Video Article

Imaging- and Flow Cytometry-based Analysis of Cell Position and the Cell Cycle in 3D Melanoma Spheroids

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URL: https://www.jove.com/video/53486
DOI: doi:10.3791/53486

Keywords: Medicine, Issue 106, Melanoma, spheroid, cell cycle, image analysis, flow cytometry analysis, 3D, cancer therapy, cancer cell biology, hypoxia, tumor sub-populations, vibratome sectioning, multicellular.

Date Published: 12/28/2015

Citation: Beaumont, K.A., Anfosso, A., Ahmed, F., Weninger, W., Haass, N.K. Imaging- and Flow Cytometry-based Analysis of Cell Position and the Cell Cycle in 3D Melanoma Spheroids. *J. Vis. Exp.* (106), e53486, doi:10.3791/53486 (2015).

Abstract

Three-dimensional (3D) tumor spheroids are utilized in cancer research as a more accurate model of the *in vivo* tumor microenvironment, compared to traditional two-dimensional (2D) cell culture. The spheroid model is able to mimic the effects of cell-cell interaction, hypoxia and nutrient deprivation, and drug penetration. One characteristic of this model is the development of a necrotic core, surrounded by a ring of G1 arrested cells, with proliferating cells on the outer layers of the spheroid. Of interest in the cancer field is how different regions of the spheroid respond to drug therapies as well as genetic or environmental manipulation. We describe here the use of the fluorescence ubiquitination cell cycle indicator (FUCCI) system along with cytometry and image analysis using commercial software to characterize the cell cycle status of cells with respect to their position inside melanoma spheroids. These methods may be used to track changes in cell cycle status, gene/protein expression or cell viability in different sub-regions of tumor spheroids over time and under different conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/53486/

Introduction

Multicellular 3D spheroids have been known as a tumor model for decades, however it is only recently that they have come into more common usage as an *in vitro* model for many solid cancers. They are increasingly being used in high-throughput drug discovery screens as an intermediate between complex, expensive and time-consuming *in vivo* models and the simple, low cost 2D monolayer model [1-6]. Studies in 2D culture are often unable to be replicated *in vivo*. Spheroid models of many types of cancer are able to mimic the growth characteristics, drug sensitivity, drug penetration, cell-cell interactions, restricted availability of oxygen and nutrients and development of necrosis that is seen *in vivo* in solid tumors [6-11]. Spheroids develop a necrotic core, a quiescent or G1 arrested region surrounding the core, and proliferating cells at the periphery of the spheroid [7]. The development of these regions may vary depending on the cell density, proliferation rate and the size of the spheroid [12]. It has been hypothesized that the cellular heterogeneity seen in these different sub-regions may contribute to cancer therapy resistance [13,14]. Therefore the ability to analyze cells in these regions separately is crucial to understanding tumor drug responses.

The fluorescence ubiquitination cell cycle indicator (FUCCI) system is based on the red (Kusabira Orange – KO) and green (Azami Green – AG) fluorescent tagging of Cdt1 and geminin, which are degraded in different phases of the cell cycle [15]. Thus cell nuclei appear red in G1, yellow in early S and green in S/G2/M phase. We describe here two complementary methods both using FUCCI to identify the cell cycle, along with the use of imaging software or a dye diffusion flow cytometry assay to determine whether cells reside in the G1 arrested center or the outer proliferating ring, and the distance of individual cells from the edge of the spheroid. These methods were developed in our previous publication, where we demonstrated that melanoma cells in hypoxic regions in the center of the spheroid or/and in the presence of targeted therapies are able to remain in G1 arrest for extended periods of time, and can re-enter the cell cycle when more favorable conditions arise [7].

Protocol

1. FUCCI Transduction and Cell Culture

1. FUCCI transduction
    1. Create cell lines stably expressing the FUCCI constructs mKO2-hCdt1 (30-120) and mAG-hGem (1-100) [15] using lentivirus co-transduction as previously described [7].
    Note: The FUCCI system is now commercially available.
    2. Generate sub-clones with bright fluorescence by single-cell sorting. Sort single cells positive for both AG and KO (yellow) by fluorescence activated cell sorting into a 96-well plate as previously described [7,16].

2. Melanoma cell culture
    1. Culture C8161 human melanoma cells as previously described [7,17].

2. 3D Spheroid Formation (as previously described [3,9])

1. Agarose plate preparation
    1. Dissolve 1.5% agarose (or noble agar) in 100 ml of Hank's balanced salt solution (HBSS) or phosphate buffered saline (PBS) by boiling in the microwave for 3-5 min with swirling. Make sure the agarose is fully dissolved.
    2. Immediately dispense 100 µl of the agarose solution per well to a flat-bottomed 96-well tissue culture plate using a multi-channel pipette.
    Note: Agarose may solidify in the tips after a short period of time, to overcome this problem tips must be changed between plates if making more than one agarose plate.
    3. Leave 96-well plate on a flat surface to harden agarose for at least 1 hr.

2. Cell preparation and overlay onto agarose
    1. Grow cells to approximately 80% confluency in a T75 flask. Wash with 10 ml of HBSS.
    2. Detach cells using 1.5 ml of 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) and resuspend in 10 ml of normal medium.
    3. Count cells using a hemocytometer [18] or other automated counting method.
    4. Resuspend cells to a final concentration of 25,000 cells/ml in normal medium.
    5. Overlay agarose wells with 200 µl of the cell suspension for a final number of 5,000 cells per well.
    6. Return plates to the cell culture incubator (5% CO_2 and 37 ˚C) to form spheroids over approximately 3 days.
    Note: The time taken to form a spheroid varies between different cell lines. Some cell lines do not form spheroids using this method at all.
    7. Image spheroid morphology with an inverted microscope using a 4X objective and a phase contrast filter. A cell line that successfully forms spheroids will produce compact, roughly spherical cell aggregates, and there should be only one spheroid per well.
    Note: Optional: Once spheroids have formed, they can be transplanted into collagen or other matrix for further analysis of growth and invasion [3,7,17]. To remove spheroids from collagen, treat with 500 µl of 2 mg/ml collagenase at 37 °C until the collagen is dissolved enough for the spheroids to come free into the media (approximately 30 min). Gently remove spheroids using a 1 ml pipette.
    8. For sectioning/imaging fix spheroids (either isolated from collagen or grown for 3-5 days on agarose) in 10% neutral buffered formalin (CAUTION: Formalin should be used in a fume cupboard) for at least 2 hr at room temperature (RT), or overnight (O/N) at 4 °C. Spheroids may be stored in HBSS at 4 °C for several days.

3. Spheroid Vibratome Sectioning

1. Dissolve 5 g of low melting point agarose in 100 ml of HBSS in a 500 ml glass bottle in a microwave. Use a medium heat setting with swirling. CAUTION: Keep the lid loose when boiling the solution so that the pressure can be released. Use protective heatproof gloves to protect hands from burns when handling the hot glass bottle.
2. Wait until the agarose cools slightly – so the bottle can be touched without burning hands .
3. Pour approximately 2 ml of agarose into the bottom of a Coulter counter cup or similar plastic mold. Immediately aspirate a fixed spheroid (see section 2.2.8) using a 1 ml pipette in the smallest volume of liquid possible. Add spheroid to the agarose. A few spheroids (up to 10) may be added per cup.
4. Cover the spheroids with 2 ml more of agarose. Do this quickly to avoid the agarose hardening before the second layer is added.
5. Allow the agarose to harden at RT in the dark for at least 3 hr. At this point cups may be stored for a short time at 4 °C with 2-3 ml of HBSS overlaid to prevent dehydration.
6. Remove agarose from the cup. Trim the agarose containing the spheroids so that it fits onto the vibratome metal block, and spheroids are close to the top of the agarose. Spheroids can be seen as small white objects inside the agarose. Super glue the agarose to the block.
7. Fill the vibratome with distilled water and mount the block in the vibratome. Set the vibratome to cut 100 µm sections using low speed (setting 2) and high vibration (setting 8). Set the cutting blade angle to 16°. Turn vibratome on, turn on the vibratome light and cut sections from the top of the agarose until 100 µm spheroid sections are cut. Place cut spheroid sections into HBSS in a 24-well plate.
8. Choose middle sections for image analysis. Mount sections on slides by transferring the section flat onto the slide using tweezers. Fill a 10 ml syringe barrel with vacuum grease; add a thin line of vacuum grease around the edges of the section in order to prevent the mounting media from running off the edges of the slide and help seal the section under the coverslip. Cover the section with mounting medium and a coverslip. Seal edges of coverslip with nail polish. Store slides at 4 °C before imaging.

4. Confocal Image Acquisition

1. Take a z-slice image of a middle FUCCI spheroid section using a 10X or 20X objective as previously described [7]. Make sure no overlap or crosstalk occurs between the Kusabira Orange2 and Azami Green. If comparing image analysis between multiple spheroid sections, keep laser power and other settings the same between samples.

5. Hoechst Dye Diffusion Assay for Flow Sorting

1. Transfer live spheroids (3-5 days after agarose seeding) to a 15 ml tube. Let spheroids gravity settle to the bottom of the tube. Several spheroids may be stained per tube. Approximately 20 spheroids are needed to gain sufficient cell numbers for flow cytometry.
2. Remove excess medium and add 1 ml of 10 µM Hoechst diluted in normal medium. Incubate spheroids at 37 °C for approximately 1 hr. Use gentle flicking to resuspend the spheroids once or twice during incubation.
   Note: The incubation time may be varied to alter how far the Hoechst dye penetrates into the spheroids. This will vary based on the spheroid size, density and cell line. For some large/dense spheroids there may be a maximum dye penetration that is less than 100%.
3. Wash the spheroids gently with 5 ml of HBSS using gravity settling.
   1. Optional: For imaging of dye penetration: Fix spheroids with 10% neutral buffered formalin for at least 2 hr at RT or O/N at 4 °C. Prepare vibratome sections, mount on slides and image on the confocal as described in steps 3 and 4 above.
4. To prepare cells for flow cytometry, trypsinize spheroids with 1 ml of 0.05% trypsin/EDTA at 37 °C for approximately 30 min with flicking every 10 min to break the spheroids apart.
   Note: Spheroids are difficult to get into single cell suspension, conditions may need to be optimized. Viability of C8161 4 day old spheroids after this process is approximately 95%.
5. Stain cells with a near-infrared live/dead stain according to the manufacturers protocol. Wash with HBSS then fix with 1 ml of 10% neutral buffered formalin for approximately 30 min. Cells may be stored in HBSS at 4 °C for several days before flow analysis.

6. Flow Cytometry Analysis of Hoechst Stained FUCCI Spheroids

1. Ensure cells are not clumped together by passing them through a cell strainer. Resuspend the Hoechst stained FUCCI spheroids in ice cold FACS wash (PBS with 5% Fetal Calf Serum) at a concentration of 1-5 x 10^6 cells/ml. Transfer 200 µl of sample to 5 ml round bottom tubes.
2. Prepare the following single colour control samples as described in 6.1: un-stained melanoma cells; adherent melanoma cells stained with 10 µM Hoechst for 1 hr; Azami Green only expressing melanoma cells; and Kusabira Orange only expressing melanoma cells.
   Note: Single colour controls may be fixed in formalin and stored in FACS wash with 0.1% Sodium Azide at 4 °C for weeks or even months.
3. Run single cell suspensions on a flow cytometry analyzer with appropriate lasers and single colour/unstained controls as previously described [7,16].
4. Use commercial cytometry software such as FlowJo to analyze the inner vs. outer spheroid cells as follows:
   1. Remove debris by gating on the main cell population in Forward Scattered Light (FSC) vs. Side Scattered Light (SSC).
   2. Remove doublets by gating on single cells using FSC (Area) vs. FSC (Height)
   3. Gate for live cells by gating on the live/dead low population.
   4. Define Hoechst high cells, gated based on the positive signal from the adherent cells stained with Hoechst, as “outer” cells.
   5. Define Hoechst low or negative cells, gated based on the signal from the un-stained control, as “inner” cells.
   6. After gating for the inner and outer populations, cells may be further gated for FUCCI red (G1), yellow (early S), green (S/G2/M) or negative (early G1).
      Note: Compensation using the single colour controls may be required to properly define the FUCCI red, yellow, green and negative populations.
5. Once the assay has been optimized and Hoechst penetration validated via confocal microscopy, run cells without fixing on a flow cell sorter as previously described [7,16] for further analysis of live cell sub-populations.

7. Image Analysis of FUCCI Spheroid Sections

1. Open software *e.g.* Volocity, choose the Quantitation ONLY configuration.
2. Create a new library. Import the FUCCI spheroid confocal image file into the software by dragging the raw data file from the confocal into the library. Alternatively, use the File >Import command.
3. Go to the measurements tab to build an image analysis protocol. The Protocol is built by dragging and dropping the appropriate commands in the correct order as follows in the protocol window.
4. Find objects in the green channel: Drag the find objects command (found in ‘Finding’) into the protocol window, select the correct channel in the find objects protocol.Add an open command (found in ‘Processing’), then a separate touching objects command (found in ‘Processing’ - this will separate cells). Exclude objects by size <50 µm (found in ‘Filtering’ - this will exclude small non-cellular objects).
   Note: Variables such as the find objects threshold, number of open iterations, the size of objects to be separated and the size of objects to exclude must be manually optimized until the object masks match the image.
5. Find objects in the red channel: Repeat find objects as above for the red channel. Green and red protocols may need to be adjusted separately.
   Note: Due to nuclei being in G2, green nuclei are often slightly larger.
6. Confirm objects found are accurate: Confirm red and green objects match the red and green nuclei in the image by turning off and on the green and red channels (using the hide or show channel command) while displaying the red and green masks found by the protocol. Feedback options (Measurements >Feedback options) may be used to modify the appearance of the object masks.

7. Find yellow objects (early S phase cells): To find yellow cells, add an intersect red with green cells command (found in 'Combining'). Exclude objects by size (<50 µm, found in 'Filtering') to exclude any small non-cellular objects.
8. Find exclusively red objects (G1 phase cells): To find exclusively red nuclei, subtract the yellow cells from the red cells (found in 'Combining'). Exclude objects by size <50 µm, (found in 'Filtering') to remove any small non-cellular objects created.
9. Find exclusively green objects (S/G2/M phase cells): Repeat for the green channel to find exclusively green.
   Note: If there are still non-cellular objects remaining, a filter population command may be added to the Exclusive green or Exclusive red protocols (found in 'Filtering') to retain only objects with a shape factor greater than 0.25. This will remove non-circular objects.
10. Find the spheroid outline: Find the spheroid outline using a find objects command (found in 'Finding') with the green or red channel (whichever has more cells around the edge of the spheroid). A much lower threshold (found in the find objects variables) will need to be used to find the spheroid outline compared to individual cells.
    1. Use a close command to join objects (found in 'Processing'), then fill holes in objects (found in 'Processing'), then use a fine filter (found in 'Filtering') to remove noise from objects. Finally, exclude objects by size to remove smaller objects <30,000 µm (depending on the spheroid size).
       Note: This protocol will need to be optimized manually till the spheroid outline is accurate. A brightfield image may also be used – however this is usually less accurate with a spheroid section, and an invert command (found in 'Combining') will need to be used.

11. Measure distances of nuclei to spheroid outline: Measure distances (found in 'Relating') from the yellow, exclusively green and exclusively red populations from the cell centroid to the edge of the spheroid outline.To visualize the minimum distances, which should be from the cell to the nearest spheroid edge, turn on show distances in the relationships tab in feedback options (Measurements >Feedback options >Relationships).
12. Save the protocol. This protocol may be re-applied to other images using the Measurements >Restore protocol command.
13. Create a measurements item (Measurements >Make Measurement Item). Data may be analyzed using the analysis functions.
    1. Go to the analysis tab in the measurements item. Go to the Analysis menu (Analysis >Analyze) and analyze the minimum distance from the cell centroid (red, green or yellow) to the spheroid edge, summarized by count and organized by population.
    2. To count the number of cells found at a certain distance from the edge create a filter (Analysis >Filter). The filter for minimum distance in **Figure 2** is based on Hoechst penetration (*e.g.* minimum distance is less than 80 gives the "outer" population). Alternatively, export raw data to a spreadsheet for further analysis.

Representative Results

There are several methods of producing tumor spheroids, this protocol uses the non-adherent growth method, where cells are cultured on agar or agarose [3,7,9]. **Figure 1** shows an example of a C8161 melanoma spheroid after 3 days on agar. C8161 spheroids form regular sized spheroids with a diameter of 500 - 600 µm (mean = 565, SD = 19, n = 3) after 3 days. Other melanoma cell lines that will form spheroids include: WM793, WM983C, WM983B, WM164, 1205lu (spheroids formed with this cell line are irregular and less dense [19]).

In order to visualize the cell cycle of individual cells within the 3D spheroid model, C8161 melanoma cells were transduced with the FUCCI system [7,15]. Due to the large size of the spheroids (up to 1 mm in diameter after 3 days on agarose and 24 hr growth in collagen for C8161), sectioning is the best option for visualizing cells in the center of the spheroid. Whole spheroids (live or fixed) may be imaged by confocal microscopy, however the confocal imaging is only able to penetrate up to approximately 150 µm, therefore a middle optical slice of the spheroid can only be obtained in spheroids with a diameter smaller than 300 µm. **Figure 2A, B** and **C** shows an example of a section through a C8161 FUCCI spheroid. A necrotic core, surrounded by G1 arrested cells, with a gradient of proliferating cells in the outer layers is evident. To identify and quantify cells based on their position in the spheroid and cell cycle status, semi-automated image analysis was performed. **Figure 2D** shows the FUCCI cell masks and spheroid outline, and **Figure 2E** shows the quantification of the numbers of red (G1) and green or yellow (S/G2/M) cells either less than 80 µm from the edge of the spheroid (outer cells) or greater than 80 µm from the spheroid edge (inner cells). This quantification shows that red cells in G1 are greatly enriched in the inner region of the spheroid, as expected.

In order to identify and potentially sort cells based on their cell cycle status and position in the spheroid, a Hoechst dye diffusion assay in combination with the FUCCI system was used**.** Incubation of whole spheroids with Hoechst dye results in a limited diffusion of the dye into the outer layers of the spheroid, this may be used to separate Hoechst positive outer layer cells from Hoechst negative inner spheroid cells via flow cytometry. **Figure 2F** shows the penetration of Hoechst up to approximately 80 µm from the spheroid edge. The Hoechst positivity distance of 80 µm from the edge was obtained by visual analysis of spheroid sections and optimization of the dye concentration and incubation time so that the Hoechst penetration closely marked the proliferating cells in the outer layers (which are largely found less than 80 µm from the edge), and did not penetrate into the G1 arrested area.The incubation time with Hoechst may be varied to obtain deeper or shallower penetration. An example of varying Hoechst penetration over time in demonstrated in **Figure 3**. **Figure 4A** shows an example of the gating for Hoechst high and low populations, while **Figure 4C** and **D** demonstrates the gating for FUCCI red, yellow and green cells. **Figure 4B** shows the quantification for the number of red (G1) and green or yellow (S/G2/M) cells either in the Hoechst high (outer cells) or Hoechst low (inner cells). Again this technique shows that red cells in G1 are greatly enriched in the inner region of the spheroid (*cf.* similarity to the image analysis in **Figure 2E**).

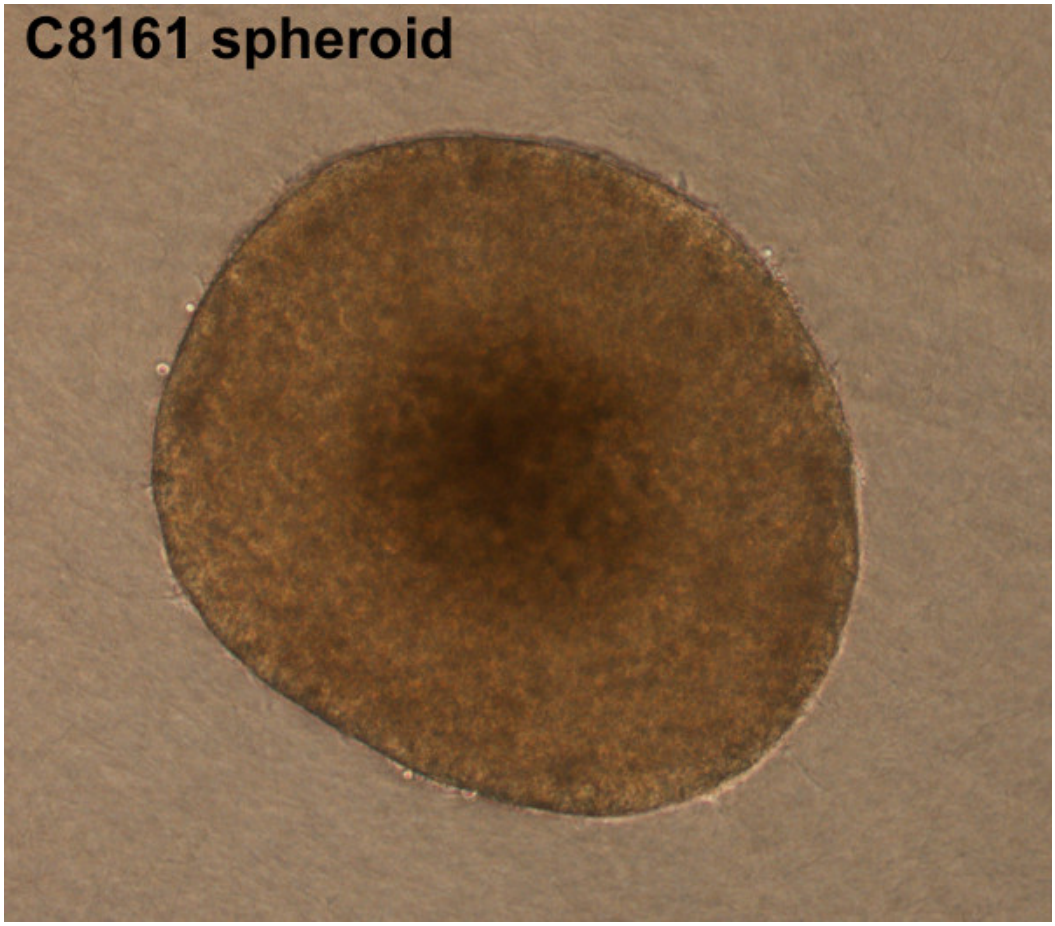

Figure 1: C8161 Spheroid. Representative phase contrast image taken at 10X magnification of a C8161 spheroid after 3 days culture on agar. Scale bar equals 100 µm. Please click here to view a larger version of this figure.

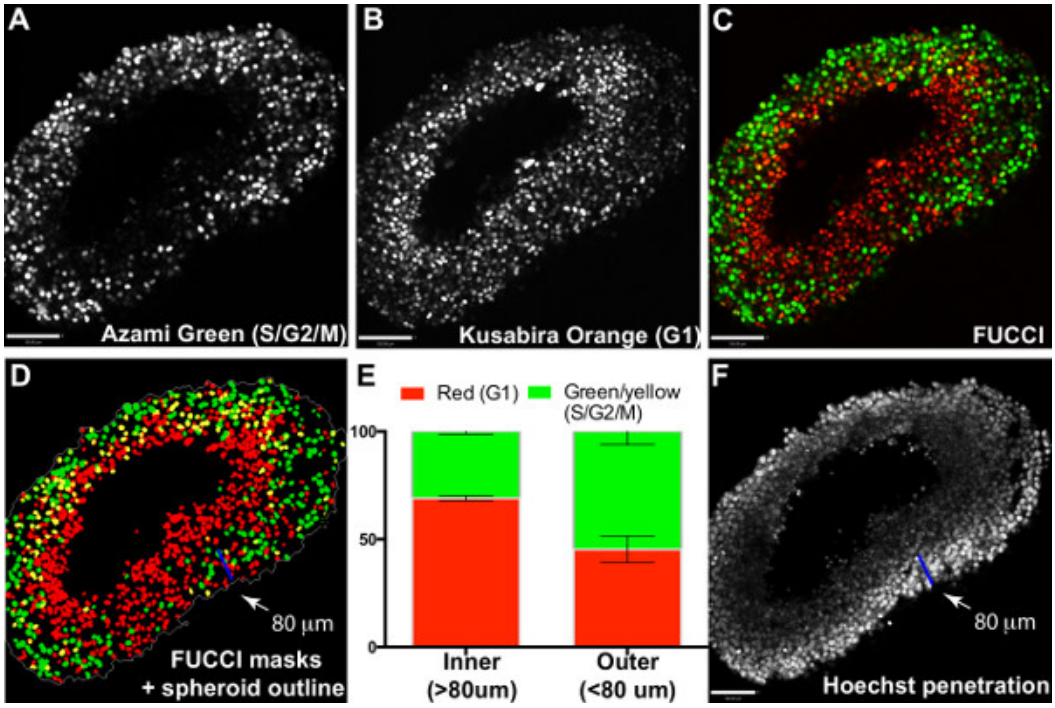

Figure 2: C8161 FUCCI Spheroid Image Analysis. Representative image of a C8161 FUCCI spheroid vibratome section taken at 20X magnification. Spheroid was cultured for three days on agarose, then a further 24 hr in collagen matrix. Confocal z-slice of (**A**) Azami Green, (**B**) Kusabira Orange2 and (**C**) FUCCI overlay. Scale bar = 100 µm. (**D**) Red and Green object masks created in Volocity, with the spheroid outline in grey. Arrow indicates 80 µm distance from the spheroid edge. (**E**) Quantification of the numbers of red (G1) and green/yellow (S/G2/M) cells within the inner (>80 µm from the spheroid edge) and outer (<80 µm from the spheroid edge) regions. Error bars represent the SD from 4 spheroid sections from 2 independent experiments. (**F**) Penetration of 10 µM Hoechst dye after 1.5 hr incubation. Scale bar = 100 µm. Please click here to view a larger version of this figure.

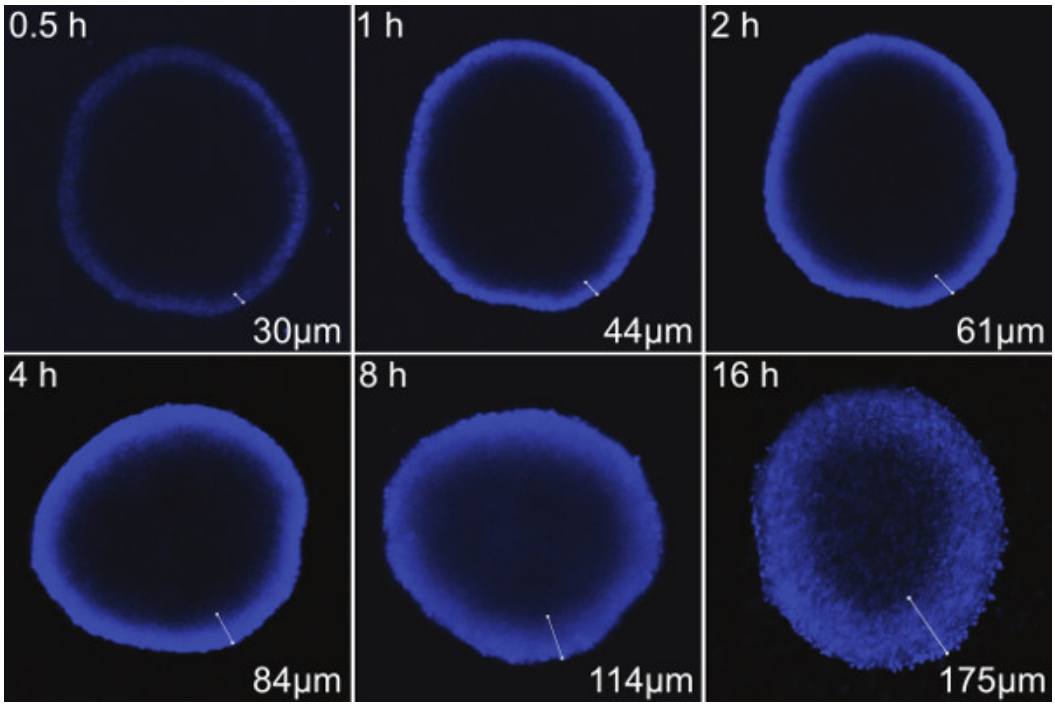

Figure 3: C8161 Spheroid Hoechst Dye Diffusion Time Course. Representative confocal z-slice images from the middle of whole C8161 spheroids cultured on agarose for 4 days and incubated with 10 µM Hoechst for the indicated times. Taken at 10X magnification. White bars indicate the approximate Hoechst penetration depth. Note that C8161 agarose spheroids are denser than the C8161 spheroids that have been implanted in collagen for 24 hr in **Figure 2**, resulting in less dye penetration at the same time point. Please click here to view a larger version of this figure.

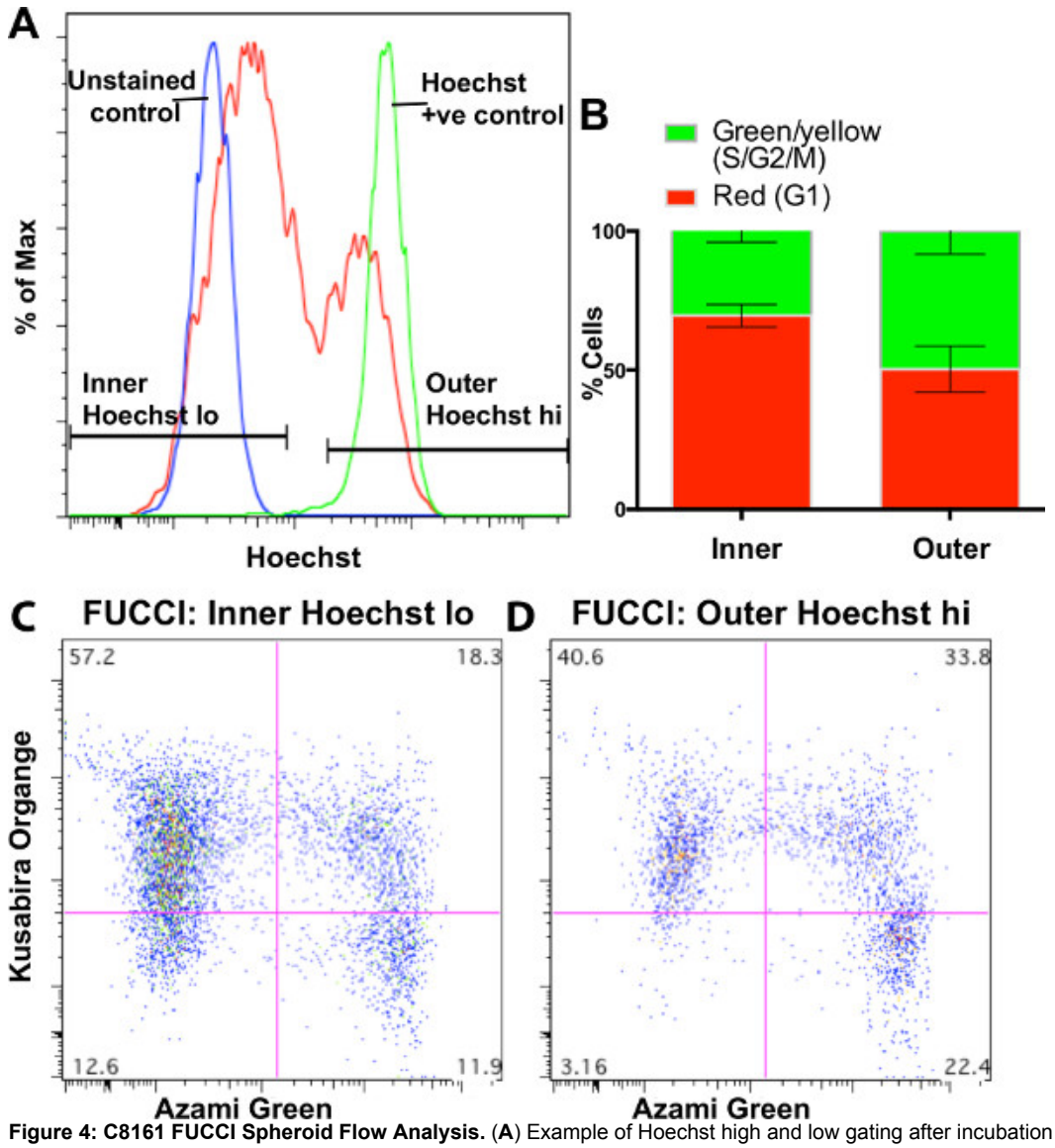

Figure 4: C8161 FUCCI Spheroid Flow Analysis. (**A**) Example of Hoechst high and low gating after incubation of 20 spheroids with 10 µM Hoechst. Blue line indicates the unstained control, green line indicates a fully stained Hoechst control. (**B**) Quantification of the numbers of red and green/yellow cells within the inner (Hoechst low) and outer (Hoechst high) populations. Error bars represent the SD from 8 independent experiments (including both live sorting and fixed cell analysis). Example of FUCCI gating for the inner (**C**) and outer (**D**) spheroid populations. Please click here to view a larger version of this figure.

Discussion

Semi-automated image analysis identified the spheroid inner G1 arrested region, and proliferating outer layers. This method may be used on live spheroids using an optical section, or in fixed spheroid sections, to identify changes in not only the cell cycle but marker expression (via immunofluorescence), cell death, or cell morphology in these different regions. Cell motility within different spheroid regions may also be quantified – if live confocal time lapse imaging along with a cell tracking image analysis step is added. Critical to image analysis is that high quality z- slice confocal images are obtained, higher resolution images allow better identification of cells. One limitation with image analysis is that it is not possible to find every cell correctly if nuclei are too dense, or if there is too much variation in the red and green intensity. FUCCI negative cells (in early G1) are not able to be found with this image analysis method, only red (G1), yellow (early S phase) and green (S/G2/M). One other drawback of the image-based analysis method described here is that it does not take into account the full 3D nature of the spheroids. Although the Volocity software can perform 3D measurements using z-stack images, confocal microscopy is unable to penetrate and image through the entire spheroid due to the large size. Multi-photon imaging may be used to obtain a z-stack of a whole spheroid for 3D measurements [7] as this technology allows penetration of up to 500 µm, although some spheroids may still be too large to image whole spheroids via this method. Another option for imaging the whole spheroid is light-sheet-based microscopy, which allows visualization of the spheroid from multiple angles and penetrates up to 200 µm inside large spheroids [20,21]. Choice of imaging strategy may be influenced by the spheroid size, cell packing density and cell type.

The Hoechst dye diffusion method for separating inner and outer cells in a multicellular spheroid via flow cytometry was first described in 1982 [22]. This method is based on the fact that the Hoechst dye diffuses slowly into the spheroid, with the outer cells being stained first. The protocol

described here combines the Hoechst method with the FUCCI system [15], which allows not only separation of inner and outer spheroid cells, but also visualization of Hoechst penetration with respect to the inner ring of G1 arrested cells. This allows accurate separation of the G1 arrested region of the spheroid. Using FUCCI alone does not allow the separation of the inner G1 arrested cells from a spheroid, given that the outer proliferating cells also contain cells in G1. A limitation is that this method only allows a crude separation of the spheroid into two regions ("outer" Hoechst positive and "inner" Hoechst negative). The benefit of this method over image analysis, is that flow cytometry allows multiple markers to be tested at once, and physical separation of live cells for further downstream analysis such as gene or protein expression, re-culturing in different environmental conditions, drug treatments or other analysis.

The FUCCI spheroid system in combination with flow cytometry and image analysis allows the identification of an inner ring of G1 arrested cells surrounding the necrotic core, and an outer layer of proliferating cells. The necrosis and G1 arrest found in the center of the spheroid is due to lack of oxygen and nutrients, and develops as the spheroid grows in size. Previously, we and others have shown co-localization of the pimonidazole hypoxia marker with the G1 arrested spheroid center [7,23]. We also demonstrate that proliferation largely occurs within approximately 100 µm from the edge of the spheroid. This matches previous studies in spheroids [12,23,24], and also correlates with the distance of proliferating cells from the nearest vasculature *in vivo* [25,26].

An alternative method that may be used to separate cells based on their position in the spheroid for flow cytometry or other analysis is sequential trypsinization [24,27]. In this method successive "shells" of the spheroid are removed by short, sequential incubation periods with trypsin at low temperatures. However using this method it is more difficult to ascertain the exact size of the outer shell (in µm thickness) that is removed compared to the Hoechst dye diffusion method, where the Hoechst penetration may be directly visualized and measured.

Separating the "inner" and "outer" cells by dye diffusion and/or image analysis can be extended to studies of FUCCI tumor xenografts in mice. However, for analysis of the cell cycle *in vivo*, the presence of vasculature should also be taken into account, as cells in a tumor may be able to access oxygen and nutrients from vasculature in the central regions.

Disclosures

PerkinElmer contributed to the costs of publication.

Acknowledgements

We thank Ms. Danae Sharp and Ms. Sheena Daignault for technical assistance. We thank Dr. Atsushi Miyawaki, RIKEN, Wako-city, Japan, for providing the FUCCI constructs, Dr. Meenhard Herlyn and Ms. Patricia Brafford, The Wistar Institute, Philadelphia, for providing cell lines, the Imaging and Flow Cytometry Facility at the Centenary Institute for outstanding technical support. We thank Mr. Chris Johnson and Dr. Andrew Barlow for Volocity software technical support. N.K.H. is a Cameron fellow of the Melanoma and Skin Cancer Research Institute, Australia. K.A.B. is a fellow of the Cancer Institute New South Wales (13/ECF/1-39). W.W. is a fellow of the Cancer Institute New South Wales (11/CDF/3-39). This work was supported by project grants RG 09-08 and RG 13-06 (Cancer Council New South Wales), 570778 and 1051996 (Priority-driven collaborative cancer research scheme/Cancer Australia/Cure Cancer Australia Foundation), 08/RFG/1-27 (Cancer Institute New South Wales), and APP1003637 and APP1084893 (National Health and Medical Research Council).

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