3-25-2009

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Evans, Conor L.; Rizvi, Imran; Hasan, Tayyaba; and de Boer, Johannes F., "In Vitro Ovarian Tumor Growth and Treatment Response Dynamics Visualized with Time-Lapse OCT Imaging" (2009). Dartmouth Scholarship. 1488.
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In vitro ovarian tumor growth and treatment response dynamics visualized with time-lapse OCT imaging

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Abstract

In vitro three-dimensional models for metastatic ovarian cancer have been useful for recapitulating the human disease. These spheroidal tumor cultures, however, can grow in excess of 1 mm in diameter, which are difficult to visualize without suitable imaging technology. Optical coherence tomography (OCT) is an ideal live imaging method for non-perturbatively visualizing these complex systems. OCT enabled detailed observations of the model at both nodular and cellular levels, revealing growth dynamics not previously observed. The development of a time-lapse OCT system, capable of automated, multidimensional acquisition, further provided insights into the growth and chemotherapeutic response of ovarian cancer.

1. Introduction

A major challenge in the field of cancer research is the difficulty in observing the growth, development, and treatment of microscale lesions in vivo. This limitation is especially evident in epithelial ovarian cancer. One of the major problems in the treatment and management of ovarian cancer is the lack of effective therapy for advanced metastatic disease, which is unfortunately presented by over 70% of women at the time of diagnosis. Although ovarian epithelial cancer is actually one of the most treatable solid tumors in its early stages, it is rarely detected at that point as the disease is often asymptomatic when it starts in the ovaries. This cancer metastasizes rapidly through an exfoliation process, where cancer cells are shed from the primary tumor into the peritoneal cavity to form micrometastatic lesions [1]. These microscopic metastases, which grow along peritoneal surfaces, are exceedingly difficult to detect clinically with existing technologies and are therefore often missed in the diagnosis and treatment of ovarian cancer. As a result, little is known about the early development of these tumor nodules, and even less is understood regarding their structural and molecular responses to therapeutic regimens.

A vast array of in vitro models for cancer growth and treatment have been developed to mimic human disease, ranging from simple monolayer cell cultures to more complex tissue constructs [2,3,4,5] and animal models [6,7,8]. Traditional cell cultures are grown as a two-dimensional
monolayer and therefore lack the architectural cues that are critical for accurately modeling tumor growth and treatment. Animal models, while excellent at recreating tumor microenvironments, are typically costly and time-consuming, making large-scale therapeutic studies difficult. Three-dimensional in vitro models can address these limitations as they are capable of recapitulating many of the cell–cell interactions needed to model tumor growth and can be developed for high-throughput experiments. A promising in vitro model for cancer studies is the multicellular tumor spheroid. These 3D culture systems are well established and have been utilized in a wide array of cancer studies, including research on radiation therapies, drug resistance, tumor invasion and angiogenesis, as well as high-throughput drug screening programs [2,3,4,5]. Recent research efforts utilize basement membrane substitutes to restore some of the cellular signals observed in vivo [9]. In this “overlay” model, cells seeded onto the surface of a matrix comprised of reconstituted basement membrane self-assemble to grow into spheroidal tumor nodules called acini that sit atop the matrix bed. An ovarian cancer overlay culture based on NIH OVCAR-5 cells generates acini that are similar in size and composition to in vivo micrometastatic nodules, and grow along the matrix surface, providing a powerful model for visualizing ovarian cancer tumor growth and treatment response [10].

In the early stages of in vitro growth, ovarian acini are small enough to be imaged using standard confocal imaging technology. However, as the acini mature, their diameters can range up to 200 μm, beyond the imaging depth of confocal microscopes. Ongoing studies investigating therapeutic regimens are generally carried out between one and two weeks of growth, at which time the acini can reach 350 μm in size. By three weeks of tumor growth, acini can grow as large as 1 mm in diameter with complex internal structures. These large spheroids, which sit atop several hundred microns of gel matrix, become unfeasible to image even with advanced multiphoton microscopy systems. This lack of suitable imaging technology has made it difficult to visualize the important structural dynamics which occur in these complex tumor models.

An ideal approach for visualizing large, structurally complex three-dimensional models is optical coherence tomography (OCT). OCT is a highly sensitive, cross-sectional imaging technique capable of visualizing the microscopic details of biological tissues. OCT has found extensive applications in fields such as ophthalmology [11,12], cardiology [13], gastroenterology [14,15] and dermatology [16]. As the optical analog of ultrasound, OCT provides structural information based on the light reflected from objects in a sample. Current state-of-the-art spectral domain OCT systems utilize large bandwidth light sources (>150 nm) capable of cellular-resolution imaging. With penetration depths exceeding 1.5 mm, OCT is particularly apt for visualizing even the largest 3D nodules. Most importantly, OCT is a non-perturbative imaging technique. The standard methods for tumor spheroid imaging require fixation and staining [3], which are highly perturbative, destructive and can introduce artifacts. With OCT imaging, cells remain viable allowing for long-term, longitudinal three-dimensional visualization of acinar growth and development. This gives OCT the potential to play a large role in understanding tumor therapeutics, where current imaging approaches only capture temporal snapshots. Finally, OCT is capable of fast imaging where the structure of dozens of acini can be captured in a few seconds. This rapid image acquisition enables high-throughput imaging experiments for drug screening applications. In this study, OCT will be shown as an ideal structural imaging technology for visualizing three-dimensional tumor models, with the capability to carry out extended, time-lapse imaging of the complex structural dynamics underlying both tumor growth and treatment response.

2. Experimental Methods

2.1. Ultrahigh resolution optical coherence tomography imaging system

Imaging experiments were performed on a custom-built, laser-scanning microscopy system (Fig. 1). A broadband Ti:Sapphire laser (Integral OCT, Femtolasers) with a bandwidth of 190
nm centered at 850 nm and output power of 110 mW provided the light for both the ultrahigh resolution SD-OCT and two-photon imaging experiments. SD-OCT [17,18] was chosen for this experiment as it has the capability to be stable over several days of continuous, automated imaging. For the OCT system, the output of the Integral OCT was fiber coupled into a SMF fiber (SMA 600) and entered into an 80:20 fiber interferometer (AC Photonics). The 80% arm of the interferometer was directed toward a reference arm, while the 20% arm was directed towards the laser-scanning microscope. A pair of galvanometric mirrors (Cambridge Technologies) were arranged in a 4f configuration to enable point-scanning of the beam in the microscope focal plane. The scanned beam was directed into a Zeiss 200M inverted microscope, reflected off a silver mirror (21004b, Chroma Technologies) mounted in the microscope turret, and finally passed through an objective lens. A .15NA Zeiss 5X EC Plan Apo objective was used for all OCT imaging sessions. The total power at the focus was 1.5 mW, and was controlled by a neutral density filter wheel (Thorlabs) in the sample arm. The reflected light from the microscope focal plane was combined with the light reflected from the reference arm in the fiber interferometer and a portion of the interference was directed towards a custom-built spectrometer. Light emerging from the fiber tip was first expanded with a telescope before passing through a transmission grating (1200 lines/mm). The dispersed spectrum was focused using a three-element lens with a focal length of 100 mm onto a Basler L104k 2048 element line-scan camera capable of running at 29.3 kHz. The spectral range of the detector was set to sample a spectral bandwidth of 122 nm. A National Instruments 1428 frame grabber board was used to read in the spectral from the line scan camera using the NI IMAQ interface. The camera readout and galvanometric mirrors were controlled using home-built software. A LabView front end provided user control of the imaging system, while custom multithreaded C libraries called by LabView allowed for rapid Fourier Transforms via the Intel IPP libraries. The software was run on an eight-core SignaTec computer utilizing a four-disk RAID array for real-time processing, visualization, and storage of incoming camera data at the full camera readout rate. The RAID array used in this experiment was comprised of four 750 GB, 7,500 RPM SATA II hard drives (Seagate Barracuda). For the OCT experiments here, the camera was run at 20 kHz with an integration time of 50 μs. The spectrum was calibrated and remapped for even spacing in frequency space using a coverslip placed between the laser aperture and the fiber coupler. An iterative method was used to obtain the wavelength-dependent dispersion for real-time compensation during data acquisition [19]. An axial resolution in tissue of 1.8 μm (n = 1.34) was measured at the −3 dB point by fitting a gaussian through the point spread function derived from the reflection off a mirror in the sample arm. Using size-calibrated polystyrene microspheres (Polysciences), the lateral resolution of the imaging system was found to be 6.7 μm. A temperature-controlled incubation stage (DH-35i, Warner Instruments) was mounted on the microscope for imaging live samples.

2.2. Two-photon microscopy system

The free-space output of the Integral OCT was directed through a broadband dispersion mirror pair (GSM201, Femtolasers), which provided −3500 fs² of dispersion pre-compensation for two-photon imaging experiments [20]. The pre-compensated beam was directed to the galvanometric mirror pair described above, before being sent into the inverted microscope. A 700 nm long-pass filter (HQ700LP, Chroma Technologies) was positioned before the microscope entrance to filter out the Ti:Sapphire superfluorescence. The beam was reflected towards the objective lens by a short-pass dichroic mirror positioned in the microscope turret (675DC-SPxr, Chroma Technologies). A .75NA Olympus 20X UPlanSApo UIS2 objective was used for all multiphoton imaging experiments as it was found to have high infrared transmission and low dispersion. The average power at the sample was 30 mW for all experiments. The lateral and axial resolutions of the two-photon system were measured, using 200 nm size-calibrated microspheres (Polysciences YG Fluoresbright Microspheres), to be 560 nm and 4.2 μm, respectively, due to moderate beam astigmatism. The objective was screwed
into a piezoelectric stack mounted on the microscope nosepiece that was used for fine axial height adjustments (PIFOC P-725.2CD, PI). Fluorescence generated in the focal volume was collected in the epifield, and passed through a set of filters (HQ450/50X, HQ620/60M, Chroma Technologies) before being focused by a 100 mm lens onto a photon-counting photomultiplier tube (H7421-40, Hamamatsu). The photon counts generated by the H7421 PMT were acquired using a National Instruments 6602 board. A LabView program was used to control the galvo mirrors and read the photon counts. In addition to providing a live image of the sample, the program was also made to collect z-stacks for three-dimensional imaging.

2.3. Time-lapse optical coherence tomography

For long-term time-lapse OCT imaging, alterations were made to the OCT system. In order to have a stable spectrum for greater than 48 hours, the output of a Broadlighter D890HP (890 nm centered, 90 nm bandwidth, Superlum) was used instead of the Ti:Sapphire. The spectrum was found to be stable for over four days after a three-hour warm-up period. As the output power of the combined diodes was only 3 mW, the acquisition speed of the OCT system was considerably reduced to allow for 300 to 600 μs of spectral integration time. The lower bandwidth SLD source resulted in an axial resolution in tissue of 3.7 μm (−3 dB, n = 1.34), while the lateral resolution was found to be 7.0 μm. A custom Labview program ran the time-lapse system continuously, with volumes recorded at user-defined intervals. The power at the sample was below 300 μW. The alignment of the spectrometer was checked regularly during time-lapse experiments. An incubation chamber was built around the Warner Instruments temperature-controlled stage to allow for longitudinal imaging of acinar growth and treatment dynamics. Pre-mixed 95% air, 5% CO₂ gas was passed through a Pyrex glass bubbler containing sterile water maintained at 100 C providing humidity to slow the evaporation of sample media. Gas flow was set to provide a slight positive pressure flow over the sample dish. A layer of mineral oil (Sigma) was also poured on top of the media in the culture dish to slow evaporation while allowing for gas exchange. As the cell media was supplemented with phenol red dye, the culture media pH was checked by observing the media color under white light illumination on the microscope.

The Live/Dead viability assay was purchased from Invitrogen and used according to the supplied protocols. The viability marker used in this experiment was Calcein Green AM ester. In its ester form, the calcein dye molecule is non-fluorescent and can easily permeate the cell membrane. Inside the cytoplasm of a viable cell, cellular esterases enzymes act to cleave the ester bond, converting calcein green AM into calcein, a polyanionic, fluorescent, and cell-membrane impermeable dye. The conversion of calcein green AM to calcein by esterase activity leads to the retention and build up of intracellular calcein, allowing the easy observation of cell viability. The nuclei of non-viable were stained by ethidium bromide, which readily penetrates the dead cells’ ruptured cell membranes and brightens upon DNA intercalation.

2.4. Three-dimensional ovarian acini culture

The three-dimensional ovarian cancer model was developed following a protocol similar to that described in Debnath et al [3]. Human ovarian carcinoma cells (OVCAR-5 cell line, Fox Chase Cancer Institute) derived from an untreated patient were used. OVCAR-5 cells were grown and maintained in cell media composed of RPMI 1640 (CellGrow, Mediatech) supplemented with 10% fetal bovine serum (Gibco) and 1% 5,000 I.U./ml penicillin/streptomycin (CellGrow, Mediatech).

The three-dimensional tissue culture was created on the coverslip surface of 20 mm aperture glass-bottomed 35 mm culture dishes (P35G-0-20-C, Mattek). This surface was evenly coated with 150 μL of reduced growth factor reconstituted basement membrane (GFR Matrigel, BD Biosciences). As Matrigel solidifies rapidly at room temperature, the coating protocol was
carried out on ice with all components chilled to 4°C or below. After 30 minutes of incubation at 37°C, 500 μL of a 15,000 cell/mL OVCAR-5 cell suspension in cell media was seeded over the 20 mm diameter Matrigel surface. The culture dishes were then incubated for 40 minutes at 37°C to allow for cell adhesion to the gel. After 40 minutes, the media volume in each culture dish was brought to 2 mL and was supplemented with 2% GFR Matrigel. The culture dishes were then maintained with 2% GFR Matrigel supplemented cell media for the duration of acinar growth.

2.5. Immunofluorescence protocol and imaging method

Acini were stained following a protocol closely derived from Debnath et al. [3], with one major exception. To prevent the degradation of the Matrigel during the staining protocol and subsequent loss of acini from the Matrigel surface, culture dishes were maintained at 37°C between all protocol steps. The exchange of buffers and staining solutions was accomplished using a micropipetter to minimize disruption of the Matrigel. Antibodies against activated caspase-3 were acquired from Cell Signaling (Asp175, 5A1E), while anti-E-cadherin antibodies were obtained from Transduction Laboratories (C20820). Alexa Fluor 488 labeled goat anti-rabbit and Alexa Fluor 568 labeled goat anti-mouse (Invitrogen) were used to counterstain the caspase-3 and E-cadherin antibodies, respectively. Alexa Fluor 568 labeled phalloidin was purchased from Invitrogen to label F-actin. DAPI (4′,6-diamidino-2-phenylindole) was obtained from Sigma and diluted for use.

Immunofluorescence controls were carried out for both caspase-3 and E-cadherin staining. In these control experiments, the same staining protocol was used, but the primary antibody incubation was replaced by a mock incubation in blocking buffer. The fluorescence-tagged secondary antibody was then added as normal following the mock incubation. For both the E-cadherin and caspase-3 controls, only a weak, poorly localized staining pattern was observed. The fluorescence intensity of the controls was found to have an average fluorescence intensity level one order of magnitude less than what was observed normally.

In order to image acini before staining with OCT and after staining with two-photon microscopy, a large 1.25X image of the culture dish surface was taken using a Zeiss 100TV inverted cell microscope equipped with a SPOT RT Slider camera (Diagnostic Instruments). This image was used as a map of the culture dish so that acini could be re-imaged following the 36 hour staining process.

2.6. Chemotherapy treatment

The most effective chemotherapeutic agents against epithelial ovarian cancer have been based on cytotoxic platinum compounds [1], such as the front-line chemotherapeutic agent CISplatin [1]. This drug interferes with the cells DNA replication process, effectively targeting fast-growing cancer cells. CISplatin is clinically administered either intravenously or, more recently, intraperitoneally (i.p). To simulate i.p administration of CISplatin using the three-dimensional ovarian cancer model, CISplatin (APP Pharmaceuticals) was added to the 2% Matrigel cell media just prior to the time-lapse imaging session. The CISplatin dose was calculated from the maximum i.p. therapeutic dose of 100 mg/m² used clinically [21], taking into account the area of the 35 mm culture dish.

2.7. Data and image processing

OCT images were acquired from a rectangular imaging region with a width to height ratio of 1:2. Ultrahigh resolution OCT volumes were streamed to the RAID, with a single volume comprising of 2048 (x pixels) × 250 (y pixels) × 2048 (spectral pixels) 10-bit values, resulting in about 2 GB of data per volume. To allow for long-term data collection and to reduce the post-processing load, time lapse volumes were acquired using a reduced y axis pixel count of
60 and reduced width to height aspect ratio of 1:6, resulting in about 450 MB of data per volume. At an acquisition rate of 2 volumes per hour, the time-lapse OCT system generates nearly 22 GB of data per day.

Data collected in the OCT experiments was processed using Matlab. Time-lapse movies were initially created in Matlab and later modified using ImageJ. Two-photon images and stacks were processed using ImageJ.

3. Results

3.1. Imaging acinar growth and composition

Immediately after seeding, early ovarian cancer acini have been observed to form through a combination of proliferation and fusion, via the migration and coalescence of individual cells as well as entire acini [10]. Figure 2 shows representative images from the later stages of acinar growth and development captured by the ultrahigh resolution SD-OCT system. After ten days of incubation, ovarian cancer cells form solid spherical structures that sit on top of the gel matrix (Fig. 2(a)). The varying height of these acini above the coverslip reflect the rough surface topography of the underlying Matrigel bed, which can be several hundreds of microns thick. Acini at this stage of growth are already quite large, with average diameters at approximately 100 μm. Unlike their far smaller breast cancer counterparts [9], ovarian cancer acini were not observed to undergo growth arrest, and continued to increase in size. By thirteen days of growth, acini can reach up to 300 μm in diameter. Beyond this time point, a subpopulation of acini remained solid spheroids and continued to slowly grow. The remaining acini, however, followed a more complex trajectory, first developing a hollow center near 17 days of growth (Fig. 2(c)) with loose scattering bodies visible in the hollow centers. By three weeks of acinar growth, the acini developed a luminal structure resembling bubbles filled with debris (Fig. 2(d)). The luminal appearance of these late-growth acini resembles luminal bodies found in advanced stage ovarian metastatic lesions in a murine model for disseminated ovarian carcinomatosis [7]. This finding indicates that the in vitro 3D model replicates the metastatic growth patterns observed in vivo, and could have biological relevance to human disease. As can be seen from the temporal progression, the developing acini grow and fuse into each other, resulting in the elongated, merged structures observed in later growth stages. These complex structural features, which span up to 1 mm in size, were only first observed during OCT imaging.

In order to understand the structural evolution of the ovarian cancer acini, the structural data collected using OCT was directly compared to the standard methodology in acini characterization, fluorescence microscopy. The contrast in OCT is derived from scattering in the sample, which can arise from numerous biological features, including cell nuclei, protein layers, and interfaces. In order to visualize the cellularity of the acinar structures, cultures of ovarian acini were plated and grown to 13, 17, and 21 days. First visualized with OCT in vitro, the samples were then immediately fixed and stained with DAPI and Alexa568-phalloidin, to visualize cell nuclei and the actin cytoskeleton, respectively. Figure 3 shows a direct comparison between living acini first imaged with OCT and the same, stained acini visualized using two-photon microscopy. The figure presents a single horizontal projection 4 μm thick through the center of each acinus in both the OCT and two-photon volumes for structural comparison. At 13 days of growth, the acinus is composed of closely packed cells with only small unfilled regions in the acinus center (Fig. 3(d)) marked by actin staining (Fig. 3(d) arrow). The densely packed cancer cells with large nuclei scatter light strongly, giving rise to the contrast observed in the OCT image. By day 17, a hollow center can be seen in both OCT and two-photon images (Figs. 3(b) and 3(e)), and small, DAPI-stained bodies can be observed in the acinus center in the fluorescence image (Fig. 3(e) arrow). The shape of these bodies appear fragmented, indicating that the cells may have undergone apoptosis [22]. By
three weeks of growth, the thin outer layer of the acinus observed in OCT is shown to be composed only of a layer of cells (Figs. 3(c) and 3(f)). The numerous actin-lined regions devoid of cells observed in the fluorescence images (in red) do not appear in the OCT data (Figs. 3(d) and 3(f) arrows), most likely due to the lower lateral resolution compared to the two-photon imaging system. Differences between the sets of images, such as the appearances of neighboring acini in the fluorescence images, are the result of fixation and staining artifacts. Other artifacts include changes in acinar shape, acinus detachment from the Matrigel surface, and significant changes to the Matrigel surface itself. The structural data collected by OCT shows the true state of the acini, as it was collected without any sample preparation or processing when the acini were still viable.

To confirm the cellular debris observed in the acini centers arise from apoptosis, acini were grown and stained for activated caspase-3, a key factor in the cellular apoptosis signaling pathway. Cells that are undergoing or have completed apoptosis will stain positive for the activated form of caspase-3. Figure 4 shows two similarly sized acini, one grown for 13 days, the other for 17 days, stained for activated caspase-3 and E-cadherin, a cell-cell adhesion protein. These figures show a 4 μm projection through the center of each acinus. In the early stages of acinar growth (Fig. 4(a)), only a few apoptotic bodies were observed within the entire acinus volume, and were distributed randomly throughout the spherical structure. By day 17, numerous apoptotic bodies appeared predominantly in the hollowing center (Fig 4(b)). The appearance of these bodies, both in time and in spatial location, matches the debris found to accumulate in large numbers at the bottom of the acinus by three weeks (Fig. 2(d)). It is interesting to point out that not all cells found in the hollow acinar center stain for activated caspase-3. This may indicate that cells first enter the luminal space, and then undergo apoptosis in response to a lack of cellular survival signals [23].

If the acini are allowed to grow for extended periods of time, the hollowing process leads to the formation of acini composed of a single cellular layer about the spheroid circumference, filled with apoptotic debris settled into the lower half of the acinus. This single wall of cells stains strongly for E-cadherin, which may serve to hold the structure together. The large volume imaging capabilities of OCT are able to easily visualize the three-dimensional structure of later stage acini, which grew to nearly 1.5 mm in diameter.

3.2. Time-lapse OCT imaging of acinar structural development

To understand how these complex structures form, a time-lapse OCT system was built to continuously follow the acinar structural evolution. As a non-perturbative, volumetric imaging technique, OCT is uniquely capable of capturing the long-term, three-dimensional structural dynamics of acinar development. Time-lapse OCT differs from the standard fixation/staining approaches used in these in vitro model experiments, where dynamics are typically inferred by analyzing temporal snapshots. The lower bandwidth Broadlighter light source was used in this experiment, providing a stable broadband spectrum over the course of 110 hours of continuous imaging. Figure 5 shows a time-lapse OCT movie built from volumes recorded every hour, revealing the nature of the structural changes occurring between 13 to 17.5 days of growth. The two large acini in the cross section both begin hollowing, with acinus C initiating structural changes almost a day after acinus A. The sinking of the acini in the movie is due to both long-term drift of the microscope stage over the course of the multi-day experiment and the thinning of the Matrigel bed.

Acinus A was observed to initially form a hollow center, suggesting that, through a combination of growth and migration, cells at the acinar core first migrated away from the core towards the outer surface by day 16. This observation agrees with the day 17 DAPI and caspase-3 staining results, which showed only a minimal amount of apoptotic bodies at the acinar center. After the formation of the initial hollow core, the small segments of the inner acinar walls are
observed to shed off into the luminal center. These cellular flakes collected in the bottom half of the acinus, generating the numerous, loosely packed apoptotic bodies seen with OCT and multiphoton imaging in Figures 2 and 4. As large chunks of cells are observed to break off into the hollow center, the time-lapse OCT movie demonstrates that this shedding process is also responsible for the thinning of the acinar walls with time.

Time-lapse OCT also enables the visualization of rare dynamics not possible with standard fix-and-stain methods, such as the migration and fusion of two acini. Acinus A in Figure 5 is initially flanked by acinus B, a small, slow-growing solid acinus nearly one fifth its diameter. As the acini develop, the small acinus migrates toward acinus A, and over the course of 40 hours, merges and fuses into the large structure. These migration events do not seem to be due to simply random motion, and have been observed to be directed towards select acini [10], perhaps due to chemotaxis. The fusion of both acini resulted in a single spheroid, strongly suggesting that the cells in both acini must be highly motile and able to undergo rapid migration within the solid structure. Understanding the nature of such events may provide important information about in vivo ovarian metastatic growth.

### 3.3. Longitudinal visualization of chemotherapy treatment response

In addition to giving insights into growth and development, the three-dimensional ovarian cancer models provide a powerful platform for modeling tumor chemotherapeutic response. Ovarian micrometastatic lesions are difficult to detect in vivo, let alone monitor pre- and post-treatment. As this model was created with the end goal of better understanding ovarian treatment response, the volumetric, non-perturbative longitudinal imaging provided by OCT is an ideal approach for visualizing large-scale therapeutic effects. Figure 6 shows the impact of a single clinical dose of one of the most common chemotherapeutic agents for ovarian cancer, CISplatin, on the ovarian cancer model. During the first 24 hours of treatment, the acini are observed to slow in growth, and did not start developing hollow centers. This result correlates well with the mechanism of CISplatin, which interferes with DNA replication by cross-linking strands of DNA [24], shutting down the cells’ ability to grow and develop.

At the end of 24 hours in the OCT time-lapse movie, numerous small vacuoles were seen to appear in the acini just prior to the major structural breakdown observed at hour 36. The outer walls of the acini lost their solid packing, and began to slowly shed away from the structures. Both large and small acini showed similar structural transformation. As the movie progresses, the outer layers of cells continued to flake away from the acini structures. By 81 hours of drug exposure, the smaller acini were observed to completely decay, while the two leftmost acini retained some structure, with decaying masses surrounding what appeared to be remaining solid bodies.

Immediately following OCT imaging, the cell culture dish was processed and stained with a Live/Dead assay to show the status of the ovarian acini. As expected from the time-lapse OCT movie, the smaller acinar structures contained mostly dead cells, stained by ethidium bromide dye. The two large leftmost acini, on the other hand, were found to contain living cores that survived the treatment (Fig. 7), stained in bright green by the cleavage of the Calcein Green AM ester in the cells’ cytoplasm. The ability to follow the treatment dynamics and visualize living structures with OCT should allow for more advanced studies in drug screening experiments.

### 4. Discussion

The structural evolution observed in these ovarian cancer acini has been classically observed in MCF-10A 3D breast cell models [23]. In these far smaller spherical bodies (about 40 μm in diameter) the hollowing process has been linked to apoptosis of cells at the acinus center to
form glandular structures reminiscent of the terminal end buds of mammary ducts. The hollowing ovarian cancer acinar structures observed here most likely result from a combination of growth, migration, and cell death. Cells at the center of the hollowing acinus may become loosely bound to their neighbors due to down-regulation of surface adhesion proteins [25]. When these cells break off into the acinar core, they lose their cell-cell contacts and subsequently undergo apoptosis in response to a lack of cell-cell and cell-matrix survival signals [26], creating the numerous apoptotic bodies that fill the luminal structure.

The flaking off of cells from the inner acinar walls observed with time-lapse OCT is suggestive of the shedding process seen in epithelial ovarian cancer. Cancer cells flake off the surface of primary ovarian cancers into the peritoneal cavity, and form metastatic lesions on surfaces in the peritoneum [25]. If the cells shedding into the acinar core are the result of a similar cellular signaling cascade, the structural evolution in these late acini may be used to model ovarian peritoneal metastasis. A study investigating this process is already underway, using a combination of OCT and multiphoton imaging.

The SD-OCT imaging system in this study utilized an ultrabroadband light source centered at 800 nm. The use of light at this wavelength was seen to affect the ultimate penetration depth of the system in a few cases. While better imaging depths could be obtained using swept-source OCT systems at 1060 nm or 1300 nm, the current generation of these light sources may not be able to maintain a stable, consistent spectrum continuously for the many days needed in time-lapse experiments. Additionally, few light sources offer bandwidths close to 200 nm. As can be observed between the time-lapse and ultrahigh resolution OCT images, higher axial resolution is better for cellular-level imaging in these model studies. While a broadband SD-OCT system was ideal for this study, emerging stable broadband light sources deeper in the infrared will significantly enhance three-dimensional tumor model imaging.

Optical coherence tomography, with its ability to rapidly visualize large structures with cellular resolution, clearly has the potential to provide new insights in the field of three-dimensional cancer models. Widefield imaging techniques cannot follow the complex growth dynamics, limiting studies of 3D models to 2D observations. Currently used 3D imaging methods, such as confocal or multiphoton microscopies, require the slow process of taking depth-resolved stacks to visualize three-dimensional structures, and typically can only be used to capture snapshots of development due to the fixation step needed for staining. Even when dyes compatible with live cell imaging are available, fluorescent labels can be perturbative. Moreover, the depth penetration of these fluorescence imaging techniques limits them to smaller objects below 500 or 600 μm in size. Confocal reflectance imaging, which can provide high resolution structural imaging deep in samples, still is limited by the time required to collect depth stacks. In contrast, the ability of OCT to collect volumes at high rates allowed for rapid surveying and characterization of hundreds of ovarian acini on a single dish in just minutes. A video-rate confocal microscope with comparable axial resolution to the system used in this work would still be nearly an order of magnitude slower than OCT in acquiring the same volume.

At the same time, it is important to point out the trade-offs inherent in using optical coherence tomography as a platform for in vitro model imaging. While the strength of OCT is its capability to optically image deep in samples nonperturbatively and without labels, the contrast in OCT is based on index of refraction changes along the optical path. Therefore, standard implementations of OCT do not have the capability of visualizing samples with molecular selectivity. While there has been some pioneering work in visualizing molecular information with OCT, most notably by Boppart and his colleagues [27,28,29], OCT remains, in practice, a structural imaging platform. Another trade-off in OCT is the choice between lateral resolution and depth of field. Since the axial resolution is dependent on the bandwidth of the light source,

*Opt Express.* Author manuscript; available in PMC 2010 March 11.
it is essentially decoupled from the constraints of optical system. Thus, unlike the case in standard microscopic techniques, high numerical aperture lenses are not required for high axial resolution. Instead, the choice of numerical aperture dictates how much of the optical path through the sample will be in focus to yield strong backscattered signal. OCT can take advantage of the large depth of field afforded by lower NA lenses to provide large volume imaging, as was done in this study. A numerical aperture of 0.15 was chosen to enable strong OCT signal along nearly 1 mm of the optical axis, allowing for the full visualization of even the largest acini. With low numerical apertures, however, comes reduced lateral resolution and the inability to resolve subcellular details. In this study, the lateral resolution of approximately 7 μm was acceptable for imaging acini composed of dozens of cells, and was still less than the size of the cells themselves (about 15 μm). The requirements of individual experiments will dictate the needed system parameters; in this work the nonperturbative, label-free structural contrast of OCT with a high depth of field was critical for both long term visualization and quickly surveying the thousands of acini that can grow on a single plate.

The rapid surveying capability is critical for 3D tumor models to achieve their ultimate usefulness as high-throughput drug screening and therapeutic monitoring models. The majority of studies on drugs and their effects are so far conducted on monolayer cell cultures, which lack many of the three-dimensional cellular and extracellular cues known to be involved in drug uptake and resistance. This deficiency can result in poor drug screens that do not accurately measure efficacy and other critical parameters. Animal models are too costly and time consuming for large-scale pharmaceutical screening, with complexity that can hinder detailed studies of drug effects. The differential drug response seen between large and small acini captured by time-lapse OCT in this study demonstrates the ability to visualize the long-term effects of therapeutics. This imaging approach could be easily scaled to allow for high-throughput, threedimensional screening of tens of thousands of acini in a single therapeutic study.

5. Conclusion

Optical coherence tomography can be a powerful imaging technique for the visualization of three-dimensional cultures of cancer. The large size, imaging depth, and structural complexity of spheroid and acini models can be rapidly imaged using OCT for high throughput studies. Most importantly, the non-perturbative nature of OCT makes it ideal for long-term time-lapse studies of tumor nodules growth and structural development. When combined with complementary molecular imaging technologies, OCT is capable of providing new insights into the biology of these model cancer systems, and ultimately cancer itself.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jon Celli and Adnan Abu-Yousif for their help with the three-dimensional tumor model and for their insightful and enlightening discussions. Hyle Park and Benjamin Vakoc were extremely helpful in the programing of the OCT data collection, processing, and rendering software. Our thanks go out to both the de Boer and Hasan groups for their helpful and stimulating conversations regarding this work. C.L.E. is supported by the Massachusetts Biomedical Research Corporation (MBRC) Tosteson Postdoctoral Fellowship. I.R. is supported by a Wellman Center for Photomedicine Fellowship. The OCT and multiphoton research components were funded by a grant from the National Institutes of Health (RO1 RR019768) to J.F.dB. The ovarian cancer model development is funded by a National Institutes of Health (R01 AR040352-09) grant to T.H.
References and links

1. Berek, JS.; Bast, RCJ. Ovarian Cancer. In: Kufe, D.; Pollack, RE.; Weichselbaum, RR.; Bast, RCJ.; Gansler, TS.; Holland, JF.; Frei, EI., editors. Cancer Medicine. BC Decker Inc.; Hamilton: 2003. p. 1831-1861.

2. Mueller-Klieser W. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. Am. Physiological Soc 1997;273:1109–1123.

3. Debnath J, Brugge JS. Modeling glandular epithelial cancers in three-dimensional cultures. Nat. Rev. Cancer 2005;5:675–88. [PubMed: 16148884]

4. Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. Annu. Rev. Cell Dev. Biol 2006;22:287–309. [PubMed: 16824016]

5. Smalley KSM, Lioni M, Herlyn M. Life isn’t flat: Taking cancer biology to the next dimension. In vitro cell. dev. biol. - Animal 2006;42:242–247.

6. Jain R, Munn L, Fukumura D. Dissecting tumour pathophysiology using intravitral microscopy. Nat. Rev. Cancer 2002;2:266–76. [PubMed: 12001988]

7. Molpus KL, Koelliker D, Atkins L, Kato DT, Buczek-Thomas J, Fuller AF Jr, Hasan T. Characterization of a xenograft model of human ovarian carcinoma which produces intraperitoneal carcinomatosis and metastases in mice. Int. J. Cancer 1996;68:588–95. [PubMed: 8938139]

8. del Carmen MG, Rizvi I, Chang Y, Moor ACE, Oliva E, Sherwood M, Pogue B, Hasan T. Synergism of Epidermal Growth Factor Receptor-Targeted Immunotherapy With Photodynamic Treatment of Ovarian Cancer In Vivo. JNCI 2005;97:1516–1524. [PubMed: 16234565]

9. Lee GY, Kenny PA, Lee EH, Bissell MJ. Three-dimensional culture models of normal and malignant breast epithelial cells. Nature Methods 2007;4:359–365. [PubMed: 17396127]

10. Rizvi I, Celi J, Evans CL, Abu-Yousif A, Spring BQ, Neuman D, Pouge B, de Boer JF, Hasan T. Adherent ovarian carcinoma cells migrate and assemble in vitro into a heterogeneous population of 3D micronodules with differential response to treatment. To be submitted.

11. Fujimoto J. Optical coherence tomography for ultrahigh resolution in vivo imaging. Nature Biotech 2003;21:1361–1367.

12. Nassif N, Cense B, Park B, Pierce M, Yun S, Bouma B, Tearney G, Chen T, de Boer JF. In vivo high-resolution video-rate spectral-domain optical coherence tomography of the human retina and optic nerve. Opt. Express 2004;12:367–376. [PubMed: 19474832]

13. Jang IK, Bouma BE, Kang DH, Park SJ, Park SW, Seung KB, Choi KB, Shishkov M, Schlendorf K, Pomerantsev E. Visualization of coronary atherosclerotic plaques in patients using optical coherence tomography: comparison with intravascular ultrasound. J. Am. Coll. Cardio 2002;39:604–609.

14. Pones JM, Brand S, Bouma BE, Tearney GJ, Compton CC, Nishioka NS. Diagnosis of Specialized Intestinal Metaplasia by Optical Coherence Tomography. Gastroenterology 2001;120:7–12. [PubMed: 11208708]

15. Bouma BE, Tearney GJ, Compton CC, Nishioka NS. High-resolution imaging of the human esophagus and stomach in vivo using optical coherence tomography. Gastroint. Endosc 1999;51:467–474. [PubMed: 10744824]

16. De Boer JF, Srinivás S, Malekafzali A, Chen ZP, Nelson J. Imaging thermally damaged tissue by Polarization Sensitive Optical Coherence Tomography. Opt. Express 1998;3:212–218. [PubMed: 19384363]

17. Fercher A, Hitzenberger C, Kamp G, El-Zaiti S. Measurement of intraocular distances by backscattering spectral interferometry. Opt. Commun 1995;117:43–48.

18. Chen T, Cense B, Pierce M, Nassif N, Park B, Yun S, White B, Bouma B, Tearney G, de Boer JF. Spectral Domain Optical Coherence Tomography Ultra-high Speed, Ultra-high Resolution Ophthalmic Imaging. Arch. Ophthalmol 2005;123:1715–1720. [PubMed: 16344444]

19. Mujat M, Park B, Cense B, Chen T, de Boer JF. Autocalibration of spectral-domain optical coherence tomography spectrometers for in vivo quantitative retinal nerve fiber layer birefringence determination. J. Biomed. Opt. 2007;12:041205. [PubMed: 17867794]

20. Denk W, Strickler J, Webb W. Two-photon laser scanning fluorescence microscopy. Science 1990;248:73–76. [PubMed: 2321027]
21. Alberts, DS.; Hess, LM.; Von Hoff, DD.; Dorr, RT. Pharmacology and therapeutics in gynecological cancer. In: Hoskins, WJ.; Perez, CA.; Young, RC.; Barakat, R.; Markman, M.; Randall, M., editors. Principles of gynecological oncology. Lippincott Williams & Wilkins; Hamilton: 2005. p. 500-501.

22. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F, et al. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death(necrosis). Cytometry 1997;27:1–20. [PubMed: 9000580]

23. Debnath J, Mills K, Collins N, Reginato M, Muthuswamy S, Brugge J. The Role of Apoptosis in Creating and Maintaining Luminal Space within Normal and Oncogene-Expressing Mammary Acini. Cell 2002;111:29–40. [PubMed: 12372298]

24. Knox R, Friedlos F, Lydall D, Roberts J. Mechanism of Cytotoxicity of Anticancer Platinum Drugs: Evidence That cis-Diamminedichloroplatinum (II) and cis-Diammine-(1, 1-cyclobutanedicarboxylato) platinum (II) Differ Only in the Kinetics of Their Interaction with DNA. Cancer Research 1986;46:1972–1979. [PubMed: 3512077]

25. Naora H, Montell D. Ovarian cancer metastasis: integrating insights from disparate model organisms. Nat. Rev. Cancer 2005;5:355–366. [PubMed: 15864277]

26. Frisch S, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell. Biol 1994;124:619–626. [PubMed: 8106557]

27. Xu C, Vinegoni C, Ralston TS, Luo W, Tan W, Boppart SA. Spectroscopic spectral-domain optical coherence microscopy. Opt. Lett 2006;31:1079–1081. [PubMed: 16625909]

28. Oldenburg AL, Gunther JR, Boppart SA. Imaging magnetically labeled cells with magnetomotive optical coherence tomography. Opt. Lett 2005;30:747–749. [PubMed: 15832926]

29. Marks DL, Boppart SA. Nonlinear interferometric vibrational imaging. Phys. Rev. Lett 2004;92:123905. [PubMed: 15089675]

Opt Express. Author manuscript; available in PMC 2010 March 11.
Fig. 1.
Ultrahigh resolution OCT and multiphoton microscopy experimental setup. The output from a Ti:Sapphire laser is either coupled into a fiber interferometer for OCT imaging or selected with a flip mirror towards a pair of dispersion compensating mirrors (DSP) for two-photon microscopy. The sample arm of the interferometer or the reflected dispersion compensated beam was coupled into an inverted beam-scanning microscope. SL, scan lens; TL, tube lens; DM, dichroic mirror; SM, silver mirror; PMT, photomultiplier tube.
Fig. 2.
OCT B-scans of acinar growth. After 10 days of growth (a) (Media 1) acini appeared as small, solid structures which continue to grow larger by day 13 (b) (Media 2). Internal structure appeared on day 17 (c) (Media 3) as the acini begin to hollow. By three weeks of growth (d) (Media 4), the developing acini were hollow, luminal structures full of highly scattering, loosely packed bodies. Each B-scan links to a movie showing a transverse scan through a 2 mm × 1mm × 360 μm volume.
Fig. 3.
Direct comparison between live OCT images and stained two-photon fluorescence images of ovarian cancer acini at different time points. All images are 4 μm thick projections taken at the acini centers. DAPI appears in blue, while Alexa568-phalloidin labeled actin is shown in red. A Day 13 acinus visualized with OCT (a) and two-photon fluorescence microscopy (d) shows a densely packed cellular structure. The hollow center seen in a day 17 acinus captured by OCT (b) matches the structure visualized using fluorescence staining (e). The thin walls of three-week old acini seen in OCT (c) are revealed to be composed of a layer of cells (f).
Fig. 4.
Ovarian cancer acini stained for activated caspase-3 (red) and E-cadherin (green). As a cell-cell adhesion molecule, E-cadherin can be seen outlining cellular contacts throughout the acini. Before acinar hollowing, very few apoptotic cells were observed, and were distributed randomly throughout the acinus volume (a). Following hollowing, cells in the luminal space begin undergo apoptosis (b), leading to the eventual filling of the luminal space with apoptotic bodies.
**Fig. 5.** (Media 5) Time-lapse OCT movie of acinar growth and development. Two neighboring B-scans were averaged. The movie runs for 92 hours, beginning on day 13 of growth. The camera was run at a rate of 3076 Hz for an integration time of 325 μs per A-line. Volumes were collected continuously, with a full 2 mm × 1 mm × 1.6 mm volume recorded every hour. Three acini are labeled in the figure, here called “A”, “B”, and “C”.
Fig. 6. (Media 6) Time-lapse OCT movie of acinar treatment with CISplatin. Two neighboring B-scans were averaged. The movie runs for 81 hours, beginning on day 13 of growth. The camera was run at a rate of 1081 Hz for an integration time of 925 μs per A-line. Volumes were collected continuously, with a full 2 mm × 1 mm × 1.6 mm volume recorded every 30 minutes.
Fig. 7.
CISplatin treated ovarian cancer acini can survive the chemotherapeutic regimen. Two acini from the time-lapse movie in Figure 6 before treatment (a) and after treatment (b), and their z-projections (c), and (d), respectively. Following treatment, both acini display solid, spherical structures (blue circles in (d)) that are revealed to be the surviving acinar cores using the Live/Dead viability assay (e). Viable cells show green fluorescence from cleaved Calcein Green AM, while dead cells are shown in red, stained by Ethidium bromide. Many of the dead cells seen in (d) were lost during the staining process, resulting in the few dead cells observed in (e).