gold standard in flow cytometry as it usually allows for accuracy, biological representativity, detection of relatively rare events and the application of statistical comparisons. In addition, elimination of doublets is an important gating strategy to obtain good quality flow cytometry data.

We established the dissociation of a pool of two large spheroids (per cell lineage) for a short incubation time in the enzyme mix and under mechanical forces (10 min, 1400 rpm, 37 °C) to compare the feasibility of dissociation of spheroids from distinct OSCC cell lines. All spheroids could be dissociated into single cells using the described method, with exception of HSC3 spheroids (Fig. 2A). H314 and SCC9 cell lines yielded the highest number of events per microliters and therefore also the largest total number of events (more than 10,000 events in each experimental repetition) (Fig. 2B and C). The mean percentage of doublets (from gate P1, Fig. 2A) was below 20% for spheroids of all cell lines, except for HSC3 (Fig. 3). Importantly, for all cell lines except for H314 it may be necessary to combine more than two 100,000 cell spheroids (or multiple smaller sized spherpoids) to obtain the minimum 10,000 events after dissociation. We think this reflects the low yield of the dissociation procedure and it is up to the individual researcher to consider if this low yield represents a possible bias for the analyses of the specific outcomes of interest.

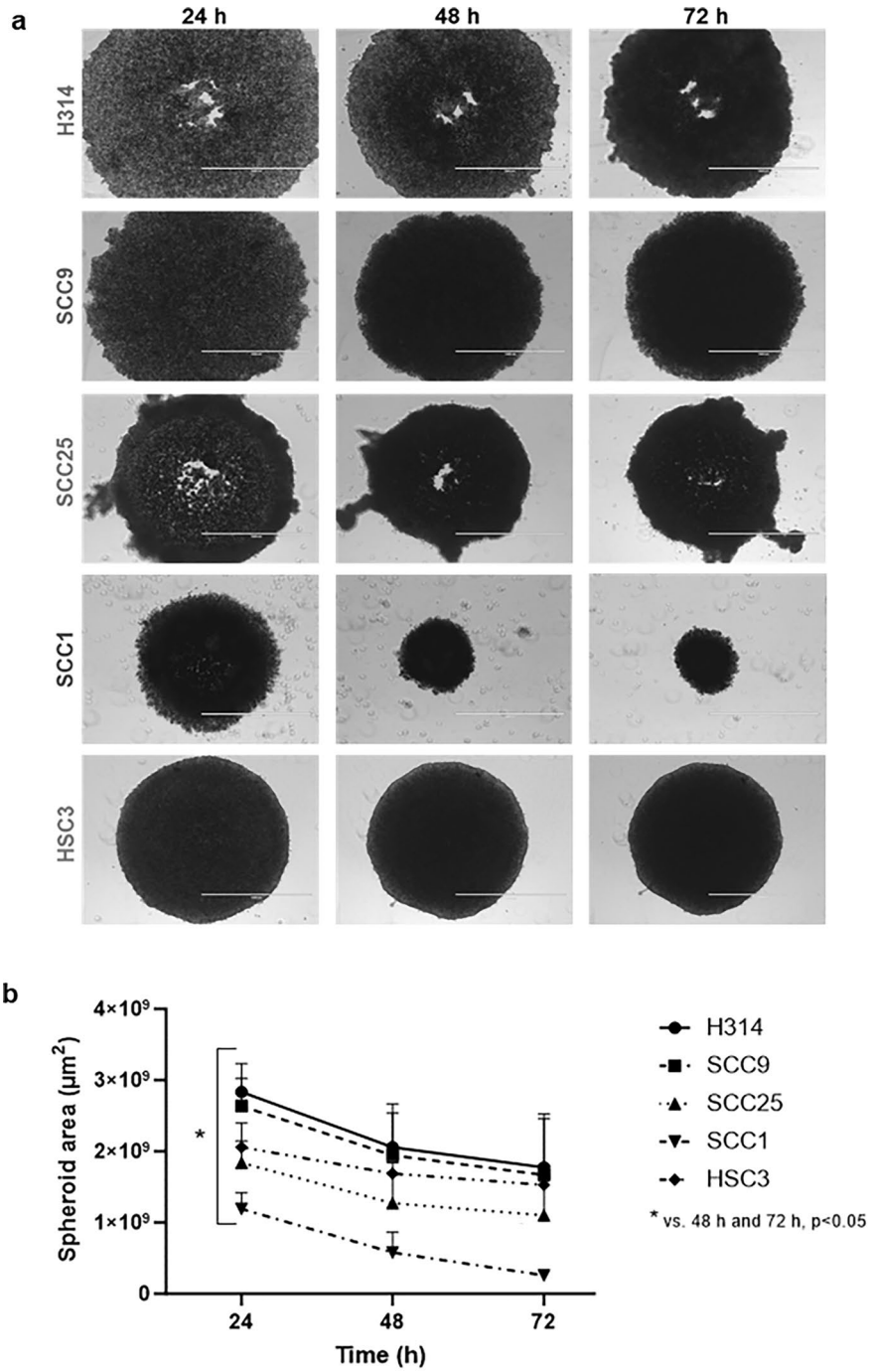

Fig. 1 Bioprinting magnetic field technique form spheroids from OSCC cell lines. **A** Representative images documented after 24 h, 48 h and 72 h of spheroid drive magnet removal showing the morphology of H314, SCC9, SCC25, SCC1 and HSC3 spheroids. H314, SCC9 and HSC3 formed spheroids with a high rate of yield, whilst SCC25 spheroids were the most irregular; SCC1 spheroids were surrounded by more cellular debris and it did not form spheroids in the third independent experimental repetition. **B** Quantification of spheroids area represented by the mean ± SD of spheroids from three independent experiments (SCC1 from two independent experiments). The size of all spheroids were continuously decreased over time. Scale bar: 1000 µm

Cell viability

Single cells from dissociated spheroids were selected after exclusion of doublets (gate P2, Fig. 3B). All OSCC cell lines presented mean percentage viability above 20%, with better results for H314 cell line (~ 70%) and SCC25 cell line (~ 60%) (Fig. 4). H314 presented the largest final total number of viable cell events (Fig. 5).

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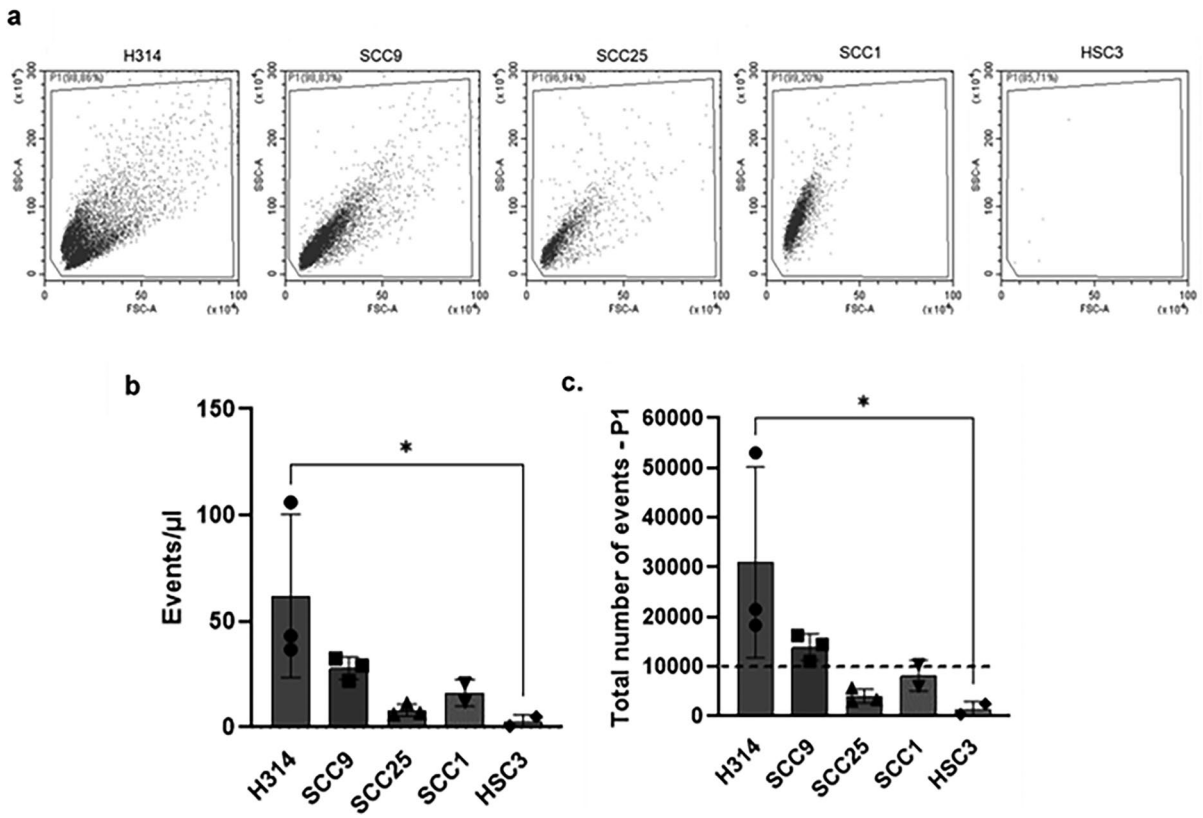

Fig. 2 Spheroids formed by OSCC cell lines could be dissociated into single cells. **A** The spread of events in representative FSC × SSC dot plots. **B** Number of events/µl. **C** Total number of events in 500 ul of cell suspension from the dissociation of two large spheroids for each cell line. Data is represented by mean ± SD from three (H314, SCC9 and SCC25) or two (SCC1 and HSC3) independent experiments. *$p < 0.05$

Discussion

Tridimensional spheroid cultures are a powerful approach to investigate various aspects of cell biology. However, the ability to investigate some specific phenotype/cell biology-related outcomes of experimental interventions in spheroid 3D cultures is somewhat limited, particularly when dissociation of the spheroid cell aggregate is required for the subsequent analyses. The subsequent investigation of phenotype and other cell biology outcomes by flow cytometry requires effective and gentle dissociation of spheroid cultures.

We tested various different enzyme-based approaches and enzymatic/mechanical combinations for dissociation of the cancer cells spheroids generated by magnetic 3D bioprinting (supplementary Table 1) and these protocols resulted either in poor cell viability or in insufficient dissociation. In this study we report a protocol for dissociation of spheroid cultures of OSCC cells using a proprietary mix of enzymes (Miltenyi Biotec) associated with mechanical disruption that yielded good results in both efficiency and cell viability. It is important to stress that the goal of this manuscript is to provide a 'starting point' for researchers interested in dissociating spheroids, and we cannot rule out the possibility that efficacy of the procedure may vary with different methods of generating spheroid cultures. We also note that the cell type influences the efficacy of dissociation. This variability is likely related with the ability of different cell lines to form spheroids, cell density/cell proliferation, production of extracellular matrix, cell-to-cell and cell-to-matrix interactions. In support to this rationale, we observed that, albeit using the same method to generate spheroids, some cell lines generate more dense and tough spheroids (e.g., HSC3), whereas spheroids of other cell lines (e.g., H314) are more 'loosely' aggregated. This phenomenon was also noted for other tumor cell lines and cancer-associated

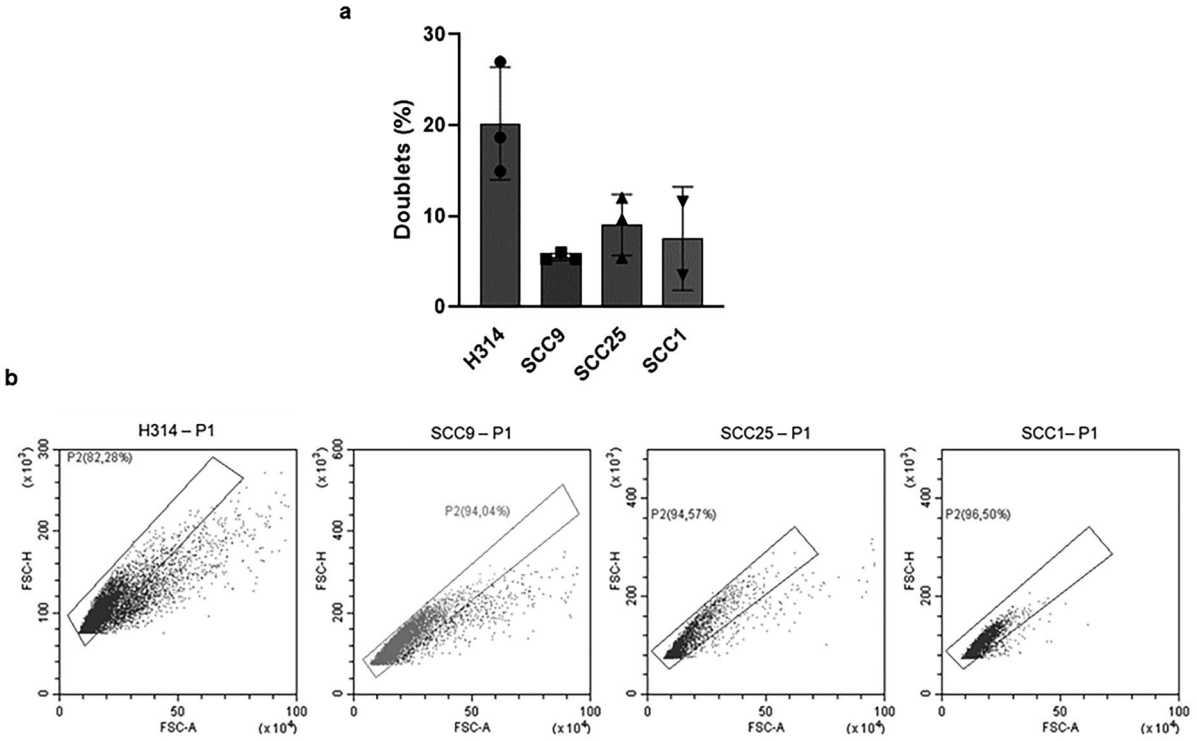

Fig. 3 Dissociation of spheroids generated low percentage of doublets. **A** Percentage of doublets from gate P1 (Fig. 2A) represented by mean ± SD from three (H314, SCC9 and SCC25) or two (SCC1) independent experiments. **B** Representative gate strategy in dot plot graphs (FSC-H × FSC-A) for exclusion of doublets

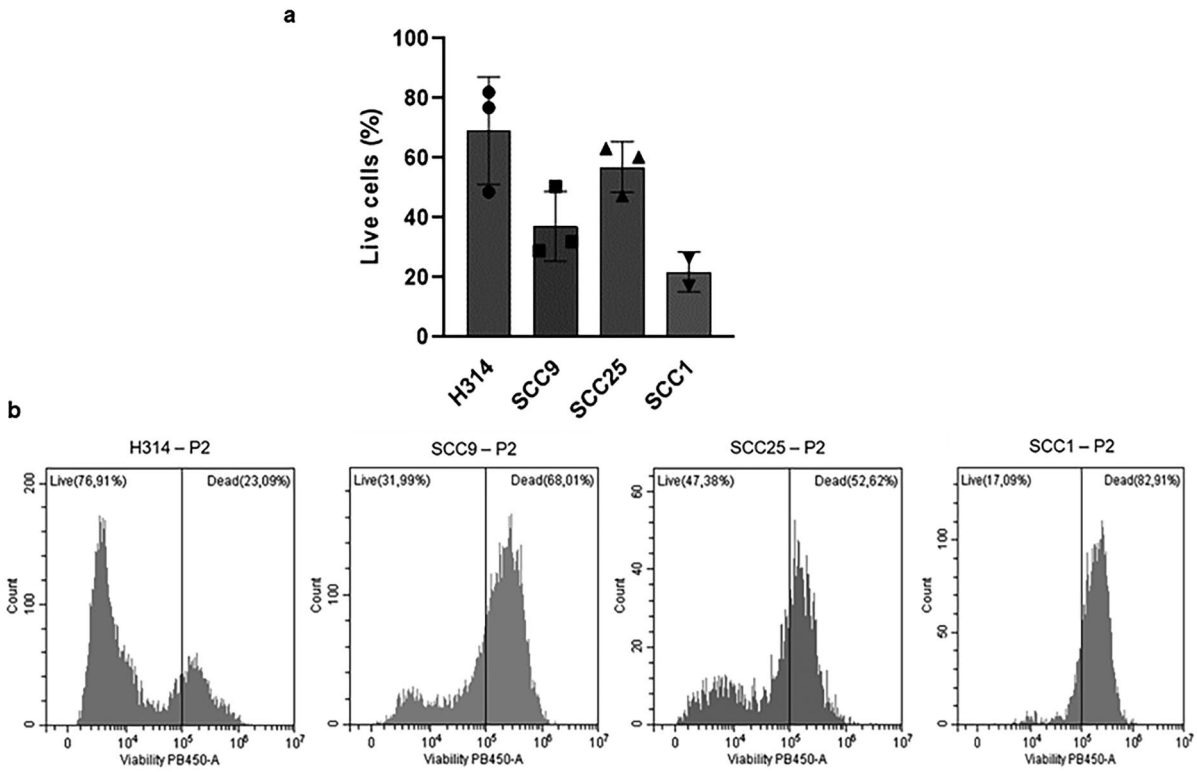

Fig. 4 Living cells after dissociation of spheroids. **A** Percentage of living cells from gate P2 (Fig. 3B) represented by mean ± SD from three (H314, SCC9 and SCC25) or two (SCC1) independent experiments. **B** Representative gate strategy in histograms graphs for exclusion of dead cells

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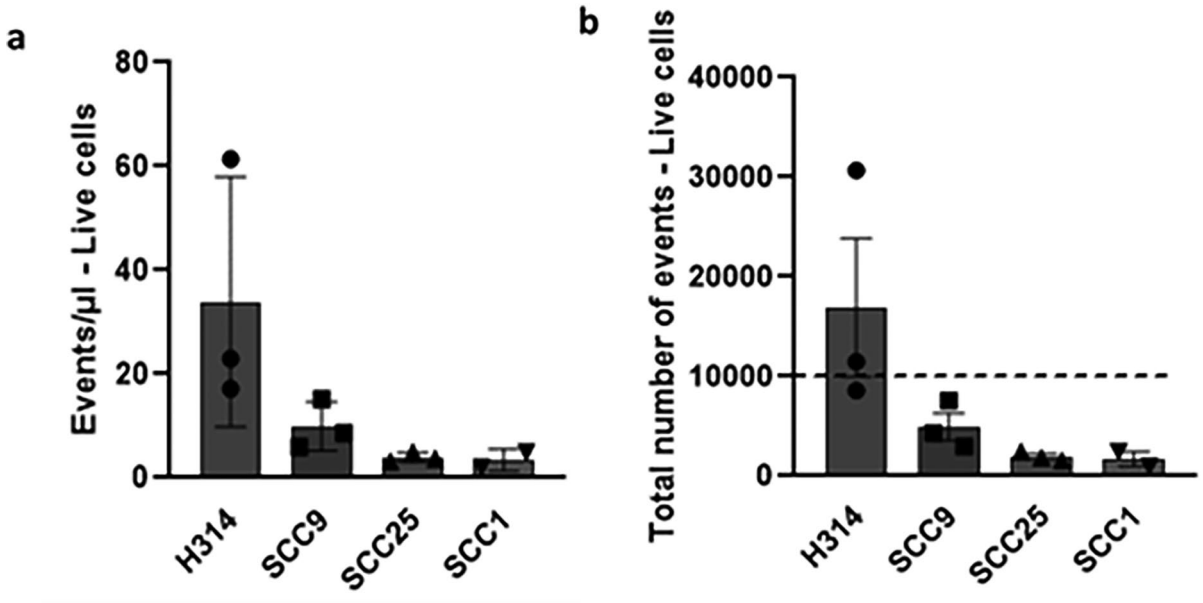

Fig. 5 Dissociation of spheroids generated a satisfactory quantity of living cells events for further downstream flow cytometry analysis. **A** Number of events/µl related to living cells. **B** Total number of events 500 ul of cell suspension related to living cells from the dissociation of two large spheroids for each cell line. Data is represented by mean ± SD from three (H314, SCC9 and SCC25) or two (SCC1) independent experiments. *$p < 0.05$

fibroblasts using the same magnetic field method for generating 3D multicellular spheroids (Hou et al. 2018). Cell type- and cell line-specific discrepancies in expression of integrins and other adhesion molecules (e.g., cadherins) as well as of ECM proteins ultimately affect cell-to-cell and cell-to-matrix contacts and the resilience of the spheroid (Burleson et al. 2004; Lin et al. 2006; Ivascu and Kubbies 2007), which subsequently influence both the efficacy and viability of the dissociating procedure. However, since it is not always feasible to customize the dissociation method for each cell line/condition there will always be a possible bias associated with cell death, cell stress or loss of membrane-bound proteins caused by the dissociation procedure. Moreover, efficacy of dissociation of 3D spheroids from the same cell type / cell line may vary depending on the very experimental conditions of interest in a given study.

In this study we used only one method to establish spheroid 3D cultures, thus the method of generating spheroids is not a variable to consider when interpreting the results. We cannot rule out the possibility that spheroids generated by different methods (e.g., hanging drop, scaffold-based, spontaneous cell aggregation, spinner cultures) (Costa et al. 2016) may yield different results with the same dissociation methods. Nevertheless, the data reported in this study may provide a starting point for researchers interested in dissociating and recovering intact and viable cells from 3D spheroid cultures for subsequent analyses.

Nevertheless, we assume that the dissociation procedure described will yield similar results in spheroid cultures established by other methods. It is important to initially consider that this method uses biocompatible iron-based nanoparticles that integrate in the bi-lipid cell membrane and upon exposure to magnetic fields generated by weak magnets (10–300 mT) promote an initial aggregation of cells in ultra-low attachment plates (Tseng et al. 2013, 2014, 2015; Castro-Chavez et al. 2013; Kim et al. 2013; Daquinag et al. 2013; Souza et al. 2010; Caleffi et al. 2021; Gaitán-Salvatella et al. 2021). The advantages of this method are its reliability, homogeneity and rapidity, as spheroids are formed within 24 h in contrast to other methods that are more labor-intensive, lengthy and, thus, expensive. Importantly, the speedy process of spheroid formation allows for the preservation of the initial cell viability and basal phenotype of the cells, which will be subsequently

shaped in the established tridimensional microenvironment, as opposed to a continuous shift in these cellular aspects associated with methods that generate spheroid cultures over longer periods (3–5 days) of time.

There is evidence that exposure to weak magnetic field (3–50 mT) for a short period of time has no effect on cell biology (Wang et al. 2009; Badr-Eldin et al. 2022), as opposed to static exposure to a moderate/high magnetic field (300 mT and up) that affected production of ROS and gene expression in endothelial cells (Potenza et al. 2010). A low intensity (e.g., 80 mT) magnetic field has been shown to affect cellular functions, however the length of continuous/static or pulsating/dynamic exposure of cells was at least 24 h (Zanoni et al. 2019; Manjua et al. 2021), which is much longer than the short period (3 h) used to promote initial cell aggregation in the 3D bioprinting method used in this study.

After 3 days in culture without exposure to a magnetic field, we think that there was no residual magnetic influence on the contact among cells, and therefore spheroids formed by this method may be no different than spheroids formed by other methods in terms of the strength of cell-to-cell interactions. Short-term exposure of cells to magnetic fields has long been used as a method to sort cells based on iron particle-conjugated antibodies without any significant effects on viability or cell biology (Lea et al. 1988; Hardwick et al. 1992; Safarík and Safaríková 1999). There is evidence indicating that the magnetic nanoparticles do not affect cell viability or biology (Tseng et al. 2014, 2015), albeit these iron-based nanoparticles can be observed on brightfield microscopy as a dark shade even after 4 or 5 days in culture, similarly to the observations in magnetic-based cell sorting. In fact, differences between the different cell lines may be attributable in part to the chosen method of generating spheroids, among other aspects such as the differential production of extracellular matrix and expression of adhesion-mediating molecules regulating cell-to-cell and cell-to-matrix contacts, and spheroids' density and dimensions.

Conclusion

In conclusion, the dissociation method described can be useful as a starting point for dissociating spheroid cultures generated using magnetic 3D bioprinting into single cells suitable for subsequent flow cytometry analysis. It may be necessary to combine more than two spheroids to obtain the minimum amount of 10,000 events for some cell lines. Nevertheless, it is not possible rule out the possibility of biases introduced by the dissociation method.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10616-025-00776-w.

Author contributions NARF—conducted experiments, data analyses and manuscript preparation; CRN—conducted experiments; RDC—conceptualization and manuscript review; CRJ—conceptualization, experimental design, supervision, manuscript preparation and review.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare that there are no conflict of interest.

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