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Self-Renewal and CSCs In Vitro Enrichment: Growth as Floating Spheres

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Abstract

Cancer stem cells (CSC) are a vital component to the progression and reoccurrence of cancers, making them a primary target of study for both fundamental understanding of cancer biology and the development of effective and targeted treatments. CSCs reside in a complex 3D microenvironment, and the 3D spheroids are an indispensable tool in tumor biology due to their 3D structure and replication of the tumor microenvironment. Within this chapter the methodology for CSC isolation, suspension culture in hanging drop model, and characterization assays for CSC are described. First, the methodology for identifying and isolating CSCs from patient tumors, ascites, or cancer cell lines is described through the use of FACS analysis. Next, a detailed description of 3D hanging drop model for generating CSC spheroids is provided, followed by maintenance and monitoring techniques for extended 3D culture. Analysis methods are described for the quantification of CSC spheroid proliferation and viability tracking, throughout culture by on-plate alamarBlue fluorescence. Additional viability assays are described utilizing confocal microscopy with Live/Dead Viability/Cytotoxicity Kit. The characterization of CSCs populations within spheroids is described through FACS analysis. Further, an immunohistochemistry procedure is described for cell-cell and cell-matrix interaction assessment. Finally, several notes and tips for successful experiments with 3D CSC spheroids on the hanging drop model are provided. These methods are not only applicable to CSCs within a variety of tumor cell types, for not only understanding the fundamental tumor biology, but also for drug screening and development of preclinical chemotherapeutic strategies.

Keywords

Spheroid; Cancer stem cells; Ovarian cancer; Hanging drop; Proliferation; Viability; Drug sensitivity

1 Introduction

Cancer stem cells (CSC) are classified as the subpopulation of tumor cells capable of tumor initiation, self-renewal, and differentiation [1, 2]. CSCs maintain a level of pluripotency which drives tumor heterogeneity [1, 3, 4]. Through heterogeneity, adaptations arise that enable better survival of cancer cells when confronted with chemotherapy drugs or other treatments such as radiation and inhibitors [1, 5]. These treatments act akin to natural selection process targeting only a select group of cells and leaving behind those resistant to

the treatment because cells with different characteristics respond differently to treatment [6]. CSCs are often more difficult to kill and thus are predicted to survive despite apparent reduction in the primary tumor. This resistance to treatment is attributed to characteristics such as overexpression of ABC transporters, enhanced aldehyde dehydrogenase activity, specific signaling pathways, response to DNA damage, epithelial to mesenchymal transition, and dormancy [6, 7]. They can make up small fractions of the bulk tumor, as little as 0.1% [8]. CSC populations thus provide the tumor with a source of not only constant bulk renewal but continued survival for a small subpopulation despite drug treatment. These CSC traits harbor poor outcomes for patients so long as CSC populations are retained. In order to improve patient outcomes, CSCs are a prime target for developing effective treatment strategies.

In vivo, tumor cells reside in a three-dimensional (3D) environment with complex structural and environmental signaling. The complexity of this habitat is difficult to mimic in traditional 2D monolayer cultures. We have previously demonstrated that by utilizing hanging drop plates, which provide a 3D environment to culture few to single cells at a time, we can recapitulate the key features of the innate tumor microenvironment. The hanging drop method has been found to more closely mimic the outcomes of drug success in vivo, providing a more accurate representation of effectiveness and patient response [9–11].

The hanging drop method provides a 3D isolated suspension environment where a small number of cells, such as rare patient-derived CSCs, can be maintained and propagated into spheroids originating from the same stem or progenitor cells. This allows for the propagation of selected cell populations over several weeks and thus is useful for the study of predicted CSC markers and treatment. Here, we demonstrate the hanging drop method to produce CSC spheroids from malignant ascites or primary tumors that have been isolated using desired CSC markers. We detail the preparation and care required for CSC spheroid plating in 384 hanging drop plates, maintenance through feeding and image tracking of spheroid formation, as well as proliferation and viability analysis. Finally, use of spheroids for quantifying drug sensitivity response is described, as well as proper embedding and sectioning techniques for immunohistochemistry analysis of CSC spheroids. We have chosen ovarian cancer as the model system for these demonstrations; however, this approach can be applied to CSCs from any cancers. Beyond ovarian cancers, we believe that this platform developed in this proposal has a wide appeal to a variety of other cancer cells and CSCs, and will prove useful for both tumor biology, as well as drug-screening studies.

2 Materials

Prepare all the solutions using ultrapure deionized water and analytical research grade reagents. Commercial antibody sources are indicated within the methods.

Following materials are required for the protocols:

DMEM and Ham's F12 basal cell culture medium, fetal bovine serum (FBS) 10%, Antibiotic/Antimycotic solution, PBS, Ammonium-Chloride-Potassium (ACK) lysis buffer, alamarblue reagent, Live/Dead Cytotoxicity kit, 4',6-diamidino-2-phenylindole (DAPI), low

melting agarose, CD133 antibody, Pluronic acid, cisplatin, ALDH1A antibody, secondary antibodies, and ALDE-FLUOR assay kit (StemCell Technologies, Vancouver, BC).

2.1 CSC Spheroids Cell Culture Medium (See Note 1)

In order to promote the formation of spheroids, and the maintenance of the CSC population, the cell culture growth medium composition is the key. For spheroids initiated with CSCs, use of a serum-free medium is strongly recommended. 50 mL of a common medium composition can be made in the manner outlined below, though this will not work for all spheroids, and some composition adjustment may be necessary for CSCs of a specific tumor. The composition noted below applies to CSCs from ovarian cancers.

1. Form a 1:1 mixture of DMEM and Ham's F12 medium in a 50 mL conical tube.
2. Place 47.150 mL of the DMEM/F12 mixture into a separate tube.
3. Add 1 mL of 50× B-27 Supplement without Vitamin A to the same tube for a 1× mixture.
4. Add 25 µL of 10 µg EGF to the tube to form a 5 ng/mL Solution.
5. Add 25 µL of 10 µg bFGF to the tube to form a 5 ng/mL Solution.
6. Add 500 µL of 100× Antibiotic-Antimycotic for a 1× Solution.
7. Add 500 µL of 100× MEM Non-Essential Amino Acids for a 1× Solution.
8. Add 500 µL of 100× Insulin-Transferrin-Selenium for a 1× Solution.
9. Add 300 µL of 200 mM L-Glutamine to the tube, to form a 1.2 mM Solution.
10. Mix all reagents together and store this serum-free CSC medium in a 4 °C fridge.

3 Methods

3.1 Isolation of CSCs Using Fluorescent-Activated Cell Sorting (FACS) from Primary Patient Malignant Ascites or Ovarian Cancer Cell Lines

Our lab isolates ovarian cancer CSCs based on a concurrent elevated activity in Aldehyde dehydrogenase (ALDH) and expression of CD133, based on previously published protocols [12], nevertheless Subheading 3.2 and following, apply to CSCs independent of tumor type and isolation method. We routinely isolate ovarian CSCs from patient ascites, primary or metastatic tumors and from several ovarian cancer cell lines. The following outlined protocol is used to isolate ovarian CSCs.

1. Centrifuge the malignant ascites, primary, or metastatic tumor sample collected under IRB approved protocols at $1000 \times g$ for 5 min (*see Note 2*).
2. Lyse the red blood cells using the ACK lysis buffer following the manufacturer's protocol. Briefly, incubate the cell pellet for 3–5 min with ACK buffer, and

¹We recommend making fresh serum-free medium every week for the CSC spheroids.

²For cell lines, trypsinize cell lines from a regular tissue culture plate and omit the ACK lysing buffer step, and proceed with the staining. For primary tissues, mechanically dissociate tissues following dissection until single-cell suspensions are obtained. The Miltenyi MACS human Tumor Cell Dissociation and Isolation Kit can also be used.

centrifuge at $300 \times g$ for 5 min. Discard the supernatant and resuspend cell pellet with neutral phosphate-buffered saline pH 7.4.

3. Filter cell suspension through 40 μm nylon filters twice, washing in between filtration and recovering cells using centrifugation.
4. Triturate cell suspension by pipetting to obtain single-cell suspensions (*see Note 3*).
5. Stain and sort by flow cytometry cell population that express both ALDH+ (Aldefluor Kit, following manufacture instruction) and CD133+. A representative FACS sorting of ovarian CSCs from patient ascites is shown in Fig. 1.

The following methods can be performed on CSCs from different tumors that have been selected with other methods and markers.

3.2 Generating Spheroids from CSCs Using the 384-Well Hanging Drop Array Plate

1. Sonicate the new hanging drop plate in a water bath for 20 min to dislodge any debris on the surface or within the wells.
2. Using gloves, rinse the plate in running DI water, periodically shaking the plate out vigorously—to remove debris incurred from injection molding and manufacture of plates.
3. Place the plate in a 0.1% Pluronic acid bath for 24 h and cover bath. Pluronic applies an amphiphilic surfactant coating to the plate preventing cell adhesion and thus promoting proper spheroid formation [13, 14].
4. Remove the plate with gloved hands from the bath and rinse the plate again in DI water, making sure to remove all water from wells.
5. Place the plate under UV light for sterilization for at least 30 min on each side. The plate is now ready to use (*see Note 4*).
6. Prepare a sterile 6-well plate to act as a humidity chamber by adding 4–5 mL of filtered DI water into each of the 6 wells. This will provide the humidity necessary to maintain the hanging drops over extended periods of time. Without proper humidification excessive evaporation of the drops is observed leading to cell death.
7. Next, place the 384 hanging drop plate atop the prepared 6-well plate and pipet 800 μL of filtered DI water into each of the border reservoirs for further humidity control.
8. Each hanging drop will contain 20 μL of volume. Plan out plate design before proceeding to plating. Be sure to leave a 2-well border around the outside edges

³A standard hub pipetting needle can also be used to break cell pellets into single-cell suspensions. Use up to gauge 23.

⁴It is important to sterilize both the faces of the hanging drop plate.

of the plate in order to avoid contact with the hydration wells. Schematic of optimal design of hanging drop plate is shown in Fig. 2.

9. Trypsinize cells as per a regular passage, and count cells using a hemocytometer or any other automated cell counter. Dilute cell concentration appropriately to the desired concentration (i.e., 100 cells in a 20 μ L drop). It is suggested that 100 cell spheroids are attempted first and working down to smaller concentrations (and even as low as 1 cell per hanging drop).
10. Mix the working cell solution using a 1000 μ L pipet before plating (*see* Note 5).
11. Pipet 20 μ L of the cell solution into desired well (Fig. 2; *see* Note 6).
12. Once all desired wells are filled, place the lid of the 6-well plate, atop the hanging drops effectively sandwiching the 384-well plate.
13. Use parafilm® to carefully seal all outside edges of plates in order to prevent additional evaporation of droplets.
14. Place the entire sandwiched plate carefully into a standard CO₂ humidified incubator (5% CO₂, 37 °C). The hanging drops will need to be fed once every other day for cell maintenance.

3.3 Maintenance and Monitoring of Ovarian CSC Spheroids in Hanging Drop Arrays

1. Carefully remove the plate from the incubator and place into sterilized space in a biological safety cabinet.
2. Remove parafilm from plate edges, being careful not to disturb the droplets (*see* Note 7).
3. Carefully carry only the 384-well plate from the biological safety cabinet to the imaging location and lower into microscope tray for live-cell imaging using the right plate adapter (standard multiwall plate adapters for microscope stages will fit the 384-well hanging drop array plate).
4. Using the scanning option at either 10 $\times$ or 20 $\times$ magnification, hanging drops can be observed and imaged on an inverted microscope (e.g., Olympus IX81, Japan, equipped with an ORCA R2 cooled CCD camera and CellSens software). Figure 3 demonstrates phase images of a patient-derived CSC spheroid developing from day 1 through day 35, initiated from a single ALDH+ CD133+ cell, isolated using flow cytometry (*see* Note 8). Figure 4a illustrates increase in spheroid size over 5 days, when initiated with 100 cells per drop.

⁵It is recommended to mix the cell suspension often in order to achieve consistent cell counts per drop, as cells tend to aggregate in solution over time, which changes the cell density in the suspension.

⁶It is recommended that the pipet tip rests on the well at an angle, since the vertical positioning tends to cause the droplet to fall. Also, it is advised to use every other well, staggering each row to avoid the unintentional merging of droplets.

⁷Be sure all parafilm is removed before attempting to take off lid as remnants of parafilm can cause sticking and disturbance of hanging drops. It is advised to hold the plate stable for this process by placing pressure on the lid.

⁸Keep the imaging time under 10–15 min, as prolonged exposure to the microscope light will cause evaporation leading to cell death, as well as increased likelihood of acquiring debris.

5. Remove the plate from imaging stage and place back into the laminar flow biological safety cabinet, atop the hydration plate.
6. Add 2 or 3 μL of medium to each well containing a droplet. 2 μL is advised for every other day. These volumes can be adjusted according to the observed droplet size as some may evaporate more than others (*see* Note 9).
7. Place the lid back on the 384-well plate and 6-well plate stack
8. Reseal edges of stack with parafilm and carefully place in an incubator.

3.4 Quantification of CSC Spheroids' Proliferation and Viability

Alamarblue is a cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. The resazurin is a nontoxic, cell permeable compound that is blue in color and nonfluorescent [15, 16]. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity [17]. Alamarblue can be used in both 2D and 3D cell culture platforms [14, 15, 17]. Typically, alamar-blue is added in a 1–10 dilution, with altered incubation times based on cell number. For wells with very low cell number (less than 500), alamarblue can be added and incubated overnight, reading the following day after 12–18 h of incubation. Higher cell densities can be read within a few hours or less. Some optimization may be needed to find the ideal incubation time for the experiment type. After incubation, fluorescence readings are collected at 560 nm excitation, and 590 nm emission within a fluorescence plate reader.

Proliferation Assay—In order to examine proliferation, alamar-blue fluorescence is collected at Day 1 of plating, and at a further day upon which the data is desired, such as Day 7. The fluorescence from the end time point day, such as Day 7, is normalized to Day 1, giving a value of change in fold proliferation (*see* Fig. 4b).

Viability Assay—Cell viability after drug treatment is evaluated at the end of the time point. The values obtained for the drug-treated group are normalized to the control group that did not receive treatment. Refer to Subheading 3.7 for more detailed protocols.

The following steps describe the addition of the alamarblue reagent and quantification of the hanging drops plates on a microplate reader.

1. Remove the plate from the incubator and carefully remove parafilm.
2. Add 2 μL of filtered alamarblue solution to each well desired for analysis. The same methodology as feeding is recommended for this step. Once alamarblue is added to a hanging drop it is not recommended for use in future analysis, due to possible degradation products; therefore, this should be taken into account when

⁹When adding medium to the hanging drop, place the pipet tip at an angle to the well to prevent unnecessary disruption of the hanging drop.

planning analysis. Often one or two rows of spheroids will be analyzed at a time to maintain the rest of the plate for further culture.

3. Place cover back atop hanging drop plate and 6-well plate stack. There is no need to reapply the parafilm the edges as the plate will need to be read on the microplate reader within a few hours.
4. Place hanging drop stack back in an incubator for 4 h. This incubation time may need to be adjusted depending on the experiment as it depends on several factors such as cell number, pH of the solution, and cell type. Specific incubation times should be experimentally determined for each case.
5. Remove the plate from the incubator and place in a sterilized biological safety cabinet.
6. Remove the lid of hanging drop stack and place face down in a biological safety cabinet.
7. Carefully remove the 384-well plate from the humidifying 6-well bottom plate and place the 384-well plate into the prepared plate reader.
8. Read the plate at 560 nm excitation and 590 nm emission within a fluorescence plate reader (e.g., Synergy HT, BioTek Instruments, Winooski, VT).
9. Once finished remove the plate from the reader and carefully place back into the biological safety cabinet atop the 6-well hydration plate.
10. Place the lid back onto the hanging drop stack and reapply the parafilm edges of the stacked plates.
11. Place the plate in an incubator for further analysis.

3.5 Confocal Microscopy to Assay Viability of the Ovarian CSC Spheroids

Our lab uses the Live/Dead Viability/Cytotoxicity Kit to assess the presence of live and dead cells in the spheroids. It is a simple and quick method for imaging and quantifying the amount of cell death within a spheroid.

1. Prepare a solution of 8 μ M Calcein AM and 16 μ M Ethidium Homodimer-1 in $1\times$ PBS. This should be prepared fresh each time, as Calcein AM is not stable for long periods of time when exposed to moisture.
2. Add 5 μ L of this solution to the 20 μ L drop on the hanging drop plate, and let it incubate for 1 h. Longer incubations may be needed for spheroids over 1000 cells. We recommend incubating on the same day as the fluorescent imaging.
3. Use a 1000 μ L pipette to carefully pull the spheroid out of the hanging drop, and place on a glass coverslip.
4. Set the z-stack parameters on a confocal microscope (Olympus IX81, equipped with a Yokogawa CSU-X1 confocal scanning laser unit, Andor iXon x3 CCD camera, and Metamorph 7.8 software) to image the entirety of the spheroid,

marking the top and bottom of the spheroid, and set the step size as per recommendations from the microscope software.

5. Select the wavelengths of 488 nm (green), and 561 nm (red), to image live and dead cells, respectively.
6. Adjust the gain and the exposure to avoid saturation of the image. Keep these settings constant throughout all spheroids.
7. Collect fluorescent images readout of the entire spheroid over the z-stack at these two wavelengths.
8. Save images from each wavelength separately to allow for more accurate quantification, and as a 16-bit TIFF image to reserve picture quality.

Figure 5 demonstrates that majority of the cells within patient-derived CSC spheroids are alive (green), with a few dead cells (red).

3.6 Characterization of Ovarian CSC Populations Within Spheroids by FACS

In order to determine ovarian CSC populations, patient-derived spheroids are harvested from 384-well hanging drop array plates, and collected in a tube. We typically assay for ALDH⁺, CD133⁺ status, but additional markers like CD44, CD117, etc. can be utilized to assay for other ovarian CSC markers using this method as well.

1. Harvest spheroids from 384-well hanging drop array plates, triturate to generate single-cell suspensions, filter through a 40 µm nylon filter and count cells.
2. Centrifuge suspension to recover cells, and follow protocols established for FACS analysis of CSCs within the spheroids (for example, as noted in Subheading 3.1).

3.7 Assessment of Drug Sensitivity and the Use of Ovarian CSC Spheroids for Preclinical Drug Testing

Screening of novel effective anti-neoplastic therapeutics is a nontrivial problem. The activity of anticancer drugs has been conventionally evaluated in 2D-cultured isogenic cancer cell lines, which fail to identify promising drugs that exhibit potency in vivo [18]. Physiological 3D models, such as the spheroids, have now been firmly established to more accurately mimic the drug sensitivity/resistance behavior of cancer cells found in solid tumors in vivo than the cancer cells cultured under conventional 2D monolayer conditions [19, 20]. Spheroids possess several in vivo features of tumors such as cell-cell interaction, hypoxia, drug penetration, response and resistance, and production/deposition of extracellular matrix [21]. Therefore, our lab utilizes spheroids to screen for novel drugs that could be effective against targeting CSCs and chemoresistant cells.

The following steps outline the treatment of the spheroids with a conventional clinically relevant chemotherapy drug for ovarian cancers, cisplatin. However, the same steps can be used for the screening of any drug compounds.

1. Seed CSCs in hanging drop plates at a seeding density of 50cells per well as outlined in Subheading 3.3.

2. Monitor cells for spheroid formation for 3 days—using live-cell microscopy as outlined in Subheading 3.4.
3. To a 20 μ L-hanging drop we typically add 2 μ L drug, so the drug to cells ratio is 1:10. For example, weigh 1 mg Cisplatin and dissolve in 1 mL of DI water to get a 1 mg/mL concentration of Cisplatin. Dilute the stock to make a final drug concentration, of 50 μ M in each hanging drop.
4. After uniform spheroids are observed under the microscope at Day 3, add 2 μ L drug to the hanging drops (*see* Note 10).
5. A standard 384-well plate can house 140 spheroids, with spheroids in alternate wells, and staggered rows. Apart from adding the drugs to majority of the wells of the hanging drop plate, 20–30 spheroids remain untreated with the drug, these wells act as a negative control. Add 2 μ L of the cell culture growth medium to the negative control spheroids.
6. Seventy two hours the addition of the drug, image the spheroids (both negative control and drug treated) on the inverted microscope.
7. Perform the alamarblue metabolic assay as described in Subheading 3.4 and normalize the fluorescence intensity of the drug-treated spheroids with the negative control.

Figure 6 shows the results of cisplatin treatment on ovarian cancer spheroids initiated with 50 cells/drop of the OVCAR3 cell line. Two different concentrations of cisplatin were dosed, 30 μ M and 50 μ M. Compared to control untreated spheroids (normalized to 100%), cisplatin-treated spheroids had reduced viabilities (60–80%).

3.8 Histological Assessment of Spheroid 3D Structure and Cell-Cell and Cell-Matrix Interactions

Given that the spheroids are 3D microtissues, confocal microscopy enabled by immunostaining and immunohistochemistry are key to study specific cell-cell and cell-matrix interactions within spheroids [22]. Embedding 3D spheroids in agarose is relatively inexpensive and easy to perform. We use low melting point agarose to envelop the spheroids to provide a stable mold for spheroids once it cools down and solidifies.

1. Make a 2% w/v low melting point agarose solution in deionized water.
2. Pipette out 50 μ L warm (40–45 °C) 2% agarose on a sterile non-reactive surface, making an agarose drop.
3. Using a pipette, harvest spheroids from their respective wells on the hanging drop array plate and embed in the agarose drop.
4. Fix the samples in 4% formalin overnight.

¹⁰It may take longer than 3 days for cells to form spheroids depending on the patient sample, in which case monitor spheroid formation using live-cell microscopy before drug treatment.

5. Remove the biopsy cassette containing samples from formalin and place in 70% ethanol. If paraffin processing for microtomy, place cassettes in 70% ethanol, and run through a tissue processor and embed in paraffin blocks.
6. If staining agarose embedded spheroids, perform a quick Glycine buffer pH 7 wash, and wash with 1× PBS.
7. Add appropriate permeabilization, blocking buffer, primary and secondary antibodies including careful wash steps in between, to visualize proteins within spheroids using confocal microscopy.

Figure 7 shows an ovarian cancer spheroid generated from 100 cells/drop. Spheroids were embedded in agarose at Day 7, fixed and stained with a primary antibody directed against ALDH1A1. The red fluorescence indicates the presence of ALDH1A1, and the nuclei are counterstained blue with DAPI.

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Fig. 1.

ALDH+ CD133+ ovarian cancer stem cells are sorted from patient ascites via fluorescent-activated cell sorting (FACS). Malignant ascites samples were filtered to remove debris, and labeled with DEAB (a negative control for Aldehyde dehydrogenase), an isotype-control for the APC antibody, or double labeled with ALDH and CD133-APC. The Y-axis indicates APC fluorescence, while the X-axis indicated ALDH activity. By gating based on the negative control samples (DEAB and APC-isotype), we can identify distinct populations within malignant ascites (derived from patient sample) that are CD133+ ALDH+ (0.15%), CD133+ ALDH- (24.62%), ALDH+ CD133- (1.58%), or ALDH- CD133- (73.65%). Ovarian cancer stem cells or OvCSCs are isolated based on ALDH+ CD133+ expression

Fig. 2.

Ideal plate layout for spheroid generation in 384-well hanging drop array plates. This experimental design features hanging drops (illustrated in *pink color*) that are staggered from each other in order to avoid droplet merging. The open access holes are represented as *white*. The experiments can be designed to have specific groups across the same row. At least two rows are recommended for use for any specific experimental group, to serve as technical replicates (20 spheroids in 2 rows)

Fig. 3.

Patient OvCSC spheroids can be grown from 1 cell/drop over 35 days in hanging drop culture. Spheroids were initiated with 1 OvCSC (isolated from patient ascites) per drop on 384-well hanging drop arrays, and monitored using phase contrast microscopy over 35 days. Cells within spheroids proliferate and aggregate, and form tight compact spherical structures. Spheroids can be maintained for over 1 month in culture. Scale bar = 100 μ m

Fig. 4.

Proliferation within OvCSC spheroids is quantified with on-plate alamarblue fluorescence detection. (a) Phase contrast images are depicted for ovarian cancer spheroids initiated with 100 cells/drop. Representative images are shown for Day 1 and Day 5, where spheroids increase in size owing to proliferation and compaction. (b) Alamarblue fluorescence was measured at Day 1 and Day 5, and represented graphically. A comparison of fluorescence at Day 5 with fluorescence at Day 1 indicated that there was a 6.1 fold proliferation within spheroids. Scale bar = 100 µm

Fig. 5.

Ovarian CSC spheroids contain majority of viable cells. Live/Dead staining was performed using the Live/Dead Cytotoxicity kit. Patient-derived CSC spheroids were incubated with calcein-AM (*green*) and ethidium homodimer (*red*) for 60 min, and fluorescence was visualized using a confocal microscope. *Green* fluorescence indicates live cells within the spheroid, while *red* fluorescence indicates dead cells within spheroids. The balance of live/dead cells indicates good viability of cells within patient CSC spheroids. Scale bar = 100 μ m

Fig. 6.

Hanging drop ovarian CSC spheroids can be successfully utilized for drug screening or treatment using alamarblue fluorescence quantification. Ovarian cancer cells (50 cells per drop) were treated with Cisplatin (30 µM and 50 µM) on day 3, after confirming the formation of spheroids. On day 7, spheroids were incubated with alamarblue and the fluorescence intensity was quantified 4 h after incubation. The drug-treated values were normalized to control. Drug-treated spheroids are less viable than control untreated spheroids

Fig. 7.

OvCSC spheroids can be histologically analyzed for CSC markers. Ovarian cancer spheroids were embedded in agarose, and stained with a rabbit polyclonal antibody directed against ALDH1A1 and a TRITC-conjugated secondary antibody. The *red* fluorescence within spheroids indicates the presence and expression of the stem cell marker, ALDH1A1. The nuclei are counterstained *blue* with DAPI, and the images were acquired on a spinning disk confocal microscope. Scale bar = 100 μ m