

Video Article

Evaporation-reducing Culture Condition Increases the Reproducibility of Multicellular Spheroid Formation in Microtiter Plates

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Abstract

Tumor models that closely imitate *in vivo* conditions are becoming increasingly popular in drug discovery and development for the screening of potential anti-cancer drugs. Multicellular tumor spheroids (MCTSes) effectively mimic the physiological conditions of solid tumors, making them excellent *in vitro* models for lead optimization and target validation. Out of the various techniques available for MCTS culture, the liquid-overlay method on agarose is one of the most inexpensive methods for MCTS generation. However, the reliable transfer of MCTS cultures using liquid-overlay for high-throughput screening may be compromised by a number of limitations, including the coating of microtiter plates (MPs) with agarose and the irreproducibility of uniform MCTS formation across wells. MPs are significantly prone to edge effects that result from excessive evaporation of medium from the exterior of the plate, preventing the use of the entire plate for drug tests. This manuscript provides detailed technical improvements to the liquid-overlay technique to increase the scalability and reproducibility of uniform MCTS formation. Additionally, details on a simple, semi-automatic, and universally applicable software tool for the evaluation of MCTS features after drug treatment is presented.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55403/>

Introduction

Cancer cells in tumors are physiologically arranged in a complex, 3-dimensional (3D) structure surrounded by extracellular matrix and interacting cells. As nearly all cells in tissues reside in a 3D environment, the need for more physiologically relevant *in vitro* tumor models that mimic tumor traits has resulted in the development of several 3D culture techniques^{1,2,3}. These models are now becoming fundamental research tools for studying the role of the tumor microenvironment on metastasis and cell response to therapeutics in 3D². Moreover, compared to 2-dimensional (2D) cell cultures⁴, 3D models allow for a better understanding of tumor-stroma interactions, which affect cell signaling pathways.

Multicellular tumor spheroids (MCTSes) of cancer cell lines are frequently used in 3D cell culture models due to their relative closeness to *in vivo* tumors. Out of the several techniques in use, the liquid-overlay technique (LOT) of MCTS generation on agarose-coated plates has gained significant interest for lead optimization and target validation^{5,6,7,8,9,10,11}. This is evident from the recent studies that were successfully able to run pilot screens of compound libraries in MCTS cultures using LOT^{6,7}. However, well-to-well variability in MCTS morphology and growth due to the evaporation-induced uneven loss of medium are common hurdles that accompany the LOT using microtiter plates (MPs). Consequently, the formation of non-uniform MCTSes compromises the significance and relevance of data from pharmacological assays^{8,12,13}. In addition to the reproducibility issues, another practical problem that affects LOT-based high-throughput assays is the coating of the MPs with agarose when using automatic liquid dispensing units. Although the dispensing unit can be kept heated to prevent the gelling of agarose, the clogging of the dispensing cassette and tubing is a potential concern for robotic systems⁶.

To overcome some of these challenges, we have recently devised a few modifications in the LOT for MCTS culture⁸. These modifications are mainly based on possible ways to prevent uneven medium loss from the MPs using instruments that are commonly found in high-throughput screening laboratories. A detailed procedure of the modified LOT for the generation of uniformly sized and reproducible MCTSes across 384-well plates (WPs) is presented here. The manuscript also presents a semi-automated routine for the evaluation of MCTS size, particularly in partially disintegrated, drug-treated MCTSes that do not have a clearly defined boundary for the measurement of cross-sectional area.

Protocol

1. Preparation of Agarose-coated Plates

1. Weigh 0.75 g of low-melting point agarose and add it to 100 mL of McCoy's 5A medium (with or without phenol red) without serum. Heat the solution in a microwave and swirl every 1-2 min to completely dissolve the agarose. Autoclave the solution to sterilize it.

- Cool the autoclaved agarose to about 70 °C and filter it through a 500 mL, 0.22 µm filter top by vacuum filtration in a laminar flow box. Aliquot the 0.75% filtered agarose solution (FAS) into smaller volumes if not using the entire solution at once. Store this ready-to-use agarose solution aseptically in a cold room or 4 °C fridge for up to 4 weeks.
- Attach a small tube with a plastic- or metal-tipped dispensing cassette to a Combi reagent dispenser and prime the cassette with 70% ethanol (EtOH) and then with sterile phosphate-buffered saline (PBS) in a flow box.
- Prior to use, heat a stored aliquot of FAS in the microwave to melt it. Prime the dispensing cassette with agarose solution and coat 384-well, tissue culture (TC)-treated "special" microplates with 15 µL of FAS. Allow the agarose in the plate to cool for 15-20 min before seeding the cells. Store the agarose-coated plates aseptically, wrapped in a polyethylene bag in a cold room or at 4 °C and away from direct light. NOTE: Avoid repeated heating of the 0.75% FAS to prevent a change in the concentration of agarose in the stock solution. Agarose-coated plates can be stored for up to 2 weeks when stored under the above-mentioned conditions. The FAS does not require heating during the coating of plates.
- Clean the dispensing cassette by priming it with 70-80 °C sterile water to remove any remaining agarose in the cassette tips and tubes.

2. Cell Culture and MCTS Formation

- Culture human colorectal carcinoma HCT116 cells, as described previously⁸.
- Remove the required number of agarose-coated, 384-well, TC-treated microplates from cold storage and equilibrate them to room temperature (RT) for 15 min.
- Prepare the Combi reagent dispenser for cell seeding by priming a standard tube dispensing cassette with 70% EtOH and sterile PBS. Adjust the seeding volume to the required µL and the dispensing speed to medium using the manual setting buttons on the dispenser. NOTE: The entire cell seeding process is performed under sterile conditions and in a laminar flow box.
- Dissociate adherent cells from the tissue culture flask using a recombinant cell-dissociation enzyme. Make a cell suspension stock in a sterile beaker with seed cells at a density of 2.5×10^4 cells/mL per well in 50 µL of complete growth medium. When seeding more than one 384-WP, stir the cells using a magnetic stirrer to prevent them from settling to the bottom of the beaker.
- Allow the plates to rest for 30 min at RT and then centrifuge for 15 min at 4 x g.
- Meanwhile, take an evaporation-reducing environmental lid and fill it with 8 mL of sterile H₂O or 5% dimethyl sulfoxide (DMSO) in the short sides (left and right) using a 5 mL pipette (**Figure 1A**). First, dispense 4 mL of filling liquid into the left-side trough by sweeping the pipette tip slowly up and down. Repeat this step with the right-side trough. NOTE: Ensure that the liquid added to the side troughs does not merge at the center of the lid, and leave a gap for gas exchange (**Figure 1A**). Adding an excess amount of H₂O results in the seepage of H₂O to the exterior of the lid and subsequently into the outer wells of the 384-well TC plates.
- Fill the liquid reservoir of the 384-well TC plate with sterile H₂O and replace the regular plate lids with the liquid-filled environmental lids (**Figure 2A**).
- Place the plates in a 37 °C rotary incubator with 95% humidity, 5% CO₂, and 20% O₂, and allow the cells to aggregate into MCTSES for 4 days. Avoid opening the incubator door for too long in the subsequent days in order to prevent the humidity level from dropping abruptly.

3. Medium Exchange Using a Robotic System

- On day 4 following MCTS formation, add 30 µL of pre-warmed medium per well using an automated microplate washer dispenser. Allow the MCTSES to grow for additional 3 days. Replace the medium regularly every 3 days, until they attain the desired size for experimentation.
- To aspirate a defined volume of medium from each well, empirically adjust the z-height of the washer manifold. Aspirate 30 µL of medium per well and replace it with 30 µL of fresh, pre-warmed medium. NOTE: Set the dispensing speed and the speed at which the washer manifold travels down into wells at the lowest rate to minimize turbulence in the wells.

4. High-content Imaging of MCTS and Semi-automated Image Analysis

- Image the MCTS in a high-content automated imaging system using a 4X air objective (N.A. 16).
- Set the exposure time to 11 ms and the binning to 4 x 4. Adjust the number and spacing of the z-stacks and the pixel binning as desired for the experiment to capture an entire MCTS per well.
- Process the images stepwise, as described in the "readme," using an in-house routine written in programming language. NOTE: The .m code and .txt readme files are available as supplementary code files (**Figure 1B**). The routine measures the MCTS area, major and minor axes, perimeter, and solidity from the 2D images.

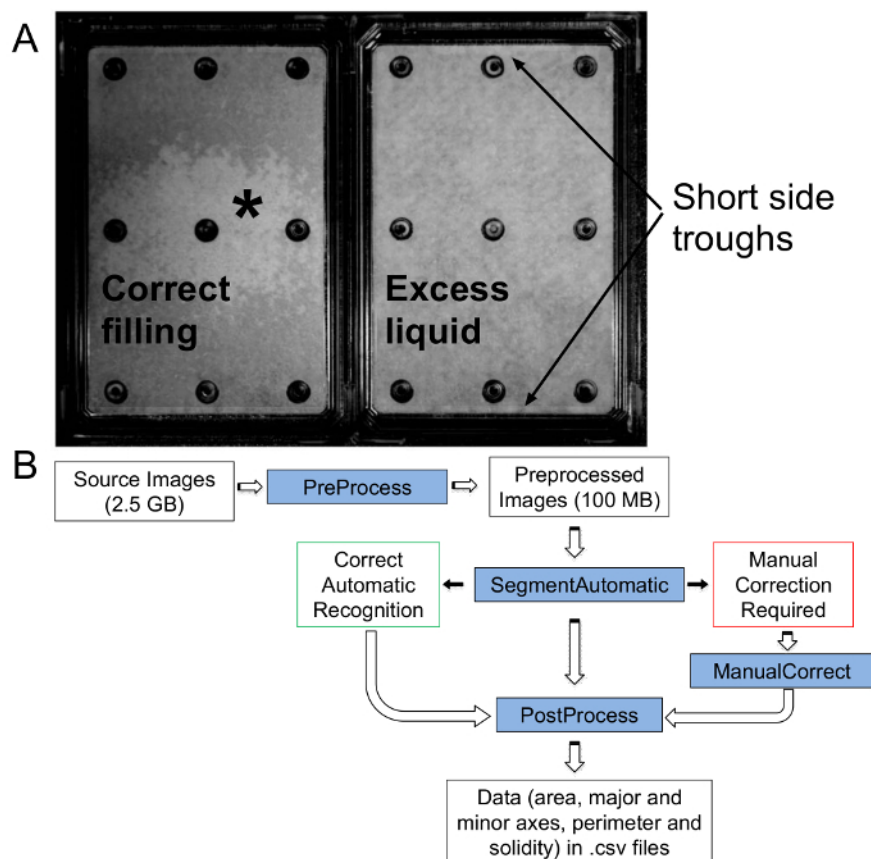


Figure 1: Preparation of environmental lids and a flowchart of the semi-automatic routine. (A) Image of an evaporation-reducing environmental lid filled with the correct (left) and excess (right) amount of filling liquid (here, 5% DMSO). The arrow indicates the short-side trough for adding liquid. The asterisk shows the gap in the middle of the lid following the addition of the correct volume of DMSO. **(B)** A workflow showing the steps involved in the semi-automated image analysis routine. [Please click here to view a larger version of this figure.](#)

Representative Results

The uneven loss of medium, particularly from peripheral wells, is a frequently encountered issue in MPs with small culture volumes. Substantially improved culture conditions, such as incubators with well-controlled temperature/humidification systems and evaporation-reducing MPs and plate lids, reduce the significant loss of medium across wells⁸. To measure the relative evaporation, equal volumes of Orange G (OG) were added to each well, and the change in OG absorbance over 3 days was recorded in plates with regular and environmental lids in standard and rotary incubators. Plate wells were divided into 6 groups based on their distance from the edge of the plate (**Figure 2A**). In groups 1-4, there was a significant change in the absorbance of OG over time in plates with regular lids in a standard incubator (**Figure 2B**). Although there was also a variation in OG absorbance from group 1 wells in plates under the environmental lid/rotary incubator combination (**Figure 2C**), the coefficients of variation (CVs) were much lower compared to those of the plates with regular lids in a standard incubator (**Figure 2D**).

The evaporation-induced uneven loss of medium is one of the potential reasons for well-to-well variability in MCTS size and assay readout in MPs⁸. However, 384-well TC plates with the environmental lid/rotary incubator combination resulted in the formation of uniform MCTSes across the 6 groups of wells (**Figure 2E**). In contrast, MCTSes in plates with the regular lid/standard incubator combination vary significantly in size and solidity (**Figure 3F**). Solidity is a measure of MCTS sphericity and gives the degree of disintegration of the MCTS. The solidity of a 2D region is the proportion of the pixels in the convex hull of the region of interest (ROI) and is determined by dividing the area of the ROI by the area of the convex hull of the ROI. Both circular and elliptic regions have a solidity of 1, and as the MCTS disintegrates, the solidity decreases. The difference in area has already been reported in Das *et al.* (2016)⁹. The volume was calculated from the major and minor axes of the MCTSes.

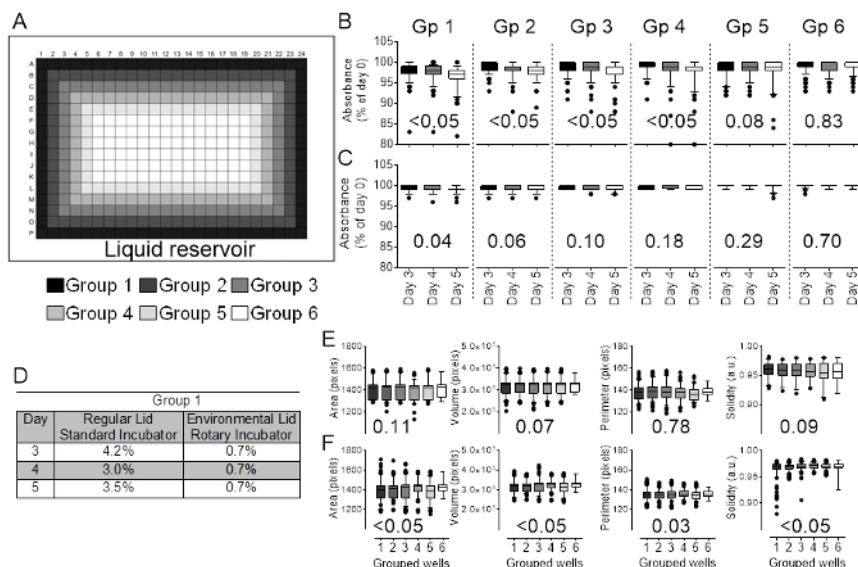


Figure 2: Plates with minimal evaporation result in increased MCTS reproducibility. (A) A plate map is presented to show the division of wells into 6 groups. (B and C) There is a significant time-dependent variation in the relative absorbance of OG from wells in groups 1-4 in plates cultured in a standard incubator with regular lids (B) and from group 1 wells in plates with environmental lids in a rotary incubator (C). Day 3-5 OG absorbances are normalized to day 0. (D) Averaged CVs of OG absorbance of group 1 wells from days 3-5 are shown for the two plate lid/incubator combinations. CVs are averages of 3 independent experiments. (E) MCTSes formed in plates with the environmental lid/rotary incubator combination did not differ significantly across the 6 groups of wells. (F) However, plates with regular lids in a standard incubator formed MCTSes of variable size. Data are shown for $n > 60$ MCTSes per group. The boxes and horizontal bars within the boxes in the boxplots represent the 25th and 75th percentiles and median, respectively. The whiskers represent the 5th and 95th percentiles, and the outliers are indicated by the aligned black dots in the boxplots. The p-values of the Kruskal-Wallis analysis are presented below each boxplot. The data are from at least 3 independent experiments per plate lid/incubator combination. [Please click here to view a larger version of this figure.](#)

To determine the potential applicability of the routine, 7 day-old MCTSes were treated with paclitaxel (PTX), vincristine (VCR), oxaliplatin (OXA), doxorubicin (DOX), and 5-fluorouracil (5-FU) at 3 different concentrations for 4 days. The drugs were obtained from the University Hospital Olomouc, Palacky University. The MCTS images were then analyzed, and the area, major and minor axes, perimeter, and solidity of treated MCTSes were compared to controls (CTLs). The major and minor axes were used to calculate the geometrical volume. There was a concentration-dependent decrease in MCTS area and volume after drug treatment (Figure 3). Although 0.001 $\mu\text{g/mL}$ VCR and 0.4 $\mu\text{g/mL}$ DOX and 5-FU resulted in increase in MCTS area, the volume was significantly increased only following 0.001 $\mu\text{g/mL}$ VCR. MCTS perimeter was significantly different only at the highest concentrations of PTX, DOX, and 5-FU, which completely affected the MCTS size. PTX and VCR at 0.25 $\mu\text{g/mL}$ and 0.06 $\mu\text{g/mL}$ and DOX and 5-FU at 100 $\mu\text{g/mL}$ resulted in a significant decrease in the solidity, indicating a complete-to-partial disintegration of the MCTSes (Figure 3).

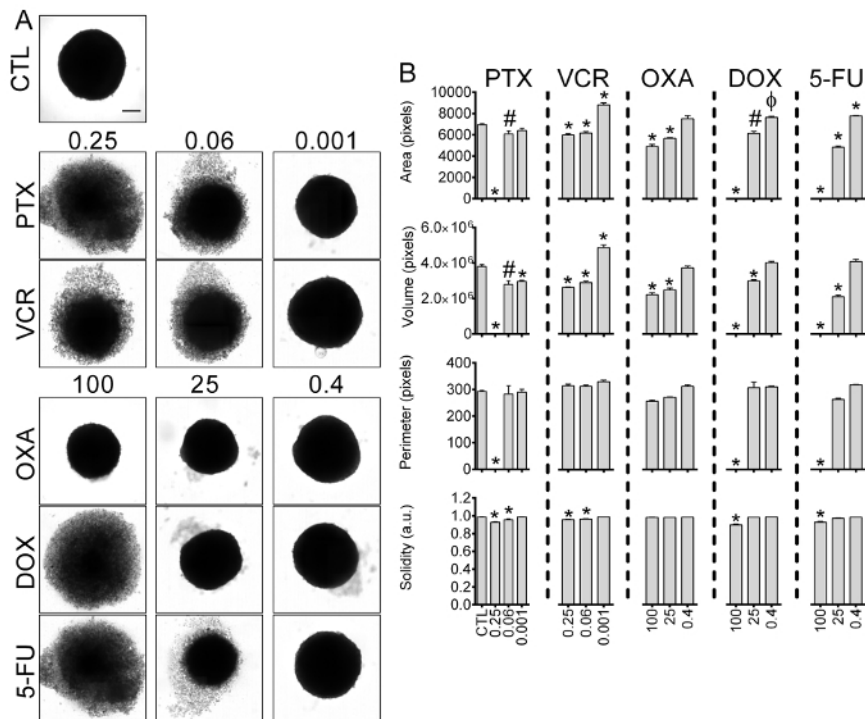


Figure 3: Measure of drug effects in MCTses by the semi-automated routine. (A) Images of CTL and drug-treated MCTses are presented. Scale bar = 10 µm. Concentrations in µg/mL are given for each image. (B) The bar graphs show a dose-dependent effect of PTX, VCR, OXA, DOX, and 5-FU on the area and volume of 7 day-old MCTses following 4 days of treatment. MCTS perimeters were significantly reduced following 0.25 µg/mL PTX and 100 µg/mL DOX and 5-FU. Drug concentrations that resulted in the complete-to-partial disintegration of MCTses significantly reduced MCTS solidity compared to CTL. The data are the mean ± SD of at least 5 MCTses from 3 independent plates. *p < 0.001, #p < 0.01, °p < 0.05 drug-treated versus CTL, one-way ANOVA with Dunnett's multiple comparison test. [Please click here to view a larger version of this figure.](#)

Discussion

Coating 384-Well TC Plates with Filtered Agarose

The standard practice in LOT is to use a 1-1.5% low-melting point agarose to coat the plates, which requires the agarose and/or dispensing unit to be kept heated to prevent the gelling of the agarose⁶. The gelling of the agarose is of potential concern while preparing multiple plates using liquid dispensing cassettes with small tubing apertures ranging between 0.2 and 0.4 mm in diameter. To overcome the potential issue of clogging the dispensing cassettes, 0.75% FAS was used, as it requires no additional heating during dispensing. Incidentally, it was also observed that a 0.75% FAS does not gel as rapidly as unfiltered 1-1.5%, or even 0.75%, agarose solution. Using 0.75% FAS, it takes less than 35 s to coat an entire 384-well TC plate without clogging the dispensing cassette. The cassette can be cleaned at the end by priming it with heated water to remove residual agarose, which could clog the tips when the cassette is not in use.

Environmental Lid and Rotary Incubator over Regular Plate Lid and Incubator

The evaporation-induced uneven loss of medium significantly affects the reproducibility of uniform MCTS formation and consequently compromises the outcome of the assays¹⁴. Surprisingly, group 1 wells of 384-well TC plates under the environmental lid/rotary incubator combination showed a statistically significant variation in OG absorbance (Figure 2C). However, a low CV of OG absorbance in group 1 wells indicates that the evaporation in the outer wells of the 384-well TC plates in the rotary incubator with environmental lids is not so excessive as to affect MCTS growth (Figure 2D). This is evident from the uniformly sized MCTses formed in group 1 wells in plates with the environmental lid/rotary incubator combination. Also, reduced loss of medium prevents well-to-well variability in drug assays from 384-well TC plates cultured in the rotary incubator with environmental lids⁸.

The presented methodology also results in the formation of uniform MCTses of a few other cancer and non-cancerous cell lines⁸. However, since all cells cannot inherently aggregate into MCTses on a non-adherent surface, this method may not be suitable for other cell lines that were not tested for the reproducibility of uniform MCTS formation. Additionally, the focus was mainly on the use of an advanced-technology incubator in combination with evaporation-reducing MPs and plate lids to increase the reproducibility of MCTS formation. Another cheap and easy solution to increase the reproducibility of MCTS formation could be the use of embryo-grade mineral oil and breathable sealing tapes or membranes, which prevent evaporation but allow normal gas exchange¹⁵. Although agarose adds extra thickness to plate bottoms, which can be a barrier to high-content imaging, the depth of field created by an agarose bottom in transmitted light imaging does not hinder the ascertainment of MCTS size and shape after drug treatment. Therefore, the presented method will be of potential interest to researchers facing reproducibility issues in the LOT of MCTS culture.

Semi-automated Image Analysis Routine

Although there are many semi-automated/automated software available for MCTS image analysis, the measurement of the sizes of complete-to-partially disintegrated MCTSeS after drug treatment is prone to errors^{16,17}. In the first step of the presented routine, the best-focused image is automatically selected from the set of z-stack images by computing the norm of the gradient of the image and selecting the one with the largest 0.999 quantile. This is a robust and noise-insensitive measure of the maximum.

Next, the darker borders are cropped and the processed images are stored at the original resolution in Portable Network Graphics format. This process reduces the total size from 2.5 GB, from imaging an entire 384-well, TC-treated plate, to approximately 100 MB. Subsequently, the center of the MCTS is found through a convolution with a circular filter of user-defined radius (we use 20 pixels; **Figure 4A**). A clear local minimum of the filtered image provides the position of the center (**Figure 4A**). Moving from this center in concentric annuli, the median grayscale value, M , in each shell is computed as a function of the shell radius, R . Thereafter, the first (substantial) maximum of the numerical derivative of the function $M(R)$ is found (**Figure 4B**). The point M_{OPT} of this maximum is taken as the optimal threshold, and the image is segmented by thresholding at this level.

The MCTS is identified as the largest central segment of the image. This procedure usually works better than the standard thresholding technique because it is able to separate the MCTS core from the disintegrating parts that often result from drug treatment (**Figure 4C and 4D**). Moreover, the approach adopted here makes implicit use of the *sphericity* of the MCTS. Simple thresholding routines, or algorithms based on active contours, do not exploit the *a priori* knowledge that a spherical object is to be found. When the MCTS is clearly detected and delineated, the maximum M_{OPT} is clear and single. The routine then measures the characteristics of the MCTS, such as the area, major and minor axes, perimeter, and solidity of the fitted ellipse, and saves a preview of the segmented image. If the maximum is not clear, and/or if multiple local maxima are present, a preview of the suggested segmentation is saved into a correction folder.

In the penultimate step, the routine walks the user through the correction folder to manually adjust the suggested segmentation by moving the threshold up or down and selecting a polygonal region of interest to cut off possible redundant parts of the mask. After this manual correction, the routine measures and saves the MCTS characteristics, as described above.

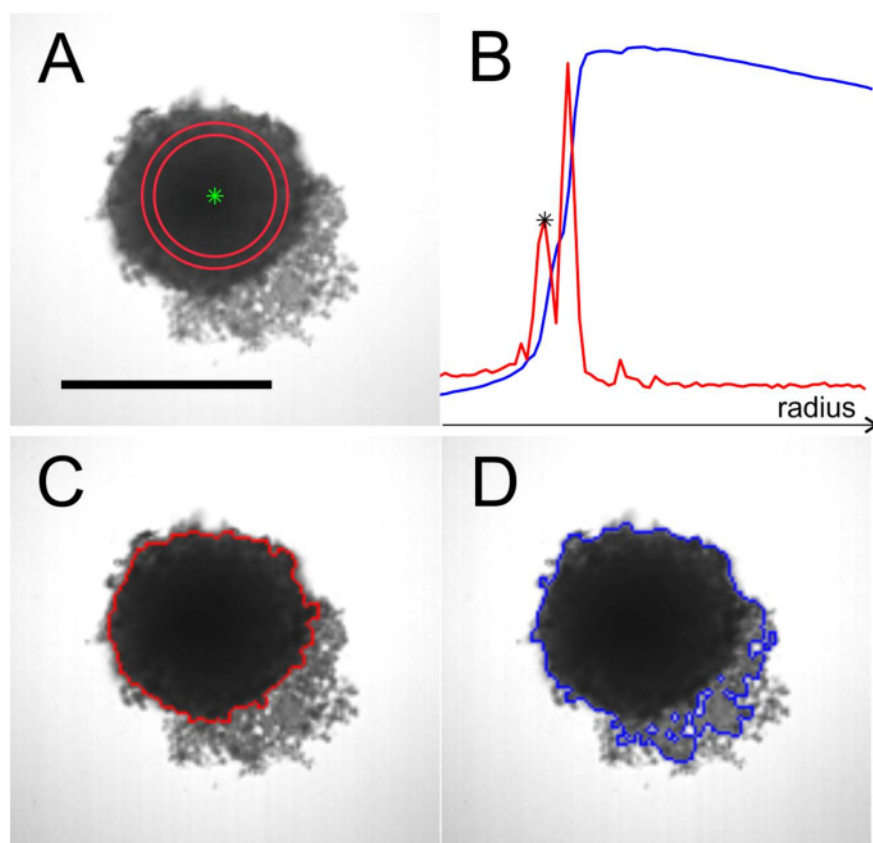


Figure 4: Illustration of the image segmentation procedure. (A) The center of the MCTS is marked with a green asterisk. An example of the annulus is drawn in red. (B) The median grayscale value in each shell is plotted as a function of the shell radius (blue line). The numerical derivative of this curve is computed (red line). The first substantial maximum of the numerical derivative is found (black asterisk). (C) The resulting correct segmentation of the image into the MCTS core and the background with the disintegrating MCTS parts. (D) Incorrect segmentation obtained by Otsu's method. 4X objective; Scale bar = 500 μ m. [Please click here to view a larger version of this figure.](#)

Disclosures

The authors have nothing to disclose.

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