Published in final edited form as:

Acta Biomater. 2020 November ; 117: 322–334. doi:10.1016/j.actbio.2020.09.042.

Non-Destructive Tumor Aggregate Morphology and Viability Quantification at Cellular Resolution, During Development and in Response to Drug

Cassandra L. Roberge^a, David M. Kingsley^a, Denzel E. Faulkner^a, Charles J. Sloat^a, Ling Wang^b, Margarida Barroso^b, Xavier Intes^a, David T. Corr^{a,*}

^aDepartment of Biomedical Engineering, Rensselaer Polytechnic Institute, 110 Eighth St., Troy, NY 12180, USA

^bDepartment of Molecular and Cellular Physiology, Albany Medical College, Albany, NY, 12208, USA

Abstract

Three-dimensional (3D) tissue-engineered *in vitro* models, particularly multicellular spheroids and organoids, have become important tools to explore disease progression and guide the development of novel therapeutic strategies. These avascular constructs are particularly powerful in oncological research due to their ability to mimic several key aspects of *in vivo* tumors, such as 3D structure and pathophysiologic gradients. Advancement of spheroid models requires characterization of critical features (i.e., size, shape, cellular density, and viability) during model development, and in response to treatment. However, evaluation of these characteristics longitudinally, quantitatively and non-invasively remains a challenge. Herein, Optical Coherence Tomography (OCT) is used as a label-free tool to assess 3D morphologies and cellular densities of tumor spheroids, generated via the liquid overlay technique. We utilize this quantitative tool to assess Matrigel's influence on spheroid morphologic development, finding that the absence of Matrigel produces flattened, disk-like aggregates rather than 3D spheroids with physiologically-relevant features. Furthermore, this technology is adapted to quantify cell number within tumor spheroids, and to discern between live and dead cells, to non-destructively provide valuable information on tissue/construct viability, as well as a proof-of-concept for longitudinal drug efficacy studies. Together, these findings demonstrate OCT as a promising noninvasive, quantitative, label-free, longitudinal and cell-based

*Corresponding author: Dr. David T. Corr Address: Rensselaer Polytechnic Institute | 110 Eighth St. Troy, NY 12180 USA | 7042, Jonsson Engineering Center, corrd@rpi.edu, Phone: 518-276-3276 Fax: 518-276-3035.

Data Availability: The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study. Data can be made available on request.

Declarations of interest: none.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

method that can assess development and drug response in 3D cellular aggregates at a mesoscopic scale.

Graphical Abstract

Keywords

multicellular tumor spheroids; optical coherence tomography; cellular resolution; morphology; viability

1. Introduction

Three-dimensional (3D) multicellular models are rapidly becoming the new paradigm for *in vitro* cultures, due in part to their increased biological relevance over traditional 2D cultures and their potential to enable major advances in tissue engineering and personalized medicine (1). Indeed, gene expression in multicellular aggregates, as well as many other biological processes, are more representative of *in vivo* conditions than those seen in 2D cultures (2). Significant advances in organotypic 3D systems have been achieved for a wide range of applications, including the development of engineered human organs (3, 4), exploring treatment options for diseases while reducing *in vivo* and animal studies (5, 6), and high-throughput screening of anti-cancer drugs for efficacy as well as toxicity (3, 5). Although there is a push to engineer larger, more elaborate tumor models with integrated vasculature, the relatively simple 3D multicellular tumor spheroid (MCTS) has been, and continues to be, widely utilized to gain insight into cancer progression and chemoresistance, and has become central for exploring novel therapeutic strategies and optimizing personalized medicine (7–9). Importantly, the biologic behavior of these relatively simple, avascular tumor models is heavily influenced by their size and shape (10–14). To maintain physiologic relevance, MCTSs need to grow to diameters of 300–500µm before they exhibit several key aspects of *in vivo* tumors, such as 3D structure, metabolic function, and pathophysiological gradients (1, 15).

Over the last two decades, a variety of fabrication approaches have been developed to produce MCTS constructs (10, 12, 16). One commonality among all these techniques is the goal of minimizing the surface attachment sites for cells to encourage direct cell-to-cell contact. These methods include spinner flasks, hanging drops, core-shelled microenvironments, or non-adherent plates, all of which have been demonstrated to enable

self-assembly cellular aggregation into compact structures. The latter, termed “liquid overlay,” serves as the current gold standard thanks to its high-throughput capabilities, ease of use, and sample reproducibility (17). This technique is well documented to produce spheroids using a variety of cancer cell types (18, 19). However, many cell lines lack the ability to compact to the extent required to be characterized as a spheroid (20). For these cell types, culture media is typically supplemented with Matrigel, a gelatinous protein mixture derived from basement membrane of mouse sarcoma cells to elicit cellular aggregation (21). However, due to its large number of undefined growth factors and significant lot-to-lot variability, the impact of such supplements on 3D aggregate morphology and cellular density is not well understood (22–24). These limitations have hindered the use of such *in vitro* models for standardized oncological applications.

The advancement of 3D MCTS models for reliable and reproducible biological assays is heavily dependent on their characterization and analysis, both at the onset of any investigation, as well as longitudinally during their development or treatment progression. Especially, spheroid volume and cellular density are key parameters that are known to affect cancer progression and therapeutic response (11, 25). However, the ability to measure these parameters is limited by the size, thickness, and irregularity of 3D MCTS. To date, the volume of 3D MCTS is mainly estimated based on their 2D cross-sectional diameters (26–30). Piccinini *et al.* underscored the importance of volume quantification in their review of seven different methods used to estimate tumor spheroid volume, concluding that each of these approaches can lead to strikingly different results (31). They highlight a novel volume estimation software based on single-plane imaging specifically for MCTSs, known as ReViSP (Reconstruction and Visualization from a Single Projection), which can reconstruct a model’s volume based only on a bright-field wide-field image of the sample. While this software provides a much-needed improvement in morphology characterization, its reliance on certain priors, such as axial rotational symmetry, limits its accuracy for topographically heterogeneous or irregularly-shaped spheroids. Huang *et al.* recently reiterated the limitations of these 2D-based methods, which were shown in practice to yield large quantification errors (32). They instead report a voxel-based approach relying on canny edge detection and manual centroid selection, specifically for tumor spheroid applications. These results highlight the continuing need for improved non-invasive and direct quantification methods capable of overcoming MCTS size, shape and heterogeneity limitations.

Biological function is another important parameter correlated with 3D tumor model morphology, due to the influence of radial depth on the diffusion of oxygen and nutrients throughout the spheroid. Obtaining information on cytotoxicity and cell viability is critical to characterizing both metabolic activity and drug response (33). This information is frequently collected through fluorescent imaging or *ex vivo* immunohistological staining (33, 34). While these methods can provide extensive information on tissue viability and properties, they are often terminal and preclude longitudinal analyses within large 3D MCTS samples. Such longitudinal analyses are highly desired as they can provide unique insights into the time course of aggregate development and maturation, as well as temporal responses to drug treatments. Despite this demand, extracting quantitative, non-destructive measurements with these characterization techniques is still an ongoing challenge. Bright-field and phase contrast microscopy are frequently used to longitudinally assess 3D MCTS

growth, yet these modalities are limited to 2D projections, missing the critical 3D morphology of these models (1, 32, 35–36). Similarly, fluorescent imaging is routinely used to evaluate structure and cellular function of 3D MCTS (33–34, 37); however, there are challenges associated with these modalities, specifically lack of penetration depth due to light attenuation, short working distances ($\sim 50\ \mu\text{m}$), limited fields-of-view, and relatively long acquisition times that can lead to destructive photobleaching (38). Light-sheet fluorescence microscopy is a promising alternative for rapid imaging with improved depth penetration, and wide fields-of-view to produce large, 3D, fluorescent images with rapid acquisition times. However, beyond the difficulty in imaging the central core of large spheroids without increased risk of photobleaching, this technique requires extensive preparation including tissue clearing and gel embedding of the sample, which precludes longitudinal imaging (39).

Alternatively, Optical Coherence Tomography (OCT) is a well-established optical imaging technique with great potential for 3D structural imaging of large cellular aggregates. It operates on the principles of partial low coherence interferometry that uses infrared light to non-destructively probe samples and generate 3D image reconstructions. OCT's few-micron axial-lateral resolution, up to several millimeters deep, enables 3D visualization at the cellular resolution required for analysis of heterogeneous MCTSs (40, 41). This platform holds potential as a rapid, non-invasive imaging tool for evaluating developing tissue-engineered samples in a label-free manner, eliminating the reliance on fluorescent labels or dyes. Indeed, a few recent studies have sought to utilize OCT's structural imaging capabilities to analyze tumor spheroid behavior. Huang *et al.* demonstrated an OCT-based technique for non-destructive detection of necrotic regions within tumor spheroids, relying on increased intrinsic optical attenuation to discriminate between live and dead cell regions (32). Other recent studies have utilized OCT for volumetric tumor model measurements over the course of drug treatment, including label-free quantification of shrinking tumor nodule volume in response to anti-cancer photodynamic and chemotherapies (42), and tumor volumetric changes in response to anti-cancer and gene-silencing drugs (43). The latter incorporated a rapid, low-sensitivity swept-source OCT platform to rapidly image 96-well culture plates ($\sim 5\text{sec/well}$), demonstrating its potential for high-throughput, longitudinal tumor model assessment (43). However, all of these prior OCT-based approaches focused their analyses on whole tumor models or large tumor regions, and thus were not able to appreciate critical events occurring during drug treatment at the level of individual cells. One notable study by Leroux *et al.* in 2016 used OCT to image cell-level events (44). This study looked at actomyosin contractility of cells within small tumor spheroids ($100\ \mu\text{m}$ diameter) and correlated the dynamic OCT signal with changing cell motility during cell aggregation. While this study analyzed cell motility, the authors observed the cells for minutes, not days, and used optically-favorable small spheroids, which may not be physiologically relevant as metastatic models. Additionally, the authors did not seek to track cell density within their small spheroids to provide broader indications of aggregate growth.

Herein, we integrate the use of OCT with Imaris image analysis software to longitudinally assess the 3D development of MCTSs with cellular resolution. These findings reveal critical information on morphological and cell density changes during tumor model progression, with direct implications on the physiological relevance and utility of MCTSs as *in vitro*

tumor models. This method is extended to non-destructively measure cell viability in MCTSs in response to anti-cancer drug treatment, revealing its potential for longitudinal assessment of new drugs and/or the efficacy of patient-tailored regimen.

2. Materials and Methods

2.1 Cell Culture.

MDA-MB-231 GFP-tagged triple-negative human breast cancer cells (MDA-MB-231-gfp) (ATCC, Manassas, VA) were grown in standard cell culture conditions (37°C, 5% CO₂, 95% Relative Humidity) in growth medium consisting of Dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U ml⁻¹ penicillin/streptomycin, and 2 mM L-glutamine.

2.2 Liquid overlay MCTS fabrication.

Cell monolayers were detached from their culture flask via standard trypsinization protocol. Cells were counted and re-suspended in media to the desired concentration of 2.5×10^5 cells/mL following previously established methods (17). Next, the cell suspension was dispensed into round bottom, non-adherent, 96 well-plates (CellStar, Greiner Bio-One) at a volume of 100 μ L per well. These suspensions were cultured with (+) or without (–) a concentration of 2.5% Matrigel (Corning, prod. #354263, Corning, NY). Plates were centrifuged for 10 min at 1000 rpm, immediately following seeding to ensure collection of the cell pellet at the bottom of the well. Plates were then incubated, and cell aggregates were cultured over a four-day maturation period, with OCT imaging performed on each day.

2.3 Bulk alginate gel encapsulation.

Tumor aggregates were prepared via liquid overlay technique, as previously described in Section 2.2. After 72 h, the resulting cell aggregates were lifted via gentle pipetting and were immediately seeded into cylindrical polydimethylsiloxane (PDMS) molds, containing a solution of 2% alginate 10% gelatin. The gels were subsequently cross-linked with 2% calcium chloride to complete encapsulation.

2.4 Tumor aggregate visualization via OCT.

A commercial Spectral Domain Optical Coherence Tomography (SDOCT) system, operating with a central wavelength of 1310 nm (TEL220C1, Thorlabs Inc. New Jersey, USA) was utilized for all OCT-based imaging. The SDOCT system has axial resolution of 5.5 and 4.2 μ m in air and water, respectively, and a lateral resolution of 5 μ m. Image collection for this study was performed at a 5.5kHz A-scan rate with a sensitivity of 101dB. Indices of refraction used in this study were 1.33 for samples in liquid medium, and 1.37 for those in alginate gel. Lateral pixel size was held constant at 1.0 μ m.

2.5 Processing of OCT images.

Volumes and cell counts were calculated using Imaris image analysis software (v9.2, Bitplane USA, Concord, MA). Following intensity thresholding to isolate the sample region within the OCT volume scan, aggregate outlines were traced slice-by-slice, and stitched

together to recreate the volume of the sample. The “spots” function in Imaris was used to identify and count objects matching the input diameter with sufficiently high intensity, thus providing a systematic estimation of cells present within the sample. Following analysis of a 2D monolayer of MDA-MB-231 cells, a spot size of 10µm was selected to match the average diameter of this cell type.

2.6 Validation of geometric measurements obtained through OCT imaging.

To establish the fidelity of this approach for volume quantification, 3D samples were assessed with OCT and compared against measurements from conventional phase contrast microscopy. While this microscopy is able to image full mesoscopic samples, it is limited to 2D images, so our validation began with 2D measurements. OCT maximum intensity projections and phase contrast microscopy images were compared for both smooth-surfaced acellular phantoms of complex geometries (3D PEGDA letters), and more topographically varied cellular samples (tumor spheroids). Measurements of height and major width were collected from 2D images for each of the acellular silicon letters, while cross-sectional areas obtained from the maximum intensity projection of OCT C-scans were compared against phase contrast microscopy images of the same sample taken at the plane of maximum diameter. This analysis was performed in both liquid and gel mediums. Bland-Altman statistical analyses were performed to compare geometric measurements obtained from phase contrast microscopy and OCT.

To extend our validation to 3D geometries, we looked next at the volumes of both the acellular PEGDA letters and cellular aggregates embedded within gels. By rotating the silicon letter (e.g., “T”) onto its side and obtaining its thickness, the volume of the silicon letter was calculated from 2D phase contrast microscopy. This volume was compared to that calculated from the 3D OCT volume scan of the same letter. To test cellular samples, MCTSs were encapsulated in gels, which were then sectioned into cubes. This allowed for rotation of the aggregates and visualization of their geometry along three principal planes, using phase contrast microscopy. Dimensions measured in phase microscopy images and dimensions from OCT maximum intensity projections in each of the principal planes were plugged into the equation for ellipsoid volumes and their resulting spheroid volume quantifications were compared. The choice to use ellipsoid formula was drawn from spheroid literature, in which this assumption is often made (28, 29, 45). The results from this “3D” phase microscopy analysis were also compared against the 3D OCT volume scans quantified with Imaris, which included no geometric assumptions. Bland-Altman statistical analyses were once again performed to compare geometric measurements obtained from phase contrast microscopy and OCT.

Once we confirmed our ability to quantify 2D/3D morphology via OCT, we directly compared our OCT-based method to the ReVisP computational method. Three samples were created for each condition (+/- Matrigel) and were imaged using both phase contrast microscopy and OCT. The ReVisP measurements made using the microscopy image were compared with the Imaris measurement made using the OCT image for each sample.

2.7 Validation of cell counts obtained with Imaris.

In order to validate the cell counts obtained from Imaris analysis, aggregates on each day were returned to cell suspension following OCT imaging and cells in the aggregate were counted via hemocytometer. To dissociate the aggregates, 80 μ L of media was removed from each well and replaced with 100 μ L of trypsin (46). During trypsinization, care was taken to ensure that cell exposure was limited to no more than 10 minutes to reduce the potential for cell death. After 10 minutes, wells were titrated for approximately 30 seconds, and the resulting cell suspensions were counted via hemocytometer using phase contrast microscopy.

2.8 Light-Sheet Microscopy of dense tumor spheroids.

All light-sheet imaging was performed using a Leica TCS SP8 DLS Microscope. GFP-expressing MDA-MB-231 aggregates were individually mounted into Leica capillary 2.5 mm sample holders (Leica Code No. 158007060) by embedding them in 1% low melting point agarose (IBI, Cat. No. IB70050). The light sheet microscope utilized a 488nm laser for GFP excitation, and a 505–540nm filter for emission.

2.9 Cell counting as a measure of drug efficacy.

We next sought to test the ability of our cell counting procedure to evaluate the cytotoxicity of a cancer drug, using live cell count as a metric. Doxorubicin, a well-known breast cancer drug, was added on day 4, at a dose concentration of 20 μ M, to samples created with and without Matrigel. This dose, much higher than the IC₅₀ value (1.24 μ M) for this cell line in monolayer (47), was intentionally selected to guarantee large amounts of cell death. Samples were continuously exposed to the drug for 24 hours, and OCT imaging was performed immediately prior to addition of the drug ($t=0$), and at 4 hours and 24-hours post addition. Six separate treated samples from the same batch were trypsinized and counted via hemocytometer at each time point. Longitudinally-tracked samples were benchmarked against these cell-dissociated results. Again, care was taken to minimize cell exposure to trypsin, enabling accurate representation of drug efficacy.

2.10 Statistical Analysis.

Two distinct statistical analyses were employed in this study. To quantify agreement between two quantitative measurements for a single sample, statistical significance was determined using Bland-Altman graphical analysis. Briefly, the Bland-Altman method provides a way to quantify the agreement between two different measurement techniques, by plotting differences in measurements between the two techniques (e.g., OCT and microscopy) against the average measurements of the techniques. Differences that fall within one standard deviation of the mean measurement, also termed the “limits of agreement,” indicate statistical agreement between the two techniques (48, 49).

For all other comparisons, statistical significance was determined by Student’s t-test. Data are shown as means $\pm$ SD. In all cases, $p < 0.01$ was used as significance cutoff, unless otherwise indicated. In instances claiming statistical similarity, additional ANOVA testing was performed to check for equality in variance and distribution.

3. Results

3.1 Validation of geometric measurements obtained by OCT.

To establish OCT as a reliable tool for quantitative morphologic assessment, we first validated our measurements made from OCT images using acellular phantoms of complex known geometries (i.e., 3D PEGDA letters; Figure 1), as well as topographically varied cellular samples (tumor spheroids; Figure 2). As shown in Figures 1 and 2, assessment with OCT imaging and Imaris image analysis accurately quantifies 2D and 3D morphology in these models. Comparison to ground truth revealed < 3% error in acellular samples and < 10% error in cellular samples analyzed in 2D. For 3D cellular validation, OCT-based analysis outperformed the ground truth by calculating volumes with no geometric assumptions. Furthermore, measurement validity was maintained for samples in both liquid and gel mediums, samples in curved wells (cellular aggregates), and those embedded within thick hydrogels. This establishes OCT's quantitative morphologic measurement, in 2D and 3D, for a wide range of common culture conditions. While previous studies report the utilization of OCT for quantitative assessment of 3D whole spheroids (27,32), none have sought to verify the accuracy of their measurements made from OCT images.

3.2 Visualization and Morphological Quantification of Developing Cellular Aggregates.

OCT-based analysis was then applied to MCTS samples to longitudinally assess their morphology, requiring whole-sample imaging to provide important information on tumor progression, especially rate of growth. Medium-sized MCTS (300–500 μm diameter) samples were prepared from human breast cancer MDA-MB-231 cells using liquid overlay, in the presence (+) or absence (–) of 2.5% Matrigel serum. Representative images of samples on days 1 and 4 (Figure 3A-D) revealed significant geometric differences between these two models: aggregates prepared without Matrigel grew to a flat, disk-like morphology, while those prepared with Matrigel aggregated into 3D spheroids after 4 days (Figure 3, Figure S1).

We further quantified the observed morphologies, measuring both volume and sphericity for a total of six samples from each condition (+/– Matrigel). The average values show no significant differences in volume between aggregates grown with or without Matrigel, on any of the 4 days (Figure 3E-F), indicating that cell proliferation was not affected by Matrigel. However, despite achieving similar volumes, there was a significant increase in sphericity with the addition of Matrigel, which agrees with our qualitative observations (Figure 3A-D).

Next, we compared our OCT-based method to the aforementioned ReVisP computational method (31), a computational method that utilizes a 2D bright-field image of an aggregate (e.g., MCTS) and, by enforcing an assumed spherical geometry, reconstructs and quantifies the spheroid volume to achieve 3D measurements from the 2D image. Direct comparison revealed that ReVisP consistently measured larger aggregate volumes compared to OCT-based measurements (Figure 4). For aggregates prepared without Matrigel, this larger volume was significantly greater than the OCT-based measurement ($p = 0.003$). This discrepancy was expected as the tumor aggregates prepared with/without Matrigel exhibited

sphericities of 0.908 ± 0.02 and 0.504 ± 0.02 , respectively, as measured by Imaris; neither matching the perfect 1.0 sphericity assumed by ReVisP. These findings illustrate that spheroids with similar projected areas can have very different volumes, and ReVisP's assumption of perfect sphericity consistently leads to an overestimation of aggregate volume, particularly for thinner, less spherical samples.

3.3 Longitudinal cell density measurement in developing tumor spheroids.

Our OCT-based Imaris technique provides measurements at cellular resolution and, in the case of minimally scattering samples such as a MCTS, we hypothesized that the OCT contrast obtained in the MCTS could correlate with overall cell population. Thus, we sought to exploit this correlation to non-destructively quantify cell density in MCTSs over the time course of aggregate development to provide valuable longitudinal 3D information on both cell proliferation and overall model growth. To assess OCT's ability to nondestructively count cells in 3D aggregates, MCTS samples prepared without Matrigel were imaged using OCT, then immediately dissociated into individual cells and counted via hemocytometer to serve as the cell count "ground truth". OCT-based cell counts, obtained using the "spots" function in Imaris, correlated strongly (trial 1: $R^2 = 0.74$, trial 2: $R^2 = 0.78$) to respective hemocytometer counts (Figure S2), highlighting OCT's ability to non-destructively approximate cell count within dense 3D cellular samples.

To explore OCT's ability to longitudinally assess aggregates during growth and development, MDA-MB-231 MCTS samples were prepared $\pm$ Matrigel ($n=6$, each), and imaged daily via OCT over a 4-day growth period (longitudinal samples; Figure 5, red lines). A reference growth curve was established by subjecting aggregates, grown under identical conditions, to dissociation and hemocytometer-counting ($n=6$ per timepoint), from the same seeding density, at each of the 4 days (growth curve samples; Figure 5, blue lines). These terminal reference cell counts were used to construct a 95% confidence interval for cell counts over the 4-day period. Average Imaris counts ($n=6$ per condition) were within the confidence interval for all samples, on each of the 4 days (Figure 5); the only exception being samples fabricated without Matrigel on Day 1, whose average fell just above the 95% interval. Cell counts for samples grown with Matrigel exhibited larger standard deviations, likely due to the increased thickness of the samples, which poses a challenge to the imaging modality. However, these counts still fell within the 95% confidence interval, demonstrating OCT's ability to longitudinally, and non-destructively count cells in developing 3D MCTSs.

To characterize the sensitivity of this Imaris-based approach to input spot diameter, we analyzed a representative Day 4 Matrigel+ sample using input diameters of 5, 10 and 20 μm , and compared to the actual cell count obtained from dissociating and hemocytometer-counting the same aggregate (Figure S3). The 10 μm spot size used throughout this study, selected based on the average cell diameter in 2D monolayer, agreed well with the actual cell count. Selecting a spot size that was too small resulted in an overestimation of cell density, whereas a larger spot size underestimated the cell count.

3.4 Benchmarking OCT-based cell counting against light-sheet microscopy.

Light-sheet fluorescent microscopy has been recently established as a powerful tool to image 3D structures, with single-cell or sub-cellular resolution (39). Thus, we sought to benchmark our OCT-based cell counting method against this state-of-the-art microscopy technique, using Matrigel-positive MCTSs, selected for their spheroidal shape (i.e., similar thickness in three dimensions), which makes them the most challenging of our samples to image and assess using OCT. After 4 days of growth, spheroids were first imaged with OCT, then embedded in agarose gel within a capillary tube, and imaged with light-sheet microscopy. All images were analyzed in Imaris using the same aforementioned cell-counting method. Three separate aggregates from each batch were dissociated and counted via hemocytometer to provide “ground truth” control cell counts for each given seeding density and growth condition. Two trials were performed with 3 spheroids each (Figure 6) and Bland-Altman statistical analyses revealed little discrepancy between methods, and tight limit-of-agreement intervals, with no statistical differences noted between the cell counts of light-sheet and OCT ($p > 0.01$). Moreover, there were no statistical differences between cell counts from either OCT or light-sheet counting, and their corresponding dissociated aggregates counted via hemocytometer. Thus, our non-destructive OCT-based approach is as accurate at counting cells as light-sheet microscopy.

3.5 Tracking cell viability in thick aggregates using OCT.

Viability testing is a critical assessment tool for MCTSs, especially for applications in drug screening. Currently, this is performed using fluorescent stains which, when imaged under fluorescence microscopy, distinguish between live and dead cells. However, fluorescent dyes do not diffuse uniformly across dense 3D MCTS of the average size used here, and the photo-bleaching destructive nature of most fluorescent microscopies precludes longitudinal analysis of these samples. Herein, we addressed whether our OCT-based cell counting could distinguish between live and dead cells in dense 3D aggregates, to provide a method to directly measure cell-scale viability in these hypoxic MCTSs in response to doxorubicin, a well-known anti-cancer drug.

Liquid overlay aggregates, prepared either with or without Matrigel (as previously described) were treated with a bolus dose of doxorubicin on day 4, and cultured in the presence of the drug for an additional 24 hours. Samples ($n=6$) were longitudinally imaged with OCT prior to drug addition, 4h post-addition, and 24h post-addition. Cell number was quantified at each time point, and these numbers were benchmarked against treated aggregates from the same batch, dissociated and analyzed at each timepoint using direct cell counting hemocytometer-based assay. Within dissociated samples, both live-cell counts and total (live+dead) cell counts were calculated, and the Imaris results were plotted against these (Figure 7). OCT-based Imaris cell counts were statistically similar to the dissociated live cell count at 0 hours and 4 hours post drug addition, for both the Matrigel-positive and Matrigel-negative groups, and statistically different from the total (live+dead) cell counts at 4 hours. Thus, OCT is able to discern between live and dead cells, within both flat, disk-like aggregates and truly 3D spheroids, to provide an accurate estimation of living cells following drug treatment.

The results were less conclusive at 24 hours after drug addition, where the Imaris counts no longer matched the dissociated live cell counts; rather, they fell in between the live and total cell counts, indicating that OCT was erroneously counting some dead cells or large cell debris as live cells. These findings reveal that OCT is able to provide accurate estimates of cell viability at early timepoints, though this accuracy decreases when a large percentage of cells are dying by necrosis.

Lastly, we sought to compare our viability approach with the prior OCT-based volume correlation methods (42, 43). Given that our Imaris-based approach can accurately estimate cell density in addition to aggregate volume, we sought to test the validity of their assumption that changes in cell viability will necessarily be reflected in changes in volume. Using our MDA-MB-231 tumor models, we measured aggregate volume and cell density and explored their correlations over the 4-day course of aggregate development, as well as in response to treatment with an anti-cancer drug (e.g., doxorubicin). As seen in Figure S4, average aggregate volumes parallel the measures for cell number at each time point during aggregate development. Additionally, when treated with doxorubicin, the reduction in aggregate volume parallels the decrease in cell viability (Figure S5).

4. Discussion

Multicellular spheroids and organoids are popular *in vitro* oncological models for investigating disease progression and high-throughput drug screening, especially towards applications in personalized and precision medicine. Advancing these models further requires characterization of critical features during model development, which is frequently accomplished using fluorescent imaging or immunohistological analyses. In contrast, OCT structural imaging offers three-dimensional, cell-scale, label-free imaging of mesoscopic models in high-resolution and, most notably, in a non-destructive manner. Herein, we applied OCT for longitudinal quantification of 3D morphology, cellular density, and cell viability of thick multicellular spheroids, during development and in response to anti-cancer drugs.

A key feature of effective MCTS models is their morphology; size and shape of the model directly influences the gradients developed, overall growth, and diffusion behaviors of the tumor. Small-sized MCTSs (diameter < 300 μm) do not experience gradient-related challenges for cell survival, large MCTSs (> 500 μm) develop necrotic cores due to lack of sufficient nutrient/waste transport, and medium-sized MCTSs (300–500 μm diameter), such as those used in this study, develop pathophysiological gradients and, consequently, hypoxic cores that mimic the pre-metastatic signaling seen *in vivo* to provide a more accurate surrogate for avascular solid tumors (50, 51). Furthermore, a lack of standardization of MCTS fabrication has led to a large spectrum of “spheroid” models, few of which are able to replicate the spherical morphology necessary to establish the physiologically-representative gradients that make these such powerful *in vitro* models. Moreover, because these models are frequently used for drug-screening applications, this discrepancy has been implicated in the high failure rate in drug discovery, where only a low percentage of investigated drugs succeed in clinical trials (52). Herein, we assessed MDA-MB-231 tumor aggregates, fabricated with or without Matrigel, to study the influence that this widely-used culture

supplement has on spheroid development. Our 3D imaging revealed major morphological differences with the addition of Matrigel, suggesting that for the MDA-MB-231 cell line presented here, Matrigel addition is critical to produce truly spherical 3D aggregates, with morphologies sufficient for *in vitro* cancer studies, yet has negligible impact on proliferation or cellular density. Such spherical geometry results in formation of proper surface-to-volume ratios, which are expected to lead to isotropic gradients over the full sample for more consistent and reproducible biological outcomes. While the addition of Matrigel generally promotes increased 3D aggregation in liquid overlay cultures, the results will likely vary among different cell types, and will be greatly influenced by the cells' innate abilities to self-aggregate.

We compared our OCT-based 3D morphologic quantification to that of ReVisP, an established approach that utilizes 2D maximum intensity projections to reconstruct aggregate volumes based on the assumption of ideal sphericity (31). ReVisP consistently overpredicted aggregate volumes, especially in the thinner, less spherical samples. Conversely, our OCT-based method accurately quantifies both surface (2D) and volumetric (3D) spheroid characteristics, without requiring any such assumptions of shape or size. Hence, the OCT-based method provides a means to directly quantify MCTS 3D morphological features, notably improving upon the established ReVisP methodology.

Our OCT-based technique was then adapted for quantification of cellular density in developing aggregates, tracking aggregate growth at a cellular-scale through model maturation. This type of information is highly desired in many tissue engineering applications, and 2D and/or 3D imaging techniques are frequently employed to interrogate engineered constructs. Two-dimensional imaging provides a limited amount of information, and 3D techniques commonly cite penetration depth and lengthy imaging times as main hurdles they must overcome for complete tissue characterization. In contrast, our proposed technique utilizes OCT to collect this information in a rapid, label-free manner. Our results agreed very well with reference values over a 4-day timecourse, for both thinner, disk-like aggregates and the thicker more challenging spheroid models. Such capabilities enable direct measurement of tumor model growth and provide indications of proliferative and metabolic activity throughout development.

OCT-measured cell densities were no different than those measured via light-sheet microscopy, the current state-of-the-art in fluorescence microscopy. Although light-sheet microscopy offers qualitatively better images, it requires a significant amount of time for sample preparation, modality calibration, and image acquisition, in addition to the notable drawback of being a terminal imaging modality due to requisite sample embedding (39, 53). Thus, for cell-counting applications, OCT offers distinct advantages, such that samples can be imaged quickly (in a matter of minutes) and non-destructively, directly in their culture well or dish, to enable label-free, longitudinal assessment of the sample during 3D tumor model progression. While we acknowledge the presence of speckle noise, inherent to OCT imaging, within our images, we do not expect that the existence of this noise had an influence on the cell counting results. The combination of averaging and the use of a Hann window apodization greatly reduced the speckle noise in our images [1]. Additionally, speckle noise occurs at the scale of a voxel within each OCT image, which was set to $(1.0 \times$

1.0 × 2.54) μm for all imaging in this study. Given that our cell counting method searches for particle sizes near 10 μm , it is highly unlikely this noise aligned such that our cell counts would be affected. To our knowledge, this is the first imaging method that can collect accurate, quantitative, and longitudinal information on cell density within dense, cellular 3D MCTS in a non-destructive manner.

We further applied this tool for cellular-resolution viability testing in tumor spheroid models challenged by a known chemotherapeutic. To a promising extent, we found that OCT was able to distinguish between live and dead cells in these thick constructs, thus providing a non-destructive alternative to directly measure cell viability, and enabling real-time, continuous drug screening and efficacy assessment. Our technique performed well at early timepoints, but provided somewhat skewed results at later timepoints when more cell death had occurred. We believe that this is most likely due to the way doxorubicin induces cell death, via both apoptosis and necrosis (55, 56). While the programmed cell death of apoptosis breaks the dying cell down into small fragments that will not be registered by the Imaris spot-function, cells in the process of dying via necrosis are most likely included in the Imaris cell count. Because OCT is a structural imaging modality, its images gain contrast from the presence of large organelles and sub-cellular structures such as cellular membranes (in various stages of rupture) or nuclei within. Given this knowledge, we believe that cells dying by apoptosis are not recognized, but those dying by necrosis may be registered by the spot-size filter, and erroneously counted by OCT as living cells. This is consistent with our observations that the discrepancy between counts increases with time; at later time points, when more dying cells are present, more necrotic cells could be falsely registered as live, resulting in an Imaris cell count that lies between the live cell and total cell count. Future studies will seek to address this hypothesis by exploring anti-cancer treatments that operate purely via apoptosis, such as Trastuzumab for the case of AU565 HER2+ breast cancer cells.

Despite these very promising results for 3D morphology and cell viability quantification, our current OCT-based approach suffers from a few shortcomings. With regards to morphologic analyses, the snaking approach required for the slice-by-slice Imaris analysis is somewhat subjective, and thus can impart a slight inter-user variability. To address this, our future work seeks to incorporate an edge-detection algorithm to objectively identify sample edges in progressive b-scan slices and remove user-based variability. Regarding cell counting, the OCT-based count is sensitive to the spot size selected within Imaris, as demonstrated in Figure S3, and the 10 μm size utilized in this study was chosen specifically based on the size of MDA-MB-231 cells. Selecting spot sizes that are too small may cause Imaris to erroneously count subcellular structures (organelles, nuclei, etc.) within an aggregate, resulting in an overestimation of cell density. Conversely, selecting spot sizes that are too large will miss counting some cell-sized structures, leading to an underestimation of cell count. Therefore, spot size is not universal across all cell types, and should be adjusted accordingly when analyzing cells of notably different size. In general, this value should be set to match the average diameter of the cells used in the aggregate or tissue-engineered construct, to ensure that Imaris is registering structures of the correct size. Addressing these limitations will improve objectivity and wider applicability of our OCT-based approach.

Lastly, given that prior OCT-based methods have relied on aggregate volume as an indirect correlative measure of cell viability (43, 44), we sought to validate the accuracy of this assumption using our cell-scale approach. We found that aggregate volumes correlated well with cell counts at every time point explored. This correlation appears to hold throughout the development of these tumor aggregates, capturing aggregate growth and cellular proliferation, in both thin aggregates and 3D spheroids. Additionally, when these aggregates were treated with a chemotherapeutic, we observed a reduction in aggregate volume that paralleled our quantified decrease in cell viability. By utilizing the same cell line and similar fabrication methods, we provide quantitative support for those prior 3D tumor spheroid studies that utilized OCT volumetric measures as qualitative indicators of cell viability (43, 44). However, despite these strong correlations, qualitative agreements are expected to significantly diverge with changes in cell density within the aggregate, such as those due to compaction, or the development of internal necrotic regions (32). This underscores the importance of directly quantifying cell viability, at the individual-cell scale, rather than relying on an indirect correlative value. The novel Imaris-based approach presented herein is able to non-destructively provide a working estimate of living cells, within both developing and drugged aggregates, regardless of natural variations in cell density. Moreover, due to OCT's depth of penetration, this method has great promise for assessing aggregate structures up to 1 mm thick, including embryoid bodies, larger tumor spheroids, and other organoids. To our knowledge, this is the first published tool for label-free and non-destructive viability quantification at a cellular resolution in dense cellular aggregates, enabling more detailed and predictive future drug efficacy studies.

4. Conclusion

In this work, OCT imaging was used with Imaris image analysis to establish a tool for quantitative multicellular spheroid assessment. We measured spheroid morphology and cellular density longitudinally, both throughout aggregate development, and in response to treatment with a known chemotherapeutic. Quantitative measurements of full aggregate morphology are often overlooked or minimized in the field, as they are difficult to obtain with common imaging modalities. However, our findings reveal distinct differences (i.e., cellular density, response to drug) between models of similar volume, yet different sphericities. These morphologic differences would not be appreciated using traditional 2D imaging, underscoring the importance of 3D visualization and quantitative characterization. Additionally, this OCT-based approach provides non-destructive cell counts, in thick 3D aggregates, enabling longitudinal viability assessment in 3D cellular constructs. Beyond this demonstration in tumor spheroids, the framework for imaging and analysis has great utility for wide-ranging biomedical applications. Herein, we provide ample validation and a robust proof-of-concept for implementing this OCT-based approach to quantitatively assess multicellular aggregates and 3D cultures (e.g., organoids, embryoid bodies) during their development, and in response to drug for efficacy assays. Additionally, the ability to measure longitudinally reduces the number of required samples and removes sample-to-sample variability between time points; ultimately leading to richer time course data and greater model predictive power. Thus, this work paves the way for quantitative, label-free,

non-destructive analyses of 3D aggregates, not previously feasible with traditional microscopy and fluorescence-based viability tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This study was supported by NIH R01 BRG CA207725 (XI/MB/DTC), and NIH R01 CA233188 (MB). Light-sheet fluorescence imaging was supported by NSF-MRI-1725984. The authors would like to thank Dr. Alena Rudkouskaya for kindly providing the Doxorubicin used in the viability testing.

References:

1. Hirschhaeuser F, et al. (2010) Multicellular tumor spheroids: an underestimated tool is catching up again. *Journal of biotechnology* 148(1):3–15. [PubMed: 20097238]
2. Abbott A (2003) Biology's new dimension. *Nature* 424:870–872. [PubMed: 12931155]
3. Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA (2009) Spheroid-based drug screen: considerations and practical approach. *Nature protocols* 4(3):309. [PubMed: 19214182]
4. Shamir ER, Ewald AJ (2014) Three-dimensional organotypic culture: experimental models of mammalian biology and disease. *Nature reviews Molecular cell biology* 15(10):647. [PubMed: 25237826]
5. Persano L, Rampazzo E, Della Puppa A, Pistollato F, Basso G (2011) The three-layer concentric model of glioblastoma: cancer stem cells, microenvironmental regulation, and therapeutic implications. *The Scientific World Journal* 11:1829–1841. [PubMed: 22125441]
6. Sharick JT, et al. (2019) Cellular metabolic heterogeneity in vivo is recapitulated in tumor organoids. *Neoplasia* 21(6):615–626. [PubMed: 31078067]
7. Bray LJ, et al. (2015) Multi-parametric hydrogels support 3d in vitro bioengineered microenvironment models of tumour angiogenesis. *Biomaterials* 53:609–620. [PubMed: 25890757]
8. Kunz-Schughart LA, Freyer JP, Hofstaedter F, Ebner R (2004) The use of 3-d cultures for high-throughput screening: the multicellular spheroid model. *Journal of biomolecular screening* 9(4):273–285. [PubMed: 15191644]
9. Song Y, et al. (2018) Patient-derived multicellular tumor spheroids towards optimized treatment for patients with hepatocellular carcinoma. *Journal of Experimental & Clinical Cancer Research* 37(1):109. [PubMed: 29801504]
10. Lin RZ, Chang HY (2008) Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal: Healthcare Nutrition Technology* 3(9–10):1172–1184.
11. Kelm JM, Timmins NE, Brown CJ, Fussenegger M, Nielsen LK (2003) Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnology and bioengineering* 83(2):173–180. [PubMed: 12768623]
12. Kingsley DM, et al. (2019) Laser-based 3D bioprinting for spatial and size control of tumor spheroids and embryoid bodies. *Acta Biomaterialia*. 95: 357–370. [PubMed: 30776506]
13. Yu L, Ni C, Grist SM, Bayly C, Cheung KC (2015) Alginate core-shell beads for simplified three-dimensional tumor spheroid culture and drug screening. *Biomedical microdevices* 17(2):33. [PubMed: 25681969]
14. Curcio E, et al. (2007) Mass transfer and metabolic reactions in hepatocyte spheroids cultured in rotating wall gas-permeable membrane system. *Biomaterials* 28(36):5487–5497. [PubMed: 17881050]
15. Mueller-Klieser W (1987) Multicellular spheroids. *Journal of cancer research and clinical oncology* 113(2):101–122. [PubMed: 3549738]

16. Sutherland RM, McCredie JA, Inch WR (1971) Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. *Journal of the National Cancer Institute* 46(1):113–120. [PubMed: 5101993]
17. Nagelkerke A, Bussink J, Sweep FC, Span PN (2013) Generation of multicellular tumor spheroids of breast cancer cells: how to go three-dimensional. *Analytical biochemistry* 437(1):17–19. [PubMed: 23435308]
18. Carlsson J, Yuhas J (1984) Liquid-overlay culture of cellular spheroids in Spheroids in cancer research. (Springer), pp. 1–23.
19. Rofstad EK, Wahl A, Davies CdL, Brustad T (1986) Growth characteristics of human melanoma multicellular spheroids in liquid-overlay culture: Comparisons with the parent tumour xenografts. *Cell Proliferation* 19(2):205–216.
20. Ivascu A, Kubbies M (2006) Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. *Journal of biomolecular screening* 11(8):922–932. [PubMed: 16973921]
21. Froehlich K, et al. (2016) Generation of multicellular breast cancer tumor spheroids: comparison of different protocols. *Journal of mammary gland biology and neoplasia* 21(3–4):89–98. [PubMed: 27518775]
22. Knight E. and Przyborski S. (2015), Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. *J. Anat.* 227: 746–756. doi:10.1111/joa.12257 [PubMed: 25411113]
23. Baker AEG et al. (2019) Benchmarking to the Gold Standard: Hyaluronan-Oxime Hydrogels Recapitulate Xenograft Models with In Vitro Breast Cancer Spheroid Culture. *Adv. Mat* 31(36): 1901166.
24. Tienderen G. et al. (2019) Recreating Tumour Complexity in a Dish: Organoid Models to Study Liver Cancer Cells and their Extracellular Environment. *Cancers*. 11(11): 1706.
25. Mueller-Klieser W, Schreiber-Klais S, Walenta S, Kreuter M (2002) Bioactivity of well-defined green tea extracts in multicellular tumor spheroids. *International journal of oncology* 21(6):1307–1315. [PubMed: 12429982]
26. Alessandri K, et al. (2013) Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. *Proceedings of the National Academy of Sciences* 110(37):14843–14848.
27. Sharma M, Verma Y, Rao K, Nair R, Gupta P (2007) Imaging growth dynamics of tumour spheroids using optical coherence tomography. *Biotechnology letters* 29(2):273–278. [PubMed: 17160349]
28. Jin HJ, Cho YH, Gu JM, Kim J, Oh YS (2011) A multicellular spheroid formation and extraction chip using removable cell trapping barriers. *Lab on a Chip* 11(1):115–119. [PubMed: 21038070]
29. Raghavan S, et al. (2016) Comparative analysis of tumor spheroid generation techniques for differential in vitro drug toxicity. *Oncotarget* 7(13):16948. [PubMed: 26918944]
30. Leroux C-E, et al. (2015) Elastography of multicellular aggregates submitted to osmo-mechanical stress. *New Journal of Physics*. 17:073035.
31. Piccinini F, Tesei A, Bevilacqua A (2016) Single-image based methods used for non-invasive volume estimation of cancer spheroids: a practical assessing approach based on entry-level equipment. *Computer methods and programs in biomedicine* 135:51–60. [PubMed: 27586479]
32. Huang Y, et al. (2017) Optical coherence tomography detects necrotic regions and volumetrically quantifies multicellular tumor spheroids. *Cancer research* 77(21):6011–6020. [PubMed: 28904062]
33. LaBarbera DV, Reid BG, Yoo BH (2012) The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert opinion on drug discovery* 7(9):819–830. [PubMed: 22788761]
34. Håkanson M, Textor M, Charnley M (2010) Engineered 3D environments to elucidate the effect of environmental parameters on drug response in cancer. *Integrative Biology* 3(1):31–38. [PubMed: 21049126]
35. Imamura Y, et al. (2015). Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. *Oncology Reports*, 33, 1837–1843. 10.3892/or.2015.3767. [PubMed: 25634491]

36. Song Y, et al. (2018) Patient-derived multicellular tumor spheroids towards optimized treatment for patients with hepatocellular carcinoma. *Journal of Experimental & Clinical Cancer Research* 37(1):109. [PubMed: 29801504]
37. Pickl M, Ries C (2009) Comparison of 3D and 2D tumor models reveals enhanced HER2 activation in 3D associated with an increased response to trastuzumab. *Oncogene* 28(3):461. [PubMed: 18978815]
38. Appel AA, Anastasio MA, Larson JC, Brey EM (2013) Imaging challenges in biomaterials and tissue engineering. *Biomaterials* 34(28):6615–6630. [PubMed: 23768903]
39. Pampaloni F, Ansari N, Stelzer EH (2013) High-resolution deep imaging of live cellular spheroids with light-sheet-based fluorescence microscopy. *Cell and tissue research* 352(1):161–177. [PubMed: 23443300]
40. Zhong H, et al. (2010) Enhancement of permeability of glycerol with ultrasound in human normal and cancer breast tissues in vitro using optical coherence tomography. *Laser Physics Letters* 7(5):388.
41. Fujimoto J, Swanson E (2016) The development, commercialization, and impact of optical coherence tomography. *Investigative ophthalmology & visual science* 57(9):OCT1–OCT13.
42. Jung Y, Klein OJ, Wang H, Evans CL. Longitudinal, label-free, quantitative tracking of cell death and viability in a 3D tumor model with OCT. *Sci Rep.* 2016;6(May):1–11. [PubMed: 28442746]
43. Tsai MT, Huang BH, Lei KF (2020) Quantitative Monitoring of the Response of Tumor Spheroids Cultured in 3D Environment by Optical Coherence Tomography. *IEEE Access.* 8:28360–6.
44. Leroux C-E, Bertillot F, Thouvenin O, Boccara A-C (2016) Intracellular dynamics measurements with full field optical coherence tomography suggest hindering effect of actomyosin contractility on organelle transport. *Biomedical Optics Express.* 7:4501. [PubMed: 27895991]
45. Piccinini F, Tesei A, Arienti C, Bevilacqua A (2015) Cancer multicellular spheroids: volume assessment from a single 2d projection. *Computer methods and programs in biomedicine* 118(2):95–106. [PubMed: 25561413]
46. Angello JC, & Hosick HL (1982). Glycosaminoglycan synthesis by mammary tumor spheroids. *Biochemical and biophysical research communications*, 107(3), 1130–1137. [PubMed: 7138518]
47. Wellcome Sanger Institute O (2018) Genomics of drug sensitivity in cancer. compound - doxorubicin.
48. Bland JM, Altman DG (1986) Statistical Methods For Assessing Agreement Between Two Methods Of Clinical Measurement. *The Lancet*, 327(8476):307–310.
49. Giavarina D (2015) Understanding Bland Altman analysis. *Biochemia. Medica* 25(2): 141–151. [PubMed: 26110027]
50. Jiang Y, Pjesivac-Grbovic J, Cantrell C, Freyer JP (2005) A multiscale model for avascular tumor growth. *Biophysical journal* 89(6):3884–3894. [PubMed: 16199495]
51. Lorenzo C, et al. (2011) Live cell division dynamics monitoring in 3D large spheroid tumor models using light sheet microscopy. *Cell division* 6(1):22. [PubMed: 22152157]
52. Langhans Sigrid (2018) Three-dimensional in vitro cell culture models in drug discovery and drug repositioning. *Frontiers in pharmac.* 9(6), doi:10.3389/fphar.2018.00006.
53. Zaroni M, et al. (2016) 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. *Scientific reports* 6:19103. [PubMed: 26752500]
54. Adabi S. et al. (2018) An Overview Of Methods To Mitigate Artifacts In Optical Coherence Tomography Imaging Of The Skin. *Ski. Res. Technol* 24: 265–273.
55. Shin HJ, et al. (2015) Doxorubicin-induced necrosis is mediated by poly-(adp-ribose) polymerase 1 (parp1) but is independent of p53. *Scientific Reports* 5:15798. [PubMed: 26522181]
56. Muir C, Adams M, Graham C (2006) Nitric oxide attenuates resistance to doxorubicin in three-dimensional aggregates of human breast carcinoma cells. *Breast cancer research and treatment* 96(2):169–176. [PubMed: 16331349]

Statement of Significance:

Tumor spheroids are powerful 3D *in vitro* models for studying disease progression and drug-screening, whose size and shape greatly influence their biologic behavior. Herein, we established a new label-free approach to non-destructively quantify 3D morphology and cell density, and utilized it to assess tumor aggregates during development and in response to drug. We observed distinct differences between aggregates of similar volume, yet different sphericities - critical differences that traditional 2D imaging cannot appreciate. We demonstrated longitudinal, label-free viability measurements in 3D aggregates, which can provide rich time-course data with enhanced predictive power, and require fewer samples. This approach is the first to quantify 3D aggregate morphology without assumptions of shape, and non-destructively measure cell density and viability in these models.

Fig. 1.

Verification of geometric measurements made by OCT in acellular samples using Bland-Altman statistical testing. (Ai) A phase-contrast microscopy image of acellular PEGDA letters. (Aii) An OCT en-face (top-view) of the PEGDA letter I. (B) Percent error calculated between measurements made with each of the defined modalities. (PCM = phase-contrast microscopy; OCT = Optical Coherence Tomography). Modalities labeled (pp) indicate quantification performed using the sample dimensions quantified in principal planes, while those labeled (vs) represent samples analyzed in Imaris using 3D OCT volume scans. The “ground truth” modality served as the measurement against which experimental measurements could be compared. All percent errors were low (< 5%) indicating excellent agreement between modalities for all measured values. (C,D) Bland-Altman plots created for compared parameters, where red lines represent average differences, and pairs of black lines show intervals of agreement. (C) Bland-Altman analyses revealed no statistical differences between phase-contrast microscopy and OCT in measurements of sample height (2D). (D) Rotating the samples orthogonally enabled 3D volume measurement with microscopy. Bland-Altman testing similarly showed no statistical differences between phase-contrast microscopy “3D” analysis and OCT volume scans in measurements.

Fig. 2. Verification of geometric measurements made by OCT in tumor aggregate samples using Bland-Altman statistical testing, as performed in Figure 1. (A) A phase-contrast microscopy image of a representative tumor aggregate in gel medium. Planar image validation of OCT was performed by collecting a volume scan of each sample and obtaining maximum intensity projection from the c-scan, shown in (B) for the presented sample. This projection is compared directly to the microscopy image. (C) Percent error calculated between measurements made with each of the defined modalities. As in the previous figure, PCM =

phase-contrast microscopy, OCT = Optical Coherence Tomography, modalities labeled (pp) indicate quantification in principal planes and plugged into the ellipsoid formula, and those labeled (vs) represent samples analyzed in Imaris using 3D OCT volume scans with no geometric assumptions. Percent errors between measurements made for cellular samples were found to be higher than in acellular, which can likely be attributed to the increased difficulty in pinpointing sample edges in these more irregularly-shaped samples. Still, percent errors were calculated to be below 10% for 2D analysis of these samples, indicating good agreement. Although phase microscopy and OCT planar-analysis were in good agreement, larger discrepancies were observed between phase microscopy and 3D OCT analysis. As the OCT analysis can be performed with no required assumptions, we believe this highlights the inaccuracy of the ellipsoidal assumption for spheroid models and underscores the importance of 3D visualization and quantification. (D,E) Bland-Altman testing showed no statistical differences in cross-sectional area measurements between microscopy and OCT in (D) liquid and (E) gel mediums. Embedding the aggregates in gel medium also enabled their rotation, such that orthogonal microscopy images could be collected to obtain a 3D volume scan with 2D phase-contrast microscopy. This process is not possible in liquid medium, hence there is not a similar validation in for volume scans in a liquid medium. (F) Despite the larger percent error observed between these measurements, statistical testing similarly showed no differences between phase-contrast microscopy “3D” analysis and OCT volume scans in measurements of sample volume in gel medium. (n=5).

Fig. 3. Longitudinal visualization of evolving sample morphology measured with OCT. (A-D) Sequential en face, side view, and isometric OCT images showing the development of a representative tumor aggregate from day 1 to 4. (A, B) Samples prepared without Matrigel formed flat disks, whereas those prepared with Matrigel (C,D) aggregated into 3D spheroids. (E,F) Longitudinal quantification showed that aggregates grown (+/-) Matrigel had similar volumes at each of the 4 days, but significantly different shapes. Matrigel (+) samples had significantly higher sphericities than those without Matrigel as early as day 1, which was maintained through day 4. (***) = $p < 0.001$ ($n=6$) (Scale bars=100 μ m).

Fig. 4.

(A) Microscopy image of representative MDA-MB-231 aggregate grown with Matrigel.

(B,C) Aggregate reconstructed in 3D using (B) our OCT-based Imaris technique or (C) ReVisP code described by Piccinini et al., respectively (31).

(D) Microscopy image of representative MDA-MB-231 aggregate grown without Matrigel.

(E,F) Aggregate reconstructed in 3D using (E) our OCT-based Imaris technique, or (F) ReVisP code. Axes values on the ReVisP images are in voxels.

(G) Quantification of aggregate volumes provided by both techniques, represented as average ± standard deviation (n = 3 per condition). Analysis revealed consistently larger volume approximations using the ReVisP technique. Paired T-testing revealed a statistical difference between the ReVisP and Imaris measurements for samples prepared with No Matrigel (p = 0.003), and no statistical difference for samples prepared with Matrigel.

Fig. 5.

Longitudinal quantification of cell density in developing tumor aggregates. (A) Representative OCT image of aggregate grown with Matrigel, including zoomed-in section highlighting OCT's cellular resolution. (B,C) Growth curves created from similarly grown terminal samples are plotted in blue, with their associated 95% confidence interval for cell counts in shaded light blue. Imaris-based cell counts for samples imaged longitudinally over 4 day are shown in red, illustrating that the mean Imaris cell counts fell within the confidence interval at every time point, with the only exception being samples grown without Matrigel at day 1, whose standard deviation falls inside the interval, but whose average falls just outside. Insets show representative aggregates from the given conditions that have been analyzed with the Imaris spots function.

Fig. 6. Comparison of cell counting abilities between OCT and light-sheet microscopy. (A,B) Representative images of a sample imaged with light-sheet and OCT, respectively. Scale bars = 200 μm . (C) Bland-Altman graph showing no statistical difference between cell counts from OCT and light-sheet microscopy for the same samples.

Fig. 7. Cell counting as a potential measure of drug efficacy. Representative Imaris images of samples at 0h, 4h, and 24h post drug addition. The graphs show longitudinal data from samples grown with and without Matrigel, revealing that at both 0h and 4h, the Imaris cell counts are statistically similar to the live cell counts calculated from terminal samples cultured and drugged under the same conditions. Additionally, at 4h, the Imaris cell counts are statistically different from the total cell count. At 24h, the Imaris cell counts fall between the live and total cell counts in both sample groups. Scale bars = 150 μ m.